

Interaction between inclusions mediated by surfactant membranes and changes in the local order of the acyl chains

Elise Azar

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Thèse de doctorat de l'Université Paris-Saclay préparée à l'Université Paris-Sud

Ecole doctorale n°564 Ecole Doctorale de Physique en Ile-de-France (EDPIF) Spécialité de doctorat : Physique

par

MLLE. ÉLISE AZAR

Interaction between inclusions mediated by surfactant membranes and changes in the local order of the acyl chains

Thèse présentée et soutenue à Orsay, le 14 Décembre 2016.

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... let there be no scales to weigh your unknown treasure; And seek not the depths of your knowledge with staff or sounding line. For self is a sea boundless and measureless. Say not, "I have found the truth," but rather, "I have found a truth." Say not, "I have found the path of the soul." Say rather, "I have met the soul walking upon my path." For the soul walks upon all paths...

Gibran Khalil Gibran - The Prophet

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Chapter 1

Introduction

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1.1 Cells and membranes: biological overview

The cell is considered as the elementary structural and functional unit in all known living organisms [1-4]. Among the vast variety of living beings, cells are separated into two classes based on their cellular properties: eukaryotic cells and prokaryotic cells.



FIGURE 1.1: Sketch representing a cut of a eukaryotic cell (typical dimension: 10 to 100 μ m). The various organelles, i.e., intracellular compartments surrounded by membranes, are indicated with a lowercase legend. This cell corresponds to an animal cell. Original illustration from Wikimedia Commons, adapted and modified.

The main difference between the two types is the presence of a nucleus in the former and its absence in the latter. Aside from these differences, cells share some common universal features. They all produce DNA, RNA and proteins, each with very specific sequences. DNA contains the information necessary to build a cell, passes this genetic information to RNA through transcription on how to make a protein, then the RNA goes to a ribosome and a polypeptide chain is made, through translation, which eventually folds into a protein. [4]. Another general feature, which constitute the basic target of our study, is the presence of permeable thin membranes that isolate the cell from its surrounding and allow the formation of individual cellular compartments known as organelles. Figure 1.1 shows a sketch of an eukaryotic cell with its various organelles, each with its specific role in the cell. From this illustration, it can be clearly seen that the membranes of organelles have various specific shapes, some being highly curved and densely packed.

The basic structure of the membrane is the same among all living cells: it is essentially a bilayer of amphipathic molecules called lipids, with inclusions such as proteins. It is estimated that in a human being, which is composed of about 10^{14} cells, the total surface of the membranes is around 100 km² [5].

Seeing how the cellular and subcellular structures have different lipid bilayer constitutions and each structure has different and specific role brings up many questions about the effective function of the lipid bilayer. Is it only a neutral background compartmentalizing the living matter or does it influence in a way or another the biological functions of the cellular molecules? And if so, what are the structural and dynamical properties involved? As a matter of fact, it has recently become clear that compartmentalization is far from being the only function of lipid bilayers. Scientists have shown special interest in the study of these membranes from the point of view of both basic and applied research and have shown that membrane lipids tend to play an active role in many biological processes that take place in the membrane or that are mediated by it: they can act as enzymes, receptors, drugs, messengers, regulators, etc. [5].

1.2 The basic structure of biological membranes

Under normal biological conditions, cellular membranes are most often found in liquidcrystalline state and more specifically in lyotropic smectic phase [6]. Briefly, liquid crystals, and as their name literally stands for, are a special form of matter, with properties between those of a crystalline solid and a liquid. More precisely, they are substances flowing like a liquid state but also having a long-range order, as in crystals [7]. This allows liquid-crystalline structures to be more dynamical and flexible than normal solids. In this Section, I will start with a brief description of the liquid crystals properties then, I will present a small outline of the membrane composition in terms of the structure of lipid molecules and proteins.

1.2.1 Lyotropic liquid crystals

The above-mentioned order, present in one coordinate direction and absent in another direction, allows the formation of different liquid-crystalline phases (mesophases, from the Greek *meso*, meaning "in between"), where this organization can be positional in one or two dimensions as in smectic and columnar phases respectively, or orientational as in a nematic phase [8].

The most striking feature of liquid crystals is their anisotropy and the resulting birefringence [9]. Between crossed polarizers, liquid crystals appear bright with different textures, unlike a conventional liquid. Aside from optical microscopy, their long-range order and symmetry make them perfect candidates for X-ray studies. As the mesophase periodicities are about an order of magnitude greater than those of atomic crystals, the scattered X-ray signal is concentrated at small angle with respect to the incident beam, a regime referred to as small angle X-ray scattering (SAXS).

Liquid crystalline materials are generally divided into two basic categories: thermotropic and lyotropic mesophases [8]. Thermotropic liquid crystal phases are usually composed of a single type of anisotropic molecule and appear only as a function of temperature change, whereas lyotropic liquid crystal phases are always mixtures of compounds and form in the presence of a suitable (isotropic) solvent and also of an additional variable, the concentration of the substance in the solvent as well as the temperature. The most



FIGURE 1.2: Sketch representing different scales of a membrane: overview of the membrane as a cell boundary, closer view of the cell membrane composition, the bilayer membrane view and finally a sketch of a single phospholipid molecule. Original illustration from Wikimedia Commons, adapted and modified.

common substances that form lyotropic liquid crystals are amphiphilic molecules called surfactants.

1.2.2 Lipids

Lipids are amphiphilic molecules composed of hydrophilic head groups and hydrophobic tails [4, 5]. The lipid head groups can be nonionic, zwitterionic or ionic. Most of the lipid molecules are based on fatty acids, in other terms, carboxylic acids with an aliphatic chain. This chain is hydrophobic, meaning that it does not dissolve in water, whereas the carboxyl group is hydrophilic and is ionized in solution at neutral and basic pH. Three types of lipids are found mainly in biological membranes: phospholipids, glycolipids and cholesterol. We will focus on phospholipids, as they are the main constituent of membranes.

Phospholipids consist of a polar head containing a phosphate group, connected to the tail via a glycerol moiety. The glycerol group is linked to the tail constituted of two fatty acids via ester bonds. The chemical structure of phospholipids is illustrated in Figures 1.2 and 1.3. Varying the lengths of the tail, as well as adding double bonds at various



FIGURE 1.3: On the left of the figure, an illustration of the chemical formula of a phospholipid deriving from two different fatty acids (an unsaturated oleic acid and a saturated palmitic acid). This phospholipid is palmitoyl-oleoyl-phosphatidilcholine, or POPC. The parts corresponding to the hydrophilic head group are indicated in blue, while the ones pertaining to the hydrophobic chains are indicated in red. On the right, the same phospholipid is represented as a space-filling model. On the right, original illustration from Wikimedia Commons, adapted and modified. On the left, illustration taken from Ref. [4].

positions, lead to a great variety of phospholipids. Moreover, one must distinguish between lipids that have charged headgroups (i.e. non-zero net charge at neutral pH) or zwitterionic headgroups (i.e. containing both a negative charge and a positive charge, which render them globally neutral) [5]. The nature of the R group attached to the phosphate determines the phospholipid type (Figure 1.3).

Phospholipids are not the only lipid type to be deriving from fatty acids. Sphingolipids arise from a fatty acid linked to sphingosine, which is a long-chain amine, thus constituting the hydrophilic head group [1]. Both phospholipids and sphingolipids can have their head groups substituted by sugars, in which case they are called glycolipids [1, 5]. In this work, we will study the influence on the interaction between embedded inclusions and on the order of surfactant chains of cholesterol, which is a very different lipid from the above mentioned. It has a steroid structure involving four steroid cycles and a short hydrocarbon side chain, and a simple hydroxyl group as its polar head group. Hence, cholesterol is a short lipid molecule with a bulky and stiff hydrophobic part and a small hydrophilic head group [5] making it an amphipathic molecule. Cholesterol is inserted with its hydroxyl group oriented toward the aqueous phase and its hydrophobic system parallel to the fatty acid tails of phospholipids [10]. Therefore, the head groups of both cholesterol and neighboring phospholipids interact via hydrogen bonds, allowing the steroid rings to interact with the top carbons of the hydrocarbon chains. This leads to a decrease in the fluidity of the membrane. The presence of cholesterol in the membrane inhibits the latter's transition to the crystalline state by preventing the hydrocarbon chains from coming together and crystallizing [10].



FIGURE 1.4: The asymmetric distribution of membrane lipid in plasma membranes. Original illustration from [1].

Among the membrane of cells and organelles we find a very vast variety of lipids (see Figure 1.1 and the second illustration of Figure 1.2). There is also often an asymmetry between the composition of the two monolayers that constitute the bilayer membrane. This asymmetry is depicted in Figure 1.4, where we have the different lipid distribution along the inner and outer layer of the membrane bilayer.

1.2.3 Surfactants: constituents for model membranes

To facilitate our study, we worked extensively with bilayers formed by one-chain surfactants, which yield more fluid (and thus easier to align) lamellar phases than lipids. Like a phospholipid, a surfactant is a molecule that has both hydrophilic (head) and hydrophobic (tail) groups. Because of its double nature, it is soluble in both organic solvents and water. Furthermore, it can adsorb at liquid-liquid or liquid-air interfaces, and thus can change their properties, most importantly their surface tension.

Surfactants can be classified according to the charge of their hydrophilic group. This charge is positive in cationic surfactants (e.g. CTAB), negative in anionic surfactants (e.g. SDS), or null in nonionic surfactants (e.g. $C_n EO_m$). There are also surfactants with dual charge, called zwitterionic (e.g. DDAO).

1.2.4 Membrane proteins

Another constituent of biological membranes are proteins. By definition, a protein is a polymer constituted of natural amino acids linked via a "peptide bond" (-CO-NH-), which is an amide bond in chemical terminology [11]. These macromolecules represent



FIGURE 1.5: The three categories of molecular-level organization needed to achieve efficient and diverse membrane protein functions. *Figure reprinted from* [14].

around 50 to 70% of the cell membrane mass. The sequence of amino acid residues in a protein is defined by the sequence of a gene, which is encoded in the genetic code and each protein has its own unique amino acid sequence forming its primary structure [12]. Biochemists have identified four distinct aspects of a protein's structures, though most fold into unique 3-dimensional structures [13]. When some sections in the primary sequence fold and form intra-molecular hydrogen bonds between the CO and NH part of the amide group they thus engender the secondary structure of the protein. Two types of secondary structures exist: the α -helix and the β -strand [13].

These structures are highly sensitive to their environment, which is obviously very different for lipid-embedded proteins and for water-soluble proteins. Therefore, to achieve efficient and diverse membrane protein functions three categories of molecular organization must be exploited [14]: structure, molecular dynamics, and environmental constraints as seen in Figure 1.5. Depending on the nature of their structure and the type of their interaction with the membrane, membrane proteins are classified as peripheral or integral [11] (see Figure 1.6). Peripheral proteins are associated to the surface of the lipid bilayer, without passing through it, via covalent bonds with the lipids of the external membrane layer, or with weak bonds such as electrostatic or van der Waals interactions with the lipid head-groups or other membrane proteins [15].

Integral proteins span the membrane. They are more likely amphiphilic molecules, with both hydrophilic and hydrophobic regions that can cross the membrane one or multiple times, as seen in Figure 1.6. The hydrophobic regions are mainly formed by amino acids with hydrophobic lateral chains (Leucine, Valine, etc) folded as α -helices and β barrels. The hydrophobic residues in each secondary structure point outward, facing the lipids, and the hydrophilic residues point inward, facing the inside of the structure. The outcome can be seen in Figure 1.6.



FIGURE 1.6: (A) Different structures and functions of membrane proteins. (B) Membrane proteins can be integral or peripheral. Integral membrane proteins come in two flavors: α -helical bundles and β -barrels. *Image reprinted from* [15]

1.2.4.1 Gramicidin

Gramicidin is a peptide with antibiotic activity. It is naturally produced by the soil bacterium *Bacillus brevis* and was discovered in 1939 by René Dubos (hence the name "Gramicidin D") [16]. Gramicidin D was one of the first commercially produced antibiotics, and the very first one to be clinically used, making a significant impact on battlefield medicine during the Second World War [17]. In 1942, Soviet researchers isolated a compound with similar antibacterial properties and thus labeled it Gramicidin S (for Soviet), but its structure is different from that isolated by Dubos (it is actually a cyclic deca-peptide) and we will not consider it further.

It had to await till 1985 for the first well-resolved structure to be solved by solution ¹H-NMR spectroscopy [18]. For about 15 years, gramicidin was the only transmembrane channel with a known structure, and hence we find in literature many studies of this molecule.

Gramicidin D is the pharmacological molecule and consists of a mixture of mainly three pentadeca-peptides: gramicidin A, B and C. These are all naturally occurring **dimers** and differ only in the residue at position 11 with the following chemical formula [17]: HCO- \mathbf{X}_L -Gly-Ala_L-Leu_D-Ala_L-Val_D-Val_L-Val_D-Trp_L-Leu_D-Y_L-Leu_D-Trp_L-Leu_D-Trp_L-NHCH₂CH₂OH where Y is Trp for gramicidin A, Phe for gramicidin B and Tyr for gramicidin C. Further, X can be Val or Ile for the three analogs. The L and D subscripts indicate left-handed and right-handed enantiomers of the amino acids. This alternating L- and D- residue structure leads to the formation of a $\beta^{6.3}$ -helix with 2.5 turns per monomer.

The natural function of the molecule (in its native environment) is not completely known,

but it has been demonstrated that gramicidin A is active primarily against Gram-positive bacteria other than the *Bacilli*, as well as select Gram-negative species [19]. It forms a trans-membrane ion transfer pore in the membrane of the bacteria, thus increasing its permeability and thereby destroying the ion gradients between the cytoplasm and the extracellular environment and finally killing the bacterium. [20-22]

The high-resolution structure of activated gramicidin was deduced by solid-state NMR [23] and then refined by molecular dynamics simulations [24]. In lipid membranes, two gramicidin monomers, one on each side of the bilayer, associate via the N-terminus to form a dimer which is stabilized by six intermolecular hydrogen bonds [25]. The dimer has a 4-Å-wide cylindrical pore hosting a single-file chain of water molecules [26] (see Figure 1.7a). As seen in Figure 1.7b, the formation of the gramicidin channel can be



FIGURE 1.7: (A) Configuration of the gramicidin channel occupied by two Na⁺ ions (represented in purple spheres at each terminal) used in the MD/FES calculations. The water molecules are represented in white and red: as spheres inside the pores and as rods outside. (B) Gramicidin channel (a) Gramicidin channel formation by trans-bilayer dimerization of two subunits, one from each bilayer leaflet. The channel formation is associated with a local bilayer deformation (b) Side and end views of a bilayer-spanning gramicidin channel, in which the carbon atoms of the two subunits are indicated in yellow and green, respectively. Image (A) is reprinted from Ref. [26] and image (B) is reprinted from Ref. [27].

associated with a local bilayer perturbation due to hydrophobic mismatch (difference in length between the hydrophobic length of the channel and that of the bilayer). Conductivity measurements have detected the typical formation and dissociation times of the channel (which are of the order of 100 ms) and found that they are directly influenced by the membrane properties [28].

We can conclude from this section that gramicidin is one of the favorite molecules for

biochemists and biophysicist due to its simple structure, easy production and selectivity. It is a convenient experimental model for membrane proteins, extensively used to gain insight into their physical and biological properties. In our case, we will use it to probe the membrane-mediated interactions in surfactant bilayers.

1.2.5 Hybrid nanoparticles: models for membrane inclusions

Nowadays, the term "nano" is very fashionable: it is often used to attract attention and suggest novelty and innovation, whether as a means to obtain funding, or even as a cool way to sell cosmetic products or detergents etc.

Despite this overuse, nano-objects play an extremely important role in current science and technology, especially due to their unique physical properties, highly influenced by the size and shape of the particles. Their synthesis, characterization and function constitue a wide and active multidisciplinary domain of research at the junction of physics, chemistry and material sciences. Nano-objects are often used as building blocks for self-assembly methods in the fabrication of new materials.

A hybrid nanocomposite is a material that consists of both organic and inorganic components. The organic-inorganic association is a relatively novel way to create new systems, where the two components are brought together to form a material that combines their properties. These hybrid materials can be used for fundamental studies but can also find many practical applications.

We will be concerned with materials obtained by dispersing solid nanoparticles in a "soft" continuous matrix of amphiphilic molecules. One should carefully choose the components to avoid any modification of the nanoparticles' properties.

The aim of our study is to prepare lyotropic L_{α} phase doped with a significant amount of (hydrophobic and charge neutral) hybrid nanoparticles, metallo-oxo-clusters, and to use these particles as probes of the membrane-protein interaction. A doped lamellar phase is an example of a hybrid liquid crystalline matrix, where surfactant bilayers are the organic component and the metallo-oxo-clusters are the inorganic one. In such a system, one should naturally consider the influence of confinement in the host phase on the inclusions in terms of the effect of the elastic and anisotropic medium on the inter-particle potential, but also the potential changes induced by the particles in the structure of the lyotropic host.

The interest of our approach resides in:

- Our use of dilute lamellar phases (formed by a non-ionic or a zwitterionic surfactant) and doped with inorganic metallo-oxo-clusters. The resulting phase is very fluid, and hence can be easily aligned.
- The nanoparticles being identical in shape and monodisperse (so that the scattering intensity can be written as the product of a form factor, only depending on the internal constitution of the particles, and a structure factor, which describes the interaction between particles

$$I(q) = S(q) \cdot |F(q)|^2$$

- The nanoparticles having sizes comparable to the lamellar period of the mesophases (approximately 1 nm), ensuring their intimate mixing.
- The high X-ray contrast of the metallic-oxo-clusters (due to their metallic core) which is a major advantage with respect to biological inclusions (such as gramicidin channels).

1.3 The physics of bilayers membranes

Under normal biological conditions, lipids and surfactants self-assemble into monolayers. In water, a second monolayer attaches to the bottom of the first one, with the head groups of each monolayer exposed to water and forming a bilayer. The stacking of parallel bilayers gives a lyotropic smectic phase. Many parameters influence the morphology of these phases. One should take in consideration the concentration of the amphiphilic molecules and their geometrical shape. A packing parameter P, also called the shape factor, determines the topology of the self-assembled structure [29], as can be seen in Table 1.1 and illustrated in Figure 1.8. P is defined as the ratio between the volume of the hydrophobic portion (v) and the product of the area of the polar head (a_0) by the length of the molecule (l_c) : $P = v/a_0 l_c$. In Figure 1.9 we show in detail the partition of a lipid into a unit cell [30]. We attribute an average cross-sectional area per lipid $\langle A \rangle$, perpendicular to the smectic phase normal. Then we separate the interlamellar repeat spacing (D) into three parts: the water spacing (D_W) , the length of lipid head groups (D_H) and the average length of lipid chains (D_C) which is the hydrocarbon thickness. The product of the area per lipid by the associated length gives a measurement of the volume occupied by each partition.

Packing parameter (v/a_0l_c)	Formed structure
P < 1/3	Spherical micelles
1/3 < P < 1/2	Cylindrical (rod like micelles)
1/2 < P < 1	Bilayers (if a_0 is small and hydrocarbon chains are bulky)
P > 1	Inverted micelles

TABLE 1.1: Packing parameters and possible structures [31].



FIGURE 1.8: Simple examples of supramolecular structures formed by amphiphilic molecules in water. (a): Amphiphilic molecules that have an effective "conical shape", with a hydrophilic head group wider than their hydrophobic chain(s), form micelles. This is typically the case for molecules with a single hydrophobic chain, such as fatty acids, but also some surfactants and detergents. (b): Amphiphilic molecules that have an effective "cylindrical shape", with a hydrophilic head group roughly as wide as their hydrophobic chain(s), form bilayers. This is typically the case for phospholipids. (c): Bilayers can spontaneously close to form vesicles. Original illustrations from Encyclopedia Britannica, adapted and modified.



FIGURE 1.9: General partition within a half unit-cell of a lipid bilayer with associated water. *Image taken from [30].*

1.3.1 Softness

One might think that the lipid or surfactant chains are rigid, but this is not always the case. At room temperature, lipid or surfactant membranes are soft objects, highly sensitive to their environment, and especially to thermal fluctuations. In the liquidcrystalline state (at physiological conditions), the lipid or surfactant hydrophobic chains are soft and can be described as moving like the legs of dancers, albeit more closely to "break dancers rather than ballet dancers" as vividly put by Kinnun [30]. Indeed, this flexibility is due to the length of the hydrophobic chain in which the C-C bonds rotate about their axis, yielding a large space of possible conformations for the chain [5].

1.3.2 Fluidity

In 1970, Frye and Edidin [32] showed that molecules could diffuse within a membrane after they succeeded in fusing together a mouse cell and a human cell tagged with fluorescent antibodies. According to the image of Singer et al. [33] in 1972, the phospholipid bilayer can be considered as a two-dimensional liquid incorporating globular assemblies of proteins and glycoproteins (model showed in the second image of Figure 1.2). Thus, the proteins and lipid molecules are able to diffuse freely in the matrix. The lateral diffusion is very rapid, so the phospholipid or surfactant molecule is able to move from one end of the monolayer to the opposite end within few seconds. Additionally, these molecules can rotate rapidly along their axes. On the other hand, the mobility of membrane proteins is very different from that of phospholipids and that is due to their large size and multiple polar regions.

Apart from lateral diffusion, lipids and surfactants undergo a transfer movement from one side of the monolayer to the opposite side. This process is knowns as "flip-flop" or transverse diffusion and is very slow and energetically unfavorable because the hydrophilic head group must pass through the hydrophobic core of the bilayer. No flip-flop has yet been observed for the proteins. In fact, the fluidity of membranes is deeply related to the conformational degrees of freedom of lipids excited at ambient temperature. More precisely, three factors affect directly the bilayer fluidity: the temperature, the lipid composition and the cholesterol content.

Below normal physiological temperatures, the lipids or surfactants can enter the gel state, in which the tails are no longer moving but are rigidly packed and form a smectic-C liquid crystal [34], see Figure 1.10. We define the transition temperature as the temperature for which the bilayers melts and cross from an ordered phase (gel phase) to a disordered phase (liquid crystalline phase).

The presence of cholesterol also affects the bilayer fluidity. As mentioned above (see $\S 1.2.2$), the cholesterol inserts itself between the phospholipids molecules, with its steroid rings parallel to the tails and its hydroxyl group oriented toward the aqueous phase, leading to a more rigid and stiff system and to lower membrane fluidity.



FIGURE 1.10: Sketches of a lipid bilayer membrane composed of saturated phospholipids. (a): Liquid phase with dancers legs. (b): Gel phase. *Illustration reproduced* from Ref. [1].

1.3.3 Elasticity

The membrane can undergo several types of deformations (Figure 1.11): it can bend or stretch (in the latter case, its local surface area changes). The energy cost of these deformations is quantified by the associated moduli. Being fluid, it does not resist shear deformation, so there is no shear modulus. Membranes have no intrinsic surface tension and in consequence the contributions of the deformations can be described macroscopically by an elastic energy. The continuum elastic model built upon these ingredients was proposed by Canham in 1970 [35] and Helfrich in 1973 [36] and is described by the effective Hamiltonian H of the membrane as:

$$H = \int_{A} dA \left[\frac{\kappa}{2} (c - c_0)^2 + \bar{\kappa} c_1 c_2 \right]$$
(1.1)

Where A is the total area of the membrane, κ is the bending rigidity of the membrane while $\bar{\kappa}$ is its Gaussian bending rigidity, c_0 is called the spontaneous curvature of the membrane, c the tensor describing the local curvature and c_1 and c_2 the eigenvalues of this tensor.

Many experiments and models have estimated the order of magnitudes of the elastic moduli for lipid bilayers [37–41], with the following results:

- The bending modulus $\kappa \simeq 10^{-19} \,\mathrm{J}$
- The stretching modulus $K_a \simeq 0.1 \,\mathrm{N/m}$
- The effective tension $\sigma \in [10^{-8}, 10^{-3}] \,\mathrm{N/m}$

The Canham-Helfrich model has been widely used to describe large-scale membrane deformation (over micron distances).



FIGURE 1.11: Schematic representation of the basic three membrane deformations: a) stretching, b) shearing, c) bending. *Image taken and modified from [42]*.

1.4 Membrane-mediated interaction between inclusions

The elucidation of membrane-mediated interaction between inclusions has been one of the main topics in biophysics research. In 1972, when Singer *et al.* [33] proposed the fluid mosaic model, proteins were described as free to diffuse in a passive lipid matrix. Shortly afterwards [43], it was recognized that membranes are not just neutral hosts but can influence the protein organization in the plane of the membrane and thus can alter many features of their biological activity. Much effort has been concentrated to probe the eventual membrane properties that mainly affect the integral membrane proteins activities and more particularly researchers have examined the effect of cholesterol [44–47] on membranes and inclusions and found that the presence of cholesterol, increases the rigidity of the membrane and in some cases decreases the activity of membrane proteins [17].

1.4.1 Lipid-protein interaction

In the environment of the membrane, lipid chains coexist with and surround the transmembrane regions of integral proteins, which are mainly hydrophobic and generally helical. This organization preserves the hydrophobic character of the inner region of membranes [48]. To satisfy this constraint, the thickness of the hydrophobic domain of the membrane must match that of the proteins within. Otherwise, exposing hydrophobic surfaces to the aqueous phase would be very costly in free energy. Any difference between these two hydrophobic lengths must then be accommodated by a distortion at the lipidprotein (or surfactant-protein) interface.

Since the lipid or surfactant chains are flexible (see \S 1.3) and proteins are much more rigid due to the stable and well-defined backbone structure, it is the bilayer that will alter its thickness to match the protein in case of a hydrophobic mismatch, as can be



seen in Figure 1.12. This local deformation of the membrane thickness occurs at the nanometer scale and yields a membrane-mediated interaction between two such proteins.

FIGURE 1.12: Illustration of hydrophobic matching: inclusions (membrane proteins) in a lipid bilayer with their corresponding hydrophobic thicknesses coinciding in (a); (b)-(d) the hydrophobic thicknesses of bilayer and inclusions are different: the overlap of the deformations around the inclusions leads to a lipid-mediated protein-protein interaction. *Image taken from [49]*.

However, the molecular details of the protein-lipid interactions and dynamics are poorly understood, although evidence emerged that integral membrane proteins are highly influenced by the lipid molecules surrounding them. For example, it was observed in the *E. coli* inner membrane that lipids (as phosphatidylglycerol) are involved in proton translocation [50]. Furthermore, using FTIR spectroscopy, Hielscher *et al.* found that delipidation leads to the loss of catalytic activity and alteration of the redox properties in the cytochrome bc_1 complex of the respiratory chain. On the other hand, experiments show that intrinsic membrane proteins also alter the properties of nearby phospholipids in the membrane. For instance, experiments using differential scanning calorimetry showed that, even at low concentration, the presence of proteins decreases the phase transitions of the membrane [51] and even the shape of the embedded proteins can induce a phase transition [52].

Based on the same concepts of probing the effect of intrinsic proteins on membranes, other studies showed evidence of an immobilized lipid layer around the intrinsic hydrophobic protein indicated as "boundary lipid" and a second region of fluid bilayer [53]. This proves that the motion of the lipid molecules are affected by the presence of a protein. This can be easily probed using the Magnetic resonance experiments. A wealth of measurements have already been done [54–56] indicating order or disorder in the acyl chains which decay with distance from protein. This topic has been studied in the second part of this thesis using NMR and WAXS.

1.4.2 Membrane-mediated interactions: theoretical and numerical approach

Many types of membrane-mediated interactions exist. They are rather directly correlated to the membrane degrees of freedom such as curvature, thickness, composition, tilt, charge etc. than to some specific chemical bonds. This simplifies the way to describe these interactions and one can use a simple model where the membrane is considered as a self-assembled system. Note that a given inclusion can couple simultaneously to several degrees of freedom. I will briefly review the effects that result from the coupling of inclusions with membrane thickness which yield short-range interactions. Long-range interactions (with a range larger than the characteristic size of the inclusions) result from constraints imposed by inclusions on the membrane curvature and shape. They can be described starting from the coarse-grained Helfrich model [36] but is outside of the scope of my thesis.

Many theoretical works have attempted to describe the free energy cost of hydrophobic matching [57–60]. The first most complete functional model was proposed by Huang [61, 62]. He was based on de Gennes' work on liquid crystal to create a theory that could be applied to small deformations occurring in a solvent free lipid bilayer and defined the Hamiltonian per unit area of the membrane.

Consider a planar bilayer coupled through hydrophobic interactions to an integral protein, the effective Hamiltonian H of the membrane can be written as follow:

$$H = \int dx dy \left[\frac{K_a}{2d_0^2} u^2 + \frac{\gamma}{4} (\nabla u)^2 + \frac{\kappa}{8} (\nabla^2 u)^2 \right]$$
(1.2)

Where u is the thickness excess of the membrane relative to its equilibrium thickness d_0 (see Figure 1.13), K_a is the stretching modulus of the membrane, d_0 its equilibrium thickness, γ its "surface tension", and κ an elastic constant associated to splay. Finally, x and y denote Cartesian coordinates on the mid-plane of the membrane.



FIGURE 1.13: Cut of a bilayer membrane (yellow) containing a protein with a hydrophobic mismatch, represented as a square (orange). The equilibrium thickness of the bilayer is d_0 , while the actual thickness is denoted by $d_0 + u$. Image taken and modified from [63]

More theoretical work in this area emerged and examined in depth equation 1.2 [64, 65]

and spent a considerable mathematical effort in theoretically analyzing the various terms of the Huang's model Hamiltonian equation [66–68]. These efforts are either continuumelasticity theories or more detailed models dealing with the lipid bilayer organization at the molecular level [69–72]. Recently many advances in numerical simulations have emerged combining many degrees of freedom [63, 73–75] but yet there isn't a complete description of the membrane deformation and mediated interaction due to the lack of experimental data.

1.4.3 Membrane-mediated interactions: experimental approach

The fluid nature of the membrane makes that any attempt to study the distribution of embedded inclusions would essentially be a statistical-mechanical one. Two-dimensional inclusion-inclusion distributions are modeled by the pair correlation function q(r) which is the probability of finding a particle at a distance r from another reference particle at the origin. It has proven very difficult to directly measure the interactions between membrane inclusions. However several attempts have determined a pair wise interaction potential, v(r), based on a measured or theoretical q(r). In particular we cite the case of Pearson et al. [59] who measured "by hand" the q(r) from the distributions of rhodopsins in diacyl-PC membranes using the freeze-fracture electron microscopy (FFEM) and then via simple liquid theory they determined a form for v(r). However it is difficult to consider that the distribution of proteins in frozen membranes corresponds well to that in the fluid state. Very recently, Casuso et al. [76] attempted to measure the in-plane potential interaction of membrane proteins using also a microscopic technique but this time a new atomic force microscopy method, the High-Speed atomic force microscopy (HS-AFM). They were able to calculate an attractive potential of several k_BT with a range of ~ 103 Å.

However, to date, the most convenient way to probe the nanoscale and measure membranemediated interactions is the small angle scattering techniques. The reason behind that is because these strategies are unoffensive and don't harm the samples and most essentially it is because the order of magnitude of both the wavelength and the system to probe is the same, the nanometer scale. In the following I will present a brief review about the most relevant studies by small angle scattering performed in that purpose.

1.4.3.1 Small-angle scattering

The first (to my knowledge) to have used X-ray scattering techniques in the view to probe the peptide-protein interaction was Blasie *et al.* in 1969 [77]. They measured the rhodopsin antibodies' radial distribution function g(r) in frog retinal discs. Around

twenty years after that, He *et al.* [78] followed the path and developed the techniques of membrane in-plane scattering with x-ray and neutron from oriented samples [48, 79–81]. The scattering curves provide information about the size and shapes of the scattered objects, as well as a direct measurement of their in-plane correlations.

However, in these studies mentioned earlier the interaction potential wasn't quantitatively measured and only one or two inclusion concentrations were investigated for each system.

Building upon this work, recent studies by Constantin *et al.* emerged covering features that haven't been approached before using variable density of inclusions and different types of: inclusions, lipids and surfactants.

My thesis is a continuity to these strategies.

In 2007, Constantin *et al.* measured the interaction potential of alamethicin pores in highly aligned dimyristoylphosphatidylcholine (DMPC) bilayers [82]. This is the first quantitative measurement of an interaction potential between pores inside a lipid bilayer. (see Figure 1.14). They used classical techniques of liquid state theories and described



FIGURE 1.14: Structure of the alamethicin pore : side cut-out view (a) and top view (b). Interaction potential between the pores (c). Illustration of the pore fluid within the plane of the membrane (d). In red, the alamethicin monomers; in blue, the central water channel and in grey the range of the interaction. Image a and b taken from ref. [83]. Image c and d taken from ref. [82]

their interaction by a hard-core model with an additional repulsive contribution with a range of 3nm and a contact value of 2.4 k_BT . This analysis was made possible due to simultaneous data treatment on series of samples at different peptide to lipid P/Lconcentrations.

Similarly, in 2009, Constantin studied the interaction between gramicidin pores in DLPC (dilauroyl-phosphatidylcholine) bilayers and also in the nonionic surfactant pentaethylene glycol monododecyl ether ($C_{12}E_5$) [84]. He also found similar results in both cases described by a hard-core model with a repulsive exponential lipid-mediated interaction, with a decay length of 2.5Å and an amplitude that decreases with the pore density. In dilute systems he measured a contact value of about $30k_BT$ (see Figure 1.15).

All of these results are in qualitative agreement with recent theoretical models ([72, 85]) On the other hand and yet still with the same objectives, Constantin *et al.* inserted inor-



FIGURE 1.15: A) Atomic configuration of the pore formed by gramicidin (balls) in a completely hydrated DMPC bilayer (lipid chains shown as green sticks and water molecules in red). B)The best results for the interaction potential V(r) within each model class. The respective χ^2 values are also indicated. Image (A) is reprinted from Ref. [86] and image (B) is reprinted from Ref. [84]

ganic nanoparticles of butyl-tin oxo clusters labeled BuSn12 (presented in section 2.1.1.1) within oriented multilayers of a zwitterionic surfactant the dimethyldodecylamine-N-oxide (DDAO) and measured the membrane-mediated interaction by SAXS [87]. Again they found an additional repulsive interaction viewed as a perturbation with respect to the hard core model and was taken into account via the random phase approximation approach. They measured a contact value of about 4 k_BT and a range of 14Å.

Further on, in order to develop a full analysis of the interactions induced by mem-



FIGURE 1.16: Interaction potential U(r) of BuSn12 particles within DDAO bilayers. The lower curve is the interaction potential of the particles in ethanol. The solid vertical line marks the hard core interaction with radius 4.5 Å .*Reprinted from Figure* 3 of reference [87]

branes between included nano-molecules, Constantin made two main improvements in 2010 [88] as to his study of 2008: he determined the complete structure factor $S(q_r, q_z)$. And secondly, he used a more sophisticated analytical model (based on the numerical solution of the Ornstein-Zernicke equation coupled to the Percus-Yevick closure) with a view to describe the structure factor in terms of an interaction potential. Similarly to the above results, he measures a repulsive interaction with a contact value of 2.2 k_BT and a range of about 10Å.

In 2011, another type of inclusion was used. This time 2 nm gold nanoparticles capped either with hexanethiol or dodecanethiol were confined within classical swollen lyotropic phases of SDS, pentanol, dodecane, water [89]. The results show that the confinement within the lamellar phase induces a new repulsive interaction whose range varies with the lamellar period and that dominates van der Waals attraction seen in bulk suspension.

1.5 Brief outline of this thesis: objectives and novelty

My work is part of the MEMINT project (funded by the ANR), aiming to probe the membrane-mediated interaction between embedded inclusions (organic and hybrid) in order to better elucidate the biophysics of the membrane and better understand the activity of important biologial molecules such as native membrane proteins and antimicrobial peptides. The activity of the latter being directly related to the environment in which they stand as the composition of the membrane from the point of view of the hydrophobic thickness, polarity, cholesterol presence, elasticity etc. rather than to some specific chemical recognition.

To do so, in the first part of my thesis (in Chapters 3 and 4) I use extensively Xray scattering from in-house setups (for the WAXS measurements) on powder samples, and synchrotron facilities (for the SAXS data) on highly aligned and oriented phases. I perform systematic studies, varying the following relevant parameters: membrane thickness, cholesterol content, temperature, degree of hydration, surfactant type, inclusions types and the inclusion density.

From the dependence of the scattered signal on the in-plane scattering vector, I determine the structure factor of the two-dimensional fluid composed of the inclusions in the surfactant multilayers, which is then analyzed by standard liquid state theory (based on the numerical solution of the Ornstein-Zernicke equation coupled to the Percus-Yevick closure) to yield the interaction potential between inclusions within the membrane. This is explained in detail in Chapter 3. Another important question is whether the inclusions in neighboring layers interact with each other. To answer it, I needed to gain access to the full structure factor of the system $S(q_r, q_z)$ using a particular experimental configuration, explained in detail in Chapter 4.

Then in the second part of my thesis, I combine wide angle X-ray scattering (WAXS) (Chapter 6) and nuclear magnetic resonance (NMR) (Chapter 5) techniques to determine respectively the variation of the positional and the orientational order parameter of the membrane lipids and surfactants after insertion of various inclusion concentrations.

Some of the problems to be addressed have already been considered before. It is therefore necessary to spell out the innovative aspects of the current research project, and the way they helped us in accomplishing the objectives.

- Performing several measurements along a **dilution line** (varying the inclusions density) and fitting all the data with the same parameters provides reliable **thermodynamic** information.
- We implemented **more elaborate analytical and numerical models** that can be calculated very easily and are amenable to nonlinear least-squares fitting. This is essential, in particular when many curves must be treated simultaneously.
- The use of **inorganic particles** as membrane probes has a number of advantages with respect to proteins. In particular, the increase in scattering contrast is crucial for liquid systems such as those under investigation.
- The use of **aligned samples** is essential for distinguishing between the organization of the particles in the plane and across the bilayers and for separating their signal from the very strong contribution of the host mesophase.
- Combining WAXS and NMR to determine respectively the **positional and the orientational order parameter** of the lipids and surfactants at various inclusions concentration.

Chapter 2

Materials and methods

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2.1 Materials and sample compositions

In this section I will present briefly the different constituents we used to create our systems in terms of membrane constituents (surfactants and lipids and cholesterol) and in terms of inclusions. The most relevant parameter used for the results and data treatment are summarized in Table 2.1.

2.1.1 Inclusions

We have used as inclusions two types of nano-objects: hybrid (organic-inorganic) nanoparticles and antimicrobial peptides. As hybrid nanoparticles, we have worked with two different types of clusters: Tin-Oxo clusters and Titanium-Oxo clusters. They consist of a perfectly defined inorganic core with organic ligands on their surface and have a nanometric size (1 - 2 nm).

2.1.1.1 Organometallic-oxo clusters

Tin-oxo cluster Organometallic oxo clusters correspond to clusters with metallic core and decorated with organic molecules such as hydrocarbon chains. In particular we use organotin oxo-clusters with the structure $\{(RSn)_{12}O_{14}(OH)_6\}^{2+}$. The synthesis was done by our collaborator François Ribot at the Laboratoire de Chimie de la Matière Condensée de Paris and the details are given in Ref. [90]. Two different tin clusters were synthesized for this work. The first one was synthesized from a reaction between butyltin hydroxide oxide with ρ -toluenesulfonic acid yielding the following cluster $\{(BuSn)_{12}O_{14}(OH)_6\}(O_3SC_6H_4CH_3)_2$ and another cluster was also used with the following complete formula $\{(BuSn)_{12}O_{14}(OH)_6\}(O_3SCF_3)_2$. The difference between the two Tin-Oxo clusters is the "cation-anion" chains and their arrangement into planes (counteranions bridging macrocations). They both consist of a tin oxide core decorated with butyl chains. In the rest of the manuscript these nanoparticles will be labeled BuSn.

Titanium-oxo clusters On the other hand the Titanium-Oxo clusters were synthesized by Laurence Rozes at the Laboratoire de Chimie de la Matière Condensée de Paris with the formula $[Ti_8O_8(OOCC_6H_5)_{16}](CH_3CN)_2H_2O$. These well-defined nanobuilding units (NBUs) are obtained upon reactions of titanium alkoxides $[Ti(OiPr)_4]$ with a large excess of benzoic acid (10:1) in acetonitrile under non-hydrolytic conditions [91]. Yellow crystalline needles are obtained at 100 °C in a closed vessel after 15 hours.

We have inserted these nanoparticles using the same protocols as for the BuSn and

the gramicidin peptides and performed X-ray measurements. We had big difficulties dispersing the particles homogeneously within the membranes and aligning them and hence the results obtained weren't viable. We will not show these results or mention these systems.

These hybrid materials have many advantages but the reasons we use them is most importantly because of their identical shape and mono-dispersity and because of their high scattering contrast due to the presence of metal atoms.

2.1.1.2 Gramicidin peptides

The gramicidin channel is a transmembrane peptide with a long history of computational and experimental study [17, 48, 56, 78, 92, 93], and serves as an appropriately simple model for the development of experimental and analytical techniques to quantitatively characterize its activity. As described in details in §6 1.2.4.1, the gramicidin is a mixture of mainly three pentadecapeptides : gramicidin A, B and C. It is formed of two monomers with a conformation of $\beta^{6.3}$ that in membranes, come together and form a channel with an inner radius of 4Å[17].

The antimicrobial peptides inclusions were bought from Sigma Aldrich.

Those inclusions were doped in membranes of various constituents. We have used lipid membranes as well as surfactant multilayers.

2.1.2 Membrane constituents

2.1.2.1 Surfactants

Dimethyldodecylamine-N-oxide (DDAO) a single-chain zwitterionic surfactant. It has only one polar atom that is able to interact with water. Still, this surfactant shows very hydrophilic properties: in mixtures with water, it self-assembles and forms normal liquid crystalline phases and micelles [94].

DDAO was purchased from Sigma-Aldrich. It was first dried in vacuum for 20 hours to remove any residual water and then dissolved in isopropanol. Its phase diagram with water has been studied and shows a lamellar $L\alpha$ phase domain for a water wt% between 10 - 30 % (Figure 2.1c).

Tetra (ethylene oxide) mono dodecyl ether $C_{12}EO_4$ is a non-ionic surfactant. It belongs to the polyoxyethylene type.

 $C_{12}EO_4$ was bought from Nikko Chemicals Ltd (Japan). The phase diagram of pure $C_{12}EO_4$ in aqueous solution has been published in [95]. At room temperature, a lamellar $L\alpha$ phase domain extends from roughly 40% to 80% w% surfactant. In my experiments, I used a concentration of 50% w% surfactant.

We have also used the Brij30 surfactant which consists mainly of $C_{12}EO_4$ with other homologues of the same C_mEO_n series . This surfactant is way cheaper and was bought from Sigma Aldrich. The reason we used both surfactants was because the BuSn particles did not disperse well in $C_{12}EO_4$ so we used the Brij30 and it actually dispersed really good and we had a very homogeneous phase. In the fallowing we refer to the $C_{12}EO_4$ as $C_{12}E_4$.

Area per molecule value for $C_{12}E_4$

Many have measured the area per molecule for $C_{12}E_4$ in different conditions. I will briefly mention some of them and justify the value I chose.

In Kurtisovski et al [96] SAXS measurements have been performed on solutions containing different water diluted lamellar phases. Each membrane is composed of a $C_i E_j$ bilayer. For each $C_i E_j$ used, they measured the area per surfactant polar head a and few other properties. For $C_{12}E_4$ the found a value of 41.1 Å². These conditions are the closest to my system (SAXS, lamellar phases).

Schmiedel et al [97] measured by SANS the surface area per amphiphile for unilamellar vesicles of $C_{12}E_4/POPC$. They found a value of 52\AA^2 for pure $C_{12}E_4$ water unilamellar vesicles and this value increases within the mixed membranes of $C_{12}E_4/POPC$.

Just like in so many other references [98] [99], the area per molecule have been measured at cmc by different techniques. In Persson paper [99], the authors investigate the surface tension isotherms for six surfactants chemically close. The surface tensions of the surfactant solutions were measured with a Kruss K12 tensiometer employing the Wilhelmy plate method. They measured the area/molecule at the cmc and obtained a 42.4 Å^2 . In [98], also using the wilhemly plate technique, at cmc the authors found 45.7 \AA^2 at 25° and 48.7 \AA^2 at 40°.

I choose 41.1Å^2 as a value for the area per molecule of $C_{12}E_4$ for my systems and that is because this surface area has been obtained in similar conditions to my experiments, using SAXS and lamellar phases of $C_{12}E_4$ and not mentioning also that the experiments are recent, done in 2007.

2.1.2.2 Lipids

I used the lipid layers only in the second part of the thesis to study the orientational order parameter of the hydrocarbon chains in presence of gramicidin inclusions. Two types of phospholipids with choline head-group were used: the 1,2-dilauroyl-sn-glycero-3-phosphatidylcholine (DLPC) and the 1,2-dimyristoyl-sn-glycero-3-phosphatidylcholine (DMPC). They are both saturated lipids with a tail of 12 carbons and 14 carbons respectively. They were both purchased from Avanti Polar Lipids Inc. (Birmingham, AL, USA).

2.1.2.3 Cholesterol

The activity of integral membrane proteins in cell membranes is believed to be very sensitive to the cholesterol content. We tried to incorporate this constituent in all the systems we created in order to compare the interaction potential between inclusions in its presence and in its absence. We purchased cholesterol from Sigma Aldrich.

	MM ~(g/mol)	$ ho~({\rm g/cm^3})$	R_{HD} (Å)	A (Å ²)
BuSn	2777 [90]	1.93 [<mark>90</mark>]	4.5 [87]	
Gram	1882 [100]	~ 1	$9.5 \ [84]$	250 [101]
$C_{12}E_4$ / Brij30	362 *	$0.946 \ *$		41.1 [96]
DDAO	229.40 *	0.84 [<mark>94</mark>]		37.8 [87]
Cholesterol	386.65 *	1.067 *		39 [102]

TABLE 2.1: Membrane constituents parameters details. MM denotes the molar mass in g/mol, ρ denotes the mass density in g/cm³, R_{HD} denotes the effective hard disk radius parameter used in our data treatment in Å, and A denotes the surface area occupied by the inclusion or by the surfactant polar head. The * denotes that the values were taken from Sigma Aldrich.

2.2 Sample preparation

Each component of the system (nanoparticle, surfactant, cholesterol) is separately dissolved in an organic solvent (isopropyl alcohol as a main solvent for all the systems except for the Titanium TiO_8 system which are dissolved in dichloromethane). The mass fraction of each component in the final solution is easy to calculate as we know both the mass of nanoparticles or the surfactant added and the mass of the solvent added. it is given by

$$m\% = m_C/(m_C + m_S)$$

where m_C and m_S are respectively the mass of the membrane component whether the nanoparticles or the surfactants and the mass of the solvent added. From the above equation and having the density of each constituent the volume fraction is thus given by:

$$\phi_C = \frac{V_C}{V_C + V_S} = \frac{m_C/\rho_C}{m_C/\rho_C + m_S/\rho_S}$$

Then according to the molar Particles to Lipids ratio (P/L), volumes of stock solutions are mixed in 4 ml tubes and sealed with parafilm then mixed with a vortexer. Each tube was beforehand rinsed with ethanol and then with Millipore water multiple times and then dried in an oven. After mixing very well the tubes containing the corresponding volumes of nanoparticles and surfactant solutions, the samples are left to dry in vacuum for a few days till the complete evaporation of the solvent. Afterwards, we obtain a powder of mixed surfactant and inclusion that we hydrate at various amounts of water in a way to obtain a fluid lamellar L α phase according to the corresponding water phase diagram for each surfactant (Figure 2.1).

Once the mixture is homogeneous after vortexing and centrifuging, the lamellar phases are then drawn into flat borosilicate capillaries of 100 μ m thick and 2 mm wide (VitroCom Inc., Mt. Lks, NJ) by aspiration with a syringe or a pump (depending on the sample viscosity) and then the capillaries are flame-sealed. In order to distinguish between the organization of the particles in-plane and across the membrane, we need to align the samples in homeotropic anchoring (with the lamellae parallel to the flat faces of the capillary). This is the crucial part before the X-ray scattering experiments. Note that, we observed for samples at low inclusions concentrations, the texture is very viscous, especially when there is a cholesterol content and thus it makes it harder for the samples to be well aligned. Hence we observe in the scattered patterns the apparition of residual peaks due to the orientation defects and membrane deformations. These samples are sucked up in the capillaries using an air pump. Whereas, at higher inclusion concentrations, the samples tend to be less viscous and yield well aligned lamellar phases. For this alignment step we used a Mettler FP52 heating stage. The samples were heated up to the isotropic phase (the transition temperature varies according to the lipid or surfactant in use) and then cooled down to the lamellar phase at a rate of $1 \, ^{\circ}C/min$ (see Figure 2.3). This temperature treatment for orientation is applied only to the membranes with hybrid inclusions. The peptide inclusions can't support high temperatures so we add excess water and we leave them to orient by themselves with time.



FIGURE 2.1: (A) and (B) present the phase diagram of $C_{12}EO_4$ according to Mitchell [95] and Strey [103] respectively. L_{α} denotes the lamellar phase; L_1 and L_2 denote water-rich and surfactant-rich micellar phases respectively; L_3 denotes a sponge phase. (C) is the DDAO-Water phase diagram according to Kocherbitov [94]. Dry denotes anhydrous surfactant, MH stands for monohydrate, Lam, Cub, and Hex stand for the lamellar, cubic, and hexagonal liquid crystalline phases, respectively, and Iso denotes isotropic solution.

2.3 Inverstigation of the lamellar systems

The first step in the elaboration of a new system is to study its phase diagram. One can vary the surfactant concentration or the nanoparticle concentration or the water percentage. The purpose remains the same, we need to identify a domain where the samples are homogeneous, in the L α phase, and hopefully contain lots of nanoparticles.

Multiple series of samples were elaborated (Table 2.2). The inclusion density number in the plane of the membrane η varies from 0.21 Å⁻² to 2.3 Å⁻² corresponding to a mass fraction of nanoparticles in the hydrated bilayers varying from 5% Wt% to 35% Wt%.

Samples more concentrated in inclusions have been prepared but they are not mentioned here as they are all biphasic. Samples with a high surfactant mass fraction greater than 90% have not been prepared because they are too viscous to work with. All membranes containing Cholesterol were prepared respecting a molar ratio $\mu = \frac{n_{Chol}}{n_{DDAO}} = 0.5$ for BuSn DDAO Cholesterol system and $\mu = \frac{n_{Chol}}{n_{DDAO}} = 0.25$ for the Gramicidin DDAO Cholesterol system. All systems were hydrated to excess water except the DDAO membranes, these systems were hydrated at a 20% wt% of water. The Gramicidin DMPC and Gramicidin DLPC samples were left to orient alone with time because they require a high transition temperature and the Gramicidin is a peptide so it would denaturate at such a high temperature. The orientation could take a year in time. In my case the samples weren't oriented maybe because the capillaries weren't well sealed and the excess water used to dry.

Samples	P/L [mol/mol]		
BuSn DDAO	0.004, 0.008, 0.009, 0.011, 0.015		
	0.020, 0.024, 0.037, 0.040, 0.044		
BuSn DDAO Cholesterol	0.010, 0.012, 0.015, 0.018, 0.020, 0.021, 0.025		
BuSn $C_{12}E_4$	$0.009\ ,\ 0.02\ ,\ 0.037\ ,\ 0.06\ ,\ 0.095\ ,0.15$		
BuSn $C_{12}E_4$ Cholesterol	0.006, 0.013, 0.020, 0.026, 0.032		
BuSn Brij30	0.03,0.05,0.085		
BuSn Brij30 Cholesterol	0.005, 0.012, 0.017, 0.024		
TiO ₈ Brij30	0.05,0.1,0.15		
Gramicidin DDAO	0.029, 0.052, 0.112, 0.174		
Gramicidin DDAO Cholesterol	0.028, 0.042, 0.067, 0.082		
Gramicidin $C_{12}E_4$	0.015, 0.037, 0.054, 0.073, 0.099		
Gramicidin $C_{12}E_4$ Cholesterol	0.010, 0.02, 0.03, 0.04, 0.05		
Gramicidin DMPC	0.1,0.03,0.05		
Gramicidin DLPC	0.1,0.03,0.05		
BuSn DLPC Cholesterol	0.009, 0.018, 0.027, 0.034, 0.042		

TABLE 2.2: List of the samples prepared and used for the various experiments

The samples are viscous liquid or gels depending on the inclusions volume fractions and with those at high surfactant fraction being less liquid-like and sometimes really very viscous (> 90% wt % of surfactant). We observe that the addition of inclusions decreases the viscosity of the lamellar phase and increases the transparency. This could be due to the fact that defects in the lamellar structure both scatter light and reduce fluidity of the lamellar phase.

We represent in Figure 2.2 the Lamellar-to-isotropic transition temperatures for the hybrid system BuSn DDAO hydrated at a 20% wt% of H_2O in absence and presence of cholesterol (Figure 2.2a and 2.2b respectively). We observe that the lamellar-to-isotropic transition decreases as the amount of inclusions in the phase increases. This

depression of the clearing temperature upon doping with nanoparticles has been observed for other doped L_{α} membranes [104, 105].



FIGURE 2.2: Lamellar-to-isotropic transition temperatures for the hybrid system BuSn DDAO hydrated at a 20% wt% of H_2O in absence (A) and presence (B) of Cholesterol.

This indicates that the presence of inclusions stabilizes the lamellar phase. We observe the same depression of the clearing temperature with addition of cholesterol but the Lamellar-to-isotropic transition temperatures are higher comparing to the same concentration of BuSn without Cholesterol. For instance, in presence of Cholesterol, the transition temperature is 130°C for molar ratio P/L = 0.015 and 118°C for P/L =0.025 whereas the transition temperature for respectively both molar ratios is 125°C and 113°C in pure DDAO membrane. There is approximately a 5°C difference between the transition temperature for the same concentration of BuSn doped in DDAO layers in presence and absence of Cholesterol. This is a clear evidence that Cholesterol rigidifies the membranes.

2.3.1 Sample textures

In the lamellar phase, samples are transparent and birefringent (Figure 2.3 and 2.4). In polarized light microscopy, the textures are typical lamellar phase textures, presenting all the characteristic defects of an L α phase like focal conics and oily streaks separating regions of homeotropic alignment of the phase on the glass walls of the flat capillaries. Figure 2.3 shows microscopic images of the DDAO membranes doped with BuSn at a molar ratio P/L = 0.04 and hydrated at 20% Wt% water while temperature orientation in a Mettler FP52 heating stage. In these images the temperature was increased at a rate of 3°C / min from room temperature to 60°C and then at a speed of 1°C / min from 60°C to 90°C. The same steps were used for the cooling stage.

In Figure 2.3a we represent the texture seen through the polarized light microscope directly after preparing the capillary and at room temperature ($\approx 20^{\circ}C$). We see how

the birefringence and the textures change along with the temperature. In Figure 2.3b and at 65° C the textures start to "elongate" and slowly start fading away at 75° C (Figure 2.3c) and then at 85° C (Figure 2.3d) we have a coexistence of the isotropic melt (black) and Smectic A bâtonnets (bright). And then at the temperature anchoring transition (Figure 2.3e and Figure 2.3f) a uniform alignment of the membrane director along the capillary's wall direction is confirmed by the transmission of nearly zero light intensity. However at the edge of the capillary we can find bright regions (Figure 2.3e) due to an opposite director or some focal conics defects in the middle of the oriented areas (Figure 2.3f).

In Figure 2.4 I represent a glimpse of some sample textures I observed under polarized light microscopy. The samples have special textures that directly allow the recognition of the cholesterol presence in the sample. Figure 2.4a, 2.4b and 2.4c show a fingerprint texture under homeotropic anchoring conditions of Gramicidin/ $C_{12}E_4$ in (A) and (B) and BuSn/ $C_{12}E_4$ /cholesterol phase. Figure 2.4a is the same as Figure 2.4b both taken at a magnification of x5 but the latter is under normal light. We always observe sample textures at both direct and polarized light.. Figure 2.4d represents a fan-shaped texture of a BuSn/ $C_{12}E_4$ /cholesterol phase. The director basically lies in the plane of the substrate and the smectic layers are curved across the fans. Figure 2.4e represents a typical focal conic texture of a BuSn $C_{12}E_4$ membrane. Figure 2.4f shows oily streaks that are characteristic of lamellar phases in a BuSn DDAO sample. Figures 2.4g and 2.4h are a cholesterol fingerprint texture with so-called cholesterol droplets (in the bottom of figure 2.4h) and a pseudo-isotropic region with an homeotropic nematic director configuration (black).



FIGURE 2.3: Observations by polarized light microscopy of the textures of the same capillary (DDAO membranes doped with P/L=0.043 of BuSn) and orientation by temperature treatment using a Metler hot stage. (A) is at 20°C, (B) at 65°C, (C) at 75°C, (D) at 85°C, (E) at 89°C, (F) at 89°C, (G) at 75°C cooling after transition and (H) at 50°C cooling after transition



FIGURE 2.4: Observations by polarized light microscopy of some textures: (A) and (B) Gramicidin/ $C_{12}E_4$ well aligned phase observed under polarized light in (A) and direct light in (B); (C) fingerprint of $BuSn/C_{12}E_4$ /Cholesterol; (D) fan shape of $BuSn/C_{12}E_4$ /Chol; (E) focal conics of $BuSn/C_{12}E_4$, (F) oily streaks of a BuSn/DDAO sample; (G) and (H) phase of a Gramicidin/ $C_{12}E_4$ /Cholesterol sample.

2.4 Experimental techniques

Polarized light optical microscopy has been used to identify mesophases by observing their textures. Small angle X-ray scattering (SAXS) has been used for the study of the organization of the inclusions and their interactions in the lamellar phase. Finally, Solid State NMR (presented in Chapter 5) and wide angle X-ray scattering (WAXS) have been used to measure the order parameter of the membrane surfactants hydrocarbon chains in presence of inclusions.

2.4.1 Polarized light optical microscopy

The polarized light microscope is useful for characterizing and observing anisotropic specimens. As the liquid crystal is optically anisotropic (i.e. birefringent), we observe a characteristic texture that corresponds to a specific mesophase (in my case, the lyotropic lamellar phase) [7]. My samples were filled inside optical glass capillaries (VitroCom Inc, Mt. Lks, N.J.), of 0.1 mm thickness and 2 mm width, flame-sealed at both ends. All capillaries were observed with an Olympus BX51 microscope using polarized light at different magnifications (x5 - x50). Textures were photographed using an Olympus (Camedia C-3030) digital camera.

It is very important to distinguish between resolution and magnification. Resolution is the ability to differentiate the smallest features in the sample from each other whereas magnification is the increase in the apparent size of an object. Mathematically, the resolution is calculated as $d_r = 0.16\lambda/NA$ [106] where d_r is the resolution (size of the smallest resolvable feature), λ is the wavelength of light, and NA is the numerical aperture of the objective.

Basic of polarized light microscopy

Light is an electromagnetic wave. When traveling in an isotropic medium with a refractive index n, it travels in all directions and at the same speed. Once launched through a polarizer, the vibrations composing this wave become linear with a fixed direction and is called polarized light. A polarizer acts like a filter to allow only light oscillating in one orientation to pass.

A fundamental configuration in polarized light microscopy is that of crossed polarizers: the sample is placed between two polarizers oriented perpendicular to each other. If the sample is isotropic, light that goes through the first polarizer is then blocked by the second one (called analyzer). When the sample is birefringent (i.e. optically anisotropic), the incident light can be described as the superposition of two linearly components, that encounter different refractive indices and hence propagate at different speeds.

The delay between these two components results in a rotation of the polarization of the beam, which can now partially cross the analyzer, thus allowing the characterization of the observed textures [107] (Figure 2.4).

Polarized light microscope configuration

There are few steps to take in consideration before each microscope utilization:

1. Place the capillary on the stage of the microscope (between the polarizer and the analyzer)

- 2. Set tungsten-halogen bulbs voltage
- 3. Center the filament and adjust its location relative to the microscope stand

4. Place a relatively high magnification objective (x20) and focus on an object with reasonably sized features and contrast. Ensure that the appropriate condenser is in place.

- 5. Center and Focus the Field diaphragm.
- 6. Ensure the lamp filament is focused and fills the field of view.
- 7. Adjust the substage aperture for optimum specimen contrast.

2.4.2 Small-angle X-ray scattering

Small-angle X-ray scattering (SAXS) is the main technique used in this study. It is a very useful tool, because it allows studying the organization of the inclusions in the lamellar phase and, indirectly, their interactions. One must distinguish between SAXS and X-ray diffraction. In the latter, the scattering comes from sets of atomic planes while in this case, the repeat distance of typical lyotropic mesophases is about 20 times greater so the scattered intensity is concentrated at a very small angle around the transmitted beam. We can easily find in the literature very detailed presentations of the general theory behind X-ray scattering, for instance we cite [108]. In this section we will only reproduce the main results that will be useful later in this study.

2.4.2.1 Basics of SAXS

X-rays are electromagnetic waves with wavelengths in the region of an Ångström (1Å = 10^{-10} m). We will be concerned with a monochromatic beam of X-rays as depicted in Figure 2.5. It is characterized by the wavelength λ , or equivalently the wavenumber $k = 2\pi/\lambda$. The orientation of \vec{k} defines the propagation direction of the wave.

In the classical description of the scattering event, the electric field of the incident X-rays exerts a force on the electronic charge of the sample, which then accelerates and radiates the scattered wave. Classically, the wavelength of the scattered wave is the same as that of the incident one, and the scattering is then necessarily elastic.

To begin, let us consider an incident beam with an intensity given by a photon flux N_i (in photons/s) and a wave vector \vec{k}_i , that hits a sample of volume V and thickness e. The scattered contribution at the angle θ is then described by a wave with a flux N_{sc} and a wave vector \vec{k}_{sc} (θ is the angle between \vec{k}_i and \vec{k}_{sc}) (see Figure 2.5). The number of scattered photons per time unit N_{sc} in the detector solid angle $\Delta\Omega$ is given by

$$N_{sc} = N_i T \frac{e}{V} \frac{\mathrm{d}\sigma}{\mathrm{d}\Omega} \bigg|_d \mathrm{d}\Omega \tag{2.1}$$

where T is the transmission of the sample and $\frac{d\sigma}{d\Omega}_{d\Omega}_{d}$ is the differential scattering crosssection of the sample. From N_{sc} (equation 2.1) we can obtain the scattered intensity per volume unit in absolute scale (i.e. in units of reciprocal length):

$$I[m^{-1}] = \frac{1}{V} \left(\frac{\mathrm{d}\sigma}{\mathrm{d}\Omega} \right)_d = \frac{N_{sc}}{N_i T} \frac{1}{e \,\mathrm{d}\Omega}$$
(2.2)

where V is the volume of the irradiated sample.



FIGURE 2.5: Geometry of scattering and definition of \vec{q}

The interest of this scale is to be independent from the experimental conditions such as the intensity of the source, the size of the beam and the dimensions of the sample. The scattered intensity $I[m^{-1}]$) varies with the scattering direction. The scattering vector is defined as $\vec{q} = \vec{k}_{sc} - \vec{k}_i$ and its amplitude is given by:

$$q = |\vec{q}| = \frac{4\pi}{\lambda} \sin \frac{\theta}{2} \tag{2.3}$$

During a scattering experiment, we probe sizes at a scale order of $2\pi/q$. To probe length scales in the direct space between 1 nm and 1 μ m we should work at a scattering vector range between $6 \, 10^{-4} \leq q \leq 0.6 \,\text{\AA}^{-1}$. Working at very small angle scattering θ , very close to the incident beam, gives access to the structural properties of the sample and allows probing sizes larger than the nanometer.

Scattering from an L_{α} phase

The idealized structure of L_{α} lyotropic mesophases consists of planar, infinitely parallel stacks of amphiphilic bilayers separated by water with a long-range order (Figure 2.6). Lamellar phases are identified by the typical signature of a smectic lattice: equally spaced peaks giving intense X-ray signals concentrated at a scattering vector modulus $q = \frac{2\pi}{d_{lam}}$ where d_{lam} is the spacing between adjacent bilayers and is usually of the order of a few nanometers.

This situation presents the simplest model to illustrate and understand the scattering from an L_{α} phase and can be clearly equivalent to scattering from a set of atomic planes in a crystal. Thus we expect equally spaced Bragg spots with a periodicity of $\frac{2\pi}{d_{lam}}$ with the q_z reciprocal space axis parallel to the bilayer normal (Figure 2.7a).



FIGURE 2.6: Simple geometrical model of an L_{α} phase. Infinite stack of flat bilayers with periodicity d_{lam} . The z direction is taken to be parallel to the bilayer normal.



FIGURE 2.7: Schematic SAXS patterns obtained from a model L_{α} phase. In the case of the aligned phase, the q_z reciprocal space axis is parallel to the bilayer normal. The "powder" sample presents an isotropic distribution of intensity in reciprocal space.

Our samples are made of lamellar phase domains with nanoparticle inclusions forming a polycrystalline powder sample. The ideal "powder" sample contains a multitude of randomly oriented small single crystals, or crystallites (in our case the lamellar phase domains). X-rays are scattered in a sphere around the sample. A cone centered on the incident beam corresponds to a single Bragg angle 2θ : The randomly oriented crystallites in an ideal sample produce a Debye diffraction cone (Figure 2.5). The director of individual domains is randomly distributed in space, giving pseudo-isotropic scattering. The cross-section for the whole powder sample depends on the modulus of the scattering vector $q = |\vec{q}|$ but not on its direction. Thus the scattering from a lamellar phase gives equally spaced rings at the same scattering vector q as the quasi-Bragg peaks would normally be (Figure 2.7).

2.5 Liquid state theory

A large part of this work consists in measuring the interaction potential between nanoobjects inserted in membranes by analyzing their scattering signal I(q), recorded as described above. I will now briefly present the formalism of this analysis, focusing on the concepts and equations I used directly. A thorough introduction to liquid state theory ca be found in Refs. [109?, 110].

2.5.1 Scattering from solutions of identical isotropic particles

In the following, I consider a system of N identical objects in the volume V (in D dimensions), at temperature T. D = 3 for objects in bulk (such as nanoparticles in a solvent) and D = 2 for objects confined within membranes (when the volume V is actually a surface area).

If the objects have spherical symmetry (D = 3) or azimuthal symmetry (D = 2), the small-angle scattering signal from a collection of identical particles can be written as the product of a form factor of the object F(q) and a structure factor S(q). The form factor expresses the contribution to the scattering of a single particle and gives access to its size and shape. The structure factor describes the interference of the waves scattered by different particles and is due to the interactions between particles, as discussed below. We will then write the scattering intensity:

$$I(q) \propto F(q)S(q) \tag{2.4}$$

where the constant of proportionality depends on the number of scattering objects in the beam.

The form factor F(q) is the signal of a single particle. As the amplitude scattered by an object is the Fourier transform of its electron density $\rho(r)$, the form factor (in D = 3) can be written as:

$$F(q) = \left| \int \rho(r) e^{-iqr} d^3r \right|^2 \tag{2.5}$$

The above formula can be used to calculate the form factor of an object if we know the distribution of its electron density in real space. For example, a sphere of constant electron density with radius R yields:

$$F(q) \propto \left[\frac{\sin(qR) - (qR)\cos(qR)}{(qR)^3}\right]^2$$
(2.6)

As the object is immersed in a medium (i.e. the solvent) of uniform electron density ρ_0 , the relevant quantity that determines the scattering power of the object is the relative electron density of the particle $\Delta \rho = \rho - \rho_0$.

$$F(q) = \left| \int (\rho(r) - \rho_0) e^{-iqr} d^3r \right|^2$$
(2.7)

In a dilute solution the particles are sufficiently far away for each other (with respect to the range of the interaction), and the waves scattered by different objects are not correlated. The structure factor is equal to one and I(q) is only proportional to the form factor. Thus scattering from a dilute solution gives access experimentally to the form factor of the particles.

2.5.1.1 Structure factor and radial distribution function

On the other hand, for more concentrated solutions the scattered intensity also depends on the interaction. The structure factor $S(\mathbf{q})$ (which is now different from one) can be related to the pair correlation function $g(\mathbf{r})$, which is the normalized probability of finding a particle at a position \mathbf{r} with respect to a reference particle fixed at the origin, so that $\rho g(\mathbf{r})$ is the local density around the reference particle and describes its influence on the neighboring particles.

In liquids, $g(\mathbf{r})$ goes to one for large r, where the reference particle has no effect. At short r, the radial distribution function is generally zero, since most particles exhibit steric interaction (cannot occupy the same space).

So if we consider the particle 0 fixed at \mathbf{r}_0 , then the number of particles in the small volume $d^D \mathbf{r}$ centered in \mathbf{r} with respect to it is: $\rho g(\mathbf{r}) d^D \mathbf{r} = dn(\mathbf{r} - \mathbf{r}_0)$, where:

$$\mathrm{d}n(\mathbf{r}) = \left\langle \sum_{i \neq 0} \delta \left[\mathbf{r} - (\mathbf{r}_i - \mathbf{r}_0) \right] \right\rangle \mathrm{d}^D \mathbf{r}$$

such that:

$$g(\mathbf{r}) = \frac{1}{\rho} \left\langle \sum_{i \neq 0} \delta \left[\mathbf{r} - (\mathbf{r}_i - \mathbf{r}_0) \right] \right\rangle = V \frac{N-1}{N} \left\langle \delta \left[\mathbf{r} - (\mathbf{r}_1 - \mathbf{r}_0) \right] \right\rangle$$
(2.8)

Radial distribution functions can be measured by means of scattering experiments, using, for example, X-rays or neutrons. These techniques give access to the static structure factor $S(\mathbf{q})$:

$$S(\mathbf{q}) = \frac{1}{N} \left\langle \sum_{ij} e^{-i\mathbf{q}(\mathbf{r}_i - \mathbf{r}_j)} \right\rangle = 1 + \frac{1}{N} \left\langle \sum_{i \neq j} e^{-i\mathbf{q}(\mathbf{r}_i - \mathbf{r}_j)} \right\rangle$$
$$= 1 + \frac{1}{N} \left\langle \int_V d^D \mathbf{r} e^{-i\mathbf{q}\mathbf{r}} \sum_{i \neq j} \delta \left[\mathbf{r} - (\mathbf{r}_i - \mathbf{r}_j) \right] \right\rangle$$
$$= 1 + \frac{N(N-1)}{N} \int_V d^D \mathbf{r} e^{-i\mathbf{q}\mathbf{r}} \left\langle \delta \left[\mathbf{r} - (\mathbf{r}_1 - \mathbf{r}_0) \right] \right\rangle = 1 + \rho \int d^D \mathbf{r} e^{-i\mathbf{r}\mathbf{q}} g(\mathbf{r})$$
(2.9)

The structure factor is closely related to the Fourier transform of the radial distribution function, but the latter tends to 1 at large distances. This baseline induces a Dirac peak centered at the origin, that we remove by subtracting the baseline from the right-hand side of (2.9) and redefining the structure factor as:

$$S'(\mathbf{q}) = S(\mathbf{q}) - \rho \delta(\mathbf{q}) = 1 + \rho \int d^D \mathbf{r} \left[g(\mathbf{r}) - 1\right] e^{-i\mathbf{r}\mathbf{q}}$$
(2.10)

Finally, we drop the prime symbol ' and introduce the isotropy of the system:

$$S(q) = 1 + \rho \int d^{D} \mathbf{r} [g(r) - 1] e^{i\mathbf{r}\mathbf{q}} = 1 + \rho h(q)$$
(2.11)

redefining S(q) as the Fourier transform of the total correlation function h(r) = g(r) - 1. Conveniently, $h(r) \to 0$ for sufficiently large r. Also, h(r) = 0 for an ideal system.

2.5.1.2 Potential of mean force

In the low-density limit, it can be shown that

$$g(r) \simeq \exp\left[-\frac{u(r)}{k_B T}\right]$$
 (2.12)

This relation can be understood as a Boltzmann distribution of the neighbor particles around the reference one. One can then expand g(r) with respect to this value:

$$g(r) = \exp\left[-\frac{u(r)}{k_B T}\right] y(r), \quad \text{with} \quad y(r) = \sum \rho^n y_n(r).$$
 (2.13)

By analogy with the low-density case, we can also write formally:

$$g(r) = \exp\left[-\frac{w(r)}{k_B T}\right]$$
(2.14)

thus defining the potential of mean force w(r) ([109], section 7.3) which is the mean work needed to bring two particles from infinity to a distance r.

2.5.2 Integral equations

At higher concentrations, where the expansion (2.13) is no longer useful, in order to calculate g(r) from a given pair potential u(r) one often uses another approach, based on the Ornstein–Zernike (OZ) integral equation for the total correlation function h(r), proposed in 1914 by Leonard Ornstein and Frits Zernike [111].

The OZ relation is based on the separation of the total correlation function h(r) between two particles 1 and 2 into two contributions: a direct correlation function c(r) and an indirect correlation $\gamma(r) = h(r) - c(r)$ due to the presence of all other particles. The density of these particles is itself described by h(r) and their influence by c(r). We can then write (for a homogeneous and isotropic system) :

$$h(r) = c(r) + \rho \int c(|\mathbf{r} - \mathbf{r}'|)h(\mathbf{r}')d^{D}\mathbf{r}'$$
(2.15)

We can formally see the OZ equation as a definition for c(r), as we can more clearly see by taking the Fourier transform of (2.15):

$$h(q) = c(q) + \rho c(q)h(q) \Rightarrow c(q) = \frac{h(q)}{1 + \rho h(q)}$$

$$(2.16)$$

As it stands, the OZ equation is not very useful since c(r) and h(r) are both unknown. Furthermore, it does not involve explicitly the pair potential u(r) of the system. We therefore need a further relation between h(r), c(r) and u(r), known as a "closure".

2.5.2.1 Closure relations

Several such closure relations are used in the literature, each one adapted to different types of interaction potential.

The mean spherical approximation (MSA). As we have seen above in Eq. (2.12), in the low-density limit $g(r) = \exp[-\beta u(r)]$, with $\beta = \frac{1}{k_B T}$. In the same limit, (2.15) reduces to $h(r) \simeq c(r)$, yielding:

$$c(r) = e^{-\beta u(r)} - 1, \qquad \rho \to 0$$
 (2.17)

or, if $\beta u(r) \ll 1$,

$$c(r) = -\beta u(r) \tag{2.18}$$

The MSA adopts this approximation at all concentrations. It describes well the behavior of hard spheres interacting via attractive and repulsive short-range potentials with an amplitude lower than k_BT .

The hypernetted chain (HNC) closure is

$$g(r) = \exp[-\beta u(r) + h(r) - c(r)]$$
(2.19)

or

$$c(r) = -\beta u(r) + h(r) - \ln[h(r) + 1]$$
(2.20)

Since $h(r) \to 0$ for $r \to \infty$, the HNC gives like in the MSA $c(r) = -\beta u(r)$. In this case the OZ equation together with the HNC can only be solved numerically. It is found by comparison with Monte Carlo simulations that the HNC scheme works especially for long-ranged potentials.

The Percus-Yevick (PY) closure is the function we adopted in all our data treatment in order to solve the Ornstein-Zernike integral equation. It uses the following closure relation :

$$c(r) = g(r) \left\{ 1 - \exp\left[\frac{u(r)}{k_B T}\right] \right\} = f(r)y(r)$$
(2.21)

with $f(r) = \exp\left[-\frac{u(r)}{k_BT}\right] - 1$ the Mayer function. This function represents a measure of the "excluded volume" of the particle and, as seen above in § 2.5.1.2, $y(r) = \sum \rho^n y_n(r)$. It is obvious from (2.21) that, due to the presence of f, $u(r) = 0 \implies c(r) = 0$, so the direct correlation function is explicitly of the same range as the potential.

The Percus-Yevick scheme works well for short-ranged potentials and very repulsive interactions. We use it in our analysis for the hard sphere potential calculations, where we consider each time that the interacted particles have a dense core. In general the OZ equation with the PY closure does not have an analytical solution. We numerically solve it using an algorithm introduced by Lado [112].

Approximation	Closure relation	Analytical results
Mean spherical approxima-	$c(r) = -\beta u(r)$	Analytical solution for
tion (MSA)		screened Coulomb poten-
		tial (not accurate for low
		densities)
Percus-Yevick (PY)	$c(r) = g(r)[1 - e^{\beta u(r)}]$	Analytical solution for
		hard spheres interacting
		only by steric repulsion
Hypernetted-chain (HNC)	$c(r) = -\beta u(r) + h(r) -$	Only numerical solution
	ln1+h(r)	

TABLE 2.3 :	Closure	relations.
---------------	---------	------------

2.5.3 Random phase approximation

The random phase approximation (RPA) is a technique much easier to use than the integral equation approach in § 2.5.2 and can be applied when the interaction potential can be written as the sum $u(r) = u_0(r) + u_l(r)$ of a reference potential $u_0(r)$ (short-ranged, in general) and an additional long-range component $u_l(r)$, to be treated as a perturbation.

The RPA is particularly useful if we have an analytical expression of the structure factor $S_0(q)$ – and hence for the direct correlation function $c_0(r)$. Fortunately, this is the case for the hard-core potential (in two or three dimensions).

The total direct correlation function is

$$c(r) = c_0(r) - \beta u_l(r)$$
 (2.22)

or, to put it differently,

$$n\beta\tilde{u}(q) = S^{-1}(q) - S_0^{-1}(q) \quad ; \quad \beta = (k_B T)^{-1}.$$
 (2.23)

This approximation is valid if the concentration or the amplitude of the long-ranged interaction are sufficiently low.

2.6 X-ray sources

X-ray setups can be classified in two categories: laboratory installations, whose X-ray source is a conventional generator or a rotating anode, and synchrotron beamlines, where



FIGURE 2.8: (A) MOMAC laboratory setup. (B) MAXS laboratory setup.

X-rays are produced by the radiation of accelerated particles. In this section I will discuss the X-ray sources I used for my experiments.

2.6.1 Laboratory sources: MAXS and MOMAC setups

When one wishes to characterize samples in SAXS or WAXS, laboratory installations remain the most accessible experiments. They often allow a first feasibility test before further study on beamlines. Both setups I employed (MOMAC and MAXS) use a rotating anode as X-ray source and are based at the Laboratoire de Physique des Solides, in Orsay. MOMAC is the result of a collaboration with the LIONS lab of the CEA Saclay; see Figures 2.8a and 2.8b.

The principle of these sources is to heat a filament of tungsten (cathode) and apply a high voltage in order to snatch electrons that will then hit a water-cooled anode. Due to electron-matter interactions, one can excite the atoms composing the anode (usually made out of copper), which by returning to their initial state will emit X-rays. In these systems, most of the kinetic energy of the electrons is dissipated as heat, and about 1 % is converted into X-rays. The power of these sources is limited by the cooling efficiency. Therefore, one often uses a cylindrical anode that rotates around an axis, so that the incident electron beam does not strike the same area continuously, spreading the heating over the entire surface [113]. The flux is then increased compared to that of a conventional generator with a fixed anode.

X-rays emitted by these sources come from two types of processes: "braking radiation" or *bremsstrahlung*, produced from the deceleration of electrons during the interaction of the radiation with matter; and X-ray fluorescence due to the de-excitation of the atoms of the anode. Bremsstrahlung has a wide spectrum in wavelength, whereas the X-ray fluorescence yields a spectrum of lines typical of the electronic transitions specific to the element composing the anode. The fluorescence X-ray radiation is several orders of magnitude more intense than that of the bremsstrahlung. The K_{α} line is typically the strongest one, located at a characteristic wavelength for each element. Hence, we use a monochromator to select this line. As there is no preferred direction for the fluorescence radiation, in practice we can only collect a small fraction of the emitted X-rays.

The MAXS setup uses a copper anode, while MOMAC uses a molybdenum one. The latter has the advantage of being more energetic, their characteristic X-rays are more penetrating and therefore they are interesting for the study of systems with high absorption (due to the presence of heavy elements). The table 2.4 describes in brief the advantages and disadvantages of each installation.

Each setup has its qualities: MOMAC has the advantage of offering high flow, and a wide range of q, quite interesting for systems whose typical sizes cover a wide range, or are not known a priori. MAXS, with its variable distance, is quite versatile but its largest disadvantage is that the mirrors are not very stable, often needing time-consuming alignment. The parasitic scattering (from air and any windows) is also stronger than on MOMAC due to the lower energy and can hide that of the samples, as we can see in Figure 2.9.

There is a difference in the curves obtained with the MAXS and MOMAC installations. As mentioned above, this is due mainly to the unstable optical setup for MAXS and to parasitic scattering, so the sample signal is weak. It is important to mention that in MOMAC the beam is under vacuum and only the sample area is exposed to air, whereas in MAXS the beam travels through air all along (Figure 2.8). In Figure 2.9c we superposed the measured intensity for the same dodecane capillary, in red using MOMAC and in blue using MAXS. We can see that in the low-q range there is a decrease in the intensity for the blue curve then it increases with almost the same profile as the red



FIGURE 2.9: Comparison of the data obtained by the MOMAC and MAXS laboratory X-ray installations.

curve but the peak is shifted, so we applied an offset. The red curve goes through a peak at $q = 1.3718 \text{ Å}^{-1}$ while the blue curve goes through a peak at $q = 1.4034 \text{ Å}^{-1}$ and then it decreases to a lower level in comparison with the red one.

The full width at half maximum (FWHM) is a parameter commonly used to describe the width of a peak on a curve . It is given by the distance between points on the curve at which the function reaches half its maximum value. For a Gaussian function, the FWHM is defined by $FWHM = 2\sqrt{2\ln 2\sigma}$, with σ the standard deviation. I calculated the FWHM by fitting a Gaussian to my data using Igor Pro and obtained FWHM_{dodecane/MAXS}=0.31 and FWHM_{dodecane/MOMAC}=0.36.

If we now look at the curves in Figure 2.9a and we compare them to Figure 2.9b we can clearly see that the profiles are very different. They both represent the scattered intensity I(q) for the same system: DDAO bilayers containing different concentrations of gramicidin. From the data presented in Figure 2.9 we conclude that the data obtained



FIGURE 2.10: Schematic presentation of a synchrotron. (source : SOLEIL)

by MAXS are not accurate. In the following, I will use exclusively data recorded using MOMAC.

2.6.2 Synchrotron Source: ESRF - D2AM line

Beamlines of third generation synchrotrons like the ESRF (European Synchrotron Radiation Facility), use as X-ray source electrons accelerated to speeds close to the speed of light. In fact, when an electron is accelerated at higher speeds, it emits a very bright radiation at a wide spectral range, confined into a cone of very small aperture and concentrated in the direction of the electrons' movement. Figure 2.10 is a schematic representation of a synchrotron. At first, the electrons are accelerated in a linear accelerator (Linac); then they go through a circular accelerator (booster) in order for them to reach the desired energy. Once done, an injection phase leads them into the storage ring, where electrons are maintained at high speed through a series of electromagnetic components such as bending magnets. The synchrotron radiation emitted by the electrons is retrieved by the beamlines placed tangentially to the storage ring.

We used the D2AM beamline at the ESRF [114]. On this bending magnet beamline, the beam intensity is about 1000 times higher than that obtained with rotating anodes. This allows the acquisition of spectra in a very short time (of the order of a few seconds to a couple of minutes), while the characteristic time to get good spectra in the lab is of the order of hours. This is the main advantage of using the synchrotron light.

D2AM has two interchangeable instruments, a diffractometer and a small-angle scattering setup, and covers a wide range of energy (5 to 25 keV). For our work, we used the small angle configuration with details shown in Table 2.4.

Setup	MAXS	MOMAC	D2AM
Anode	Cu	Mo	-
$\lambda(\mathrm{nm})$	0.1542	0.0709	0.11 (variable)
E(keV)	8 (K _{α} Cu)	$17 (K_{\alpha} Mo)$	11 (variable)
Flux (photons/s)	5×10^7	10×10^{7}	10^{11}
Beam size (μm^2)	800×800	1000×1000	150×125
Distance D	variable $(6 \text{ to } 20 \text{ cm})$	Fixed (75 cm)	variable
Range of $q(\text{\AA}^{-1})$	5×10^{-3} - 2.6	2×10^{-2} - 3.2	6×10^{-4} - 0.6 (variable)
Detector	CCD	Image plate	CCD
Pixel size (μm^2)	240×240	100×100	50×50

TABLE 2.4: Comparison of the X-ray scattering equipments used for our measurements.

2.7 Distance calibration

The sample-to-detector distance D must be known accurately in order to calculate the associated q values.

2.7.1 MAXS setup calibration

With MAXS (Figure 2.12), the calibration is easily done because the detector is perpendicular to the beam. We use lead(II) dithiolate, which has a lamellar structure. From the scattering pattern (Figure 2.11) we can measure the radii R_n of the diffraction rings in pixels. Knowing the size of the pixels of the detector, we can obtain R_n in meters and thus determine q_n .

Lead alkanethiolates provide a series of sharp lines, that can be used for calibrating Xray setups over a wide q-range. We used the compound $(n-C_{18}H_{37}S)_2Pb$, synthesized according to Tiers [115] and presenting as a yellow crystalline powder. It consists of a C18 aliphatic chain with a thiol and a lead counterion head, making its structure look like a fatty acid with a heavy metal ion instead. It forms a lamellar crystal which gives several diffraction orders (Figure 2.11a), all belonging to the same lattice planes and corresponding to a stack of crystalline bilayers with peaks evenly spaced at a periodicity $d_1 = 50.2\text{ Å}$ (Figure 2.11c). Therefore, in Figure 2.11 we can see very well the first-order sharp lines in Figure 2.11a and the corresponding peaks in Figure 2.11c in terms of q. Diffraction results in eight evenly spaced peaks in the range between 0.1 and 1 Å^{-1} : the first peak is found at 0.125 Å^{-1} . Higher precision is obtained by measuring higher diffraction orders, e.g. n = 4 or n = 5. The correspondence is given by Bragg's law:

$$2d_1 \sin \theta_n = n\lambda \tag{2.24}$$



FIGURE 2.11: Scattering pattern of lead(II) dithiolate.

yielding

$$\sin \theta_n = n \frac{\lambda}{2d_1} = n \frac{\lambda}{4\pi} q_1$$

where n is the diffraction order, λ is the wavelength of the X-rays, d_1 is the layer spacing and θ is the half-angle between the incident ray and the scattered one. The angle θ can be simply determined geometrically from the diffraction cones that we intercept by a flat detector (2.11b) via the following formula:

$$\tan 2\theta_n = \frac{R_n}{D} \tag{2.25}$$

and thus from the formulas above and with $q_n = n 2\pi/d_1$:

$$D = \frac{R_n}{\tan\left[2 \arcsin\left(\frac{q_n \times \lambda}{4\pi}\right)\right]} = \frac{R_n}{\tan\left[2 \arcsin\left(\frac{nq_1 \times \lambda}{4\pi}\right)\right]}$$
(2.26)

From Figure 2.11a, $R_5 = 1.032$ cm (48 μ m pixel size and 215 pixels in radius) the sampleto-detector distance calculated from equation 2.26 is D = 6.66 cm. (Figure 2.12).



FIGURE 2.12: MAXS setup.

2.7.2 MOMAC setup calibration

 K_{α} emission lines result from electron transitions between an L shell, with principal quantum number 2, and a K shell with principal number equal to 1. This line is actually a doublet, with slightly different energies (for molybdenum, $\lambda_{K\alpha_1} = 0.709319$ Å and $\lambda_{K\alpha_2} = 0.713607$ Å). We use this splitting and the diffraction of α -quartz to calibrate the sample-to-detector distance D for our experiments.

We know that D should be close to 74 cm, because the detector is fixed and only the sample holder is variable (Figure 2.13). An important factor to take in consideration is that the detector is actually slightly tilted to an angle of $\beta = 83^{\circ}$, so that the different diffraction orders appear in the images as ellipses, instead of circles so the treatment is more complicated, as described in Figure 2.14d.

From the geometry of the setup we have:

$$\tan(2\theta) = \frac{R_{horizontal} \times \sin\beta}{D - R_{horizontal} \times \cos\beta}$$
(2.27)

which gives

$$R_{vertical} = D \times \tan(2\theta) \tag{2.28}$$

and

$$R_{horizontal} = \frac{R_{vertical} \times D}{R_{vertical} \times \cos\beta + D \times \sin\beta}$$
(2.29)

We should take in consideration the pixel size of the detector which is in our case $100 \ \mu m$. Now we have the theoretical values of R and we should measure the experimental values and compare them. We use imageb software to open the 2D diffraction pattern and simply by defining the center of the pattern we can easily measure the radius of the rings in pixel.

The α -quartz sample is a powder that has been previously ground down to particles of μ m cross-section. The powder is placed inside a round capillary, 1 mm diameter. It is homogeneous and the crystallites are randomly distributed inside. We measure the quartz capillary and we apply the q radial average from the scattered image and we introduce a starting value for the distance (the detector angle β is fixed) and we specify the pixel size and the center of the detector (Figure 2.14a). Using a Python routine, we obtain the average radial file that contains the values of the wave vector q and the scattered intensity I(q) (Figure 2.14b).



FIGURE 2.13: MOMAC setup.



The graph in Figure 2.14b) shows two single peaks at $q_{010} = 1.47 \text{ Å}^{-1}$ and $q_{011} = 1.87 \text{ Å}^{-1}$ and then doublets starting from $q = 2.5 \text{ Å}^{-1}$ with one of the doublet peak being more intense than the other. In each doublet, the stronger peak corresponds to K_{α_1} and the weaker one to K_{α_2} . I determine the q value for each of the peaks and

hkl	$q_{th}(\text{\AA}^{-1})$
010	1.477
011	1.879
110	2.557
012	2.754
111	2.809
020	2.953

TABLE 2.5: Theoretical q values for each hkl index of $\alpha\text{-quartz.}$

compare them to the theoretical ones. I adjust D in order to match q_{th} and q_{exp} .

2.8 Corrections

A complete SAXS measurement does not only consist in recording the scattered signal. Several corrections must be applied before we can use the data. Here I will list some imperfections I faced during my experiments and the corrections I applied to improve the data quality.

2.8.1 Detector correction

Depending on the detection mode, some detectors have imperfections. Those can be divided into two categories, intensity distortions and geometry distortions. Intensity distortions are deviations in the amount of measured intensity, and geometry distortions are deviations in the location of the detected intensity.

One factor to take in consideration for a reliable evaluation of the data is the range within which the measured intensity is proportional to the number of incoming photons (linearity window). All detectors have a limit above which they exhibit non-linearity.

A very important distortion is the electronic noise of the detector which consists of the addition of a mostly homogeneous background (especially in the case of CCD detector). To compensate for this, we measure what we call a "dark-current" image, an acquisition of the detector signal without an incoming X-ray beam, at an exposure time equal to the sample measurements, since this noise may have a constant but also a time-dependent component.

The last detector distortion I will mention is the detection of a large intensity localized in a single spot (also known as a "zinger"), due for instance to cosmic rays. When the acquisition time is long, we split it into several shorter images. If a pixel has an abnormally high value in one image with respect to the other images, it is discounted before averaging [116].

2.8.2 Transmission correction

Most samples absorb a fraction of the photon beam radiation. Therefore, the absorption must be determined for each sample and must be corrected for to allow the subtraction of a reference signal (see below) and proper normalization. We assess it by measuring the X-ray beam flux values I_0 before, and I_1 after inserting the sample capillary, respectively. The ratio of the two is the transmission factor T ranging from 0 to 1:

$$T = \frac{I_1}{I_0}$$
(2.30)

2.8.3 Background correction

Besides the sample of interest, other elements of the setup also scatter X-rays: the air in the beam path (our instruments are not totally under vacuum), the slits and windows, but also the capillary and even the solvents in the samples. This scattering contribution is added to the detected signal and must be subtracted before analysis.

The correction consists in doing a measurement with exactly the same conditions with a reference sample. In other words, one measures the scattering of a capillary (identical in size to that of the samples) containing the solvent we used, at the same exposure time as for the normal sample, and subtracts this reference intensity from the sample signal after correcting for the transmission, which can be different.

Part I

Interactions between inclusions embedded in surfactant layers
Chapter 3

Membrane-mediated interaction between inclusions in absence of interlayer interaction

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3.1 Introduction

In this Chapter we present small-angle x-ray scattering (SAXS) measurements on highly oriented multilayer samples of surfactants doped with different inclusions, performed at the bending magnet beamline BM02 (D2AM) of the European Synchrotron Radiation Facility (ESRF) in Grenoble, France. The x-ray energy was 11 keV, to minimize absorption problems and beam damage. Background subtraction, regrouping and normalization were done following standard procedures using the bm2img program developed at the D2AM station (more details in chapter 2 section 2.8).

For each type of system (nature of the inclusion and membrane composition) we studied dilution lines (with at least three concentration values) as a function of temperature. In particular, we monitored the effect of the hydrophobic thickness on the interaction potential.

The data are described as the product of the form factor of the particle $|F(q)|^2$ with the structure factor of the two-dimensional fluid, S(q). The former is obtained as the Fourier transform of the electron density, while the latter is computed in the framework of standard liquid state theory (integral equations of the Ornstein-Zernike type, with a Percus-Yevick or HNC closure relation) by an iterative numerical calculation based on a potential V(r) containing a hard-core repulsion and an additional "soft" component representing the membrane-mediated interaction.



3.2 SAXS measurement

FIGURE 3.1: The in-plane SAXS experimental setup while using the heating stage. The latter contains 8 holes in the center of which we stick the capillary region of interest

The flat capillaries were scotched against the heating stage, as shown in Figure 3.1. The stage has eight holes, and the region of interest of each capillary is placed at the center of each hole. The capillaries were scanned in the beam to find well-aligned domains (where the intensity of the residual Bragg reflections was as low as possible). All scans were done starting at room temperature and then going up by $10^{\circ}C$. After each temperature variation, we waited for 10 minutes before any signal acquisition, in order for the sample to stabilize at that temperature.

The SAXS measurements were performed with the incident beam perpendicular to the flat face of the capillary (parallel to the layer normal, which we take along the z axis.) Thus, the scattering vector q is mostly contained in the (x, y) plane of the layers $(q_z \simeq 0, up$ to the curvature of the Ewald sphere) and the measured scattered signal I(q) probes inhomogeneities of the electron density in this plane. yielding the diffuse signal scattered by the 2D fluid of inclusions in the plane of the layers $I(\mathbf{q}_r)$. Since the bilayers form a two-dimensional liquid, the scattering pattern exhibits azimuthal symmetry: $I = I(q_r = |\mathbf{q}_r|)$.

3.3 Structure factor

We checked that the samples are homogeneous and that the inclusions are well dispersed inside the layers. The temperature treatment we perform in-house before the synchrotron run in order to align the sample in homeotropic anchoring is crucial for eliminating the (very strong) Bragg contribution of misaligned domains.

In the absence of such Bragg contamination and after subtracting the scattering profile from a reference capillary (in our case, water), the remaining signal is due to the twodimensional fluid formed by the inclusions. Furthermore, as the inclusions are monodisperse and identical in shape and size (up to an azimuthal averaging), we can consider the scattering intensity as the product of a structure factor multiplied by a (rotationally averaged) form factor [117], yielding $I(q_r) = S(q_r) \cdot F(q_r)$, with:

$$S(q_r) = \frac{1}{N} \left\langle \left| \sum_{k=1}^{N-1} \exp(-i\mathbf{q}_r \, \mathbf{r}_k) \right|^2 \right\rangle$$
(3.1)

where N is the number of objects and object "0" is taken as the origin of the coordinates. As explained in the previous chapter (section 2.5.1), the form factor $F(q_r)$ is the squared modulus of the Fourier transform of the electron density $\rho(r)$ of the scattering object (see Eq. 2.5). In the case of gramicidin inclusions, the in-plane form factor adopted is the one computed by Constantin in ref. [84] from the atomic configuration of the gramicidin helical dimer pore structure used in the Molecular Dynamics (MD) simulation

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FIGURE 3.2: A) In-plane form factor of the gramicidin channel [84]. B) Form factor of BuSn inclusions, experimental data obtained on a dilute BuSn/propan-2-ol solution at 5W%.

of deGroot *et al.* (Figure 3.2a). In the case of BuSn inclusions, we consider that the form factor of the particles is dominated by their inorganic core, as the scattering of the butyl chains around it is similar to that of the dodecyl chains within the bilayers and to that of propan-2-ol in solution. This assumption is confirmed by the intensity at higher scattering vectors $(q > 0.5 \text{ Å}^{-1})$, obtained with a dilute solution of BuSn at 5 W% in propan-2-ol solvent, as can be seen in Figure 3.2b. That signal is well described by the form factor of a sphere $|F(R,q)|^2$, with a radius $R = 4.5 \pm 0.2$ Å, in good agreement with the average radius of the tin oxide core estimated from the crystallographic data. The form factors in Figure 3.2 were used to treat the data throughout this thesis.

The intensity is divided by the form factor to yield the two-dimensional structure factor $S(q_r)$ of the fluid formed by the inclusions in the plane of the membrane. Some examples are shown in Figure 3.3a.

Despite all our precautions, the sample alignment is not always perfect and sometimes lamellar defects persist and give rise to residual peaks, as can be seen in Figure 3.3 for $q\sim$ 0.11 Å^{-1} . We discard these points by applying a mask, as well as the ones in the shade of the beamstop and those above 0.4\AA^{-1} where the samples signal strongly decreases and it becomes difficult to subtract the parasitic background (e.g. the contribution of the kapton windows). Nevertheless, the first peak of the structure factor is properly measured for all samples, as we will see below.



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FIGURE 3.3: Experimental structure factors at multiple temperature as a function of inclusions concentration for in (A) Gramicidin/ $C_{12}E_4$ and in (B) for BuSn/DDAO systems

3.4 Model

In this section I will describe the approach I used to treat all our data (systems with both type of inclusions, gramicidin and BuSn, at all the experimental temperatures). In the following section I will use as an illustration the gramicidin- $C_{12}E_4$ system at three different inclusion concentrations, performed at T=30°. The procedures applied to this system were used for all the data discussed below. It is noteworthy that, throughout this chapter, we consider that there is no interlayer interaction (between inclusions in different layers). This assumption will be discarded in the next Chapter.

3.4.1 Hard-disk model

The simplest model for the interaction of inclusions in membranes is that of hard disks confined in the plane. Such an analysis has already been performed by [118] for gramicidin in DLPC bilayers at P/L=1/10 and by [84] for gramicidin in $C_{12}E_5$ and DLPC and DDAO at multiple values of P/L and by [88] for BuSn in DDAO multilayers. As a first step, we analyzed all the curves using the two-dimensional structure factor $S_{HD}(qr)$, given by the simple analytical expression obtained by [119] using the "fundamental measure" approach:

$$S_{HD}^{-1}(q) = 1 + 4\eta \left[A \left(\frac{J_1(qR)}{qR} \right)^2 + B \frac{J_0(qR)J_1(qR)}{qR} + G \frac{J_1(2qR)}{qR} \right]$$
(3.2)

where q is the scattering vector in the plane of the layers, R is the hard disk radius, $\eta = n\pi R^2$ the surface fraction (with n being the numerical density of the disks) and J_k the Bessel function of the first kind and order k. The prefactors are given by:

$$G = (1 - \eta)^{-3/2}$$
$$\chi = \frac{1 + \eta}{(1 - \eta)^3}$$
$$A = \eta^{-1} [1 + (2\eta - 1)\chi + 2\eta G]$$
$$B = \eta^{-1} [(1 - \eta)\chi - 1 - 3\eta G]$$

During the fit, the number density of the pores n_{pore} is fixed at the experimental value (determined by the preparation) and the effective hard-disk radius varies freely. We can see in Figure 3.4 in dashed lines the hard disk fit for three different concentrations of gramicidin inclusions in $C_{12}E_4$.

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FIGURE 3.4: Experimental structure factor of Gramicidin inclusions in $C_{12}E_4$ membranes at 30°C for three concentrations.

As found previously by Constantin et al [84, 87, 88], although it fits very well the individual curves the hard disk model is not satisfactory since the interaction radius decreases with P/L, sign of an additional soft repulsive interaction. This effect can be understood by noting that, at low concentration, the pressure of the fluid of inclusions is low, and the objects can stay well away from each other. As the density increases, so does the pressure, which can now overcome the repulsive potential and the particles are pushed closer together, for a smaller effective radius.

3.4.2 Additional interaction

In the plane of the membrane, one should calculate the structure factor for a hard core with radius $R_{HD} = 9.5$ Å for the gramicidin peptides and $R_{HD} = 4.5$ Å for the BuSn nanoparticles adding an additional exponential "soft" potential

$$V(r) = u \exp\left[-\frac{1}{2}\left(\frac{r-2R_{HD}}{\xi}\right)\right] \qquad r > 2R_{HD} \tag{3.3}$$

where r is the distance between the pore centers. The structure factor S(q) is defined by four parameters: the hard-core radius R_{HD} , the number density n_{pore} , as well as the amplitude u and the decay length ξ of the additional component. The first two parameters are known and will be kept fixed, while the last two are allowed to vary in order to optimize the fit to the experimental data.

We calculate S(q) using the method of Lado [120], implemented as an IGOR PRO

function. Briefly, the method provides an iterative solution to the Ornstein-Zernike equation with the Percus-Yevick closure (explained in detail in Chapter $2, \S 2.5.1$).

3.4.2.1 Lado algorithm

The structure factor is calculated practically via the algorithm introduced by Lado [120], based on the Ornstein-Zernike equation with the Percus-Yevick closure, see § 2.5.2. In the following, I will use the relevant equations without further comment and proceed directly to the Lado algorithm.

We introduce the indirect correlation function $\gamma(r)$ as:

$$\gamma(r) = h(r) - c(r) \tag{3.4}$$

yielding

$$\gamma(q) = \frac{\rho c^2(q)}{1 - \rho c(q)}.$$
(3.5)

The Percus-Yevick relation writes:

$$c(r) = [1 + \gamma(r)][e^{-\beta ur} - 1]$$
(3.6)

The Lado algorithm yields the structure factor S(q) by iterating (3.5) and (3.6) (and switching between direct and reciprocal space by numerical Fourier transform). Note that S(q) is nothing but h(q) + 1, and that ρ in equation 3.5 is the number density n. The algorithm is implemented as a procedure in the IGOR PRO program. First, we use a function to set up and create waves that we will use during the procedure (the real space waves to store the indirect correlation function $\gamma(r)$, the direct correlation function c(r), the radial distribution function g(r) etc. and the reciprocal space waves to store the $\gamma(q)$, c(q), g(q), S(q) etc. This setup function takes as argument the existing q scale and the number density wave. Then we can proceed with the Lado algorithm function which takes as argument three waves. The first wave contains the hard disk radius, the maximum potential intensity U at contact (in kT units) and the potential range ξ (in Å). The second argument is the wave that will store the S(q) model and the third is the q wave.

We modeled the additional interaction by a decreasing exponential, as follows:

$$V(r) = U \cdot fr \cdot \exp\left(-\frac{r-2R}{\xi}\right)$$
(3.7)

where fr was introduced in Ref. [84] as an "effective fraction" of the interaction amplitude as a function of n:

$$fr(n) = \left(\frac{n_{max} - n}{n_{max} - n_{min}}\right)^2 \tag{3.8}$$

where n_{max} is the number density of the highest concentration used in the system and n_{min} is the lowest number density of inclusions. The function fr represents a crude way of accounting for multi-body effects, in particular the fact that, as n increases, the hydrophobic thickness of the membrane approaches that of the inclusions and the resulting elastic interaction is reduced.

Since the disks cannot come closer than a distance 2R corresponding to the core diameter, we set V(r < 2R) = 1000 kT. Then, we initialize $\gamma(r)$ to 0 and obtain c(r) from Eq. 3.6. Then we apply a direct Fourier transform on c(r) to yield c(q), after which we compute $\gamma(q)$ from Eq. 3.5 and apply an inverse Fourier transform to yield the next version of $\gamma(r)$. This procedure is iterated until convergence, when we take S(q) = 1 + h(q). Once the equations are solved, the S(q) model is drawn as can be seen in Figure 3.4. A comparison between the S(q) model and the experimental S(q) data yields the goodnessof-fit function χ^2 for different values of the fit parameters (see Figure 3.5 as an example). From the χ^2 matrix function we find the best combination of interaction intensity and decay that fit our data.

3.4.2.2 Second virial coefficient

As pointed out by Noro and Frenkel [121], for hard core particles with an additional short-range interaction the structure factor does not depend on the details of the potential, but rather on an effective parameter: the second virial coefficient, B_2 . For a two-dimensional fluid, B_2 is defined as :

$$B_2 = \frac{1}{2} \int_{\mathbb{R}^2} d^2 \mathbf{r} \left[1 - e^{-\beta u(r)} \right]$$
(3.9)

The gramicidin pores in the membranes are cylindrical, thus there is no angular dependence in the interaction potential. The only important variable is the center-to-center distance between inclusions, so the B_2 expression simplifies to:

$$B_2 = \frac{1}{2} \int_0^\infty 2\pi r dr \left[1 - e^{-\beta u(r)} \right]$$
(3.10)

Furthermore, in our case the interaction contains a hard core from r = 0 to r = R, with an additional potential u(r) for r > R. The second virial coefficient can thus be written as:

$$B_2 = 2\pi R^2 + \pi \int_{2R}^{\infty} r dr \left[1 - e^{-\beta u(r)} \right]$$
(3.11)

where we can separate the hard core contribution $b_0 = 2\pi R^2$. Using the normalization $b_2 = B_2/b_0$ we finally obtain

$$b_2 = 1 + \frac{1}{2R^2} \int_{2R}^{\infty} r dr \left[1 - e^{-\beta u(r)} \right]$$
(3.12)



FIGURE 3.5: Goodness-of-fit function χ^2 for different values of the parameters U_0 and ξ for the C₁₂E₄ membranes fitted with the virial coefficient b_2 .

We implemented these equations in Igor as a MATRIXOP command. An example is given in Figure 3.5. In this figure we plotted the Goodness-of-fit function χ^2 , for different values of the parameters U_0 and ξ , fitted with the virial coefficient b_2 for the gramicidin embedded in $C_{12}E_4$ membranes. We clearly see that the minimum for χ^2 is found at different combinations of U_0 and ξ but which correspond to the same value of the virial coefficient : $(U_0 = 16k_BT; \xi = 1.5\text{Å}); (U_0 = 5.5k_BT; \xi = 2\text{Å}); (U_0 = 4.9k_BT;$ $\xi = 2.5\text{Å})$ corresponding to $b_2 = 1.6$. In other terms, one must keep in mind that what matters is not the values of U_0 and ξ taken separately but rather on their combination described by the virial coefficient b_2 .

3.4.2.3 Data verification with the RPA method

Now that we have calculated the interaction potential numerically using the Lado algorithm, we can validate our results by comparing them to interaction potentials calculated analytically using the random phase approximation (RPA). The latter is explained in chapter 2 in section 2.5.3. It is a very easy technique based on the following equation:

$$n\beta\tilde{u}(q) = S^{-1}(q) - S_0^{-1}(q) \quad ; \quad \beta = (k_B T)^{-1}.$$
 (3.13)

 $S^{-1}(q)$ denotes the reciprocal of the experimental structure factor obtained for each inclusion concentration and $S_0^{-1}(q)$ is the reciprocal of the hard disk model with the physical radius R_{HD} of the object, see § 3.4.2.

We plot the U(q) calculated analytically with the RPA method along with the U(q) obtained by Lado. In fact the lado procedure presented earlier yields a V(r) in the real space described in our case by a decreasing exponential, so to access the interaction potential in the reciprocal space of the 2D system formed by the inclusions within the bilayer, we perform the Fourier transform of the V(r) as follows:

$$\tilde{U}(q) = \int_{\Re} d^2 \vec{r} \, e^{-i\vec{q} \cdot \vec{r}} \, U(\vec{r}) = \int_0^{2\pi} d\phi \int_0^{\infty} r dr \, e^{-iqr\cos\phi} \, U(r)$$
$$= \int_0^{\infty} r dr \, U(r) \int_0^{2\pi} d\phi \, e^{-iqr\cos\phi}$$
$$= 2\pi \int_0^{\infty} r dr \, U(r) \, J_0(q_r)$$

where

$$\tilde{U}(r) = U_{(0)} e^{-\overline{\xi}} = U_0 \cdot \exp\left(\frac{2W}{\xi}\right) e^{-\overline{\xi}}$$
$$\tilde{U}(q) = 2\pi \xi^2 U_{(0)} \int_0^\infty dt \, t \, e^{-t} \, J_0(q \, \xi \, . \, t)$$

$$\tilde{U}(q) = 2\pi U_0 \exp\left(\frac{2R}{\xi}\right) \xi^2 \int_0^\infty dt \, t \, e^{-t} \, J_0(q \, \xi \, . \, t) \tag{3.14}$$

 U_0 denotes the amplitude of the interaction potential at contact, $U_{(0)}$ is the amplitude of the interaction potential at the origin, ξ is the decay length and R is the hard core radius. We use in eq. 3.14 the combination of U_0 and ξ obtained in the Lado procedure and append the model to the U(q) calculated by the RPA method for data comparison. We apply this process to all our data series.

3.5 Results

3.5.1 Gramicidin/ $C_{12}E_4$

For gramicidin channels in $C_{12}E_4$ bilayers, the samples contain 50 % W% of H_2O and three different number density of inclusions were used: n = 0.00074, 0.00098 and 0.0015 Å⁻², corresponding to P/L values of respectively 0.037, 0.053, 0.099. In figure 3.6 we can see the experimental structure factors, shown as dots for the three concentrations

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		$q_{max} (Å)^{-1}$		
Temp	n (Å ⁻²)	0.00074	0.00098	0.0015
30°		0.232	0.249	0.295
40°		0.234	0.249	0.298
50°		0.237	0.255	0.303
60°		0.236	0.26	0.22

TABLE 3.1: Position of the gramicidin structure factor maximum q_{max} at the different temperatures for the three number densities n in Figure 3.6.

(in red, black and green from the lowest to the highest concentration respectively), in dashed lines the hard disk fit done with a variable radius fit parameter and a fixed number density and in solid line the model fit obtained by the Lado procedure. These structure factors were obtained at four different temperatures, from 30°C up to 60°C.



FIGURE 3.6: Experimental structure factors of gramicidin inclusions in $C_{12}E_4$ membranes at four temperatures: the dots represent the experimental S(q) at the three concentrations (in red, black and green from the lowest to the highest concentration respectively), the dashed lines the hard disk fit and the full lines the model fit obtained by the Lado procedure.

Looking at the curves it is very clear that for each temperature: the structure factor differs for the three concentrations and the peak position q_{max} increases with the concentration, as can be seen in Table 3.1. Furthermore, as the temperature increases the peak flattens, signaling a decrease in the interaction.

The hard-disk model, shown in dashed line, does not fit well the experimental curves, especially at higher temperature. The model obtained with an additional soft interaction fits well the experimental data for the three concentrations at 30°C. Then, with increasing temperature, the model of the highest concentration fits badly the experimental S(q) and this is due to the interaction fraction fr, defined previously in Equation 3.8, that was fixed to zero for the highest concentration. For all our simulations with the various membranes and inclusions, we have used the following fr values : $\{1, 0.5, 0\}$ for respectively the lowest, intermediate and highest concentration of inclusions. For systems with 4 inclusions concentration the range of fr used is: $\{1, 0.5, 0.25, 0\}$. The best model fits at each temperature using these fr values were obtained for the following interaction amplitude and its corresponding decay length summarized in Figure 3.7:

- T=30°C : $U_0 = 9 k_B T$; $\xi = 1.5 \text{ Å}$; $\chi^2 = 0.1$
- T=40°C : $U_0 = 6 k_B T$; $\xi = 2 \text{ Å}$; $\chi^2 = 0.4$
- T=50°C : $U_0 = 2 \ k_B T$; $\xi = 5 \ \text{\AA}$; $\chi^2 = 0.8$
- T=60°C : $U_0 = 1.5 \ k_B T$; $\xi = 11 \ \text{\AA}$; $\chi^2 = 5.6$



FIGURE 3.7: Comparison between the interaction potentials estimated via the Lado algorithm for gramicidin channels in $C_{12}E_4$ membranes at different temperatures.

From these values, one can deduce that the interaction weakens at higher temperature. We observe that the potential decreases with temperature whereas the range increases. This result can be explained by the fact that at high temperatures the elastic constants of the membrane decrease. These results for the in-plane interaction potential of gramicidin channels in $C_{12}E_4$ membranes are summarized in Figure 3.7. The parameters of Equation 3.7 are the potential value at contact $U_0 = u(2R)$ (a distance indicated on the graph) and the exponential decay length ξ , which can be found by $\xi = x_{int} - 2R$, where x_{int} is the intercept of the tangent at contact. An example is plotted for $T = 50^{\circ}$ C in Figure 3.7.

We see that when ξ increases the tangent at the origin is less steep and thus the potential decreases more slowly, covering a wider range.

To find the best fit, I have done many tests using different fr values, for two different estimated areas per $C_{12}E_4$ surfactant. An example is given in Table 3.2 for these different tests results at T=30°C and 40°C. We see clearly the dependence of the potential on this interaction fraction parameter. We have different values of the potential intensity and range for each fr and for each area per surfactant. The potential is larger at $A = 46 \text{ Å}^2$ than at 41.1 Å^2 , whereas the exponential decay is independent of A.

I finally used the area per surfactant $A = 41.1 \text{ Å}^2$ as justified in the previous chapter (Chapter 2, § 2.1.2.1) and the following fr values for all our systems $\{1,0.5,0\}$.

Previously we have presented the results of the interaction potentials obtained via the Lado procedure (numerical method). In order to check our results, we calculate the interaction potential via an analytical process the RPA method and plot the resulted U(q) along with a model. The latter is nothing but the Fourier transform of the interaction potential measured via the Lado procedure as explained in details earlier in § 3.4.2.3. In Figure 3.8 we show the potential U(q) for each series of inclusion at the four temperatures. These graphs show a weakening in the potential intensity as a function of temperature. At high temperature of 60°C the U(q) of the highest concentration is negative but still generally the different potentials follow the trace of the Lado model. More particularly, we observe that the Lado model describes best the U(q) calculated by RPA for the lowest inclusion density. Note that the RPA is valid for sufficient low concentration and for long-ranged interaction at low amplitude.

In the literature, this membrane-mediated interaction is generally explained in terms of the membrane deformation induced by the inclusions, in particular by locally changing its hydrophobic thickness to match that of the inclusion (hydrophobic matching). This deformation has a certain lateral extension (determined by the elastic properties of the membrane) and therefore induces an in-plane interaction between inclusions in the plane of the membrane with a comparable range.

In 1983, Elliott *et al.* estimated the hydrophobic length of gramicidin as $h_G = 22$ Å and recently Kurtisovski *et al.* measured the aliphatic chain lengths L of some C_iE_j-water lamellar phases [96] and found $L_{C_{12}E_4} = 8.6$ Å resulting to a hydrophobic length of

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FIGURE 3.8: Comparison between the interaction potentials estimated via the RPA approximation for the three gramicidin concentrations in $C_{12}E_4$ membranes.

	$T=30^{\circ}C$				T=4	0°C		
	A = 4	1.1\AA^2	A = 4	46\AA^2	A = 41	1.1\AA^2	A = 4	6\AA^2
fr	U_0	ξ	U_0	ξ	U_0	ξ	U_0	ξ
a) {1,0.5,0}	9	1.5	13.5	1.5	6	2	10	1.5
b) $\{1,1,1\}$	7	1.5	15	1.5	5	2	11	2
c) {0.7,0.4,0.03}	7	2.5			4.5	3		
d) $\{0.8, 0.4, 0.07\}$			6	2.3			4.5	2.5
e) $\{0.43, 0.2, 0\}$	21.5	1.5			14	2		

TABLE 3.2: The different U_0 and ξ prefactor values obtained at 30°C and 40°C for the different simulations tests performed using different fr values and for both estimated surfactant polar head area 41.1 Å² and 46 Å². In a) and b) the fr values were estimated, in c) the values were calculated using equation 3.8 from the number density n, in d) and e) the fr was calculated from Ref. [84].

 $h_{C_{12}E_4} = 17.2$ Å. I have experimentally measured the thickness of $C_{12}E_4$ membranes by SAXS using the Bragg peak position for a sample of pure $C_{12}E_4$ membranes hydrated with 50% W% of H₂O and for the $C_{12}E_4$ membranes embedded at the different concentrations of inclusions, see Table 3.3. I measured $q_0(\text{\AA}^{-1})$ of the Bragg peak, and then

determined the membrane thickness $\delta = \Phi_{mb} \cdot d$ where Φ_{mb} is the membrane volume fraction and d is the periodicity $d = 2\pi/q_0$.

n $[10^{-2} \text{ Å}^{-2}]$	δ [Å]
0	29.089
0.074	25.730
0.098	25.732
0.15	25.19

TABLE 3.3: Variation of the $C_{12}E_4$ membrane thickness δ with the gramicidin pore density n.

We measured a thickness of 29Å in pure $C_{12}E_4$ membranes and then adding gramicidin reduces the thickness to 25Å. This is a proof that the $C_{12}E_4$ membranes are deformed to match the hydrophobic thickness of gramicidin pores.

Recently, Constantin measured the in-plane interaction for gramicidin channels at room temperature (~ 22°C) using the same method [84]. He fitted his experimental data well using the decreasing exponential model. The best fits were obtained for $U_0 =$ $31.5 \pm 10k_BT$ and $\xi = 2.5 \pm 0.5$ Å for DLPC bilayers and similarly $U_0 = 27 \pm 10k_BT$ and $\xi = 2.75 \pm 0.5$ Å for C₁₂E₅ bilayers. In both cases, his amplitudes and ranges are larger than what I measured at T=30°C, although one would expect similar values for C₁₂E₄ and C₁₂E₅. However, surfactants of the C_iE_j family are very sensitive to the temperature, so a 10°C difference can change the membrane properties considerably.

3.5.2 Gramicidin/ $C_{12}E_4$ /cholesterol

For gramicidin/ $C_{12}E_4$ /cholesterol, the system is hydrated at 50 % W% of H₂O and three different number density of inclusions were used: n = 0.0005, 0.00075 and 0.001 Å⁻² corresponding to P/L values of respectively 0.02, 0.03 and 0.04. Note that we have used 1 molecule of cholesterol for two molecules of $C_{12}E_4$. In Figure 3.9 we show as dots the experimental structure factors for the three concentrations (in red, black and green from the lowest to the highest, respectively), as dashed lines the hard disk fit done with a variable radius fit parameter and a fixed number density and as solid line the model fit using the Lado procedure. The structure factors presented were obtained at three different temperatures $T = 40^{\circ}$ C, 50°C and 60°.

In the presence of cholesterol, the shape of the curves changes significantly. The peak is wide and not very well defined, so that its position cannot be precisely quantified, but we observe the same tendency as in the previous system: a shift towards higher q as the density increases and as the temperature rises. We also see that neither the hard disk nor the Lado model fit the curves well at high temperature, especially since the

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FIGURE 3.9: Experimental structure factors of gramicidin inclusions in a bilayer mixture of $C_{12}E_4$ /cholesterol at three temperatures: the dots represent the experimental S(q) for the three concentrations (in red, black and green from the lowest to the highest, respectively), the dashed lines are the hard disk fit and the solid lines are the model fit obtained by the Lado procedure.

structure factor at the highest density has a different shape at $T=60^{\circ}$, a temperature close to the phase transition of the $C_{12}E_4$ lamellar phase, as can been seen in the phase diagram [95] (see Figure 2.1a).

The fit of the structure factor with a hard core plus an additional interaction, yielded the amplitudes and decay lengths summarized in Figure 3.10. The potential is almost zero.

- T=40°C : $U_0 = 0.5 \ k_B T$; $\xi = 0.5 \ \text{\AA}$; $\chi^2 = 1.5$
- T=50°C : $U_0 = 0.5 \ k_B T$; $\xi = 0.5 \ \text{\AA}$; $\chi^2 = 1.6$
- T=60°C : $U_0 = 0.2 \ k_B T$; $\xi = 0.2 \ \text{\AA}$; $\chi^2 = 2.41$

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FIGURE 3.10: Interaction potentials estimated via the Lado algorithm for gramicidin channels in $C_{12}E_4$ membranes in the presence of cholesterol at different temperatures.

n $[10^{-2} \text{ Å}^{-2}]$	δ [Å]
0	30.23
0.054	40.81
0.075	46.86
0.1	44.69

TABLE 3.4: Variation of the C₁₂E₄/Cholesterol membrane thickness δ with the gramicidin pore density n.

In an attempt to verify our data, we calculated the interaction potential via the RPA method. U(q) shown in Figure 3.11. We see that the $\tilde{U}(q)$ of the Lado potential is null and does not fit with the U(q) from the RPA. Here again, as in the gramicidin/C₁₂E₄ we have a negative potential for the highest pore density at T=60°.

This almost nul interaction potential can be justified by the fact that adding cholesterol expands the membrane thickness. So maybe in our case the hydrophobic length of the membrane corresponds to that of gramicidin and thus there is no hydrophobic matching and no membrane-mediated interaction. To verify this assumption about the expanding membrane thickness, we calculated the membrane thickness δ from our SAXS measurements for the three concentrations at 40°C we find an increasing membrane thickness and validate our assumption as shown in Table 3.4. We pass from a thickness of 30Å for pure C₁₂E₄/Cholesterol membrane to a thickness of 44.69Å at the highest inclusion density. We observe an increase in the membrane thickness at the second inclusion concentration, and then a decrease at the third and highest density. This effect is also observed in the gramicidn/C₁₂E₄.

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FIGURE 3.11: Comparison between the interaction potentials calculated via the RPA approximation for the three gramicidin concentrations in $C_{12}E_4$ membranes in presence of cholesterol plotted along $\tilde{U}(q)$ resulted from the Lado procedure

3.5.3 BuSn/C₁₂E₄

For BuSn $/C_{12}E_4$, the samples are hydrated at 50 % W% of H₂O and four different number densities of inclusions were initially used: n = 0.0004, 0.001, 0.0018 and 0.003 Å⁻², corresponding to P/L values of respectively 0.0096, 0.022, 0.037 and 0.062. In Figure 3.12 we show the experimental structure factors as solid lines for the four concentrations (in blue, green, black and red from the lowest to the highest value, respectively) and as dashed lines the hard disk fit; The lines with dots show the model fit with the Lado procedure. These structure factors were obtained at four different temperatures, from 30° C up to 60° C.

By simple inspection the structure factors, we can see the same tendency as in the previous systems in terms of variation with concentration and temperature. The hard disk model fits shown on the graphs were performed with a free number density fit parameter. The fits obtained correspond better to the experimental curves than the model with the additional interaction. In fact, after preparing the BuSn $C_{12}E_4$ membranes, I

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FIGURE 3.12: Experimental structure factors of BuSn inclusions in $C_{12}E_4$ bilayers at four temperatures: solid lines, experimental S(q) values at the four concentrations (in red, black, green and blue from the lowest to the highest value, respectively). Dashed lines show the hard disk fit and lines with dots show the model fit obtained by the Lado procedure.

observed via polarized light microscopy that the particles are not well dispersed in the samples. For this reason, I decided to prepare BuSn inclusions in Brij30 membranes. In the latter, the samples were very well homogeneous and were perfectly aligned into homeotropic anchoring. The problem is that we did not perform the aquisition for the Brij30 BuSn membranes at enough time. The scattered intensity obtained is too low to be treated. Regardless, I present the data of $BuSn/C_{12}E_4$ since I had already treated them and tried to figure out if we can extract some information. So since the particles are not well dispersed, this is why we fit the curves with a free number density fit parameter and obtain a value of around 0.0003 \AA^{-2} for the four samples meaning that the concentration of the inclusions is almost the same in the four systems. The Lado fits shown in Figure 3.12 are quite bad. The corresponding amplitudes, decay lengths and goodness-of-fit χ^2 values are presented below and summarized in Figure 3.13. We observe a decrease in the interaction potential amplitude and decay length with a high value of χ^2 .

- T=30°C : $U_0 = 2 \ k_B T$; $\xi = 15 \ \text{\AA}$; $\chi^2 = 23.4$
- T=40°C : $U_0 = 1.5 \ k_B T$; $\xi = 15 \ \text{\AA}$; $\chi^2 = 22.2$
- T=50°C : $U_0 = 1.5 \ k_B T$; $\xi = 15 \ \text{\AA}$; $\chi^2 = 21.78$
- T=60°C : $U_0 = 1 \ k_B T$; $\xi = 8 \ \text{\AA}$; $\chi^2 = 23$



FIGURE 3.13: Interaction potentials estimated via the Lado algorithm for BuSn inclusions in $C_{12}E_4$ membranes at different temperatures.

On the other hand, the potential estimated via the RPA approximation in Figure 3.14 show very good affinity with the Lado $\tilde{U}(q)$ model. Let us now compare the two types of inclusions (gramicidin and BuSn) embedded in $C_{12}E_4$ membranes at the same membrane hydration level. The interaction potential results are different which is expected since the geometry of the membrane around the inclusion is different: gramicidin resembles a cylinder with an effective radius R = 9.5 Å, and a hydrophobic thickness slightly larger than that of the membrane, while the BuSn can be seen as small spheres with radius R = 4.5 Å embedded in the alkyl medium of the membrane.

3.5.4 BuSn/C₁₂E₄/cholesterol

For BuSn/C₁₂E₄/cholesterol, the system is hydrated at 50 % W% of H₂O and four different number density of inclusions were used: n = 0.00016, 0.00051, 0.00066 and 0.00083 Å⁻² corresponding to P/L values of respectively 0.00637, 0.0197, 0.0259 and 0.032. Note that we have a ratio of one molecule of cholesterol over two molecules of C₁₂E₄. In Figure 3.15 we show the experimental structure factors as solid lines for the

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FIGURE 3.14: Interaction potentials estimated via the RPA approximation for the three BuSn concentrations in $C_{12}E_4$ membranes.

four concentrations (in blue, green, black and red from the lowest to the highest value, respectively), as dashed lines the hard disk fit done with a variable radius fit parameter and a fixed number density and as lines with dots the model fit obtained by the Lado procedure. These structure factors are presented at six experimental temperatures, from 30°C up to 80°C. Except for the highest density value, both fits agree well with the experimental data. The best fit of this system at each temperature was obtained for the amplitudes and decay lengths summarized in Figure 3.16:

- T=30°C : $U_0 = 12 \ k_B T$; $\xi = 9 \ \text{\AA}$; $\chi^2 = 25$
- T=40°C : $U_0 = 10 \ k_B T$; $\xi = 10 \ \text{\AA}$; $\chi^2 = 0.82$
- T=50°C : $U_0 = 15 \ k_B T$; $\xi = 5 \ \text{\AA}$; $\chi^2 = 0.99$
- T=60°C : $U_0 = 11 \ k_B T$; $\xi = 5 \ \text{\AA}$; $\chi^2 = 1.22$
- T=70°C : $U_0 = 11 \ k_B T$; $\xi = 6 \ \text{\AA}$; $\chi^2 = 1.04$
- T=80°C : $U_0 = 4 k_B T$; $\xi = 4 \text{ Å}$; $\chi^2 = 0.66$



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FIGURE 3.15: Experimental structure factors of BuSn inclusions in bilayers consisting of $C_{12}E_4$ /Cholesterol, at six temperatures: the solid lines are the experimental S(q) of the three concentrations (in red, black, green and blue from the lowest to the highest value, respectively), the dashed lines are the hard disk fits and the lines with dots are the model fits using the Lado procedure.

For $T = 30 \,^{\circ}\text{C}$ we have a very high value for the goodness-of-fit χ^2 . If we discard this temperature, we see that the potential increases from 10 $k_{\text{B}}T$ at $T = 40 \,^{\circ}\text{C}$ to 15

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FIGURE 3.16: Interaction potentials estimated via the Lado algorithm for BuSn inclusions in $C_{12}E_4$ membranes in the presence of cholesterol at different temperatures.

 k_BT at T = 50 °C and then drops to 4 k_BT at T = 80 °C. Similarly, the decay length decreases along the temperature. Furthermore, as a function of temperature, we see in Figure 3.17 that the fastest to tend to zero are the highest temperatures and we seem to have two different behaviors separated between low temperatures (for T = 30 °C and 40 °C) and high temperatures (for T = 50 °C to T = 70 °C). The potential of T=80°C is weaker. An important comparison to be made is between the potentials obtained for the gramicidin/C₁₂E₄/cholesterol system, where we have almost null potential $U_0 = 0.5$ k_BT ; $\xi = 0.5$ and the same membrane mixture embedded with BuSn we have high interaction potential. Note that in Figure 3.17 we have a very good agreement between the U(q) calculated by the RPA approximation and the $\tilde{U}(q)$ calculated by Lado. $\tilde{U}(q)$ describes well the U(q) of the lowest inclusion concentration.

3.5.5 BuSn/Brij30/Cholesterol

For BuSn/Brij30/cholesterol, the system is hydrated at 50 % W% of H₂O and four different values of the number density of inclusions were used: n = 0.0002, 0.0004, 0.0006 and 0.0008 Å⁻², corresponding to P/L values of respectively 0.0054, 0.0117, 0.0172 and 0.0237. Note that in this case we have prepared a ratio of 1 molecule of cholesterol to 4 molecules of Brij30. In Figure 3.18 we show the experimental structure factors as solid lines for the four concentrations (in blue, green, black and red from the lowest to the highest value, respectively), as dashed lines the hard disk fit done with a variable radius fit parameter and a fixed number density and as lines with dots the model fit obtained by the Lado procedure. These structure factors are presented at three experimental temperatures 30°C, 50°C and 70°C.

As for all our cholesterol mixtures, the curves have a particular shape, with a very wide peak. In Figure 3.18 we see that the structure factor at the lowest concentration



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FIGURE 3.17: Interaction potentials estimated via the RPA approximation for the three BuSn concentrations in $C_{12}E_4$ membranes in the presence of cholesterol.

looks more like the curves in absence of cholesterol and we see that for this curve the calculated U(q) by RPA method in Figure 3.20 is in bad agreement with the $\tilde{U}(q)$ model calculated by Lado method.

The structure factor fits for the BuSn/Brij30/cholesterol system, both with the hard disk model and the Lado procedure, do not match very well the experimental data

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FIGURE 3.18: Experimental structure factors of BuSn inclusions in bilayers of Brij30/Cholesterol, at three temperatures: the dots are the experimental S(q) at the three concentrations (in red, black and green from the lowest to the highest value, respectively), the dashed lines are the hard disk fit and the solid lines correspond to the model fit obtained by the Lado procedure.

(Figure 3.18). The parameters for the interaction model and the corresponding goodnessof-fit function χ^2 are presented below and summarized in Figure 3.19.

- T=30°C : $U_0 = 3 k_B T$; $\xi = 10$; $\chi^2 = 10.9$
- T=50°C : $U_0 = 10 \ k_B T$; $\xi = 5 \ \text{\AA}$; $\chi^2 = 10.12$
- T=70°C : $U_0 = 11 \ k_B T$; $\xi = 5 \ \text{\AA}$; $\chi^2 = 10$

In this case we have a different behavior then observed before, the interaction potential amplitude increases along the temperature while the decay length decreases.

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FIGURE 3.20: Interaction potentials estimated via the RPA approximation for the four BuSn concentrations in Brij30 membranes in the presence of cholesterol.

3.6 Discussion and Conclusion

To summarize the results previously presented in this chapter, we have shown so far that the in-plane interaction, between both BuSn hybrid inclusions and gramicidin peptides in $C_{12}E_4$ membranes with and without cholesterol, decreases along the temperature. For Gramicidin/ $C_{12}E_4$, $V_0(r)$ drops from $9k_BT$ at 30°C to $1.5k_BT$ at 60°C while its range goes up from 1.5Å to 11Å. Once adding cholesterol to the latter system, we measure almost no interaction, a potential of an amplitude $U_0(r) = 0.5k_BT$. For the pure $C_{12}E_4$ membranes embedded with gramicidin, the temperature reduces the interaction due to the decrease of the elastic constants and thus of the interaction (see Figure 3.21a for a schematic representation of the membrane geometry). But in the case of cholesterol, it is known that cholesterol tends to rigidify and reduce the membranes and so increase the elastic constant. Hence we were expecting a stronger interaction. But it is not the case here. It could be explained by the fact that cholesterol decreases the hydrophobic mismatch and so the bilayer thickness is then close to that of the gramicidin pore and thus the interaction decreases (see Figure 3.21b for a schematic representation of the membrane geometry).



FIGURE 3.21: Schematic representation of the $C_{12}E_4$ membrane embedded with gramicidin (A) in absence of cholesterol and (B) in presence of cholesterol

We also measured the membrane thickness in both cases and found that in absence of cholesterol the membrane thickness decreases from 29 Å in pure $C_{12}E_4$ membranes to 25Å at the highest concentration of inclusion. In presence of cholesterol, we have the opposite effect, the membrane increases from 31 Å in pure $C_{12}E_4$ /cholesterol membranes to 44.69 Å at the highest concentration of inclusion. In both cases we have an increase of the membrane thickness when passing from the lowest inclusion concentration to the medium one and then a decrease when passing to the third and highest inclusion concentration.

Moreover, we studied the BuSn $C_{12}E_4$ Cholesterol and the BuSn Brij30 Cholesterol. In both cases we have a decrease decay length but the interaction amplitude decreases $U_0 = 12k_BT$ at 30°C to $U_0 = 4k_BT$ at 80°C for the BuSn $C_{12}E_4$ Cholesterol wherease the amplitude increases from $U_0 = 3k_BT$ at 30°C to $U_0 = 11k_BT$ at 70°C for the BuSn Brij30 Cholesterol.

As we can see Brij30 and $C_{12}E_4$ membranes don't yield the same results when adding BuSn in presence of cholesterol though these two molecules are chemically very close with the presence of impurities in the case of Brij30. Additionally, the latter is sold as an alternative for $C_{12}E_4$ since it is much cheaper. Although I have previously encountered this difference between the two molecules when preparing the membrane samples. Actually I found that these two molecules have a different maximum inclusion concentration dispersion capacity. When preparing the membranes, as mentioned previously in chapter 2, section 2.1.2.1, the BuSn particles do not disperse well for a density higher than $n = 0.0003 \text{Å}^{-2}$ while Brij30 membranes can contain a concentration of inclusion up to $n = 0.0018 \text{Å}^{-2}$. So this difference in the interaction potential can be well justified. Additionally, we have used a different ratio of cholesterol content in each sample, we have one molecule of cholesterol over two molecules of $C_{12}E_4$ while we prepared samples with one molecule of cholesterol over four molecules of Brij30. This shows that the smallest details in the membrane composition like small impurities can have important effect on the interaction.

Furthermore, looking at the $C_{12}E_4$ Cholesterol membranes embedded with two different inclusions yield different interaction potential between these inclusions. More particularly, the BuSn hybrid particles which are localized in the vicinity of the membrane, separate the two layers from the inside and thus induce a thickening in the membrane and hence an interaction potential varying from $U_0 = 12k_BT$ at 30°C to $U_0 = 4k_BT$ at 80°C (see Figure 3.22). However, for the gramicidin pore with a transmembrane hydrophobic domain, the cholesterol presence already increases the hydrophobic length of the membrane and it seems that the thickness corresponds to the hydrophobic dimension of the pores and thus we have a much reduced interaction potential of $U_0 = 0.5k_BT$ (Figure 3.21a). Thereupon, we can validate that the membrane geometry, the inclusions shape, the cholesterol content all are parameters that influence directly the membrane-mediated interaction between inclusions in membranes.

To conclude, we were able to measure the membrane-mediated interaction potential in the plane of the membrane and quantify it using the Ornstein-Zernike integral equation with the Percus-Yevick closure for five different systems at several inclusion concentrations, as a function of temperature. We showed that this potential varies with the temperature and with the membrane composition. We also proved that, for the same membrane composition, different inclusions (that induce different deformations) yield clearly different interaction potentials.



FIGURE 3.22: Schematic representation of a $C_{12}E_4$ /Cholesterol membrane embedded with BuSn inclusions

Chapter 4

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4.1 Introduction

The data presented in Chapter 3 was obtained in normal incidence, a configuration that only gives access to the (q_x, q_y) plane of reciprocal space. If the inclusions only interact within the (x, y) plane of the bilayer via a potential $V_0(r)$, this information is sufficient to fully characterize V_0 , which is the main focus of our study. If, however, the nano-objects also interact via a potential $V_1(r)$ with their counterparts in neighboring layers of the lamellar stack, i.e. at different heights z, the variation of the structure factor S with q_z is also needed, not only to determine $V_1(r)$, but also –and more importantly– to accurately measure $V_0(r)$. Indeed, as we will see below, the two types of interaction are intertwined and only a model for the full structure factor $S(\vec{q})$ can separate their effects.

In this Chapter, we measure $S(\vec{q})$ for systems with interlayer interaction and extract both the in-plane and the interlayer interactions, $V_0(r)$ and $V_1(r)$.

4.2 Scattering geometry and data transformation

The experimental configuration is modified simply by turning the sample with respect to the incoming beam, and thus gaining access to the complete structure factor $S(\vec{q})$. The aligned sample was oriented with an angle α between its normal \vec{n} and the incident x-ray beam (with wave vector \vec{k}_i). In fact, the normal incidence configuration is a special case of this technique, with $\alpha = 0$.

Both techniques use the same instrument, described in § 2.6, and the same sample capillaries, prepared according to § 2.2. The scattering pattern was recorded on a 2D detector of size X = 1340 pixels and Y = 1300 pixels. A photograph of the experimental setup is shown in Figure 4.1. A point on the 2D detector uniquely defines an outgoing wave vector \vec{k}_o for the scattered signal, and thus the scattering vector is $\vec{q} = \vec{k}_o - \vec{k}_i$ (Figure 4.2). In the reference system of the sample, \vec{q} generally has components both in the plane of the layers and along the normal, denoted by \vec{q}_r and q_z respectively.



FIGURE 4.1: Photos of the SAXS experiment setup at the BM02 (D2AM) beamline of the ESRF.

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FIGURE 4.2: Scattering setup. D is the detector. S is the sample. The dashed lines show the sample orientation in the off-plane scattering geometry.

The Fourier transform of the density from the scattering of our lamellar phases can be depicted in Figure 4.3. Scattering in reciprocal space (purple cylinder in Figure 4.3) is located essentially in two regions: first, the Bragg peaks (along q_z , presented as black dots in Figure 4.3) coming from the layers stack and second a cylindrical region, related to the particles confined in the lamellae. Scattering intensities are recorded when the reciprocal space intersect with the Ewald sphere. The scattering angles are small hence only a smaller region of the Ewald sphere is accessible. It is actually almost flat and thus its effect is minimized. Consequently we consider the tangential plane instead of the Ewald sphere. In the in-plane technique with $\alpha = 0$ we probe the plane within the layers along q_r only, the Ewald sphere is contained in the $(q_x - q_y)$ plane and we have a circular ring. Whereas when the sample is tilted we probe outside the in-plane of the layers. When $\alpha = 90^{\circ}$ we probe along q_z only (Bragg peaks). For $0 < \alpha < 90^{\circ}$, the scattering vectors scan both q_r and q_z and thus we have access to more information.

So even the off-plane scattering does not cover the entire reciprocal space and the accessible range is a triangle-shaped region with an angle 2α at the origin [88, 122] (Figure 4.3, 4.4). As an illustration, I show in Figure 4.4 the background-subtracted intensity for gramicidin inclusions in DDAO at a density $n = 1.024 \, 10^{-3} \,\text{\AA}^{-2}$, for a tilt angle $\alpha = 40^{\circ}$.

The signal recorded in the plane of the detector, as a function of the pixel position (X, Y), must be converted into the q_r and q_z components of the scattering vector. From the geometry of the setup (Figure 4.2), q_z is only given by the X coordinate in the image:

$$q_z = (X/A)\sin\alpha \tag{4.1}$$



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FIGURE 4.3: Schematic representation of the scattering of the lamellar phases (blue aligned lines) and the intersection of the Ewald sphere with the reciprocal space, the latter represented as a purple cylinder :(A) for $\alpha = 0$ and (B) for $\alpha \neq 0$. The black dots represent the Bragg peaks

and thus

$$(X/A) = q_z / \sin \alpha \tag{4.2}$$

while q_r combines X and Y via:

$$q_r^2 = (X/A)^2 + (Y/A)^2 \cos^2 \alpha \tag{4.3}$$

and thus yields:

$$(Y/A) = \sqrt{q_r^2 - (q_z/\tan\alpha)^2}$$
 (4.4)

where A is a conversion constant from pixel number to the physical units of reciprocal space (Å⁻¹). Using these relations we can now project the scattering pattern into the (q_r, q_z) plane in order to analyze it. Instead of finding the reciprocal space position of

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FIGURE 4.4: Scattering pattern recorded at a tilt angle $\alpha = 40^{\circ}$. In dashed red lines we present the reciprocal plane defined by the 2α angle at the origin, the horizontal lines represent the q_r coordinates of the corresponding pixels and the ellipsoidal line represent the q_z coordinates passing through the peaks

each physical pixel, it is more convenient [122] to use a regular spacing in q_r and q_z and "regrid" the recorded data to this matrix using Eqs. (4.2) and (4.4).

Two more transformations need to be accounted for: First, the rotation axis is not perfectly vertical (as in Figure 4.2), but rather rotated by an angle Φ (clearly visible in Figure 4.4) and the origin of reciprocal space (given by the primary beam position) is in (X_b, Y_b) . For each pixel in reciprocal space (q_r, q_z) , the corresponding position on the detector is:

$$X' = X_b + X\cos\Phi + Y\sin\Phi \tag{4.5}$$

and

$$Y' = Y_b + Y\cos\Phi - X\sin\Phi \tag{4.6}$$

The final intensity value $I(q_r, q_z)$ is the average over the nine pixels closest to (X', Y'). Figure 4.5 shows the regridding of the data in Figure 4.4.
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FIGURE 4.5: Regridding of the scattering pattern in Figure 4.4. The red dotted lines at constant q_r and q_z correspond to the dotted lines in that image.

4.3 Data treatment

Once the pattern is regridded, we can proceed to the data treatment and analysis. I will use as a demonstration the patterns for the gramicidin/DDAO system at P/L = 0.052, with an incident angle $\alpha = 40^{\circ}$. The same theory is applied to the BuSn inclusions.

In the analysis of scattering data from a multilayer inclusions-surfactant sample, the system is simplified as identical particles confined in evenly-spaced parallel layers. As seen in § 2.5.1, the form factor is the Fourier transform of the electron density contrast $\rho(r)$ with respect to the surfactant background, Eq. (2.5).

BuSn inclusions have full spherical symmetry, and their form factor is well described by a solid sphere with radius R = 4.5 Å, see Eq. (2.6).

For gramicidin channels, the situation is slightly more complicated, but the object has azimuthal symmetry and the form factor can be factorized into two parts that depend only on q_r and q_z , respectively:

$$F(q_r, q_z) = F_r(q_r) \cdot F_z(q_z) \tag{4.7}$$

with $F_r(q_r)$ containing the dependence on $q_r = \sqrt{q_x^2 + q_y^2}$ (in the (x, y) plane of the membrane) and $F_z(q_z)$ describes the variation along q_z .

We used for $F_r^2(q_r)$ the numerical profile obtained by Constantin [84] from the molecular simulation data of de Groot et al. [86]; the curve is shown in Figure 4.6a. For the z

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FIGURE 4.6: A) In-plane form factor of the gramicidin channel $F_r^2(q_r)$ from [84], B) complete form factor $F^2(q_r, q_z)$ of the gramicidin channel C) Form factor of the BuSn particles.

dependence we assume a cylindrical shape, yielding $F_z(q_z) = \frac{\sin(q_z L/2)}{q_z L/2}$, with L = 22 Å the length of the channel [123].

4.3.1 Structure factor in the lamellar phase

Theory

The structure factor describes the distribution of the pores in the layers. In our case, the phase is anisotropic and so is the structure factor $S(q_r, q_z)$, which depends on both the absolute value of the in-plane scattering vector $q_r = \sqrt{q_x^2 + q_y^2}$ (but not on its orientation, since we assume that the inclusions form a liquid in the plane of the layers) and on the scattering vector along the membrane normal q_z .

Since the particles are confined in equidistant layers at precise positions along z, the structure factor can be written as a series of partial structure factors S_{0m} [81, 88]:

$$S(q_r, q_z) = S_0(q_r) + 2\sum_{m=1}^{\infty} \cos(q_z dm) S_m(q_r),$$
(4.8)

where d is the layer spacing and each partial structure factor S_m describing the interaction between particles situated m layers away:

$$S_m(q_r) = \delta_{0m} + 2\pi\rho \int_0^\infty r dr J_0(rq_r) [g_m(r) - 1], \qquad (4.9)$$

where δ_{0m} is the Kronecker symbol, $m \geq 0$ and $g_m(r)$ is the (normalized) probability of finding a particle in bilayer m at an in-plane distance r from a reference particle in bilayer 0. Equation (4.8) is valid for samples with an interlayer distance rigorously equal to d. However, in fluid lamellar phases this distance varies as a result of thermal fluctuations and other defects, an effect we account for by a Lorentzian factor:

$$S(q_r, q_z) = S_0(q_r) + 2\frac{\cos(q_z d)}{1 + (q_z \sigma)^2} S_1(q_r)$$
(4.10)

where σ is a "disorder" parameter and $S_1(q_r)$ is the structure factor between inclusions in adjacent layers: e.g. layer 0 and layers ± 1 . We describe it by a Gaussian function:

$$S_1 = A_1 \exp\left[-\frac{(q - q_{max})^2}{2\Delta q^2}\right]$$
 (4.11)

We will see below that these two partial structure factors $S_0(q)$ and $S_1(q)$ are enough to describe the experimental data, so there is no need to go beyond m = 1.

Data treatment

The regridded intensity $I(q_r, q_z)$ is divided by the form factor $F^2(q_r, q_z)$ of the particle to yield the full structure factor $S(q_r, q_z)$, based on the analysis in the preceding section. As an illustration, we show in Figure 4.7a the structure factor of gramicidin inclusions in DDAO membranes at P/L = 0.052, for an incidence angle $\alpha = 40^{\circ}$ obtained by dividing the regridded intensity in Figure 4.5 by the form factor calculated according to Eq. 4.7 and shown in Figure 4.6b. A modulation along q_z is clearly visible, indicating the presence of the S_1 component in the general structure factor equation (4.8) and hence an interaction between layers. This modulation is analyzed by making a cut along q_z in the structure factor and fitting it with Eq. 4.10 explained in the section below and shown in Figure 4.7b. The agreement is quite good, showing that the structure factor is dominated by its partial components S_0 and S_1 .



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FIGURE 4.7: (A) The structure factor obtained from dividing the regridded intensity in Figure 4.5 by the form factor calculated according to Eq. 4.7. (B) Cut of the structure factor above along q_z for $q_r = 0.23\text{\AA}^{-1}$ (peak position) and fitted with Eq. 4.10.

Absorption correction The scattering signal should be symmetric under $q_z \Rightarrow -q_z$, but we can see clearly in Figure 4.7b that the modulation is not symmetric and it displays a linear slope, probably due to the difference in the path length of the scattered beam through the sample between the positive and negative values of X in Figure 4.2. This

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FIGURE 4.8: Structure factor (from Figure 4.7a) after absorption correction.

effect depends on the scattering direction, the sample thickness and the tilt angle, but we choose to account for it simply by adding a linear background to the model (4.10):

$$A\frac{\cos(q_z d)}{(1+q_z \sigma)^2} + a + bq_z$$

This linear contribution (with the values of a and b obtained by fitting the data) is then subtracted from the whole matrix.

Although the functions of interest are $S_0(q_r)$ and $S_1(q_r)$, experimentally the most accessible quantity is the equatorial cut through reciprocal space:

$$S_{eq} = S(q_r, q_z = 0) = S_0(q_r) + 2S_1(q_r)$$
(4.12)

This is simply the structure factor at normal incidence ($\alpha = 0^{\circ}$), the most reliable configuration since the background subtraction and absorption correction are easiest to perform. Furthermore, the images are isotropic and the angular integration makes for very good signal statistics. To obtain S_1 , we therefore subtract S_{eq} from the experimental $S(q_r, q_z)$ as shown in Figure 4.9a:

$$S(q_r, q_z) - S(q_r, q_z = 0) = 2S_1(q_r) \left[\frac{\cos(q_z d)}{(1 + q_z \sigma)^2} - 1\right]$$
(4.13)

and then we fit it with a version of Eq 4.10. The best fit for the data in Figure 4.9a is given in the image on the right. The agreement between data and the fit is very good, as one can see in the cuts at constant q_r and q_z (Figures 4.9d and 4.9c, respectively). We conclude that the model is accurate: the model (4.11) describes well the q_r dependence



and the q_z oscillation is well accounted for: there is no need to include higher-order partial structure factors in (4.10).

FIGURE 4.9: (A) Comparison between the experimental data (color scale) and the fit (contour levels) for $S(q_r, q_z) - S(q_r, q_z = 0)$. (B) The model of (A) obtained from the fit parameters shown at the same color scale. (C) Cut along q_z at $q_r = 0.23\text{\AA}^{-1}$ through the experimental data and in the fit. (D) Cut along q_r for $q_z = 0.1\text{\AA}^{-1}$.

This treatment yields $S_1(q_r)$, which can then be subtracted from the equatorial structure factor $S_{eq}(q_r)$ to yield $S_0(q_r)$. Figure 4.10 shows the $S_1(q_r)$ at $\alpha = 40^{\circ}$ and 60° and $S_{eq}(q_r)$ functions.

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FIGURE 4.10: Partial structure factors $S_{eq}(q_r)$ and $S_1(q_r)$ at $\alpha = 40^{\circ}$ and 60° .

4.3.2 Interaction within the layer

Once the experimental data for the partial structure factors $S_0(q_r)$ and $S_1(q_z)$ are available, it can be described in terms of the interaction potentials $V_0(r)$ and $V_1(r)$ via well-known methods in the theory of liquids, described in § 2.5.2 and § 2.5.2.1 of Chapter 2. Below I describe the algorithm I used, which is based on the one for the study of the in-plane interaction (see § 3.4.2.1) but extended to take into account the interaction between different layers.

4.3.2.1 Implementation

To begin with, let us consider a system of n layers $\{0,1, ..., n-1\}$ with periodic boundary conditions (i.e. bilayer n is identical to bilayer 0). Let us rewrite the relevant functions (already introduced in Chapter 2) for this system:

- The total correlation function,

$$h_{\alpha\beta}(r) = g_{\alpha\beta}(r) - 1 \tag{4.14}$$

- The Ornstein-Zernike relation,

$$h_{\alpha\beta}(r) = c_{\alpha\beta}(r) + \sum_{\gamma} \rho_{\gamma} \int d^2 \vec{s} \, c_{\alpha\gamma} \left(|\vec{s}| \right) h_{\gamma\beta} \left(|\vec{r} - \vec{s}| \right)$$
(4.15)

where $c_{\alpha\beta}(r)$ defines the direct correlation function c(r) between layers α and β . ρ_{γ} is the number density in the plane of the layers and γ runs over all layers. - The Percus-Yevick closure,

$$c_{\alpha\beta}(r) = \left[1 + h_{\alpha\beta}(r)\right] \left[1 - \exp\left(\frac{V_{\alpha\beta}}{k_B T}\right)\right]$$
(4.16)

where $V_{\alpha\beta}$ defines the interaction potential between particles situated in layers α and β . These equations will be solved numerically. The procedures are based on the work of Lebowitz and Baxter [124, 125]. We will use the notations introduced by Baxter [125], except for the interaction potential that we denote as V(r) (instead of ϕ) following our previous notation.

Here we give an example for n = 3 layers, with periodic boundary conditions: $3 \equiv 0$ and $-1 \equiv 2$. The number density $\rho = \rho_0 = \rho_1 = \rho_2$ supposing that the environment is identical for all layers. Equations 4.15 and 4.16 then write as:

in reciprocal space:
$$h_{00}(q) = c_{00}(q) + \rho \left(c_{00}h_{00} + 2c_{01}h_{01} \right)$$
 (4.17a)

$$h_{01}(q) = c_{01}(q) + \rho \left(c_{00}h_{01} + c_{01}h_{00} + c_{01}h_{01} \right)$$
(4.17b)

in real space:
$$c_{00}(r) = [1 + h_{00}(r)] \left[1 - \exp\left(\frac{V_0}{k_B T}\right) \right]$$
 (4.17c)

$$c_{01}(r) = [1 + h_{01}(r)] \left[1 - \exp\left(\frac{V_1}{k_B T}\right) \right]$$
 (4.17d)

Following Lado [120], we will work with the indirect correlation function $\gamma_{\alpha\beta}(r) = h_{\alpha\beta}(r) - c_{\alpha\beta}(r)$, for convergence reasons. Rewriting (4.17) in these terms yields in reciprocal space:

$$\gamma_{00}(q) = \rho \left[c_{00} \left(\gamma_{00} + c_{00} \right) + 2c_{01} \left(\gamma_{01} + c_{01} \right) \right]$$
(4.18a)

$$\gamma_{01}(q) = \rho \left[c_{00} \left(\gamma_{01} + c_{01} \right) + c_{01} \left(\gamma_{00} + c_{00} \right) + c_{01} \left(\gamma_{01} + c_{01} \right) \right]$$
(4.18b)

while in real space:

$$c_{00}(r) = (1 + \gamma_{00} + c_{00}) \left(1 - e^{V_0/k_B T} \right) \implies c_{00} - c_{00} \left(1 - e^{V_0/k_B T} \right) = (1 + \gamma_{00}) \left(1 - e^{V_0/k_B T} \right)$$
$$\implies c_{00} e^{V_0/k_B T} = (1 + \gamma_{00}) \left(1 - e^{V_0/k_B T} \right)$$

and therefore,

$$c_{00}(r) = (1 + \gamma_{00}) \left(e^{-\frac{V_0}{k_B T}} - 1 \right)$$
(4.19a)

$$c_{01}(r) = (1 + \gamma_{01}) \left(e^{-\frac{V_0}{k_B T}} - 1 \right)$$
(4.19b)

We start by applying (4.19a) and (4.19b) in real space and then solve (4.18a) and (4.18b) in reciprocal space:

$$\gamma_{00} \left(1 - \rho c_{00} \right) = \rho \left(c_{00}^2 + 2c_{01}^2 \right) + 2\rho c_{01} \gamma_{01}$$
(4.20a)

$$0 = \gamma_{01} \left(\rho c_{00} + \rho c_{01} - 1 \right) + \rho c_{01} \gamma_{00} + \rho \left(2c_{00} c_{01} + c_{01}^2 \right).$$
(4.20b)

Dividing both equations by ρ yields:

$$\gamma_{00} \left(c_{00} - 1/\rho \right) + 2\gamma_{01}c_{01} + \left(c_{00}^2 + 2c_{01}^2 \right) = 0 \tag{4.21a}$$

$$\gamma_{00}c_{01} + \gamma_{01}\left(c_{00} + c_{01} - 1/\rho\right) + \left(2c_{00}c_{01} + c_{01}^2\right) = 0 \tag{4.21b}$$

To eliminate the term γ_{00} we subtract (4.21b) and (4.21a) after multiplication by $c_{00}-1/\rho$ and c_{01} , respectively:

$$\begin{split} \gamma_{00}c_{01}\left(c_{00}-1/\rho\right) &+ \gamma_{01}\left(c_{00}-1/\rho\right)\left(c_{00}+c_{01}-1/\rho\right) + \left(c_{00}-1/\rho\right)\left(2c_{00}c_{01}+c_{01}^{2}\right) \\ &- \gamma_{00}c_{01}\left(c_{00}-1/\rho\right) - \gamma_{01}.2c_{01}^{2} - c_{01}\left(c_{00}^{2}+2c_{01}^{2}\right) = 0 \implies \\ \gamma_{01}\left[\left(c_{00}-1/\rho\right)^{2} + c_{01}\left(c_{00}-1/\rho\right) - 2c_{01}^{2}\right] + 2\left(c_{00}^{2}c_{01}+c_{00}c_{01}^{2} - \frac{2c_{00}c_{01}}{\rho} - \frac{c_{01}^{2}}{\rho} - c_{00}^{2}c_{01} - 2c_{01}^{3} = 0 \\ \implies \gamma_{01}\left[\left(c_{00}-1/\rho\right)^{2} + c_{01}\left(c_{00}-1/\rho\right) - 2c_{01}^{2}\right] = 2c_{01}^{3} + \frac{c_{01}^{2}}{\rho} + \frac{2c_{00}c_{01}}{\rho} - c_{00}^{2}c_{01} - c_{00}c_{01}^{2} \\ &= c_{01}\left[2c_{01}^{2} + \frac{c_{01}}{\rho} + \frac{2c_{00}}{\rho} - c_{00}^{2} - c_{00}c_{01}\right] \\ &= c_{01}\left[2c_{01}^{2} + \frac{c_{01}}{\rho} + \frac{2c_{00}}{\rho} + \frac{1}{\rho}\left(c_{00}+c_{01}\right) - c_{00}\left(c_{00}+c_{01}\right)\right] \end{split}$$

$$\implies \gamma_{01} = \frac{c_{01} \left[2c_{01}^2 + \frac{c_{00}}{\rho} + \frac{1}{\rho} \left(c_{00} + c_{01} \right) - c_{00} \left(c_{00} + c_{01} \right) \right]}{\left(c_{00} - 1/\rho \right)^2 + c_{01} \left(c_{00} - 1/\rho \right) - 2c_{01}^2}$$
(4.22)

To eliminate γ_{01} we proceed as follows: $(c_{00} + c_{01} - 1/\rho) \cdot (4.21a) - 2c_{01} \cdot (4.21b)$:

$$\gamma_{00} (c_{00} + c_{01} - 1/\rho) (c_{00} - 1/\rho) + (c_{00} + c_{01} - 1/\rho) (c_{00}^2 + 2c_{01}^2) - \gamma_{00} c_{01}^2 - 2c_{01} (2c_{00}c_{01} + c_{01}^2) = 0$$

$$\gamma_{00} \left[\left(c_{00} - 1/\rho \right)^2 + c_{01} \left(c_{00} - 1/\rho \right) - 2c_{01}^2 \right] = 2c_{01} \left(2c_{00}c_{01} + c_{01}^2 \right) - \left(c_{00} + c_{01} - 1/\rho \right) \left(c_{00}^2 + 2c_{01}^2 \right) = 4c_{00}c_{01}^2 + 2c_{01}^3 - c_{00}^2 \left(c_{00} + c_{01} - 1/\rho \right) - 2c_{00}c_{01}^2 - 2c_{01}^3 + 2c_{01}^2/\rho = 2c_{01}^2 \left(c_{00} + 1/\rho \right) - c_{00}^2 \left(c_{00} - 1/\rho \right) - c_{00}^2 c_{01}$$

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$$\implies \gamma_{00} = \frac{-c_{00}^2 \left(c_{00} + c_{01} - 1/\rho\right) + 2c_{01}^2 \left(c_{00} + 1/\rho\right)}{\left(c_{00} - 1/\rho\right)^2 + c_{01} \left(c_{00} - 1/\rho\right) - 2c_{01}^2}$$
(4.23)

We solved the equations for a number of layers n = 3, 4, 5 and 7. Below we give without proof the solutions for n = 7:

$$\gamma_{00} = \frac{\rho c_{00}^2}{1 - \rho c_{00}} \left[1 + 2 \left(\frac{c_{01}}{c_{00}} \right)^2 \frac{1}{\left(1 - \rho c_{00} \right)^2 - \rho^2 c_{01}^2 \left[2 + \frac{1 - \rho c_{00}}{1 - \rho c_{00} - \frac{\rho^2 c_{01}^2}{1 - \rho c_{00} - \rho c_{01}} \right]} \right]$$
(4.24)

and

$$\gamma_{01} = c_{01} \left[\frac{1}{(1 - \rho c_{00})^2 - \rho^2 c_{01}^2 \left[2 + \frac{1 - \rho c_{00}}{1 - \rho c_{00} - \frac{\rho^2 c_{01}^2}{1 - \rho c_{00} - \rho c_{01}} \right]} - 1 \right]$$
(4.25)

From Equation 2.11 in Chapter 2, the partial structure factors S_0 and S_1 are simply:

$$S_0(q) = 1 + h_{00}(q) \tag{4.26}$$

$$S_1(q) = h_{01}(q) \tag{4.27}$$

The procedure is iterative and stops once convergence is reached. At first we enter initial guesses for the potential intensity and range (U_0, U_1, ξ_0, ξ_1) with two fixed parameters, the hard disk radius of the inclusion which is R = 4.5 Å in the case of BuSn and 9.5 Å for the gramicidin, and the number density n of inclusions which is calculated in each case as follows:

$$n_{gramicidin} = \frac{1}{A_P + \left(\frac{L}{P}\right)A_L} \tag{4.28}$$

with A_P the surface occupied by the inclusion and A_L the area per lipid or surfactant.

$$n_{BuSn} = \frac{1}{A_{BuSn}} \tag{4.29}$$

with,
$$A_{BuSn} = \frac{1}{2} \frac{\nu_L}{\nu_{BuSn}} A_L$$

where ν_L and ν_{BuSn} denote respectively the mole number of the lipid or surfactant and BuSn. Outside the hard core, the interaction potential in the plane of the membrane is described by an exponential:

$$V_0(r) = U_0 fr(n) \exp\left[-\frac{r-2R}{\xi}\right]$$
 (4.30)

with $fr = (1 - Kn)^2$ the effective fraction of the interaction amplitude seen in the previous chapter. On the other hand, we consider $V_1(r)$ independent of the inclusion concentration:

$$V_1(r) = -U_1\left(\frac{r}{\xi} - 1\right)$$
(4.31)

The calculated S_{eq} and S_1 are compared to the experimental results, yielding the goodness-of-fit χ^2 . We minimize χ^2 using the Optimize function in Igor Pro, with the simulated annealing method.

4.4 Results

Using the technique described above in § 4.2 we first measured the following systems: BuSn/DDAO/Cholesterol and gramicidin/ $C_{12}E_4$ with $\alpha = 38^{\circ}$. We were not able to reach a wider angle due to the limitations of the X-ray setup.

Later on another run, we measured gramicidin/DDAO, gramicidin/DDAO/Cholesterol, BuSn/C₁₂E₄, BuSn/Brij30 and BuSn/Brij30/Cholesterol at $\alpha = 30^{\circ}, 40^{\circ}$, and 60° . We followed the data treatment procedures in § 4.3. We detected no interlayer interaction for inclusions inserted in C₁₂E₄ and Brij30 membranes, as we did not observe modulations along q_z in the experimental structure factor. This is due to the hydration level in those systems. In fact the DDAO membranes are hydrated with 20% W% of H₂O while the C₁₂E₄ and Brij30 membranes are hydrated with 50% W% of H₂O so in the latter case the layers are more separated and spaced hence the fact that we do not observe interaction.

We will then focus in this chapter on the results obtained for DDAO bilayers.

4.4.1 Gramicidin/DDAO

In this section I will present results for the interaction potential of Gramicidin inclusions inserted within DDAO membranes at three concentrations : P/L = 0.052, 0.112 and 0.174. For the lowest concentration used we have 20 molecules of DDAO per molecule of gramicidin with a surface density of $\eta = A_{gramicidin} n = 0.255$ and 9 molecules of DDAO per 1 molecule of gramicidin in the second concentration and a surface density of $\eta = 0.425$.

At a very advanced stage of our data treatment we realized that the highest concentration P/L=0.174 can't be well fitted with the Hard disk model or the Lado procedures. The models do not converge with the experimental curve when fixing the number density calculated according to our concentration. So I assumed there is a problem with the number density and tried to fit the curve by fixing only the hard disk radius to 9.5 Å and keeping the number density as a free fit parameter. Doing so yielded a good fit with a density number of the same value as the second curve. I decided that this might be the maximum concentration the membrane can support, and I assign to the third curve the same number density as the second curve. In the following we consider $n = 1.75 \times 10^{-3} \text{\AA}^2$ as the highest density and we continue the data analysis procedures.



FIGURE 4.11: Experimental partial structure factors S_{eq} and S_1 for the gramicidin/D-DAO system at $\alpha = 40^{\circ}$ and 60° (black lines) and fits (red lines) using three forms of $V_0(r)$ for comparison: (A) decreasing exponential, (B) Gaussian centered at the origin, (C) Gaussian centered at contact.

As illustrated in Figure 4.10, I summarized in Figure 4.11 the partial structure factors S_{eq} and S_1 for the gramicidin/DDAO system, measured at a tilt angle $\alpha = 40^{\circ}$ and 60° . These structure factors were fitted with the models obtained by the Lado procedure, as explained in § 4.3.2.1. I used three different functional forms for $V_0(r)$ in an attempt to find the best fit results (shown as red lines). $V_1(r)$ is described by a linear form as in Eq. 4.31. In Figure 4.11a we used the exponential form of $V_0(r)$ defined in Eq. 4.30.

$$V_0(r) = U_0 fr(n) \exp\left[-\frac{r-2R}{\xi}\right]$$
(4.32)

In Figure 4.11b we used the Gaussian centered at the origin

$$V_0(r) = U_o fr(n) \exp\left[-\frac{1}{2}\left(\frac{r}{\xi}\right)^2\right]$$
(4.33)

and in Figure 4.11c we used the Gaussian centered at contact

$$V_0(r) = U_c fr(n) \exp\left[-\frac{1}{2} \left(\frac{r-2R}{\xi}\right)^2\right]$$
(4.34)

The best fits from the three forms of $V_0(r)$ are obtained for the following parameter combinations:

- Exponential : $U_0 = 1 k_{\rm B} T$; $\xi_0 = 5 \text{ Å}$; $U_1 = 3 k_{\rm B} T$; $\xi_1 = 6 \text{ Å}$; $\chi^2 = 11.01$; fr(n) = 1
- Gaussian centered at origin : $U_o = 2 k_B T$; $\xi_o = 13$ Å; $U_1 = 4 k_B T$; $\xi_1 = 5$ Å; $\chi^2 = 11.91$; $fr = (1 250 n)^2$
- Gaussian centered at contact : $U_c = 0.72 k_B T$; $\xi_0 = 5$ Å; $U_1 = 3 k_B T$; $\xi_1 = 6$ Å; $\chi^2 = 11.58$; $fr = (1 300 n)^2$

The corresponding potentials are shown in Figure 4.12.

The best fit in this case was obtained with the exponential form, but the difference in χ^2 is minimal and the potentials are very close. The amplitude of the in-plane interaction potential is low (one or a couple of $k_B T$) and both the in-plane and inter-layer potential are short-ranged.

Let us look at the S_1 at $\alpha = 40^{\circ}$ for the first and second curve we have the impression that the experimental S_1 trough reduces when passing from n = 0.001 Å² to n = 0.00175 Å², against what we intuitively expect. We expect generally that the interaction increases with the concentration. In our case, it seems to be the opposite. This brings up some questions mark about the need to measure the interlayer potential with a version of $V_1(r)$ that varies along the concentration.

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FIGURE 4.12: Best results for the three forms used for $V_0(r)$ in solid line and corresponding χ^2 values for Gramicidin/DDAO system. $V_1(r)$ is plotted as a linear curve in dashed line.

I also performed X-ray measurements on the gramicidin/DDAO system as a function of temperature. Since the temperature-controlled setup does not allow changing the incidence angle (which is fixed at $\alpha = 0$), I do not have access to the interlayer interaction. I therefore use the best $V_1(r)$ measured at room temperature and assume that it does not vary with temperature. I therefore fix $U_1 = 3 k_{\rm B}T$ and $\xi_1 = 6$ Å. For the in-plane potential I use the exponential form eq. (4.32); the only free parameters are U_0 and ξ (see Figure 4.14).

I did the measurements at eight different temperatures, starting at 30°C and going up to 100°C in steps of 10°C. After cooling, I re-measured at 30°C. The experimental structure factors (black lines) fitted with an exponential form of $V_0(r)$ (red lines) are presented in Figure 4.13.

Unlike what we have seen in the previous chapter, we do not observe major variations between the experimental structure factors at different temperatures. Accordingly, the resulting interaction potentials $V_0(r)$ displayed in Figure 4.14 overlap for the different temperatures. We have obtained the following combination of $U_0(r)$ and ξ at a fixed $U_1 = 3 k_{\rm B}T$ and $\xi_1 = 6 \text{ Å}$:

- T=30°C : $U_0 = 1.83 \ k_B T$; $\xi = 1.1 \text{\AA}$; $\chi^2 = 12.58$; fr = (1 336.7 n)
- T=40°C : $U_0 = 6.19 \ k_B T$; $\xi = 0.44 \text{\AA}$; $\chi^2 = 21.9$; $fr = (1 75.8 \ n)$
- T=50°C : $U_0 = 2.74 \ k_B T$; $\xi = 0.74 \text{ Å}$; $\chi^2 = 22.6$; $fr = (1 279 \ n)$

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P/L	$n \; [10^{-3} \text{\AA}^{-2}]$	η
0.027	0.48	0.11
0.042	0.68	0.17
0.067	0.99	0.25
0.082	1.15	0.29

TABLE 4.1: Gramicidin concentrations in DDAO/cholesterol membranes. P/L is the molar ratio of gramicidin to DDAO and cholesterol, n is the number density and η is the surface density of the inclusions.

- T=60°C : $U_0 = 2.51 \ k_B T$; $\xi = 0.79 \text{\AA}$; $\chi^2 = 30.38$; fr = (1 292 n)
- T=70°C : $U_0 = 2.34 \ k_B T$; $\xi = 0.84 \text{\AA}$; $\chi^2 = 20.65$; $fr = (1 265 \ n)$
- T=80°C 100°C: $U_0 = 1.98 \ k_B T$; $\xi = 1\text{\AA}$; $\chi^2 = 25$; fr = (1 250 n)

This lack of change is not entirely surprising, since the DDAO (a zwitterionic molecule) is less affected by temperature than nonionic surfactants such as C_12E_4 . For instance, at the concentration I used (20 w% of water), the phase diagram in the literature [94] indicates a very high transition temperature, around 140°C. I obtained similar values, as explained in Chapter 2, § 2.3. However, the more surprising result is that the gramicidin channels seem to remain stable even at high temperatures.

4.4.2 Gramicidin/DDAO/cholesterol

In this section I will present both the off-plane measurements at room temperature and those at normal incidence done as a function of temperature for the gramicidin/DDAO/-cholesterol system.

We used the following concentrations P/L = 0.027; 0.042; 0.067; and 0.082, where the molar ratio P/L is defined as the number of moles of gramicidin divided by that of moles of DDAO and cholesterol, noting that I used 1 mol of cholesterol for 4 moles of DDAO. The surface density for all samples is given in Table 4.1.

The in-plane and inter-plane structure factors are presented in Figure 4.15. Once again, I fitted the data using the same three forms of $V_0(r)$: decreasing exponential (4.30), Gaussian centered at contact (4.34) and Gaussian centered at the origin (4.33). The best fits are shown as red lines in Figure 4.15 and were obtained for the following U and ξ combinations:

• Exponential: $U_0 = 5.59 k_{\rm B}T$; $\xi_0 = 1.06$ Å; $U_1 = 1.22 k_{\rm B}T$; $\xi_1 = 24.98$ Å; $\chi^2 = 2.9448$; fr = (1 - 265 n)

- Gaussian centered at the origin: $U_o = 1.785 k_{\rm B}T$; $\xi_o = 12.47$ Å; $U_1 = 1.112 k_{\rm B}T$; $\xi_1 = 28.58$ Å; $\chi^2 = 4.67$; fr = (1 332 n)
- Gaussian centered at contact: $U_c = 2.9 k_B T$; $\xi_0 = 1.31 \text{ Å}$; $U_1 = 1.189 k_B T$; $\xi_1 = 24.52 \text{ Å}$; $\chi^2 = 2.84$; fr = (1 400 n)

The corresponding potential are shown in Figure 4.16.

This time the best fit was obtained using the Gaussian centered at contact shown in Figure 4.15c, but the shape of the curve is very similar to that of the best exponential fit, except very close to contact. In the structure factors we see that the S_1 is null for the second and third gramicidin concentration and we only observe a very small trench at the lowest concentration, yielding an interlayer potential of $U_1 = 1 k_B T$ and a range of 24.5 Å, which correspond to the values found by Constantin [88] in BuSn/DDAO.

In-plane SAXS measurements were performed on the gramicidin/DDAO/cholesterol samples at 4 different temperatures, starting at 30° and going up to 60° in steps of 10°. I then redid the measurement at 30° to check for reversibility and lack of sample damage. For the data treatment, I considered again that the interlayer potential $V_1(r)$ does not vary with the inclusion concentration and temperature and kept it fixed at its value measured above at room temperature with the off-plane SAXS method: $U_1 = 1.189 k_B T$ and $\xi = 24.52 \text{ Å}$. The experimental structure factors are shown as black lines in Figure 4.17 and the best fits are shown in red lines. I used the Gaussian centered at contact to describe the $V_0(r)$ at the different temperatures since it best described the potential at room temperature (see above). The results for $V_0(r)$ are shown in Figure 4.18. The best fits were obtained for a $fr = (1 - 400 n)^2$. The potential decreases with temperature:

- T=30°C initial : $U_0 = 2.92 \ k_B T$; $\xi = 1.35 \text{\AA}$; $\chi^2 = 11.48$
- T=40°C : $U_0 = 1.57 \ k_B T$; $\xi = 1.68 \text{\AA}$; $\chi^2 = 18.65$
- T=50°C : $U_0 = 1.54 \ k_B T$; $\xi = 1.4 \text{\AA}$; $\chi^2 = 2.25$
- T=60°C : $U_0 = 1.137 \ k_B T$; $\xi = 1.5 \text{\AA}$; $\chi^2 = 1.86$
- T=30°C final : $U_0 = 3.21 \ k_B T$; $\xi = 1 \text{\AA}$; $\chi^2 = 11.08$

We can note that raising the temperature up to 60° C does not seem to affect the peptide inclusions. We measured the same interaction potential at the initial temperature of 30° C and the final temperature of 30° C (after performing the measurements at 40,50 and 60° C). The structure factors are similar, except for the highest concentration where the structure peak has moved to lower q.

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P/L	$n [10^{-3} \text{\AA}^{-2}]$
0.012	0.31
0.015	0.39
0.018	0.47
0.0195	0.52
0.021	0.55
0.022	0.57

TABLE 4.2: Concentrations of BuSn nanoparticles embedded in DDAO/cholesterol membranes. P/L is the molar ratio of nanoparticles to membrane molecules and n is the number density.

We observe that the interaction potential between the gramicidin pores decreases with the temperature raise from 30°C to 60°C and we are able to remeasure the same potential at 30°C at the end of the temperature variation experiments, very clear in Figure 4.18. Note that at the initial temperature of 30°C the potential calculated and the range are the same found at room temperature via the off-plane saxs $U_0 = 2.9k_BT$ and $\xi = 1.3$ Å.

4.4.3 BuSn/DDAO/cholesterol

To verify the influence of the inclusion properties on the membrane-mediated interaction we used the same membranes of DDAO/cholesterol and inserted BuSn nanoparticles at the concentrations found in Table 4.2. The corresponding experimental structure factors S_{eq} and S_1 are presented in Figure 4.19 as black lines. We observe again the experimental S_1 trough reducing as the inclusions concentration increases and, as already seen in the previous chapter, the curves shape are similar for BuSn/Brij30/cholesterol and BuSn/C₁₂E₄/cholesterol. Here again, we fitted the structure factors using the three forms of $V_0(r)$: decreasing exponential (4.30), Gaussian centered at contact (4.34) and Gaussian centered at the origin (4.33).

The best fits are shown as red lines in Figure 4.19 for the following parameter combinations:

- Exponential : $U_0 = 10k_{\rm B}T$; $\xi_0 = 6.135$ Å ; $U_1 = 5k_{\rm B}T$; $\xi_1 = 16.24$ Å ; $\chi^2 = 9.87$; fr = 1
- Gaussian centered at origin : $U_o = 6.95k_{\rm B}T$; $\xi_o = 11.11$ Å ; $U_1 = 5k_{\rm B}T$; $\xi_1 = 16.32$ Å ; $\chi^2 = 9.84$; fr = 1
- Gaussian centered at contact : $U_c = 5.91k_{\rm B}T$; $\xi_0 = 7.64$ Å ; $U_1 = 5k_{\rm B}T$; $\xi_1 = 16.19$ Å ; $\chi^2 = 9.82$; fr = (1 - 400 n)

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$\overline{P/L}$	$n \; [10^{-3} \text{\AA}^{-2}]$
0.008	0.43
0.110	0.58
0.015	0.81
0.021	1.11
0.024	1.27
0.037	1.97
0.040	2.13
0.044	2.31

TABLE 4.3: Concentrations of BuSn nanoparticles embedded in DDAO membranes. P/L is the molar ratio of nanoparticles to DDAO molecules and n is the number density.

The corresponding potentials are shown in Figure 4.20.

The Gaussian centered at contact seems to fit best our data with $U_c = 5.91 \ k_{\rm B}T$ and $\xi_0 = 7.64$ Å, similar to the results of Constantin in Ref. [88], where he measures for BuSn/DDAO $U_c = 5.0 \pm 0.6 \ k_{\rm B}T$ and $\xi = 9.3 \pm 0.7$ Å.

4.4.4 BuSn/DDAO

In 2010, Constantin [88] measured the membrane-mediated interaction between BuSn inclusions in the plane of DDAO membranes and between the neighboring layers at room temperature using both in-plane and off-plane SAXS measurements.

I completed the study of this system by temperature measurements using in-plane SAXS (with the x-ray beam normal to the layers). I used the same concentrations as in Ref. [88], listed in Table 4.3.

I present here the structure factors of the nanoparticles as a function of concentration and at eight different temperatures, starting at 30°C and going up to 100°C in steps of 10°C, followed by another acquisition at 30°C after cooling down, to check the stability of the sample. To avoid excessive graph mass, I will only show the results at 30°C, 50°C, 70°C and 100°C. Since we know that an interlayer interaction exists between the inclusions, I account for it in our data treatment by a $V_1(r)$ that does not vary with temperature and inclusion concentration and is defined by $U_1 = 1k_BT$ and $\xi = 25$ Å, as measured in Ref. [88].

The $V_0(r)$ has been described in Ref. [88] by a Gaussian centered at contact and in two different scenarios: including the fr(n) dependence of the potential on the particle concentration or not. I took in consideration only the first case (with dependence) but described the $V_0(r)$ using two functional forms, for comparison: a decreasing exponential (4.30) and a Gaussian centered at contact (4.34). In both cases, the model structure factors are plotted in red along the experimental data (shown in black) in Figures 4.21 and 4.22, respectively.

To begin with, we clearly see that the structure peak (saturation point) shifts to higher q as the concentration increases fitting very well with the model for temperatures less than T= 50°C. For high concentrations ($n > 0.00127 \text{ Å}^{-2}$) at higher temperatures (T > 70 °C), the peak (saturation point) is broadened and the model fits rather badly (Figure 4.21 and 4.22).

The best fit parameters U and ξ obtained in each cases for the different temperatures are given below; the corresponding potentials are plotted in Figure 4.23.

When the interaction potential is described by an exponential it yields the following amplitude and range with fr = (1 - 250 n):

- T=30°C : $U_0 = 2.24 \ k_B T$; $\xi = 6.5 \ \text{\AA}$; $\chi^2 = 1.0$
- T=50°C : $U_0 = 1.34 \ k_B T$; $\xi = 6.5 \ \text{\AA}$; $\chi^2 = 2.73$
- T=70°C : $U_0 = 1.70 \ k_B T$; $\xi = 7.8 \ \text{\AA}$; $\chi^2 = 7.2$
- T=100°C : $U_0 = 1.31 \ k_B T$; $\xi = 8 \ \text{\AA}$; $\chi^2 = 25$

When the interaction potential is described as a Gaussian centered at contact it yields the following amplitude and range:

- T=30°C : $U_c = 1.58 \ k_B T$; $\xi = 6.58 \ \text{\AA}$; $\chi^2 = 0.75$; $fr = (1 207 \ n)^2$
- T=50°C : $U_c = 1.019 \ k_B T$; $\xi = 7.53 \ \text{\AA}$; $\chi^2 = 2.57$; $fr = (1 185 \ n)^2$
- T=70°C : $U_c = 1.17 \ k_B T$; $\xi = 7.27 \ \text{\AA}$; $\chi^2 = 6.32$; $fr = (1 230 \ n)^2$
- T=100°C : $U_c = 0.89 \ k_B T$; $\xi = 7.8 \ \text{\AA}$; $\chi^2 = 24.13$; $fr = (1 250 \ n)^2$

In both cases the interaction potential decreases along the temperature whereas the range slightly increases from 6.5 Å to 8 Å. We pass from a goodness-of-fit value $\chi^2 = 1$ to $\chi^2 = 25$, which means that the model fits badly the experimental data at high temperature.



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FIGURE 4.13: Experimental structure factors of gramicidin/DDAO measured at different temperatures (black lines) and fits (red lines) with a fixed interlayer interaction $V_1(r)$ and an exponential in-plane potential $V_0(r)$ using to Lado procedure.

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FIGURE 4.14: Comparison of the in-plane interaction potential between gramicidin inclusions in DDAO membranes at different temperatures.



FIGURE 4.15: Experimental partial structure factors S_{eq} and S_1 (black lines) for the gramicidin/DDAO/cholesterol system at $\alpha = 40^{\circ}$ and 60° and fits (red lines) with three forms of $V_0(r)$ for comparison: (A) decreasing exponential, (B) Gaussian centered at the origin, (C) Gaussian centered at contact.



FIGURE 4.16: Best results for the three functional forms used for $V_0(r)$ and corresponding χ^2 values for the gramicidin/DDAO/cholesterol system.



FIGURE 4.17: Experimental structure factors of gramicidin/DDAO/cholesterol measured at different temperatures (black lines) and fits (red lines) with a fixed interlayer interaction and an exponential in-plane interaction.

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FIGURE 4.18: Comparison of the in-plane interaction potential between gramicidin inclusions in DDAO/cholesterol membranes at different temperatures.



FIGURE 4.19: Experimental partial structure factors S_{eq} and S_1 for the BuSn/DDAO/cholesterol system at $\alpha = 38^{\circ}$ (black lines) and fits (red lines) with three forms of $V_0(r)$ for comparison: (A) decreasing exponential, (B) Gaussian centered at the origin, (C) Gaussian centered at contact.

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FIGURE 4.20: Best results for the three forms used for $V_0(r)$ and corresponding χ^2 values for the BuSn/DDAO/cholesterol system.



FIGURE 4.21: Experimental structure factors for BuSn/DDAO measured at different temperatures (black lines) and fits (red lines) with a fixed interlayer interaction and an exponential in-plane interaction.



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FIGURE 4.22: Experimental structure factors for BuSn/DDAO measured at different temperatures (black lines) and fits (red lines) with a fixed interlayer interaction and an in-plane interaction described by a Gaussian centered at contact.



FIGURE 4.23: Comparison of the in-plane interaction potential between BuSn inclusions at different temperatures in DDAO membranes, described in (A) by an exponential form and in (B) by a Gaussian centered at contact.

4.5 Discussion

To summarize, the main results of this work are the interaction potentials of gramicidin and BuSn inclusions in lamellar phases, $V_0(r)$ the in-plane interaction potential and $V_1(r)$ in the neighboring layers. we measured the latters by off-plane SAXS experiments for three different systems using three forms of $V_0(r)$ and the best results were described by a Gaussian centered at contact with the following combination of the amplitude and range :

- Gramicidin/DDAO : $U_c = 0.72 \text{ k}_{\text{B}}\text{T}$; $\xi_0 = 5 \text{ Å}$; $U_1 = 4 \text{ k}_{\text{B}}\text{T}$; $\xi_1 = 5 \text{ Å}$; $\chi^2 = 11.58$; fr = (1 300 n)
- Gramicidin/DDAO/Cholesterol : $U_c = 2.9 \text{ k}_{\text{B}}\text{T}$; $\xi_0 = 1.31 \text{ Å}$; $U_1 = 1.189 \text{ k}_{\text{B}}\text{T}$; $\xi_1 = 24.52 \text{ Å}$; $\chi^2 = 2.84$; fr = (1 - 400 n)
- BuSn/DDAO/Cholesterol : $U_c = 5.91 \text{ k}_{\text{B}}\text{T}$; $\xi_0 = 7.64 \text{ Å}$; $U_1 = 5 \text{ k}_{\text{B}}\text{T}$; $\xi_1 = 16.19 \text{ Å}$; $\chi^2 = 9.82$; fr = (1 400 n)

The in-plane and inter-plane interaction potentials are small, of few k_BT and short ranged for the in-plane potentials and of longer range for the interlayer potentials.

On the other hand we observe for the gramicidin inclusions that U_c is bigger when inserted within DDAO/Cholesterol membranes whereas their interaction with the neighboring pores is stronger in cholesterol-free DDAO membranes.

Comparing the results for the same cholesterol content of DDAO membranes with gramicidin inclusion on one hand and BuSn inclusions on another hand, we can validate our hypothesis which is that the interaction potential between BuSn inclusions is stronger than between gramicidin pores. Since the presence of gramicidin and cholesterol induces an increase of the hydrophobic length of the membrane to adapt to that of gramicidin whereas, the BuSn in the vicinity of the bilayer induce a more important perturbation of the hydrophobic length.

We also measured the in-plane interaction potential in presence of an interlayer interaction as a function of temperature and inclusion concentrations. We considered that this interlayer interaction is independent of the temperature and fixed it to that found at room temperature via the off-plane SAXS measurements. We found that the interaction potential between gramicidin pores and between BuSn particles decreases as a function of temperature in DDAO and DDAO/Cholesterol membranes. In the all later cases, the interaction potential weakens slightly, we pass from a $U_c = 1.58k_BT$ at 30°C to $U_c = 0.9k_BT$ at 100°C for BuSn DDAO ; as for gramicidin/DDAO we pass from $U_c = 1.83k_BT$ at 40°C to $U_c = 2.34k_BT$ at 70°C and finally we pass from $U_c = 2.9k_BT$ at 30°C to $U_c = 1.1k_BT$ at 60°C for Gramicidin/DDAO/Cholesterol. The change is very small especially for BuSn/DDAO. The same for the interaction range ξ it slightly changes by increasing from 6Å for BuSn/DDAO to 8Å and from 1.35Å to 1.5Å for gramicidin/DDAO/Cholesterol. Furthermore, we note that the interaction potential and range obtained at T=30°C is the exact found at room temperature via the off-plane SAXS for the gramicidin/DDAO/Cholesterol.

Comparing to the previous results obtained in the previous chapter (Chapter 3 where we had no interlayer interaction, both the in-plane interaction potential and the interaction range decreased along the temperature for $BuSn/C_{12}E_4$, $BuSn/C_{12}E_4$ /Cholesterol. Only for the Gramicidin/ $C_{12}E_4$ we observed an increase in the interaction range and for the BuSn/Brij30/Cholesterol an increase of the potential along the temperature.

Another very remarkable finding we had was trying to make all fits for all the systems at fr = 1 and obtaining almost the same results as for fr(n). Let's take for example the gramicidin/DDAO/Cholesterol system. We obtain using the off-plane saxs measurement the exact value of $V_0(r)$ and $V_1(r)$ for the three different forms of $V_0(r)$ but with a goodness-of-fit function χ^2 slightly higher: in the exponential form we have a $\chi^2 = 2.94$ for $fr = (1 - 265 n)^2$ whereas $\chi^2 = 3.2$ for fr = 1. For the Gaussian centered at contact form, $\chi^2 = 2.84$ for $fr = (1 - 400 n)^2$ while $\chi^2 = 4$ for fr = 1. For the Gaussian centered at origin form we measure $\chi^2 = 4.67$ for $fr = (1 - 332 n)^2$ while $\chi^2 = 6$ for fr = 1. The same concept for the measurement of the interaction potential along the temperature, we obtain the same values for U_c and ξ at a slightly higher value of χ^2 for the Gramicidin/DDAO/Cholesterol system. As for the BuSn/DDAO/system, for fr = 1 we don't have the exact same value of U_c and ξ but we have very similar ones. For instance, at T=30° we measure $\chi^2 = 0.75$ for $fr = (1 - 207 n)^2$ with $U_c = 1.58 k_B T$ whereas $\chi^2 = 1.6$ for fr = 1 with $U_c = 2k_BT$. For T=50°, $\chi^2 = 2.57$ for $fr = (1-185 n)^2$ with $U_c = 1.019k_BT$ while $\chi^2 = 2.9$ for fr = 1 with $U_c = 1.5k_BT$. At T=70°, $\chi^2 = 6.32$ for $fr = (1 - 230 n)^2$ with $U_c = 1.17k_BT$ whereas $\chi^2 = 7.6$ for fr = 1 with $U_c = 2k_BT$. Finally at T=100°, $\chi^2 = 24.13$ for $fr = (1 - 250 n)^2$ with $U_c = 0.89 k_B T$ whereas $\chi^2 = 23.7$ for fr = 1 with $U_c = 1.4k_BT$. So again we repeat that we observe the same tendency for all the measured systems.

We attempted to compare our results with previous experimental data in ref. [88] where the author studies the interaction potential in two cases for BuSn/DDAO samples. In the first case where he considers that the interaction potential between BuSn inclusions do not vary along the particle concentration, he measures $U_c = 2.2k_BT$ and $\xi = 10.8\text{\AA}$ at room temperature. I measure the same value for the potential at T=30° and for fr = 1 with $U_c = 2k_BT$ and $\xi = 7\text{\AA}$. As for the second case, where the author considers that the potential does depend on the particle concentrations, he finds a $U_c = 5k_BT$ and $\xi = 9.3\text{\AA}$ with a $fr = (1 - 154.35 n)^2$ and a $\chi^2 = 1.00$. Using the same value for fr, I measure $U_c = 2.07k_BT$ and $\xi = 7\text{\AA}$ and a $\chi^2 = 1.05$.

I tried to check the in-plane interaction potential in presence of cholesterol for the BuSn/DDAO/Cholesterol system at the same fr values and try to compare it with the author values for the DDAO/BuSn. I measure for BuSn/DDAO/Cholesterol : $U_c = 8k_BT$ and $\xi = 7\text{\AA}$ for $fr = 1-154.35 n)^2$, close to the results of ref. [88], and $U_c = 1k_BT$; $\xi = 1\text{\AA}$ for fr = 1.

4.6 Conclusion

To conclude, we were able to measure the in-plane and inter-plane interaction potential between two different inclusions, BuSn hybrid inclusions and gramicidin peptides inclusions embedded in DDAO lamellar phases in presence and absence of cholesterol at different particles concentration. We find that we have small-ranged potentials of few k_BT . On the other hand we consider that the interlayer interaction do not vary along the temperature and particle concentration and we measure the in-plane potential along the temperature. We find that this potential slightly decreases while the range slightly increases as a function of temperature. Another very remarkable finding, was obtaining similar results for the inplane interaction potential with fr = 1 or fr(n) which means that the fr parameter does not improve the data normalization and its use is not necessary.

Part II

Effect of inclusions on the local order of the acyl chains

Chapter 5

Effect of inclusions on the orientational order of the acyl chains

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5.3.1	Conclusion

In this chapter I will study the effect of gramicidin A inclusions on the orientational order of DDAO and $C_{12}EO_4$ acyl chains using the Nuclear Magnetic Resonance (NMR) technique. The orientational order parameter S_{C-H} is the average value of the orientational degree of the C-H bond in respect to the bilayer normal (\vec{n}) , see Figure 5.1.



FIGURE 5.1: Schematic representation of a phospholipid molecule with the axes systems needed for the description of the orientational order parameter. Image taken and modified from [126]

5.1 Introduction

The principles of nuclear magnetic resonance spectroscopy are founded on a diverse body of experimental and theoretical work. Many have developed excellent treatments on general NMR theory, for instance, Abragam [127] and Slichter [128]. One can also find some specialized treatments of liquid-crystal NMR by Vold and Vold [129] and Brown [130].

Nuclear magnetic resonance (NMR) spectroscopy is a useful, versatile technique for the detection of molecular structure and composition that relies on measuring the interactions of nuclei with an applied magnetic field. It can be used with liquid, solid or gas samples, with compounds of various masses and compositions. It has been used in a diverse range of research areas, including organic, inorganic, and physical chemistry, medicine, biochemistry, protein science, and the food industry [127].

All nuclei possess an intrinsic property known as spin (that we will denote by I), a form of angular momentum, which may have an integer or half-integer value in units of $\hbar = h/(2\pi)$, where h is Planck's constant.

Nuclei also have an intrinsic magnetic moment μ , which is proportional to the spin [128]:

$$\mu = \gamma I \hbar \tag{5.1}$$

via the magnetogyric ratio γ , which is a proportionality constant unique to each nucleus; for example, the magnetogyric ratio of protons, $\gamma_{1H} = 26.7522 \times 10^7 \, rad \, s^{-1}T^{-1}$, is the largest one among commonly examined nuclei. Thus, its magnetic moment $\mu_p =$ $2.7982 \, \mu_B$, where $\mu_B = \frac{e\hbar}{2m_e}$ is the Bohr magneton and m_e is the mass of the electron. When a magnetic moment $\vec{\mu}$ is placed in an external (static) magnetic field $\vec{B_0}$ parallel to the z axis, the field will exert a torque on $\vec{\mu}$, tending to align it along its direction. Unlike a classical dipole, however, the magnetic dipole of a nucleus will not necessarily line up exactly with the field, but rather assume one of (2I + 1) orientations (corresponding to the possible values for the spin component along the field) and precess around $\vec{B_0}$ at a frequency ν_0 , commonly named after Larmor.

In the particular case of spin-1/2 (e.g. the proton and ${}^{13}C$), the moment has two possible states: spin-up, where $\mu_z > 0$ (along the field) or spin-down, with $\mu_z < 0$ (antiparallel to the magnetic field). The spin-up state has a magnetic energy of $-\mu_z B_0$, while the spin-up has a higher energy, $+\mu_z B_0$. The energy difference between the two quantum states is:

$$\Delta E = 2\mu_z B_0 = 2\gamma \frac{\hbar}{2} B_0 = \gamma \hbar B_0 \tag{5.2}$$

The number of nuclei in each spin state can be described by the Boltzmann distribution:

$$\frac{N_{down}}{N_{up}} = \exp\left(-\frac{\Delta E}{kT}\right) \tag{5.3}$$

where $N_{up, down}$ are the spin populations in the up- and down- states; $k = 1.3805 \times 10^{-23} J/K$ is the Boltzmann constant and T is the temperature in K.

For protons at room temperature in a field with $B_0 = 10 T$, the Boltzmann factor $\frac{\Delta E}{kT} \simeq 7 \, 10^{-5}$.

5.1.1 Signal generation

The sample under study is subjected to the permanent magnetic field B_0 and to a radio frequency (RF) signal generated by a coil. The coil is excited by an oscillating current and hence generates a magnetic field with the same frequency. If the frequency is close to a resonance value, it will excite the transition of nuclei between the spin-up and spin-down states. The excited nuclei then precess about the \vec{B}_0 axis at their individual resonance frequencies, creating an oscillating magnetic field and thus RF signals that are then analyzed by the computer software into the free induction decay (FID). The FID, which is a time-domain spectrum, is converted into the usual frequency-domain NMR spectrum by Fourier transformation. The pulse is repeated as many times as necessary to allow the signals to be identified from the background noise.

5.1.2 The NMR spectrum

Chemical shift The chemical shift δ of a nucleus is related to the resonance frequency of that particular nucleus [127], i.e. to the frequency at which the nucleus transitions between the spin-up and spin-down quantum states. Simply put, the NMR spectrometer is a machine that allows us to determine the chemical shift of the nucleus in its environment, allowing us to differentiate between the nuclei of the same element found at different positions in the molecule of interest.

In relative terms, δ is defined by the resonance frequency expressed with reference to a standard compound, rendering it independent of the spectrometer frequency. The scale is made more manageable by expressing it in parts per million (ppm):

$$\delta = \frac{\nu - \nu_0}{\nu_0} \times 10^6 \tag{5.4}$$

where ν and ν_0 are the resonance frequencies of the nucleus under investigation, in its actual environment and in a reference compound, respectively. The chemical shift can have multiple origins:

The electron cloud also reacts to the applied field, so that the nucleus experiences an effective field value:

$$\vec{B}_{\rm eff} = \vec{B}_0 + \vec{B}_{\rm induced} \tag{5.5}$$

The electron-generated field B_{induced} is proportional to the external applied field B_0 , with a proportionality constant which can be negative (shielding) or positive (deshielding). The nuclei are said to be shielded when a larger external magnetic field is required to get them to resonate. These nuclei will appear more upfield on spectrum. On the other hand, the deshielding effect exists when the nuclei resonate under lower external magnetic field. They will appear more downfield on the NMR spectrum.

The splitting of the peaks into multiple peaks (or signals) is known as spin-spin coupling. This is a direct result of the interaction between the neighboring nuclei.

Coupling constant Spin-spin coupling is the interaction that takes place between neighboring, non-equivalent NMR-active nuclei on a given molecule. The interaction leads to the splitting of spectral lines in an NMR spectrum and the separation distance between the spectral lines is given by the coupling constant J. Two very important parameters affect the magnitude of the coupling constant J: the distance between atoms and the angle between atoms. In the following I will take the example of hydrogen atoms for simple demonstration.

Distance between atoms For two different hydrogen atoms on the same molecule, the coupling constant decreases with their distance, as illustrated in Scheme 5.1. On molecule 1, the separation distance between H_1 and H_2 is given by three σ bonds, while in molecule 2 H_1 is found four σ bonds apart from H_3 . Because the separation distance in molecule 1 is lower than in molecule 2, the magnitude of the coupling constant will be greater for molecule 1 than for molecule 2. As an order of magnitude, the range for the coupling constant J_1 will be about 2 - 30 Hz and J_2 about 0 - 1 Hz.



SCHEME 5.1: Example for the effect of distance on the magnitude of J.

Angle between atoms The orientation of the two different hydrogen atoms with respect to one another also affects the magnitude of the coupling constant. For instance, the magnitude of J between two adjacent hydrogen atoms for cis and trans (see the example in Scheme 5.2 for ethene) is different because of the difference in orientation (angle). For cis ethene J = 6 - 12 Hz, while for the trans configuration J = 12 - 18 Hz.



SCHEME 5.2: Adjacent hydrogen atoms for the cis and trans configurations of ethene.

The dihedral angle between two different H atoms in an alkane also affects the magnitude of J. As illustrated in Scheme 5.3, its value depends on the dihedral angle between H_1 and H_2 . The Karplus equation describes how the orientation between the two dihedral angles in the alkane affects the value of J:

$${}^{3}J = A + B\cos\theta + C\cos^{2}\theta \tag{5.6}$$

where ${}^{3}J$ is the three-bond spin-spin coupling constant; A = 2 Hz, B = -1 Hz, and C = 10 Hz are constant coefficients and θ is the dihedral angle. When $\theta = 0$ or 180° , H_{1} and H_{2} are aligned along the same plane and the value of J is relatively large, while it reaches its minimum when the angle is close to 90° .


SCHEME 5.3: Possible orientations of hydrogen atoms in an alkane.

5.1.3 NMR experiments

A large variety of NMR experiments exists, to be chosen depending on the information we are seeking, on the nature of the sample and, of course, on the instruments available. The most commonly used, and simplest, NMR experiments are the 1D proton and the 1D carbon spectra, routinely used in organic synthetic laboratories to quickly produce confirmation of structures of novel compounds. Further structural detail can be obtained by using more advanced 2D experiments such as NOESY, COSY or HSQC.

NMR measures not only carbon and hydrogen but basically any atom with a non-nul spin. The most commonly measured nuclides beside ${}^{13}C$ and ${}^{1}H$ are ${}^{31}P, {}^{19}F, {}^{15}N$ and ${}^{29}Si$.

5.1.3.1 Solid-state NMR spectroscopy

The main difference between solution-state and solid-state NMR is that in the liquid state the molecules tumble very quickly and many interactions get averaged during time (for instance, dipolar interactions average to zero and cannot be measured in this configuration). This gives for each atom one sharp signal. However, as the system of interest increases in size, the peaks get broader since the rotational diffusion slows down. It is at this stage that solid NMR steps in.

Now that so many interactions became visible and measured very broad peaks are obtained, so in order to analyze the peaks the most common method is to artificially introduce an orientation and spin the sample around an axis very fast (several thousand times a second). In this way, many interactions get averaged again around the rotation axis. The coupling can be expressed in terms of cosine functions and they conveniently vanish while spinning at an angle $\theta_m = 54.74^\circ$ to the B_0 axis. This angle corresponds to the average of the x, y and z axes.

Since its invention, magic-angle spinning (MAS) has been used with a wide variety of compounds, including catalysts, polymers and biomolecules, especially membrane proteins [131]. MAS-NMR is mostly used for the analysis of native and model membranes, membrane proteins, and lipid/protein interactions [55, 132]. The resonance peaks of the

MAS-NMR are similar to those of solution spectra and enable the analysis of proteins in complex environments [133]. Unlike solution-state spectroscopy, there is no upper limit to the molecular mass of proteins that can be analyzed. Despite the obvious advantage of being able to study a large variety of samples in various states, there are some drawbacks to the MAS technique. The resolution of the spectrum is lower than that obtained with solution-state NMR, and thus it requires greater amounts of sample due to the decrease in sensitivity. [134].

There may also be problems associated with localized heating of the sample due to high spinning speeds, and with non-uniform distribution of the sample due to high centrifugal forces. This problem may be overcome to a certain extent with the use of rotor inserts which help position a small amount of sample at the correct position in the NMR coils to achieve results with maximum efficiency. The signal-to-noise ratio found with MAS may be improved using the cross-polarization (CP) or the refocused INEPT techniques, which allow the transfer of polarization from abundant spins (such as protons) to rare spins (^{13}C or ^{15}N , for instance), thus improving detection of these rare nuclei [134]. The MAS experiment may be further improved by using high-power decoupling, which simplifies the spectrum by decoupling protons from rare nuclei. By spinning the sample, one loses information on lipid conformations and dynamics in the bilayer, due to the averaging out of anisotropic interactions. However, these interactions can be "recoupled", as we will see below with the DROSS experiment.

Solid-state NMR spectroscopy enjoys an exceptional position in the field of membrane research [135]. The chemical shift measured by NMR is highly sensitive to hydrophobic and electrostatic interactions providing information about phase composition not accessible with other methodologies [136]. Additionally, the measurements of segmental order parameters obtained from the chemical shifts allow an estimation of domain sizes in the biomembranes (formed for example by lipids and cholesterol) system as a function of temperature and composition [135].

In the current study we present a novel application of MAS dipolar recoupling to record simultaneously the isotopic ${}^{13}C$ chemical shifts (at natural isotopic abundance) and segmental order parameters for surfactant and lipid membranes doped with gramicidin at two temperatures: $30^{\circ}C$ and $5^{\circ}C$. These temperatures were chosen based on the phase transition temperature in each corresponding phase diagram of the systems.

Dipolar Recoupling On-Axis with Scaling and Shape Preservation - DROSS is an NMR technique that measures ${}^{13}C - {}^{1}H$ dipolar couplings in solids. The widths of the dipolar

couplings are used to produce order parameter measurements as follows:

$$S_{CH} = \frac{W_{CH}^{averaged}}{W_{CH}^{static}} = \frac{W_{CH}^{measured}}{\chi \times W_{CH}^{static}}$$
(5.7)

where S_{CH} is the ¹³C –¹H order parameter, W_{CH}^{static} , $W_{CH}^{averaged}$, $W_{CH}^{measured}$ are the static, averaged and measured widths of the dipolar splitting, and χ is the DROSS scaling factor (equal to 0.393). W_{CH}^{static} is around 20 kHz, but another way to measure it is to use equation 5.7 with $W_{CH}^{measured}$ for a known S_{CH} value (see below).

Equation 5.7 is derived from the effective Hamiltonian equation [137]:

$$H^{eff} = \overline{\langle \omega_D \rangle} 2I_z S_z \tag{5.8}$$

where

$$\overline{\langle \omega_D \rangle} = \epsilon \pi J_{CH} + \chi \langle b_{CH} \rangle \frac{1}{2} (3\cos^2\beta - 1)$$
(5.9)

with β as the angle between the bilayer normal and the direction of the static magnetic field, χ and ϵ are the scale factors (ranging from 0 to 0.393 and from 0.797 to 0 respectively) and

$$\left\langle b_{CH} \right\rangle = -b_{CH} S_{CH} \tag{5.10}$$

as the motionally averaged heteronuclear dipolar coupling, where

$$S_{CH} = \frac{1}{2} \left\langle 3\cos^2\theta - 1 \right\rangle \tag{5.11}$$

is the ¹³C –¹H dipolar order parameter, $b_{CH} = (\mu_0/4\pi)(\gamma_c\gamma_h\hbar/r_{CH}^3)$ is the rigid lattice dipolar coupling, and θ is the average angle between the internuclear vector and motional axis [137].

5.1.3.2 Advantage of DROSS

There are a number of advantages to the use of DROSS over other methods: it has greater sensitivity than deuterium NMR, there is therefore no need for isotopic enrichment and it can be used to analyse natural abundance membranes, which is not possible for deuterium NMR [55, 133, 137]. It also yields the information for all the carbons at once, with straightforward assignment, whereas Deuterium NMR would require labelling of each deuteron at a time, since the resolution between two deuterons is very small. However, the precision in the order parameter values calculated for deuterium NMR is higher than the values calculated for DROSS because the ${}^{13}C - {}^{1}H$ dipolar couplings are about an order of magnitude smaller than the deuterium quadrupolar coupling [138]. Last but not least, the possibility of obtaining, via a simple equation, the order parameter from the dipolar coupling width in Hz makes it a much easier NMR technique comparing to the others.

In principle, the DROSS experiments are implemented with a $4-\pi$ pulse sequence applied to either the ${}^{13}C$ or the ${}^{1}H$ nuclei; however, it is found that the latter case yields distorted line shapes. Therefore, the $4-\pi$ pulse recoupling scheme is applied to the ${}^{13}C$ nuclei as described in the Figure 5.2.



FIGURE 5.2: Pulse timing diagram for the separated local field MAS experiment DROSS.

Sample preparation is the same as for the SAXS experiments, except for the use of D_2O instead of H_2O . We prepared four different membrane systems doped with gramicidin, denoted in the following by DMPC/gA, DLPC/gA, $C_{12}E_4$ /gA and DDAO/gA. All experiments on DMPC were performed at 30°C. This is 7°C above the gel to liquidcrystal phase transition of the lipid membrane. At this temperature, one is able to remove the strong (~ 10 kHz) homonuclear ¹H - ¹H dipolar couplings revealing the residual homonuclear interaction, i.e. spinning sidebands, and isotopic chemical shifts of the center band.

5.1.3.3 NMR methods

NMR experiments with DMPC, DLPC and $C_{12}E_4$ were performed with a Bruker AVANCE DMX400-WB NMR spectrometer (¹H resonance at 400 MHz, ¹³C resonance at 100 MHz) using a Bruker 4-mm MAS probe. NMR experiments for DDAO were performed with a

Bruker AVANCE 300-WB spectrometer (¹H resonance at 300 MHz, ¹³C resonance at 75 MHz) using a Bruker 4-mm MAS probe. All experiments were performed at 30°C and only DLPC was also performed at 5°C. Samples are loaded into 4 mm diameter rotors and fitted with finned caps. Spinning speeds are normally between 2.5 and 12 kHz.

The DROSS pulse sequence [137] with a scaling factor $\chi = 0.393$ was used with carefully set pulse lengths and refocused insensitive nuclei enhanced by polarization transfer (IN-EPT) [137, 138] with delays set to 1/8J and 1/4J and a J value of 125 Hz. The spinning rate was set to 5 kHz, the typical pulse lengths were ¹³C (90°) = $3\mu s$, ${}^{1}H(90^{\circ}) = 2.5\mu s$ and ¹H two-pulse phase-modulation (TPPM) decoupling at 50 kHz with a phase modulation angle of 15°. For the 2D spectra, 64 free induction decays were acquired, with 64 to 512 scans summed, a recycle delay of 3 s, a spectral width of 32 kHz and 8,000 complex points. The total acquisition time was between 2 and 14 hours. The data were treated using the Bruker TopSpin 3.2 software.

5.2 Results

5.2.1 Data treatment procedure

5.2.1.1 1D measurements

First we checked the ${}^{13}C$ 1D spectra zgig and refinept before proceeding with the 2D analysis. The refinept, or refocused INEPT (Insensitive Nuclei Enhanced by Polarization Transfer) method consists of transferring dipolar modulated ${}^{1}H$ magnetization to the directly bound ${}^{13}C$ aliphatic nuclei. The zgig ${}^{13}C$ shows a normal spectrum in which we excite the carbon by sending a pulse and thus the proton is decoupled and we detect the signal. Which means that, in the case of zgig, the integral of each peak reflects the number of carbons of the lipid.

Because the DROSS experiments rely on a refocused INEPT transfer between ${}^{1}H$ and ${}^{13}C$, and because refocused INEPT is sensitive to the dynamics of carbons [55], we first compare these two 1D spectra in order to check that the DROSS experiment will be efficient for all carbons. The INEPT polarization transfer efficiently occurs in the liquid-ordered, liquid-disordered, or isotropic phase, resulting in an increase in ${}^{13}C$ intensity. The transfer will not be effective in the solid-ordered phase, resulting in a null spectrum. So the comparison between the two 1D allows us to estimate the percentage of the carbon intensity loss. Chupin [139] labeled the DOPC lipid at its carbon position 11, right after the double bond in the carbon chain, and studied the behavior of this atom in presence of gramicidin. He shows that the effect of adding gramicidin results in an L_{α} to H_{II}

transition, with a coexistence of both phases. So if the gramicidin separates the bilayer in two phases, a liquid-crystalline fluid phase and a gel phase then the gel phase would not contribute to our DROSS spectra because we cannot measure it. This is the essential role of doing the 1D spectra integrals, to verify that there aren't parts of the bilayers that have "disappeared" from the Dross only because the gramicidin has "gelified" and frozen the motion of some lipids. If such a "freezing" should occur, we expect it to affect only the lipid molecules in direct contact with the gramicidin molecule (also known as annular lipids).



FIGURE 5.3: 1D 13 C spectrum of DDAO.

In the software, we manually select the peak region in the 1D spectra of refinept and 1D spectra of zgig and integrate it (an exemple is given in Figure 5.3). The integral value is an estimation of the carbon intensity value. Then, for each peak of both spectra we measure its surface area R. R is a ratio between the integral value of refinept over the integral value from zgig for each corresponding peak.

$$R = \frac{Area_{DWrefineptcpd}}{Area_{zgig}}$$

Then we plot R as a function of temperature. The best R value is around 4 and it shouldn't be less than 1. If R = 1 then it means that applying the INEPT method doesn't improve the spectra. We didn't have any R value less than 1. The average R was around 1.7. It means that the contribution of the inept method is good.

5.2.1.2 Resonance assignment

Resonance assignments are made for the fatty acyl segments and methyl groups using the $C(\omega - n)$ convention, where n is the total number of segments increasing from the terminal methylene segment, $C\omega$, to the upper carbonyl segment C1. This representation permits a segment-by-segment comparison of the chain regions. Backbone regions are assigned according to the stereospecific nomenclature (sn) convention for the glycerol moiety. Phosphocholine headgroup carbons are given Greek (α, β, γ) letter designations.

5.2.1.3 2D measurements

2D ${}^{1}H - {}^{13}C$ DROSS experiments were performed for all lipid samples at 30°C and one sample at 5°C at different gramicidin concentration. The spectra obtained provide information for the headgroups and carbonyl chain order parameters. (e.g. Figure 5.4). For each ${}^{13}C$ resonance, 1D spectra slices were extracted from the 2D spectra (e.g. Figure 5.5). Assignment of the ${}^{13}C$ resonances followed that of previously published data ([126, 137, 140–142]). The internal reference was chosen to be the acyl chain terminal ${}^{13}CH_3$ resonance assigned to 14 ppm for all lipids and surfactants studied here.

Order parameters were extracted from the 2D DROSS spectra by measuring the dipolar splittings of the Pake doublet at each carbon site (Shown in red in Figure 5.5). This splitting was converted into a dipolar coupling by taking the scaling factor χ into account as explained in Equation 5.7. The absolute value of the segmental order parameter is an additional scaling factor χ' of the static dipolar coupling into the measured dipolar coupling. Since the static dipolar coupling, on the order of 20 kHz, is not known with a high precision for each carbon, we have adjusted it empirically in the case of DMPC, by comparing it to previously determined values.

In the following I explain in detail and with schematic examples how we practically determine the order parameter for each carbon from the experimental 2D ${}^{1}H - {}^{13}C$ DROSS spectra. I use as demonstration reference the $C_{12}E_4$ / gA system with P/L = 0.118.





Figure 5.4 depicts a 2D plane of a DROSS spectrum for the $C_{12}E_4/\text{gA}$ system with P/L = 0.118 in the liquid crystalline state at 30°. The F_2 frequency dimension (horizontal) shows the isotopic ¹³C chemical shift (δ) spectra obtained under MAS. Along the F_1 frequency axis (vertical), the DROSS spectra contain Pake doublets corresponding to the ¹³C $-^1$ H dipolar couplings (the red curve on the graph is a Pake doublet that represents the dipolar coupling for the C3). The dipolar slices correspond to each of the isotopic ¹³C chemical shift positions and assigned according to Scheme 5.4. So briefly in this step we select each of the interaction peaks in order to measure the dipolar coupling coupling as in Figure 5.5. Here we have to carefully select the right doublet of the dipolar

In the case of lipids (DLPC and DMPC), the resonance in the glycerol backbone and the upper part of the acyl chains, especially C2 and C3 methylenes, often present several large splittings at each position but only the most intense ones are reported here, when they are measurable. They are due to the various atomic environment around those particular ${}^{1}H - {}^{13}C$ nuclei.

coupling.

For each 2D DROSS NMR spectrum, dipolar coupling slices were extracted at each carbon position. Figure 5.6 shows a set of such representative slices and visualizes the

variation of the same carbon depending on the concentration of doped gramicidin and the surfactant or lipid type. Data of the measurable values of those dipolar coupling for each system are available in the following tables (Table 5.3, 5.5, 5.4, 5.6, 5.7).



FIGURE 5.5: Measure of the dipolar coupling ω on a 2D DROSS spectrum for $C_{12}E_4$ /gA with P/L = 0.118

In Figure 5.6 I show the dipolar coupling resonance slices for each assigned carbon in order to visualize the variation of the same carbon depending on the concentration of doped gramicidin and the surfactant or lipid type.

Let us take for instance the C_{w-1} dipolar coupling slices in Figure 5.6b for DLPC at 5°C and 30°C. The ω -slices are different according to each temperature for each concentration and also for the same temperature, the ω -slices are different depending on the concentration. The dipolar coupling spectrum is larger. For example, at the C₂ position for DLPC and DMPC DROSS detects three splittings, one due to the sn - 1 chain and two for the two inequivalent protons of the sn - 2 chain. It has been called the "spectral fingerprint" of phospholipids, and is almost independant of the physical state or chemical composition ([138]). We have shown the splittings of the C3 which was better measurable in Figure 5.6e. Similarly, order parameters in the lipid headgroup are, at a constant ionic strength, relatively independent of the sample phase. We can see that very well in Figure 5.6f, 5.6g, 5.6h (Table 5.3, 5.4, 5.5). The representative differences in order profiles of one lipid to another and of one surfactant to another are in the acyl chains.



FIGURE 5.6: Dipolar coupling slices.

5.2.1.4 Order parameter measurement

The DMPC/gA system has been extensively studied and even the DLPC/gA system using the Deuterium ²H NMR [51, 54, 126, 141, 143, 144]. So with our experiments on these two systems we do not provide new information rather than a new alternative method for the measurement of the magnitudes and signs of ${}^{13}C - {}^{1}H$ dipolar couplings. And most important is using their results as reference for the calculation of S_{CH}. I used the DROSS equation:

$$S_{CH} = \frac{W_{CH}^{measured}}{\chi \times W_{CH}^{static}} = \frac{\omega(Hz)}{\chi'}$$
(5.12)

with ω being the width of the measured dipolar coupling (as shown in Figure 5.5) splitting and χ' being the scaling factor. The scaling factor is a constant that should be determined and divided by our measured dipolar couplings data for all our experiments. In the following I will present the method I followed in order to determine this scaling factor χ' .

First we proceed with the assignment of the ${}^{13}C$ resonances by comparison with literature data. We take as reference the lipid headgroup $\gamma - CH_3$ resonance assigned to 51 ppm. The DROSS technique cannot properly discriminate all the carbons. We have obtained the resonance of C_{ω} , $C_{\omega-1}$, $C_{\omega-2}$, sn-1, sn-2, sn-3, C3, C2, C(4-n), but due to the uncertainty we only took in consideration the following resonances in Table 5.3 to Table 5.5 for DMPC and DLPC.

Since DMPC has been extensively studied as I mentioned above, I relied on those studies to compare the resonances I measured experimentally for the DMPC with the order parameter values obtained in the above references and estimated the scaling factor constant χ' in Equation 5.12 and thus used it to determine the order parameter in the rest of my experiments.

In Table 5.1 I present the order values for C_3 , $C_{\omega-1}$, $C_{\omega-2}$ measured by Douliez (Table 1 of Ref. [126]), Leftin (Table S1 of Ref. [140]) and Gross et al. (Table 1 of Ref. [137]).

Carbon label	Leftin	Douliez	Gross	S_{Avg}	stdv	$\omega_{measured}$	χ'
C_3	0.19	0.221	0.19	0.2	0.018	$1010\ \pm 10$	5040 ± 50
$C_{\omega-2}$	0.12	0.15	0.11	0.126	0.02	670 ± 10	$5290\ \pm 80$
$C_{\omega-1}$	0.09	0.121	0.09	0.1	0.018	$610\ \pm 10$	6080 ± 100
C_{β}		0.03	0.03	0.03	0	130 ± 10	4330 ± 300
						χ'_{Avg}	5200 ± 100

TABLE 5.1: Comparison of order parameter values for DMPC bilayers at 30°C.

From the three references I took the order parameters and determined their average value S_{Avg} in Table 5.1. Using the cross-multiplication I try to determine the scaling factor that allows me to obtain the same value. For example, the $S_{Avg} = 0.2$ for C₃. I measured for C₃ with pure DMPC membranes $\omega_{measured} = 1010 \pm 10 \, Hz$ (Table 5.3). So in order to obtain the $S_{Avg} = 0.2$ I need to divide my width by $5040 \pm 50 \, Hz$. I proceeded similarly for the rest of the DMPC resonances. Let us describe the C_{ω -2} case for an additional example. The average value of the order parameter $S_{Avg} = 0.126$, from the three references. My experimentally measured dipolar coupling for the C_{ω -2} is $\omega_{measured} = 670 \pm 10 \, Hz$ (Table 5.3). By cross-multiplication I obtain $\chi' = 5290 \pm 80 \, Hz$.

I then computed the average value of the four values of the scaling factor χ'_{Avg} and then calculated the order parameter $|S_{CH}|$ (Equation 5.12) for each of the five values of χ' in Table 5.1 and plotted them.

To calculate the uncertainty in the mean of χ'_{Ava} , I used the formula:

$$\Delta \chi'_{Avg} = \frac{\chi'_{max} - \chi'_{min}}{2\sqrt{N}}, \quad \text{with} \quad N = 4$$

I then determined the order parameter for pure DMPC membranes by dividing my own experimentally measured dipolar coupling $\omega_{measured}$ with each of the calculated scaling factor constants χ' .

	$S_{CH} = \frac{\omega_{measured}}{\chi'}$										
	via $\chi' = 5040$	via $\chi' = 5290$	via $\chi' = 6080$	via $\chi' = 4330$	via $\chi' = 5200$						
C_3	0.2 ± 0.003	0.191 ± 0.003	0.166 ± 0.003	0.233 ± 0.016	0.195 ± 0.003						
$C_{\omega-2}$	$0.133\ {\pm}0.002$	$0.127\ {\pm}0.003$	$0.110\ {\pm}0.002$	$0.155\ {\pm}0.011$	$0.129\ {\pm}0.002$						
$C_{\omega-1}$	$0.121\ {\pm}0.002$	$0.115\ {\pm}0.003$	0.1 ± 0.002	0.141 ± 0.01	$0.118\ {\pm}0.002$						
C_{β}	$0.026\ {\pm}0.002$	$0.025\ {\pm}0.002$	$0.021\ {\pm}0.002$	$0.03\ {\pm}0.003$	$0.025\ {\pm}0.002$						

The results are shown in Table 5.2 and the plot in Figure 5.7.

TABLE 5.2: DMPC order parameters determined using the scaling factor χ' calculated in Table 5.1.

The graph in Figure 5.7 shows the order parameter profiles for each of the following carbons of the DMPC bilayers: C_3 , $C_{\omega-2}$, $C_{\omega-1}$ and C_{β} . The scaling factor χ' used to divide the measured dipolar coupling is defined in Table 5.1. The average value of the reference order parameter is represented as red crosses. The values of the order parameter calculated by dividing the measured dipolar coupling through the scaling factor χ' obtained respectively from the order profiles of: $C_{\omega-2}$ (purple dots), $C_{\omega-1}$ (blue dots), C_3 (green dots), C_{β} (brown dots), and the average of the different χ' values represented in black.



FIGURE 5.7: Comparison between order parameters S_{CH} calculated using different scaling factor χ' in Table 5.2 and the reference order parameter S_{Avg} in Table 5.1.

The average value of the four calculated scaling factor χ'_{Avg} is in better agreement with the reference data (in red) than the individual estimates. Thus, for the rest of the experiments, the order parameter is determined using Equation 5.12 with the scaling factor $\chi' = 5200 \pm 100 \, Hz$ and $\Delta S = \frac{1}{5200} \sqrt{100 + (100)^2 \times S^2}$ from Equation ??, using a typical value $\Delta \omega = 10 \, Hz$.

5.2.1.5 Results

The calculated ${}^{1}H - {}^{13}C$ order parameter values were plotted for all biomembranes. The S_{CH} for DLPC were plotted separately for each temperature. A general observation can be made for all systems at 30°C: order parameters are modified with the concentration of gramicidin, we generally observe an increase and it's very clear for each of the lower carbons (C_{ω} , $C_{\omega-1}$, $C_{\omega-2}$), it decreases in some particular cases detailed later. Note that higher temperatures boost the lipids movements and thus increases the order, but in our case the DLPC at 5°C has close results to the DMPC at 30°C. This result is expected since these temperatures for both lipids represent the same relative temperature, a little bit above their respective transition temperature.

For DMPC/gA, as shown in Figure 5.8, the order parameter increases for a ratio of P/L = 0.06 and then decreases for the P/L = 0.115 for the C_{α} , C_{ω} and $C_{\omega-1}$ whereas

it increases depending on the peptide concentration for the C₃ and C_{ω -2}. for C_{β} at P/L = 0.115 the order parameter decreases below that of pure DMPC.

In DLPC mixtures at 30°C (Figure 5.10) the order parameter of the lower acyl chain increases with P/L compared to the pure lipid, reaching almost the same values for both P/L = 0.053 and P/L = 0.112 while in the headgroup the order profile varies. We observe for C_{β} the same behavior as for DMPC, the $|S_{CH}|$ is below that of pure DLPC at both concentration of doped peptide and we find it also for the C_{α} at P/L = 0.112.

For DLPC at 5°C (Figure 5.9) the order parameter remains unchanged for the $C_{\omega-1}$, while for the C_{ω} at a P/L = 0.112 it gets higher but at a lower value than for $C_{\omega-1}$. Furthermore, the C₃ represents 3 splittings with high order profile for the higher concentration of doped peptide at P/L=0.112. This behavior of the C₃, representative of the upper part of the acyl chain, close to the glycerol backbone, is commonly observed for the DMPC, DLPC and C₁₂E₄.

In the case of $C_{12}E_4$ (Figure 5.11) and DDAO (Figure 5.12), the surfactants used for these experiments, we have the remarquable increasing order parameter in the lower acyl chain carbons, the $C_{\omega} C_{\omega-1} C_{\omega-2}$ depending on the gramicidin concentration for each, and also we observe this increasing profile for the headgroup and the C_3 and C_2 , especially in the case of DDAO. As for the temperature depending, We only performed two temperatures for the DLPC/Gramicidin biomembranes, one at 5°C and another measurement at 30°C. We can see very clearly in table 5.4 and table 5.5 that the $|S_{CH}|$ values are higher in the 30°C experiment, especially for the $C\omega_{-1} C\omega_{-2}$, C_3 and C_{α} which is not expected.



The chemical shifts are measured and assigned in Table 5.3.

$\begin{bmatrix} 0 & 1 & 1 & 1 & 1 & 2 \\ \hline 0 & 1 & 1 & 1 \\ \hline 0 & 1 & 1 & 1 \\ \hline 0 & 1 & 1 & 1 \\ \hline 0 & 1 & 1 & 1 \\ \hline 0 & 1 & 1 & 1 \\ \hline 0 & 1 & 1 & 1 \\ \hline 0 & 1 & 1 & 1 \\ \hline 0 & 1 & 1 & 1 \\ \hline 0 & 1 & 1 & 1 \\ \hline 0 & 1 & 1 & 1 \\ \hline 0 & 1 & 1 & 1 \\ \hline 0 & 1 & 1 & 1 \\ \hline 0 & 1 & 1 & 1 \\ \hline 0 & 1 & 1 & 1 \\ \hline 0 & 1 & 1 \\ \hline 0 & 1 & 1 & 1 \\ \hline 0 & 1 & 1 & 1 \\ \hline 0 & 1 \\ \hline 0 & 1 & 1 \\ \hline 0 & 1 & 1 \\ \hline 0 & 1 \\ \hline 0 & 1 \\ \hline 0 & 1 & 1 \\ \hline 0 & 1 \\ $							
Carbon label	$\delta(\text{ppm})$	ú	$J \pm 10 (Hz)$	i)	$S_{CH} \pm 2.10^{-3}$		
		P/L =	P/L =	P/L =	P/L =	P/L =	P/L =
		0	0.06	0.0115	0	0.06	0.0115
C_{ω}	11	0	100	0	0	0.02	0
$C_{\omega-1}$	20	610	700	640	0.12	0.14	0.12
$C_{\omega-2}$	29	670	900	870	0.13	0.17	0.17
C3	22	400	390	500	0.08	0.08	0.1
C3	22	700	720	740	0.14	0.14	0.14
C3	22	1010	1010	1280	0.19	0.19	0.25
C_{α}	57	260	315	270	0.05	0.06	0.05
C_{β}	63	130	170	93	0.03	0.03	0.02
C_{γ}	51	0	0	0	0	0	0

TABLE 5.3: Summary of our experimental results for the DMPC/gA system at P/L = 0, 0.06 and 0.0115 at 30°C. δ is the chemical shift in ppm, ω is the measured dipolar coupling in Hz and S_{CH} is the order parameter. For both parameters, the uncertainty is given after the "±" sign.



Carbon label	$\delta(\text{ppm})$	$\omega \pm 10 \text{ (Hz)}$			$S_{CH} \pm 2.10^{-3}$			
		P/L =	P/L =	P/L =	P/L =	P/L =	P/L =	
		0	0.053	0.0112	0	0.053	0.0112	
C_{ω}	10	0	0	280	0	0	0.05	
$C_{\omega-1}$	20	590	550	550	0.11	0.11	0.11	
$C_{\omega-2}$	29	580	620	540	0.11	0.12	0.10	
C3	22	360	365	405	0.07	0.07	0.08	
C3	22	705	705	790	0.14	0.14	0.15	
C3	22	1005	1050	1330	0.19	0.20	0.26	
C_{α}	56	250	310	300	0.05	0.06	0.06	
C_{β}	63	225	200	190	0.04	0.04	0.04	
C_{γ}	0	0	0	0	0	0	0	

TABLE 5.4: Summary of our experimental results for the DLPC/gA system at P/L = 0, 0.053 and 0.0112 at 5°C. Column labels as in Table 5.3.



FIGURE 5.9: Order parameter for DLPC Bilayers at $5^{\circ}C$.

Carbon label	$\delta(\text{ppm})$	$\omega \pm 10 (Hz)$			$S_{CH} \pm 2.10^{-3}$			
		P/L =	P/L =	P/L =	P/L =	P/L =	P/L =	
		0	0.053	0.0112	0	0.053	0.0112	
C_{ω}	10		90	115		0.02	0.02	
$C_{\omega-1}$	20	550	720	740	0.11	0.14	0.14	
$C_{\omega-2}$	29	740	975	985	0.14	0.19	0.19	
C3	22	395	475	0	0.08	0.09	0	
C3	22	785	765		0.15	0.15		
C3	22	1235	1150		0.24	0.22		
C_{α}	56	360	340	315	0.07	0.07	0.06	
C_{β}	63	210	170	160	0.04	0.03	0.03	
C_{γ}	51	0	0	0	0	0	0	

TABLE 5.5: Summary of our experimental results for the DLPC/gA system at P/L = 0, 0.053 and 0.0112 at 30°C. Column labels as in Table 5.3.



FIGURE 5.10: Order parameter for DLPC Bilayers at $30^{\circ}C$.

Carbon label	$\delta(\text{ppm})$	μ	$\omega \pm 10 (Hz)$			$S_{CH} \pm 2.10^{-3}$			
		P/L =	P/L =	P/L =	P/L =	P/L =	P/L =		
		0	0.015	0.073	0	0.015	0.073		
C_{ω}	14	0	0	0	0	0	0		
$C_{\omega-1}$	23	210	235	335	0.04	0.05	0.06		
$C_{\omega-2}$	32	260	355	360	0.05	0.07	0.07		
C1	71	440	260	400	0.08	0.05	0.08		
C_{β}	72	0	520	0	0	0.1	0		
C_{γ}	0	0	0	0	0	0	0		

TABLE 5.6: Summary of our experimental results for the $C_{12}E_4$ /gA system at P/L = 0, 0.015 and 0.073 at 30°C. Column labels as in Table 5.3.



FIGURE 5.11: Order parameter for $C_{12}E_4$ bilayers at $30^{\circ}C$.

Carbon label	$\delta(\text{ppm})$	ú	$\begin{array}{c c c c c c c c c c c c c c c c c c c $		$_{CH} \pm 2.10^{\circ}$	$c_H \pm 2.10^{-3}$		
		P/L =	P/L =	P/L =	P/L =	P/L =	P/L =	
		0	0.053	0.112	0	0.053	0.112	
C_{ω}	14	210	235	350	0.04	0.05	0.07	
$C_{\omega-1}$	23	515	690	810	0.10	0.13	0.16	
C_2	25	0	270	200	0	0.05	0.04	
C_3	27	160	185	0	0.03	0.04	0	
$C_{\omega-2}$	32	640	840	1125	0.12	0.16	0.22	
CH ₃ headgroup	58	90	260	320	0.02	0.05	0.06	

TABLE 5.7: Summary of our experimental results for the DDAO/gA system at P/L = 0, 0.053 and 0.112 at $30^{\circ}C$.



FIGURE 5.12: Order parameter for DDAO Bilayers at $30^{\circ}C$.

5.3 Discussion

Our results demonstrate that, at constant temperature (30°C), order parameters increase with the concentration of gramicidin, and it's very clear particularly for the lower carbons ($C\omega$, $C\omega_{-1}$, $C\omega_{-2}$). This is a sign of higher order in the plane of the bilayers, which means that adding gramicidin rigidifies the membrane. In many cases we observe a decrease of the order parameter to a value similar to the case of pure membranes after adding a certain concentration of inclusions.

Rice and Oldfield [141], specifically labeled DMPC with deuterium at certain positions and measured the order parameter in the presence of different gramicidin A concentrations. They studied in detail lipids labeled at position 14, for a wide range of gramicidin concentration (Figures 1 and 2 of Ref.[141]). We cannot make a direct comparison, since our technique is not sensitive to position 14 of DMPC (the coupling constant is always zero). However, in Figure 4 of the same reference, the authors show the deuterium quadrupole-echo Fourier-transform NMR spectra for all the carbon positions along the chain in the presence of P/L=0.15 of gramicidin (a P/L higher than our maximum concentration) and the data shows two splittings for the carbon C_2 , as found in our experiments. They conclude that adding up to P/L=0.15 gA increases the order parameter. As the gramicidin content of the bilayer is increased above P/L=0.4, the quadrupole splittings collapses and vanishes beyond P/L=0.5 of gramicidin. But we observe for the bottom carbons of the acyl chains (C_{ω} and $C_{\omega-1}$) an increase of the S_{CH} for the low concentration used and then a decrease back to the inclusion-free order parameter for a P/L=0.115. We also observe the similar effect for the headgroup carbon C_{α} .

Morrow and Davis [51] studied the temperature dependence of the first moment M_1 of an ²*H*-NMR experiment for gramicidin-DPPC-d₆₂, and gramicidin-DMPC-d₅₄ mixtures fully deuterated. The first moment provides information on the whole spectrum, its behavior with added gramicidin or with a temperature change. In Figures 5 and 10 of their paper, they show the temperature dependence of M_1 for gramicidin-DLPC and gramicidin-DMPC. The M_1 curve of a heating process is a reverse "S" shape, with the transition temperature at the center of the "S". Added gramicidin has a strong disordering effect just below the transition temperature and a weaker ordering effect just above the pure lipid transition temperature. In other words, adding gramicidin decreases the M_1 in the gel phase and then increases it in the fluid phase. Knowledge of M_1 , and thus of the average orientational order parameter, allows one to calculate approximately the thickness of the hydrophobic region of the bilayer as a function of gramicidin content [145]. He observes an augmentation of ≈ 1 Å.

De Planque and co-workers [54] investigated the effect of some peptides in different PC bilayers (DLPC, DMPC, DPPC and DSPC). He finds that the order parameter increases of 30% in the case of DLPC, 25% for DMPC, 5% for DPPC and decreases 10% for DSPC which is due to a hydrophobic mismatch that perturbs the bilayer in a systematic manner without apparent peptide aggregation which we can observe in Figure 3 of his paper. De Planque discusses also the bilayer thickness in these 4 mixtures and measures an expanding thickness of 6 Å for DLPC and 4 Å for DMPC.

Douliez [126], Leftin [140], Morrow [51] and De Planque [54] have measured the bilayer thickness and realized that it increases with the gramicidin concentration doped in the bilayer. We tried to measure the bilayer thickness for our measurements but it is too complicated to achieve with only the order parameter values of 5 carbons for each lipid.

Greger Orädd 1995 determined the(DDAO)/water/gramicidin D diagram by ²H NMR [146]. He discovers that further addition of gramicidin above the "ideal" stoichiometry of the L_{α} phase results in changes of the order parameter profile of DDAO. This is reflected in an increased ordering of the headgroup and a decreased in the ordering of the terminal part of the chain, an effect we also observe in Figure 5.12. We have an obvious raise in the order parameter of the headgroup as we increase the content of gA but what's new in our case is that we measure an increase in the terminal part of the tail. Indeed the increase is less prominent as we go down in the chain, $S_{\omega-2} > S_{\omega-1} > S_{\omega}$ but at the level of each carbon the order parameter increases as a function of inclusion concentrations.

Cornell and Separovic, 1988 in Figures 3 and 4 show that there exists an important difference between the DMPC and DDAO molecules, because for the former there is an increased ordering observed for the acyl chains and a decreased ordering in the headgroup whereas we find as mentioned earlier an increase of S_{α} for the low concentration and then it decreases back to its original fluidity for higher concentration, while in C_{β} it is unchanged for the low concentration but decreases beyond the order of the original pure DMPC membrane (Figure 5.8). The profile of the S_{α} is the same for the two terminal carbon of the tail. But in DDAO we have a decrease in S_{CH} in all parts of the measured acyl chains.

Per-Ola Quist 1998 performed a slow spinning ${}^{13}C$ NMR on a gramicidin A in a DDAO membrane. Quist used the intensity of the spinning sidebands from ${}^{13}C$ in the backbone carbonyls to determine the residual chemical shift tensor. The observed shifts indicate the secondary structure for gramicidin A which is a β -sheet-like and its orientation in the membrane. Quist also showed that the zwitterionic surfactant DDAO improves the resolution in the ${}^{1}H$ and ${}^{13}C$ NMR spectrum.

5.3.1 Conclusion

In the current study we present a novel application of MAS dipolar recoupling, the Dipolar Recoupling On-Axis with Scaling and Shape Preservation - DROSS, to record simultaneously the isotopic ${}^{13}C$ chemical shifts (at natural abundance) for surfactant and lipid membranes doped with two concentrations of gramicidin. Via a simple equation, the widths in Hz of the dipolar coupling produce the segmental order parameter measurements of the latter doped lipid and surfactant membranes. So the data treatment is very simple for nonspecialists and the sample preparation is very easy since there is no need for isotopic enrichment, and yet with a simple equation we can have a quantitative measurement of the order parameter , all these facts make this technique

ideal to probe and study new molecules and be able to compare the results with the ones obtained with other similar molecules. We were able to study the order parameter of DDAO and $C_{12}E_4$ acyl chains in membranes doped of gramicidin. These two surfactants are rarely studied by NMR and we performed the same measurements on DMPC and DLPC to validate our technique.

We observe that order profiles significantly increase along the acyl chains when adding gramicidin, except in the case of DMPC where the order profile globally increases with the addition of P/L=0.05 of gramicidin and then decreases at P/L=0.11. This peculiar effect was already qualitatively observed by Rice and Oldfield, at the ω position by ^{2}H NMR [141], and by Cornell and Keniry, measuring the carbonyl CSA by ^{13}C NMR [147]. The increase is larger in DLPC than in DMPC, as already observed by De Planque by ^{2}H NMR with P/L=0.03 gramicidin [54] but in both cases we only observe an increase compared to the inclusion free order. The order parameter is in fact does not depend on raise in the inclusion concentration. The increase is mostly significant in DDAO, as observed by Orädd et al. by ²H NMR [146] and in the case of $C_{12}E_4$ we observe a modest raise in the order parameter with as for DMPC and DLPC, no dependence on the P/L is found. In the headgroup region, effects are generally smaller, within the error bar, except for DDAO where we show that gramicidin has the same effect as in the acyl chains. We must insist on the fact that the order of the acyl chains for DMPC, DLPC and $C_{12}E_4$ increases when adding gramicidin but is independent on the P/L whereas it is significant in the case of DDAO.

Consequently, we show that gramicidin generally rigidifies the acyl chains of DLPC, DDAO and $C_{12}E_4$, as well as the headgroup region of DDAO. In the case of DMPC, gramicidin first rigidifies the acyl chains, but more peptide tends to destabilize the membrane and return it to its original fluidity.

Chapter 6

Effect of inclusions on the positional order between acyl chains

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6.3	Conclusion 166

In this chapter I will study the effect of gramicidin A inclusions on the positional order between DDAO and $C_{12}EO_4$ acyl chains using wide angle X-ray scattering technique. We use this technique because at wide angle we can observe the interaction between the chains. In particular we obtain at $q \approx 0.4$ nm⁻¹ a distinctive peak of the chains and thus we will characterize the degree of positional order by the position and width of this peak.

We study gramicidin A channels inserted within bilayers composed of single-chain surfactants with zwitterionic or nonionic head groups: dodecyl dimethyl amine oxide (DDAO) in the presence and absence of cholesterol and tetraethyleneglycol monododecyl ether $C_{12}EO_4$.

All the experiments presented in this chapter were realized on the two laboratory sources presented in § 2.6.1: MAXS and MOMAC.



FIGURE 6.1: MOMAC setup using the heated bath

We started by using the MAXS setup because, at that time, it was the only one that allowed using a heated sample holder. Once we found that the data was contaminated by parasitic scattering (as explained in Chapter 2), we switched to the MOMAC experiment and found a way to set up the temperature control as shown in Figure 6.1.

To this end we employed a new sample holder, devised and fabricated by Denis Petermann from the MATRIX group of the lab. The holder has ten sample positions and is isolated with kapton foil windows to prevent losses by convection and to keep the temperature constant along the capillary. Water from the heat bath circulates through channels in the aluminum body.

The stock samples used for the WAXS experiments were the same as for the SAXS series, see Table 2.2. The filling process was different, since I used round glass capillaires (Capillary Tube Supplies Ltd, (CTS)), 2 mm in inner diameter and with a wall thickness of 10 microns, flared at the top and sealed at the bottom. With a microspatula, I repeatedly inserted small amounts of sample through the top end and centrifuged the capillary each time until reaching a height of about 1.5 cm.

6.1 Results

The exposure time was 720 s for all the measurements. For each series I first acquired an image in the absence of the X-ray beam to estimate the detection noise due to the detector itself. I refer to this signal as a "dark current"¹ and denote its integrated intensity by I_{dark} . Other calibration acquisitions (with beam) include: a background using no capillary, one using an empty capillary and a water capillary. The data treatment has been done taking in consideration all the corrections I mentioned in the section above.

After measuring the samples I apply the radial averaging to the scattering patterns using a Python script (Figure 2.14a). The radial averaging saves the raw data in a file with 4 columns each corresponding to the point number or radius, the wave vector q, the intensity I_{raw} and its uncertainty. I then use Igor Pro to display and plot the results. For each sample I follow a multi-step procedure to obtain the corresponding corrected intensity I(q):

1. Subtract the dark current

$$I_1 = I_{raw} - I_{dark}$$

2. Divide by the transmission factor

$$I_2 = I_1 / T$$

3. Subtract the background

$$I_3 = I_2 - I_2$$
(capillary)

4. Subtract the water signal, in proportion to the water content of the sample

$$I_4 = I_3 - \phi_W \times I_3$$
(water)

where the intensities I_2 (capillary) and I_3 (water) had first been corrected themselves.

Each sample was measured at nine temperatures. I started at room temperature (close to 20° C), went down respectively to 0° C, 10° C, 15° C and then up to 30° C, 40° C, 50° C, 60° C, finally returned to 20° to check the stability of the samples, position denoted as "20° (return)". The three sample series and their component concentrations are

¹This term is appropriate for CCD detectors, such as those used on MAXS and D2AM, but not for the image plate used on MOMAC, which is based on a different detection principle. For simplicity, I will apply the same name to all of them.

DDAO/Gramicidin						
P/L	0	0.03	0.053	0.112	0.178	
DDAO - Cholesterol /Gramicidin						
P/L	0	0.028	0.042	0.067	0.082	
$C_{12}E_4$ / Gramicidin						
P/L	0	0.0148	0.037	0.053	0.073	0.099

TABLE 6.1: The three sample series. The molar peptide-to-lipid ratio P/L is the number of gramicidin monomers divided by that of surfactant molecules.

described in Table 6.1: two series based on DDAO (with and without cholesterol, respectively) and one $C_{12}E_4$ series.

Some examples of integrated intensities I(q) are shown in Figures 6.2, 6.3 and 6.4. Figure 6.2a and Figure 6.4a illustrate respectively the profile of DDAO at P/L=0.178 and DDAO Cholesterol for P/L=0.082 at various temperatures, while Figure 6.2b and Figure 6.4c illustrate the various profiles of the DDAO sample series at $T = 50^{\circ}C$ in the absence and presence of cholesterol.

Similarly, in Figure 6.3a and Figure 6.3b we visualize I(q) for the C₁₂E₄-Gramicidin system at P/L = 0.073 for all temperatures and for all P/L at $T = 20^{\circ}C$.

The scattered signal obtained for all three systems (Figures 6.2, 6.3, 6.4) exhibits a broad peak centered around 14 nm⁻¹. This WAXS peak is due to the correlation between the hydrocarbon chains of bilayer membranes in the liquid crystalline L_{α} state, and has been known since the 1960's [78, 148–152].

Even at the lowest temperatures, we do not find sharp peaks in any of the three surfactant systems studied, which are thus always in the liquid crystalline phase. The fact the wide-angle peak in our case is broad indicates that the in-plane packing of the chains within the surfactant bilayer is disordered, and can be viewed as a liquid [148].

Our main concern in this study is the effect of the gramicidin content on the liquid order of the chains, in each of the three membranes compositions. I will present each system separately and conclude by a global discussion of all the observations and comparison with the literature in § 6.2.

It should be noted that the (positional) order measured here is quite distinct from the (orientational) order parameter determined by NMR in Chapter 5 for distinct carbons along the alkyl chain, although they can exhibit similar tendencies (for instance, in a gel phase both the positional and the orientational order are generally higher than in the liquid crystalline phase).



FIGURE 6.2: Scattered signal I(q) for DDAO bilayers, as a function of temperature for the most concentrated sample, with P/L = 0.178 (A) and for all concentrations at $T = 50^{\circ}C$ (B).

The information obtained from the WAXS patterns is the structure factor of the alkyl chains (with the main peak at 14 nm^{-1} as the most distinctive feature), in the same way that the SAXS data analyzed in Part I yields the structure factor of the inclusions. Instead of performing a full-curve analysis in terms of liquid state theory, here we will only characterize the degree of positional order by the position and width of the peak.

In all systems, the best fit for the main peak was obtained using a Gaussian function:

$$I(q) = I_0 \exp\left[-\frac{(q-q_0)^2}{2\sigma^2}\right]$$
(6.1)



FIGURE 6.3: Scattered signal I(q) for $C_{12}EO_4$ bilayers, as a function of temperature for a sample with P/L = 0.073 (A) and as a function of concentration at room temperature: $T = 20^{\circ}C$ (B).

with σ the standard deviation. We use Igor Pro (with the package **Multi-peak Fitting** 2), where the **gauss** fit function is defined slightly differently, in terms of the width $w = \sqrt{2} \sigma^2$.

A parameter commonly used to describe the width of a peak on a curve is the Half Width at Half Maximum (HWHM), which is half the value of the Full Width at Half Maximum (FWHM), the latter being defined as the distance between points on the curve at which the function reaches half its maximum value. For a Gaussian, FWHM = $2\sqrt{2 \ln 2} \sigma$ and

²The function **Gauss1D** has the same form as **gauss**. On the other hand, the normalized Gaussian function **Gauss** is defined in terms of σ , as in Eq. (6.1)!

thus

$$HWHM = \sqrt{2\ln 2}\,\sigma = \sqrt{\ln 2}\,w \tag{6.2}$$

Following a common practice in the literature, we also compute an "in-plane spacing", defined by analogy with a Bragg peak as $2\pi/q_0$ and expressed in Å. One should however be very careful when interpreting this quantity as a physical length in the system (in particular as an average distance between first neighbors) because, unlike in crystalline systems, its value depends not only on the concentration, but also on the interaction between objects!

To determine the parameters of interest, first we define on the graph the range of points over which the Gaussian fit will be performed and estimate manually the peaks (the main one and other scattering contributions to be discussed below). For each selected peak there are three parameters: the location (q_0) , the width $(w = \sqrt{2}\sigma)$ and the height I_0 ; there are also three parameters that describe a common quadratic background. For the main peak, we store q_0 compute HWHM according to equation 6.2. The results are shown in Figure 6.5a for the DDAO Gramicidin system, in Figure 6.5b for DDAO Cholesterol Gramicidin and in Figure 6.5c for the $C_{12}E_4$ Gramicidin.

6.1.1 $C_{12}EO_4$

Position As an example, we observe a small decrease of q_0 with the temperature at P/L=0.073 (Figure 6.3a), as well as a very slight increase with P/L at 20°C, as seen in Figure 6.3b. We can observe the overall WAXS peak position shift of the whole $C_{12}EO_4$ membranes as a function of temperature and inclusions in Figure 6.8 where the temperature dependence is very obvious for each P/L. Comparing the value in absence of inclusion, the in-plane spacing decreases after adding gramicidin at a P/L=0.015 but remains almost the same for the different gramicidin content, with a small decrease at high P/L, showing no significant influence of the inclusions on the $C_{12}E_4$ membranes.

Width This conclusion is confirmed by the very modest change in the HWHM values presented in Figure 6.5c. At P/L = 0, the HWHM is very close to 2.6nm^{-1} for all temperatures. As the gramicidin content increases, we observe a small gap between the different temperatures: the width stays constant or increases for the lower temperatures (up to about $40^{\circ}C$) and decreases for the higher ones. This gap widens at high gramicidin content (P/L > 0.07).

6.1.2 DDAO

In the case of DDAO, the influence of gramicidin content is more notable than for $C_{12}EO_4$ and the behavior is richer, especially in the presence of cholesterol.

Position Without cholesterol, the DDAO WAXS peaks coincide for the different temperatures at a given inclusion concentration (e.g. in Figure 6.2a at P/L = 0.178) whereas the profiles differ according to the gramicidin concentration for a given temperature (see Figure 6.2b).

These observations differ in presence of cholesterol where for one concentration of gramicidin inclusions (e.g. case of P/L = 0.082 in Figure 6.4a) at different temperatures, we observe two families in which the spectra are quasi identical: one group at low temperatures $(0 - 30^{\circ}C)$ and another distinct group at higher temperatures $(40 - 60^{\circ}C)$. At $20^{\circ}C$ (Figure 6.4b), the peaks for DDAO Cholesterol tend to superpose for P/L > 0.028, whereas at $50^{\circ}C$ (Figure 6.4c) the peak profiles differ and vary with P/L.

We note that the main peak for DDAO (Figure 6.6) is slightly shifted towards lower wave vectors compared to that of $C_{12}EO_4$, by about 2.5 % (Figure 6.8). For instance, at 50°C $q_0(C_{12}E_4) = 13.87 \text{ nm}^{-1}$, while $q_0(DDAO) = 13.57 \text{ nm}^{-1}$; at 20°C $q_0(C_{12}E_4) =$ 14.21 nm⁻¹ and $q_0(DDAO) = 13.81 \text{ nm}^{-1}$. Finally, at 30°C $q_0(C_{12}E_4) = 14.08 \text{ nm}^{-1}$ and $q_0(DDAO) = 13.73 \text{ nm}^{-1}$. At the position of the main peak, the scattered signal is dominated by the contribution of the tails, which have the same chemical nature (single dodecyl chains) in both surfactants. However, unlike in pure alkanes, the local order of the tails is also influenced by the head group: a larger area per molecule A_m should yield a smaller q_0 . Surprisingly, the best estimate for this parameter is *larger* $(A_m = 41.1\text{\AA}^2)$ [96] for $C_{12}E_4$ than for DDAO $(A_m = 37.8\text{\AA}^2)^{-3}$ by about 8 %. This apparent contradiction could of course be due to errors in the values of A_m , but also to the possible discrepancy (already alluded to above) between $2\pi/q_0$ and an average distance.

For the DDAO system, the peak occurs at much lower q_0 with cholesterol than without: $q_0 = 12.77 \text{ nm}^{-1}$ at $20^{\circ}C$, 12.62 nm^{-1} at $30^{\circ}C$ and 12.28 nm^{-1} at $50^{\circ}C$. Thus, the cholesterol expands DDAO bilayers, in contrast with the condensing effect observed in lipid membranes [154, 155]. More detailed molecular-scale studies (for instance, using the NMR techniques employed in Chapter 5) would be needed to understand this phenomenon.

³To our knowledge, A_m has not been measured directly for DDAO. We use literature values [153] for the molecular volume V_m and the monolayer thickness d_m and evaluate $A_m = V_m/d_m$.

In DDAO systems, without (Figure 6.6) and with cholesterol (Figure 6.7), we observe a small shift of the main peak toward higher q with increasing concentration, revealing a dependence of the in-plane order of the chains with temperature and gramicidin content. The in-plane distance (with or without cholesterol) increases with temperature and decreases markedly with the gramicidin content.

A characteristic of DDAO systems is the presence of a small peak at $q \sim 8.4 \text{ nm}^{-1}$ for P/L > 0.04. This peak cannot be detected in the absence of gramicidin or for low P/L, with or without cholesterol. In the presence of cholesterol, another notable feature is the appearance of a peak at a slightly larger $q \sim 17.6 \text{ nm}^{-1}$ (i.e., shorter distance) with respect to the main peak. This side peak also increases with the peptide content and its location corresponds to a distance of 3.5 Å. We tentatively assign both these signals to the inner structure of the peptide, but their positions do not coincide with that of the helix pitch of the gramicidin channel, which was measured at $4.7 \pm 0.2 \text{ Å}$ in lipid bilayers of comparable thickness [156].

Though the head groups are less structured than the tails [157], their contribution to the total scattering intensity is not negligible. Using numerical simulations, Sega et al. [149] predict a contribution to the scattering signal due to the head groups, which should be dominant in the range $q < 11 \text{ nm}^{-1}$, coherent with the signal at $q \sim 8.4 \text{ nm}^{-1}$. However, it is not clear why the presence of gramicidin would increase the order of the head groups and thus enhance the scattering.

Width Without cholesterol, the width of the main peak in DDAO membranes is little affected by a temperature change, at least between 0 and $60^{\circ}C$. Without gramicidin, we observe two distinct HWHM values: $\sim 2.38 \text{ nm}^{-1}$ at the lower temperatures (between $0^{\circ}C$ and room temperature) and $\sim 2.5 \text{ nm}^{-1}$ for higher temperatures (between 30 and $60^{\circ}C$), but this gap closes with the addition of gramicidin, and at high P/L only an insignificant difference of 0.05 nm^{-1} persists (Figure 6.5a).

On the other hand, at a given temperature the HWHM does vary as a function of P/L. This change is sigmoidal, with an average HWHM of $\sim 2.4 \,\mathrm{nm^{-1}}$ for P/L < 0.05 and $\sim 2.7 \,\mathrm{nm^{-1}}$ for P/L > 0.11. Thus, above this concentration, the gramicidin decreases slightly the positional order of the chains.

An opposite effect is observed in the presence of cholesterol (Figure 6.5b), where at high temperature $(40 - 60^{\circ}C)$ the HWHM drops with the P/L: for instance, from 2.37 nm^{-1} to 2.08 nm^{-1} at $60^{\circ}C$. At low temperature $(0 - 30^{\circ}C)$ there is no systematic dependence on P/L.

Overall, we can conclude that gramicidin addition has an effect that differs according to the membrane composition. The temperature has a significant influence only in the presence of cholesterol.



FIGURE 6.4: Scattered signal I(q) for DDAO Cholesterol bilayers, as a function of temperature for the most concentrated sample, with P/L = 0.082 (A) and for all concentrations at $T = 40^{\circ}$ (B).



FIGURE 6.5: HWHM as a function of the concentration P/L, for all measured temperatures. DDAO bilayers (A), DDAO-Cholesterol bilayers (B) and $C_{12}E_4$ bilayers



FIGURE 6.6: In-plane spacing of DDAO bilayers containing gramicidin as a function of the concentration P/L, for all measured temperatures.



FIGURE 6.7: In-plane spacing of DDAO cholesterol bilayers containing gramicidin as a function of the concentration P/L, for all measured temperatures.


FIGURE 6.8: In-plane spacing of $C_{12}EO_4$ bilayers containing gramicidin as a function of the concentration P/L, for all measured temperatures.

6.2 Discussion

As for many molecules containing hydrocarbon chains, the x-ray scattering signal of lipid bilayers exhibits a distinctive peak with position $q_0 \sim 14 \,\mathrm{nm^{-1}}$, indicative of the packing of these chains in the middle of the membrane. We used the position and width of this peak as probes for the liquid order of the chains under the effect of gramicidin content.

The effect of peptide inclusions on the chain peak has been studied for decades [158]. Systematic investigations have shown that some AMPs (e.g. magainin) have a very strong disrupting effect on the local order of the chains: the chain signal disappears almost completely for a modest concentration of inclusions [159–161]. With other peptides, the changes in peak position and width are more subtle [162] and can even lead to a sharper chain peak (as for the SARS coronavirus E protein [163]).

In all systems and over the temperature range from 0 to $60^{\circ}C$, the peak is broad, indicating that the alkyl chains are in the liquid crystalline state. There are however subtle differences between the different compositions, as detailed below.

In $C_{12}E_4$ membranes, the peak position q_0 decreases very slightly with temperature, while the HWHM is almost unchanged by temperature or gramicidin content.

For DDAO (without cholesterol), q_0 also decreases with the temperature rise at a given P/L, but increases with P/L at fixed temperature. On adding gramicidin, the HWHM increases slightly with a sigmoidal dependence on P/L. Thus, a high gramicidin concentration $P/L \ge 0.1$ reduces the positional order of the chains in DDAO bilayers.

The opposite behavior is measured in DDAO membranes with cholesterol. Adding gramicidin inclusions have two distinct behaviors depending on the temperature. For low temperatures (between $0^{\circ}C$ and $30^{\circ}C$) we have a small peptide concentration dependence and a clear temperature correlation, whereas at high temperatures (between $40^{\circ}C$ and $60^{\circ}C$) we have a strong decrease in the HWHM in presence of inclusions depending only with the P/L content without any variation with the temperature rise. Since at P/L=0 the HWHM value is very close for the different temperatures then we can conclude that adding gramicidin to a membrane containing cholesterol helps rigidify it.

Another remarkable observation with DDAO bilayers, with and without cholesterol, is the presence of a short peak at smaller angles for $q \sim 8.4 \text{ nm}^{-1}$ for P/L > 0.04. This peak doesn't exist for low P/L or for pure DDAO, with or without Cholesterol membranes which means that it is due to the peptide content contribution.

In presence of cholesterol, another notable feature is the presence of a spike at higher angle located at slightly larger wavevectors with respect to the tails' peak, at $q \sim 17.6$ nm⁻¹. These spikes are marked with increasing peptide content. The peak's location corresponds to a distance of 3.5Å which coincides with the axial distance between adjacent residues in a beta sheet. And according to [17, 25] the most preferred (thermodynamically stable) conformation of the gramicidin dimer channel in membranes is a single stranded $\beta^{6.3}$.

The latter observations are not directly related to the order parameter but are relevant to be mentioned.

6.3 Conclusion

We have proved that inserting gramicidin in bilayers modify the local order of the constituent acyl chains depending on multiple factors, particularly we studied the influence of the membrane composition and temperature on the local order. We showed that the temperature has a significant influence only in the presence of cholesterol. Furthermore, we showed that not only it influence the acyl chain's order but also, adding gramicidin increases the order of the DDAO head groups and thus enhance the scattering. Additionally, we were able to discover the apparition of small side peaks for low and high q with respect to the main peak and only from a certain P/L in DDAO membranes and DDAO/Cholesterol. While the gramicidin content seem to notably influence the behavior of DDAO, especially in the presence of cholesterol, we do not find such a major influence on the $C_{12}E_4$ membranes.

Chapter 7

Conclusion

The aim of this study was probing the interaction of membrane inclusions with their environment, in our case lipid or surfactant bilayers. Inserting nano-objects within the membrane has two types of effects: a bilayer deformation (a collective effect, involving many molecules, over several nanometers), but also local variations in the positional and orientational order parameter of its constituents (lipid or surfactant molecules), that occur at a "microscopic" scale, involving an individual molecule and possibly its closest neighbors. We studied both these effects, as discussed below.

The bilayer deformation engenders a membrane-mediated interaction between the inclusions, with a range of nanometer order. The most adapted technique is X-ray scattering, since it is non-destructive, performs a statistical average over time and space and the wavelength used is of the same order of magnitude as the space scales to be probed. The measurements were performed at a synchrotron facility (the D2AM beamline of the ESRF), affording very good flux and resolution.

We performed systematic studies in order to quantify the interaction potential between two types of inclusions in several kinds of membranes and tried to elucidate the influence of the relevant parameters: the type of lipids or surfactants, the cholesterol content, the hydration degree, the type of inclusions and the membrane thickness.

To study the local properties of the molecules in the bilayer, we also used X-ray scattering, but on in-house setups and at larger angles, in order to quantify the positional order of the acyl chains of the lipids and surfactants, at different inclusions concentrations.

The orientational order of the various segments of the acyl chains was determined by NMR measurements, performed using the DROSS (Dipolar Recoupling On-Axis with Scaling and Shape Preservation) technique. In the first part of the thesis we measured the membrane-mediated interaction potential in the plane of the membrane at multiple inclusions concentration and as a function of temperature for eight different systems, among which three systems showed an interaction between inclusions in adjacent membranes, and five systems where the nano-objects only interacted with neighbors in the same membrane.

For systems with no inter-membrane interaction, we performed small-angle x-ray scattering (SAXS) in normal incidence (with an incident beam parallel to the layer normal) on highly oriented multilayer samples of various surfactants, doped with different inclusions at multiple temperatures. The analysis yielded the diffuse signal scattered by the 2D fluid of inclusions in the plane of the layers $I(q_r)$ (at $q_z = 0$). The data was described as the product of the form factor of the particle $|F(q)|^2$ with the structure factor of the two-dimensional fluid, S(q). While the former was obtained as the Fourier transform of the atomic coordinates, the latter was obtained in the framework of standard liquid state theory using the integral equations of the Ornstein-Zernicke with the Percus-Yevick closure by an iterative numerical calculation based on a potential V(r)containing a hard-core repulsion and an additional "soft" component representing the membrane-mediated interaction.

We showed that this potential varies with the temperature and with the membrane composition. It decreases when both the density of inclusions across the membrane and also the temperature increases due to the decrease of the membrane elastic constants. We also proved that for the same membrane mixture we don't have the same deformations and results when using different inclusions. So the geometry of the membrane plays an important role on its degrees of freedom. In fact the gramicidin hydrophobic thickness is higher than the surfactant bilayer thickness used in this thesis (in the case of both DDAO and $C_{12}E_4$) which induces a hydrophobic mismatch and thus a perturbation in the membrane resulting in an interaction potential. As for BuSn, the small inclusions are placed in the vicinity of the membrane and tend to separate the two monolayers of a bilayer from the inside and thus perturb the membrane and we have an interaction potential. We also studied the effect of cholesterol content on the interaction-potential along the temperature and inclusions concentration. We found that with gramicidin in membrane-free of cholesterol the interaction potential is much higher than in presence of cholesterol, whereas for the BuSn hybrid inclusions we found the opposite effect. We explained this by the fact in absence of cholesterol, we have a hydrophobic mismatch when adding the gramicidin inclusion as just explained earlier, but once we add cholesterol, the hydrophobic thickness of the membrane increases and matches that of the gramicidin inclusion and results in a hydrophobic matching and thus no interaction is seen. On the other side, when adding cholesterol for BuSn embedded membranes, the

increase in the bilayer thickness induce a higher interaction potential as the BuSn in the middle of the membrane tends to separate even more the monolayers.

When the particles also interact with inclusions in neighboring membranes of the lamellar stack, we changed the geometry by turning the flat sample with respect to the incoming beam, and thus gaining access to the complete structure factor $S(q_r, q_z)$. We described it in terms of the interaction potentials $V_0(r)$ (in-plane potential) and $V_1(r)$ (interplane potential), based on the same integral equations applied to this case with a more elaborate model. We found that the membrane properties highly affect the inter-layer interaction between inclusions, particularly the presence of cholesterol.

We also found that the degree of hydration and probably the surfactant type also play a major role on this type of interaction. We did not identify any interlayer interaction between inclusions embedded in $C_{12}E_4$ membranes, whereas in DDAO membranes we measured interactions between gramicidin and BuSn inclusions in neighboring layers. Though both surfactants have the same hydrophobic length, the $C_{12}E_4$ membranes were more hydrated (50 W% H₂O) than DDAO membranes (20 W% H₂O) so the water layer between the inclusion layers is thicker, reducing the interaction in $C_{12}E_4$ membranes.

In the second part of this thesis, we investigated the influence of gramicidin channels on the local order of acyl chains of two types of lipid (DLPC and DMPC) and two surfactants DDAO and $C_{12}E_4$. We combined two complementary techniques: wide-angle X-ray scattering (WAXS) which gives access to the positional order between neighboring chains and nuclear magnetic resonance (NMR) which is sensitive to the orientational order of chain segments, thus yielding a comprehensive picture of the state of the membrane as a function of the concentration of inclusions.

With NMR we observe that order profiles increase along the acyl chains when adding a first concentration of gramicidin but is independent on P/L for DLPC and $C_{12}E_4$. The increase is mostly significant with the concentration of the pore in the case of DDAO. For DMPC we found that the order profile globally increases with the addition of P/L = 0.05 of gramicidin and then decreases at P/L = 0.11. For the lipids, the increase is larger in DLPC than in DMPC. In the headgroup region, effects are generally smaller –within the error bar– except for DDAO where we show that gramicidin has the same effect as in the acyl chains. Consequently, we show that gramicidin generally rigidifies the acyl chains of DLPC, DDAO and $C_{12}E_4$, as well as the headgroup region of DDAO. In the case of DMPC, gramicidin first rigidifies the acyl chains, but more peptide tends to destabilize the membrane and return it to its original fluidity.

Using WAXS we observe a broadening in the wide-angle peak an indicative of a disorder in the in-plane packing of the molecules within the bilayer, as in the fluid lamellar $L\alpha$ phase. In both cases with DDAO and with DDAO Cholesterol we have a small shift in the wide-angle peak toward upper angles at the higher concentrations, revealing a pronounced temperature and inclusion content dependence of the in-plane structure, with the average separation between surfactant molecules increasing with temperature and decreasing markedly with the inclusion content. In the case of $C_{12}E_4$ we have no significant change with the gramicidin concentration but we observe a proportional variation of the in-plane spacing with the temperature.

Comparing the effect of gramicidin inclusions on the local order of DMPC, DLPC, DDAO and $C_{12}E_4$ acyl chains at 30°C in terms of the orientational order parameter measured using NMR and of the positional order degree using WAXS yield similar results except for DDAO surfactant.

We measured by NMR that the orientational order parameter for DMPC increases when adding P/L=0.05 and slightly decreases at P/L=0.01. This behavior was also measured by WAXS for the positional order parameter at both P/L values. Similarly, we measured for DLPC acyl chains the same orientational and positional order profiles where the order increases for P/L=0.05 and remains the same when adding P/L=0.1 gramicidin. As for the $C_{12}E_4$ surfactant acyl chains, we found a modest raise in both the orientational and the positional order parameters when adding the gramicidin peptide with no dependence on the P/L molar ratio. Only for DDAO we found that adding gramicidin significantly increases the orientational order and decreases the positional order degree.

In few words, we probed the interaction potential between inclusions within the same layer and between adjacent layers of two surfactants types and found that the interaction potential can be described by a decreasing exponential as a function of inclusion concentrations, temperature and cholesterol content. Furthermore we showed that inserting peptides inclusions within the membrane rigidifies the acyl chains and modifies their local order.

Perspectives

This thesis opens a number of perspectives, such as extending the composition range of accessible systems (in terms of inclusions and membrane components), applying complementary techniques or using more refined analysis approaches.

In particular, future work should:

• Our Waxs and NMR results showed that inclusions perturb modestly the bilayer. This is a very important finding which allows further elaboration of elastic models in the presence of inclusions by using the same elastic constants obtained in models without these inclusions

- Current collaboration with Paolo Galatola and Jean-Baptiste Fournier (Florent Bories thesis subject) : fit our results with the collaborators' theoretical and analytical models to describe our experimental potentials with the elastic constants of the bilayer
- Use two types of inclusions in the same membrane to try to be the closest to the biological membrane composition. We can also use two types of surfactants or lipids, in the presence or absence of cholesterol.
- Further investigate the cholesterol content effect on the membrane perturbation in presence of inclusions and as a function of temperature by performing off-plane saxs at different experimental temperature.
- Use of surfactants with different hydrophobic length to probe the hydrophobic matching effect.
- Use peptide that can be easily aligned so we can have better homeotropic anchoring, for example peptides that can be aligned via electric or magnetic fields.
- Study the activity of the gramicidin pore as a function of the different parameters elucidated in this thesis and try to extrapolate these results to membrane proteins.
- Use our experimental results as models to further elaborate numerical simulations. This is done by describing in detail the membrane elasticity at the nanoscale in terms of the relevant material parameters and correlate their value with our inplane interaction potential $V_0(r)$.
- Perform DROSS NMR on samples in presence of cholesterol and study the effect of cholesterol on the acyl chains order
- Perform DROSS NMR on a wider range of samples by using the BuSn inclusions, adding cholesterol (to both the BuSn and gramicidin membranes), and do those measurements at different temperatures.

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Résumé

Etude de l'interaction entre des nano-inclusions médiée par des membranes de surfactant et des changements du paramètre d'ordre local des chaînes alkyles

Pendant les dernières décennies les chercheurs ont étudié l'interaction entre des inclusions membranaires avec leur environnement et plus particulièrement avec la bicouche lipidique. On trouve dans la litérature beaucoup d'études théoriques et de simulations numériques sur ce sujet mais très peu d'expériences ont été menés là-dessus. D'où le but de ma thèse.

Le dopage de nano-objets dans les membranes a deux types d'effets: une déformation de la bicouche (effet collectif impliquant plusieurs molécules sur une échelle de quelques nanomètres), mais aussi des variations locales du paramètre d'ordre au niveau de la position et l'orientation des constituents membranaires (molécules de lipides ou de tensioactifs) qui ont lieu à une échelle microscopique impliquant une seule molécule ou bien la molécule voisine la plus proche. Nous avons étudié ces deux effets et nous présenterons brièvement les résultats les plus marquants.

La déformation de la bicouche engendre une interaction entre les inclusions médiée par la membrane sur une échelle de l'ordre du nanomètres. La technique la plus adaptée est la diffusion des rayons X, une technique non destructive qui effectue une moyenne statistique sur le temps et l'espace en utilisant une gamme de longueur d'onde de la même ordre de grandeur que celle du milieu sondé. Les mesures ont été effectuées au synchrotron (à l'ESRF sur la ligne de lumière D2AM).

Nous avons effectué des études systématiques afin de quantifier le potentiel d'interaction entre deux types d'inclusions dans plusieurs types de membranes dans le but d'élucider l'influence des paramètres pertinents suivants: la concentration de particules dopantes, le type de tensioactif, l'épaisseur de la membrane, le contenu en cholestérol, la température, le degré d'hydratation. Afin d'étudier les propriétés locales des molécules dans la bicouche, nous avons aussi eut recours à la diffusion des rayons X, mais en utilisant le montage de notre laboratoire à plus grands angles. Ceci nous permet de quantifier l'ordre positionnel des chaînes alkyles, des lipides et des tensioactifs, à différentes concentrations d'inclusions.

L'ordre orientationel des différents segments de la chaîne alkyle a été déterminé par des mesures de résonance magnétique nucléaire en effectuant la technique de DROSS (Dipolar Recoupling On-Axis with Scaling and Shape Preservation).

Dans la première partie de cette thèse nous avons mesuré le potentiel d'interaction médié par la membrane à plusieurs concentrations d'inclusions et à différentes températures dans le plan même de la membrane pour huit systèmes différents. Parmis ces derniers nous avons trouvé trois systèmes présentant une interaction entre les inclusions dans les couches membranaires adjacentes et puis cinq systèmes dans lesquels les nano-objets dopés interagissent seulement avec les molécules voisines dans le plan de la même bicouche.

Pour les systèmes sans interactions entre les couches, nous avons effectué de la diffusion des rayons X aux petits angles (SAXS) en incidence normale (avec le rayon incident parallèle à la normale à la couche), sur des multicouches hautement alignées formées par des tensioactifs différents et dopées dans chaque cas par un système d'inclusions différent et ceci à plusieurs températures. L'analyse des clichés de diffraction donne le signal diffusé par le fluide bi-dimensionnel formé par les particules dans le plan des couches $I(q_r)$ (à $q_z = 0$). Vu que les inclusion dans les membranes forment un ensemble d'objets identiques et avec une même orientation (jusqu'à une moyenne azimutale), nous pouvons appliquer la séparation classique de l'intensité diffusée en un facteur de structure S(q)multiplié par un facteur de forme $F(q)^2$. Ce dernier est la transformée de Fourier des coordonnée atomiques et le premier est obtenu selon la méthode de Lado et implémentée sous la forme d'une fonction Igor Pro. Cette méthode détermine une solution itérative de l'équation Ornstein-Zernicke, avec la relation de fermeture de Percus-Yevick basée sur un potentiel V(r) contenant une répulsion de type disque dure avec une composante supplémentaire qui représente l'interaction médiée par les membranes.

Nous avons montré que ce potentiel varie avec la température et la composition de la membrane (Figure 7.1). Il diminue quand la densité d'inclusions le long de la membrane et la température diminuent ceci à cause de la diminution des constantes élastiques de la membrane. Nous avons aussi montré que pour une même composition membranaire nous n'obtenons pas les mêmes déformations et les mêmes résultats quand nous utilisions des inclusions différentes. Ceci montre que la géométrie de la membrane joue un rôle très important sur ses degrés de liberté. Plus particuliérement, le pore de gramicidine a une épaisseur haydrophobe plus grande que celle de la membrane des surfactants

utilisés (DDAO ou $C_{12}E_4$) donc une fois inséré, nous avons un "hydrophobic mismatch" et le pore perturbe la membrane ce qui induit l'interaction. En ce qui concerne les nanoparticules de BuSn, elles vont se placer au sein de la membrane et séparer les deux monocouches de l'intérieur et ainsi induire une interaction. Ensuite, nous avons étudié l'effet du contenu en cholesterol sur le potentiel d'interaction en fonction de la température et de la concentration en inclusions. Nous avons montré que dans le cas de la gramicidine dopée dans des membranes sans cholesterol, le potentiel d'interaction est plus élevé que celui en présence du cholesterol, alors que nous avons trouvé l'effet opposé en dopant du BuSn.



FIGURE 7.1: Potentiels d'interaction estimés par l'algorithm de Lado à différentes temperatures pour: (A) gramicidine/ $C_{12}E_4$; (B) gramicidine/ $C_{12}E_4$ /cholesterol; (C) BuSn/ $C_{12}E_4$; (D) BuSn/ $C_{12}E_4$ /cholesterol

Dans le cas où les particules interagissent avec des inclusions dans les bicouches voisines, nous avons changé la géométrie en tournant l'échantillon plat le long de la direction du rayon incident, et ainsi nous avons accès au facteur de structure complet $S(q_r, q_z)$. Nous l'avons décrit en terme des potentiels d'interaction $V_0(r)$ (potentiel dans le plan) et $V_1(r)$ (potentiel entre les plans), basé sur les mêmes équations intégrales appliquées dans notres cas en utilisant un model plus élaboré. Nous avons trouvé que les propriétés de la membrane, et surtout la présence du cholesterol, affectent énormément l'interaction inter-lamellaire entre les inclusions. Nous avons aussi trouvé que le degré d'hydratation et probablement aussi le type de tensioactif joue tous de même un rôle majeur dans ce type d'interaction (Figure 7.2). Nous n'avons pas identifié d'interaction inter-lamellaire entre les inclusions dopées dans des membranes de $C_{12}E_4$, alors que dans le cas des membranes de DDAO, nous avons mesuré des interactions entre des inclusions de gramicidine et de BuSn dans des couches voisines.

Les deux tensioactifs ont la même épaisseur hydrophobique, cependant les membranes de $C_{12}E_4$ étaient plus hydratées (50 W% H₂O) que les membranes de DDAO (20 W% H₂O). Donc la couche d'eau entre les couches dopées est plus épaisse dans le cas des membranes de $C_{12}E_4$ réduissant ainsi l'interaction entre ses couches.



FIGURE 7.2: Comparaison des potentiels d'interactions dans le plan à différentes températures pour: (A) gramicidine/DDAO; (B)gramicidine/DDAO/cholesterol; (C) BuSn/DDAO

Dans la deuxième partie de cette thèse, nous avons examiné l'influence des pores de gramicidine sur l'ordre local des chaînes d'alkyles de deux types de lipides (DLPC et DMPC) et de deux tensioactifs (DDAO et $C_{12}E_4$). Pour cette fin, nous avons combiné deux techniques complémentaires: La diffusion des rayons X aux grans angles (WAXS) qui donne accès à l'ordre positionnel entre les chaînes voisines, et la résonance magnétique nucléaire (RMN) qui est très sensible à l'ordre orientationnel des segments de chaînes ainsi rend une image compréhensive de l'état de la membrane en fonction de la concentration d'inclusions.

Par RMN (voir Figure 7.3), nous avons observé que les profiles des paramètres d'ordre augmente le long des chaînes d'alkyles une fois une concentration en gramicidine est ajoutée mais cette augmentation est indépendente du rapport P/L pour DLPC et $C_{12}E_4$. Dans le cas de DMPC on observe que le profile d'ordre croît en ajoutant une concentration de P/L = 0.05 de gramicidine et puis décroît à P/L = 0.11. La croissance est plus grande dans le cas de DLPC que DMPC. L'ordre des chaînes alkyles croît significativement dans le cas du DDAO et ceci en fonction de la concentration d'inclusion de gramicidine. Dans la région des têtes polaires, les effets sont généralement réduits -dans les limites des barres d'erreur- à l'exception du DDAO pour lequel nous avons montré que la gramicidine a le même effet que sur les chaînes d'alkyles. Par conséquence, nous avons montré que la gramicidine tend généralement à rigidifier les chaînes d'alkyles du DLPC, DDAO et $C_{12}E_4$, de même que les têtes de DDAO. Dans le cas de DMPC, la gramicidine rigidifie d'abord les chaînes d'alkyles, mais ensuite l'ajout de plus de peptides tend à déstabiliser la membrane et la rammener à son état de fluiditié principal.



FIGURE 7.3: Comparaison des paramètres d'ordre orientationel à 30°C pour: (A) gramicidine/DMPC; (B)gramicidine/DLPC; (C) gramicidine/C₁₂E₄; (D) gramicidine/D-DAO

Par WAXS (voir Figure 7.4), nous observons un élargissement du pic des grands angles indiquant un désordre dans l'empilement planaire des molécules au sein de la bicouche, comme dans une phase lamellaire, L_{α} . Dans les deux cas du DDAO avec et sans cholesterol, et à haute concentration d'inclusions, nous avons un petit déplacement du pic des grands angles vers les plus grands angles. Ceci montre une dépendance en température et en inclusions prononcée de la structure se trouvant dans le plan de la membrane avec une séparation moyenne entre les molécules de tensioactifs qui augmente avec avec la température et notamment diminiue avec la concentration en inclusions. Dans le cas du $C_{12}E_4$, nous avons aucun changement significatif en fonction du contenu en gramicidine mais nous trouvons une variation de l'espacement dans le plan en fonction de la température.



FIGURE 7.4: Comparaison des mi-largeurs à mi-hauteur mesurées à différentes tempértures et á différentes concentrations de gramicidine pour: (A) gramicidine/D-DAO; (B)gramicidine/DDAO/Cholesterol; (C) gramicidine/ $C_{12}E_4$

Pour conclure, nous avons sondé le potentiel d'interaction entre des inclusions au sein d'une même couche et entre des couches adjacentes. Nous avons trouvé que le potentiel d'interaction peut être décrit par une exponentielle décroissante, en fonction de la concentration en inclusions et de la température. En plus, nous avons montré que l'insertion de peptides au sein des membranes rigidifie les chaînes d'alkyles et modifie leur ordre local. Enfin cette thèse ouvre de nombreuses perspectives tant dans le fait d'étendre la gamme de composition des systèmes (en terme d'inclusions et de composants membranaires), appliquer des techniques complémentaires et utiliser des approches plus raffinées pour l'analyse des données. Plus particulièrement, nous avons montré par WAXS et RMN que les inclusions perturbent peu les membranes. Ce résultat est très important et permet d'élaborer des modèles élastiques de membranes en présence d'inclusions tout en utilisant les mêmes constantes élastiques obtenues en abscence de ces inclusions.

D'autre part aussi une collaboration avec Jean Baptiste Fournier et Paolo Galatola est en place (aussi dans le terme du sujet de thèse de Florent Bories) afin de fitter nos résultats par leur modèles théoriques et analytiques afin de décrire nos potentiels d'iteraction expérimentales obtenus pendant cette thése par les constantes élastiques de la biouche. On peut ajouter aussi pour un futur travail: Utiliser deux types d'inclusions au sein d'une même membrane, aussi deux types de tensioactifs ou de lipides avec et sans cholesterol, afin d'être le plus proche possible de la composition membranaire biologique. On peut encore aller plus loin en effectuant du saxs hors-plan sur des échantillons avec du cholesterol et étudier son effet sur la perturbation de la membrane aussi en variant la température et la concentration en inclusions. De même il faudra utiliser des tensioactifs avec différentes épaisseur hydrophobe afin d'examiner plus en détail l'effet du décalage hydrophobe. En continuité, il est essentiel d'étudier l'activité du pore de gramicidine en fonctions des différents paramètres élucidés dans cette thèse dans une tentative d'extrapoler les résultats aux protéines membranaires. En plus, il faudra utiliser nos résultats expérimentaux comme modèle pour élaborer davantage les approches et les simulations numériques. D'autre part, effectuer la technique de rmn DROSS sur les échantillons en présence de cholesterol et en utilisant aussi les inclusions de BuSn et en plus à différentes températures permettra étude détaillée de ces paramètres sur l'ordre local des chaînes d'alkyles.



Title : Interaction between inclusions mediated by surfactant membranes and changes in the local order of the acyl chains

Keywords : Mediated interaction, inclusions, bilayers, order parameter, SAXS, DROSS-NMR, WAXS

Abstract : The aim of this study was probing the interaction of membrane inclusions with their environment, in our case lipid or surfactant bilayers. Inserting nano-objects within the membrane has two types of effects: a bilayer deformation which engenders a membrane-mediated interaction between the inclusions at the nanometer scale. And a second effect defined by local variations in the positional and orientational order parameter of the membrane constituents that occur at a "microscopic" scale. In order to study the membranemeditated interaction between embedded inclusions we performed a systematic investigation on highly aligned and oriented lamellar phases using Small-Angle X-ray Scattering (SAXS). The oriented lamellar phases are essential to provide a strong scattering signal but on the other hand might also cause an eventual interaction between the layers. So a very important information is whether the particles only interact within the plane of their own layer, or also with inclusions in neighboring layers in the lamellar stack. So we studied as well the interaction potential in the plane of the membrane in the presence of an interlayer interaction between neighbor inclusions. All of these studies were done by varying the following relevant parameters: membrane thickness, cholesterol content, temperature, degree of hydration, surfactant type, inclusions types and the inclusion density. The interaction potential was obtained from the structure factor of the scattered samples in the framework of standard liquid state theory using the integral equations of the Ornstein-Zernicke with the

Percus-Yevick closure by an iterative numerical calculation based on a potential V(r) containing a hard-core repulsion and an additional "soft" component representing the membrane-mediated interaction. We found that the interaction potential decreases with the temperature and the inclusion content. We also found that the cholesterol plays a major role on the interaction since it changes the hydrophobic thickness of the membranes but its effect varies according to the type of inclusion used. We found that the membrane geometry is important to the effect of cholesterol on the interaction between the inclusions. Additionally the hydration degree plays an important role on the interaction type. Only at low hydration, the layers are closer and the inclusions are able to interact with their counterparts in neighboring layers.

In order to have a comprehensive picture of the state of the membrane as a function of the concentration of inclusions, we combined two complementary techniques: wide-angle X-ray scattering (WAXS) which gives access to the positional order between neighboring chains and a nuclear magnetic resonance technique"DROSS-NMR" which is sensitive to the orientational order of chain segments. Overall we have small modifications in the local order in terms of position and orientation when adding gramicidin especially in the case of DMPC, DLPC and $C_{12}E_4$. Only in the case of DDAO, the gramicidin content seem to notably influence the acyl chains by decreasing their positional order and increasing their orientational order

Titre : Etude de l'interaction entre des nano-inclusions médiée par des membranes de surfactant et des changements du paramètre d'ordre local des chaînes alkyles

Mots clefs : Interaction médiée par les membranes, inclusions, paramètre d'ordre, SAXS, RMN, WAXS

Résumé : Pendant les dernières décennies les chercheurs ont étudié l'interaction entre des inclusions membranaires avec leur environnement et plus particulièrement avec la bicouche lipidique. On trouve dans la litérature beaucoup d'études théoriques et de simulations numériques sur ce sujet mais très peu d'expériences ont été menés là-dessus.

Nous avons effectué des études systématiques afin de quantifier le potentiel d'interaction entre deux types d'inclusions au sein de la même couche et entre des couches adjacentes de plusieurs types de membranes ceci en variant surtout la concentration de particules dopantes, mais aussi d'autres paramètres pertinents : le type de tensioactif, l' épaisseur de la membrane, le contenu en cholestérol, la température, le degré d'hydratation. Pour cette fin nous avons utilisé la diffraction des rayons X aux petits angles et nous avons constaté que

le potentiel d'interaction peut être décrit par une exponentielle décroissante en fonction de la concentration et de la température d'inclusion et qu'il dépend largement de la teneur en cholestérol et du degré d'hydratation. D'autre part, nous avons étudié l'effet de la gramicidine, un peptitde membranaire, sur l'ordre local des chaînes alkyles de lipides et de tensioactifs. Cette étude a été menée en utilisant deux techniques différentes: en premier lieu la résonnance magnétique nucléaire, (DROSS-NMR) qui permet de détecter le changement d'ordre dans l'orientation des chaînes alkyles, et en second lieu par diffraction des rayons X aux grands angles afin de déterminer le changement d'ordre dans la position des chaînes alkyles. Nous avons trouvé que le dopage de la gramicidin dans les membranes rigidifie les chaînes alkyles et dans un cas aussi les têtes polaires et en plus induit une modification de l'ordre local de ces chaînes.

