EFFECTS OF PHYSICAL AND CHEMICAL PARAMETERS ON THE BEHAVIOR OF SUPPORTED LIPID BILAYERS STUDIED BY AFM

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to my family, Federica and Alfredo, which fully supported me during these three PhD years by understanding and encouraging all my steps.
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MOTIVATION

My PhD activity has been focused on the investigation of how chemical (such as pH and ionic strength) and physical (such as temperature and pressure) parameters could affect the behavior of a particular model of biological membranes and on the understanding of which interactions could be involved. I concentrated on Supported Lipid Bilayers (SLBs), which means planar lipid bilayers deposited on smooth solid substrates such as mica and silicon oxide. I chose a particular lipid mixture (POPE:POPG 3:1) which mimics the bacterial biological membrane in order to elucidate what happens in living cells when, for example, they are exposed to pH gradients in pathological conditions or when the ionic strength changes, for instance, due to pump actions. It is also to be noted that such variations may influence both the lipids conformational state and their interactions with proteins, which in turns, due to these conformational changes, may alter their functions.

All this work rises by the fact that, instead of what was believed in past, biological membranes are no more considered to be a uniform matrix which only hosts proteins but a interactive network that is able to modulate many cell functions including proteins behavior. The main focus of the work is that of characterizing the main phase transition of SLBs model systems. The reason for this choice is that, even if biological membranes are usually found in the fluid phase in physiological conditions, the solid phase is usually within reach with small variations in physical or chemical parameters. In particular, one of the main parameters which might be relevant for inducing local lipid phase changes is pH. I also concentrated on the mechanical properties of the lipid bilayer as can be obtained by Atomic Force Spectroscopy studies. Even in this case I focused my activity on the characterization of the mechanical properties across the lipid phase transition. The strong motivation for this aspect is that the mechanical properties of the lipid bilayer, especially when studied on the nanoscale, might be very relevant to the activity of membrane proteins. In fact, membrane proteins usually perform their physiological activity by changing their conformation within the lipid bilayer. A modification of the lipid bilayer mechanical properties, such as lateral compression, might influence the free energy of the membrane proteins altering the population of the different conformational states.
In section 1 an overview of the biological membranes and their functions will be introduced; moreover an outline of the simplified systems (model systems) used to perform the experiments of the present thesis will be given.

In section 2 all materials (such as the supports for our model system) and techniques (such as Atomic Force Microscopy and Force Spectroscopy) that have been used during all the experiments will be discussed.

In section 3 all the results achieved will be analyzed and discussed.

In section 4 all the results will be summarized and commented.

A part of the obtained results has been the object of a publication and another publication, including the results on Force Spectroscopy, is at the moment in preparation.

Supported lipid bilayers on mica and silicon oxide: comparison of the main phase transition behavior.
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1 INTRODUCTION

1.1 THE BIOLOGICAL MEMBRANES

Biological membranes are essential for the organization of cells and therefore for life. They both separate each compartment in the cells and protect them from the environment (fig. 1). Membranes also work as shield against undesirable agents and by keeping important molecules on the inside. An important feature of biological membranes is that they are not static barriers but active ones, in fact, it is known that molecules, ions and signals continuously across the membranes. The structure of biological membranes is a hydrophobic layer made of phospholipids, disposed in two leaflets, which have hydrophilic heads that face the outside and the inside environment and hydrophobic tails that face those of the other leaflet.

This structure is also called lipid bilayer and it separates two aqueous compartments and all the molecules present outside and inside this structures are not soluble within them. It is worth saying that due to the fact that the biological membrane is a flexible structure it allows both the growth and the movements of cell but also the insertion and the operation of protein machinery. The low dielectric constant of the biological membrane gives us the opportunity to study the signaling, the transport and the energy transduction of these complex structures in order to better understand all their mechanisms. To better understand the membrane functions one should first analyze its structure which regulates fundamental properties such as fluidity, permeability and potential.

Due to the fact that all living organisms obey to physical and chemical laws, a depth knowledge of them will give a powerful tool to understand most of the processes, but what is essential for all of these processes is that they have to follow the second law of thermodynamics: processes must result in a net decrease in free energy in order to occur spontaneously. All metabolic processes require free energy changes, for instance the distribution of compounds across the membrane in response to concentration gradients and membrane potentials.

In this thesis a complete overview of all chemical and physical processes which regulate the membrane will be given as well as the explanation of all available tools to understand these processes.
The first scientists that observed that biological membranes had a hydrophobic character, but proposed a wrong lipid bilayer model, were Danielli and Davson in 1935; their lipid bilayer concept did not establish the location of the protein components of the membrane and proteins were proposed to be stucked on to the membrane surface (fig 2).

This was not compatible because proteins are responsible for moving molecules and messages across membranes and they could not perform those functions without being a integral part of the membrane itself. This realization gave rise to the concept of integral and peripheral proteins. Peripheral proteins are loosely associated with the membrane and
located on the surface of the lipid bilayer. Integral proteins are inserted into the membrane and pass all the way across the membrane. Originally, the integral proteins were thought to form a well defined matrix with the lipid bilayer filling in the spaces in between. What came out few years later was that many proteins are not rigidly fixed in the membrane, but can diffuse across the surfaces of cells relatively easily and independently. At this point membranes were considered as fluid structures with proteins and lipids arranged in their thermodynamically most favorable structure.

It is now clear that lipids build a bilayer and provide the matrix in which the integral membrane proteins float. The proteins are oriented so that their hydrophobic surfaces are immersed in the hydrophobic interior of the lipid bilayer and Hydrophilic amino acids are exposed only in the aqueous regions on either side of the membrane. Because lipids and proteins are not held together by bonds, they are free to diffuse and move independently within the plane of the membrane. Singer and Nicolson (1972) exposed this view in the fluid mosaic model (fig. 3).

It has been long recognized that the classical Singer–Nicholson fluid-mosaic model (fig. 3a), while remaining valid as a basic concept, provides a drastically oversimplified picture of the true nature of biological membranes.

Nowadays, one regards biological membranes rather to be heterogenous than homogenous[3](fig. 3b), and the correct folding and/or physiological activity of many membrane proteins are dependent on the nature of the phospholipids that constitute the bilayer[4].

Fig 3. Representations of the fluid mosaic model of biological membrane (a) and of the heterogenous model (b).
Many different functions are performed by biological membranes in all kinds of cells. They can be divided, however, into four classes based on differences in their fundamental energetics.

Cell membranes that belong to the first class are those that are called prokaryotic, such as the inner mitochondrial membrane and the thylakoid membrane of chloroplasts. The second class is that of plasma membranes of animal cells. In the third class are included the plasma membranes of plant and fungal cells. The fourth class of membranes is that of the vacuolar system membranes. This comprises the membranes of Golgi-derived organelles including lysosomes, endosomes, secretory vesicles in animal cells and peroxisomes, vacuoles and tonoplasts in plants and fungi.

As it has been stated before biological membranes are asymmetric, the two sides differ one from each other because of the way the membrane is synthesized; so it is possible to say that the membrane has a functional polarity. In animal and plant cells, the plasma membrane and vacuolar membranes share a common synthetic pathway. The proteins are inserted into the endoplasmic reticulum. The membranes are transferred to the Golgi apparatus where carbohydrates are attached and processed. The membranes are also sorted and leave the Golgi targeted to their final destinations. This process has a number of consequences. First, the proteins are inserted with a defined orientation into the membranes, i.e. from the cytoplasmic side of the membrane. Second, the carbohydrates are attached only to the interior of the Golgi. In this way, the carbohydrates face only the interior of organelles and the exterior of the cell; they do not appear on the cytosolic side of a membrane. This, along with the processes of exocytosis and endocytosis, demonstrate that the interior of vacuolar organelles is equivalent to the exterior of the cell in terms of membrane polarity.

As regards bacterial cells, the proteins and lipids that are synthesized inside the cell are then inserted into the membrane. The mitochondrial inner membrane as well as the thylakoid one biosynthesis are more complex because some of the proteins that are synthesized in the cytoplasm and then inserted into the organelle. This results in the fact that proteins are placed in the membrane with a defined orientation and giving the membrane polarity.

What characterizes the fluid mosaic model is the spontaneous assembly of the lipid bilayer with the proteins that are oriented in the way that can accommodate their own hydrophobic and hydrophilic surfaces, retaining the asymmetry of the membrane. The protein structure is determined by the amino acid sequence and the direction where that sequence was inserted into the membrane. Further processing of the protein, such as the attachment of
carbohydrates, is also asymmetrical because the important enzymes are placed either to
one side of the membrane or the other. The molecules transport occurs in defined
directions and this, in turn, creates the membrane potential, a polarity that plays a
fundamental role in many membrane processes.

1.1.1 MEMBRANE COMPONENTS AND THEIR FUNCTIONS

As it has been claimed before about cellular membranes, they establish a hydrophobic
barrier between the inside and outside environment of the cell. What changes in cellular
membranes is their lipid composition; the plasma membrane exhibits a transverse lipid
compositional asymmetry and is enriched in sphingolipids and sterols, whereas the
endoplasmic reticulum (ER) displays a symmetrical transbilayer lipid distribution and
contains low levels of these two lipids.

These dynamic and highly complex structures, that only measure few nanometers, are
made of two main components: 1) phospholipids, that are lipid molecules held together by
hydrophobic interactions that constitute a two-dimensional space, and 2) proteins, that are
embedded within phospholipids or partially associated with them. The study of the
physicochemical properties of the phospholipids is important for the understanding of
many physiological and pharmacological events.

Lipids can be classified into five different categories: 1) Fatty acids, 2) triacyl glycerol, 3)
glycerophospholipids, 4) sphingolipids and 5) steroids.

1) Fatty acids are carboxylic acids with a long hydrocarbon chain. The hydrocarbon chain
may have one or more double bonds in it or may be saturated, i.e. absence of any
double bonds along the hydrocarbon chain. Those which contain 14-20 carbons are
widely found in plants and animals, among them C16 (palmitic), C18 (stearic, oleic,
linoleic) are the predominant ones.

2) Triacyl glycerols or triglycerides are fatty acid esters of glycerol; their function is to be
energy reservoirs in plants and animals, therefore they are the most abundant form of
lipids. However they are not components of cellular membrane.
The major classes of lipids that constitute the cellular membranes are the glycerophospholipids, sphingolipids and cholesterol (a class of steroids).

3) Glycerophospholipids consist of a glycerol-3-phosphate esterified with fatty acids at the C1 and C2 positions. The phosphoryl group at the C3 position in addition is attached to another group ‘x’.

4) Sphingolipids are usually a derivative of sphingosine a C18 amino acid. The N-acyl fatty acid derivatives of sphingosine known as ceramides are the parent compounds of most sphingolipids. Sphingolipids can be further classified as sphingomyelins (ceramides containing a phosphocholine or phosphoethanolamine head group), cerebrocides (containing a head group with a single sugar unit) and gangliocides (ceramides attached with oligosaccharides that contain at least one sialic acid residue). Both glycerophospholipids and sphingolipids have a polar head group and non polar tails made up of acyl chains.

5) Other important constituents of the membranes are sterols like cholesterol.

The lipids that constitute the bilayer can exist in different phases depending on the temperature and the composition. The phospholipids and the sphingolipids both have a melting temperature ($T_M$) below which they exist in a solid (gel) state and above which they exist in a liquid like state\cite{5}. The lipids in the gel state are packed more tightly and have an ordered array and are referred to be in a liquid crystalline phase (lc) while the lipids in the liquid state are loosely packed and are free to move around.

In this thesis the liquid phase term will be replaced by the liquid disordered phase (ld) one, while the term solid state will be replaced by the solid ordered phase one. Different lipids forming the membrane have a different $T_M$ and this suggests that the heterogeneity in the membrane composition can lead to a segregation of the constituent lipids into different phases in the membrane itself. The presence of cholesterol and lipids having high $T_M$ (such as sphingolipids) in the membrane favors the formation of an intermediate liquid ordered (lo) phase in which the acyl chains of the lipids are ordered as in the gel state but also have a greater lateral mobility\cite{2,4,6}, this condition will be referred with the term intermediate phase. It is worth stressing that the study of the physicochemical properties of the phospholipids that constitute biomembranes is crucial for understanding many physiological and pharmacological events.
Another important component of biological membranes are proteins, many of the membrane proteins interact preferentially with certain lipids present in the membrane and this preferential interaction can favor the formation of lipid domains. The interaction of proteins with the lipids can be based on its charge or due to its hydrophobicity. The hydrophobic portion of the proteins that is exposed to the lipid can often be larger than the membrane thickness and in this case the membrane lipid or proteins have to change their conformation in order to minimize the hydrophobic mismatch. The proteins can stretch themselves when the membrane thickness is smaller or they can fit themselves within the membrane by tilting their transmembrane helices or by taking a different conformation.
Also lipids can stretch their acyl chains\cite{7}, in the presence of several lipids in the membrane the proteins may preferentially interact with a particular lipid which has a similar hydrophobic length or an opposite charge. The protein-lipid interaction can act as a mechanism to maintain the proteins stability. Ryba and Marsh (1992) have studied the stability of rhodopsin in PC bilayers. Ca$^{2+}$ - ATPase and Na$^+$/K$^+$ - ATPase for example showed an optimum activity when they were reconstituted in PC bilayers of length about 18 carbons\cite{8}. The enzyme activity was found to increase by the addition of decane which decreases the membrane thickness, while the activity decreased with the addition of lipids with longer chains. This can be ascribed to the fact that the hydrophobic matching can regulate the activity of proteins\cite{9}. Mouritsen and Bloom (1984) have proposed the “Mattress Model” that describes the adaptation of proteins and their segregation due to hydrophobic mismatch\cite{9}. Proteins that interact preferentially with saturated acyl chains have a tendency to move to the ordered phase of the DRMs (detergent-resistant membranes). Several GPI (Glycosylphosphatidylinositol) anchored proteins (which contain a saturated acyl chain) were found to be enriched in the cholesterol-sphingolipid domains which are detergent resistant. The Src-family kinases, endothelial NO synthase, caveolin, influenza hemagglutinin are some DRM proteins linked to saturated acyl chains.

Many biological membranes are asymmetric with respect to lipid distributions across the bilayer, this asymmetry is maintained by a set of lipid translocases (“flippases”) which are ATP-dependent integral membrane ABC transporters. Several classes of lipid-anchored membrane proteins are also asymmetrically distributed across plasma membranes of eukaryotic cells. Biosynthetic pathways are responsible for establishing the asymmetry of these classes of proteins.

Few words must be spent on cholesterol that has been recognized as a major modulator of lateral membrane structure, it interacts with different lipids in different ways creating transient structures that can phase separate into domains with different structural and dynamical properties. These domains are sometimes also called “lipid rafts”, they have been hypothesized (but never really strictly proven) to be the organizing platforms for transducing signals from ligand-activated receptors on the extracellular side of the membrane to downstream effector proteins that reside on the intracellular side of the plasma membrane.
1.1.2 MODEL MEMBRANES

**Supported lipid bilayers (SLB)**

When one uses the term membrane model it is easy to get confused as regard the meaning of the word “model”. As membrane model one assumes a theoretical interpretation of the system making it ideal; whereas the term model membrane is referred to a simpler system than the original, but real. For example, studying lipid bilayers made of few lipids simulates the cell membrane, that instead has many kinds of lipid and is more complex, ad it is easier. It is possible to say that membrane models are what I am thinking of but membrane models allow me to study a complex system in a simple way. For my experiments I choosed, as model membrane, the so called Supported Lipid Bilayer; the term “Supported” indicates that the Lipid Bilayer is placed onto a support, which can be made of different materials such as mica or silicon dioxide for using surface specific analytical techniques such as Atomic Force Microscopy. In order to support a high quality membrane (i.e. little or no defects and high lipid mobility) the surface should be hydrophilic, smooth, and clean. In solid supported systems membrane fluidity is maintained by a 10–20 Å layer of trapped water between the substrate and the bilayer\[10,11\] (Fig. 5).

![Fig 5. A schematic representation of a Supported Lipid Bilayer](image)

My artificial bilayer was made of two particular phospholipids, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), that is zwitterionic, and 1 palmitoyl-2-oleoyl-sn-
glycero-3-[phospho-rac-(1-glycerol)] (POPG), that has a net negative charge, (fig. 6) in a weight ratio of 3:1; they are the major lipid components of bacterial membranes and are considered a suitable model system to study the biological membrane behavior.

For instance, the inner membrane of Escherichia coli contains 70–80% PE, 20–25% PG, and 5% cardiolipin\textsuperscript{[12]}. Comparative studies on PE and PC bilayers revealed that the hydrocarbon chain order is higher in PE than in PC bilayers\textsuperscript{[13,14]} due to a smaller cross sectional area of the PE molecule\textsuperscript{[15]}. In effect, the main-phase transition temperature of bilayers composed of PEs is higher than that of equivalent PGs\textsuperscript{[16]}. The smaller cross sectional area of PE originates from a smaller head group (the ammonium group is small and a small number of bound water molecules\textsuperscript{[17]}. This stems from the ability of the PE ammonium group, to form hydrogen (H) bonds with phosphate and carbonyl oxygen atoms of adjacent PE molecules. These intermolecular H-bonds replace some of the PE-water H-bonds and significantly strengthen interlipid contacts.

\textbf{Fig 6.} Schematic representation of the two lipids used for the experiments

PE--PE intermolecular H-bonds were observed both experimentally\textsuperscript{[18,19]} and in molecular dynamics (MD) simulations\textsuperscript{[19,20]}. Similarly to the ammonium group of PE, the hydroxyl group of PG has a potential to form intermolecular H-bonds. The occurrence of PG--PG H-bonds in a PG monolayer\textsuperscript{[21,22]} and bilayer\textsuperscript{[23]} was suggested by experimental studies. Limited formation of such bonds was demonstrated in MD simulation of a PG monolayer\textsuperscript{[23]}. These interlipid interactions via H-bonding are evidently weakened by electrostatic repulsion of negatively charged PGs, since only at high ionic strength is the main-phase transition temperature of a PG bilayer
similar to that of an equivalent PC bilayer\textsuperscript{[24]}. At low ionic strength, the gel-liquid transition displays a complex thermal behavior\textsuperscript{[24]}. This alteration in the head group composition is the way of preserving the stability and low permeability of the membrane by increasing the average phospholipid head group area and presumably the chain order. The way in which a negatively charged PG head group could promote a higher order of chains in the membrane is not that obvious. Nevertheless, a higher order of PG than PC chains was observed in a MD simulation study of mixed lipid PC-PG monolayers\textsuperscript{[25]}. In this study, a MD simulation was applied to investigate the properties of a bilayer built of PE and PG molecules in the proportion 3:1. Mixed phospholipid-charged bilayers, which provide a good model for bacterial membranes, are studied much less frequently than bilayers made of zwitterionic phospholipids that constitute models of eukaryotic membranes. Indeed, the majority of lipid bilayers studied by MD simulation are uncharged single-component bilayers built predominantly of PC\textsuperscript{[26]} with only a few built of PE\textsuperscript{[20,27-29]}. Only recently the bilayers\textsuperscript{[30-35]} and monolayers\textsuperscript{[25]} composed of charged or mixtures of uncharged and charged lipids have also been simulated. Those simulations demonstrated that the properties of such bilayers are dominated by electrostatic interactions among lipid head groups, ions, and water at the membrane/water interface.

\textit{SLB preparation techniques and physical properties}

There are three general methods for the formation of supported phospholipid bilayers: 1) the first method involves the transfer of a lower leaflet of lipids from the air–water interface by the Langmuir–Blodgett technique (Fig. 7a). This is followed by the transfer of an upper leaflet by the Langmuir–Schaefer procedure, which involves horizontally dipping the substrate to create the second layer. 2) A second method of supported bilayer formation is the adsorption and fusion of vesicles from an aqueous suspension to the substrate surface\textsuperscript{[35,37]} (Fig. 7b). 3) Also, a combination of the two methods can be employed by first transferring a monolayer via the Langmuir–Blodgett technique followed by vesicle fusion to form the upper layer\textsuperscript{[38]}(Fig. 7c).
Fig 7. Common techniques for the formation of supported lipid bilayers. (a) The Langmuir–Blodgett technique is carried out by pulling a hydrophilic substrate through a lipid monolayer and sequentially pushing it horizontally through another lipid monolayer. (b) Vesicles in solution adsorb and spontaneously fuse to the surface to form a solid supported lipid bilayer. (c) A combination of the Langmuir–Blodgett and vesicle fusion processes.

Each of the three deposition methods has its particular advantages and disadvantages. The transfer of amphiphilic molecules from the air–water interface to a solid substrate dates back to the 1920s[39,40]. Tamm and McConnell were the first to apply this technology to form supported phospholipid bilayers by sequential monolayer transfer onto quartz, glass, and oxidized silicon substrates[10]. This method is useful for the formation of asymmetric bilayers[41]; however, it is difficult if not impossible to incorporate transmembrane proteins into the lipid bilayer with this technique because prior to transfer portions of the proteins within the monolayer these are exposed to air and can become irreversibly denatured[38]. The adsorption and fusion of small unilaminar vesicles (SUVs) is one of the easiest and most versatile means for forming solid supported phospholipid bilayers (Fig. 7b). SUVs can be prepared by a plethora of methods. The simplest involves the extrusion of multilaminar vesicles through porous polycarbonate membranes at high pressure[42-44]. Another method is the sonication and ultracentrifugation of aqueous lipid suspensions[45]. The incorporation of transmembrane proteins into SUVs requires a gentler process such as detergent removal via dialysis[46,47]. Factors affecting the adsorption and fusion of SUVs to solid supports include: the vesicle composition, size, surface charge, surface roughness, surface cleanliness, solution pH, ionic strength, and the osmotic pressure of the vesicles[48,49]. The process begins with the adsorption of vesicles from the
bulk solution onto the substrate (Fig. 8). In the early stages, SUVs may fuse with one another\cite{49}.

![Fig 8](image)

**Fig 8.** Schematic representation of the early stages of lipid bilayer's formation. (a) vesicles adsorb onto the support. (b) The vesicles fusion on the surface leads to an increase of the vesicle radius. Consequently the vesicle becomes more crushed and breaks. (c) From vesicles's breaking R•R, a bilayer is formed. (d) An adsorbed vesicle\cite{49}.

The vesicles then rupture and fuse to the substrate planar supported bilayers in a process that depends upon the chemistry of the individual lipids\cite{50}. The adsorption process can be accelerated by the presence of divalent cations such Ca2+ and Mg2+\cite{41}. Fusion of SUVs to the substrate can also be enhanced by heating\cite{51}, creating an osmotic gradient across the vesicle membrane\cite{48}, and by the addition of fusogenic agents such as polyethylene glycol\cite{52}. Although the exact mechanism of bilayer formation from the adsorption and fusion of SUVs is not fully understood, mathematical modeling of the system has shown good agreement with experimental results\cite{53}.

The main advantage in using solid supports is clearly an increase in robustness and stability of the phospholipid bilayer membrane. Almost equally important is the ability to probe interactions that occur at the membrane surface with powerful analytical techniques that are surface specific (e.g. atomic force microscopy, quartz crystal microbalance, surface plasmon resonance, vibrational sum frequency spectroscopy, etc.). While solid
supported phospholipid bilayers are somewhat limited in terms of their substrate compatibility, their major disadvantage is that the supported membrane is not truly decoupled from the underlying substrate. Indeed, the system may not prevent transmembrane proteins from interacting unfavorably with the underlying substrate. Such interactions with the surface can cause proteins in the membrane to become immobile and hinder their function[54].

**Lipid Bilayer Phase Transition**

Depending on the thermodynamic variables such as pressure and temperature, the lipid bilayer can undergo different phase transitions, the most important two are represented in fig.9. In the solid-ordered phase the lipid hydrophobic tails are ordered and stretched and the molecules disposition shows a crystal order. In the liquid-disordered phase the molecules lateral order is casual, the distance among the molecules is bigger and the hydrophobic tails are disordered. The liquid-disordered phase appears to a higher temperature than the solid one. During the transition from liquid-disordered to solid-ordered, the lipid bilayer undergoes an area and volume decrease, and a thickness increase.

The transition temperature, also known with the name melting temperature ($T_M$), can be defined as the temperature to which the 50% of the lipids is in the first phase and the other 50% is in the second. For a one lipid system the phase transition is highly cooperative; this means that one lipid melting affects the melting of another one. A synthetic bilayer made of a single lipid kind switches from a liquid state to a solid one at a particular freezing point, the melting temperature ($T_M$). When lipids with different melting temperatures are mixed the mixture phase diagram contains a region where the solid-ordered and liquid-disordered phase coexist. This means that the system is less cooperative with respect to a bilayer made of only one lipid.

![Fig 10](http://openlearn.open.ac.uk/mod/oucontent/view.php?id=397829&printable=1)

**Fig 10.** Potential energy for a C-C bond rotation. In the picture are shown three energy minimums: gauche+, trans, e gauche. Both gauche conformations are specular images.

The mechanism which allows the phase transition can be better explained by reasoning about the C-C bonds present on the alkyl chains. When the alkyl chain is saturated a
rotation around the C-C bond is possible (fig 10). If no C-C bond is rotated one is in the all-trans conformation, in this case the system has its energy minimum. If the bonds are rotated one is in the gauche configuration; to rotate one bond in the gauche configuration a barrier of 14.65 kJ/mol must be overcome\textsuperscript{[55]}. The probability to form a gauche conformation depends on the temperature.

![Fig 11. Trans conformation of alkyl chains in solid ordered phase.](image)

In the gel (solid-ordered) phase the alkyl chains are in their more stable conformation that is the all-trans one. In this conformation the chains are totally stretched and form a highly ordered bilayer (FIG 11). If one gradually rises the temperature, lipids acquire energy that allows them to move from the all-trans configuration to the gauche one, that is more disordered and that covers a bigger volume (FIG 12).

![Fig 12. Gauche conformation of alkyl chains in liquid disordered phase.](image)
The chains melting process is influenced by the competition effects balance. By an entropic point of view the disordered chains are more favoured rather than the highly ordered ones; the Van der Waals interactions, however, increase with the presence of ordered chains. The solid and liquid phase coexistence leads to a domains lateral organization, so it will be possible to have gel phase and liquid phase bilayer portions. The presence of an interface among the domains can explain, in some cases, the mechanism which allows the bilayer to affect proteins properties; but also proteins affect the properties of the lipid bilayer. Given the reciprocal influence between the lipid bilayer and proteins it is not corrected to separately deal with their functions and properties inside the cell. The mechanisms that regulate the membrane functioning are, hence, complex and their interpretation deserves care and attention. Before studying the behavior of the proteins that are immersed in the lipid bilayer it is important and necessary to characterize the bilayer without the proteins, in this way it is possible to analyze the cell membrane mechanisms.

**pH-effect on the phase transition**

Temperature has been used to trigger lipid phase transitions. Biological systems, however, are remarkably constant in temperature; hence conformational changes in vivo must be caused by variables other than temperature. Given this it has been seen how lipid phase transitions could be triggered at constant temperature by monovalent and divalent cations and by changes in pH. Such effects are to be expected for charged lipid bilayers because the electrostatic free energy $\Gamma$, of the system, changes at $T_t$ as a result of bilayer expansion. Therefore, all parameters affecting the value of $\Gamma$ can be expected to alter the transition (or melting) temperature. In a certain temperature range these variables may thus be used to induce the phase transition at a constant temperature.

For a reversible phase transition at constant pressure the molar free energies of the two states are equal at the critical temperature $T_t$. Therefore $\Delta H = T \cdot \Delta S$, where $\Delta S$ denotes the entropy change and $\Delta H$ the enthalpy change, or the heat absorbed at the ordered $\rightarrow$ fluid phase transition. $\Delta H$ may be written as a sum of a non electrostatic term, $\Delta H^o$, and an electrostatic term $\Delta \Gamma = \Gamma(\text{fluid}) - \Gamma(\text{ordered})$. The change in $T_t$, caused by electrostatic effects is given by $\Delta T_t = T_t - T_t^o = \Delta \Gamma / \Delta S$, where $T_t$ is the observed transition temperature.
and $T^\circ_1 = \Delta H^\circ/\Delta S$ is the value of $T_1$, in the absence of electrostatic interactions. If the membrane charges are assumed to be uniformly distributed over the membrane surface, $\Delta \Gamma$ can be expressed in terms of the difference in the electrical double layer energy, $\phi$, of the two states. $\phi$ may be calculated on the basis of the Gouy-Chapman theory\textsuperscript{[57]}. For a 1:1 electrolyte and high surface potentials the free energy of the interface per cm$^2$ is

$$\phi = 2(kT/e)(\sigma'/f) \quad \text{Eq. 1}$$

independent of the electrolyte concentration. Half of this value is obtained for 2:1 electrolytes. In this expression $k$, $T$, and $e$ have their usual meaning; $f$ is the area of one lipid molecule and $\sigma'$ is the charge per polar group (specific charge). For phosphatidic acid (PA), $\sigma'$ is connected the degree of dissociation, $\alpha$, of the phosphate groups by $\sigma' = e \cdot \alpha$.

$\sigma'/f$ is equivalent to the surface charge density, $\sigma$. Using the Eq. 1 and denoting by $\Delta f = f_2 - f_1$ the increase in molecular area at the ordered $\rightarrow$ fluid phase transition, one calculates for the change in electrostatic free energy per mol (cal mol$^{-1}$):

$$\Delta \Gamma = -2RT \left( \frac{\Delta f}{f_1} - \frac{(\Delta f)^2}{f_1 f_2} \right) \frac{\sigma'}{e} \quad \text{Eq. 2}$$

Here $\sigma' e = \alpha$; $f_1$ and $f_2$ are the molecular areas for $T < T_1$ and $T > T_1$, and $R$ denotes the gas constant (1.987 cal/mol). Hence, the corresponding change in $T_1$ is:

$$\Delta T_1 = \frac{\Delta \Gamma}{\Delta S} = -\gamma \alpha \quad \text{with} \quad \gamma = 2 \frac{RT}{f_1} \left( \frac{\Delta f}{f_1} - \frac{(\Delta f)^2}{f_1 f_2} \right) \quad \text{Eq. 3}$$

Thus, for highly charged bilayers $T_1$ is expected to decrease linearly with increasing surface charge ($\sigma' = e \cdot \alpha$) or increasing $\alpha$. The physical picture of the charge-induced phase transition is quite simple. The electrostatic free energy of the fluid bilayer is smaller than in the ordered state ($\Delta \Gamma$ is negative) and therefore an increase in surface charge favors the fluid state.

$\sigma$ can be varied (a) by changing the pH or (b) by the adsorption of cations to negatively charged lipids. In (a) $T_1$ is expected to decrease with increasing pH, whereas in (b) $T_1$ increases. The ionic strength is not included explicitly in Eq. 3\textsuperscript{[58]}. 25
1.1.3 DIFFERENT MODEL MEMBRANES

When one uses the term membrane model it is easy to get confused as regard the meaning of the word “model”. As membrane model one assumes a theoretical interpretation of the system making it ideal; whereas the term model membrane is referred to a simpler system than the original, but real. For example, studying lipid bilayers made of few lipids simulates the cell membrane, that instead has many kinds of lipid and is more complex, ad it is easier. It is possible to say that membrane models are what I am thinking of but membrane models allow me to study a complex system in a simple way.

Fig 13. Schematic representations of the three kinds of model lipid bilayers; (a) liposomes, (b) black lipid membrane, (c) supported lipid bilayers.

Lipid bilayers can be created artificially in the lab to allow researchers to perform experiments that cannot be done with natural bilayers. These synthetic systems are called model lipid bilayers. There are many different types of model bilayers, each having experimental advantages and disadvantages. They can be made with either synthetic or natural lipids. The first kind of model bilayer are called liposomes (fig 13a), they are artificially prepared vesicles made of lipid bilayers. The black lipid membrane is the second kind of model bilayer, it derives its name from its appearance by optical microscopy. When Mueller et al. observed the formation of the first black lipid membranes\cite{59,60} from extracted brain lipids, they noted interference bands giving rise to color in the membrane. This interference effect disappeared during the thinning of the painted lipid mass and is thought to indicate the formation of a single bilayer membrane, as shown in Fig. 13b\cite{61}. There are several methods of producing black lipid membranes but all involve the formation of a membrane over a small aperture usually less then 1 mm in diameter. The hole is formed in a hydrophobic material such as
polyethylene or Teflon and is usually part of a wall separating two compartments that can be filled with aqueous solution, each containing a reference electrode. Two of the most popular methods for BLM formation involve the painting of the lipid solution over the aperture and the formation of a folded bilayer. The result of either method is a bilayer suspended over the aperture with an aqueous compartment on each side.

Typically a 1%–2% phospholipid solution in an organic solvent, such as n-decane or squalene, is painted across the hole under an aqueous solution. The deposited lipid mass thins as it spreads, forming the black lipid membrane. This methodology has remained basically unchanged over the decades. The formation of folded lipid bilayers requires a cell with two compartments separated by a small aperture and the solution levels in each compartment must be controlled independently. Both compartments are filled with the desired aqueous solution and a monolayer of phospholipid material is spread on top of one of the compartments. The solution level in the compartment containing the lipid monolayer is slowly lowered below the aperture and raised again. This deposits a monolayer on each pass to form the completed bilayer membrane. Since their advent, black lipid membranes have been used to investigate various biophysical processes. One of the most important is the formation of ion channels in phospholipid bilayers by peptides, proteins, antibiotics, and other pore-forming biomolecules. Of particular interest for creating nanodevices is the insertion of single protein pores for use as stochastic sensors.

As noted above, black lipid membranes are suspended in solution and there are no unwanted interferences of the membrane with an underlying support. The absence of such a support also means that transmembrane proteins suspended within the phospholipid bilayer remain fully mobile and active. However, this also limits the lifetime of the bilayer due to poor stability of the membrane. The methods of detection that can be employed with black lipid membranes are also typically limited. Usually electrical conduction and simple light microscopy are used; however, more recently investigators have begun to utilize more sophisticated optical techniques.

The third kind of model bilayer is the so called Supported Lipid bilayer, that is a sheet that lays flat on a solid substrate such that only the upper face of the bilayer is exposed to free solution (fig 13c). One advantage of this layout is its stability. SLBs will remain largely intact even when subject to high flow rates or vibration and, unlike black lipid membranes, the presence of holes will not destroy the entire bilayer. Because of this stability, experiments lasting weeks and even months are possible with supported bilayers while BLM experiments are usually limited to hours. Another advantage of the supported bilayer
is that, because it is on a flat hard surface, it is amenable to a number of characterization tools which would be impossible or would offer lower resolution if performed on a freely floating sample. One of the clearest examples of this advantage is the use of mechanical probing techniques which require a direct physical interaction with the sample such as Atomic force microscopy (AFM). Many modern fluorescence microscopy techniques such as total internal reflection fluorescence microscopy (TIRF) and surface plasmon resonance (SPR) also require a rigidly-supported planar surface. One of the primary limitations of supported bilayers is the possibility of unwanted interactions with the substrate[54]. Although supported bilayers generally do not directly touch the substrate surface, they are separated by only a very thin water gap. The size and nature of this gap depends on the substrate material and lipid species but is generally about 1 nm. Because this layer is so thin, there are often problem when incorporating integral membrane proteins, which can become denatured on the substrate surface.
2 MATERIALS AND METHODS

In this chapter the experimental methods and the sample preparation are explained. The technique mainly used is the AFM, but also the Langmuir Blodgett one will be discussed. The set up of each technique will be explained as well as the sample preparation.

2.1 Atomic Force Microscopy

The atomic force microscope (AFM) belongs to the broad family of scanning probe microscopes in which a proximal probe is exploited for investigating properties of surfaces with subnanometer resolution. The AFM, initially developed to overcome the limitations of its ancestor, the scanning tunneling microscope (STM), in imaging non-conducting samples, immediately attracted the attention of the biophysical community. At the beginning the emphasis was mainly on the improved imaging resolution compared to that of optical microscopy, but, soon after, it became clear that AFM was much more than just a high-resolution microscope. The possibilities of spectroscopic analysis, surface modification and molecular manipulation gave rise to a real breakthrough in the realm of AFM use. In biological applications, the most appealing advantage of the AFM as a high-resolution microscope in comparison with other techniques such as SEM and TEM, is that it allows measurements of native biological samples in physiological like conditions, avoiding complex sample preparation procedures and artifacts connected to them. The use of mild imaging conditions opened the way to dynamic studies in which conformational changes and molecular interactions could be followed in real time at single-molecule level. The set of samples of biological interest studied by AFM ranges nowadays from the smallest biomolecules, such as phospholipids, proteins, DNA, RNA, to subcellular structures (e.g. membranes), all the way down to living cells and tissues. Not only structural properties can be investigated, but also mechanical or chemical and functional properties are the focus of many AFM applications.
2.1.1 Physical principles of the Atomic Force Microscope

The AFM works by scanning, in a raster fashion, a very tiny tip mounted at the end of a flexible microcantilever in gentle touch with the sample. This relative motion is performed with sub-Angstrom accuracy by a piezoelectric actuator (usually a tube). Interacting with the sample the cantilever deflects and the tip–sample interaction can be monitored with high resolution exploiting a laser beam impinging on the back of the cantilever\cite{78}. The beam is reflected towards a split photodetector configuring an optical lever which amplifies cantilever deflections. In almost all operating modes, a feedback circuit, connected to the cantilever deflection sensor, keeps tip–sample interaction at a fixed value controlling the tip–sample distance. The amount of feedback signal, measured at each scanning point of a 2D matrix, concurs to form a 3D reconstruction of the sample topography which is usually displayed as an image. In fig. 14 a scheme of an AFM is reported in a typical configuration for biological applications, where it is coupled with an optical microscope to simultaneously acquire an optical image and the surface topography with the AFM.

**Fig 14.** Scheme of an AFM coupled with an inverted optical microscope.
The scanning probe is the heart of the AFM, as for every scanning probe-based technique. The most common cantilevers used nowadays are realized by exploiting silicon micromachining technology[79-81]. In figure 15(a) a scanning electron microscope image of a silicon nitride microfabricated cantilever chip, usually used for contact AFM, is shown. At the end of a cantilever a tiny pyramid, the tip, is integrated (fig. 15b). Depending on the imaging mode adopted, different types of cantilevers and tips may be used. When the AFM is operated in contact mode, the stiffness of the cantilever should be as low as possible (less than the interatomic spring constant of atoms in a solid), whereas in dynamic operation modes higher values for the spring constant help us reduce noise and instabilities. Figure 15c reports an image of a silicon cantilever and tip, usually used in dynamic operation modes, and a magnified view of the integrated silicon tip is shown in figure 15d.

Typical spring constants for AFM cantilevers range from 0.01 N m$^{-1}$ to 100 N m$^{-1}$, enabling a force sensitivity down to $10^{-11}$ N.

![Fig 15. Scanning electron microscope images of microfabricated AFM cantilevers and tips. (a) Silicon nitride cantilevers and (b) high magnification of the tip with oxide-sharpened apex; (c) silicon cantilever and (d) high magnification of the silicon tip.](image-url)
The limit in force sensitivity is related to an interplay between thermal, electrical and optical noise. The use of carbon nanotubes as AFM tips, fig 16, has represented a great breakthrough in terms of resolution\textsuperscript{[82-85]}. Carbon nanotube tips possess a high aspect ratio, mechanical robustness, small diameter and a well-defined surface chemistry. They can be chemically functionalized, and appear to be the ideal probe for biological applications of the AFM requiring high resolution, particularly in the case of structural biology. The high-resolution imaging capabilities of carbon nanotube tips have been demonstrated on a variety of biological samples, such as DNA, IgG, IgM, GroES, SWI/SNF. The resolution attainable with carbon nanotube tips is comparable with that of other ultimate resolution imaging techniques such as cryogenic electron microscopy, but carbon nanotubes offer also the possibility to be functionalized, exposing, thus, a well-defined chemical group or chemisorbed biomolecule. This opportunity can be exploited to study the spatial distribution of chemical functional groups or complementary biomolecules in a sample.

\textbf{Fig 16.} Multiwall carbon nanotube tip attached to the end of single-crystal silicon tip. The inset is a higher magnification view of the same tip rotated 180° relative to the main image (bar = 1 \textmu m)\textsuperscript{[84]}.

The classification of the possible operation modes is strictly related to the region of the force field between the tip and the sample spanned by the tip during imaging. Considering a non-linear force field composed of a repulsive short range force (<1 nm) and an attractive long range one (van der Waals force, < 100 nm), depending on the force
experienced by the tip–cantilever ensemble, three working methods for the atomic force microscope can be defined. If the force between the tip and the sample is always repulsive and the tip is constantly in contact with the sample the microscope works in the contact mode; in this case the cantilever deflection is due to the Van der Waals repulsive forces and a low spring constant for the cantilever is required in order to avoid an extreme pressure on the sample. It is possible to operate in three different ways:

- **Force mode**: keeping the tip at constant height the cantilever deflection follows the sample surface topography and in order to achieve an image the output signal of the photodiode is used. Moreover by the output signal it is possible to calculate the power. This method is extremely sensitive but, on the other hand, each surface irregularity can produce great forces that damage both tip and sample;

- **Height mode**: The risk of the previous method can be avoided by sending the photodiode signal, by a feedback circuit, to the piezoelectric that change the height of the tip to keep the force constant. In order to achieve an image the same signal is send to the piezoelectric that gives the height variation of the tip.

- **error signal or deflection mode**: varying the tip-sample distance, keeping the feedback turned on, it is possible to achieve an image even using the photodiode output signal, in this case the observed image is the derivative of each point of the constant force image.

If the tip experiences only an attractive force with the sample and it never touches the sample, the usually called true-non-contact mode is being used. Keeping the tip few nanometers far from the sample surface it is possible to reduce the lateral forces and the strong interactions effects that usually damage soft samples. The tip oscillates with frequency close to its resonance frequency and a higher spring constant is preferred.

If the tip experiences both the attractive and the repulsive force with the sample, the tapping mode is used. The cantilever oscillates at its resonance frequency or close to it so that the tip only has a transient contact with the sample surface, in this way friction lateral forces, which could damage the sample or alterate the data or slow the tip, are not applied. The applied force to the tip can be adsorbed by most of the samples without any damages but a high spring constant is required, so that the cantilever has the right
potential energy to retract after that the sample is touched. In order to have an image, changes of the oscillation amplitude are recorded. An extension of the tapping mode that provides informations on the surface structure at the nm scale, often not well characterized by other SPM techniques, is the phase imaging. Mapping the cantilever phase oscillation, during the scanning, it is possible to track both the sample topography and other features such as viscosity, elasticity and chemical composition variations.

AFM imaging modes of interest for biological application will now be described. The first imaging mode which has been developed is the contact mode, in which the tip is constantly in gentle touch with the sample surface\textsuperscript{[86]}]. In this imaging mode the applied force, hence cantilever deflection, is kept constant by the feedback system while the tip scans the surface. Images are created by recording the piezo-vertical position required to keep the force constant. This mode of operation can be used under aqueous environments allowing a reduction of the interaction force between the tip and the sample with respect to operation in air. The interaction force reduction comes from the removal of capillary forces due to the presence of a thin water layer on surfaces in air\textsuperscript{[87]}. This mode is the one of choice as far as flat and rather rigid samples are involved (e.g. reconstituted membranes, 2D protein crystals) enabling the highest resolution level. Sometimes, image artifacts, such as tilt of the surface or drift of the scanner perpendicular to the surface, dominate the topography image over real topographical features, especially when imaging is performed over large areas with slow scan rate. To avoid low-frequency artifacts the error mode can be used\textsuperscript{[88]}, in which the cantilever deflection signal is recorded keeping the feedback response time as fast as possible. The error signal gives a measure of how well the feedback system is maintaining the desired deflection set point. Due to the finite response time of the feedback loop, high-frequency signals cannot be completely compensated, and the cantilever will not be always at the same deflection value, especially in the case of high-frequency signals, generating a compensation error, which can be exploited to obtain an image rich in information. Moreover, tilt and vertical drift of the scanner generally have a low frequency and do not contribute to the error-mode image. When the tip is scanned in contact with the sample, lateral forces arise which cause a torsion of the cantilever. The torsion can be monitored by the signal from lateral segments of the photodiode\textsuperscript{[89]}. Recording the torsion of the cantilever, surface distribution of different chemical functionalities, which result in different friction or adhesion properties with the tip, can be mapped. This operation mode is usually referred to as lateral force mode or, when chemically modified tips are used to measure differences between areas of distinct
chemical properties, the technique is referred to as ‘chemical force microscopy’\[^{90}\]. Fig 17 shows a scheme of this operating mode.

**Fig 17.** Operating principle of the lateral force mode. The signal from the lateral segments of the photodetector is monitored while the tip is scanned on the surface in the direction perpendicular to the major axis of the cantilever. Both the trace and retrace signals are recorded to avoid possible interference of the topographic signal with the lateral force signal. Indeed, a pure lateral force should give an inverted signal in the trace and retrace scan of the tip. Subtracting the two signals (trace and retrace) highlights the contrast coming only from lateral forces.

A drawback of the contact mode is the development of dragging forces associated with the lateral movement of the tip in contact with the sample. This problem is particularly evident in the case of biological samples, which are usually loosely bound to the substrate and easily damageable. To overcome this problem another mode of operation, the intermittent contact mode, has been developed (fig 18)\[^{91}\].

In this case the cantilever is oscillated at a frequency near its resonance and the oscillation amplitude is monitored. Starting from a free oscillation amplitude, when the cantilever approaches the sample and starts to hit its surface, the oscillation amplitude is damped.

By recording the feedback signal required to keep the amplitude constant, the topography of the sample surface can be obtained. The tip being only intermittently in contact with the
sample, the dragging forces during scanning are greatly reduced\[92\]. In the intermittent contact mode the tip goes through both the attractive and the repulsive regions of the tip–sample force field during oscillation.

Fig 18. Schematic representation of an AFM tip operating in the intermittent contact mode.

The operation set point can be chosen so as to make attractive forces the dominant ones reducing damage to the sample and sometimes increasing resolution\[93\]. Technically, the cantilever can be oscillated by two methods\[94\]: acoustically or magnetically. In the first case the cantilever is oscillated by a piezo-actuator almost always in contact with the cantilever supporting chip, whereas in the second case the cantilever is oscillated by means of an alternating magnetic field which acts on a magnetically susceptible film deposited on the backside of the cantilever\[95\]. Furthermore, important information on the viscoelastic properties of the sample can be retrieved by monitoring the phase difference between the cantilever driving signal and the output oscillation signal\[96\]. In particular, the phase shift is strictly related to the amount of energy dissipated in the tip–sample contact and a mapping of the phase shift on a sample surface allows us to identify regions of different interaction properties\[97,98\]. The development of intermittent contact modes of operation in liquid configured an important breakthrough for the application of the AFM in biology, especially in the case where single loosely immobilized molecules have to be imaged\[99,100\]. Imaging at high resolution (about 1 nm) by intermittent contact mode AFM in liquid on biological samples has been reported\[101\], allowing us also to retrieve information about the interaction forces from the phase signal\[102\]. Dealing with the intermittent contact mode in liquid, a new technique which has been recently introduced for controlling in a better way the force applied by the tip is active quality factor control (active-Q)\[103,104\]. This
is a technique which allows us to increase the otherwise low quality factor for the oscillating cantilever in liquid and correspondingly to decrease the force applied by the tip on the sample.

Apical curvature radii of commercially available tips can be as small as 2 nm. However, spatial resolution is not simply related to this aspect; rather, it depends also on the type of sample being imaged. At variance with the scanning tunneling microscope, in the AFM the interaction developing between probe and sample is not limited to the two nearest atoms of the tip and sample; long-range forces can also play a significant role, involving a huge number of atoms contributing to the contrast formation mechanism. Even if true atomic resolution images have been obtained by the AFM on layered samples\cite{105}, this resolution level is only possible in particular cases. As a general rule, the harder and flatter the sample the higher the spatial resolution achievable. Often, the resolution obtained is higher than that expected on the basis of the nominal value of the apical curvature radius, due to the presence of nano asperities which may play the role of the true imaging tip\cite{106}.

The dependence of AFM resolution on the softness of the sample is of remarkable importance especially for biological samples, usually very soft and damageable during scans. Spatial resolution down to sub nanometer level on biological samples has been obtained in the case of proteins in reconstituted membranes, which show a closely packed 2D molecular array; however, in the case of isolated proteins the resolution drops to a few nanometers. The resolution attainable is even worse, being in the range of a few tens of nanometers, in the case of living cells, due to the softness of the cell membrane. Moreover, the surface chemistry of the tips, usually not well defined, may limit the application of AFM in the investigation of biological samples. However, resolution is not only a question of tip dimensions. For example, the accurate control of the interaction between the tip and sample in dynamic operation modes, balancing the contribution of attractive and repulsive forces, can greatly enhance resolution\cite{93}.

AFM is a powerful technique also for studying supported lipid films\cite{107,108}. Its potentialities come from the capacity of imaging lipid films at high lateral and vertical resolution in a liquid environment, the ability of investigating local mechanical properties and interaction force and the possibility of using the AFM tip as a nano tool to locally modify the films. Samples are usually prepared by using a Langmuir–Blodgett trough which allows us to transfer lipid films from the air–water interface to a solid support. Both monolayers and multilayers can be transferred by this method. Alternatively, lipid bilayers, which represent a unique model system for biological membranes, can be formed by fusing lipid vesicle on the substrate\cite{109,110}. AFM has been employed for studying films obtained both from a
single-component lipid and from a mixture of different lipids. In these cases AFM is an excellent technique to characterize morphologically the obtained films, including the possibility of resolving defects on the nanometre scale and phase separation between different lipids. The possibility of identifying the presence of different phases is related to the high vertical resolution of AFM and the possibility of distinguishing areas with different mechanical or surface charge properties. The interest in lipid films is also connected to the possibility of using them as a support for immobilizing other biomolecules, either by inserting them in the membrane or by anchoring them at their top.

AFM offers the unique possibility of studying structural defects with nanometer-scale lateral resolution. The influence of chemical and physical external agents such as solvents, ions and temperature on the morphology of the supported layers has been elucidated in the case of symmetric and asymmetric bilayers. The effect of temperature on the topology of supported bilayers in relation to the gel/fluid phase\[^{[111]}\], on the lipid-loss process\[^{[112]}\] and on the ripple phase structure and kinetics of one and two-component lipid bilayers\[^{[113]}\] has been studied. The occurrence of the ripple phase, characterized by large-scale height modulation, is clearly recognized in AFM images due to the high vertical resolution of the technique. Lateral segregation, due to phase separation, is easily observed by AFM in the case of single-component films, of a mixture of different lipids and in the case of the interaction of the film with other biomolecules. Bilayers in the solid phase differ from bilayers in the liquid phase, the former having a thickness greater than the latter. AFM studies on lipid membranes also include the role of membranes as support for inserting or immobilizing other biomolecules such as ion channels (such as KcsA, KvAP).

2.1.2 Temperature-controlled and pH-dependent AFM measurements

Temperature-controlled AFM measurements can provide valuable informations about changes in structural and physical properties of bilayers during phase transitions. All the experiments have been performed with a Bioscope equipped with a Nanoscope IIIA controller (Veeco Metrology, USA). It has also been constructed a temperature-controlled stage based on a circulating water bath on which it has been possible to mount the Bioscope head (Fig 19). Imaging was performed in tapping mode at a scan rate of 1-2
lines/s using triangular silicon nitride cantilevers (Olympus OMCL-TR400PB-1, Japan) with a nominal spring constant of 0.09 N/m and a resonance frequency in liquid between 8-9 kHz. The force applied to the membrane was adjusted to the lowest possible value allowing reproducible imaging.

![Bioscope equipped with a Nanoscope IIIA controller](image)

**Fig 19.** A Bioscope equipped with a Nanoscope IIIA controller (Veeco Metrology, USA) and with a temperature-controlled stage based on a circulating water bath on which it is possible to mount the Bioscope head.

In this thesis the presented data correspond to cooling and heating scans of the lipid bilayers. The sample temperature was monitored by a digital thermometer Fluke 16 (Fluke, Italy) equipped with a small K-thermocouple probe (Thermocoax GmbH, Germany) in direct contact with the imaging buffer (150 mM KCl, 10 mM potassium dihydrogen citrate, pH 7). The sample temperature was changed in steps of 2°C. After each temperature step, the lipid bilayer was allowed to equilibrate before acquiring an image at a constant temperature. In order to assure the equilibrium condition, consecutive AFM scans were performed until no perceptible changes were observed in two subsequent images. If a heating scan was performed after a cooling one, the same general trend was observed but with a hysteretic behavior. In general the cooling scans gave the best reproducibility. This behavior has been found also in DMPC supported bilayers. This has been attributed to very long relaxation times for solid phases which introduce residual lateral stress in the supported bilayers and made their behavior strongly dependent on the thermal resistance.
of the sample. After an analysis performed on a specific area of the lipid bilayer, the AFM
tip was moved to an area, which was not previously imaged, in order to verify that the
observed features were not influenced by the repeated scans\textsuperscript{[114]}. Moreover, to study the
pH-induced isothermal phase transition of the SLB, the system was initially equilibrated at
the desired temperature and pH then a syringe pump (PHD 2000, Harvard Apparatus,
USA) was used to perform buffer exchange in the sample cell. A flux rate of around 150 μl/
min was established and after a ten-fold exchange of the initial buffer volume was stopped.
The slow exchange rate ensured a constant buffer temperature during the buffer
exchange. It has been done a calibration measurement without the Bioscope head in order
to allow the estimation of the buffer’s pH value during the exchange.
The calibration consisted in measuring the pH value after every 1 ml exchanged (this
amount was two times greater than that present in cell) starting from a pH value of 7 until a
pH value of 3:

<table>
<thead>
<tr>
<th>ml EXchanged</th>
<th>pH VALUE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 ml</td>
<td>4.87</td>
</tr>
<tr>
<td>2 ml</td>
<td>3.85</td>
</tr>
<tr>
<td>3 ml</td>
<td>3.38</td>
</tr>
<tr>
<td>4 ml</td>
<td>3.20</td>
</tr>
<tr>
<td>5 ml</td>
<td>3.12</td>
</tr>
<tr>
<td>6 ml</td>
<td>3.06</td>
</tr>
<tr>
<td>7 ml</td>
<td>3.03</td>
</tr>
<tr>
<td>8 ml</td>
<td>3.01</td>
</tr>
<tr>
<td>9 ml</td>
<td>3</td>
</tr>
</tbody>
</table>

![Graph showing pH values vs. buffer exchanged](image)
2.1.3 Force-volume measurements

Force spectroscopy is a precise analysis of the sample achieved by measuring the cantilever deflection as a function of the distance $z$ between the probe and the sample surface. The force-volume technique collects all force curves on a sample area. A force spectrum $f(z)$ shows the force evolution as a function of $z$ at a specific location on the sample. The general shape of a force spectrum is shown on Fig. 20.

![Fig 20. A schematic representation of an approach (solid line) and retraction (dashed line) curves of a typical force-volume measure][115].

The force intensity is extracted from the relative measurements of the cantilever deflection as a function of the relative motion of the probe $z$, where the largest $z$-value stands for the reference probe position whose location is the most distant from the sample. The force profile is made of two curves of the probe:

1) **Approach curve**: In region A, no interaction occurs as the cantilever is far from the sample, this region allows to define the zero value of the forces. The interactions present
in region B are related to surface forces (electrostatic, Van der Waals) and their sign can be either negative (adhesion between the probe and the surface) or positive (repulsion). The contact between the probe and the sample is reached between regions B and C, and region C describes the mechanical interactions of the cantilever and/or the sample. As regards a non deformable sample, this behavior is mainly due to the linear deformation of the cantilever; whereas for a deformable sample the compression and/or the indentation processes lead to linear or non linear behaviors.

2) Retraction curve: During the retraction, the presence of an hysteresis between the approach and retraction curves is due to the viscoelastic properties of the sample (region D). For non deformable surfaces, this hysteresis is equal to zero. In region E, important adhesion forces may be embedded in the retraction curve depending on the surface of contact, the contact duration, and mainly on the surface energy between the sample and the probe. This region, for micro-organisms, is composed of numerous discontinuities. By reproducing the previous precise analysis and by scanning the sample surface, a force-volume image \( f(x, y, z) \) is obtained. This image is formed of the collection of force spectra \( f(z) \) on a grid \((x, y)\) representing the sample surface (see Fig. 21).

![Fig 21. Recording of a force-volume image on a grid \((x, y)\) representing the sample surface.](image-url)
For the visualization of the 3D image one considers each force spectrum separately and then estimates the contact point between the probe and the sample. From this, it is possible to perform a 2D topographical reconstruction of the sample.

Force curves can also be used to investigate the elastic properties of a material, by measuring the force required to indent or deform the surface. Force volumes can, therefore, be used to produce micro-elasticity maps of the sample that show local variations in surface stiffness. Different fabrication processes and treatments of materials can result in inhomogeneities within the material, resulting in different values of the Young’s modulus and the elasticity maps is able to identify these defects. Similarly, elasticity measurements of biological cells, whose stiffness changes in response to many factors, can identify rearrangements of cytoskeletal elements and other cellular components.

I performed experiments both on SLBs on mica and on silicon and the sample preparation is the same described in section 2.3.

2.1.4 Image Analysis

For the image analysis the acquired height images were used, then, these were converted into graphical files using a software called WSxM (Nanotec Electronica, Spain)\[116\]. Then, these graphical files were opened with Adobe Photoshop C3 (Adobe, USA) and the different lipid domains were manually marked and the respective areas were calculated in order to determine the fraction of each with respect to the total bilayer area.

Everything that could ensure that the imaged areas were representative samples of the entire bilayer and that no imaged-area-dependent information was obtained from the analysis has been done. A series of scans (10μm x 10μm) were performed in the same region on the bilayer in order to explore a total area of 100 μm² (it has been seen that this was the right area which ensured a good representation of what was happening all over the membrane).

The force curves of force-volume measurements were analyzed by a home made program which extracts the force-curves files. The program is composed of two different parts:

extract.exe: that differently extracts the ascii data
**map_distro:** that is the main part. **Map** contains the indentation informations with respect to the spatial position. In other words, it tries to create an image that is similar to the Nanoscope program one but that can be managed with Mathematica.

**Distro** loses all the informations about the spatial resolution, but allows to reconstruct distributions which reflect the indentation value histogram. This general approach allows to identify the average indentation value (or values in case there were more phases) by a Gaussian curve fit executed by a statistical program (such as Origin or Igor pro).

### 2.2 Sample Preparation

The lipids 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE) and 1-palmitoyl-2-oleoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] (sodium salt) (POPG) were purchased from Avanti Polar Lipids (Alabaster, USA). Stock solutions (in CHCl₃) were mixed to obtain the desired lipid molar ratio. Then the chloroform was evaporated under a flow of nitrogen while heating the sample in a water bath at 50 °C. Thereafter, the sample was kept under vacuum (10⁻² mbar) for at least 4 hours to remove the remaining chloroform molecules. Afterward, the lipids were rehydrated in a storage solution of 450 mM KCl, 25 mM Hepes, pH 7. The sample was stirred at about 30 °C for 1 hour. During this time the sample was vortexed at least two times. The transition midpoint temperatures of pure small unilamellar liposomes of POPG and POPE at neutral pH are -5 and 25 °C respectively[117].

### 2.3 Supported Lipid bilayers preparation

The SLBs were prepared by the vesicle fusion technique. Either a freshly cleaved piece of mica (SPI Supplies/Structure Probe, Inc., USA) or a cleaned silicon dioxide support was placed on the AFM stage (see fig 19). With respect of details of the experiment, SLBs were prepared by either fixing the support with a ring of polydimethylsiloxane (PDMS) and then a polytetrafluoroethylene (Teflon) one or using a fresh piece of mica that was previously attached to a teflon sheet glued onto a metal disc. Then, in the case of the two rings,
amount of 500 μl of storing buffer was previously added both on mica and on silicon and the lipid suspension was sonicated for 30 seconds with the imaging buffer in an ultrasonic bath to obtain small unilamellar vesicles (SUVs). Then the SUVs were injected in order to obtain a final lipid concentration of either 0.25 mg/ml (mica) or 0.5 mg/ml (silicon dioxide). In the case of the fresh piece of mica attached to a teflon sheet glued onto a metal disc only 70 μl of storing buffer and sonicated sample were used. The vesicle solution was equilibrated at the temperature of interest together with the storing buffer and also in this case the SUVs concentration was 0.25 mg/ml. In both cases the lipid suspension was incubated for 15 minutes. Then, in the case on two rings, the storing and the imaging buffer was only exchanged for the imaging buffer (150 mM KCl, 10 mM potassium dihydrogen citrate at pH of 7, 5.6 or 3) and in the case of the simple piece of mica attached to a teflon sheet glued onto a metal disc only the storing buffer with the sample was exchanged for the imaging one in order to eliminate the unruptured vesicles. Then the sample temperature was adjusted to the desired starting temperature and the lipid bilayer system was allowed to equilibrate.

The interaction energy between a lipid bilayer and a substrate results from the balance of mainly three terms: 1) the van der Waals interaction energy which results in an attractive force at any distance, 2) the double layer interaction that depends on the surface charge of the two interacting surfaces; mica has a negative surface charge in solution and the bilayer surface charge depends on the lipids. The decaying length of the double layer force is exponentially related to the presence of electrolytes in solution via the Debye screening length, whose magnitude decreases upon an increase of the ionic strength of the solution. Both the van der Waals and the double layer interactions are described by the DLVO (Derjaguin-Landau-Verwey-Overbeek) force. The DLVO theory does not describe accurately the interaction forces at high ionic strength as the one we used for SLB assembling where the electrostatic forces are strongly screened. 3) The hydration interaction[94], another short-range force, that in solution is present between two surfaces. In our case, at small separation distance between a mica surface and a lipid bilayer, this force is repulsive. It can be described in different ways as a consequence of stable structured water layers in the proximity of a surface, especially in the case of a solid surface, or as a force due to entropic factors, namely thermal fluctuations. The second description applies mainly to soft interfaces such as lipid bilayers. In the case of lipid bilayers it can be divided in two motional contributions: 1) protrusions of the lipid headgroups and 2) collective undulations of the bilayers. All these contributions depend on temperature and are inversely proportional to the mechanical parameters of the surfaces.
In our context the mechanical parameters of interest are the bending and the area expansion modulus of the lipid bilayer. It is worth saying that the undulation contribution also depends on the tension in the bilayer. It follows that in the proximal leaflet a solid ordered domain, due to its different mechanical properties with respect to the liquid disordered phase, has a stronger interaction with the substrate than liquid disordered domains. The interplay of all forces acting between a lipid bilayer and a solid substrate is quite complicated and all the forces depend on the environmental conditions such as pH, electrolyte type and concentrations, temperature and the hydrophilicity of the interacting surfaces. Nonetheless, the overall balance of the interaction energies between the lipid bilayer and the substrate leads to an equilibrium distance between them. All involved forces have a strong dependence on distance. Thus the substrate exerts its influence on the bilayer with an interaction stronger on the proximal leaflet than on the distal one. In our studies we only concentrated on the ionic strength of the solution and we did not take into consideration specific ion effects, even if they are ubiquitous in biology.

**Preparation of silicon dioxide supports**

Silicon dioxide supports were first cleaned with water and then with detergent. Then it was thoroughly washed with bidistilled water (18 MΩ cm) and dried under N₂ stream. Afterwards, the support was exposed to a piranha solution (H₂O₂:H₂SO₄ 1:3), washed extensively with millipore water, dried under N₂ stream and exposed to an oxygen plasma discharge in a Plasma Cleaner (Diener Electronic GmbH, Germany). The preparation procedure produced a silicon oxide surface with a rms roughness of 0.20 nm compared to the rms roughness of 0.05 nm of mica.

2.4 Langmuir-blodgett films

A Langmuir–Blodgett film is made of one or more monolayers of an organic material deposited from the surface of a liquid onto a solid substrate by immersing (or emersing) this substrate into (or from) the liquid subphase. As a consequence a monolayer is
adsorbed homogeneously with each immersion or emersion step, in this way it is possible to achieve films with very accurate thickness. This thickness is accurate because the thickness of each monolayer is known and can therefore be added to find the total thickness of a Langmuir-Blodgett Film.

The monolayers are assembled vertically and are usually composed of amphiphilic molecules (such as phospholipids) with a hydrophilic head and a hydrophobic tail. When the lipid concentration is less than critical micellar concentration (CMC), they arrange themselves as shown in Fig. 22 below.

This tendency can be explained by surface-energy considerations. Since the tails are hydrophobic, their exposure to air is favoured over that to water. Similarly, since the heads are hydrophilic, the head-water interaction is more favourable than air-water interaction. The overall effect is reduction in the surface energy (or equivalently, surface tension of water).

![Fig 22. A schematic illustration showing the components of an amphiphile (left), and the orientation of an amphiphile adopted at an interface (right).(from K S V Instruments LTD)](image)

For very small concentrations, far less than critical micellar concentration phospholipids execute a random motion on the water-air interface. This motion can be thought to be similar to the motion of ideal gas molecules enclosed in a container. The corresponding thermodynamic variables for the surfactant system are:
- surface pressure (\(\Pi\))
- surface area (A)
- number of surfactant molecules (in our case phospholipids) (N)
This system behaves similarly to a gas in a container. The density of surfactant molecules as well as the surface pressure increase upon reducing the surface area $A$ (‘compression’ of the ‘gas’). Further compression of the surfactant molecules on the surface shows behavior similar to phase transitions. The ‘gas’ gets compressed into ‘liquid’ and ultimately into a perfectly closed packed array of the surfactant molecules on the surface corresponding to a ‘solid’ state. Instruments like Langmuir-Blodgett trough can be used to quantify such phenomena.

Adding a monolayer to the surface reduces the surface tension, and the surface pressure, $\Pi$ is given by the following equation: $\Pi = \gamma_0 - \gamma$ (where, $\gamma_0$ is equal to the surface tension of the water and $\gamma$ is the surface tension due to the monolayer).

Experimentally, the surface pressure is usually measured using the Wilhelmy plate (fig 23). A pressure sensor/electro-balance arrangement detects the pressure exerted by the monolayer.

![Fig 23. A schematic representation of a Wilhelmy plate. (from K S V Instruments LTD www.ksvlt.com)](image)

A simple force balance on the plate leads to the following equation for the surface pressure:

$$\Pi = -\Delta \gamma = - \left[ \frac{\Delta F}{2(t_p + w_p)} \right] \approx - \frac{\Delta F}{2w_p}$$  

Eq. 4

only when $w_p > t_p$
Here, \( w_p \) and \( t_p \) are the dimensions of the plate, and \( \Delta F \) is the difference in forces. The Wilhelmy plate gives pressure – area isotherms that show phase transition-like behavior of the LB films, (see figure 24). In the gaseous phase, there is minimal pressure increase for a decrease in area. This continues until the first transition occurs and there is a proportional increase in pressure with decreasing area. Moving into the solid region is accompanied by another sharp transition to a more severe area dependent pressure. This trend continues up to a point where the molecules are relatively close packed and have very little room to move. Applying an increasing pressure at this point causes the monolayer to become unstable and destroy the monolayer.

The most important indicator of the monolayer properties of an amphiphilic material is given by measuring the surface pressure as a function of the area of water surface available to each molecule. This is carried out at constant temperature and is known as a surface pressure - area isotherm or simply “isotherm”. Usually an isotherm is recorded by compressing the film (reducing the area with the barriers) at a constant rate while continuously monitoring the surface pressure. Depending on the material being studied, repeated compressions and expansions may be necessary to achieve a reproducible trace.

A number of distinct regions is immediately apparent on examining the isotherm in fig 24. These regions are called phases. As one can see when the monolayer is compressed it can pass through several different phases which are identified as discontinuities in the isotherm. The phase behavior of the monolayer is mainly determined by the physical and chemical properties of the amphiphile, the subphase temperature and the subphase composition. For example, various monolayer states exist depending on the length of the hydrocarbon chain length and the magnitude of other cohesive and repulsive forces existing between head groups. An increase in the chain length increases the attraction between molecules, condensing the \( \Pi \)-A-isotherm. On the other hand, if an ionasible amphiphile is used the ionisation of the head groups induces repulsive forces tending to oppose phase transitions.
In general the monolayers exist in the gaseous state (G) and can, on compression, undergo a phase transition to the liquid-expanded state (L1). Upon further compression, the L1 phase undergoes a transition to the liquid-condensed state (L2), and at even higher densities the monolayer finally reaches the solid state (S). If the monolayer is further compressed after reaching the S state the monolayer will collapse into three-dimensional structures.

The collapse is generally seen as a rapid decrease in the surface pressure or as a horizontal break in the isotherm if the monolayer is in a liquid state. There are also many other critical points in a $P$-$A$-isotherm such as the molecular area at which an initial, pronounced increase in the surface pressure is observed, $A_i$, and the surface pressures at which phase transitions occur between the L1 and L2 state and the L2 and S state.

A Langmuir-Blodgett trough is a laboratory apparatus that is used to compress monolayers of molecules on the surface of a given subphase (usually water) and measures surface phenomena due to this compression. It can also be used to deposit single or multiple monolayers on a solid substrate.

The trough is made of plastics that are insoluble in ordinary solvents, such as teflon (polytetrafluoroethylene).
The system to compress or expand the monolayers throughout the development of the LB trough is made of movable Teflon barrier blocks that slide parallel to the walls of the trough and are in contact with the top of the fluid (fig 25).

![Fig 25. Schematic illustration of a Langmuir film balance with a Wilhelmy plate electro-balance measuring the surface pressure, and barriers for reducing the available surface area.](image)

An important property of the system is its surface pressure (the surface tension of the pure subphase minus the surface tension of the subphase with amphiphiles floating on surface) which varies with the molecular area. The surface pressure – molecular area isotherm is one of the important indicators of monolayer properties. Additionally, it is important to maintain constant surface pressure during deposition in order to obtain uniform LB films.

Any type of surface experiment requires maximal cleanliness and purity of components. Even small contaminations can have substantial effects on results. If an aqueous subphase is used, the water must be purified to remove organics and deionized to a resistivity not less than 18 MΩ·cm. Impurities as small as 1ppm can radically change the behavior of a monolayer. To eliminate contamination from the air, the LB trough can be enclosed in a clean room. The trough set-up may also be mounted on a vibration isolation table, to further stabilize the monolayer. The exact calibration of the electro-balance is also very important for force measurements. Experimental preparation requires that the trough and barriers be thoroughly cleaned by a solvent such as ethanol to remove any residual organics. The liquid subphase is added to a height such that the meniscus just touches the barriers. Often it is necessary to aspirate the surface of the liquid in order to remove any last remaining impurities. The amphiphilic molecules dissolved in solvent are slowly
dropped onto the liquid surface using a micro-syringe, with care being taken to spread it uniformly across the surface. Some time must be taken to allow for evaporation of the solvent, and the spreading of the amphiphile. The Wilhelmy plate to be used must be absolutely clean. The Wilhelmy plate is then mounted on the electrobalance such that it is immersed perpendicular to the surface of the liquid and a uniform meniscus is achieved (fig 26).

The transfer of a monolayer to a substrate is a delicate process dependent on many factors. These include the direction and speed of the substrate, the surface pressure, composition, temperature, and pH of the subphase. One method involves a dipping arm which holds the substrate and can be programmed to pass through the interface from top to bottom or bottom to top at a set speed (fig 26). For dipping starting from below the liquid surface, the substrate should be hydrophilic, and for dipping starting above the liquid surface, the substrate should be hydrophobic. Multilayers can be achieved by successive dipping through alternating monolayers.

![Fig 26. Monolayer transfer onto a substrate after film compression. The substrate is moving from bottom to top and is hydrophilically coated since the polar head groups are adhering to the surface.](image)

A typical experiment was conducted on a NIMA 601 M Langmuir-trough as follows:
1) As first step the desired working temperature was set up on the heat bath attached to the trough by two tubes, 2) then, as the desired temperature was reached the flow was stopped, 3) after this the trough was cleaned by ethanol and a subphase was added (ddH₂O or buffer), 4) the temperature of the subphase was allowed to equilibrate at the desired temperature, 5) and from the monolayer menu were set respectively: the molecular weight of the sample (718.01 for POPE, 744.5 for POPE:POPG 1:1 and 731.2 for POPE:POPG 3:1), the concentration of the sample (1 mg/ml), the volume of the sample
(5 μl) and the barrier speed (10 cm²/min), 6) before starting the measure the subphase was completely cleaned from impurities, 7) then the sample was placed, by a 10 μl Hamilton siringe, over the subphase, 8) to start the compression the “isotherm” key was pressed and the barriers were stopped before one could observe a collapse (generally before a pressure value of 55 mN/m), 9) the dipping procedure was started.

The dipping procedure consisted in: 1) cleaving both sides of a mica piece, 2) attach the mica piece to a standard dipper, 3) place the dipper well above the surface and placing the mica piece parallel and between the barriers, 4) then the “creep down” key was pressed until the mica piece was almost covered by subphase, only the part attached to the deeper was left outside, 5) finally the “creep up” key was pressed (fig 27) and the speed of creeping up was set to 5 mm/min, 6) once the mica piece was outside was attached to an iron disc, by a double sided tape, and analyzed by the AFM.

![Fig 27. Transferring graph of a POPE:POPG 3:1 monolayer onto a mica square.](image-url)
3 RESULTS AND DISCUSSION

3.1 Phase transition in Supported Lipid Bilayers

It has been seen that vesicle fusion, of POPE:POPG 3:1, on mica performed at 34,5°C produces almost continuous lipid bilayers\[^{114}\], whereas on silicon dioxide the optimal temperature shifts to 33°C. After preparing the lipid bilayer, the storing buffer (450mM KCl, 25mM Hepes, pH 7) was exchanged both for pure water and for the imaging buffer (150mM KCl, 10mM potassium dihydrogen citrate, pH 7) by extensive rinsing and all the system was equilibrated for 15 minutes. The presence of a few defects (holes) in the lipid bilayer allowed to measure the height of the bilayer with respect to the substrate (mica or silicon dioxide).

The behavior of the supported bilayer was studied decreasing the temperature by steps of 2°C. For each step a series of AFM images was acquired until the system reached an equilibrium state evident from no changes on the nanometer scale between two consecutive scans. At this point the image corresponding to the temperature of interest was definitely acquired.

The most relevant interaction between the substrate and the lipid bilayer is the electrostatic interaction between the charged mica surface and the lipid head groups. On the basis of this consideration we investigated the behavior of lipid bilayers of the same compositions in buffers with different ionic strengths. It was suggested that increasing the ionic strength the connected Debye length decrease should lower the electrostatic interaction between the substrate and the lipid bilayer resulting in a coupled transition of the two leaflets as is usually observed in liposomes\[^{52}\].

3.1.1 Phase transition on mica

In literature It has been shown that POPE:POPG (3:1) SLBs on mica underwent a split temperature-induced phase transition when they are prepared at incubation temperatures
lower than that of their main phase transition\textsuperscript{[114]}. Indeed, two transitions corresponding to the two leaflets behaving independently are observed upon a temperature change across the transition region. This gives rise to what has been called an intermediate phase of the bilayer, in which one leaflet is in the liquid disordered phase and the other is in the solid ordered one. The presence of a substrate induces a vertical symmetry breaking on the two bilayer leaflets\textsuperscript{[97]}. This is even true in the case of the presence of both a imaging solution (fig 28) and pure water solution (fig 29).

**Fig 28.** AFM images sequence acquired starting from an incubation temperature of 20.1°C and after raised up to 30.1°C. The scan size is 10μmX10μm and the SLB is prepared using a 150mM KCl, 10mM potassium dihydrogen citrate pH 7 buffer solution. The lipid bilayer is in a liquid disordered phase (a). Decreasing the temperature there is the formation of intermediate phase domains (b). Then the intermediate phase becomes solid ordered and starts to cover most of the area (c).

In particular, recent simulation studies by the Faller’s group demonstrated that in supported lipid bilayers the surface density of lipid molecules in the two leaflets differs, being higher in the proximal one\textsuperscript{[98,99]}. This phenomenon influences also the order parameters of the lipids in the leaflets and the lateral pressure profile. The substrate
seems to act mainly on the proximal leaflet. In fact, the lipid density in the distal leaflet is very similar to what is expected for an unsupported bilayer. These findings give support to the interpretation of the lower temperature transition as due to the distal leaflet and the higher one as due to the proximal one\cite{89, 114}. The higher surface density of the lipids in the proximal leaflet can explain the increased transition temperature according to the Clausius-Clayperon relation assuming a transition at constant area\cite{100}. In this case, a change in surface tension is reflected in a variation of the melting temperature for each leaflet. Moreover, the more rigid solid ordered domains are entropically favored near the rigid substrate, where undulation suppression occurs.

Figure 29 reports a series of images from a temperature of 34.5°C to a temperature of 9.5°C. Lipid domains about (0.7 ± 0.2) nm higher than the surrounding lipids started to appear at 30.4°C and upon cooling they grew until they almost entirely covered the lipid bilayer. Upon further temperature decrease, the lipid bilayer appeared stable until new domains started to appear at 17.3°C. Again the height of the newly developed domains was (0.7 ± 0.2) nm. Continuing the temperature sweep, the new domains grew and extended almost all over the lipid bilayer at a final temperature of 9.5°C. Along with the nucleation and growth of the domains, holes in the lipid bilayer expanded by decreasing temperature, with the most pronounced expansion corresponding to the second domains appearance.

![Fig 29](image)

**Fig 29.** Preparing the SLB at temperature below the lipid mixture main phase transition temperature one obtains a decoupling of the two bilayer leaflets phase transition even using a pure water solution. At first one leaflet undergoes a phase transition and first after completion the other one starts to change its phase.

Incubation temperature: 20°C\cite{117}.
Fig 30 displays the plots of the fractional area occupancy for the “intermediate phase” in the 150 mM KCl case compared to pure water. Even if the two transitions were closer to each other, they were still separated. For the proximal leaflet and distal leaflet it has been obtained from a van’t Hoff analysis the $T_0 = 23.6^\circ C$ and $T_0 = 18.3^\circ C$, respectively. In both measurements the intermediate phase reached a fractional area near one. The different transition temperature means that the ionic strength is able to modify the behavior of a solid supported lipid bilayer.

![Fig 30. A schematic representation of the fractional area occupancy for the “intermediate phase” in the 150 mM KCl case compared to pure water](image)

By preparing the SLB at a temperature higher than the main phase transition, the bilayer undergoes a coupled phase transition (fig. 31) which starts at the temperature of the first transition encountered during temperature variation (high temperature transition for the temperature down-scan and lower temperature transition for the temperature up-scan)\cite{114}. This behavior can be attributed to a modulation of the interleaflet coupling strength upon variation of the incubation temperature. An increased incubation temperature leads to a decrease in the lipid surface density. Considering the asymmetry in the surface density between the two leaflets it might be that the low incubation temperature gives rise to a strong difference in the lipid densities of the two leaflets preventing the dynamic interdigitation mechanism of the lipid hydrophobic tails. Indeed, the dynamic interdigitation
of the lipid chains is considered one of the main phenomena causing interleaflet coupling [92]. This interdigitation could be considered as a phenomenon which increases the entropy of the lipid chains and the entropy loss due to a restricted dynamic interdigitation could be the driving force for maintaining a coupling between the two monolayers. In the specific case of a SLB, Merkel et al. found that increasing the packing density of the proximal layer an increase in the diffusion coefficient for the distal layer was obtained, probably due to a decreased interdigitation effect [88].

Fig 31. AFM images sequence acquired starting from an incubation temperature of 34.1°C and with a scan size of 10μmX10μm. The SLB is prepared at a pH value of 3 and is in a liquid disordered phase (a). Decreasing the temperature there is directly the formation of solid ordered domains (b). The solid ordered phase has almost covered most of the area (c).
3.1.2 Phase transition on silicon oxide

To better understand the role of the substrate in the SLB behavior the same bilayer was studied on a different support. A POPE:POPG (3:1) bilayer was assembled on a silicon dioxide surface by the vesicle fusion technique incubating the sample at 33°C. Fig 32 shows a sequence of AFM images (from 32a to 32d) of a SLB obtained at different temperatures on the same sample area. Starting from a temperature of 30°C, upon decreasing the temperature, at 21°C a transition from the liquid disordered to the solid ordered phase starts. This is characterized by the growth of domains in the lipid bilayers higher than the surrounding lipid disordered membrane. Small areas of the lipid bilayers remain in the liquid disordered phase even at a temperature down to 16°C. Analyzing the line section of an image in the transition region it is possible to measure the difference in height between the two phases (figure 32e). The bilayer’s height of 1.4 ± 0.2 nm can be attributed to both leaflets that undergo a coupled phase transition to the solid ordered phase\[114\]. In figure 32f the integral of the inverted transition enthalpy (a measure of the solid ordered fraction) calculated from the heat capacity profile obtained using Differential Scanning Calorimetry of Small Unilamellar Vesicles of the same lipid composition (see inset to fig. 32f) is reported along with the fractional occupancy of the solid ordered phase obtained from the AFM images as a function of temperature. The shape of the inverted enthalpy and the solid ordered fraction obtained by AFM images agrees well. They both show a cooperative transition with a half width of 3°C. Still, the bilayer/substrate interaction shifts the SLB transition temperature up by about 2.5 °C. It is to be stressed that the fractional occupancy of the solid ordered phase can have a thermodynamic sense only if it represents a series of equilibrium states. The possibility of observing equilibrium states by AFM is strictly connected with the kinetics of lipid domain formation which, on its turn, depends on the diffusion properties of the lipids\[102\]. In the case of a coupled phase transition of a SLB on mica it has been not possible to produce a plot of the fractional occupancy because the kinetics of domain formation was very slow (in the order of hours)\[114\]. In the case of SLBs on silicon oxide, equilibrium states upon a temperature change were obtained for time intervals in the order of minutes. This behaviour allowed obtaining a plot like the one shown in figure 32e.
**Fig 32.** Melting transition behavior of SLBs on silicon oxide and comparison with SUVs. (a-d) Sequence of AFM images (image size: 7.5 μm x 7.5 μm) showing the temperature induced phase transition of a SLB on silicon oxide. Down to a temperature of 22°C no domain formation was visible. Domains started to appear at 21°C and extended upon further cooling of the sample. (e) The line section along the solid line of image (b) reveals a step height between the two domains of 1.4 ± 0.2 nm. (f) The black solid line, representing the inverse transition enthalpy, is compared to the solid ordered fraction (open circles, dashed curve is a guide for the eye) of the SLB. The transition on the silicon oxide support happens at a slightly higher temperature than the one of the SUVs. Inset: excess heat capacity of SUVs of POPE:POPG 3:1 sample at pH 7 obtained by DSC.

The different kinetics on the two substrates (mica and silicon dioxide) could be related to the different diffusion coefficient of the lipids in the bilayer. In favor of this hypothesis, for other systems (SLBs of DPPC) comparing results from the literature, it can be found that the diffusion coefficient of the lipids on mica is significantly lower than on silicon oxide [103,10]. In order to understand the effect of the incubation temperature on the SLB phase transition behavior on silicon oxide with respect to mica, a new SLB on silicon oxide was incubated at 10°C. Then, the temperature was increased to 33°C and the phase transition was monitored upon a temperature decrease. Also in this case the main phase transition induced by temperature occurred in a coupled manner between the two leaflets and the fractional occupancy of the solid phase as a function of temperature is very similar to the previous case (figure 32e). This means that, at variance with what happens for SLBs on mica, the temperature-induced phase transition for a SLB on silicon oxide does not depend upon the preparation temperature of the membrane. As pointed out above the main transition temperature obtained from the supported lipid bilayer is very similar to what has been obtained by DSC on small unilamellar vesicles. This result agrees with those
obtained by Tamm and McConnell for a DPPC bilayer supported on oxidized silicon by measuring the lateral diffusion of a fluorescent lipid probe\cite{10}. In the case of the same lipid system on mica the starting of the main transition temperature occurred at a temperature significantly higher than that obtained by DSC. Fig 33 reports a scheme of the behavior of SLBs on mica and silicon oxide as a function of the SLB incubation temperature. In figure 33a it is shown that if a SLB is prepared on mica at an incubation temperature lower than its melting transition, three possible phases for the bilayer can occur.

**Fig 33.** Sketch of the behavior observed for SLBs on mica and silicon oxide substrates. We show a cartoon of the situation, a representative AFM image (image size: 10 μm x 10 μm) of the SLB and a line section along the solid line of the corresponding image. (a) Preparing a SLB on mica at a temperature below the melting transition regime leads to a decoupling of the two leaflet’s transitions. The step heights between different domains are in the order of 0.7 nm each. (b) Increasing the incubation temperature above the main phase transition temperature couples the transitions of the two bilayer leaflets. The step height between the domains is 1.4 nm. The lowest level in the line section represents the mica level. (c) On silicon oxide, independently of the incubation temperature, there is always a coupled transition. The step height is again in the order of 1.4 nm.
As stated before these phases have been called liquid disordered, intermediate and solid ordered phase. This behavior is a consequence of the absence of interleaflet coupling in the bilayer. If the bilayer is assembled on mica at a temperature higher than its melting transition (fig. 33b) the presence of interleaflet coupling removes the possibility of the intermediate phase and only domains in perfect registry occur. If a SLB is assembled on silicon oxide (fig. 33c), independently from the incubation temperature, the bilayer leaflets are always coupled.

Several studies by Atomic Force Microscopy on the phase transition behavior of SLBs established that the conversion from the liquid disorder phase to the solid ordered phase occurred at significantly higher temperature with respect to freely suspended bilayers, at least for one of the two leaflets if two separate transitions are observed\cite{89,114,100}. This aspect can be ascribed to the interaction between the bilayer, especially the proximal leaflet, and the substrate. The behavior observed on silicon oxide can be interpreted by assuming that the interleaflet coupling is stronger than the interaction of the proximal leaflet with the support. The mica and the silicon oxide surfaces have similar charge in the conditions used for assembling and studying the SLB. A different electrostatic force between the substrate and the bilayer cannot be evoked to explain the observed differences. The overall interaction between the substrate and the membrane is the result of many types of forces\cite{91}. The main contributions come from the van der Waals attractive interaction and the repulsive interactions resulting from both the undulation forces and the hydration forces. The balance between these forces produces a minimum in the potential energy which assures the stability of the lipid bilayer. All involved forces have a strong dependence on the separation distance between the two interacting bodies. Of special interest to our case is the effect of the substrate surface topography on the interaction potential energy. The silicon oxide surface is characterized by a higher roughness than mica. A higher roughness is related to a higher effective surface area which could in principle lead to increased adhesion energy if the bilayer was able to exactly follow the contour of the surface. However, this latter aspect would require a high bending of the lipid bilayer resulting in an increased energy for the bilayer. Due to this higher energetic cost and to the nonlinear dependence of the interaction forces on the distance between the substrate and the membrane, the adhesion energy of the membrane to the substrate decreases in the case of a rough surface\cite{104}. The presence of a rough surface resembles the presence of fluctuations in the bilayer, reducing the probability for the bilayer to lie close to the substrate and, consequently, decreasing the adhesion energy between the membrane and the support per unit of projected area. These considerations agree with
experimental results in which it is clearly observed that SLB on silicon oxide are less stable than on mica and the borders between the SLB and the bare substrate are easily disturbed by the scanning AFM tip.

3.1.3 Phase transition of SLBs as a function of pH

In bilayers composed of charged lipids, the main phase transition results in a variation of the electrostatic free energy due to a difference in the surface charge density following expansion or contraction of the bilayer surface. By varying pH, it is possible to modify the surface charge of the bilayer and to alter the electrostatic free energy. If the charge density increases, a decrease of the main phase transition temperature is expected[94]. The main phase transition temperature of the POPE:POPG 3:1 mixture has a strong dependence upon pH due to the presence of the charged PG head group[95]. Performing experiments with a pH value of 3 (fig 34) what quickly came out was that the transition temperature was higher than that at pH 7.
Fig 34. Temperature variation at constant pH value of 3 of a supported lipid bilayer. (a) the bilayer is in the liquid disordered phase at a temperature of 29.8°C; (b) as the temperature decreases it is possible to observe the nucleation of intermediate phase domains; (c) still decreasing the temperature the same domains become solid ordered, (d) the straight line is required to measure the different step heights of the domains.

Another interesting thing was that there were two transition temperatures and this was in contrast with other systems such as liposomes where there was a single transition temperature. Given this it is possible to conclude that the substrate influences the behavior of the bilayer inducing a decoupling of the two leaflets.

In order to achieve a coupling of the two leaflets of the bilayer it has been raised the temperature and, by keeping constant the pH, decreased the temperature and what happened was exactly what it was expected (fig.35).

Fig 35. pH dependence of the transition behavior. (a) Comparison of the excess heat capacity curves of POPE:POPG 3:1 SUVs measured at pH 3 and 7. Increasing pH leads to a shift of the transition region to higher temperatures. (Inset) Linear dependence of the transition midpoint temperature on pH. (b and c) AFM images of a SLB of POPE:POPG 3:1 incubated at 34 °C on mica (image size: 10 μm x 10 μm). Formation of
solid ordered domains was induced by lowering the temperature. At a pH of 3 domains started to form at 27.0 (± 0.5 °C. The time lapse between images b and c was 15 min. (d) The line section along the solid line of image c reveals that the transition of the two leaflets is coupled. The step height has a figure of 1.4 ± 0.2 nm.

This behavior can be attributed to a modulation of the interleaflet coupling strength upon variation of incubation temperature which leads to a decrease in the lipid surface density (surface density is higher in the proximal leaflet). Considering the asymmetry in the surface density between the two leaflets it might be that low incubation temperature gives rise to a strong difference in the density of the two leaflets preventing the interdigitation mechanism of the hydrophobic tails. The interdigitation of the lipid chains is considered one of the main phenomena causing interleaflet coupling.

Due to the fact that biological systems are remarkably constant in temperature and a phase transition induced by a pH variation has a biologically relevant interest[94,96], it was decided to study by AFM the behavior of a SLB on mica as a function of pH. All the supported bilayers were prepared at high temperature in order to obtain a single transition. Figure 35a shows the DSC measurements over the phase transition region of POPE:POPG (3:1) Small unilamellar Vesicles for different pH values (7 and 3). An increase of the transition temperature is observed by decreasing pH as expected from the protonation of the PG head group. Figure 35b shows a series of images of a SLB on mica by varying the temperature at a pH 3 and 7. The solid ordered phase starts appearing at 24.5°C at pH 7 and at 28.0°C at pH 3. This behavior confirms the dependence of the transition temperature on pH for a POPE:POPG 3:1 SLB on mica. Figure 35c illustrates the behavior on silicon oxide. Both a series of images and the fractional occupancy of the solid ordered phase are reported in this case. It is evident an increase of the transition temperature in the case of pH 3 with respect to pH 7 which means a stabilization of the solid ordered phase by decreasing pH. The obtained results clearly highlight that SLBs mirror, as long as the main phase transition is concerned, the behavior observed for liposomes of the same lipid composition.

After establishing the dependence of the phase transition temperature in SLBs upon pH variations, it was studied the possibility of observing by time-lapse AFM an isothermal phase transition induced by a change in pH. An SLB on mica at pH 7 was prepared by using an incubation temperature of 33°C and by adjusting the temperature at 27°C, an intermediate value between the two transition temperatures at pH 7 and 3 (see fig 36a).
In these conditions the bilayer was in the liquid disordered phase. After that, imaging buffer was exchanged for the same buffer but at pH 3 while imaging the bilayer by AFM.

Fig 36 shows a sequence of images obtained while the pH was changed from 7 to 3 and then back to 7. After an exchange of buffer which assures a pH of 4 in the sample cell, the formation of domains was observed, they initially were in the coupled solid ordered phase and progressively changed to the intermediate state. It has to be noted that during the transition from the coupled solid domains to the intermediate domains only the domains which undergo this transition significantly grow. In the case of domains remaining in the solid ordered phase, their size does not appreciably change between two consecutive scans. Moreover, the total solid area of the domain of fig 36b (considering the sum over the two leaflets) is similar to the area in the solid ordered phase of the same domain in fig 36c (14 μm² vs 15 μm²). This behavior can be ascribed to a lipid flip-flop movement which brings phospholipids which are in the solid ordered configuration to the opposite leaflet, while being substituted for lipids in the liquid disordered phase similarly to what has been observed in literature[90]. The new solid ordered domains occupying only one leaflet grow until they reach an equilibrium state. If the solution pH is changed back to 7, the previously formed domains disappear. This behavior assures that the domain effectively had formed as a consequence of the change in pH.

![Fig 36. pH-induced domain formation. (a) Traces of the heat capacity of POPE:POPG 3:1 SUVs at pH of 7 and 3. The curves have been corrected for the effect of the mica support which leads to an increase of the melting temperature by about 5°C. (b) The AFM image (image size: 10 μm x 10 μm) at a pH of 7 does not show any lateral heterogeneity. The membrane was prepared at high temperature to ensure a coupling of both leaflets. (c) Starting the buffer exchange, at some point (after reaching a pH of about 4) domains started]
to form. The different height levels in domain 1 indicate that the transition was not fully coupled. (d) Following the evolution of domain 1, it becomes clear that a flip-flop mechanism was present. The initial solid ordered domain changed to an intermediate domain. (e-f) After finally reaching a pH of 3, the buffer was again exchanged for a buffer at pH 7. The intermediate domains vanished demonstrating the reversibility of the transition.

In an attempt to establish which of the two leaflets was involved in the observed phase transition, it has been performed an experiment in which, once the pH-induced phase transition had occurred by changing the solution pH from 7 to 5.6, the sample temperature was decreased. It has been observed that a second phase transition appeared at the expected temperature for a bilayer at pH 5.6 without leaflet coupling (fig 37). This lower transition temperature was attributed to the distal leaflet and it occurred at a temperature similar to what was observed for liposomes of the same lipid composition from DSC measurements\[114,88,99].

Fig 37. pH and temperature change. Sequence of AFM images of a SLB of POPE:POPG 3:1 incubated at a temperature of 34°C on mica (image size: 20 μm x 20 μm). (a) At a temperature of 27.5°C at pH 7 the bilayer is in its liquid disordered phase. (b) At a pH 5.6 intermediate domains started to appear (c-e). After equilibration, we started to decrease the temperature from 27.5°C to 19.5°C. The intermediate domains enlarged until they almost completely covered a leaflet. (f) By further decreasing the temperature a second transition started. This transition happened at a temperature as expected for the distal leaflet at a pH of 5.6.
In light of this behavior it can be argued that the first transition occurred in the proximal leaflet and the pH variation caused the loosing of the interleaflet coupling in the bilayer. The flip-flop phenomenon in lipid bilayers is a fundamental process whose study is unfortunately hampered by many technical problems. In particular its rate has been determined with contradictory results\[118\]. It is believed that it is essentially a slow process unless it is catalyzed by proteins, but, under some circumstances, it can greatly increase its rate. One of these circumstances is related to the lipid phase transition. It has been demonstrated that at the main phase transition the flip-flop rate strongly increases\[117\]. Another case in which flip-flop movement can be enhanced is the presence of a pH-gradient across the lipid bilayer\[120\]. The exchange of pH on the SLB can result in a pH gradient across the bilayer. At pH 3 the phosphodiester group of PG starts to be protonated (pKa ~ 2.9) and the PG head group turns out to be neutral. As a consequence POPG in the distal leaflet could move to the proximal leaflet where it can release protons and contribute to reduce the pH gradient. Moreover, lipids in the solid order phase are entropically favored in the proximal leaflet due to undulation suppression near to the substrate. As can be traced from the AFM images it seems that the flip-flop mechanism proceeds mainly at the interface between the solid ordered and liquid disordered domains. The flip-flop mechanism could modify the lipid density in the two leaflets destroying the interleaflet coupling which was initially assured by the high temperature incubation of the SLB.

3.1.4 Force curves on supported lipid bilayers

In recent years the study of mechanical properties of lipid bilayers have been used as an important tool to understand how these can influence the functioning of membrane proteins. One of the first who investigated the lipid bilayer material properties was Helfrich\[121\]. These properties are relevant for the pure lipid bilayer per se (they influence the shape and fluctuations of the bilayer) but they are also relevant in the context of non specific protein lipid interactions. The interest in the lipid modulation of the activity of membrane
proteins prompted a strong attention to the investigation of the mechanical properties of lipid bilayers. In this sense Atomic Force Microscopy (AFM) emerged as a powerful technique which allowed both the topological characterization of lipid bilayers and their mechanical properties analysis.

The study of the mechanical properties of lipid bilayers by the AFM is usually performed in the force spectroscopy technique. The tip of the AFM is pressed on a supported lipid bilayer until it indents the bilayer and punches through it contacting the solid support (fig 38). It has been demonstrated that the force required to punch through the bilayer is on the nN scale (fig 38a, 38b) and it depends on the environmental conditions such as the ionic strength, temperature and pH. It has been suggested that the indentation force on a lipid bilayer can be considered as a fingerprint of the specific lipid bilayer composition.

Fig 38. Schematic representation of (a) force curve Deflection vs displacement (Z measured) (B) force curve deflection vs separation (Z actual) (c) interaction between the AFM tip and the lipid bilayer during the indentation process,
3.1.5 Indentation theory

From a theoretical point of view, different approaches have been proposed to interpret the AFM indentation experiments. It has been proposed that the rupture of the bilayer by the tip is a process with an energy barrier that must be overcome to activate the process itself. The activation energy corresponds to that required for the formation of a hole in the film, which is large enough to initiate tip penetration.

This process can be divided into four steps: 1) the film and the tip are separated (there are no forces that are acting on the tip and the energy of the bilayer is $U_i$), 2) the tip and the bilayer get into contact (the bilayer energy is still $U_i$), 3) the base of the cantilever moves further down and the cantilever is deflected, at this point the tip exerts a force on the bilayer (the applied force reduces the energy barrier and the bilayer becomes unstable under the pressure and there is a rupture of the bilayer itself), 4) the tip penetrates the film and gets in contact with the solid support (at this position the distance between tip and sample becomes equal to 0 the energy of the bilayer is $U_f$) (fig. 39).

**Fig 39.** Schematic representation of the energy of the lipid bilayer underneath an AFM tip vs the distance of the tip.
Once the tip got in contact with the bilayer the pressure on the bilayer increases and $U_i$ increases. The $U_i$ increase reduces the activation energy and this increases the probability of a bilayer rupture. If the activation barrier is of the order of $k_BT$, the reaction quickly occurs once it has become thermodynamically favorable ($U_f < U_i$), where $k_B$ and $T$ are Boltzmann’s constant and temperature.

In Butt’s article two specific models have been proposed to describe the activation process: 1) the continuum nucleation model, 2) the molecular model.

In the first model (where one has focused the attention) a thin homogeneous film between the support and the tip is considered. The tip end is supposed to have a parabolic shape with a radius $R$ while the film is supposed to be, laterally, in a liquid state, but vertically its structure is well defined. Assuming that due to thermal fluctuations a small circular hole of radius $R_h$ is formed in the two-dimensional fluid layer under the tip, the energy of the hole should be:

$$U = 2\pi r_h \Gamma + \pi r_h^2 \left( S - \frac{F}{2\pi R} \right)$$  \hspace{1cm} \text{Eq. 5}

The first term, $2\pi r_h \Gamma$, represents the free energy associated with the unsaturated bonds of the molecules at the periphery of the hole. $\Gamma$ is the line tension. The second term, $\pi r_h^2 S$, is the change in interfacial free energy that is proportional to the area of the hole. The parameter $S$ is referred as “spreading pressure” because it can be used to quantify the tendency of the film to spread into the gap between the tip and substrate.

$S$ can be determined by different interfaces, thus, assuming that the experiment is performed in liquid, there will be: the tip-film interface energy ($\gamma_{TF}$), the tip-liquid interface energy ($\gamma_{TL}$), the substrate-film interface energy ($\gamma_{SF}$), the substrate-liquid interface energy ($\gamma_{SF}$). Once the hole is formed, the tip-film and substrate-film interfaces are replaced by the corresponding interfaces with the liquid and become ($\gamma_{TF}$) and ($\gamma_{SF}$).

Now instead of taking into account the elastic relaxation energy of the two solid surfaces upon the hole formation, one has to calculate the elastic energy of the film. Because the elastic constants of tip and substrate are much higher than the elasticity of the film, under compression, more elastic energy is stored in the film than in the confining solids. To calculate the elastic energy of the film it must be taken into account the elastic foundation
model where the film is modeled by many springs that do not interact, i.e., shear between adjacent springs is ignored. The effective spring constant per unit area is $E/h$, with Young’s modulus $E$. As long as the process is elastic one has not to consider whether molecules tilt or are really compressed and the slight thinning is seen as a reduction of springs per unit area. Because would only mean a small correction it can be ignored and Young’s modulus can be interpreted as an effective value. Given this the indentation $\delta$ at a given radial position $r$ of the elastic foundation model is:

$$\delta = \delta_0 - \frac{r^2}{2R} \quad \text{for} \quad r \leq a = \sqrt{2\delta_0 R}. \quad \text{Eq. 6}$$

where $a$ is the peripheral radius of the contact area. The maximal indentation in the center $\delta_0$ and the applied force $F$ are related by the following equation:

$$\delta_0 = \sqrt{\frac{hF}{\pi ER}}. \quad \text{Eq. 7}$$

It is possible to calculate the elastic energy stored in a small section of the film of radius $r_h$, in case the considered area is much smaller than the contact area ($r_h \ll a$), this elastic energy is:

$$\pi r_h^2 \frac{h}{2} \frac{E}{h} \delta_0^2 = \pi r_h^2 \frac{F}{\pi R}. \quad \text{Eq. 8}$$

Once a hole of radius $r_h$ is formed, this energy is released and this is the reason of the minus sign in the first equation. Applying a force reduces the energy $U$ and once the force exceeds $2\pi RS$ the energy $U(r_h)$ shows a maximum at a certain critical radius $r_C$.

$$r_C = \frac{2\pi R \Gamma}{F - 2\pi RS}. \quad \text{Eq. 9}$$

The maximal energy, which is the activation energy, is:

$$\Delta U = U(r_C) = \frac{2\pi^2 \Gamma^2 R}{F - 2\pi RS}. \quad \text{Eq. 10}$$
The maximal energy thus decreases as the force is increased. Holes with a radius larger than $r_C$ have an increase in size, a film rupture, and a tip breakthrough.

### 3.1.6 Phase transition vs indentation force

It is now clear that the force spectroscopy experiments provide mechanical information at the nanometer scale, which is the relevant range for the mutual non-specific interactions between the proteins and the lipids hosting the protein. Even if the effects of many parameters on the force required for the tip/bilayer punch through have been studied, it should be useful to establish a connection between the obtained results for the thermodynamic properties of the lipid bilayer and mechanical ones. Moreover, it might be worth correlating and analyzing the macroscopic and microscopic properties in order to understand the functional properties of membranes. In this thesis it has been tried to connect the results achieved from AFM indentation experiments with thermodynamic properties of the lipid bilayer and to show that the phase transition region of the lipid bilayer represents a region of mechanical anomalous behavior which can have effects on the behavior of membrane proteins.

As a first step towards understanding the relationship between thermodynamic properties of lipid bilayer and the indentation experiments performed by AFM the behavior of the threshold force for punching through the lipid bilayer as a function of temperature has been studied. What has came out has been that the threshold force for penetration depends on the type of lipid molecules forming the bilayer. The study of a lipid phase transition in the presence of mixtures of phospholipids usually implies a redistribution of molecules in different domains. To avoid measurements of the punch-through force affected by a variation of lipid environment probes, it has been decided to measure the phase transition of a one component lipid bilayer. It has been chosen POPE as lipid component because its behavior has been well characterized in literature\textsuperscript{114} in the context of the main phase transition and because it represents one of the major components of bacterial lipid membranes.

Fig 40 reports the trends of the punch-through force in two experiments performed on a POPE supported lipid bilayer in distilled water. It has been demonstrated that in distilled water a supported lipid bilayer subjected to a temperature variation undergoes two
uncoupled phase transitions due to the independent behavior of the two leaflets composing the bilayer.

The uncoupled phase transitions configuration has been preferred due to the faster equilibration time of the system. During the measurements, the phase state of the lipid bilayer was always checked by performing AFM constant force imaging of the bilayer. The punch-through force outside the phase transition region increases while the temperature decreases.

![Image of phase transition behavior](image)

**Fig 40.** A schematic representation of the phase transition behavior of a POPE bilayer. It is possible to note how the indentation force (as consequence of the deflection variation) decreases as the bilayer reaches the melting temperature.

The punch-through event can be described as the overtaking of an activation energy which decreases on applying a force to the bilayer\(^{[127]}\). The event is so an activated process whose rate constant can be expressed by an Arrhenius formulation:

\[
k(t) = Ae^{-\Delta U(T)/kT}
\]

Eq. 11
k(t) is here expressed as a function of time, but can be also expressed as a function of force considering that during a force curve executed with a given speed the force increases with time. Usually, a linear increase with time is assumed. However, the indentation of the tip in the lipid bilayer leads to a non linear variation of the applied force with time. A is the pre-exponential factor which in the Arrhenius formalism is associated with the frequency of attempts to cross the barrier. In the theory of Butt et al.\cite{127} \Delta U is the activation energy for the punch-through process. Usually, in chemical reactions the activation energy is considered constant with respect to temperature. In the specific case of a lipid bilayer pressed by an AFM tip the height of the barrier depends on the temperature by different parameters, such as the number of molecules underneath the tip due to surface area variation of phospholipids and variation of the spring constant of each molecule due to conformational modification (trans-gauche conformation). As a result of these effects, the variation of k is not simply linear with temperature. The increase of the punch-through force while decreasing temperature is consistent with an increase of the surface density of lipids. This effect is added to the usual dependence of an activated process with temperature. While decreasing the temperature, a trend inversion in the punch-through force is observed. By imaging the lipid bilayer at the temperature corresponding to the trend inversion the appearance of new domains occurs. This appearance allows to relate the trend inversion of the punch-through force to the development of a phase transition in the bilayer.

It is possible to establish a connection between the softening of the lipid bilayer and the fluctuation in area which strongly increase in the phase transition region by considering the lipid bilayer a soft matter system, thus the lipid bilayer fluctuations and structure become strongly related. It has been demonstrated that fluctuations of many lipid parameters are related to the excess heat capacity of lipid bilayers\cite{128}. Macroscopic fluctuations become strongly enhanced in the phase transition region and their effects on the mechanical properties of the lipid bilayer are scale dependent.

Taking into account the continuum nucleation theory, it can be supposed that thermal fluctuations, in this case area fluctuations, might lead to the formation of a hole under the tip. The energy of the hole depends on some physical parameters of the bilayer that, in the specific case of a cantilever indenting the bilayer, is the force applied by the tip.

At the beginning of all the process, however, there is the spontaneous formation of area variation of the single lipids by fluctuations. It has been demonstrated that the conductance of a lipid bilayer in the absence of proteinaceous ion channels is strongly
dependent on the phase state of the bilayer. In particular, the conductance strongly increases in the phase transition region.

Another phenomenon which is usually observed in experiments performed on supported lipid bilayer is that the punch-through force increases with the ionic strength of the solution (fig 41). This effect is usually attributed to the interaction of ions in solution with the head group of the phospholipids. In fact, an increase of the ionic strength leads to a lateral compaction of the lipids and an increase of the interaction force between the lipids.

![Graph showing the dependence of indentation force on ionic strength](image.png)

*Fig 41. A schematic representation of the dependence of the indentation (as a consequence of the deflection variation) force upon increasing the ionic strength in a POPE lipid bilayer.*

### 3.1.7 Langmuir-Blodgett experiments and imaging

As can be argued by the force spectroscopy study on the POPE bilayer, one may say that there is a different behavior of the latter when the ionic strength is changed. To better understand the variation of the indentation force with the ionic strength we performed langmuir-blodgett experiments on POPE monolayers as a function of the ionic strength of the subphase.
We first characterized the isotherm of a POPE monolayer and we transferred on a mica substrate the monolayer at different pressures in order to study the morphology of the film. After depositing the mica piece onto an iron stub, it was analyzed by the AFM. The LC (liquid condensed) domains shape depends on the balance of the interaction forces among the polar heads dipoles and the line tension at the interface between different phase regions. In conditions far from critical points domains tend to assume a circular shape which minimizes the interface region. In fig 42 a typical isotherm behavior has been characterized, by acquiring images, to show the condition of the POPE monolayer during each step of the compression.

![Image of isotherm and AFM images](image)

**Fig 42.** Domains structure of a POPE monolayer in a distilled water subphase and at a temperature of 16°C. As described in the image analysis section these images have been elaborated by the free license software WSxM (Nanotec Electronica S.L. – Spain)

The isotherms allow doing observations and thermodynamic calculations. As the temperature increases also the transition pressure between $L_E$ (Liquid expanded) and $L_C$ (Liquid condensed) phases increases. Exploiting the isotherms one can calculate the, the compressibility of the sample.
The behavior of a POPE monolayer under different ionic concentrations has been studied (see fig 43) by performing different isotherms and, from each isotherm, it has been possible to calculate the compressibility (fig 44).

**Fig 43.** POPE isotherms performed with increasing ionic strength of the subphase.

**Fig 44.** Compressibility graphs of the previous isotherms.
From these results one could say that if with the indentation force it is possible to observe a different behavior of a POPE bilayer as the ionic strength increases, in the case of the compressibility no significant differences are observed. So it is reasonable thinking that when one performs force spectroscopy studies there could be other effects that may influence the obtained force values.

A next necessary step will be the study of all these properties by also using different lipid mixture such as POPE:POPG 3:1 and POPE:POPG 1:1.
In this thesis I studied some of the physical parameters that may influence the behavior of solid supported lipid bilayers as far as their main phase transition is concerned. The results point to a SLB model system as a structure in which the presence of the substrate can play a major role especially on the properties of the proximal leaflet. The SLB model system has been questioned because an independent and uncoupled behavior of the two leaflets has been observed for the main phase transition, at variance with what is observed for other lipid systems such as liposomes[129]. It has been demonstrated that it is possible to tune the preparation conditions of SLBs to reproduce a coupled behavior of the two leaflets for a mixed bilayer. This is accomplished by using electrolytes in the solution and by incubating the substrate with a solution at a temperature higher than that of the main phase transition of the lipids in liposomes. In the context of this study, for the SLBs, two interactions have to be considered: the interaction of the bilayer, mainly the proximal leaflet, with the support and the interleaflet coupling. The preparation temperature modifies the physical properties of one or both leaflets so that the interleaflet coupling is strengthened when the bilayer is prepared at high temperature. This may happen via a variation in the lipid density in the leaflets that may have a consequence on interdigitation possibilities and in the lateral tension. At the moment, it is not possible to state whether this is the same mechanism by which the same type of domain is observed in liposomes[129]. A general observation is that when transbilayer symmetric domains are observed, they tend to grow with a slow kinetics and nanometric scale domains are not stable—at variance with the case in which they are present only in one leaflet. However, in biological membranes, functional domains are on the nanoscale dimension[130]. This means that the study of supported lipid bilayers can have a biological relevance for understanding the behavior of membranes. Hence, the substrate might simulate the role that elements such as the cytoskeleton can have on the cell membrane. This includes both the compositional asymmetry and the reduced mobility of the lipids. Moreover, the possibility of tuning the behavior of SLBs opens the way to the study of membrane proteins in this model system. In particular, protein interactions with the lipids and their distribution relative to lateral heterogeneity of the lipid bilayer induced by temperature, pH, or other physical parameters, can be studied[131].
It has been also demonstrated that the substrate supporting a lipid bilayer can have a strong influence on the behavior of the bilayer. In particular, increasing the roughness of the substrate (in the case of silicon) reduces the interactions between the support and the membrane. The reduced interaction modifies the phase transition behavior of the SLB, making it more similar to a freely suspended bilayer, as confirmed by DSC measurements. The lower interaction makes the interleaflet coupling stronger, as deduced from the observed formation of domains in register between the two leaflets on silicon oxide independently of the incubation temperature. These results make the SLB on silicon oxide a more exploitable substrate to study protein/lipid interactions due to increased lipid mobility with respect to the mica support and lower probability for the membrane proteins to get stacked on the underlying solid surface. A pH difference shifts the transition temperature of SLBs as expected from DSC experiments. In contrast, if the pH is changed once the lipid bilayer is already formed, even if the bilayer has been assembled to undergo a coupled phase transition in register, the pH-induced phase transition involves only one leaflet. This could be the behavior also of a freely suspended bilayer when it is exposed to a transmembrane pH gradient. The biological relevance of this finding is connected to the possibility, experienced by many biological membranes, to be exposed to a pH gradient. The consequences of this fact could also be a loss of the interleaflet coupling in the lipid bilayer.

Membrane mechanics plays a pivotal role in many biological processes such as exo- and endocytosis and cell shape/volume regulations. In our force-volume experiments we tried to understand the nano mechanical properties of the lipid bilayer with respect to the temperature and the ionic strength.

The motions in membranes range from conformational transitions of the lipid hydrocarbon tails on picosecond scales to bending of 10-mm-sized patches extending to several milliseconds. The former region is accessible through spectroscopic methods\cite{132}, whereas the latter can be studied by microscopy\cite{133}. The understanding of how collective mesoscopic motion emerges from microscopic atomic interactions comes from the observation that biological membranes are not rigid, but behave more like liquid crystals. They exhibit a very high flexibility, which enables thermally excited undulatory and peristaltic (thickness fluctuation) motions\cite{134}.

As regard the nature of the main transition, it is still a topic of considerable controversy; It is generally assumed that the transition is of first order involving a considerable transition enthalpy\cite{135} although every experimental quantity measured varies continuously throughout the transition region. Based on careful equilibrium calorimetric measurements,
Biltonen (1990) has recently argued that the transition is effectively continuous (or weakly first order) and only a vanishing small amount of heat is involved. The effectively continuous character of the transition is reflected in the behavior of the thermal response functions, such as specific heat\textsuperscript{[136]} and lateral compressibility\textsuperscript{[137]}, which display apparent divergencies as the transition temperature is approached. These signals enhanced density fluctuations at length scales much larger than the intermolecular distances.

The biophysical motivation for studying the weak-first order nature of the main transition in lipid bilayers is formed by the observation that the accompanying density fluctuations may trigger also a number of membrane processes, such as passive permeation of e.g., ions\textsuperscript{[138,139]}, exchange of molecules between different membrane systems\textsuperscript{[140]}, and activity of certain membrane bound enzymes\textsuperscript{[141]}.

By using force spectroscopy on supported lipid bilayers, in this thesis it has been demonstrated that the mechanical properties of the lipid bilayer undergo a softening when the bilayer is in the phase transition region. This behavior might be relevant for the activity of membrane proteins. In fact, both the occupancy of the different protein conformations and the kinetics of the transitions might be strongly affected by a softening of the lipid bilayer in the phase transition region.

In the langmuir-blodgett studies it has been possible to demonstrate that the monolayer of POPE in millipore water, 10 mM KCl, 50 mM KCl and 150 mM KCl subphase had almost the same behavior with respect to the transition pressure; in fact all the isotherms had a transition pressure around 30 mN/m\textsuperscript{−1}. As regards the compressibility no significative differences, with respect to the different ionic strength, have been observed.

The fact that with the force spectroscopy technique it has been possible to observe a POPE behavior which changed as one moved to another technique, such as Langmuir-blodgett, would suggest that when one performs force spectroscopy studies there could be other parameters that may be relevant apart from the interaction of the lipid head groups as modified by the ions in solution.

A next necessary step will be the study of all these properties by also using different lipid mixture such as POPE:POPG 3:1 and POPE:POPG 1:1.
6 REFERENCES


[34] Cascales et al., (1996)


[133] Evans, (1991)


