Universitat de Barcelona

Facultat de Química

Departament de Química Física

STRUCTURE AND PHYSICOCHEMICAL PROPERTIES OF PHOSPHOLIPIDIC MONOLAYERS AND BILAYERS. LB AND AFM STUDIES

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TÍTOL DE LA TESI

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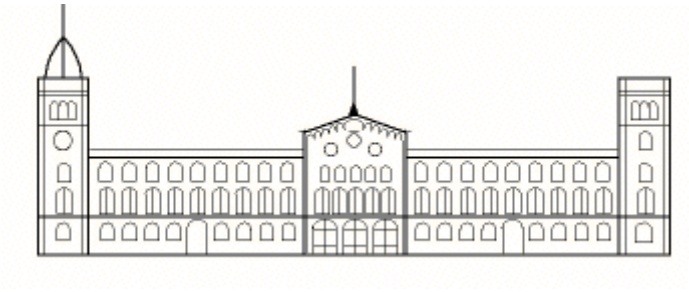
NOM DELS DIRECTORS: **FAUST SANZ CARRASCO**
JORDI HERNÁNDEZ BORRELL



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Memòria presentada pel llicenciat en Física per la Universitat de Barcelona **Òscar Domènech Cabrera** per optar al Grau de DOCTOR

El treball que es presenta ha estat supervistat i co-dirigit pel **Dr. Faust Sanz Carrasco**, catedràtic d'universitat del Departament de Química Física de la Facultat de Química, i pel **Dr. Jordi Hernández Borrell**, professor titular del Departament de Fisicoquímica de la Facultat de Farmàcia. Aquest treball ha estat realitzat en els laboratoris del Departament de Fisicoquímica de la Facultat de Farmàcia i en els Serveis Científico-Tècnics de la Universitat de Barcelona.

Vist i plau dels directors

Faust Sanz Carrasco **Jordi Hernández Borrell**

El doctorant

Òscar Domènech Cabrera

Amb pena per les oportunitats
i persones perdudes,
però amb esperança per les
coses bones que han d'arribar.

Contents

Outline

Presentation

One of the first principles of the Universe is the conservation of energy: *energy is neither created nor destroyed*. From photon absorption by atoms to the generation of ATP in a cell, energy is transformed continuously. If one atom does not absorb any photon from radiation field it remains in the ground state but if cells do not produce energy they die.

The ATP (*adenosine triphosphate*) is a molecule which is produced by incorporation of one inorganic phosphorous to an ADP (*adenosine diphosphate*) molecule during the process of oxidative phosphorylation. The chain of reactions leading to the eventual production of energy occurs in very specific suborganelles of the cell, e. g., mitochondrion or chloroplasts.

Mitochondria are organelles present in many eukaryotic cells. A very distinctive characteristic of these organelles is the "inner" membrane, which is, in physicochemical terms, completely different to the "outer" membrane. Cytochrome *c*, a peripheral membrane protein involved in the respiratory chain where ATP is generated, can be found in the inner bilayer. Whether *cyt c* is released from the bilayer ATP generation does not occur. This results in the death of the cell.

Due to the relevance of *cyt c* in the energy generation in the cell, it becomes relevant to get insight in the properties of the lipid environment where interacts. Moreover, this information may provide information which may lead to understand the release of *cyt c* during apoptosis and eventually to prevent cell death.

Personal motivation and evolution

When I decided to apply my knowledge in Physics to the Biophysics or Biology it was quite hard to me to work in a laboratory at the Faculty of Pharmacy. During my degree in Physics I did not use any biological material and my formation in these kinds of issues was nearly zero. At the beginning, this Ph.D. Thesis work was focussed to model membrane characterization using synthetic phospholipids. At that moment, year 2001, our group had a spectrofluorimeter where I began to form myself in fluorescence studies, especially with surface fluorescent probes, as ANS or Laudan® and Prodan®, interaction with liposomes. Then the group acquire a Nima® Langmuir Blodgett through where I mastered my monolayer training and improved my skills in Langmuir Blodgett films extraction. Parallel to monolayer experiments I was carrying out my experimental work of DEA, *" Nanomodification by anodic oxidation of a silicon (111) surface with the atomic force microscope"*, where I became an auto user of the atomic force microscope. After these inorganic studies I applied my new AFM knowledge to the soft matter. I began with the Langmuir Blodgett films characterization on mica and in air, but its biologic interest is restricted to very particular systems. SPBs formation was the natural evolution to characterize specific biological membranes. The visualization of SPBs under physiological conditions made the system more attractive. During the two last years of the Ph.D. my work was focused in the interactions of a small redox protein, *cyt c*, with the model membrane evaluated previously. Although some results were accomplished new experiments remain to be performed in the future.

Structure

When I begin to write my Thesis manuscript my directors suggest me that I could write it in English for a major scientific diffusion of my work, so I took their suggestion and transformed it into a personal challenge. I has not been innovative with its structure and it is structured as a typical Thesis. Chapter I is a wide Introduction where it is summarized the basis of the Langmuir Blodgett films, the biological membrane and the atomic force microscope as well as its applications. This chapter ends with a review of

Nanotechnology at the present in the world. In Chapter II, it is presented the Objectives that motivate this investigation. Chapter III contents the Material used during this work while Chapter IV is a compendium of the Experimental Methods used. Chapters V and VI are the Results and Discussion sections, respectively and Chapter VII is reserved for the Conclusions obtained from these discussions. In Chapter VIII are cited all references that appear in the text. At the end of the work there are several appendices with the symbols and abbreviations, my published articles, the submitted ones and an abstract of the Thesis in Catalan.

Chapter I. Introduction

I.1. LANGMUIR-BLODGETT FILMS

I.1.1. Short history of LB films

From antiquity, oil-water immiscibility has attracted the attention of men. One of the first scientific to write about the effects of oil on water was Pliny the Elder (23-79) in his encyclopedic work, Natural History: '*... sea water is made smooth by oil, and so divers sprinkle oil on their face because it calms the rough element…'*. The idea that oil could calm troubled waters became part of the folklore.

It was not until 1774 when the first documented experiment was performed by Benjamin Franklin (1706-1790). As he wrote at the *Philosophical Transactions of the Royal Society*, ' *... At length being at Clapman where there is, on the common, a large pond, which I observed to be one day very rough with the wind, I fetch out a cruet of oil, and drop a little of it on the water. I saw it spread itself with surprising swiftness upon the surface; but the effect of smoothing the waves was not produced; for I had applied it first on the leeward side of the pond, where the waves were largest, and the wind drove my oil back upon the shore. I then went to the windward side, where they began to form; and the oil, though not more than a teaspoonful, produced an instant calm over a space of several yards square, which spread amazingly, and extended itself gradually till it reached the less side, making all that a quarter of the pond, perhaps half an acre, as smooth as a looking glass… If a drop of oil is put on a polished marble table, or on a looking glass that lies horizontally; the drop remains in place, spreading very little. But when put on a water it spreads instantly many feet round, becoming so thin as to produce prismatic colors, for a considerable space, and beyond them so much thinner as to be invisible, except in its effect of smoothing the waves at a much greater distance'*. Franklin made some quantitative calculations. If one teaspoon (2 mL) was

spread on a half an acre of water, the resultant film width must be less than 2 nm. Although the experiment was quite important, for the very first time materials at the nanometric scale were measured, scientific community did not realize the relevance of his work.

One century later, in 1890, Franklin's experiment was resumed by Lord Rayleigh (John Williams Strutt) (1842-1919). From Rayleigh's experiments it was established that the film over the water would have the width of one molecule, between one or two nanometer. Although the results were published, few scientists were in agreement with him. Parallely to Lord Rayleight experiment, a German woman called Agnes Pockles (1862-1935) developed a rudimentary surface balance in her kitchen sink, which she used to determine surface contamination as a function of area of the surface for different oils. These experiments were published in *Nature* [Pockles, 1891; Pockles, 1892] and from ever since the quantitative investigation of fatty acids, alcohols and ethers monolayers began.

At the end of 1910's and beginning of 1920's Irwing Langmuir (1881-1957), an employed of the General Electric laboratories working on molecular monolayers, was the first to perform systematic studies of the interaction of different lipids with water [Langmuir, 1917]. From his experiments, Langmuir developed the *Theory of Surface Phenomena*, which in particular explains the behaviour of surfactant compounds at the surface of water subphase. These studies reached its highest recognition when he received the Nobel Prize award in Chemistry in 1932. In 1920 Langmuir reported the first transfer of fatty acid to a solid support. However, the first documented experiment of this kind of transference was performed by Katherine Blodgett (1898-1979) [Blodgett, 1935]. Since then, deposited monolayers of whatever thickness are known as *Langmuir-Blodgett films* (LB films).

In spite of the new perspectives that this technique introduced, scientists did not started to know its advantages near half a century after. The first international conference on LB films was held in 1979 and since then the use of the technique has been increasing widely among scientists working in different fields of research.

I.1.2. Surface tension

It is well known that when a gas is confined in a well defined volume tends to occupy all the available space. If in the same volume there are two different gases only separated by an impermeable wall, each one will occupy its half of the total volume. If the wall is removed and enough time is assured, the two gases will occupy the whole volume. This effect is not produced when we introduce a gas and a liquid in the same volume. In isothermal conditions and below the evaporation temperature of the liquid, the liquid will be in the bottom of the closed space while the gas will occupy the remaining volume. The gas will not occupy the whole volume due to the existence of a boundary or interface between the gas and the liquid. This surface is spontaneously created and can be understood as a consequence of balance between forces. Molecules in the bulk of the liquid feel similar attraction forces from all its neighbours (Figure 1) while the molecules at the interface undergo an unbalanced force directed to the liquid bulk.

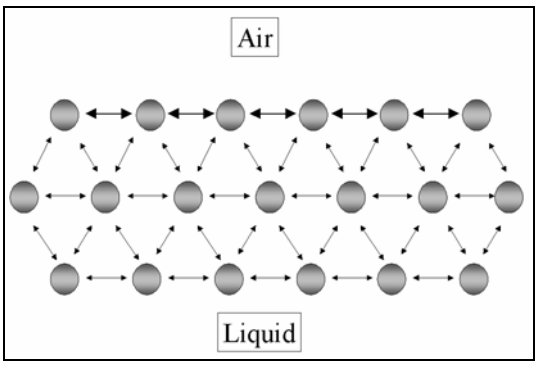


Figure 1. Force balances in the surface of a liquid.

This force produces that molecules on the surface feel more attractive forces between them than with the gas molecules that produces an excess of energy that favour the surface contraction. This energy is interpreted as the Gibbs energy needed to generate new surface being therefore an example of the useful work of a system with an interface. The energy per area unit to generate more surface is called surface tension and its symbol, according IUPAC recommendations is γ [Costa, 2004]. Formerly, the unit for γ is dynes·cm^{-1} that is normalized to the more convenient mN·m^{-1}, also according to IUPAC recommendations. γ values are always positive. Negative values of γ would indicate that it is more favourable the creation of new surface. Then new roughness

would appear at the surface and, with enough time, would disappear. At room temperature due to the Brownian movement, there is a surface roughness of 0.3 nm (2 molecular diameters) that not disappear because it is a continuous process.

Examples of γ values may be from 20 $mN \cdot m^{-1}$ for petroleum or 72-73 $mN \cdot m^{-1}$ for pure water at room temperature. These γ values are, of course, dependent of the temperature and the purity of the liquid.

I.1.3. Surface pressure

Agents that could reduce the surface tension can be considered, as a first approach, as an ideal gas in two dimensions. The surface pressure [Adamson, 1982] is defined as,

$$\pi = \gamma_0 - \gamma \qquad \text{(I)}$$

where γ and γ_0 are the surface tension in presence and absence, respectively, of the surface active agent. These molecules could be of different types, being mainly classified in inorganic particles (silicon oxide) and organic particles (surfactants).

I.1.4. Amphiphilic agents

Amphiphilic agents are molecules with two different regions differentiated by its hydrophobicity. One region is hydrophobic, formed by one or more hydrocarbon or fluorocarbon chains. The other is a hydrophilic region formed by a polar head. Normally this polar head is formed by OH, COOH, NH^{3+} groups.

Surface active agents (surfactants) are amphiphilic molecules that are able to diminish the surface tension. The surface tension decrease produced by surfactants is highly dependent on its structure. Some surfactants can form spontaneously structures at the interface of the liquid while others can be dissolved in the bulk phase without interaction with the interface.

I.1.5. Phospholipids

Phospholipids are amphiphilic molecules. Two hydrocarbons chains, acyl chains, form its hydrophobic region while a phosphate group, with one or more of the groups mentioned above, constitute the polar head.

Quantitatively, phospholipids are the most abundant lipids present in biological membranes. Phospholipids exert a double function: they form the boundary that keeps apart the cytoplasmic region of the cell from the environment and they are the matrix where proteins are embedded. A schematic structure of one phospholipid is represented in Figure 2.

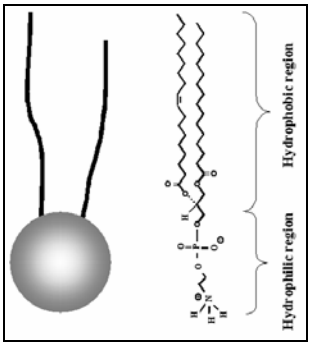


Figure 2. Phospholipid schematic representation.

Phospholipids, under specific conditions, can form a monomolecular layer, *monolayer*, at the interface between a liquid and air. In monolayers, polar heads are oriented towards the liquid with the hydrocarbon chains oriented to the air. In this position, phospholipids can keep its integrity due to its amphiphilic nature. Then, different phospholipids molecules, depending on its surface concentration, can interact with themselves. Surface pressure monitored as a function of the area occupied by each phospholipid molecule, under isothermal conditions, is named *isotherms*.

I.1.6. Isotherms of phospholipids

Phospholipid isotherms provide information on the physical state of the monolayer at a fixed temperature. Isotherms are obtained compressing laterally the monolayer at

constant velocity while the surface pressure is monitored. This process is depicted in Figure 3.

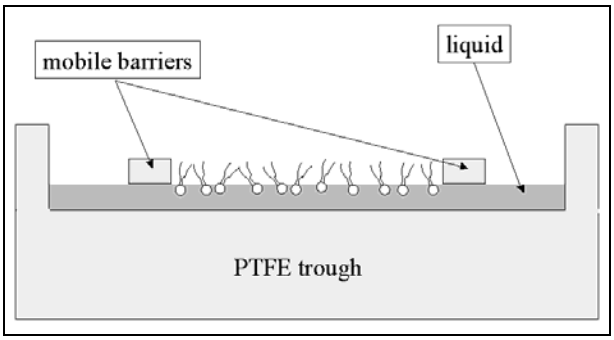


Figure 3. Schematic representation of phospholipids during an isotherm.

Two features of the isotherm are characteristic of each phospholipid, points for which the surface pressure is different to zero and the surface pressure of collapse. The area per molecule at low surface pressures strongly depends on the physical state of the molecule at the first stages of the experiment. Firstly, the phospholipids are apart from others and the thermal energy (k_BT) allows a random distribution of the hydrocarbon chains. If phospholipid molecules are compressed laterally, the specific area generated by the movement of the acyl chains promoted the interaction between the molecules (Figure 4). At this point the surface pressure is not zero anymore.

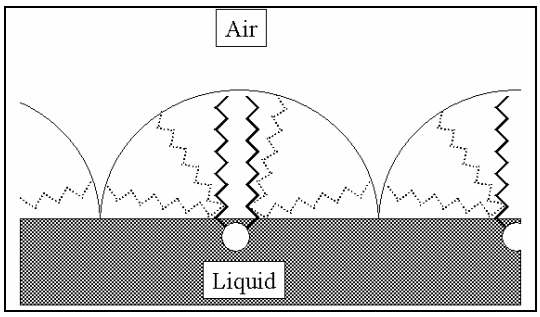


Figure 4. Interaction of specific areas of phospholipids.

The area per molecule decreases while lateral compression increases the surface pressure. This process will continue up to the collapse surface pressure (π_c). Surface pressure and area per molecule at the collapse are considered a fingerprint of the phospholipid. These values do not depend on the physical state of the molecule at the beginning of the experiment. At collapse surface pressure, phospholipids experiment a pressure too high and they can not stand in monomolecular form. The monolayer breaks

up and material can be pushed down to the bulk liquid phase or up to the air forming phospholipid multilayers.

To understand the effect of the collapse it is possible to achieve some quantitative analysis between the equivalence of pressure and surface pressure. If a surface pressure of **30 mN·m^{-1}** is applied to a monolayer with 10 cm in width and 3 nm in height, the lateral pressure is near **100 atmospheres**. So, at high surface pressure the monolayer collapses due to the excessive pressure exerted on the monolayer.

I.1.7. LB films extraction

In the previous section, the isothermal process of compression has been featured from zero up to the collapse pressure. If lateral compression is stopped at a surface pressure below the collapse and the surface pressure is hold up the monolayer can be transferred to a solid support. LB films extraction [Blodgett, 1935] depends on the nature of the support. Being hydrophobic, the support or substrate is immersed in the monolayer when the monolayer reach the surface pressure desired. If the support is hydrophilic, the support is immersed in the liquid phase before spreading the phospholipids on the surface. Then the monolayer is compressed to the desired surface pressure and the support is pulled out from the liquid phase keeping constant the surface pressure [Petty, 1996]. This last process is represented in Figure 5.

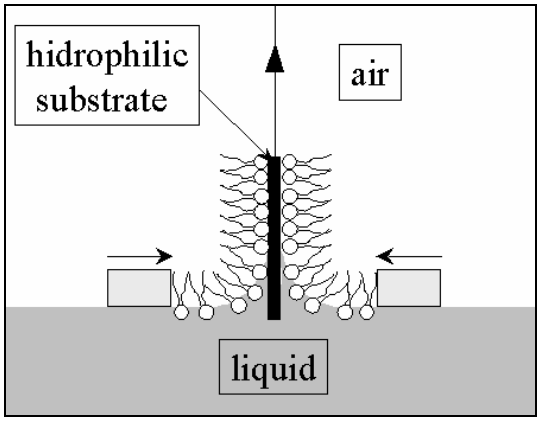


Figure 5. LB film extraction in a hydrophilic support.

I.1.8. Principle of Equivalent States

Many studies have been achieved to compare surface pressure in a phospholipid monolayer with lateral pressure in the phospholipid bilayer in liposomes [Thuren et al., 1986; Kontiila et al., 1988; Feng, 1999]. The idea that physical properties of monolayers at a surface pressure can be compared with bilayers is commonly know in the field as the *Principle of Equivalent States* [Peterson et al., 1992; Peterson and Kenn, 1994]. Nowadays, many researchers accept that physical properties of liposomes can be compared with monolayers at a surface pressure of 30 $mN \cdot m^{-1}$ [Marsh, 1996; Cevc and Marsh, 1987].

I.2. BIOLOGICAL MEMBRANES

In the previous section a model membrane of one dimension has been considered. Although this model is really understood and used to obtain thermodynamical properties of compounds there are few examples in the nature where the one dimensional model stands [Perez-Gil and Keough, 1998]. In any case three dimensional models, vesicles, are convenient to mimic the natural membrane structure.

I.2.1. Cell membrane

To carry out the chemical reactions necessaries to keep cells under physiological activity, both eukaryotic and prokaryotic cells need a specific internal medium. This medium is kept with the help of the cell membrane, which provides the boundaries with the external medium or environment. This membrane has, among others, basic functions as:

- ✓ isolate selectively the content of the cell from the external environment.
- ✓ regulate the exchange of substances between the interior and exterior of the cell.
- ✓ cell communication and possible fusion.
- ✓ keep constant the osmotic pressure.
- ✓ …

Some cells can present more than one membrane that keep apart inner cell regions. Each membrane has different composition to perform its function. Some examples are the membrane of different organelles as mitochondria, endoplasmic reticulum, nucleus, etc.

I.2.1.1.Membrane composition

The composition of natural membranes is very different among cells but all of them have a composition of around ~40 % in weight of lipids and another ~60 % in weight of proteins [Guidotti, 1972; Lotan and Nicholson 1981].

The major components in membranes, in molar fraction, are lipids and among them phospholipids are the most numerous. Phospholipids have a polar group (hydrophilic region) bound by a glycerol to two fatty acid tails (hydrophobic region). In Figure 6 a representative phospholipid model is shown. The polar group contains NH_3^+ and one acyl chain is straight (saturated) and the other presents a double bound (unsaturated). This kink, normally cys, influences the packing and the movement in the lateral plane of the membrane.

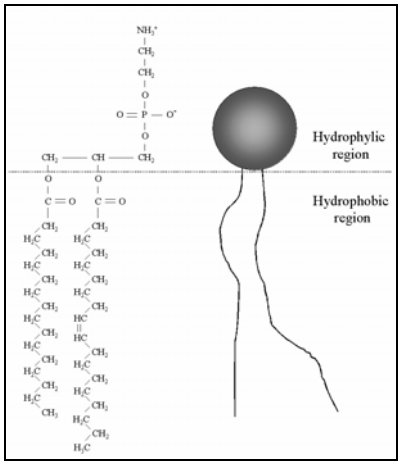


Figure 6. Schema of a phospholipid.

Length and saturation of the fatty acid tails and the difference in head groups can influence phospholipid packing in the membrane. Table I summarizes the lipid compositions of several biological membranes.

Table I. Approximate lipid compositions of different cell membranes [Alberts et al., 2002].

Lipid	Percentage of total lipid by weight					
	Liver cell plasma membrane	**Red blood cell plasma membrane**	**Myelin**	**Mitochondrion (inner and outer membranes)**	**Endoplasmic reticulum**	***E.coli* bacterium**
Cholesterol	17	23	22	3	6	0
PE	7	18	15	25	17	70
PS	4	7	9	2	5	Traces
PC	24	17	10	39	40	0
Sphingomyelin	19	18	8	0	5	0
Glycolipids	7	3	28	Traces	Traces	0
Others	22	13	8	21	27	30

The amphipathic nature of phospholipids, that means the balance between the hydrophobic (formed by the acyl chains) and the hydrophilic (the polar headgroups) regions, is the reason that they form bilayers and other aggregated structures in aqueous solutions. This is a consequence of the so called *hydrophobic effect* [Tandford, 1980].

Although membrane proteins are fewer in number than lipids, they are more specific and are responsible of the half of the weight of the membrane. Basically, membrane proteins can be classified in integral (intrinsic) and peripheral (extrinsic). Integral proteins penetrate the membrane and only can be removed by disruption of the membrane, such as dissolving it with detergents. Peripheral proteins do not penetrate the membrane. They are usually bound forming ionic or hydrophilic/hydrophobic links to membrane structures. They can be removed from the membrane by mild treatment, such as shaking with a dilute salt solution.

I.2.1.2. The phospholipid bilayer

Phospholipid nature and distribution in aqueous environment was not understood until Langmuir experiments [Langmuir, 1917]. By using the Langmuir technique, E. Gorter and F. Grendel performed an outstanding experiment where they used lipids of red blood cells dissolved with acetone. They were able to show that lipids could form a double monolayer and the surface area of the lipids extracted from the red blood cells

was about twice the surface area of the cells themselves. From these two conclusions they formulate the first accepted model for a biomembrane suggesting that red blood cells were covered by a layer of fatty substances that was two molecules thick [Gorter and Grendel, 1925]. This model did not take into account the existence of integral or peripheral proteins.

A more realistic membrane model accepted by the majority of scientists was proposed by Danielli and Davson in 1935 [Danielli and Davson, 1935]. In the model of Danielli lipids were arranged as in Gorter model and proteins were adsorbed on the bilayer making a ”sandwich of lipids”. However in this model membranes were static.

Membranes are not solid sheets that support proteins. Phospholipids and proteins are in a mobile fashion forming a fluidic mosaic [Singer and Nicholson, 1972]. This model, presently under close inspection by the scientific community [Engelman, 2005], retains the concepts of the previous models but suggests that the proteins float within the lipid bilayer. In this model pieces of the membrane can rip off without creating a hole because all the components in the membrane are in constant motion. So proteins are not anchored to the membrane.

I.2.1.3. Membrane fluidity

Dynamics and fluidity of the membrane is mainly due to temperature and phospholipid bilayer composition. As discussed in section I.2.1.1. phospholipid mobility can be influenced by length and saturation of the acyl chains. In general, phospholipid fluidity became higher with short and unsatured hydrocarbon chains while polar heads can modify membrane fluidity if they are able to establish some sort of bonds between neighbours. This is the case of phosphatidylethanolamine (PE) head groups that can form hydrogen bounds between them [Hauser et al., 1981]. Fluidity of membranes is well understood in models formed with synthetic phospholipids. A bilayer formed with only one type of these synthetic phospholipids presents a characteristic transition temperature (T_m). Above its T_m the bilayer is in a two-dimensional crystalline state (L_α) and below T_m they are in a gel state (L_β) [Houslay and Stanley, 1982]. In Figure 7 it is represented a schematic phospholipid bilayer where individual phospholipids are in

different states. In Figure 7A the phospholipids are in a two-dimensional crystalline state and hydrocarbon chains display high fluidity. In Figure 7B the same phospholipid bilayer is in a gel state. In this last figure phospholipid acyl chains are stiffer conferring a minor fluidity to the bilayer. It is also remarkable that bilayers in a gel state present usually a higher height than in a two-dimensional crystalline state.

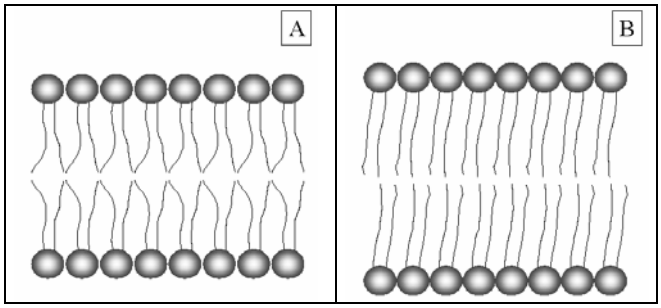


Figure 7. Phospholipid bilayer A) above and B) below its T_m.

Membrane fluidity should be seen as the average of the fluidity of different lipids that constitute the membrane modified by the presence of proteins that are embedded or adsorbed in it. At hostile temperatures the cell physiology manages to modify its membrane lipid composition synthesizing phospholipids with more or less cis-double bonds. It also can synthesize or erase proteins that can adjust the membrane fluidity.

Simplest membrane can be formed by a phospholipid mobile bilayer, with polar heads towards hydrophilic regions, which acts as the matrix where proteins are embedded (integral proteins) or adsorbed (peripheral proteins) (Figure 8).

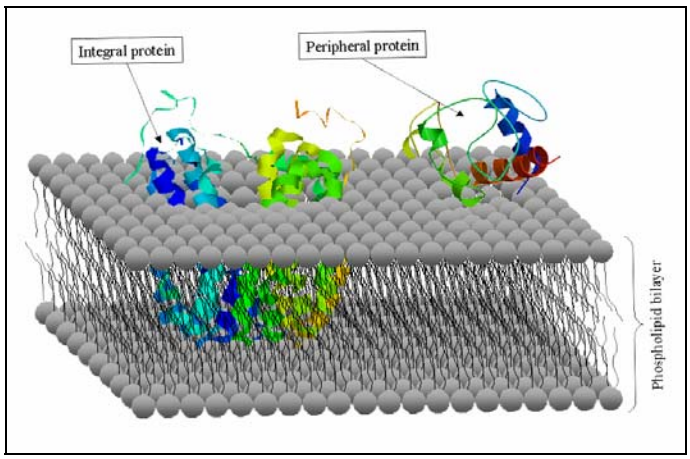


Figure 8. Schematic representation of a model membrane.

I.2.1.4. Phospholipid distribution

Usually phospholipid molecules are randomly mixed in the monolayer (*leaflet*) in which they reside. In some cases a phospholipid aggregation can be formed in the membrane, normally when phospholipids present long and unsaturated fatty acid tails. In these cases forces between adjacent molecules are strong enough to hold them together forming microdomains [Almeida et al., 2005; Binder et al., 2003]. This is the case of cholesterol and sphyngolipids that form the so called *lipid rafts*. Their physicochemical properties allow lipids to adapt to the irregular surface presented by the proteins [Alberts et al., 2003]. In Figure 9 we have depicted a cartoon where proteins are only embedded in one lipid microdomain.

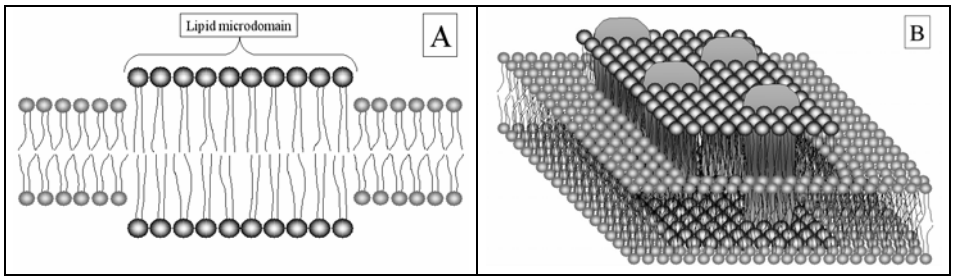


Figure 9. Lipid microdomain A) transversal section, B) 3D representation with proteins segregated in it.

Microdomains are relevant for protein organization in the membrane and have attracted the attention of many researchers. Related with that, many proteins need a particular lipid environment for their physiological activity [Naslavsky et al., 1997; Brown, 1998; Simons and Toomre, 2000]. These microdomains appear to be crucial for protein insertion and function.

Usually phospholipids of the two leaflets, inner and outer, are asymmetrically distributed (Figure 10): the outer leaflet is constituted mainly by phosphatidylcholine and sphyngomyelin while the inner membrane is formed by phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine (eukaryotic organelles) or phosphatidylglycerol (prokaryotic membranes) [Bretscher, 1973; Vance and Steenbergen, 2005]. It is remarkable that the net charged phospholipids in eukaryotic organelles are mainly located in the inner leaflet generating a difference in charge between the two leaflets. This contributes to the intermembrane difference potential.

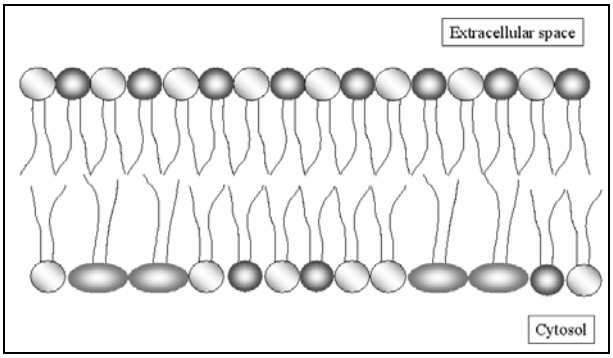


Figure 10. Asymmetrical distribution of phospholipids in the membrane.

This lipid asymmetry is used to distinguish between live or dead cells. Under physiological conditions cells do not present phosphatidylserine in the external leaflet, but when the apoptosis process begins (programmed cell death) PS is translocated from the inner to the outer leaflet [Alberts et al., 2003]. Surface membrane potential is then suddenly modified; the macrophages can not identify the cell and the phagocytosis starts. PS translocation in apoptotic cells can be carried out by two ways: inactivation of the phospholipid translocator that normally transports this lipid from the noncytosolic to the cytosolic monolayer or activation of a nonspecific translocator that transfers phospholipids nonspecifically in both directions between the two monolayers.

I.2.2. Model membranes

Biological membranes are quite complex due to the different type of lipids and proteins that constitute them and show too many degrees of freedom to characterize it. Model membranes are very useful to investigate the membrane because they allow the reduction and control of the number of variables.

I.2.2.1. Micelles

Phospholipids are amphiphilic molecules that form bilayers in aqueous solution spontaneously, but under certain specific conditions, low phospholipid concentration or some geometrical restrictions [Israelachvili, 1994], can self-assemble into other structures.

Micelles are one of these structures where the phospholipid polar heads enclose the phospholipid hydrophobic region. Micelles can form spherical and tubular structures. Figure 11 shows a phospholipid micelle and a phospholipid tubular micelle.

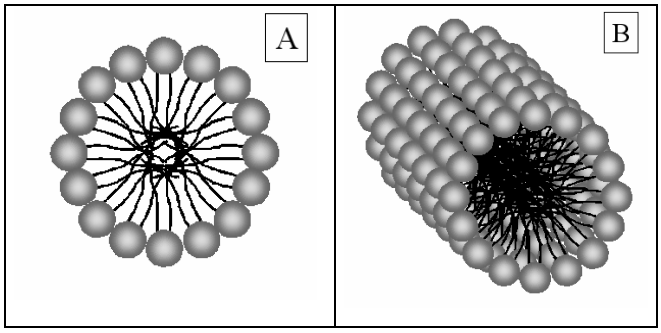


Figure 11. Representation of A) phospholipid micelle and B) phospholipid tubular micelle.

Phospholipids are arranged in micelles or bilayer below and above the critical micelle concentration (c.m.c.). This concentration is between one to eight micromolar of the phospholipid for PE and PC, respectively [Cristianes et al., 2000]. Usually phospholipid concentration used for practical purposes is greater than these values, so bilayer structures are favoured.

I.2.2.2. Hexagonal (H_{II}) phases

Another non bilayer structure that phospholipids can adopt is the hexagonal II (H_{II}) phase [Tate et al., 1991]. In this case phospholipid polar heads are close to others and hydrocarbon chains are facing the aqueous solution. In this structure phospholipids are arranged in cylinders in which the polar head groups of the phospholipid molecules surround a narrow aqueous channel [Cullis and Kruijff, 1979, Cullis et al., 1983]. Figure 12 shows a representative phospholipid H_{II} phase in two and three dimensions.

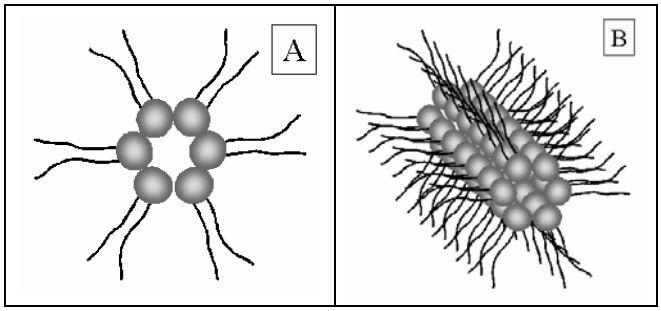


Figure 12. Phospholipid H_{II} structure A) 2D and B) 3D view.

H_{II} phases are formed by phospholipids with small headgroups, such as PEs, and unsaturated hydrocarbon chains that results in conically shaped structures that favours the negative surface curvature rather than the planar bilayer structure. This can be predicted from the value of the critical packing parameter for PEs (>1) [Cevc and Marsh, 1987]. H_{II} phases can be found in isolated phospholipids but to present, they have not been observed in biological membranes. It is however believed that they have a crucial role in membrane fusion and division [Epand, 1998]. One phospholipid that accomplishes this condition is the homo- (dioleyl) and heteroacid (palmitoyl-oleoyl) unsaturated phosphatidylethanolamine (PE) molecule under certain conditions of temperature [Epand and Bottega, 1988]. It is worth noting that phospholipids that usually form bilayers can turn into H_{II} phase in presence of divalent cations. Such is the case of pure cardiolipin (CL) in presence of calcium [Vasilenko et al., 1982].

I.2.2.3. Liposomes

Phospholipids form bilayers spontaneously in aqueous environment due to its amphiphilic nature. The most stable structure under these conditions, for lipids with critical packing parameters ~ 1, is a hollow spherical structure. Phospholipid polar heads are oriented towards the inner and the outer aqueous media protecting the hydrophobic region of the polar environment. The ideal liposome structure is represented in Figure 13.

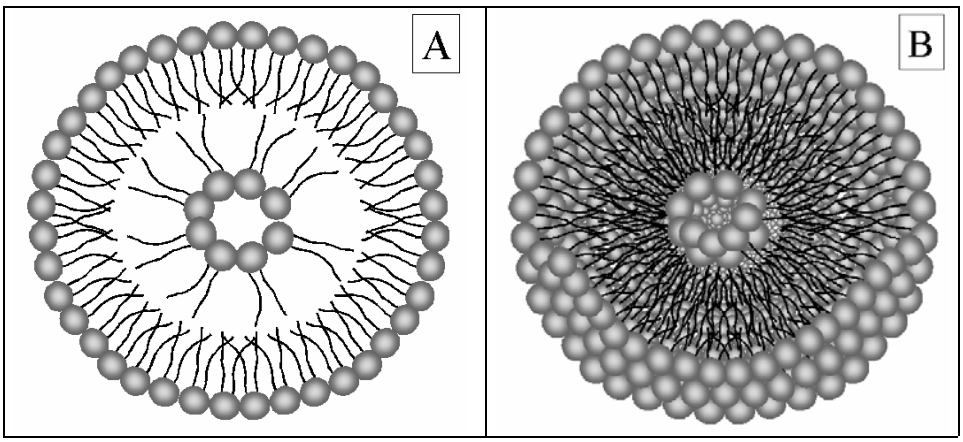


Figure 13. Phospholipid liposome A) section and B) 3D view.

Liposomes are classified according to IUPAC recommendations as:

- Multilamellar large vesicles (MLV). These liposomes are constituted by concentric spherical phospholipid bilayers with aqueous media between consecutive bilayers. MLVs are the liposomes formed spontaneously when a dry phospholipid film is hydrated. They have large dispersion in its diameter size from 400 nm to more than one micrometer.

- Large unilamellar vesicles (LUV). These liposomes are constituted by only one phospholipid bilayer with a large aqueous inner space. LUVs can be obtained by extruding them through special filters of desired diameter. Liposomes size depends on filter cut-off and properties of the phospholipid specie but usually they adopt diameters ranging from ~ 100 to 400 nm.

- Small unilamellar vesicles (SUV). These liposomes are constituted by only one phospholipid bilayer with a small aqueous space in its centre. SUVs can be obtained by sonication. They present diameters ranging from ~ 25 to 40 nm. This great radius curvature is the reason that SUVs present higher lateral surface pressure between molecules than the other types.

Table II summarizes the main physical parameters of the different kind of liposomes.

Table II. Representative parameters for different liposomes [Vance and Vance, 1996].

	Diameter (nm)	**Molar relationship IM/OM**	**Inner volume (μL/μmol)**	**Molecules per vesicle (x 10^3)**	**Vesicles per μmol of lipid (x 10^{11})**
SUV	25	0.46	0.3	4.8	1300
LUV	100	0.85	2.5	97	62
MLV	500	0.97	15.0	2600	2.3

Liposomes are the most popular model membranes used to investigate processes as drug delivery [Allen and Cullis, 2004; Merino et al., 2002], protein reconstitution into liposomes [Rigaud et al., 2003; Merino et al., 2005a], two dimensional protein crystallization [Hasler et al., 1998; Merino-Montero, et al., 2006] and others.

I.2.2.4. Supported Planar Bilayers

Supported planar bilayers (SPBs) have emerged in the last decade as one of the most suitable models to mimic lipid bilayers [Sackmann, 1996; Schneider et al., 2000; Groves and Dustin, 2003]. SPBs can be obtained in three ways:

- Black Lipid Bilayer: membrane is formed over an aperture and both sides are accessible [Benz et al., 1978; Henkart and Blumenthal, 1975]. This method was one of the first used to perform studies of protein-lipid interactions in lipid bilayers.

- Langmuir Blodgett films: Two consecutive monolayers can be extracted on a solid support to form a bilayer [Petty, 1996].

- Liposome extension: obtained by deposition of liposomes suspension on a flat support [Jass et al., 2000; Raviakine and Brisson, 2000]. With enough time they begin to fusion due to surface lipid coalescence forming a single bilayer structure on the solid support.

I.2.3. The mitochondrion

The mitochondrion (from Greek *mitos* thread + *khondrion* granule) is an organelle present in most eukaryotic cells. Each cell contains many mitochondria and they can occupy as 25 % of the volume of the cytoplasm. Many studies demonstrate that mitochondrion has an eubacterial origin [Cavalier-Smith, 1987, Martin and Muller, 1998] where the protomitochondrion established a symbiosis relationship with protoeuckaryotic cells. The most apparent consequence of the integration of mitochondria within the eukaryotic cell is the gross reduction in size and gene content of the eubacterial symbiont's genome [Lang et al., 1997]. In short, mitochondria have an own structure and function in the cell.

II.2.3.1. Mitochondrion structure and composition

At first sight the mitochondrion seems as a bacterium into the cytosol. Mitochondria are cylinder like structures with a mean diameter value of 0.5 to 1 μm. Each mitochondrion has two well differentiated membranes: the outer and the inner membranes separated by the intermembrane space. Inner membrane encloses a space called matrix where many enzymes and DNA are present. Inner membrane is folded forming cristae that penetrate into the matrix, so surface area of the inner membrane can be several times greater than the outer membrane surface area. Figure 14 shows a scheme of a mitochondrion.

Outer membrane. This membrane encloses all the organelle and presents a high proportion of membrane proteins called porins. Porins are unspecific aqueous channels that allow the transport of molecules of less than 5,000 daltons through the bilayer. Greater molecules only can reach the intermembrane space by active transport [Cramer and Knaff, 1991]. This bilayer presents a high proportion of zwitterionic phospholipids as PC and PE.

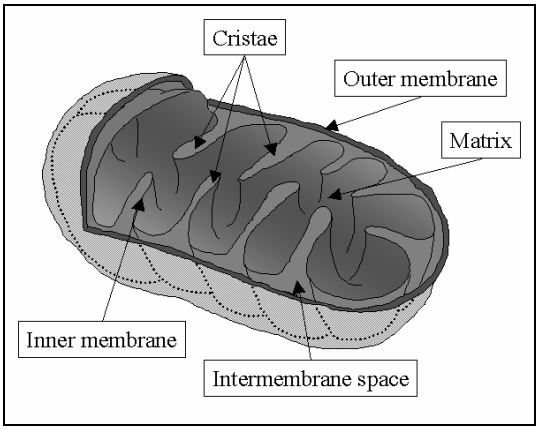


Figure 14. Mitochondrion structure.

Intermembrane space. It is the space that separates the outer and inner membranes. Its composition is chemically equivalent to the cytosol with some other molecules.

Inner membrane. This membrane is quite different from outer membrane. It is highly impermeable to ions and molecules require specific transporters to pass through the membrane. Inner membrane of the mitochondrion is highly specialized with proteins that can convert organic materials into energy as ATP. In this membrane we found the

enzymes that participate in the *oxidative phosphorylation* process that generates energy to the cell. To produce more energy, the inner membrane is folded into cristae conferring a larger surface area, so more enzymes can be embedded and more energy can be generated. Cells with great requirements of energy, as liver and heart cells, have longer cristae than other cells.

Inner membrane of the mitochondrion has a high proportion of the phospholipid CL. Bearing two negative charges, CL is indeed a double phospholipid, diphosphatidylglycerol, with multiple unsaturated hydrocarbon chains that confer a high hydrophobicity to the phospholipid bilayer. There are a lot of types of cardiolipins differentiated by their hydrocarbon chains. Table III summarizes the proportion of fatty acids that compound a commercial CL. It is illustrative to see that the major proportion of CL it is formed by two phosphatidylglycerols with hydrocarbon chains of 18 carbon atoms with 2 unsaturations in each one.

Table III. Fatty acid content of cardiolipin from heart tissue.

Fatty Acid Content of cardiolipin (%)					
16:0	**16:1**	**18:1**	**18:2**	**18:3**	**Others**
0.8	1.6	8.2	86.6	1.0	2.8

Matrix. The matrix is the space enclosed by the inner membrane where the mitochondrial DNA, ribosomes, tRNA and hundred of enzymes can be found. The major functions of these enzymes are the oxidation of pyruvate and fatty acids and the citric acid cycle [Alberts et al., 2003].

Table IV shows the differences in composition between the inner and the outer membrane of mitochondria for heart tissues. Notice that CL is only present in the inner membrane while PC is the main phospholipid head group that constitutes the outer membrane.

Table IV. Lipid composition of the inner and the outer mitochondrial membrane [Daum, 1985].

		Percentage of total phospholipids				
		CL	**PE**	**PC**	**PI**	**Others**
Heart	IM	25	38	27	3	7
	OM	< 1	28	56	9	6

I.2.3.2. Cytochrome *c*

Cytochrome *c* (*cyt c*) is a peripheral protein loosely attached to the inner mitochondrial membrane. It is an essential protein in the electron transfer chain. It is located between two intrinsic proteins, *cyt c* reductase and *cyt c* oxidase, transporting one electron at a time from *cyt c* reductase to *cyt c* oxidase [Alberts et al., 2003].

Cyt c is a heme protein with 104 amino acids with a molecular mass of 12,384 daltons. One *cyt c* sequence is shown in Figure 15 where 24 aminoacids presents positive charge while only 12 aminoacids presents negative charge. Furthermore *cyt c* presents an isoelectric point of 10 [Malmgren et al., 1978], so *cyt c* is mainly a positive charged protein under physiological conditions.

Gly	Asp	Val	Glu	Lys	Gly	Lys	Lys	Ile	Phe	Val	Gln	Lys	Cys	Ala	Gln	Cys	His	Thr	Val
Glu	Lys	Gly	Gly	Lys	His	Lys	Thr	Gly	Pro	Asn	Leu	His	Gly	Leu	Phe	Gly	Arg	Lys	Thr
Gly	Gln	Ala	Pro	Gly	Phe	Thr	Tyr	Thr	Asp	Ala	Asn	Lys	Asn	Lys	Gly	Ile	Thr	Trp	Lys
Glu	Glu	Thr	Leu	Met	Glu	Tyr	Leu	Glu	Asn	Pro	Lys	Lys	Tyr	Ile	Pro	Gly	Thr	Lys	Met
Ile	Phe	Ala	Gly	Ile	Lys	Lys	Lys	Thr	Glu	Arg	Glu	Asp	Leu	Ile	Ala	Tyr	Leu	Lys	Lys
Ala	Thr	Asn	Glu																

Figure 15. Horse heart *cyt c* amino acid sequence.

Cyt c presents a single heme group covalently attached to Cys^{14} and Cys^{17}. Figure 16 shows a three dimensional X-ray diffraction image of *cyt c* with a prosthetic group (protoporphyrin Ix containing Fe)[Bushnell et al., 1990]. This *cyt c* crystal presents a $P4_3$ space group with a resolution of 1.90 Å with parameters: *a:* 58.40 Å; *b:* 58.40 Å; *c:* 42.09 Å and angles $\alpha = \beta = \gamma = 90^{\circ}$.

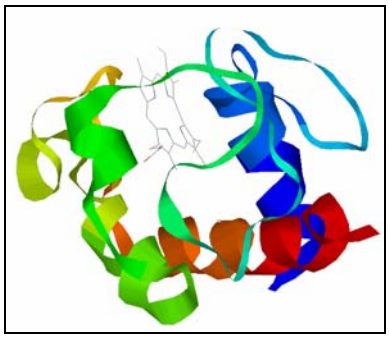


Figure 16. X-ray structure of horse heart *cyt c*.

Although *cyt c* is not crystallized in natural membranes, 3D crystal parameters can be taken as a first approach to the real *cyt c* conformation in the membrane.

I.2.4. Apoptosis

When cells are injured or not more needed in the organism they are destroyed by a tightly regulated cell suicide process known as programmed cell death or apoptosis. During organism development apoptosis is necessary for tissues and organs to acquire their unique structures and functions. In a healthy adult human billions of cells die in the bone marrow and intestine every hour and cells in the skin membrane between fingers in the human fetus undergo apoptosis to form them correctly. Apoptosis is the process that regulates the correct cell division. If a cell is damaged and can not undergo apoptosis it can originate a cancer if divides without restrictions.

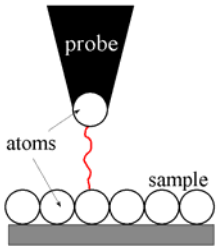
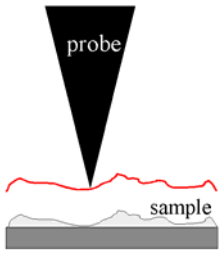
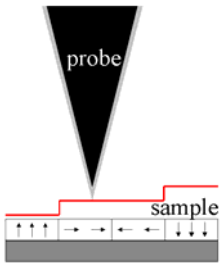
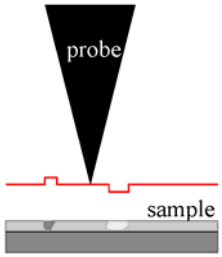
Apoptosis is understood as successive processes that causes the death of the cell. It is well known that the death of the cell is produced by a proteolytic caspase cascade but nowadays it is not well understood the mechanisms that originate this cascade. There are many works that focused the beginning of the cascade with the release of *cyt c* from the inner mitochondrial membrane [Jiang and Wang, 2000; Jiang and Wang, 2004; Garrido et al., 2006].

Although it is demonstrated that *cyt c* can begin the process that originates the caspase cascade it is not well known how *cyt c* can be released from the inner mitochondrial membrane. Some authors suggest that one protein of the BCL-2 family, Bid protein, can promote the formation of porous in membranes [Gross et al., 1999; Luo et al., 1998; Brustovetsky et al, 2003] through *cyt c* could be released from the inner membrane of the mitochondrion. But others authors suggest that an unusual concentration of calcium is the responsible of *cyt c* releases form the inner membrane of the mitochondrion [Andreyev and Fiskum, 1999; Brustovetsky et al, 2002; Mattson and Chan, 2003]. This Ca^{2+} concentration can activate the mitochondrial permeability transition (mPT) (permeability to solutes of molecular mass of ~ 1,500 daltons) and produce the swelling of the mitochondrion. Then *cyt c* can be released from the inner mitochondrial membrane through the porous.

I.3. SCANNING PROBE MICROSCOPIES

Scanning probe microscopies (SPM) are a wide group of microscopies where an ultra sharp probe scans line by line the surface of samples [Colton et al., 1997]. This probe measures the local interaction in the near-field region and registers the value of the interaction for each position. The analysis and composition of these lines enables the generation of a three dimensional image. Table V lists the most common SPM microscopies: scanning tunneling microscopy (STM), atomic force microscopy (AFM), magnetic force microscopy (MFM) and lateral force microscopy (LFM).

Table V. Comparison between the most SPM techniques used.

	STM	**AFM**	**MFM**	**LFM**
Sample	Conducting	Conducting / not conducting	Magnetic	Conducting / not conducting
Interaction	Tunneling current	Interatomic and intermolecular forces	Magnetic forces	Frictional forces
Measuring	Changes in surface electronic state density	Changes in surface topography	Changes in surface magnetic field	Changes in surface lateral forces
Image				
Detection	Sample-probe current	Probe/scanner displacement due to sample roughness	Sample-probe magnetic field difference	Changes in the probe tilt to scan the sample perpendicularly

I.3.1. Preceding

The beginning of SPMs was around 1982 when a short article appeared in the April issue of *Physics Today* [Binning and Roher, 1982]. This article was signed by Heinrich Rohrer and Gerd Binnig from the IBM's Zurich Research Center in Rüschlikon. Authors can managed to obtain traces of gold surfaces where the subtle rises and falls of the traces corresponded to atomic-scale steps and corrugations on the surface. Although results were sufficiently clear, Binnig and Rohrer were not quite ready to claim they were seeing atoms. These traces were the first images from a new instrument called the

scanning tunneling microscope (STM). Any uncertainties were dispelled when Binnig, Rohrer, Christoph Gerber, and Edmund Wiebel published a *Physical Review Letters* paper [Binning et al., 1982] that included an image showing a repeating pattern of the silicon 7-by-7 surface. Later Binning said, "it was a little too much to feel joy" while Quate said, "that 7-by-7 image of silicon was the revolution".

Revolution continued without stop:

- 1986. Rohrer and Binnig shared the Nobel Prize for their invention.
- 1986. Binning, Quate and Gerber created the atomic force microscope.
- 1987. Martin and co-workers introduced non-contact mode.
- 1989. Paul and Helen Hansma, of the University of California, reported the first AFM-made video of a biological polymerization process.
- 1990. Eigler and colleagues, at IBM Almaden, made headlines by manoeuvring 35 xenon atoms into Big Blue's famous company acronym atop a surface of crystalline nickel.
- 1992. Bustamante and co-workers, from the University of Oregon, showed routine and reproducible DNA molecules with AFM.
- 1993. Zohng and co-workers developed Tapping Mode®.
- 1994. P. Hansma and colleagues, from the University of California, were the first in use Tapping Mode® in liquids.
- 1995. D. J. Muller and co-workers, from Basel University, Switzerland, reported force-induced conformational changes in bacteriorodhopsin.
- …

I.3.2. Atomic Force Microscopy

From its invention in 1986 by Binning, Gerber and Quate [Binning et al., 1986], AFM has been one of the most usefully microscope technique to investigate at molecular and atomic resolution.

II.3.3. AFM applications

There are many AFM applications, from determination of the silicon atomic structure to the evaluation of the bacterial turgor pressure [Arnoldi et al, 2000]. All of them can be gathered in three main groups:

I.3.3.1. Inorganic applications

These applications were the first that AFM was used for. In the original paper [Binning et al., 1986], Binning and co-workers evaluated a ceramic (Al_2O_3) surface in air. After that, AFM was used to visualize inorganic surfaces that STM could not observe because they were not conducting, as is the case of ceramics or semiconductors [Albertch and Quate, 1987].

Due to the fact that AFM is able to evaluate small forces between the tip and the sample, it can visualize individual entities if the forces are in the short range [Giessibl et al., 2000].

But AFM is not only a technique of sample topography visualization. AFM tip can be used as a probe to manipulate the sample. One of the first experiments was performed scratching a layer of polymer with the tip [Majumdar et al., 1992]. Revolution began with the local anodic oxidation of surfaces [Day and Allee, 1993]. Local anodic oxidation can be performed in STM too [Dagata et al., 1990], but only on conducting or semiconducting samples. AFM enables the growth of oxides with lateral resolution of tens of nanometers and a thickness of about one nanometer in non conducting samples as Si_3N_4 [Chien et al., 2001]. These oxides could be used as masks for selective etching to transfer the pattern into the substrate [Snow and Campbell, 1994; Campbell and Snow, 1998] or to nanofabricate devices at molecular or atomic scale [Minne et al., 1995; Gwo, 2001].

Local oxidation of surfaces with AFM or STM could be the answer to the magnetic data storage problem. Nowadays, dominant method in microelectronics industry to storage data is the magnetic hard disk, but storage capacity of these devices will become

stagnant by technological limitations. Anodic oxidation with AFM has been demonstrate an areal density of 1.6 Tbits/in^2 (~ 250 Gbit/cm^2) [Cooper et al., 1999], or what is the same, ten times greater than the actually areal density performed with a fully integrated magnetic recording head and multi-layer antiferromagnetic coupled (AFC) disc.

I.3.3.2. Biological applications

Usually, organic molecules are nonconducting so they must be modified to work with them in EM, SEM, TEM, STM …, where the environment is quite different from the natural. One choice is the optical microscopy, but its resolution is nearly the micrometer. AFM can visualize samples in a biomimetic environment with higher resolutions and, in addition, when biological processes are in progress [Muller et al., 1996]. It is one of the most powerful tools to determine the surface topography of native biomolecules at subnanometer resolution [Muller et al., 1995b; Schabert et al., 1995].

Only two years after AFM invention the first paper with biological applications was published [Worcester, et al. 1988]. Since then, biological applications of AFM has been increased spectacularly:

DNA: First unquestionable image of DNA is attributed to Carlos Bustamante [Bustamante et al., 1992]. Before him A.L. Weisenhorn [Weisenhorn et al., 1990b] and H.G. Hansma [Hansma et al., 1991] had obtained DNA images but with poor reproducibility. This first evidence of DNA observation was the reference point for new experiments: DNA visualization under aqueous environment [Lyubchenko et al., 1993], force evaluation between complementary DNA strands [Lee et al., 1994], DNA degradation by enzyme DNAse I [Benzanilla et al., 1994], immobilization of DNA and RNA polymerase on the dish and *in situ* visualization of the transcription [Kasas et al., 1997], location of protein-DNA binding sites [Jett et al., 2000], etc.

Proteins: AFM is used to study proteins in two main ways: i) topographic surface protein visualization or ii) using the tip as a probe to obtain physic properties of single proteins. Visualization of proteins can be performed in its natural membrane as single or

aggregates. Single or aggregated protein visualization on flat solid substrates [Weisenhorn et al., 1990a; Hallett et al., 1995; Blackley et al., 2000] is usually the first step to protein characterization. Once protein is localized on the surface its structure and physic properties can be achieved by force spectroscopy pushing the tip down and then retracting while the protein is stretched up [Mueller et al., 1999; Viani et al., 1999; Muller et al., 2002]. Proteins can be visualized in it is natural membrane environment or reconstituted into model membranes. The most studied membrane is the "purple membrane" where the transmembrane protein bacteriorhodopsine is crystallized naturally [Muller et al., 1995b; Moller et al., 2000; Worcester et al, 1988]. Besides, proteins can be evaluated reconstituted into a lipidic matrix that mimic the biological membrane [Mou et al., 1995; Merino et al., 2005a; Merino et al., 2005b]. If the lipid to protein ratio (LPR) is the appropriate proteins can be crystallized in two dimensions [Jap et al., 1992; Lebeau et al., 2001] and high AFM resolution images of single proteins can be obtained [Muller et al., 1995a; Heymann et al., 1997; Merino-Montero et al., 2006]. The best images of membrane proteins have been acquired from 2D protein crystals, so they are the suitable arrangement to analyze protein structure and function.

Supported membranes: Supported membranes on solid supports can be achieved by using two different procedures: by the so called vesicle-fusion technique [Brian and McConnell, 1984] or by Langmuir-Blodgett technique [Blodgett, 1935].

With the LB technique lipid mono- or multilayers can be obtained, simply by single or multiple extractions on convenient supports. This technique, in combination with AFM, has enabled the visualization of individual phospholipid headgroups [Egger et al., 1990] as well as the coexistence of the liquid expanded (LE) and liquid condensed (LC) in monolayers [Yang et al., 1994; Zhai and Kleijn, 1997], phase separation [Dufrene et al., 1997; DeWolf et al., 1999], interaction between mixed lipid monolayers and glycolipids [Vié et al., 1998; Vogel et al., 1998], with cholesterol [Milhiet et al., 2001], surface properties of lipid-protein mixtures [Kernen et al., 1998; Cruz et al., 2004], etc.

Vesicle-fusion technique is based in the vesicle deposition onto a flat solid support and subsequently lipid extension and fusion to form supported planar bilayers (SPBs)

[Reviakine and Brisson, 2000]. This technique allows a reproducible method to obtain large bilayers extensions on the support. There are two main fields of studies where SPBs are used: those delineated to study the bilayer properties and those delineated to study the effect of molecules on the bilayer. In the first we can include studies of bilayer viscosity, spacing and thickness, surface physics properties, etc. [Butt et al., 2005] being the most relevant at this moment in time the observation and characterization of lipid domains formed by laterally phospholipids segregation [Tokumasu et al., 2003; Milhiet et al., 2003]. In the second field we can found studies on bilayer deformation due to drug incorporation [Merino et al., 2003; Berquand et al., 2004], resistant detergent bilayers [Rinia et al., 2001], degradation of lipid bilayers by phospholipase A_2 [Grandbois et al., 1998], etc.

Cells: In spite of cells are already observable with EM the AFM operational conditions, that is physiological environment conditions, allow the visualization of living cells. Some examples are diatoms [Crawford et al., 2001], surface topography of *Saccharomyces cerevisiae* [Ahimou et al., 2003], polymer adsorption onto mouth human cells [Patel et al., 2000], surface topography of *Escherichia coli* [Bolshakova et al., 2001], etc.

I.3.3.3. Combined applications

Nanotechnology is a field where biology can be present but requires of the physics principles to obtain measurable responses, so it can be though as a convergence of both worlds where physics and biology met. This is, for instance, the case of *biosensors*. A biosensor can be defined as a compact analytical device incorporating a biological or biologically-derived sensing element either integrated within or intimately associated with a physicochemical transducer.

A biosensor is a compendium of a biological detector that transmits a signal to a physicochemical device. This device transforms the biological signal to a usual measurable electrical signal. One of the most used electronic devices, as a part of a biosensor, is the Ion-Sensitive Field Effect Transistor (ISFET) [Volotovsky and Kim, 1998; Dzyadevich et al., 1999]. An example of biosensor is represented in Figure 17. If

only one protein is located in the gap the signal would be of individual protein translocation of its substrate. Substrate would modify the composition of the ISFET channel producing a differential signal between the anode and the cathode. This last signal would be the measurable signal that indicates the presence of protein substrate in the solution. Great signals could be achieved if more proteins are in the gap, but then the signal is the integration of all the individual substrate translocations.

AFM can be used to determine the distribution of biomolecules on the sensor surface [Hegner et al., 1993; Merino et al., 2005a]. In the previous example AFM can be used to determine the position of proteins in the lipid matrix, if they are uniformly distributed or prefer one lipid domain to be integrated in, etc.

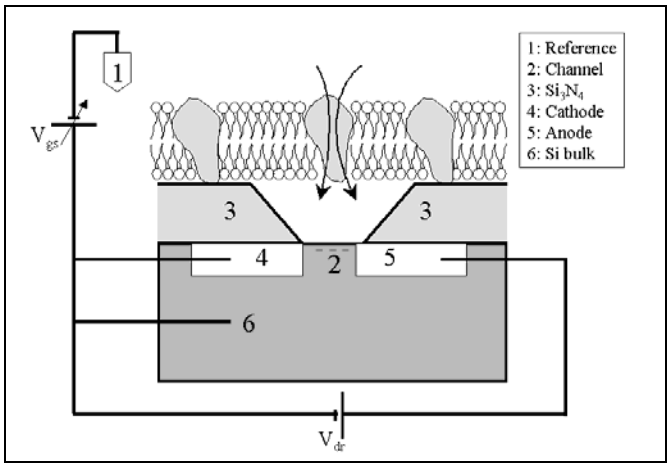


Figure 17. Schematic representation of a biosensor.

However, AFM it is not only used to visualize the organic part of the biosensor. AFM has evolved in a new technique in this field: the Scanning Capacitance Microscope (SCM). SCM is performed in an Atomic Force Microscope with an ultrahigh frequency resonant capacitance sensor connected to a grounded tip via a transmission line, which is attached to an UHF capacitance sensor. The tip sample capacitance and variations in it loads the end of the transmission line and changes the resonant frequency of the system. SCM has proven to be a quick and convenient method for direct imaging of submicron devices and a promising technique for two dimensional dopant profiling.

I.4. NANOTECHNOLOGY

Nanotechnology is technology in a nanometric scale. *Nano* prefix, small from the Greek, means the thousand millionth portion of the desired unit. So, one nanometer, *nm*, corresponds to 10^{-9} m. Then, one human hair has 200,000 nm in width, a human cell near 10,000 nm and one bacterium is close to 1,500 nm. The human immunodeficiency virus (HIV) has a mean diameter value of 100 nm, one large protein is around 10 nm, and a DNA chain has a width of 1 nm or 8 oxygen atoms in a single line.

Materials control at nanometric scale has become an interdisciplinary research in physics, chemistry, material science, engineering and biology. Nanotechnology can be defined as the research, development and processing of materials, devices and systems which structure must be lower than the 100 nm to an accurate performance. The first time the word nanotechnology appears was in the *International Conference on Production Engineering*, in 1974 in Tokyo, during the conference *'On the basic concept of Nanotechnology*' from Norio Taniguchi.

I.4.1. Nanotechnology in the world

Throughout last decade has existed a rapid increase of interest of the public investment for this field of investigation, going on from approximately 400 million € in year 1997 to more than 3 billion € at present. Although it is difficult to estimate exactly the deprived contribution to the activities of R&D in the field of the nanotechnologies, it had been expected that the contribution might approach 2 billion €. Therefore, there would be invested 5 billions € to the investigation in nanotechnologies during 2005 all over the world. But the public fund distributions are not homogenously distributed (Figure 18). The three major public investors in nanotechnologies are Japan, The United States of America and the European Community.

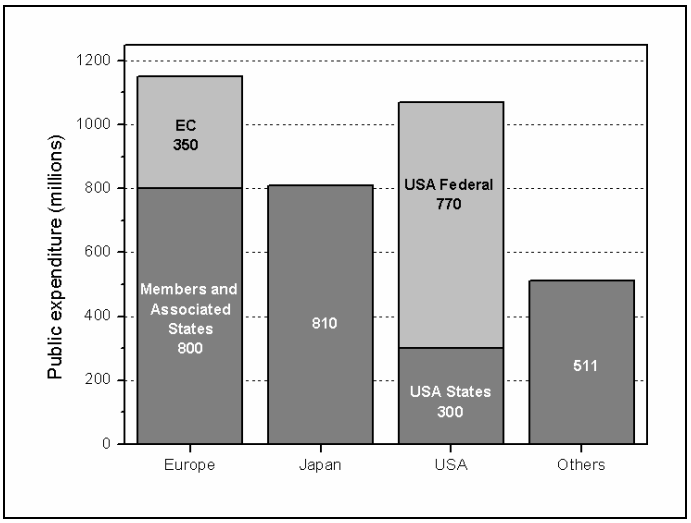


Figure 18. Global levels of the public expenditure in 2003 considering 1€=1$.

Whereas Japan was already considering priority the investigation in nanotechnologies in 2001 and the USA creates the *National Nanotechnology Initiative* (NNI) in 2000, Spain in its *Plan Nacional de Investigación Científica, Desarrollo e Inovación Tecnológica* from 2000-2003 considered '*The field of the nanotechnology is at present in the state of 'incipient emergency' directed towards to the biotechnology and at laboratory level, with the corresponding exceptions. The nanotechnologies, even considered with big interest, should be of lower priority than microtechnology.*'

But not all the whole budget comes from the public administration. In 2004 the American enterprises invested $1,700 millions (46%), Asians near $1,400 millions (36%), Europeans contributed with $650 millions (17%), and enterprises of other regions contributed only with $40 million (< 1%).

Figure 19 shows the public contribution per capita of different countries where Spain is in a precarious position. Unfortunately, Spain is one of the developed economies that destine less resource to activities of research, development and innovation (we dedicate 1.03 % of our Gross Domestic Product whereas Europe dedicates 1.99 %).

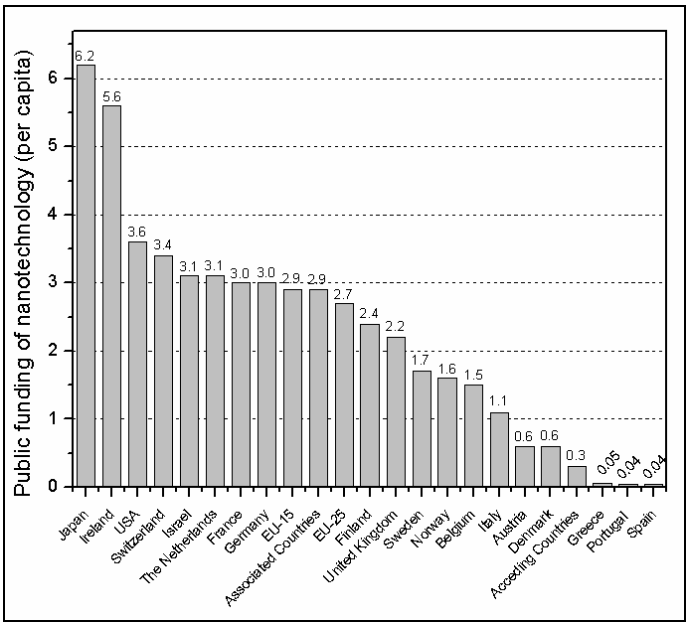


Figure 19. Comparison of the levels of financing in nanotechnologies between different countries on a base per capita of 2003.

I.4.2. Nanotechnology applications

Quoting K.E. Drexler, creator of the theory of the nanotechnology: '… nanotechnology will allow the bottom-up manufacturing' [Drexler, 1986]. So macroscopic devices could be produced atom by atom. Defects and dislocation could be erased from the beginning because the atoms will be in specific positions. Perfect alloys could be performed as well as monocrystals.

Nanoworld cannot be understood by a single science, but a compendium of them. So applications are interdisciplinary and can contribute to solve many of the problems facing today's society. Some of them are summarized below.

I.4.2.1. Computational applications

Data storage can be improved with very high recording densities (e.g. 1 Terabit/inch^2). Another huge field of investigation is the chips manufacturing. The fabrication of nanotransistors will produce more rapid processes of information. If the second law of Moore is taken into account: '*the number of transistors that fit in a chip doubles every 18 months'*. Some experts announce that in 10 years the capacity of the

computers will stagnate due to the type of manufacture of current chips, a *top-down* manufacturing. It is believed that the minimal size of a chip will be 70 nm in the year 2010. If chips would be manufactured with a *bottom-up* technology the transistors would be autoassembled molecules and the molecular computer could become reality.

I.4.2.2. Medical applications

Early diagnosis of illness could be realized by miniaturized diagnosis. New medical instrumentation and materials used in medicine will improve biocompatibility and bioactivity of implants as well as diminish the sequels produced in operation, e.g. disappearance of scars by nanosutures. Self-organizing scaffolds pave the way for new generation of tissue engineering and biomimetic materials, with the long-term potential of synthesizing organ replacement.

I.4.2.3. Security applications

Detection of biological or chemical agents with a high specificity will provide an early warning against them. Protection of property, such false documentation or banknotes, could be achieved by nano-tagging.

I.4.2.4. Science materials applications

Development of new materials, e.g. scratchproof or unwettable materials, could improve the performance of machines in extreme conditions. Selective grafting of organic molecules through surface nanostructuring is expected to impact upon the fabrication of biosensors and molecular electronic devices. Advances in material science will allow the fabrication of engines at molecular scale. These engines will be the mainstays of the industry of the future. They will be the scalpel of the doctors in the erasing of tumours as well as in neurosurgery with minimum impact to the patient during operations. Engines will repair tissues at molecular scale and close injuries from inside. In the future, molecular engines will be able to repair the DNA molecules increasing the live expectancy.

I.4.3. Present and future perspectives

Actually, some new products have been marketed. Among others, electric components, scratch-free paint, heart valves and bandages, sports components, sun creams and stain resistant fabrics. Analysts estimate that the market for such products is currently around 2.5 billion € but could rise to hundreds of billions € by 2010 and one trillion thereafter[†].

As summary, Figure 20 shows the evolution of the dimensional work scale as a function of the time, from 40's to a near future.

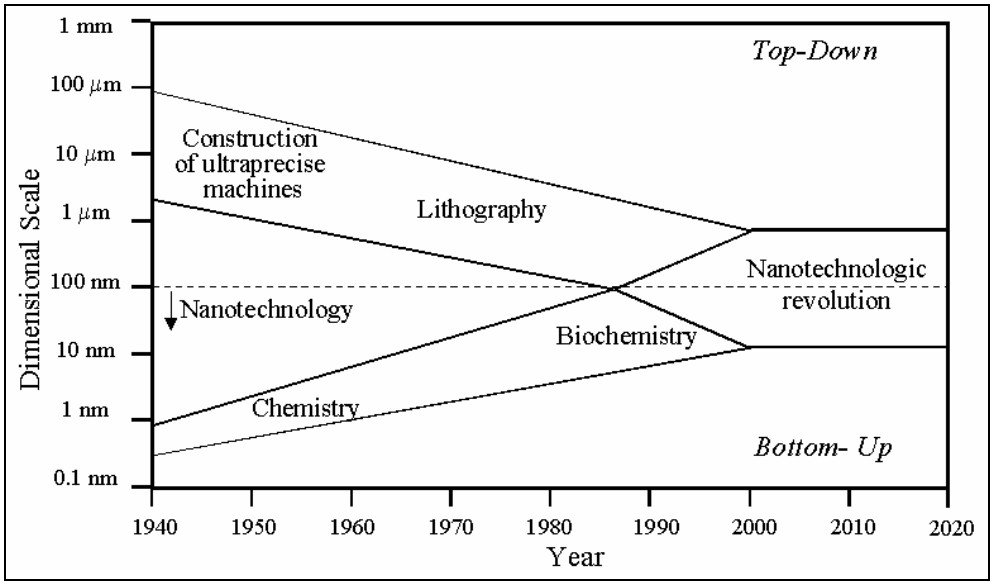


Figure 20. Evolution of the dimensional work scale of different science fields.

Component miniaturization allows the construction of ultraprecise machines from 50's. Afterwards, the electron-beam lithography allowed the modification of structures bellow micrometer reducing the size of the performed objects. In a parallel way, chemistry began to understand the laws that govern the individual molecular association up to coming to biochemistry who study the autoassembling of complex functional units. Both work philosophies, the bottom-up and the top-down, will converge in a close future and will be able to understand the macroscopic world with the concepts established in the nanoworld with models and mathematic simulations.

[†] From "New Dimensions for Manufacturing: A UK Strategy for Nanotechnology" DTI (2002) page 24.