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PII: S0005-2736(20)30355-2

DOI: https://doi.org/10.1016/j.bbamem.2020.183512

Reference: BBAMEM 183512

To appear in: *BBA - Biomembranes*

Received date: 7 September 2020

Revised date: 11 November 2020

Accepted date: 12 November 2020

Please cite this article as: M.A. Frias and E.A. Disalvo, Breakdown of classical paradigms in relation to membrane structure and functions, *BBA - Biomembranes* (2018), https://doi.org/10.1016/j.bbamem.2020.183512

BREAKDOWN OF CLASSICAL PARADIGMS IN RELATION TO MEMBRANE STRUCTURE AND FUNCTIONS.

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Summary: 18755 words, 10 figures and 2 tables.

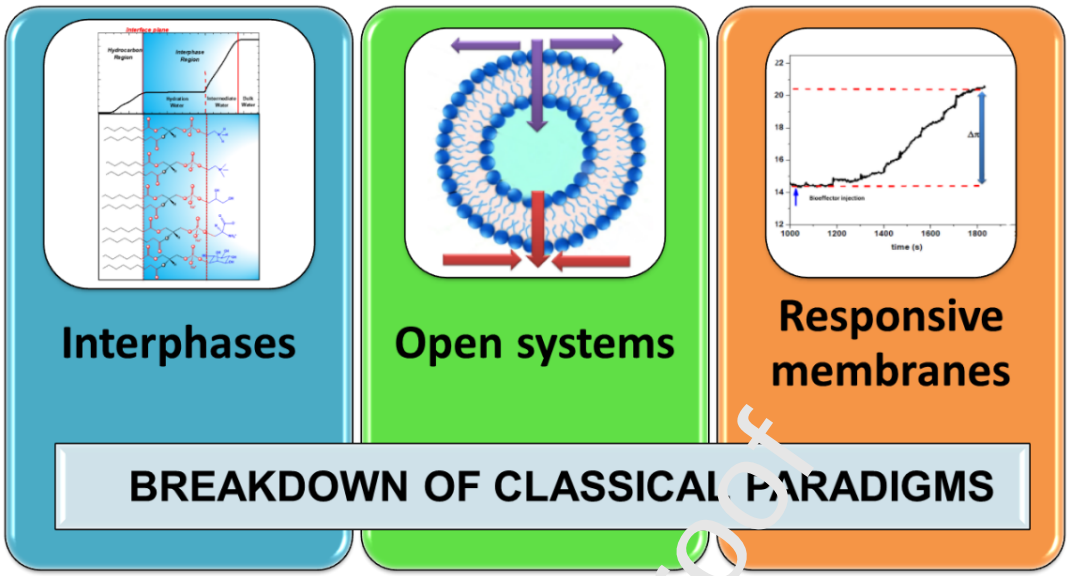
Abstract:
Updates of the mosaic fluid membrane model implicitly sustain the paradigms that bilayers are closed systems conserving a state of fluidity and behaving as a dielectric slab. All of them are a consequence of disregarding water as part of the membrane structure and its essential role in the thermodynamics and kinetics of membrane response to bioeffectors.
A correlation of the thermodynamic properties with the structural features of water makes possible to introduce the lipid membrane as a responsive structure due to the relaxation of water rearrangements in the kinetics of bioeffectors´ interactions.
This analysis concludes that the lipid membranes are open systems and, according to thermodynamic of irreversible formalism, bilayers and monolayers can be reasonable compared under controlled conditions. The inclusion of water in the complex structure makes feasible to reconsider the concept of dielectric slab and fluidity.

Keywords: model membranes, lipid interphases, water in membranes, thermodynamic response, relaxation processes.

Highlights:

- Membrane as a complex system includes water.
- Bilayers and monolayers are equivalent under thermodynamic grounds.
- Membrane is a responsive material.
- Membrane response derives from rearrangement of water organization.

Graphical Abstract



Abbreviations: PC: phosphatidylcholine; DMPC: 1,2-dimiristoyl-*sn*-glycero-3-phosphocholine; Laurdan: 6-dodecanoyl-2-dimethyl aminonaphthalene; Tc: Transition temperature; GP: Generalized Polarization, SA: stearyl amine

Acknowledgements: This work was funded by grants from ANPCyT (PICT 2015-1111), CONICET (PIP 2016 010) and UNSE (23/A227). EAD and MAF are members of the permanent research career of CONICET. The authors are grateful to Dr. L.G. Mohtar, M.F. Martini and A. Hollmann for the critical reading of the manuscript and to L. Frias for his technical support in the design of the figures.

Contents

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1. Cell membranes and bilayer structure in the context of cell behaviour.

Cells are complex systems in regard to function and crowded ones in regard to structure. Complexity is understood in the sense that the constituents are interconnected and interdependency, cooperativity and synergism are properties inherent to the functionality and response of the whole system to the environmental conditions. Crowding means that cell components are in close contact with each other in an organized way in a constrained media.

Most probably, complexity and crowding are interrelated concepts because the interdependent connexion is likely produced by signals between the components and this, in terms of efficiency (the cellular economicity in biological definition and maximal work in thermodynamic terms) implies close contacts. In this regard, here is where interfacial properties of the macromolecular and supramolecular structures take relevance much more when such structures are stabilized in water [1-5]. A number of reviews of both crowding and confinement effects have appeared during the last years [6].

In consequence, cells are not an aqueous concentrated broth of macromolecules floating in a bag closed by the membrane but rather they keep a specific location in the whole matrix. In this context, the visualization of the membrane as a physical barrier containing an aqueous dispersion is questionable and instead it must be reconsidered as a *structural integral part of the whole cell complex*.

One of the central studies in cell biology is related to the structure and function of cell membranes as an independent suprastructure. In this view, the membrane is visualized as an autonomous entity with a selective permeability that regulates the exchange of matter between two aqueous media, one in the exterior and other in the interior of the cell. Special systems were postulated to explain the passage of water and specific molecules such as amino acids, sugars and other biologically essential compounds. Following this line of thoughts, the membrane is conceived as an

impermeable support in which specific proteinaceous components are responsible of the exchange of matter. These were described as pores, facilitated transport or active transport [7].

To give sustain to the multiplicity of functions and considering the richness and variety of components, several cell membranes models have been proposed to explain experimental facts. Among them, the Singer and Nicholson fluid mosaic membrane model (SNFMMM) has been accepted as the more versatile and on this base several improvements or modifications have been extensively discussed [8-13]. However, all of them rest upon the implicit paradigm that the membrane is a closed and autonomous supramolecular structure and therefore membrane is taken as an independent system obeying classical laws of homogeneous and large phases such as Van´t Hoff for diluted solutions and Henry law for solubility properties. This view has been favoured by the investigations carried out in experimental models that are formed spontaneously when isolated lipids are dispersed in water due to its amphipathic character. These aggregates are considered as biomimetic systems of cell membranes due to the facility to study its physical-chemical properties with controlled composition [14-17]. In this regard, monolayers and bilayers have been used alternatively to study different processes. Nonetheless, the comparison of the results and equivalence between the two systems have been a matter of debate. Thus, controversial proposals have emerged due to the fact that it is not clear in what conditions bilayers and monolayers are in the same thermodynamic state in order to make the comparison of a given phenomenon in the two systems feasible [18, 19].

It is not the purpose of this review to describe the chronology of the membrane models that have appeared in literature in the last 40 years. Excellent reviews have been published in that sense, trying to improve or validate the Singer and Nicholson model [9, 13, 20]. However, in order to point out and put into relevance the current paradigms under which this model and its modifications lie, a short summary is presented. Once this is clear, a rebuttal of the classical paradigms and its consequences on membrane behaviour will be presented and new ones will be proposed.

2.-The SNFMMM and its modifications.

The mosaic fluid model proposed by Singer and Nicholson has been taken as a paradigmatic representation of the membrane considering the lipid bilayer as the backbone where integral and peripheral proteins are inserted. All models and its modification admit that the backbone structure of cell membranes is the lipid bilayer mainly constituted by phospholipids that expose the hydrophilic head group to water and exclude the hydrophobic acyl chains from water in the bilayer interior.

The central ideas of the SNFMMM reformulations are based on the introduction of heterogeneities in the membrane plane due to the coexistence of different kinds of lipids; the ability of some components to stabilize in its pure form as non bilayered structures; the presence of packing defects between the lipids due to curvature, domains, nanodomains, described with different names (rafts, pockets, protrusions, etc.); the inclusion of high levels of proteins and considerations of dynamics and fluctuations [9, 21-27].

In all of them, the bilayer is considered as the thermodynamic stable phase and its reactivity to exogenous bioeffectors (defined as protein or peptide insertion or any solute in the adjacent media that adsorbs, inserts, penetrates or permeates the membrane at different levels) is judged by the appearance of a final end product in which lipids fit with each other according to its geometrical shape (cylinder, conical, inverted conical) that gives the sufficient flexibility to adapt to the peptide or to the protein. For instance, the possible formation of non-bilayer structures is based on the presence of conic shaped of the pure dry lipid such as phosphatidilethanolamines, which is extrapolated to be maintained in the hydrated lipid, in a mixture with other lipids and in the presence of proteins [16, 21].

The concurrence of new microscopic methodologies (fluorescence, AFM among others) has allowed to visualize the plane of the membrane as composed by complex clusters of different types of lipids that organize laterally. These formations are described by rafts or domains in a mesoscopic dimension. A large discussion on the functionality of these domains or rafts has appeared in the last years [28-33].

The models for membrane-bioeffector interaction described with geometrical criteria are not predictive. It is not clear at present if the properties conferred to the membrane are due to the heterogeneities themselves or to the contact between them [34].

The observation of lateral heterogeneities suggests a separation between ordered and disordered regions [35]. This has been ascribed to specific intermolecular

interactions and lattice deformation in the membrane plane. Moreover, lipids and proteins exhibit interactions associated with a hydrophobic matching condition that can lead to elastic distortions of the membrane matrix. This type of phenomenon, in turn, gives rise to tensions between lipids and proteins, resulting in clustering of specific lipid molecules around a protein or lipid-mediated protein–protein interactions [36-38].

It is interesting to point out that membrane lateral heterogeneities, taken as structural receptors of bioeffectors, allow to introduce a new classification of compounds named linefactants, that are able to insert in the boundary separating two regions in the lipid monolayer [39, 40]. In previous papers, this type of compounds was named as “molecular harpoons” to explain its insertion to the membrane and disrupt it, in a mechanism closed to detergency [41, 42]. For example, lysophosphatidylcholine (lyso PC) is described as a conic molecule and hence its lysolitic action could be explained because its conical shape fits into a conical bilayer defect [43]. However, the effectivity of the lyso compound depends on the membrane hydration state because its action increases when a hypertonic stress is applied to the membrane. [44, 45]. A molecular view of the heterogeneities produced by osmosis (defect) was provided by analysing the hydration state of the carbonyl groups (CO) by Fourier transformed infrared spectroscopy (FTIR) [46].

These results put into relevance that the action of an external compound depends not only on its geometrical shape, but essentially on the hydration membrane state. In particular, hydration plays a relevant role in the interfacial free energy, either global due to phase state or locally by the presence of packing defects.

That is, the efficiency of the action is determined by the energetic state of the membrane surface at the moment the process initiates. This particular state has been referred to as the propensity of the membrane to evolve to some particular state or structure [47]. This propensity is just a surface free energy profile that should be redefined in terms of a membrane model that is beyond the current paradigms such as closed and autonomous phase. Thus, the membrane is responsive according to the conditions at which it is subjected that determines its thermodynamic potential to react. Under this view, few details are available at microscopic level in regard to packing defects generated by lateral contact of different lipids; protrusions from the membrane plane; local, spontaneous or induced

curvatures or reorientations of molecular residues described macroscopically as clusters, rafts and domains.

Although it is recognized that biological membranes are not mere walls, they are described as the milieu in which "important events in the physiology and pathology of the cell" takes place (sic. Goñi [9]). Implicitly, the membrane is taken as a support of the important events but not as an integral part of the functionality. However, lipids themselves have a role much more relevant than a mere support of proteins [48, 49].

The amphiphilic nature of phospholipids plays a key role in the formation of aggregates in water. All models and its modifications admit that the bilayer array is the backbone structure of cell membranes. The phospholipids expose the hydrophilic head group to water and exclude the hydrophobic acyl chains to the bilayer interior and pack laterally according to its geometrical shape. In particular, the bilayer is formed by phosphatidylcholine (PC) which is described as a molecule with a cylindrical shape [16]. However, a point of much more importance but less emphasized in the analysis of the physical chemical properties of lipid membranes is that the geometrical shape is stabilized in the aggregates by sequestering defined amounts of water with a peculiar -H- bond arrangement. Thermodynamically stable aggregates of PCs admit up to a limit of around 22-24 water molecules per lipid above the phase transition temperature which results in a bilayer with an averaged area per lipid of 64 $Å^2$ and a thickness of around 40 Å [14, 50].

All the proposals to update the mosaic fluid membrane model sustain, directly or indirectly, the classical paradigms such as a dielectric slab, fluidity, closed system. All of them, as it will be described, are a consequence of disregarding water as part of the membrane structure and its essential role for the thermodynamic and kinetic properties which are the key for membrane response in a dynamic picture.

The deviation from the predictions settled by these paradigms has been attempted to be resolved by introducing entities that has not been experimentally demonstrated: translocons, defects, etc. [51-53]. Several deviations from the predictions within this approach are tackled by postulating models in which intermediate structures are added or supposed to be formed, in addition to an extreme tendency to ascribe relevant phenomena to proteins given the lipid the role of an inert supporting media. The problem with these approaches is that: the formation of intermediate structures is controversial; the unique input of proteins neglecting lipids is debatable; to consider lipids only as a support material is against the principle of cell economicity

given the great variety of lipids in a living cell. Last, but not least, the complex lipid mixtures with or without proteins have unique mechano-chemical properties not found in other materials [54-56].

Along the corrections of the models to explain deviations from the expected behaviour, predicted by laws for macroscopic systems, the classical models do not give satisfactory explanations on the reactivity of the membrane, that is, its response to changes in the adjacent media. Much less if membrane is considered as in integral part of the cell with interconnections with the cytosol. This is covered by considering that the membrane behaves as a dynamic structure in opposition to a static one (long term structures). However, it is not clear if the short-term structure applies to the whole structure, to part of them, if the different parts have different relaxation times and mean time of living. Moreover, how these mean life times are compatible with the thermodynamic response is unclear.

This breakdown of the classical paradigms makes necessary to have a new frame of reference for the biological membrane its structure, components, functions and interrelation with other cell supra structures. For these reasons, the purpose of this review is to install a refreshing view of the lipid membrane properties in which water presence is not ignored. Moreover, the interdependence of lipids and water gives as a result a novel physical chemical system that should be analysed "ab initio" avoiding the analogies and comparisons with macroscopic systems, such as oily macroscopic liquids and thick autonomous phases. In this regard, there are two ill-defined examples: one, the lipid bilayer is treated as a macroscopic phase of low dielectric permittivity and the other, lipid monolayers are resembled to a bidimensional gas on the water surface. Both views have weak thermodynamic foundations and are contradictory in themselves. Moreover, they make the comparison between lipid bilayers and monolayers as biomimetic systems incompatible. These partial views hindered to have a picture of the membrane properties compatible to its function in a complex system such as living cells

This review is organized as follows. In the first part the five common paradigms implicit in all current models are presented and rebutted. In the second part, new concepts about the membrane as a thermodynamic system will be given. In the third part, a correlation of the thermodynamic properties with the structural features will be discussed.

3. Classical paradigms implicit in current model membranes.

The current view of the structure and dynamics of the "fluid mosaic" model [8] was influenced by the double layer of phospholipids proposed by Danielli and Davson [57]. The interpretation of membrane phenomena based on this view is the extrapolation of the thermodynamic laws valid for three-dimensional phases - large enough to neglect interfaces- to one composed of two layers of molecules in which the dimensions of the interfaces are equivalent to the whole phase.

This approach has installed a series of paradigms as follows below:

1.- the membrane is a non-polar slab uniform in the direction normal to the membrane plane disregarding interphases.*

2.- the membrane is a dielectric slab in which partition can occur in the "bulk" of the membrane taken this as a pure hydrocarbon phase.

3.- the concept of fluidity is related to the viscosity properties of homogeneous phases.

4.- the membrane is taken as a closed system, i.e. no exchange of membrane components with the adjacent media takes place.

5.- inherent to the last point, composition of the membrane is described in terms of lipid and protein constituents. Water, which is the stabilizer, is not taken into account as an active component.

Although, theses paradigms are known to be non-representative for lipid membranes and functions, they are not explicitly included in the current membrane models.

The *re-evaluation and updating* of these paradigms on the simple base of considering water as part of the structure makes possible to introduce two new properties of the lipid membranes extensive to more complex systems such as: the membrane as a responsive structure and the kinetic relaxation processes taking place in them.

This conceptual frame brings different consequences in the interpretation of lipid membrane processes. One of them is to resolve the apparent incompatibility of analysis in lipid monolayers spread on an air-water interphase with those obtained using closed vesicles as experimental model systems.

* the difference between interface and interphases will be a point of discussion.

4. Interfaces and interphases. The excluded volume concept.

Phospholipids, glycolipids, and sterols organize spontaneously in a double layer, or bilayer in water due to its amphipathic character [8, 9, 57]. In this process, PC which is one of the major components of lipid membranes stabilizes sequestering around 22-24 water molecules per lipid above the phase transition temperature [14, 58, 59].

The current visualizations of the membrane take it as a rather uniform structure in the direction normal to the membrane plane. A single scheme defines an imaginary plane dividing the hydrophobic portions from the polar moieties oriented to the outer, aqueous space. This plane runs along the ester union of the phospholipid's groups, and contains the CO groups [60, 61]. This ideal plane is defined as the interface (spelled with C), and has no structural meaning, although mathematically responds to the Gibbs definition of surface tension.

The physical view along this definition has several drawbacks. First, it has been shown that the order of the acyl chains changes from the CH_2 in position 1 near the head group to the end methyl group deep in the bilayer [62, 63]. The order parameter describes different degrees of freedom of the methylene groups according to its position in the chains. The membrane is ordered up to the first four carbon atoms (4 C) and is progressively disordered from 4 C to the end methyl group. This point puts into relevance the importance of the head groups to impose an ordered anisotropic structure. On the other hand, the polar region is defined as a homogeneous aqueous media in which no distinction is made between water arrangements in the adjacencies of the polar groups and the bulk water. That is, the dielectric properties are the same in the polar head groups region and in water. Experimental evidences make clear that this is not the case [64-66].

These two evidences make clear that the interface has no meaning in terms of the physical description. In contrast, from the CO groups´ region to the external plane of the phosphate groups a bidimensional phase composed of hydrated polar groups can be identified. This is called the interphase (spelled with ph and s) and describes a phase interposed between two other phases (hydrocarbon region and bulk water). This is in accordance to the definition of the interphase as a bidimensional solution of hydrated head groups in water [67] (Fig 1). This region is not only structurally important but also defines the thermodynamics of the bilayers. The layer of hydrated

groups and its coexistence with labile water makes that this region can be treated as a lattice solution [68].

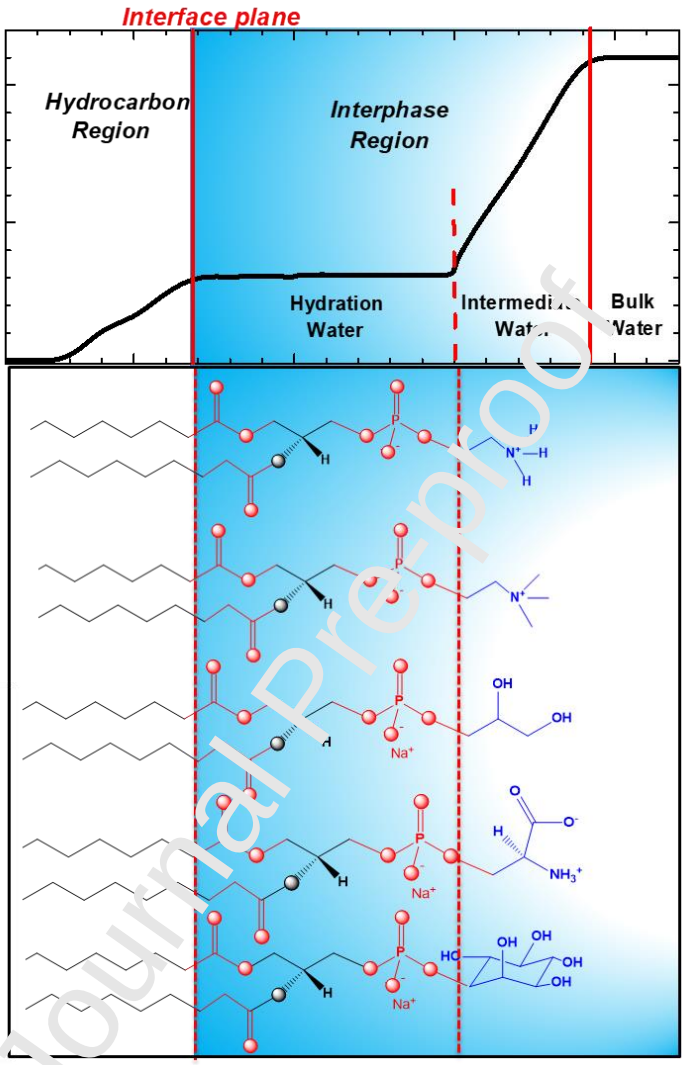

Fig. 1. Schematic description of the membrane region in which the difference between interface (an imaginary plane) and the interphase (a region of peculiar physical chemical properties) are detailed.

A crucial concept in the definition of interphases, to introduce a new approach for understanding cell matrix as a complex system, is the excluded volume. It is defined in a general way as universal and entirely non-specific interactions having the potential to significantly modulate the kinetics and equilibrium of a large number of macromolecular reactions taking place in physiological fluid media [69]. Excluded

volume effects can be classified according to its origin as macromolecular and supramolecular crowding in which the contact between one of them and another takes place through the environmental organization of the so called “solvent”. It is precisely, the case that solvent is water, a mysterious liquid with unexpected properties which versatility impose essential features in the thermodynamically and structural properties of cell components [70, 71]. Macromolecules and supramolecular aggregates preclude a macromolecular confinement of the solvent attributed to a fixed (or confined) boundary around them.

In the particular case of membranes, this excluded volume is due to the sequestering of water by the polar groups and the retention of water corresponds to the interphase region defined above in figure 1. The excluded volume concept is a crucial point to understand the membrane properties both dynamically and statically as will be shown below. The excluded volume concept allows to describe the effective area of the lipid when hydrated in an aggregate as a bilayer. It has been an issue to distribute water in the lipid aggregates to derive area per molecule considering the bilayer thickness values measured by X-ray diffraction [59, 72, 73]. The point is that the stable value in each condition is achieved by repulsion forces between the lipids against the attractive ones [74]. The repulsive forces are due to water around the lipid structure and can vary with lateral pressure or water activity changes (see section 7). Moreover, the actual lipid volume is derived introducing the molar water volume that may vary according to the type of water-lipid interaction [75, 76].

A pioneer vision of excluded volume in membranes was first introduced in analysing permeability properties. The bilayer thickness contains about 20 Å of excluded volume ascribed to water immobilized by the head groups [77] that was considered as part of the membrane structure in which confined water contributed to the permeability barrier for aqueous solutes. The excluded volume was found to be a function of the phase state of the lipids, curvature of the lipid aggregates, surface charges and presence of adsorbed ions such as Ca^{2+} among others [78-80].

In terms of interphases and its role in adhesion and adsorption phenomena, the excluded volume is clearly related to the repulsion forces between bilayers described as hydration forces or dipole potential [81-85]. The origin of those forces is related to water polarized at the interphase in a great extent, in addition to the constitutive dipoles such as carbonyl groups [86, 87].

The excluded volume is useful to describe the geometrical property of the lipid molecule considering the hydration water in the definition of the packing parameter,

$$P = \frac{v}{a.l}$$

where v is the molecular volume, a is the cross-sectional area of the headgroup, and l is the length of the molecule [17, 88]

However, the lipid molecule is a dynamic structure and cannot be assigned in terms of shape as such, and the geometric parameters should therefore be considered as average molecular properties. It turns out that the fluctuations around the average value can be assigned to the fact that excluded volume can be reduced or expand according to the mechanical and chemical forces at which the membrane is subjected. For instance, lateral pressure, changes in water activity (osmosis) or solute that may interact with the polar groups displacing water from the lipid hydration shell. This point will be discussed again in section 7 and 8.

Finally, some points must be marked in figure 1. The schematic picture represents only a monolayer of the bilayer, that is facing bulk water. However, if this analysis is made in a cell membrane, one monolayer is facing the cytosol that may have specific properties since water can be organized different than the external bulk water. A discussion of how this kind of water may affect the water membrane organization and vice versa, how the membrane itself may induce order towards the cytosol is open to discussion.

5. The membrane as a dielectric slab.

The acceptance that the lipid bilayer is composed by lamellar lipids derived in the frequent consideration that the membrane is an optimum insulator and such as it would be non-compatible with cell function, i.e. *life*. The relevant biological role is ascribed to proteins inserted in the bilayer implicitly denotes that lipids is taken as a dielectric slab with little participation of water in the bilayer structure [48, 89].

In consequence, one of the most inspected subjects, in order to explain a sealed membrane with specific proteins inserted in it has been protein membrane interaction. This derived in the need to understand how different amino acids and residues can stabilize in the membrane, taken it as a preformed oily phase.

This description of the cell membrane explained several experimental findings showing that different components can permeate cell and lipid membranes according

to its solubility in organic phases such as octanol [90]. This resource has been a precursor of the SNFMMM [91-93].

A thermodynamic description based on the non-polar character of the membrane core limited between the two ideal planes described by the interfaces in figure 1 considers the water-membrane partition of the solutes, in particular amino acids, according to the so-called hydrophobic scale [90].

Table I shows non polar and neutral amino acids partition energies from glycine to tryptophan, calculated from the partition of homologous peptides. A negative free energy value of partition can be explained by a dominating positive entropy change due to hydrophobic interactions, since no variation in the formation of -H bonds are observed.

The inspection of the molecular structure of these amino acids indicates that the entropy changes increase with the non-polar chain length.

Amino acids	**Free energy (kJ/mol)**	**Hydrophobic centre**	**Equivalent -H bonds**
Glycine	0	0	0
Methionine	-1.05	2.- methylene	0.26
Cysteine	-1.05	1.- methylene	0.26
Isoleucine	-1.25	2.- CH_3 1.- CH 1.- CH_2	0.31
Leucine	-1.67	2.- CH_3 1.- CH 1.- CH_2	0.42
Tyrosine	-3.76	1.- Methylene 1.- hydroxyphenyl	0.94
Phenyl alanine	-5.02	1.- Methylene 1.- Phenyl	1.25
Tryptophan	-7.53	1.- Methylene 1.- Indol	1.88

Table I.- Free Energy Partition in lipid membranes of hydrophobic and neutral amino acids (taken from ref 90)

Therefore, the negative free energy change can be ascribed to a reorganization of water from ordered clusters to less coordinated water molecules. This effect is sometimes recalled as a classical hydrophobic effect [94].

According to Table II, polar and charged amino acids have positive free energy of partition which would make thermodynamically impossible the transference from water to the membrane. However, arginine and other amino acids have been found to be able to incorporate the membrane [95-99]. As the molecular structure of these amino acids does not justify the entropic change due to water organization, the free energy rectification in order to turn it negative must include a negative term in enthalpy. In other words, the process should be driven energetically instead of entropically. This enthalpic change can be ascribed to the formation of -H bonds.

In Table II, an estimation of the numbers of H bonds that each amino acid can form in order to compensate the free energy change is shown. Histidine, Lysine. Aspartic and glutamic acids show the formation of one or two H bonds. This implies a negative enthalpic change of around 4-8 kJ/mol considering 4 kJ/mol as the energy of the H bonds.

Amino acids	Free energy (kJ/mol)	Hydrophobic centre	Equivalent -H bonds
Glycine	0	0	0
Valine	0.04	1.- CH 2.- CH_3	0.01
Serine	0.21	1.- CH_2	0.05
Threonine	0.21	1.- CH 1.- CH_3	0.05
Alanine	0.42	1.- CH_3	0.10
Histidine	0.42	1.-CH_2	0.10
Asparagine	1.25	1.- CH_2	0.31
Proline	1.67	NH	0.42
Glutamine	2.51	2.- CH_2	0.64
Arginine	3.34	3.- CH_2	0.83
Histidine+	4.18	1.- CH_2	1.04
Lysine	4.18	4.-CH_2	1.04
Aspartic acid	5.02	1.-CH_2	1.25
Glutamic acid	7.90	2.- CH_2	2.00

Table II, Free Energy Partition in lipid membranes of charged and polar amino acids (taken from ref 90)

It is also observed in Table I and II that taking glycine as reference, the partition amino acids free energy can shift from energy driven to entropic driven processes across a value of free energy equal to zero. At ΔG=0, an entropy - enthalpy compensation occurs that recalls to the case in which a phase equilibrium is reached. That is, solute can exchange freely between both phases as water molecules do in the liquid vapour equilibrium. In this condition, it is interesting and conceptually rich to inspect the entropy - enthalpy compensation for different solutes (Fig. 2). The enthalpic - entropic compensation denotes that data are organized by structural considerations in different families of compounds according to molecular features [100, 101].

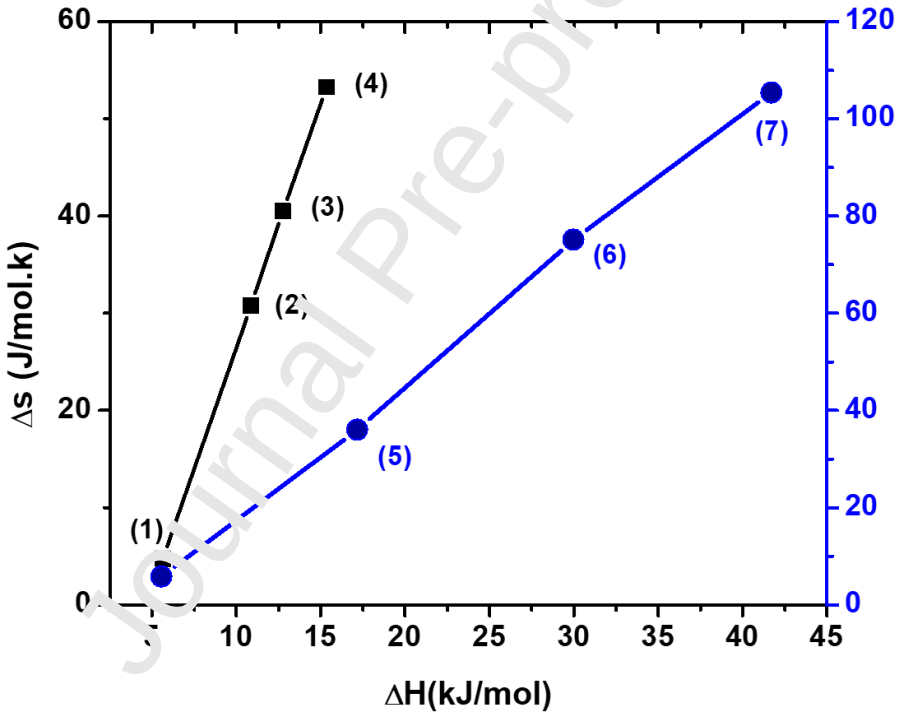

Fig. 2. Entropy - Enthalpy compensation of different families of H-bound compounds. Black symbols correspond to urea (1), ethanol (2), propanol (3) and ter butanol (4); Blue symbols correspond to urea (1), ethylene glycol (5), glycerol (6) and erythritol (7).

First family (black symbols: 1, 2, 3, 4 correspond to urea, ethanol, propanol and terbutanol compounds respectively) represents compounds in which maintaining one

-OH, methylene groups are added in a tetrahedral array. In this case, the increase in entropy with methylene addition is 4.7, 30.8, 40.5 and 53.2 J/mol.K while the enthalpy increase is between 5.6 and 15.4 kJ/mol.

The second family (blue symbols: 1, 5, 6, 7 correspond to urea, ethylene glycol, glycerol and erythritol respectively) corresponds to molecules in which the hydrophobic-hydrophilic balance is maintained. The addition of one $-CH_2$ to the chain is compensated by the addition of one -OH group. In this case, the entropy changes per $-CH_2OH$ is between 5.9 and 105.4 J/mol.K and for the enthalpy 5.6 and 41.8 kJ/mol, respectively.

The first family corresponds to compounds in which the hydrophobicity increases at nearly constant -H bond formation. This would correspond to the classical hydrophobic effect characterized by a large change in entropy. The second family shows compounds in which the ability to form -H bonds increases at nearly constant hydrophobicity. It is observed that, in this case, the entropic change is much lower in comparison to the compounds in the first family. In contrast, the values for enthalpy are larger for similar values of entropy. This is described as non-classical hydrophobic effect [102]. The enthalpic entropic variations according to molecular features of the amino acids means that membrane solvent properties are not homogeneous.

A peculiar case is urea that fits well in the two families. This may be due to the particular property of urea to form H bonds and being a water disrupting compound [103-106].

Although experimentally few information is available, a picture of the importance of water in the partition of amino acids in lipid bilayers has been proposed by molecular dynamics [97]. Different amino acids have different distribution and localization along the membrane thickness. Lys, Glu, Asp and Arg may be located at the centre of the membrane. Phe has a broad distribution in the membrane and Trp and Tyr localize strongly at the interfacial region. The simulations provide a way to correct and predict the penetration of amino acids or peptides that were not possible to explain by the widely used hydrophobicity scale [90, 107]. According to this approach partitioning of charged and polar side chains of amino acids is accompanied by water, which is assumed to be located in defects in the lipid matrix. These defects allow to connect the side chains to bulk water. It is claimed that water defects dominate the energetic of partitioning in contraposition to the simple partitioning between water and a pure

(dehydrated) hydrophobic phase. This distribution is an important feature to consider the thermodynamics of lipid–protein interactions because they determine the driving force for processes of insertion. However, although the role of water is emphasized, not relevant details about the organization of water pockets in those defects nor, their dimensions and physical chemical properties are known. The presence of water affects the energetics of the amino acid stabilization in the membrane but the water amino acid ratio in the lipids has not still been calculated.

The most striking finding is the possible formation of large water pockets that hydrate the amino acid polar and charged residues along the acyl chains penetration. The stability of these pockets is determined by a balance between the cost of forming a defect in the membrane and the energy gained by hydrating the polar side chain.

The proposal of the formation of water pockets in the membrane phase is feasible if water is admitted in the membrane structure. According to the current paradigms, solute partition in the membrane obeys the Henry´s law in which membrane is taken as a homogeneous and continuous oily phase. This law describes the partition coefficient as a difference in the standard chemical potential of the solute in water and the solute in the oily phase, taken both as pure phases. However, there are several drawbacks in applying directly this law to membrane phenomena. Firstly, the law is valid for solutes that behaves as ideal gases in the gas phase. More precisely, deviations from Henry´s law are certainly expected in crowded systems as those described above. Secondly, the phases in contact, in which the solute dissolves, should be completely immiscible. This is also an ideal condition but can be easily removed if phases are large enough to neglect the region which they are in contact with (i.e. the interphase). It is immediate to realize that this is far from the case of a lipid membrane in which the dimension of the interphase (as defined in figure 1) is similar to the so-called bulk of the membrane (hydrocarbon phase).

The solubility of solutes under a new conceptual view of the membrane, should recall that membranes are considered permeable to water. This has been demonstrated by different experimental methodologies, such as diffusion of radioactively labelled molecules and massive transport driven by osmosis [108]. So, water is present, at least transiently, in the membrane phase.

The point is more complicated when polar solutes are considered. For instance, glycerol or acetic acid can diffuse from one water solution to other through an oily phase [109]. However, diffusion implies the entrance of water to the oily phase. That

is, thermodynamically speaking the solute forms a mixture avid for water and therefore a ternary system water-oil-solute is accomplished.

This phenomenon is also observed in lipid membranes. The permeation of a solute such as erythritol, glycerol or urea in isotonic conditions produces a water penetration along with the solute diffusion. As a result, a defined amount of water copermeates with the solute [110, 111].

Therefore, in order to consider the whole information available to explain membrane properties in regard to solute penetration (operationally called partition) in a real condition, the system has to be considered as a mixture of lipids and water. The question that merges now is if these mixtures are microscopically different along the membrane to give confined regions with different solubility properties. The point is how water is distributed between the hydrocarbon chains and head groups.

In this condition, the difference in the partition should be ascribed to the influence of water as a component of the membrane structure. Thus, the failure of the characterization of the lipid membrane as a dielectric slab is derived from the classical hydrophobic effect driven by a change of entropy by the loss of structured water molecules around apolar solute to interact with the hydrocarbon phase.

The presence of water in the lipid matrix results in rheological, peculiar mechanical properties such as fluid, elastic, and deformable structures and the paradigm that the lipid membrane acts as a generic low dielectric slab should be disregarded.

6. The fluidity concept. Water between chains.

Fluidity of membranes has been taken as a measure of the microviscosity. A fluid phase is characterized by a poor lipid packing mainly of the acyl lipid chains and thus a low microviscosity. In short, high fluidity has been identified as a liquid like state and low fluidity as a rigid, likely solid media. Pure lipid membranes present a transition from a solid (gel) phase to a liquid crystalline (fluid) phase at a temperature determined by acyl chain length, unsaturation, head group nature and hydration level [112]. The transition from the solid gel state to the liquid crystalline is concomitant with an increase in the water lipid ratio from 7 to 22-25 water per phosphatidylcholine and changes in the area per lipid and thickness [58, 113-115].

The so-called gel-fluid phase transition can be operationally determined by several techniques: turbidity, refractive index, X-ray diffraction parameters (thickness and area), dipole potential, fluorescence with located probes, electron paramagnetic

resonance (EPR) with located probes, calorimetry and FTIR among others [115-120]. For brevity reasons, and in the frame of this review only the refractive index changes and the FTIR results will be described.

The refractive index is a measure of the density of the material. Its variations in a lipid suspension can be followed by turbidity changes in which an abrupt decrease is observed at the phase transition temperature. This increase in transparency of the sample is a direct consequence of the decrease in lipid membrane density which can be described as an expansion of the membrane lattice due to the concomitant increase in water. The decrease in turbidity is counterintuitive since the expansion would suggest an increase in the particle (liposome) diameter and hence turbidity should increase [118]. This observation denotes that macroscopic changes are due to modifications in the nature of the lipid membrane at microscopic level that deserves detailed inspection.

Above the transition temperature, lipids in a bilayer have several degrees of freedom e.g. diffusion along the plane of the membrane, rotation around an axis perpendicular to the membrane plane, fluctuations in and out with respect to the plane of the membrane as a cork in water (protrusions), wobbling among others. These degrees of freedom are a consequence of the balance between intermolecular interactions in the lattice and the thermal energy [68]. When the last predominates, fusion occurs. The fusion temperature of dry lipids decreases in a large extent when lipids are hydrated. Interestingly, compounds that may replace water in the dry lattice decrease the temperature of fusion in an extent comparable to that occurring in hydrated lipids [121-123].

The generalization of the term fluidity has installed the paradigm that in the liquid crystalline phase, the membrane state is similar for different lipid matrixes. In this direction, the postulation of water pockets in the membrane defects as described in the previous section - as one of the resources to explain polar and charged amino acids penetration that do not follow the hydrophobic scale- can be verified experimentally applying FTIR analysis. As known, the frequency of a given group (ν) is directly related to the strength of the chemical bond (k), according to Eq. (1)

$$\nu = 1/2\,\pi.\sqrt{\frac{k}{\mu}} \qquad (1)$$

where μ is the reduced mass

The symmetric vibrational frequency of -CH bonds in methylene groups in the phospholipid's acyl chains centred around 2850 cm^{-1} in the gel state, increases when the phospholipids aqueous dispersions go through the phase transition temperature (Fig. 3).

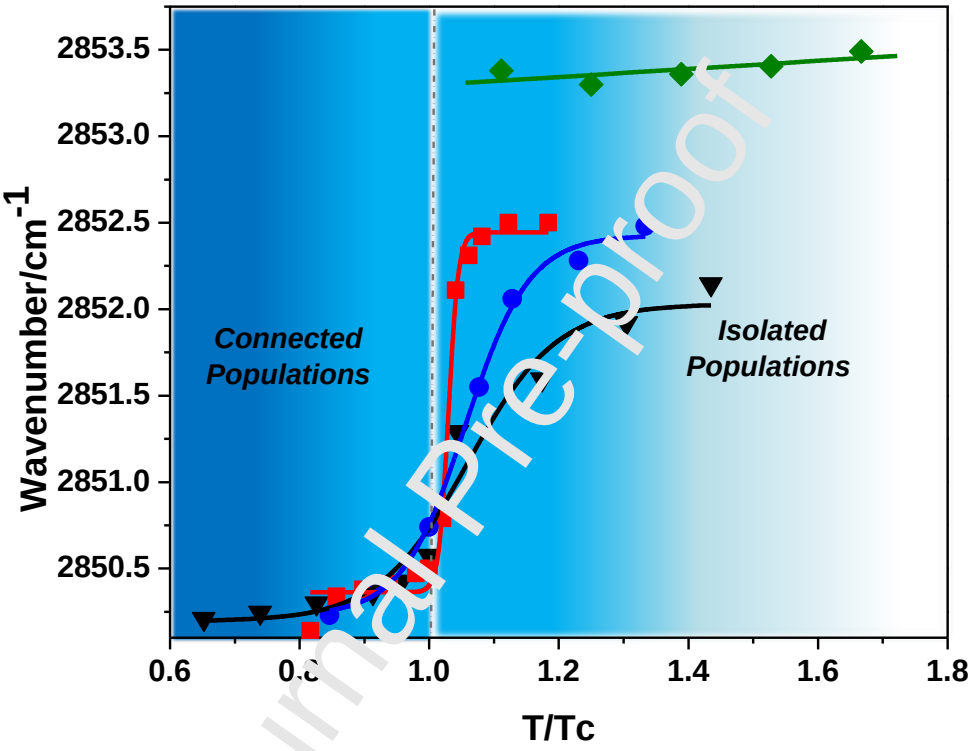

Fig. 3. Frequency shift of methylene symmetric stretching mode (-CH_2) with temperature. DMPC (black symbols), DPPC (blue symbols), DMPE (red symbols), DOPC (green symbols). Adapted from ref 124.

The frequency increase is plotted as a function of the reduced temperature of the lipids (T/T_c) for a better visualization. This plot allows to compare lipids of different chemical structure. As observed, the difference in the gel state is negligible in an excess of water. However, above the transition temperature the frequency increases according to chain length (DMPC to DPPC), head group (DMPE), and presence of double bonds (DOPC). The low frequency values in the gel state are explained because chains are in contact with each other due to the packing and hence the

strength of the bond is weaker. These populations of methylene groups are described as connected ones [124]. Temperature increases the degrees of freedom producing a separation of the acyl chains due to a decrease in the lateral interactions. In consequence, an increase in frequency is produced according to Eq. (1). As stated above, the water content increases at the phase transition. Hence, it is immediate to conclude that the spaces between chain residues above T_c are filled with water.

In order to corroborate the point that differences in frequencies in the so-called fluid phase reflects the state of the CH_2 residues in terms of water inclusion, the dependence of the CH_2 frequency with water activity is presented in figure 4 for temperatures below and above T_c.

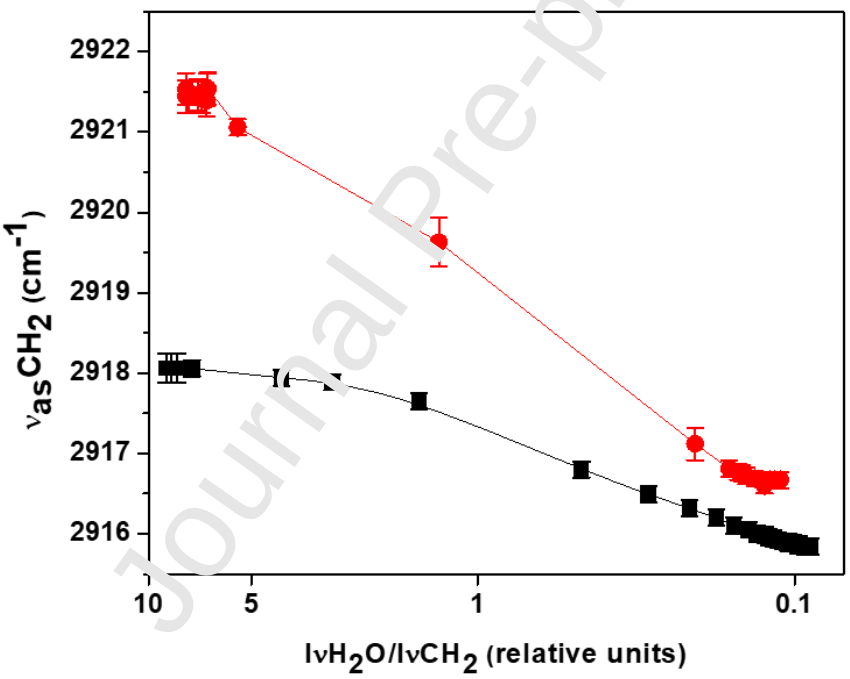

Fig. 4. Frequency shift of -CH_2 asymmetric stretching modes of DPPC as a function of hydration at 22 °C (black squares) and 50°C (red circles). Note that hydration decreases from left to right. $I\nu H_2O/I\nu CH_2$ represents the relative hydration parameter (Ref 126).

It can be concluded that the connected populations appear at low water ratio and isolated ones at high. The amount of water in the chain region is concomitant with the hydration of the phosphate groups and modulated by the presence of carbonyl

groups (CO)[68, 125, 126]. This means that in the so-called fluid region, the state of the CH_2 differs with the type of lipids. In consequence, the description of the membrane state by fluidity as a generalized term is misleading.

Therefore, the membrane is not a pure hydrocarbon phase and, in consequence, density (as derived from refractive index), elastic, mechanical and dielectric properties of cell and lipid membranes will be particularly affected.

One of the cell membranes components that may regulate the phase state is cholesterol (Chol) by reducing and eliminating the enthalpy of the L_α/L_β phase transition and by changing the mechanical rigidity and cohesiveness [127, 128] .

Both effects have been ascribed to inhibition of the rotational degrees of freedom described by the CH_2 vibration states in figure 4. As pointed out before, the hydrocarbon chain degrees of freedom are linked to the presence of water in between the acyl chains.

In parallel to this, different fluorescent methodologies indicate that cholesterol affects the emission Laurdan properties both in isotropic and anisotropic media which is ascribed to water membrane organization [65, 129-132].

In this regard, lipid chain order and the amount and dynamics of water molecules at the glycerol backbone and acyl chain regions of the membrane have been demonstrated by NMR [133] and FTIR spectroscopies [134].

Thus, it is likely that cholesterol provides a more bulk-like environment for the interfacial water molecules, due to an enhancement of local water density, a reduction in their orientational degrees of freedom and an increase in the number of hydrogen bonds at the hydrogen bond network interphase [125, 135]

This effect of cholesterol on water content will be put into relevance in sections 8 and 9 were the responsiveness of the membrane to bioeffector is analyzed.

It is concluded that in the gel phase in the excess of water most of the CH_2 population are connected with each other and above them the isolated population depends on the type of lipid. Taken together with the conclusions described in section 4 and 5, *water appears as a critical component to define the membrane phase state and its structural properties.*

The description of the membrane as a complex system in which water is a singular component and the concomitant thermodynamic properties will be discussed in the next section.

7. The membrane as an open system.

The evidences provided in the previous sections and the overwhelming amount of information obtained using NMR, X-ray diffraction and FTIR spectroscopy clearly demonstrates that water is a primary factor in membrane structure [136-142]. However, no model has included it as part of the membrane matrix and much less has considered its thermodynamic properties in terms of membrane response and membrane dynamics.

The amphipathic structure of phospholipids allows to stabilize them in aggregates that has been used as biomimetic membrane systems. In one case, when dry lipids are mixed with water, they spontaneously organize themselves in bilayers, e.g. liposome formation. The bilayer in aqueous medium is taken as the thermodynamically stable phase of the membrane. On the other case, when a chloroformic solution of lipids are spread on an air water surface a monolayer is formed [143, 144].

The stabilization of lipid bilayers in water due to hydrophobic forces has been explained on thermodynamic grounds as a consequence of the entropic changes induced in water structure [58]. The formation of a monolayer on the air-water surface has considered the lipids as a gas spread on an inert solvent [143, 145]. The information gathered from monolayers are the area per lipid and the surface pressure (surface tension changes). In bilayers, mostly accomplished in closed vesicles, volume changes due to water and solute fluxes can be measured. [18, 146]. How these data fit one with the other can be a matter of debate and controversial results in terms of comparison have merged.

Being water an exchangeable component of lipid membranes, as shown by permeability studies, the lipid interphase defined in section 3 should be treated as an open system and hence, thermodynamic of irreversible processes is the adequate frame of reference [147]. This thermodynamic analysis is general enough to satisfy the equivalence of monolayers and bilayers in terms of interfacial properties and the conditions in which topological accidents appear to produce reactive sites and the propagation of such interactions.

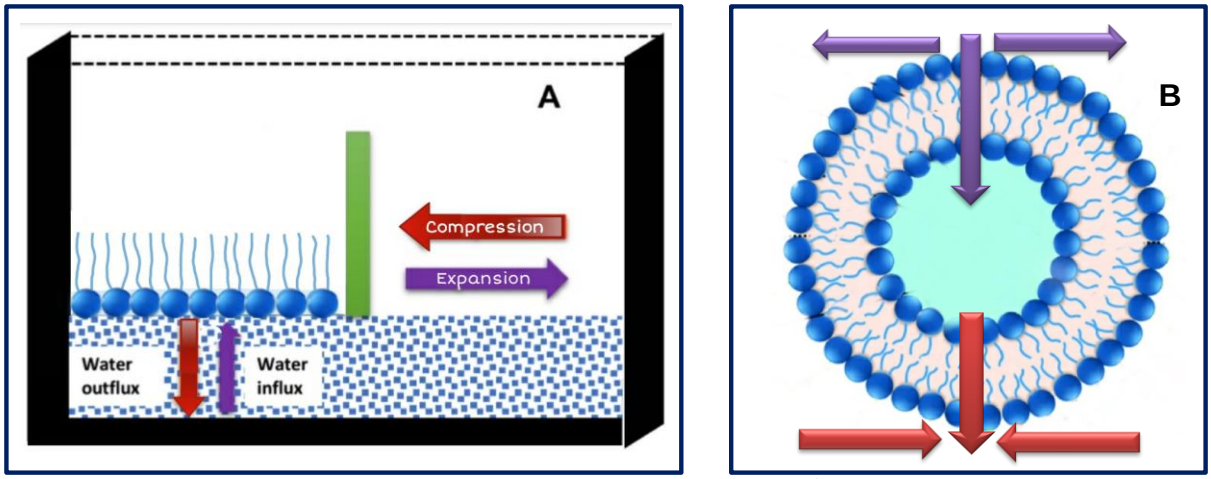

Fig. 5. (A) Expansion (violet arrow) and compression (red arrow) of a lipid monolayer with a mobile barrier. (B) Shrinkage swelling of lipid vesicles under hypertonic and hypotonic stress Expansion and water influx (violet arrow) and compression and water efflux (red arrow).

The similitude of the expansion/compression processes in monolayers and bilayers are schematically shown in figure 5. In part A, a lipid monolayer spread on the air water surface is compressed by displacing a mobile barrier in a Langmuir balance (horizontal red arrow). The external pressure is counteracted by the resistance of the lipids to compress and opposes to the barrier movement. This defines the surface pressure described as the difference between the surface tension of pure water (γ^0) and of water with the lipid (γ)

$$\pi = \gamma^0 - \gamma$$

The displacement of the barrier is a mechanical work done on the surface, defined as

$$W = f.\Delta l = \left(\frac{f}{l}\right) \Delta A = \gamma \Delta A \qquad (2)$$

where *f* is the force applied along a distance Δl, γ is the surface tension and ΔA the change in area. The difference of the work to create the same increment of area ΔA in pure water and on the surface with lipids is

$$W = (\gamma^0 - \gamma)\, \Delta A = \pi \Delta A \qquad (3)$$

In the scheme, the compression of the lipids produces a decrease in the area per lipid, i.e. an increase in the surface lipid concentration (Γ_L). As the lipid amount is

constant, the compression produces a squeezing of water from the lipids pointed as a water efflux (vertical red arrow in the figure). When the external pressure on the barrier is released, the differences in water activity between the bulk and the monolayer due to the lipid concentration produces a water flux into it (vertical violet arrow) producing an expansion (horizontal blue arrow). Thus, an equilibrium point is achieved at a given surface pressure and water activity. This point defines a state of the monolayer at constant T.

In part B, a similar phenomenon can be described. If closed vesicles are dispersed in hypertonic media with respect to the internal one (by increasing the concentration of an impermeant solute in the external media), an efflux of water is produced with a consequently volume decrease (compression, red arrows). In contrast, when dispersed in hypotonic media a swelling is produced (expansion, violet arrows). Thus, the surface pressure (surface tension) and the osmotic gradient (chemical potential of water) are the intensive properties that produce changes in its conjugated extensive ones such as area and water content, respectively [148].

The two phenomena are equivalent under controlled conditions. In the first case, a lipid-lipid friction counteracts the area changes induced by pressure. In the second, a water-water friction modulates the water volume flux driven by the difference in water chemical potential. However, surface pressure also produces water fluxes and the chemical potential differences derives in area changes.

7.1.- Thermodynamic of Irreversible Processes (TIP)

According to the Thermodynamic of Irreversible Processes (TIP) formalism, the crossed processes are due to the coupling of mechanical (π) and chemical ($\Delta\mu_w$) phenomena that can be described as the total area variation (J_a) given by:

$$J_a = I_{Lw}\Gamma_w\Delta\mu_w - I_{LL}\pi \qquad (4)$$

and the total flux of water (J_w) by

$$J_w = -I_{ww}\Gamma_w\Delta\mu_w + I_{wL}\pi \qquad (5)$$

Γ_w is defined as the surface water concentration. The system is completely defined by four coefficients in the absence of other forces: the mobility of water in water (l_{ww}),

the mobility of water in lipids (l_{wL}), the mobility of lipids in lipids (l_{LL}) and the mobility of lipids in water (l_{Lw}). The first three ones have been experimentally determined. The cross coefficients that relate area changes with water chemical potential and water flux with surface pressure as non-conjugated forces are identified as the diffusion coefficient of water in lipids and those of lipids in water, respectively [149].

Thus, Eq. (4) indicates that the fixing of a surface pressure as a controlled independent variable determines univocally a value of water activity at the monolayer interphase. In turn, Eq. (5) states that in bilayers, in which the independent controlled experimental variable is the osmotic pressure, a value of it determines also univocally the value of the surface pressure.

Although interactions between lipids in the monolayer plane are taking into account when the monolayer is considered as a van der Waals gas, it is not enough to reproduce the experimental surface pressure *vs* area per molecule curves [149-151]. This is due because the interaction of lipids with water in the subphase is ignored, i.e. crossed coefficients in Eq. (4) and (5) are taken as zero.

A master equation for both systems can be obtained considering Eq. (4) and (5), at the steady state

$$\pi = -\frac{I_{Lw} + I_{ww}}{I_{LL}} \Gamma_w \Delta\mu_w \qquad (6)$$

When experimental curves are fitted considering the coefficients in terms of self-diffusion of water in water, lipid diffusion in lipids, and water in lipids, the only adjustable coefficient is lipid in water that may vary along the compression curve. With this criterion experimental curve π/area per lipid are fitted satisfactorily below and above the coexistence region of liquid expanded and liquid condensed states [149].

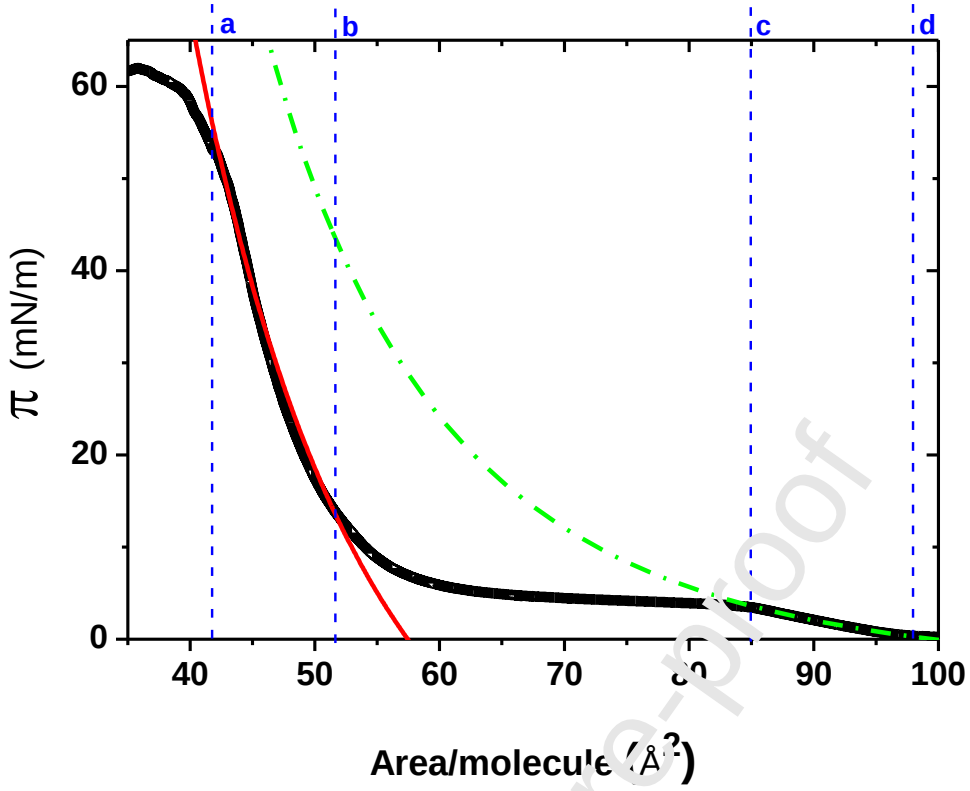

Fig. 6. Surface pressure/area per molecule isotherm for DPPC at 25 °C. Black solid line: experimental data; fitting according to Eq. (6) below (red curve) and above (green curve) coexistence region. Region between ab lines corresponds to pure liquid condensed state and region between cd lines to pure liquid expanded state (Adapted from ref 149).

When the cross-coefficients are zero and l_{ww} and l_{LL} are equal and introducing

$$\Delta\mu_w = -RT \ln a_w$$

Eq. (6) reduces to Eq. (7)

$$\pi = -\Gamma_w RT \ln a_w \qquad (7)$$

proposed by Defay-Prigogine, where a_w is defined as the water activity at the interphase. Thus, the lipid interphase is considered as a bidimensional solution of hydrated head groups as described in terms of excluded volume in section 3 [67, 152, 153].

The formalism is also suitable to fit swelling-shrinkage processes in closed vesicles. Consequently, this formalism can also be applied to lipid vesicles subjected to an osmotic unbalance between the inner and the outer compartments. The water

volume flux produces a concomitant membrane expansion or compression and the same reasoning is applied.

7.2.- Comparison between monolayers and bilayers.

Taken together this new approach for lipid membranes on thermodynamic grounds allows to fill the gap in the controversy between the validity of monolayers and bilayers as experimental model systems for lipid interphases and defines the lipid membrane state that is responsible of the responsive structure as will be described in the next section

The comparison between monolayers and bilayers is not only for an operational convenience but to define, as precisely as possible, the thermodynamic state of the membrane in both systems in order to compare a given process. For that reason, it is of interest to define the range at which a phenomenon occurs along the π/ area curve, which is the system with a better thermodynamic access.

In order to define the state of the membrane in terms of experimental conditions, it is important to emphasize two aspects to make the comparison reasonable. In first place, π/A curves are obtained by displacing a barrier that is perturbing mechanically the system. In order to consider each point of the curve as a defined state closed to equilibrium a quasi-static process must be carried out. This means that, the rate of barrier displacement should be slow enough to allow the system to reach equilibrium rapidly. This procedure allows to define each point of the curve as an "equilibrium" state of the lipid in terms of water activity and surface tension (free energy) at constant temperature, in the absence of any other forces acting on the system. Many assays in literature are not comparable because the rate of the barriers is not the same or is too fast [150]. The equivalence between monolayers and bilayers has been accepted by the finding that at a surface pressure ca. 30 mN/m, the activity of phospholipase A_2 is the same as that in bilayers. Thus, this value has been taken as the surface pressure in a spontaneously formed bilayer. However, the assays do not take into account the osmotic state of the liposomes (i.e. water activity) at which the enzyme activity was measured [154-157].

Recently, monolayers and bilayers have been compared using Laurdan fluorescence as an indicator of polarity and hydration [158]. The results indicate that a similar GP value is found for monolayers and supported bilayers of DOPC, at a surface pressure of around 26 mN/m. A similar analysis done in DPPC monolayers and

bilayers in the liquid condensed state shows that GP values are the same at a surface pressure of 28 mN/m. However, monolayers and bilayers of DPPC in the liquid condensed state give a value of GP (0.6) different for that for DOPC (-0.25) in the liquid expanded state suggesting that interphase organization is different at the same surface pressure of membrane in the different phase states.

These surface pressure values are near to those reported previously for the equivalence in the hydrolytic action of PLA_2 in PC monolayers and bilayers [154]. The observation that in both, lipids monolayers and bilayers, have similar surface pressure for a given GP denotes that the hydration is equivalent in both conformational states, independent of the lipids. The changes in Laurdan GP along the surface pressure/area curve reflect changes in hydration at the interphase as schematically shown in Figure 5 and stated by equation 6.

In figure 6, at low density states (large areas per lipid or high water/lipid ratio) monolayers are described to be in the liquid expanded state and the bilayers in the $L\alpha$ phase (usually described by the misleading term fluid phase). The high-density state corresponds to monolayers in the liquid condensed state (low area per lipid) and bilayers in the so-called gel state (low water/lipid ratio). At low density states, the lipid-lipid interaction is minimum and the lipids are fully hydrated. Then, the cross coefficient in equation 6, l_{Lw}, represents the lipid diffusion with its complete hydration shell in a free water bidimensional layer.

At low density states, water diffusion (l_{ww}) dominates in Eq. (6). However, this diffusion is affected by the interaction of water with lipids (l_{wL}) which may vary if lipids are below or above the phase transition temperature. On the other hand, l_{Lw} changes at the high-density state since lipids diffuse frictioning their hydration shells. At this stage, it may be considered that no free water is beyond the hydration shell. Mechanical properties of cell membranes have been only considered in a model in which cytoskeleton and the glycocalyx affect membrane organization [159].

In conclusion comparison of monolayers and bilayers can be done for the different mechano-chemical states in which low and high density can be defined without introducing any topological change such as collapse of the lipid head group and the presence of curvature. This region is denoted in figure 6 between lines a and d. The meaning of these limits will be discussed in the next section.

7.3.- Topological deviations and defects.

In order to determine the range of density at which monolayers and bilayers could be thermodynamically equivalent, it is necessary to define the lipid states in extreme conditions.

At large areas in monolayers, cohesion forces are neglectable and no monolayer is formed (beyond line d in figure 6). This state is comparable to that in which the lipid vesicles are subjected to drastic hypotonic solutions in which the permeability barrier properties are lost and the content is leaked. On the other hand, at low areas (high pressure), monolayers collapse and in the bilayers the hypertonicity makes the membrane to compress and form protrusions and extrusions in which high curvature regions can be formed (line a in figure 6). The surface properties of these regions are completely changed and unknown [160].

The collapse of the monolayers and the bilayers not only occurs when lipids are squeezed by an increase in lateral pressure and a decrease of the lateral space. It can be also thought as a result of the reduction of the excluded volume of hydration water that may be displaced or distorted, affecting the hydrogen bonding distribution and hence interfacial tension.

Taking into account these features, the overlapping and crossed coefficients described in Section 7.1, are only valid for certain range of surface/area curves. The monolayer at areas higher than 25 $Å^2$ does not present coherence (line d figure 6). At low area, the limit of pressure at which lipid abandon the planar geometry is represented by line a in figure 6. Beyond this point, without reaching the collapse pressure, compression can distort hydration shells, reorient carbonyl and phosphate groups, and cause local curvature. All these changes will affect the excess of free energy of the interphase and therefore its response to bind effectors. The regions between lines a and d are those in which monolayers and bilayers can be reasonable compared.

This also can be a consequence of the collapse of the structure according to the water expelled or absorbed in a given adsorption process. The interaction process is accompanied (coupled) to changes in the structural pattern of the membrane at different levels (head groups, carbonyls, acyl chains) in relation to water arrangements and lipid-lipid contact.

As described above, many improvements of the current models have introduced heterogeneities in the membrane plane obeying to the complex lipid composition

[161, 162]. Thus, topological features, defects, non-bilayer structures, curvature are introduced as a result of the finding of visually identified morphologies in the membrane plane, as a consequence of powerful microscopic methodologies. So far, structures are explained on geometrical criteria considering the stabilization at long time of the events [163].

The current view of membranes sees the bilayer as formed by heterogeneous patches ("domains"), enriched in certain lipids and proteins with diameters ranging between 0.1 and 1.0 μm which characteristic functional properties are ascribed to [9, 164].

The coexisting lipid domains in membranes gave place to a theory, in which the shape and size of a given domain would be the result of an equilibrium between line tension and electrostatic dipole–dipole interactions. Line tension, that has units of force, is the linear equivalent of surface tension (units of force/length) for a one-dimensional interface, i.e. it represents the interfacial energy. Large line tensions favour large domains with compact (ideally circular) shapes, while large dipole–dipole repulsion forces favour small domains and/or domains with extended, e.g. flower-like, shapes [165-168]. How surface pressure affects the contact of incompressible domains or if the surface pressure deforms such domains or if there is a combination of both effects is not known. In terms of confined regions and crowded systems it becomes imperative to elaborate hypothesis in which the contacts and deformability are due to the water arrangements.

The correspondence between monolayers and bilayers from a thermodynamic point of view is based on the criterion that the membrane is an open system with respect to water as one of the components. The possibility to understand on a unique thermodynamic frame the behaviour of monolayers under mechanical stress and of bilayers in an osmotic stress allows to analyse with a new perspective the dynamical properties of lipid interphases. By this, it is not only understood that the components have the possibility to rotate, protrude, flip-flop or exchange with the external media, but also that they may form transient structures and fluctuations in local arrangements. The point is that, taken in consideration the exchange of water with the media in both systems by different forces (surface pressure or water gradient), the mechanisms of insertion of external solute bioeffectors of different nature can be related with such fluctuations and dynamical properties. The inclusion of fluctuations

in terms of the hydration state of lipid membranes give place to define the membrane as a “reactant” material.

The process described in Figures 5 and 6, is produced by the dissipation occurring in isothermal conditions due to the relaxation of the surface tension and the water chemical potential. In artificial systems such as monolayers and bilayers the process run towards equilibrium and once there an external force must be applied to return the system to the initial conditions. To hold the system in a given state (surface pressure/area pair or a surface pressure /water activity state) a constant external force must be maintained on it (for instance a pressure on the barrier).

However, in a cell it is possible that this condition can be accomplished due to the coupling of other elements of the cell that accounts for the recovery stage in a cycle. These elements can be the cytoskeleton and/or the coupling with the cytosol considered as an organized structure in contrast to a dilute ionic solution. These two elements might operate on the recovery of the membrane properties to start a new cycle at expense of energy possible in a steady metabolic state. This means that the restoration forces would make the system to recover, probably to the proximities of the initial state giving place to oscillatory phenomena in which energetic and entropic contributions interplay [169-171]. In the present review we restrict our analysis for dissipative processes in artificial system. The consideration of oscillatory phenomena mostly due to the presence of cytosol or a cytoskeleton will be the aim of a next work.

8. The responsive membrane.

It has been postulated that membranes are responsive materials [172, 173]. By this, it is understood that membrane can react to physical and chemical perturbations (temperature, pressure, electric fields, chemicals) changing the physical properties, such as, hydration, thickness, density and polarity.

The response of a membrane to bioeffectors (by this it is understood any compound in the external media that interacts with the lipid membrane) is usually measured in lipid monolayers in terms of the departure from the steady state fixed in the curve of figure 6, at point given by a pair (surface pressure/area per lipid) that in fact corresponds to a pair surface pressure/water activity according to equation 6. (Fig. 7A).

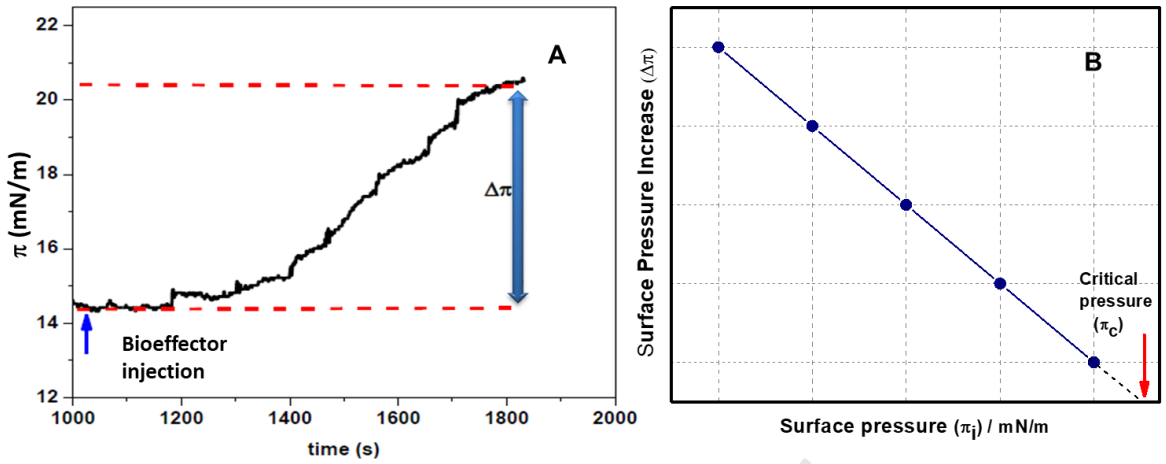

Fig. 7. (A) Surface pressure changes after the injection of a bioeffector to the subphase of a DMPC monolayer at 20 °C and an initial surface pressure of 15 mN/m; (B) Surface pressure response of a DMPC monolayer at different initial surface pressures (π_i).

In a less evident way the responsiveness can also be measured in bilayers following the change in the refractive index in a liposome dispersion which reflects the change in density of the lipid membrane by the copermeation of permeant solutes with water [118, 174, 175].

In order to make clear the contrast of the current ideas based on geometrical criteria and the thermodynamic view, let us consider the arrow and the bow metaphor.

In the first one, the arrow should fit in a pre-existing specific site in the target. Arrow and hole in the target should have complementary shapes as the key and lock model. This approach is valid for any tension in the bow. The other view is that the tension in the bow allows the arrow to produce by itself a hole in the target. The tension in the bow is equivalent to the surface free energy excess produced by the different states of the bilayer/monolayer described by a given surface pressure/area per molecule pair in the curve of figure 6.

The tension has been insinuated in the definition of propensity [47], and it is worthwhile to point out that this is a description of the driving forces that operates on the system. These forces are gradients created by differences in temperature, chemical potential, pressure, surface tension and electric potential. The relaxation of these forces produces a decrease of the system energy to a final equilibrium or a partial one.

For the sake of simplicity let us focus on two forces: chemical potential of one of the membrane components (water) and surface tension of the membrane. As lipids are confined to the lipid membrane being this a monolayer or a bilayer, the variation in chemical composition is given by water. The changes in water in the membrane directly affects the chemical potential of the lipids, i.e. the interactions between them. This will be reflected in the tension created at the interphase.

The point here is that water can exchange between bulk water and membrane phase in a monolayer or a bilayer as well. The TPI formalism described above can, in principle, be useful to correlate results employing monolayers and bilayers as experimental systems to study the reactivity to bioeffectors.

8.1.- Deviations from equilibrium

The bilayer structure is taken as the equilibrium state, a departure of it, is described by the formation of non-bilayer structures. Many experimental observations support the idea that, *in certain circumstances, a small region of a cell membrane may transiently adopt a non-bilayer architecture* [16, 176, 177], but the conditions and regions in which they occur are not defined. Moreover, in spite of this uncertainty, it is speculated that those transient structures may imply novel physiological meanings for lipid phases. The observation that some isolated lipids may stabilize in non-bilayer structures is extrapolated to cell membranes accepting the coexistence of both lamellar and non-lamellar lipids. The lamellar structure can be, at least locally in time and space, easily disrupted by a variety of events (protein insertion, electrical or chemical gradients, etc.). This makes cell membranes potentially responsive to stimuli. This response is measured by the structural organization appearing as a consequence of the process, i.e. the final product. *In fact, the probability of incorporation of a given bioeffector (peptide, protein, or any other compounds of biological action) to a preformed membrane structure is validated by the formation of a new structure explained by the matching of the spatial distribution of the proteins and of the lipids, taking these last ones as flexible to adapt to non-bilayer structures.*

However, the state of the membrane susceptible to trigger the process " ab initio" is not considered. The propensity to a phenomenon to take place has been analysed from the effector side (the arrow in the metaphor) but shallowly from the membrane state (the bow tension). In fact, in both cases, the process depends on the membrane and the bioeffector molecular properties before the event occurs. In other words, membrane needs some kind of preparation (the tension in the bow) to accept

the bioeffector. This does not imply a pre-existent site in the target (as in the metaphor) but the interplay of attractive and repulsive interactions between bioeffector and water; water and membrane, bioeffector and membrane.

In thermodynamic terms, the propensity is just the excess of free energy that a system has accumulated in order to evolve to a state of lower energy. In practical terms, the propensity is due to the surface tension of the membrane originated by particular arrangements of H bonds of surfaces in contact with each other.

A measure of this response is shown in figure 7 A, in which the addition of solutes to the subphase of a monolayer stabilized at an initial surface pressure π_i produces a subsequent increase in surface pressure to reach a new stable higher value. It has been proposed, under geometrical and excluded volume considerations that the effector can insert in spaces between the lipids created at large areas which consequently promotes an increase in the packing of the lipid's monolayer. However, although this interpretation can be reasonable to explain the final accommodation of the lipid in the presence of the bioeffector, it does not explain the origin of the driving force that makes the effector to insert into the monolayer.

The increase in surface pressure is a consequence of the decrease in surface tension, i.e., a decrease in surface free energy with respect to that of the lipid water interphase equilibrated in the absence of the effector. The surface tension change can only be ascribed to water given its ability to concert hydrogen bonds [178].

In part B of figure 7, it is observed that the response decreases with the increase in the initial surface pressure. The critical pressure (π_c), at which there is no longer response, represents the state of the monolayer beyond which there is no thermodynamic response. If this state is considered as the maximum approach of the hydrated lipids, surface pressure larger than 40-45 mN/m corresponds to extremely low water activity. At this critical pressure, free water beyond the hydration shell is not expected. Hence, the response of the monolayer is given by the amount of water outside the first tight hydration shell of the lipids [87, 179].

The interpretation of the interphase as a bidimensional solution considers that polar head groups are dissolved with its hydration shell [67, 152, 153]. In this approach, the bioeffector dissolves in the interphase water decreasing the water activity. This generates a gradient of water chemical potential between the interphase and bulk promoting a water entrance by osmosis which produces a surface area increase.

Thus, lipid surface pressure increases with water activity in accordance to Defay Prigogine interphase model described in the previous section (Eq. 7). However, the water entrance to the membrane interphase can be taken as just a variation of the geometrical interpretation if some considerations on the water state are not analysed.

The inspection of figure 6, indicates that in the regions in which there is no phase coexistence (plain LC or LE phases), a small increase in area implies a drastic change in surface pressure, i.e. in surface tension. An increase in area of only 3-4 $Å^2$ implies a decrease in surface pressure from 45 to 35 mN/m. This difference denotes that water state available for the bioeffector to interact is not a question of dimension but of free energy accumulation. This implies a qualitatively important change of the surface in terms of surface tension due to water properties.

Data in figure 7B can be replotted in terms of the difference between the critical surface pressure and the initial surface pressure (Fig. 8)

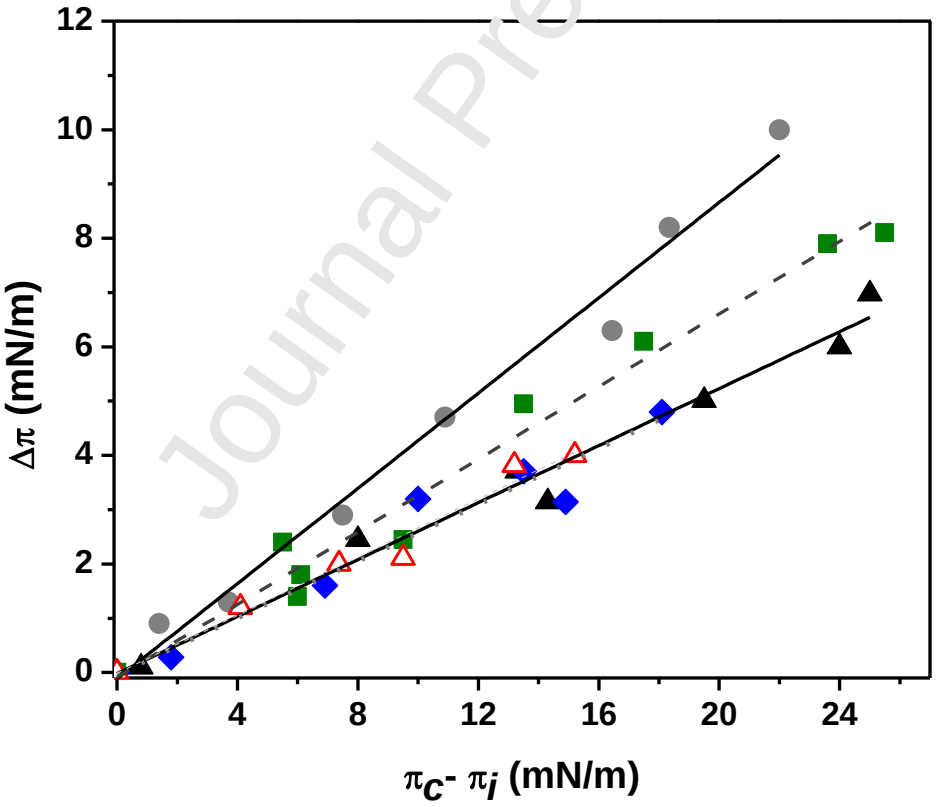

Fig. 8. Perturbation of lipid monolayers *vs.* the decrease of the initial surface pressure with respect to the critical values. (▲) DMPC, (△) DMPE, (♦) DPPC, (■) DOPC, (●) DPhPC.

The slope of each the curves (K) in figure 8 is directly related to the difference in chemical potential of water between bulk (μ_w) in monolayer in the presence of bioeffector (μ_{wp}) with respect to that difference in the absence of it (μ_w - μ_{wc}) (Eq. 8).

$$K = \frac{(\mu_w - \mu_{wp})}{(\mu_w - \mu_{wc})} \qquad (8)$$

The slope changes according to the acyl chain structure (length, unsaturation and CO groups) which is congruent with the increase in isolated populations put into relevance by FTIR in figure 3. This correlation is shown in figure 9.

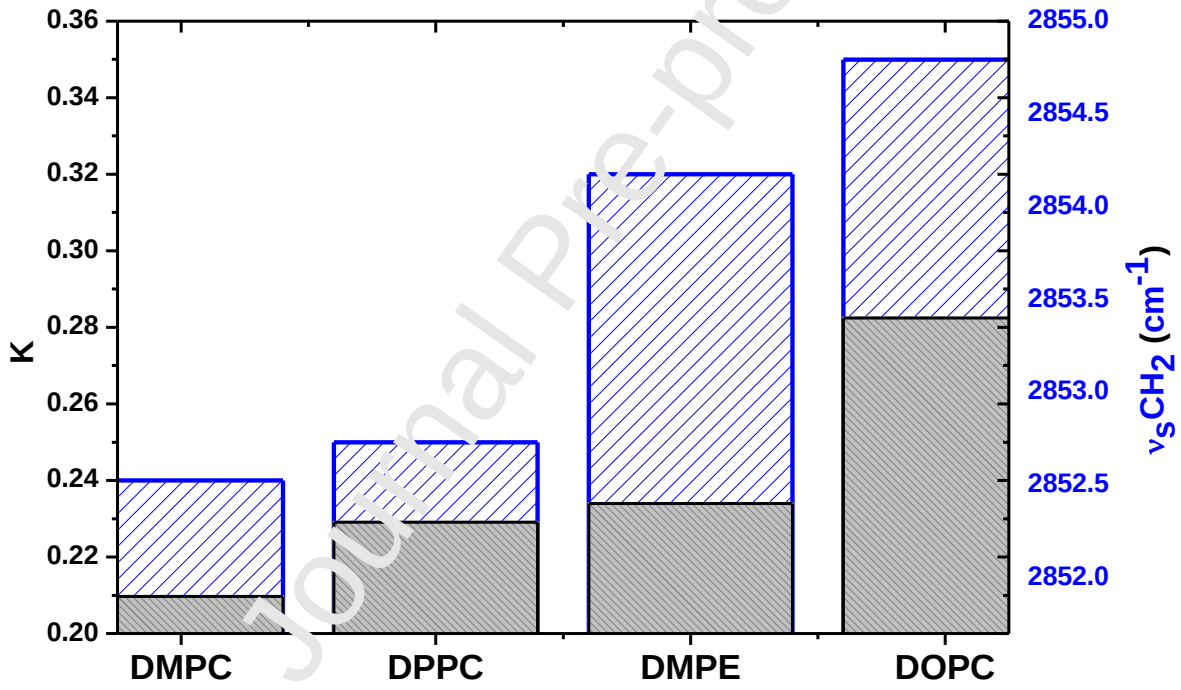

Fig. 9. Correlation between the relative difference in water chemical potential given by Eq. (8) (K) with CH_2 frequency stretching reflecting the isolated populations of CH_2 for different lipids.

That is, the water available for the membrane response is related to the water organized at the acyl chain regions, i.e. confined water.

In other words, the propensity of the membrane to react when it is chased by a bioeffector is given by the water quality in the surrounding of the hydrated lipids. This water confined between the hydrated head groups has different surface tension and

dielectric properties than pure water since the H bond arrangements are influenced by the lipid contours [180-182].

9. Relaxation processes.

Thermodynamics is a fundamental tool to understand the stability of a system but it must be also taken into account that around equilibrium, fluctuations may occur. Each of these fluctuations can be amplified by the presence of a bioeffector and the response of the system to attain a new equilibrium is governed by the kinetics. In this regard, several aspects should be considered that involve simple and complex mechanisms most related to structural changes along the process and ultimately related to water lipid arrangements.

The kinetic profile of the evolution of the system, observed in figure 7A, from an initial state (π_i) given by a surface pressure/water activity value to a final one (π) can be plotted in terms of the solution of the second Fick´s law.

The changes in bioeffector surface concentration (Γ) follows the Ward and Tordai equation:

$$\Gamma = 2\frac{C_b}{\pi^{1/2}}\, D^{1/2}t^{1/2} \qquad (9)$$

where C_b is the bulk concentration, and D is the diffusion coefficient of the bioeffector.

The surface pressure can be expressed in terms of interfacial concentration (Γ) by

$$\Pi = \Gamma RT \qquad (10)$$

where T is temperature in kelvin and R is the gas constant.

Therefore, the surface pressure is a function of time as expressed by a second Fick's law solution for molecules diffusing from bulk solution at a distance x towards the boundary surface of the monolayer according to

$$\Pi = \frac{2RTC_b}{\pi^{1/2}}\, D^{1/2}t^{1/2} \qquad (11)$$

where the bulk concentration (C_b) remains effectively constant during the process. Then, the rate processes can be generally defined by the relaxation coefficient n by Eq. (12) [183].

$$\Delta\Pi = C * t^n \qquad (12)$$

where C= $\frac{2RTC_b}{\pi^{1/2}} D^{1/2}$

For n=0.5, the processes consider a constant diffusion coefficient following Fick´s law and it is represented by a straight line in figure 10 A. Larger values than 0.5 indicate that the diffusion coefficient varies along the process which may be related to a modification of the lipid matrix.

In order to show the dependence of the relaxation coefficient with the membrane state (determined by surface pressure and water activity) the effect of surface pressure and cholesterol ratio on the value of n is analysed.

In figure10 A, it is observed that the kinetic pattern evolves from a non fickean (n> 0.5) to a fickean (n=0.5) process when going from low to high pressures.

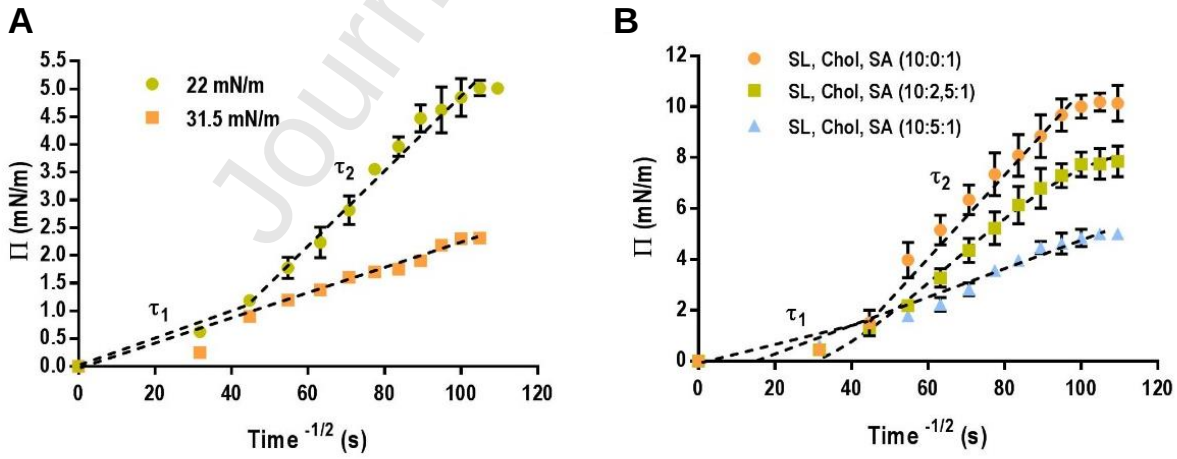

Fig.10. Effect on the diffusion pattern in the interaction of a surface protein with PC/SA monolayers with different: A) Surface pressure 22 mN/m (green circles) and 31.5 mN/m (orange squares) and B) different Chol ratio: Chol 0 (orange circles); 2,5 Chol (green squares); 5 Chol (blue triangles) molar ratios; (Adapted from Ref 153.)

The effect of the presence of water on the n value can be also observed when Chol ratio is varied in the membrane. As discussed above the increase in cholesterol decreases water in the membrane [129, 130, 184]. In figure 10 B, it is clear that the increase of cholesterol displaces the behaviour to a fickean process. The explanation of this observation is congruent with that assayed above for the surface pressure.

Considering that the surface pressure on the monolayer fixes the interfacial water activity, it may be concluded that the fickean process appears when water is decreased in the membrane either by surface pressure increase or cholesterol increase. In other words, the relaxation implied in a non fickean process is related to the presence of water and its reorganization. Taken together, the relaxation process can be ascribed to water in the system confined between the lipids.

10.- Conclusions.

Due to its dimension´- two molecular leaflets and no more than 2-3 layers of water molecules per lipid - bilayers cannot be considered as a true phase in thermodynamic terms, i.e. a region of space throughout which all physical properties of a material are uniform.

There are strong evidences that uniformity is not present both at macroscopic and microscopic scale along the direction normal to the membrane surface. The first is revealed when the incorporation of solutes does not obey the Henry.s law, the second, when the water content at hydrocarbon links are analyzed with FTIR spectroscopy.

The so-called concept of fluid phase - which is ascribed to be the most frequent state of cell membranes- exhibit liquid-crystalline properties and in consequence it is claimed to be uniform. However, according to the molecular structure of acyl chains (Fig. 3), hydrocarbon region properties are not the same for different acyl chains due to its different ability to capture water.

Lipid bilayer inhomogeneities are noticed along the hydrocarbon region and in the polar interphase, considering this as an aqueous bidimensional solution of hydrated polar head groups. The organization of water molecules along lipids can therefore be visualized as those facing hydrocarbon walls and those forming part in the first and the second hydration shell in the polar groups. This gives a complex energetic map of the membrane surface. As a consequence, a dynamic behaviour makes structures to fluctuate around a given one, and simultaneous fluctuations in different regions can occur in a coordinated way. According to the nature and strength of the interaction of the bioeffectors, the fluctuations can be

displaced changing transiently the local structure and /or to propagate to give a global phenomenon (synergism).

The kinetic patterns deviating from a single diffusional process - in which the matrix is maintained unaltered - show a clear dependence with water content as derived from its behaviour with surface pressure and cholesterol (Fig.10). Thus, this strongly suggests that fluctuations are due to water structural reorganization in the insertion processes.

The relation of surface pressure in monolayers and bilayers cannot be only operative. It also implies an important conceptual frame since they are related with the interfacial water activity. This is to say that variation in the surface pressure are indeed changes in the water surface activity due to rearrangements of water and H bonds between water and lipids.

In spite of the effort to validate the Singer and Nicolson model on the basis of the information obtained by optical methods, none of them takes into account the thermodynamics implications of the domains observed. Moreover, the paradigms discussed in this review are not explicitly considered in the schematic descriptions of membrane models. It is clear that the present analysis has been done on the basis of data obtained in model systems composed by only one or two lipids. A challenge in the next future is to extent this analysis to complex mixtures in which such heterogeneities were observed. However, few information is available in model systems in order to accomplished such aim.

The standard cartoon of lipid membranes as a two lipid leaflets is not enough to describe the relevant role that membrane has in biological processes. This review has focused on physical chemical process of lipid interphases, mainly its thermodynamics response. The evolution of the living systems has also its thermodynamic background. The formation of autocatalytic ensembles in compartmentalized systems i.e. a membrane of amphyphilic compounds enclosing a specific media is due to water entropic changes. Evolution made that such barrier could adapt to let in and out compounds of chemical reactions in a pre-biotic stage. to allow the cell to maintain a steady state preserving a low entropy interior at the cost of energy flow [185]. They conform extended surfaces in which chemical reactions take place much faster that in tridimensional bulk solution.

The compartmentalization enclosed water that may acquire properties different than a bulk phase due to the presence of macromolecules and the membranes. In a kind

of physical symbiosis, membrane affects water properties and viceversa, water affects membrane ones [186].

This speculation brings about some cautions in the visualization of the membrane as an open system. It is usually understood that open is due to exchange of matter. However, the point raised in the previous point also denotes that membrane must be coupled to other elements of the cell to be operative (i.e, a device that may give place to oscillations around a point to maintain a steady state at low entropy at expense of exchange of minimum energy. The approach that cytosol may be organized to accomplish this function is a possibility to be discussed. If this is so the state of water in the cell is a matter of importance and asymmetry generated by this condition cannot be disregarded in cell membrane function.

The inclusion of the thermodynamic response put into relevance the collective properties of the membranes in terms of its composition and interaction with its aqueous environment. In this context, as cells may regulate membrane composition, they may, by extension, modulate water activity in terms of energy and structure. In this regard, lipidomics show the complexity of plasma membranes in lipid composition and its implication in signaling processes. In this view lipidomics may derive in aquaomics for which it is understood that water acts as a mirror of lipids species and vice versa water properties determine lipid organization [68, 187]. How do cells manage to tune these interphase properties to accomplished cell function is a matter of future and exciting discussion.

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