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Structural and thermodynamic properties of water–membrane interphases: Significance for peptide/membrane interactions

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ABSTRACT

Water appears as a common intermediary in the mechanisms of interaction of proteins and polypeptides with membranes of different lipid composition. In this review, how water modulates the interaction of peptides and proteins with lipid membranes is discussed by correlating the thermodynamic response and the structural changes of water at the membrane interphases.
The thermodynamic properties of the lipid–protein interaction are governed by changes in the water activity of monolayers of different lipid composition according to the lateral surface pressure. In this context, different water populations can be characterized below and above the phase transition temperature in relation to the CH_2 conformers' states in the acyl chains.
According to water species present at the interphase, lipid membrane acts as a water state regulator, which determines the interfacial water domains in the surface. It is proposed that those domains are formed by the contact between lipids themselves and between lipids and the water phase, which are needed to trigger adsorption–insertion processes. The water domains are essential to maintain functional dynamical properties and are formed by water beyond the hydration shell of the lipid head groups. These confined water domains probably carries information in local units in relation to the lipid composition thus accounting for the link between lipidomics and aquaomics. The analysis of these results contributes to a new insight of the lipid bilayer as a non-autonomous, responsive (reactive) structure that correlates with the dynamical properties of a living system.

Contents

1. Introduction. Membranes in a crowded system

The irreversible changes produced by death promoted by the partial or total drying of cells and the programmed cell death triggered by dehydration called the attention to the balance between levels of water in biological structures in order to fulfill physiological functions [1,2]. In

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this regard, it is known that cell structure can be preserved when cells are dried in the presence of solutes that may replace water and help to its recovery. Several sugars and sugar amino acids seem to be suitable for these purposes, such as trehalose, sucrose and arbutin [3,6–11]. However, although nearly the same structural parameters than those characterizing fully hydrated systems are maintained in a dry protein or membrane in the presence of those compounds [3], cells do not grow in a dehydrated state. Thus, even if structure is preserved, water must be present in the liquid state to gain functionality. In this sense, functionality means thermodynamic propensity to respond to external agents. This is a question of surface free energy achieved by hydrated states in the cell structure.

Analysis of hydration dynamics focused on self-diffusion rates and dielectric constants as a function of crowding show significant changes in both structure and dynamics of water under highly crowded conditions. The structure of water is altered mostly beyond the first solvation shell [4]. Thus, as cytoplasm is crowded of macromolecules, in order to achieve functionality and efficiency, it is hard to understand that there is no contact between the intracellular material and the membrane.

In contact with interfaces, interacting with ionic species and/or with large organic molecules, water does not behave in the same manner as it does in the pure bulk liquid. Water dynamics are fundamental to many processes such as protein folding and proton transport [5]. Thus, it is reasonable that, as a complex system, the properties of cells should emerge from the interaction and interrelationship of the surface constituent's parts, such as membranes and macromolecules. A simple calculation shows that more than 40% of the mean volume of a cell is occupied by macromolecules and internal particles. In the remaining spaces, the mean distance between ions in a 150 mM KCl solution is, at most, 3–4 water molecules [1]. Therefore, it is plausible that the performance of a cell should consider a connectivity between the surface properties of the membrane and the so called “aqueous soluble” macromolecules. In this regard, water properties in the vicinity of lipid membranes and proteins would play a unique role as a frame of reference for cooperativity and synergistic phenomena. In order to fit the membrane response to cell performance, the concept of a membrane as a thermodynamic and structural entity in a complex system imbibed in water should be revised.

2. Water or membranes

The classical picture of a cell is a compartmentalized system in which the membrane is the barrier of contention and selectivity of the cellular material [12]. In this classical view, the core of the membrane, the lipid bilayer, is described as an autonomous rigid phase in which partition rules the thermodynamics. From the electrical standpoint, the bilayer is considered as a slab of low dielectric permitivitty that should be impermeable to water, ions and polar solutes. Hence, transport processes appear to be favored by the insertion of proteins to carry out specific permeation. In this context, water is for biologists as the canvas for the painters. It appears merely as the support solvent in which the cell structures dissolve, aggregate, organize and stabilize. In other words, the aqueous environment plays a passive role while function is carried out by macromolecules dispersed in it.

The concept of living cells as a membranous bag containing an aqueous solution, was first seriously challenged by Troschin [13]. Later, Ling in 1962 [14] proposed that most of the water inside a cell was polarized as multilayers located on protein surfaces, being an extremely poor solvent for ions. K^+ was accumulated by normally metabolizing cells because under those conditions the carboxyl groups of proteins preferentially associated with K^+ ions rather than Na^+. This theory, the association-induction hypothesis, had great explanatory potential but nuclear magnetic resonance (NMR) measurements of water protons in tissues revealed that relatively few water molecules, which were quite strongly immobilized, exchanged rapidly with normal water molecules. NMR interpretation depends upon the model chosen to describe the state of water. Thus, the description of intracellular water in terms of a few bound molecules exchanging rapidly with normal liquid water may not strictly correspond to reality. However, this hypothesis cannot be dismissed without independent experimental evidence obtained by other methodologies beside NMR [15,16].

In conclusion, neither the cell as a membrane bag nor the association-induction hypothesis describes completely the structure–function relation of living cells with experimental backgrounds and on a solid thermodynamic ground. In the first case, cells are mostly considered as composed by proteins and membranes with conformational properties not related to water. In the second, it is hard to reconcile the response of cells to external stresses and perturbations without considering the influence of the different hydrophilic and hydrophobic surfaces of biological components on the thermodynamic and structural properties of water.

Both of these two reductionist approaches put aside the role of water as part of the cell structures and the thermodynamic properties that biological surfaces may confer to it, respectively. In terms of thermodynamics, it is usually invoked, that biological phenomena should not be described by means of classical thermodynamics because biological systems are in the stationary state. This is important when considering the exchange of matter across the membrane in which a coupling between internal chemical process and the transport of matter is achieved. However, this complex matter is outside the scope of this review. With the same criterion, in the context of the present discussion, the key role of water in the thermodynamics of cell membrane response should be considered in terms of surface phenomena.

Formalisms of classical thermodynamics usually employed to understand processes in biological systems, are based on equations derived for gaseous systems in a large volume disregarding surface phenomena and in the absence of fields operating on it. Moreover, this thermodynamic formalism applied to solutions mostly considers water as a continuous solvent where macroscopic properties are thought to be still valid.

In consequence, interaction of solutes with lipid membranes is understood as a partition between the bulk aqueous phase and the bulk membrane. In this approach, bulk membrane is a low dielectric phase, ascribed to the hydrocarbon phase excluding water. Therefore, highly hydrophobic solutes are expected to dissolve in the lipid matrix while polar and charged molecules should be excluded from it.

Partition is a bulk phenomenon and hence the solubility ratio is achieved considering that membrane and water are isotropic pure solvents. The partition constant is given by the difference in the standard chemical potential (free energy) of the solute in water and the solute in membrane.

Two main process of partitioning has been described: the classical hydrophobic effect and the non-classical hydrophobic one. The reasons for these differences come from the relative contributions of enthalpy and entropy to transfer free energies: the classical effect is driven by a positive entropy change and the non classical by a negative enthalpy change.

The classical hydrophobic effect arises from the tendency of nonpolar molecules to avoid contact with water. This approach makes the hydrocarbon core of lipid bilayers a favorable environment for nonpolar solutes [17]. The hydrophobic effect is generally considered to arise from the release of ordered water molecules around the solute's nonpolar surface, which is the source of positive entropy.

For bulk phases at room temperature, entropy arising from the hydrophobic effect is dominant, whereas for bilayers enthalpy is often dominant [17–19]. Thus, the free energy of transfer of nonpolar solutes from water to lipid bilayers is often dominated by a large negative enthalpy rather than the large positive entropy expected from the hydrophobic effect. One reason invoked to explain why partitioning into lipid bilayers is much more complicated than bulk-phase partitioning, has been the anisotropic and heterogeneous nature of bilayers [18–20].

The enthalpy-driven partitioning, referred to as the "nonclassical" hydrophobic effect [19,20], appears to be a unique feature of solute–bilayer interactions. This has been questioned suggesting that the nonclassical hydrophobic effect is in reality the "bilayer effect" [28]. By this imprecise definition, it is thought that the bilayer is not equivalent to a bulk hydrocarbon phase since it consists of a slab of two layers of molecules sandwiched by two polar regions. Structurally, the free energy minimum should be the result of the balance of diverse molecular interactions, hydrophilic and hydrophobic moieties of the solute with different regions (polar and non polar) of the bilayer. Because even very low concentrations of bound peptides cause significant changes in bilayer thickness [21,22], it is reasonable to assume that the presence of bound peptides disturbs this balance, with thermodynamic consequences.

Enthalpy–entropy compensation is a general feature of processes in biological systems. A simple thermodynamic argument suggests that enthalpy–entropy compensation is a general property of weak intermolecular interactions, and that the two contributions to the free energy should nearly balance out for a hydrogen bond at 300 K [23].

Enthalpy–entropy compensation is almost complete for associations involving water at around 300 K that is ubiquitous in the chemistry of living systems. The term refers essentially to the specific linear relationship found to exist between the change in enthalpy and the change in entropy in many biological processes, especially those occurring in aqueous solution and involving changes in hydrogen bonding. This may be ascribed in principle, to a given distribution of water clustered between the acyl chains (classical hydrophobic effect) and bound by H-bonds to the polar head group regions (non-classical hydrophobic effect) in the lipid matrix [18–20,23–25].

In this context, the enthalpic vs the entropic changes in the dissolution of different OH bonding solutes in lipid membranes have been analyzed in a previous review [26]. Different alcohols can be grouped into three families: one corresponding to compounds in which the hydrophilicity remains constant (only one OH in all of them) and hydrophobicity grows with the addition of CH_3 groups in a tetrahedral array, such as in the series ethanol–propanol–isobutanol (Fig. 1A).

The second family corresponds to solutes in which the size increases maintaining the hydrophobic–hydrophilic balance due to the addition of a CH_2 with an OH group. This family includes ethyleneglycol, glycerol, erythritol (Fig. 1B). The intersection of these two series is urea, which has profound effects on water structure and hydrophobic interactions. In the third family (Fig. 1B), hydrophobicity character grows with the chain length of monoalcohols from methanol, ethanol and butanol in a nonlinear pattern [26,27].

The simple analysis of these curves indicates that the extrapolation to $\Delta H = 0$ gives negative entropy for the solute which have the ability to form hydrogen bonds (ΔS: −2.6 cal/mol·K for the first family and −5.4 cal/mol·K for the second). However, positive values (ΔS: +5 cal/mol.K) are obtained with alcohols in which the hydrophobicity grows at expense of the chain length increase (the third family). This denotes that a classical hydrophobic effect can be possible (in the absence of other interactions) only when nonpolar chains can be extended along the lipid acyl chains. The hydrophobic effect is related to the large negative heat capacity associated with the dehydration of nonpolar surfaces [24].

If hydrocarbon groups are added to form bulky molecules maintaining the H bonding capability (such as the first series of tetrahedral molecules) non classical hydrophobic effect (enthalpy driven effect) is found, the same as that observed for solutes in which the hydrophilic and hydrophobic balance is not altered in the series of the second family.

In the light of the entropic values, it is clear then that hydrophilic compounds according to its size may stabilize in different regions of the lipid membrane in comparison to long hydrocarbon chains due to the different nature of the molecular interactions with the membrane. Positive entropy means hydrophobic and short range intermolecular forces and enthalpic driven processes (negative entropic change) would be related to the formation of hydrogen bonds between the H

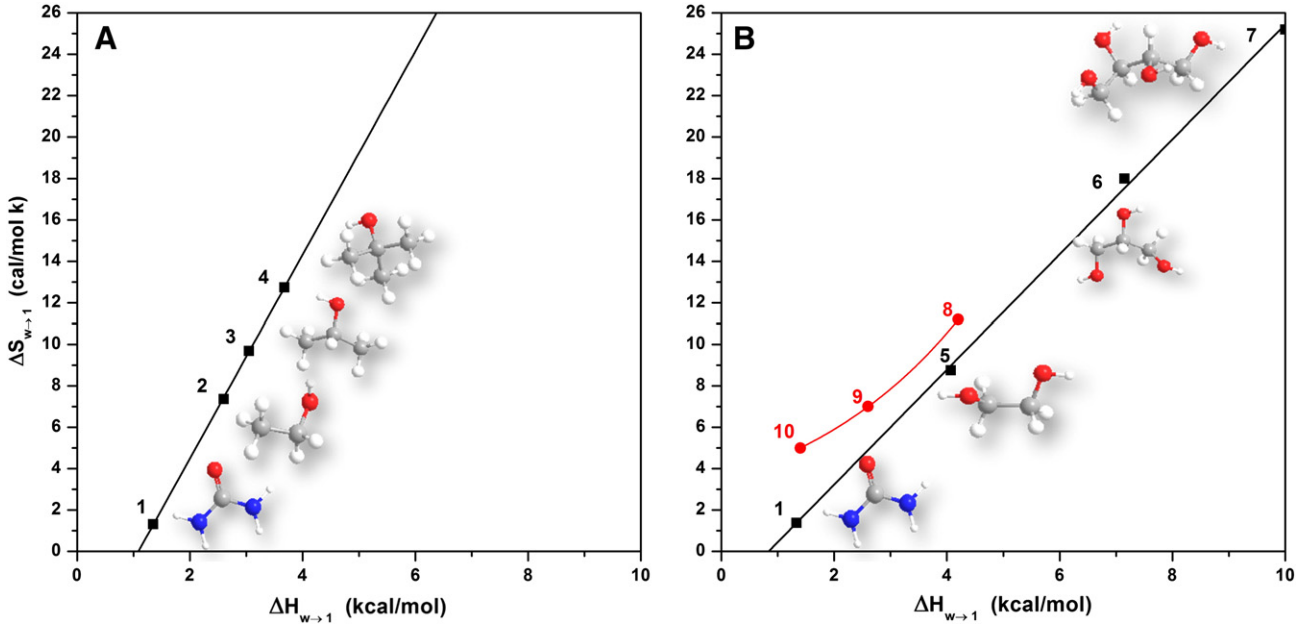

Fig. 1. Entropic–enthalpic compensation of the partition of different families of polyalcohols in DMPC bilayers. A) First family: Urea (1), ethanol (2), propanol (3), isobutanol (4). This family maintains only one H bonding groups (OH) while increasing the hydrophobic character and the molecular volume in a tetrahedral array. Slope: 0.0047; ΔS: −5.4 cal/mol·K. B) Second family: Urea (1), ethylenglycol (5), glycerol (6), erythritol (7). The hydrophobic–hydrophilic character is maintained along the series, while the size of the molecule increases by adding one HCOH residues. Slope: 0.003; ΔS: −2.6 cal/mol·K. Third family: Methanol (8), propanol (9), butanol (10). This group maintains only one H bonding group (OH) but the size of the molecule increases by increasing the chain length in one CH_2 in the series. ΔS: +5 cal/mol·K. 1st and 2nd families would correspond to those obeying a non-classical hydrophobic partition for which the entropic change is negative. 3rd family would correspond to a classical hydrophobic partition (entropy driven partition).

donor of the compound and the H-acceptor of the membrane. Whether this interaction implies replacement or mediation of water molecules is a matter of a deeper analysis [28–31].

Thus, different solutes partition in different regions of the bilayer according to their size and polarity. On the other hand, molecular dynamics calculation has suggested that partitioning of charged and polar side chains is accompanied by water defects connecting the side chains to bulk water [28–30]. The energetics of partitioning cannot be considered as a simple partitioning between water and a hydrophobic phase, at least for some amino acids. Lys, Glu, and Asp become uncharged well before reaching the center of the membrane, but Arg may be either charged or uncharged at the center of the membrane. This has been explained by suggesting the formation of water defects in the membrane phase. In addition, Phe has a broad distribution in the membrane but Trp and Tyr localize strongly to the interfacial region, specifically at the carbonyl group level [31]. Taken together, these two pictures suggest that the bilayer has different degrees of affinity by the different amino acids and thus it cannot be considered as a homogeneous solvent. Moreover, the specific role of hydration sites such as carbonyl groups should be considered. This will be further analyzed in Sections 6 and 7.

These inferences and the possibility of water clusters in lipid membranes motivate a closer inspection of the water distribution along the membrane thickness and topology in two directions. The first one is in terms of membrane structure, in regard to the location of water in the lipid matrix and its relation to the lipid chemical residues. The second one is to consider the thermodynamic properties of these water-restricted domains in regard to the interaction of compounds with the membrane.

Several assumptions are usually made on the water location in the bilayer structure that greatly influences the final numbers of the area and thickness of lipid bilayers [32]. In terms of thermodynamics, water may differ in its solvent properties according to its interaction with different types of surfaces [33].

Israelachvili and Wennestrom [34], Israelachvili [35] and Pashley et al. [36] measured a long-range effect on water structure at hydrophobic surfaces, which was shown to have profoundly modified solvent properties over distances up to 2 to 3 nm [37]. In this context, the thermodynamic properties of the hydrated membrane surfaces requires a particular inspection that should be related to the structural properties of water imposed by the heterogeneity of the membrane composition under the approach of restricted media. Thus, water would be responsible not only for self-assembly of protein–membrane ensembles, but also for the response of cells to different stresses as well.

The relationship between the functional activities of the biological structures and the lability of the water ensembles at the lipid surfaces has not received considerable attention. Two points are usually disregarded. The first is that the bilayer thickness depends on the lipid heterogeneity because it includes a hydrophilic region in which the phosphates and several esterified groups (choline, ethanolamine, glycerol and inositol) protrude into the water media. The second is that the membrane may expose regions of different polarities to water according to the lateral pressure of its components. In each of them, the abilities of water to form hydrogen bonds with itself and between the membrane groups appear as a relevant issue from the point of view of surface thermodynamics and small systems.

The complexity imposed by the presence of water in the lipid membrane structure explains the need to revise the concept of membrane in terms of its response to environmental perturbations. This is fundamental to inspect the properties having in mind its biological relevance.

In contrast to biopolymers (proteins and nucleic acids that contain information in its covalent structure), lipids manifest its biological functional response as complex mixtures [38]. In this context, the lipid heterogeneity together with the complex hydrogen bond network, extended between lipids and lipids with water, give a versatile matrix with the ability to respond to multiple physicochemical stimuli.

The lateral organization of the lipids in domains may be due to direct interactions between head groups and hydrocarbon chains, but water is an additional essential component. Details of water location and its properties in these restricted domains are scarce. The great variety of lipid composition and the multiple combinations in mixtures acquires relevance and functional meaning. As far as the properties of water microenvironments may be changed by the protrusion of the different polar moieties into the water phase, lipid species may generate new different water species each of them identified by the type of interaction they may have with its neighbor water molecules and/or chemical groups of the lipids. Thus, at this point, lipidomics approaches to aquaomics. In other words, lipid species would give an in-print on water with specific thermodynamic features for membrane response.

Despite extensive work, experimental data about the structure of water and the network of hydrogen bonds at the polar interface of lipid membranes is still a matter of debate [39–43]. In this context, several aspects of lipid hydration and its thermodynamic properties have not been completely rationalized.

In a quite extensive review, the emphasis is made on considering the lateral surface properties across the lipid thickness. Special attention was given to the heterogeneity of lipids in the membrane plane and the lateral coexistence of lipid domains. From these studies, some indications about water penetration can be inferred along the interpretation of response of fluorescent probes [12].

Thus, water by itself may constitute domains not homogeneously distributed along the interphase. Water immediately adjacent to the glycerol backbone, the side groups and the hydrocarbon chains, has a lower activity than in a zone of similar size in the bulk solution and would constitute regions with different excess surface free energy due to the membrane group–water interaction.

To decipher the biological relevant membrane surface properties, the stability of the different arrays of water around the different membrane groups and its dynamical properties should be clarified. This includes namely: water as part of the membrane structure, the definition of the lipid interphases, the identification of the sites of hydration at the membrane surface; the synergism of their hydration and its modulation according to the lipid species (usually found in biological membranes in terms of head group and fatty acid chains).

In this review, we discuss the electrical, the thermodynamic and the structural properties of lipid water interphases and the consequences of the perturbations that the interaction of aqueous soluble proteins or peptides may produce on them. The analysis of these results might contribute to a new insight of the lipid bilayer as a non-autonomous, responsive (reactive) structure that correlates with the dynamical properties of a living system.

To say that the membrane acts as a non-autonomous phase means that the membrane (i.e. the lipid bilayer) is a phase whose properties depend on the phase which it is in contact with (i.e. water), opposite to the traditional view of an autonomous, rigid nonpolar slab sandwiched by bulk water phases. This property has been denoted as responsive membranes by Sparr and Wennestrom [44]. This definition implies that the phenomena occurring at membrane level occurs with the change in the membrane structure, that is, a response of the membrane to some component of the adjacent media, reflected in the thermodynamics and the kinetics of the protein–membrane interaction. The change in the membrane structure may be local with propagation to the whole structure in the plane and along the thickness with different degrees of cooperativity and synergism. In this regard, it should be recalled that the arguments given to understand the difference between classical and non-classical hydrophobic effects, lay on the possibility that bound peptides disturb the bilayer thickness, with its thermodynamic consequences due to the interaction within the hydrophilic and/or hydrophobic regions [22,27].

3. Water in membranes: the excluded volume and the hydration forces

Water in membranes is an old problem in membrane biophysics. The presence of water was recognized in several studies as contributing to the bilayer permeability barrier properties for nonelectrolytes [45], as the origin of the repulsion hydration forces between membrane surfaces [46–48] and as a substantial part of the surface membrane potential (dipole polarization and potential) [49–51]. In addition, based on the changes in the dielectric properties of bilayers, it has been postulated by Simon and McIntosh that water penetrates deeply into the membrane interior [52].

The fact that water in membrane structure may be adjacent to surfaces of different polarities has stimulated the idea that water may be in different structural arrangements such as low density (highly hydrogen bonded waters) and high density (non bonded waters) structures. FTIR spectroscopy has provided some evidences of these two states of water [40,41,53,55].

One of the consequences of the classical model of membranes and the application of classical thermodynamics (in the terms given in the previous section) is to consider that solute penetration (and permeability) is mainly driven by a partition phenomena, i.e. the differential solubility of a given solute between the bulk water and the bulk of the membrane, this one usually considered as a pure hydrocarbon phase. We explained in Fig. 1 the differences between partition in a homogeneous phase and in the membrane phase. This view implies that, on the structural side, the membrane is a nonpolar phase and, on a thermodynamic approach adsorption (surface phenomena) is disregarded. In contrast, measurements of the polar solute partition in lipid bilayers of multilamellar and sonicated vesicles, led to the conclusion that permeability barrier properties of lipid membranes are determined to a large extent by the non solvent properties of the water layers located at each side of the membrane leaflet [45]. These two regions at the sides of the bilayers are about 1 nm thick and are composed of 18–20 water molecules per lipid in phosphatidylcholines [56]. This region, denoted as an excluded volume for solutes, was consistent with X-ray diffraction and NMR measurements [47,56]. These results are in agreement with the findings cited above of Wiggins and van Ryn [37], in the sense that membrane perturbation on water structure extends a few Å from the surface and change its solvent properties. The size of these excluded volumes contribute to the hydration force measured between membranes upon mutual approach to interbilayer distances smaller than a few nanometers [46–48,57].

Depending on the head group, phospholipids bind approx. 0.5–3 water molecules/lipid very tightly (E = 40 kJ/mol) [57–59]. Phosphate groups saturate when water/lipid ratio is around 6 [60]. Interaction between lipids appear to be nearly the same as in excess water when approximately 10 water molecules/PC is reached, as judged by T_m and lipid rotational mobility, whereas the bilayer appears more or less saturated at 22 water molecules/PC [56]. These results account for different membrane groups with different extents of hydration.

A molecular dynamical analysis indicates that waters in a hydrated lipid bilayer can be classified into four dynamically connected water layers. A detailed analysis of the water dynamics within these four regions shows that there exists a cooperative molecular motion between the hydration waters and the DMPC lipid molecules [61].

Therefore, it would be expected that at different hydration degrees different structural water arrangements would stabilize structurally different surface arrangements. This point will be discussed in the last section.

4. The definition of the membrane interphase

In order to proceed further with the determination of the properties of lipid membranes in which water is part of the structure, an updated definition of the regions of the membrane is required. The regions of 1 nm thick at each side of the bilayer, denoted as an excluded volume for solutes in the previous section, contain around 18–20 water molecules per lipid in phosphatidylcholines [45,56]. The presence of those interphases may be considered as an ulterior refinement of the membrane model, since from the structural point of view it can be described as a new composite element consisting of the lipid bilayer itself and the regions of hydrated groups at each side. Moreover, the admittance of the presence of hydrated surfaces, oblige to revise their thermodynamic properties, as we will discuss in Section 6.

Both for structural and thermodynamic purposes, we will identify as the water interphase as the region confined between two ideal planes: one located at the limit between the hydrocarbon core and the polar head groups, usually named the water–hydrocarbon interface, and an external plane tangent to the hydration shell of the polar head groups [11]. A schematic description of these regions and planes is shown in Fig. 2.

The carbonyl groups of the ester bond of the phospholipid are located at an inner plane and the interphase is enclosed between this plane and that defined by the tangent to the excluded volume of the hydration shell of phosphates. This schematic representation takes into account the protrusion of the different polar moieties such choline, serine, ethanolamine, glycerol into the water phase which may induce different water arrangements at the interphase region. The planes are the boundaries of the interphase, which is defined as a phase composed by the polar head groups of the great variety of the lipid found in cells with its hydration shells imbibed in water as ions in a solution. The anisotropy of the region located in the plane of the membrane and the small bilayer thickness makes of this region a bidimensional water solution where at least two kinds of water can be distinguished: I. confined water, buried in the first carbon of the hydrophobic chains. This water varies with the lateral compression due to thermotropic transition or to isothermal expansion or compression, II. hydration water around the polar head groups (tightly bound, high density) and III. water beyond the hydration shell (loosely bound, low density water). The probable number of water molecule bound to different chemical groups is detailed in Table 1. In addition, increase of water due to membrane expansion is also denoted. The variation of the amount of hydration water and confined water due to membrane expansion is schematically illustrated in Fig. 3.

This preliminary distinction between two kinds of water arrangements is relevant to understand functional biological properties within the frame of surface thermodynamics. On one hand, as said above, the great variety of lipids in membranes and the multiple combinations in mixtures may change the protrusion of the different polar moieties into the water phase affecting the dimensions and properties of the lipid interphase region and the magnitude and properties of the hydration shell. Each hydrophilic moiety will organize water according to the stereochemistry of the groups and thus, lipidomics will determine the water interfacial properties.

In order to justify how the inclusion of these interphases determines the response of lipid membrane and therefore to demonstrate that no model of lipid membrane employed to mimic biological response can disregard them, we analyze further the electrical, thermodynamic and structural properties of these interphase regions in the following sections.

5. Electrical properties of the lipid membrane interphases

As said above, the formation of pockets filled with water has been postulated in order to explain the insertion of polar peptides and aminoacids into lipid membranes [62,63]. These defects can be formed in relation to membrane deformation, such as expansion–contraction processes and membrane phase state. However, it is not clear, neither structurally nor energetically how they may be formed considering the definition of membrane given above. Moreover, the possibility to increase water in the membrane due to expansion (and its extrusion due to contraction) was postulated in Table 1 and Figs. 2 and 3 but

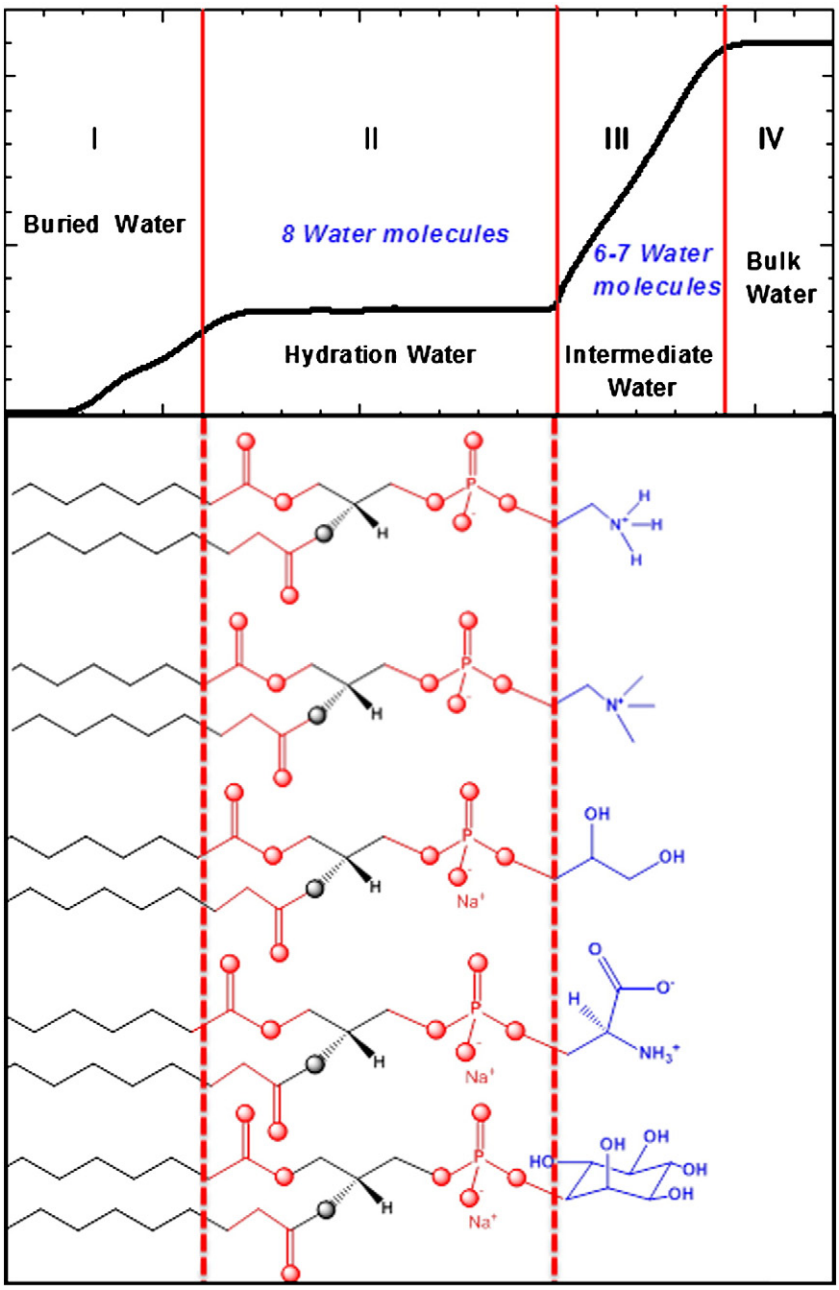

Fig. 2. The membrane interphase region. Region I contains only the hydrophobic lipid residues and contains buried water (confined water). This region may vary according to packing and phase state of the lipids (see Fig. 3) Region II begins at the carbonyl groups. In this region, the total system density increases dramatically due to the tight hydration shell of charged groups such as phosphates. It is chemically the most diverse region, containing both hydrophobic and hydrophilic components and hydration water. This region can be considered as a binary solution in which polar head groups with their hydration shells are imbibed in water (second hydration shell). Region III contains the groups bound to the phosphates that protrude into the aqueous phase. In this region, water may preserve its tetrahedral arrangement due to its binding to OH groups or by the adjacency of non polar moieties such as cholines (clathrates). Region IV is non perturbed bulk water.
Partially adapted from ref [61].

no further experimental evidence was offered. In the following, experiments of electrical capacitance in monolayers seem to justify this variation of water content in monolayers.

In terms of the classical model, lipids organized in a bilayer present a specific capacitance ($C_m = C/A$) given by

$$C_m = \varepsilon\varepsilon_o/d_m \quad (1)$$

where ε_o is the permittivity in vacuum and d_m the monolayer thickness of the permittivity ε.

This relation describes a membrane with a single element in which ε would correspond to the dielectric permittivity in the hydrocarbon

Table 1
Distribution of water molecules in the region described in Fig. 2.

Region I	Region II	Region III	Total
After expansion	4–6		
Carbonyl region		Fixed: 7–9 variable upon expansion–contraction: 4	
Fixed at phosphate (hard core)	6–7		6–7
			17–22

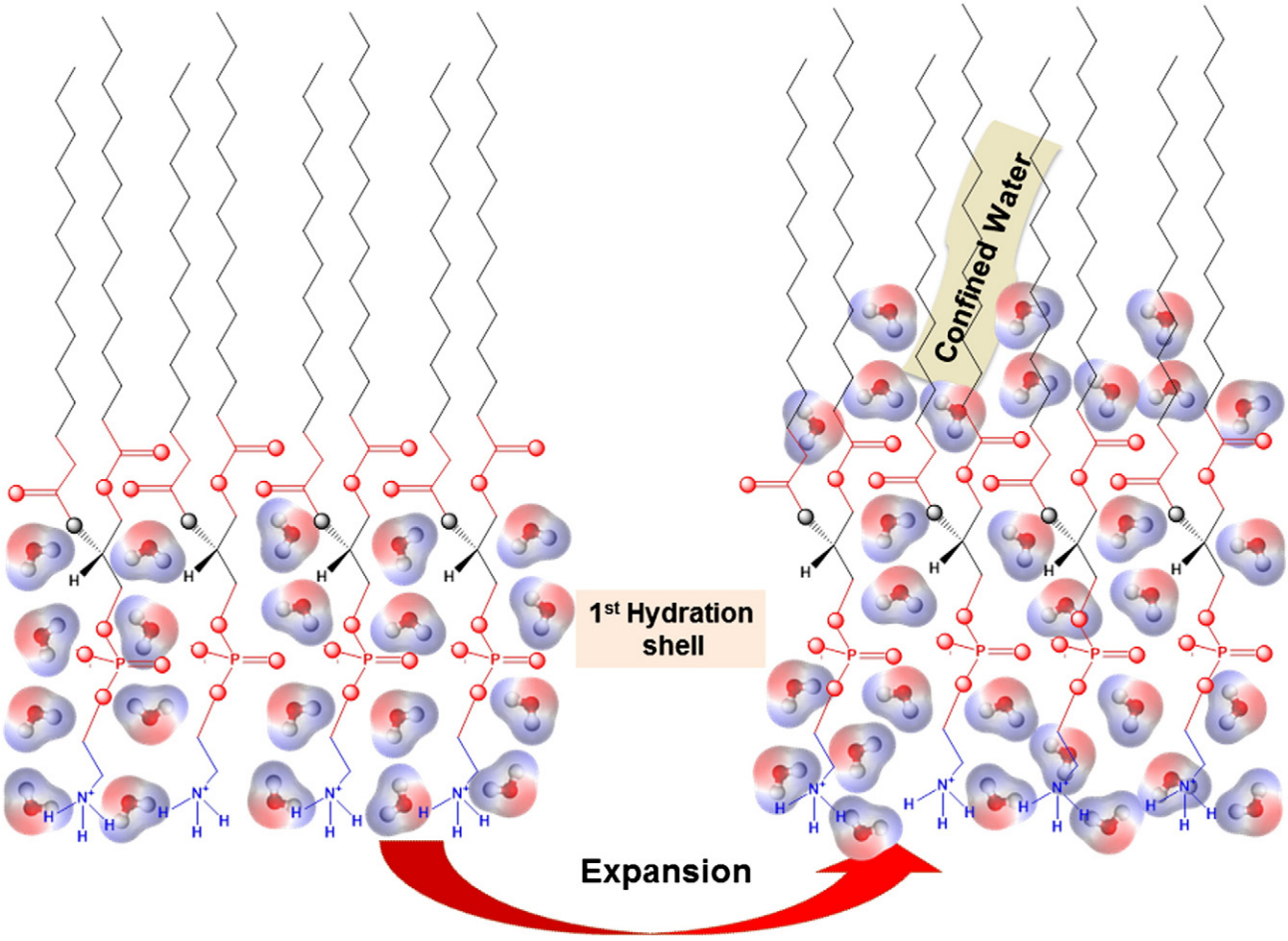

Fig. 3. Hydration and confined water in a lipid monolayer upon lateral expansion.

region. However, a composite membrane, as that discussed in the previous section and shown in Fig. 2, that includes the water interphases, cannot be described by a single dielectric constant. The capacitance of the bilayer (or a monolayer) is, therefore, a consequence of the organization of each of the components with different dielectric properties, including water.

Lipids can be spread on the surface of a Hg drop and behave analogously as lipids spread on an air–water interphase (Fig. 4) [64]. The changes of capacitance of these monolayers can be measured by means of cyclic voltammetry. In this condition, the capacitance of the monolayer is half that obtained with a bilayer (Fig. 4B). Thus, lipid monolayers on Hg are an adequate experimental model for lipid membranes.

The increase or the decrease of the volume drop allows to expand or to compress the monolayer, respectively. The current intensity of the central peak in the voltammogram of Fig. 5 increases continuously with the electrode area to reach a plateau at an area per molecule of about 67 $Å^2$. When the monolayer packing is altered, changes in capacitance of lipid monolayers spread on Hg would be a way to detect the formation of domains since water can have access to the metal surface to give a quantitatively different capacitance value [65].

The intensity reaches a plateau when the capacitance shows a critical break at an area that depends on the phase state and the type of lipids (also shown in Fig. 5). After the break, the capacitance varies linearly with the area with a slope that corresponds to the specific capacitance of the lipid-free Hg surface. This means that a slight expansion above the area per lipid at saturation, corresponding to the hydration shell, gives access to the aqueous electrolyte to the metal, as implied in Fig. 3.

Thus, the capacitance increase with area is understandable since more water spaces of the same specific capacitance corresponding to the water–Hg interface are formed with expansion. The intensity at the plateau can be ascribed to the predominance of large lipid-free electrode areas.

The break point for DMPC is observed, at 23 °C, but not in DMPE although both lipids are in the liquid condensed state at that temperature. However, it must be noticed that DMPC at 23 °C is the lipids are very near its phase transition ($T_m = 24$ °C). When the expansion of DMPE is done at 48 °C, near the phase transition ($T_m = 52$ °C) a break is also found for DMPE (data not shown). These results indicate that the break by expansion of a monolayer can only be achieved near the phase transition, a condition in which defects in packing can be found. These defects would act as precursors of the areas expanded beyond the hydration shell of the lipids.

The current intensity increase is parallel to the shift of the potential peak to more negative values with an isothermal expansion at 23 °C (Fig. 6A). The central peak potential of DMPC reaches a value $E_p = -0.83$ V at 74 $Å^2$/molecule. The more negative potential means that a higher input of energy is necessary to produce the change reflected in current and capacitance. The same value of potential is reached by increasing the temperature above the phase transition at constant area (Fig. 6B). This means that a similar lipid state can be achieved by isothermal expansion of the liquid condensed state or by a thermotropic transition at constant area. This may imply that in both processes hydration may be involved in the local organization of the lipid groups with similar energy requirements.

In order to put into relevance the role of water, it is of interest to inspect the capacitance changes in relation to area in DMPC and DMPE

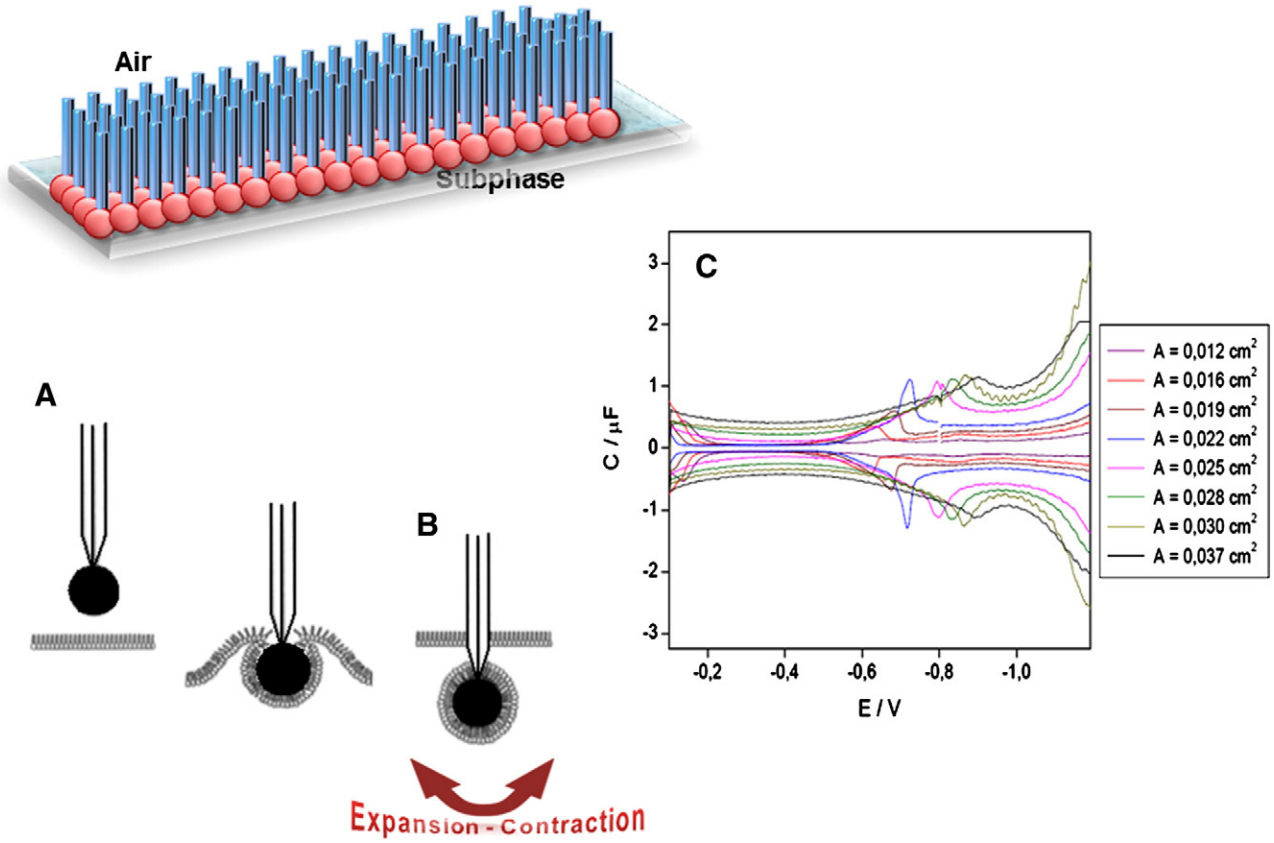

Fig. 4. Capacitance measures in a lipid monolayer formed on a Hg drop. A) A lipid monolayer formed on an air–water interphase is transferred to the surface of a Hg drop. B) Expansion and contraction of the monolayer are produced by volume changes of the drop. C) Capacitance peaks obtained in voltammograms at different areas of the drop.

monolayers, which have different degrees of hydration [66,67]. The specific capacitance of the monolayer increases with the increase in surface pressure i.e. the inverse of the area (Fig. 7) in both lipids. The capacitance does not change when it is measured in a monolayer for which the surface pressure has reached a critical value. The minimum area increase necessary to observe a capacitance change within the experimental error is less than 5% above the area of the lipids with its hydration shell. That is, the change in specific capacitance can occur when the surface pressure of the lipids packed with its hydration shell is slightly relaxed. Thus, capacitance change is possible when water in membranes is beyond the hydration shells as shown in the scheme of Fig. 3.

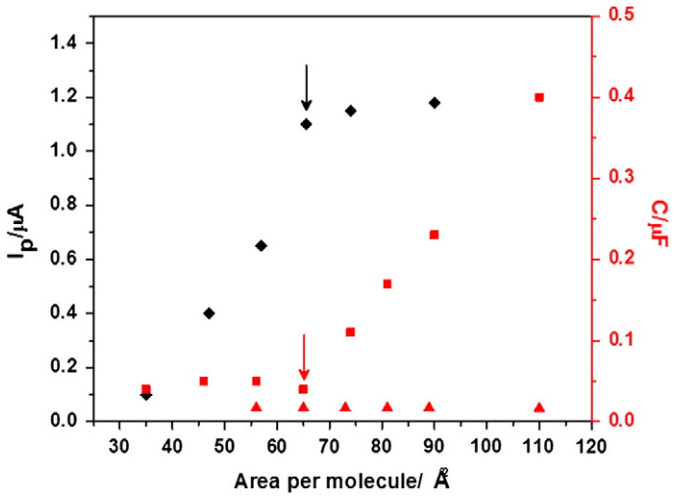

Fig. 5. Intensity and electrical capacitance of a DMPC monolayer on Hg as a function of the area per molecule. Current intensity at the peak potential (♦) and capacitance as a function of the area per molecule in DMPC (■) and DMPE (▲) monolayers at 23 °C.

The critical point (or cut-off) at which it occurs depends on the type of head group. The critical pressure for both lipids is denoted in Fig. 7, being that of DMPE below that of DMPC. In this condition, the expansion–contraction of the monolayer on Hg allows us to determine a critical point at which water can penetrate the lipids and reach the Hg surface. The expansion needed to obtain a significant increase in capacitance is around 5% of the lipid area beyond the area corresponding to lipids with its complete hydration shell. Considering the expansion of the monolayer as in a homogeneous material in which the volume modulus $k = A \cdot d$ is conserved, the corresponding thickness is 26 Å. The difference with the thickness in fluid state (30 Å) is 4.0 Å, the distance of water depth penetration as it has been reported elsewhere by impedance measures [52].

Water defects can be formed by expansion if the membrane is near the phase transition temperature. This implies that the coexistence of gel and liquid crystalline domains would favor the expansion. In other words, further growing of water domains is possible if packing defects preexist. This can be extended to lipid mixtures in which defects are present in the contact of different lipid phase components.

6. The critical cohesion. Water beyond the hydration shell

As described above, water can form a domain beyond the hydration shell of the phospholipids. Thus, it is reasonable to analyze its consequences on the peptide and protein interaction. The perturbation of the initial surface pressure of the monolayer is quantitated by the difference of the final surface pressure at equilibrium and the initial surface pressure before the addition (Fig. 8A).

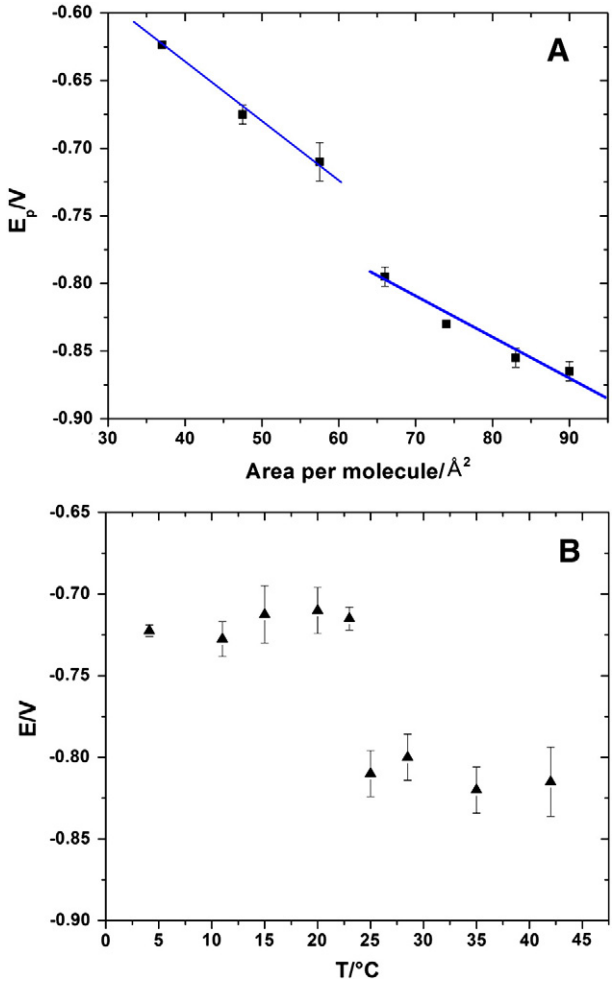

Fig. 6. Isothermal expansion and thermotropic transition in a DMPC monolayer on Hg. A) Capacitance potential peak as a function of the area per lipid at 23 °C. B) Capacitance potential peak at constant area as a function of temperature. The potential shift in part A from −0.72 V to −0.82 V is observed in an area range between 57 and 64 Å^2 which corresponds to lipids in the liquid condensed state and in the liquid expanded state, respectively, according to the plot in part B.

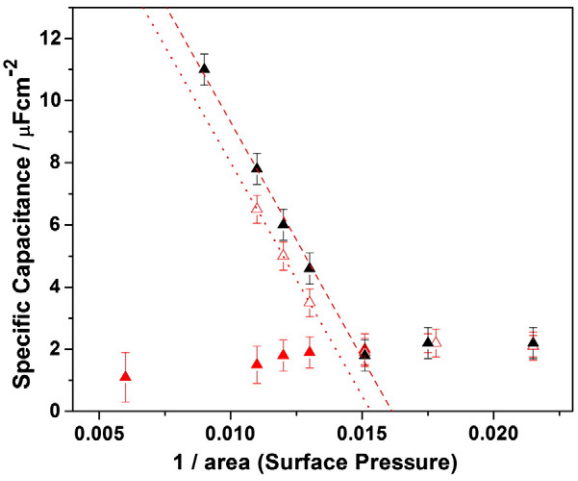

Fig. 7. Specific capacitance of the monolayer with the inverse of the area (c.a. the surface pressure) for DMPC at 23 °C (▲) , DMPE at 23 °C (▲) and DMPE at 48 °C (△). The inverse of the area is proportional to the surface pressure according to Π = n/A. RT.

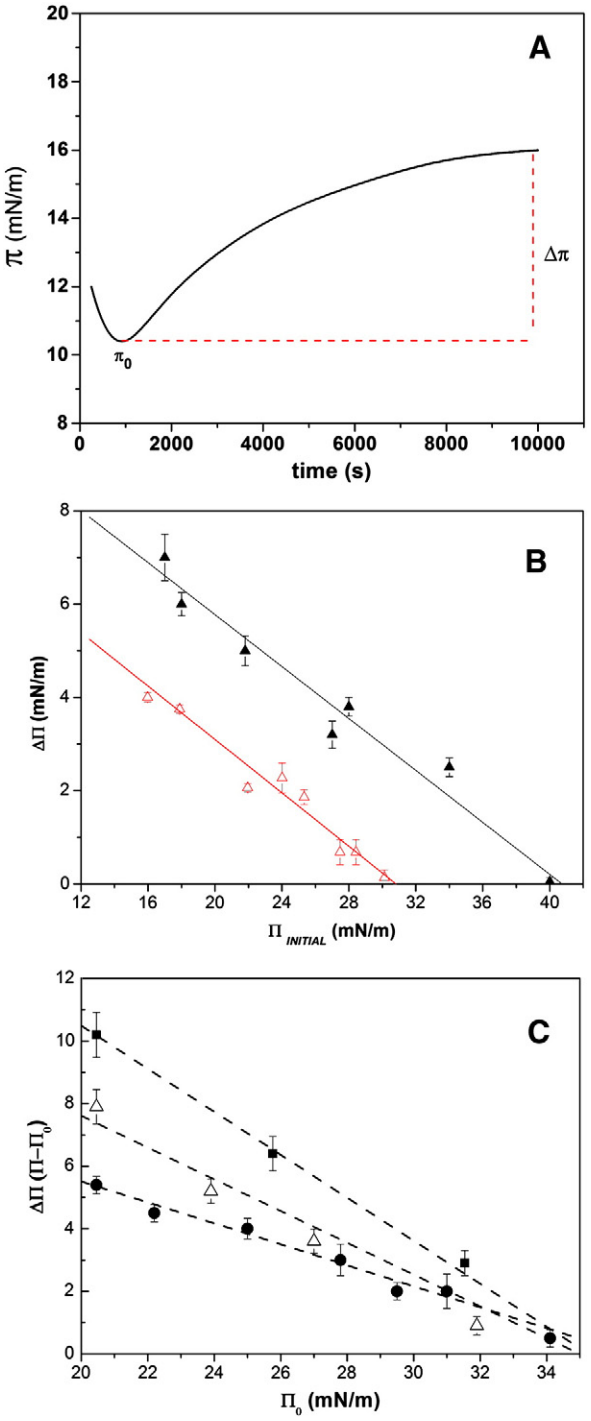

Fig. 8. Perturbation induced by the addition of proteins to the subphase of monolayers. A) Typical trace of the surface pressure increase of a monolayer of DMPC after the addition of a protein or a peptide to the subphase at an initial surface pressure (Π). B) Perturbation (ΔΠ) of DMPC (▲) and DMPE (△) monolayers at equilibrium as a function of different initial surface pressures (Π). C) Perturbation of DMPC monolayers with increasing ratios of cholesterol (ΔΠ) at different initial surface pressures (Π). (■) 0% cholesterol, (△) 2.5% cholesterol, (●) 5% cholesterol.

A classical plot, frequently used to evaluate the interactions of substances dissolved in the subphase with the lipid monolayer, is the Δπ vs π curves as shown in Fig. 8B and C.

The plot indicates that the perturbation Δπ decreases with the increase in the initial surface pressure and no perturbation is observed at a critical value ($π_c$).

The cut-off depends on the composition of the lipid head group region: phosphocholine, phosphoethanolamine, presence of stearylamine (Fig. 8B and Table 2). In turn, the slope of the curves varies with the unsaturation of the acyl chains and with the presence of cholesterol (Fig. 8C and Table 2).

The observation of Fig. 8 denotes that the perturbation of the monolayer by the solute in the subphase is produced when the monolayer surface pressure is below that corresponding to the saturation packing area of the lipids. The decrease in protein perturbation with the increase in surface pressure is concomitant with the decrease in the specific capacitance ascribed to the entrance of water by expansion shown in Fig. 7.

If we recall the data obtained with capacitance in the previous section, it is immediate to correlate the changes in capacitance by the area increase with the access of water to the monolayer. This access of water explained by polar head group dipole rearrangement against lateral forces and hydration can be also the reason for protein interaction. However, in both cases, the area increase is only around 4–12% of the initial area, i.e. few water molecules which makes difficult to explain the penetration of a bulky group of the protein under geometrical considerations. In contrast, it is more feasible that the slight expansion would promote an accumulation of water in small defects in which groups of the lipids are exposed to the aqueous phase thus changing the surface free energy; that is the surface tension.

The thermodynamic basis of this conclusion in relation to aminoacids, peptides and proteins will be discussed in the next section.

7. Thermodynamic properties of the lipid interphase

The relaxation of surface pressure at which the excess of water beyond the hydration shell becomes significant can be described by the difference $(\Pi - \Pi_c)$ between the initial surface pressure Π with respect to the critical one at the cut off Π_c. This difference, taken from the plots shown in Fig. 8, represents the excess of water that enters into the monolayer interphase beyond the hydration shell obtained for the lipids at the area of saturation. This is equivalent to the excess of water at which the increase in capacitance was observed in Fig. 5. At each of these differences, the proteins or peptides injected in the subphase produce a perturbation ($\Delta\Pi$). In Fig. 9, the plots of the perturbation of the monolayer by different peptides and proteins in the subphase vs. the surface pressure decrement i.e. the excess of water, are shown. It is clearly observed that for membranes with the same interphase composition (such PC:Stearylamine) the slope decreases with the increase in cholesterol and increases with the unsaturation and branching of the acyl chains. Correspondingly, the perturbation is reduced when the monolayer is compressed; i.e. water beyond the hydration shell is extruded.

One interesting point shown in Fig. 9B is that for similar interphase composition (similar head groups) the slope varies when carbonyl groups are depleted. This denotes that carbonyl groups at the water–hydrocarbon interface contribute to the nonpolar phase properties of the membrane. This result is congruent with the image in which most of the aminoacids stabilize in the region of those chemical groups. The variation between ester lipids and ether lipids has a similar consequence on membrane sensitivity to peptide or protein perturbation than changes in cholesterol or unsaturation. This will be important in the consideration of the carbonyl groups as hydration sites in the following section.

Table 2
Slopes and cut off of the Δπ vs π curves for different lipids.

Lipid	M	Cut-off (mN/m)
DMPC	0.263	41.5
DPPC	0.259	39.5
DOPC	0.336	41.5
DPhPC	0.429	39.6
DMPE	0.266	30.8

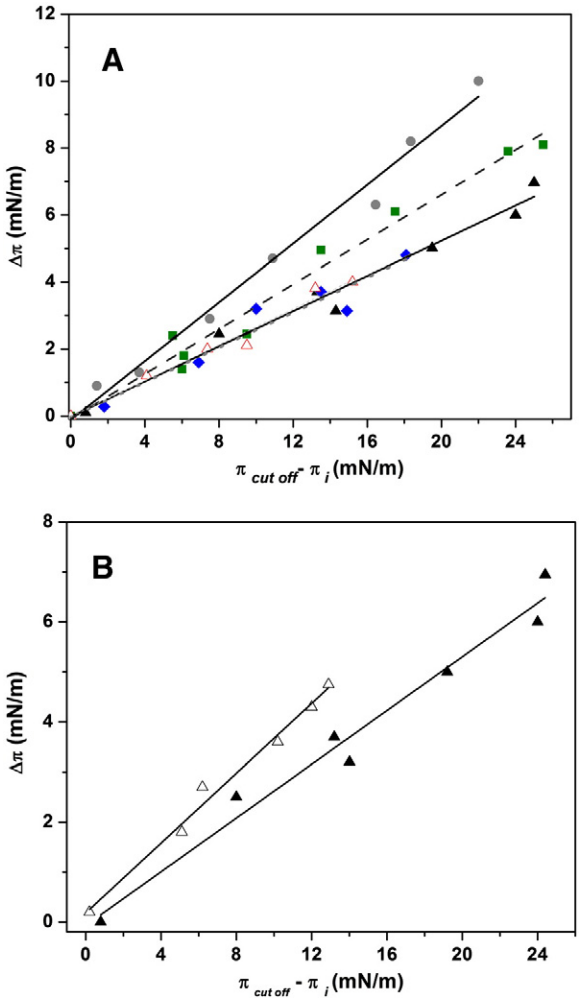

Fig. 9. Perturbation induced by proteins dissolved in the subphase of monolayers with different chain composition as a function of the excess of water at the monolayer interphase. A) DPPC (◆), DMPC (▲), DMPE (△), DOPC (■), DPhPC (○). B) ETHER (△) AND ESTER (▲) PCs. The surface pressure excess is calculated as the difference between the cut-off pressure (Π_c) and the corresponding initial pressure (Π) according to Fig. 8.

A monolayer can be considered as a closed system in relation to the lipid components. However, it can exchange water with the external media. This can be achieved by extruding water by osmosis or by decreasing water activity by the dissolution of a solute in the membrane interphase. In the more general way, a solute could be amino acid residues belonging to the surface of a protein. In both cases, compression or expansion of the monolayer will cause the interphase to increase or decrease, with proportional changes of the interaction with water of the membrane material.

When lipids are spread on the air–water surface, monolayers are formed due to the equilibrium between the internal pressure in the hydrocarbon core balanced by the interfacial tension at the water polar region. In other words, unbalances in the interphase region propagates to the whole membrane structure, i.e. the polar region plays an outstanding role in membrane response. This, in principle, can be assigned to the water properties in that region. The polar region occupies a space due to hydration that excludes water from the solution. Thus, the activity of water at the interphase is lower than in the bulk phase.

The curves shown in Fig. 9 can be explained with a thermodynamic analysis considering the definition of membrane interphase given in

Section 4. According to Defay–Prigogine an interphase of this kind can be considered as a bidimensional solution of hydrated polar head groups [68,69].

The lipid composition causes the interfacial tension to be different for different lipid packing densities. When a monolayer of lipids is formed on water, different values for surface pressure can be obtained for each degree of coverage of the water surface by the lipids. The interactions of water molecules of the bulk are obviously different from those water molecules at the air–water interface. The presence of the head groups and the exposure of the acyl chains of the lipids give place to the interfacial tension. As the monolayer is a system free to equilibrate, the equilibrium area per lipid is reached when the internal pressure of the monolayer counteracts the interfacial tension. Thus, the monolayer responds when the lateral pressure changes or when the water–surface interaction is altered.

The interfacial tension acts in the interphase, while much of the interlipid repulsion and consequent internal pressure involves the hydrocarbon part of the interface [46,51]. This means that the equilibrium balance can be displaced either by acting at the hydrocarbon core or at the polar head groups, as well. Therefore one has to consider the lateral pressure profile, at different points along the z-axis, (oriented along the bilayer normal). The slopes of Fig. 9 depend on cholesterol and the saturation/unsaturation ratio or depletion of carbonyl groups. In all cases, these variations would affect the water activity in the membrane.

The surface pressure of an insoluble monolayer is a direct measure of the surface water activity [70]. Thus, the surface tension of pure water can be defined as

$$\gamma^0 A = RT \ln\left(\frac{a_w^i}{a_w^b}\right) \tag{2}$$

where γ^0 is the surface tension of pure water, A is the average area per mole of water in the interphase region, $a_w^i$is the activity of water in the interphase of pure water and a_w^b is that in the bulk phase. When a monolayer is spread on the water surface the surface tension changes to

$$\gamma A = RT \ln\left(\frac{a_w^L}{a_w^b}\right) \tag{3}$$

where a_w^L is the surface water activity in the presence of lipids, i.e. in the interphase region.

Thus, the difference between the surface tension of pure water (γ^0) and surface tension of water with lipid forming a monolayer (γ), i.e. the surface pressure of the monolayer (π) is expressed as a function of the surface water activities as:

$$\pi = (\gamma^o - \gamma)A = RT \ln a_w^i / a_w^L. \tag{4}$$

This equation clearly denotes that the surface pressure (π) increases when a_w^L decreases from below 1 and becomes zero when $a_w^i = a_w^L$, i.e. the activity of pure water when lipids coverage is zero. In that condition, $\gamma = \gamma^0$.

The plots of Fig. 9 can be phenomenologically described by

$$\Delta\pi = k(\pi_c - \pi) \tag{5}$$

where $\Delta\pi$ defines the perturbation of the initial surface pressure of the monolayer induced by the proteins added to the subphase and the difference ($\pi_c - \pi$) stands for the excess of water beyond the hydration shell at saturation. The value of the slope k is clearly a function of the acyl chain composition including the presence of carbonyl groups, according to data in Fig. 9A and B. Specifically, the increase in branching or unsaturation and the depletion of cholesterol and carbonyl groups increase the slope (Table 3). We must notice the values of the slopes are similar for a similar acyl chain composition. A direct conclusion could be that the magnitude of the perturbation is related to the kink formation due to the rotational isomers of the acyl chains and the cooperativity [71,72]. However, since those membrane conformers imply water penetration [55], it is plausible to analyze these results in terms of the effects that those lipid components may cause on the water activity of the surface, following the hypothesis of Damoradan [70] and the formalism of Defay-Prigogine [69].

Table 3
Slopes of the plots from Fig. 9 for different proteins.
From Refs: [118–120].

Membrane composition	K	Cut off (mN/m)	Protein
DMPC	0.264	41.5	Aqueous protease
DMPE	0.266	30.8	Aqueous protease
Di(ether)PC	0.351	31.8	Aqueous protease
Di(ether)PE	0.282	29.4	Aqueous protease
DPPC	0.259	39.5	Aqueous protease
DOPC	0.336	41.5	Aqueous protease
DPhPC	0.428	39.6	Aqueous protease
PC:SA(10:1)	0.685	35.18	Bacterial S-layer
PC:Chol:SA(10:2.5:1)	0.519	34.6	Bacterial S-layer
PC:Chol:SA(10:5:1)	0.328	36.64	Bacterial S-layer

Considering Eqs. (2) and (3) the constant k can be expressed as a function of the water activity at different conditions as:

$$k = \frac{\left(\ln a_w^L - \ln a_w^p\right)}{\left(\ln a_w^L - \ln a_w^{Lc}\right)}. \tag{6}$$

The increase of the slopes due to the increase of unsaturation or the depletion of cholesterol, as shown in Fig. 9 and in Table 3, means that the protein insertion depends on the difference in a_w^L with respect toa_w^P, for a given departure from a_w^{Lc}.

Multiplying and dividing by RT and knowing that chemical potential (μ) can be defined as: $\mu = \mu^0 + RT \ln a$

$$k = \frac{\left(\mu_w - \mu_{wp}\right)}{\left(\mu_w - \mu_{wc}\right)}. \tag{7}$$

Assuming that there are no significant differences between the standard chemical potentials in the different conditions, Eq. (7) clearly denotes that the process is driven by the difference in the chemical potential of water in the different states of the interphase.

For a given value of $\mu_w - \mu_{wc}$, the perturbation is greater with the unsaturation and cholesterol depletion, which is reflected in a greater difference between the chemical potential of water at the pure lipid interphase that grows with the increase in water spaces.

The change in surface pressure, i.e. chemical potential of water of 6–8 mN/m is equivalent to an energy change for protein adsorption of 6 kJ/mol, which amounts to the energy of one H-bond and is 6 times higher than a dispersion force. These numbers are indicative that the changes in surface pressure (surface tension) are energetically comparable with reported values for protein–membrane interaction. These interactions would take place within the water-accessible region, that is, about three methylene groups. The energy of the interaction calculated for 100 Å^2 amounts to an equivalent of two CH_2 groups. Thus, the energy changes measured by surface tension are related to the lipid membrane groups exposed to water that determines the water activity at the interphase. The value agrees with literature data on hydrocarbons and amphiphiles where the group contributions per methylene of $\Delta\Delta G_0$ were two chains, burying 20% of the surface [73].

Moreover, the difference ($\pi_c - \pi$) can be expressed as the tension T, which is defined by Evans and Skalak [74] as the negative change in surface pressure, taking π_c as the natural value of surface pressure at force-free equilibrium.

In conclusion, the k values, affected by chain conformation and packing on the interaction enthalpy, serves to explain the perturbation of lipid interphases by considering the water activity variations concomitant with phase state, mechanical stress and hydric stress.

It is remarkable that the cut off and the slopes are a direct function of the head group (interphase region) and of the acyl chain (hydrocarbon phase composition) respectively, for different types of proteins, peptides and aminoacids (Table 3). This suggests that a corresponding membrane state governs the process for any kind of interacting compound. It must be emphasized that thermodynamically significant changes for response of membranes to peptide perturbations are those produced by expansion from the surface pressure force free equilibrium. Therefore, the structural counterpart of these responses is given by the decrease in the saturation/unsaturation ratio, the decrease of cholesterol level and/or the carbonyl depletion (ether vs ester phospholipids). In other words, an excess of free energy can be obtained in relation to water organization around the lipids by modulating by metabolic factors affecting membrane composition.

On the other hand, osmotic stress in which volume increases and membrane expands can be also a physical factor that may affect the peptide perturbation. Mechanical stress may affect packing and therefore water distribution and reciprocally hydration stress (osmotic stress) may modify packing.

8. The structural properties of water at the lipid interphase

It has been recognized that water may penetrate the lipid bilayer to a depth located at the region of the carbonyl groups [52,75–77]. Moreover, topological variations such as curvature, ripples and phase coexistence, gives place to different distributions of hydrated and non-hydrated carbonyl groups in phosphatidylcholines [77].

Allowance of water in the membrane structure has been useful to give a rough interpretation of the insertion of highly hydrophilic amino acid moieties in regions suggested as water pockets [62,63]. Penetration of amphipathic helices into the hydrophobic interior of phospholipid membranes has been described by a mechanism denoted as “snorkeling”. The “snorkel effect” of interfacial amino acids, such lysine or arginine, that bear a charge distribution is favored by the long hydrocarbon side chains that may deep into the acyl chain region. The highly positively charged guanidinium can only enter the membrane provided water domains are present or formed as a consequence of the amino acid interaction with membrane groups [31,78,79]. Observations of buried water molecules in phospholipid membranes predict water oriented at the lipid head group and apolar alkyl chains [80]. One possibility is that water is oriented with the dipole pointing to the phospholipid tails by electrostatic interaction [81]. As denoted in the previous section, peptides need a particular state of water activity, modulated by composition and physical conditions (surface pressure and temperature), to be active in the membrane response. It is assumed that defects can give place to restricted regions with water with an excess free energy that favors the interaction. However, a direct measure of the water structure in those pockets in relation with the lipid states of the acyl chains has not been so far analyzed. Thus, a closer inspection of the water properties at the different regions of the membrane is required.

Let us first resume some of the most relevant properties of water.

8.1. Cohesive character of water

Liquid water at normal pressures and temperatures (atmospheric pressure and 25 ºC) is a network of molecules interconnected by hydrogen bonds that extends in clusters of five water molecules in a tetrahedral array. The energy of the H-bond is generally accepted to be between 1.5 and 5 kcal/mol. The cooperativity of the hydrogen bond network confers to water unique thermal, interfacial and dielectric properties. Among them, the surface tension around 72.8 mN/m is relevant for the foregoing discussion.

The formation of hydrogen bonds in the direction of the angle of the H – O – H and the lone pair between an H-donor and an H-acceptor water molecule gives place to an ice-like open structure, identified as Low Density Water (LDW). When the hydrogen bonds collapse or bend, molecules approach to each other increasing the density. This is called the High Density Water (HDW) [53,82].

Accordingly, equilibrium between these two structural clusters may be displaced by changing the temperature, the type of surfaces and the kind of solutes dissolved in the water media. An increase in pressure on the LDW would induce HDW, somehow as a fusion process. The attractive forces, exerted on water by ions at high charge density when water orients the negative end toward the cation forming a hard-core hydration shell, generate a similar pressure increase. The difference in pressure (tension) would be caused by the balance between the strength of attraction of the surface to a given cluster of water molecule around a given group (ion, polar or non polar residue) in comparison to the attraction imposed by the water network. In a surface, the overall balance will depend on the nature of the groups exposed to water and protruding to the bulk water phase.

8.2. Water near lipid membrane surfaces

Can surface membrane groups stabilize LDW or HDW domains? To what extent and in which conditions can different lipid molecules affect the equilibrium between them?

The concept of different types of perturbed water arranged in different layers has been commonly accepted in describing the hydration of lipid membranes. Israelachvili and Wennerstrom [34] suggested that the properties of the water beyond the primary hydration layer are essentially the same as those of bulk water and thus there are virtually no further hydration layers of structured water except the first one. However, they still may present different thermodynamic properties. An indication in this direction is the observation that peptide perturbation can only be possible with a slight expansion beyond the hydration non-perturbed state of the phospholipids (see previous sections).

Perturbed water gives rise to a typical quadrupole splitting of the ^{2}H-NMR spectrum if the lipids are hydrated with heavy water [15,83]. The change of the splitting width, with the hydration degree, indicates that the first five water molecules added to dipalmitoylphosphatidylcholine form a sort of inner hydration “shell” possessing a much larger splitting width than additional, ambient water. Similar information is derived by the analysis of the asymmetric stretching frequencies of the phosphatidylcholines phosphate groups by Fourier Transform Infrared Spectroscopy [60,84]. The frequency corresponding to the phosphate group in anhydrous state decreases with the number of water molecules, which stabilizes for a ratio of around six water molecules per lipid. The decrease in the frequency is ascribed to the formation of H bonds of the P═O group with water.

On the other hand, the presence of water beyond the hydration shell of the phospholipid head groups has been described as confined water or water in restricted domains [11]. The properties of these confined regions of water have been ascribed to the influence of the adjacent wall in several materials [85,86]. In the case of lipid membranes, these water molecules are probably confined between hydrocarbon chains, mainly at the first carbon atoms and carbonyl groups [76].

This description is in accordance with the distinction between hydration water and confined water, shown in the previous paragraph by the difference ($\pi_c - \pi$).

In the presence of lipids, a 3400 cm^{-1} peak and the shoulder at 3250 cm^{-1} were attributed to O – H stretches of water molecules in an effective H-bonded structure (“network water”) whereas the weak high frequency shoulder near 3600 cm^{-1} was assigned to water in a structure of disturbed hydrogen bonds (“multimer water”). Hence, according to Binder [40,41] water forms transient microdomains of

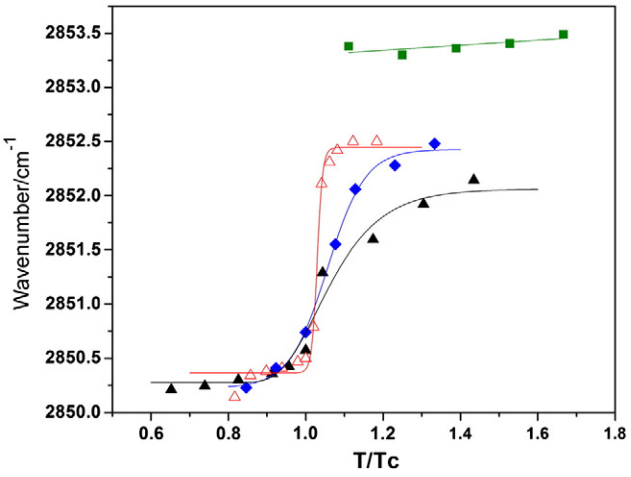

Fig. 10. Phase transition of different lipids measured as frequency of the CH_2 stretching mode as a function of the reduced temperature. DOPC (■), DMPE (△), DPPC (◆), DMPC (▲).

two possible states: low density "network" water of high connectivity and higher density "multimer" water of lower connectivity.

The network water is most likely involved in extended transient water networks forming up to four hydrogen bonds per water [40,41,87]. Thermotropic and lyotropic measurements are interpreted in terms of the significant larger fraction of multimeric water adsorbed by the liquid–crystalline phase in comparison to the gel state. Thus, lipid melting would give rise to the transfer of water from the network state into the multimeric state in considerable amounts [88,89]. Low connectivity domains would prevail in liquid crystalline phases and high connectivity in gel state. However, the relationship between this transference with the chemical nature of the lipid surface groups and its thermodynamic behavior is still unknown.

It is well known that at the phase transition temperature, lipid membranes reach a higher state of disorder ascribed to the increase in the trans-gauche isomers in the lipid acyl chains [90,91]. Concomitant to this increase in chain mobility, the water amount present in the bilayer increases from 7 to 20 water molecules per lipid in the case of phosphatidylcholines and from 4 to 7 in phosphatidylethanolamines with little variation with the chain length [67].

Different methods have been employed to measure the transition temperature. Some of them, such as differential scanning calorimetry, provide the total enthalpy change in the phase transition that can be interpreted in terms of the cooperative units of the molecules, where main contributors are the acyl chain residues [73]. Another method such as fluorescent anisotropy, uses probes inserted in the hydrocarbon region and allows reaching qualitatively similar conclusions [92].

Anisotropy measured at the level of CH_2 residues by DPH denotes that the gel state of DMPE and DMPC do not substantially differ. However, the anisotropy above the phase transition is higher for DMPC (0.5) in comparison to DMPE (0.1) [93].

The several methods used to determine phase transition in lipid bilayers distinguish three main states, the gel state the fluid state and the transition temperature. The global cooperativity of the transition is measured mainly by DSC [73,90]. The steep decrease in order parameter is accompanied by an increase in polarity as shown by Laurdan experiments [94,95]. Change in polarity is ascribed to an increase in water in the membrane phase. The generalized polarization in the gel state of DMPC and DMPE is 0.6–0.7 and above T_t, −0.1 and 0.2 for PC and PE respectively. GP values are related with the number and motional freedom of the water molecules around the fluorescence probe, assumed to reflect indirectly the state of the lipid environment. The phase transition is accomplished with a reduction of the membrane thickness and area per lipid increase as determined by SAXS [32] and electrical capacitance [96]. The decrease of the dipole potential in monolayers suggests concomitant changes in water organization [97]. More indirect methods are the changes in turbidity or refractive index that may be related to changes in the membrane density due to water penetration [98,99].

The rotational isomers of the CH_2 residues along the C–C bonds result in an elevated number of configurations and thus, low order parameter, as visualized by electron paramagnetic resonance (EPR) [91]. This order decrease is noticeable beyond the first 4–5 carbon

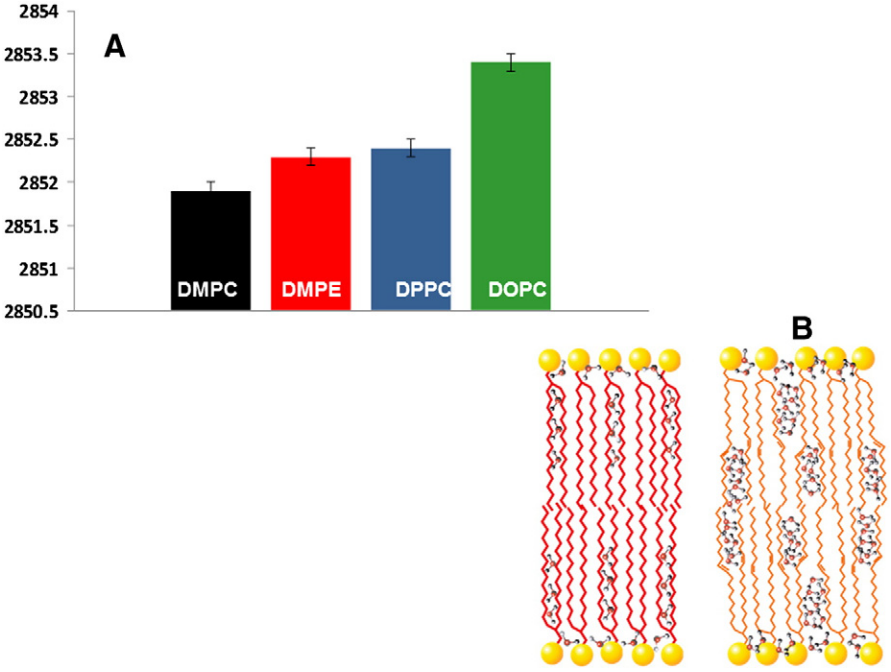

Fig. 11.) A) Frequency of CH_2 at constant temperature as a function of the acyl chain composition. B) Schematic representation of water stabilized in lipid membranes. B) Schematic representation of water stabilized in lipid membranes.

atoms of the acyl chains. The method that provides a visualization of the membrane state at molecular level is FTIR. The information is obtained by the shift of a few nanometers to higher values at the phase transition temperature of the frequency corresponding to the CH_2 groups [100]. In addition, the shift to higher frequencies of the νCH_2 symmetric stretching as a function of the water content has been also reported for DMPC [101].

The results of Fig. 10A show that frequencies of different lipids show at the reduced temperature an increase in the νCH_2 asymmetric stretching frequency. These changes correspond to a decrease in the lateral CH_2 interactions in the gel phase when the membrane goes to the liquid crystalline state. However, it must be noticed that this final value is not the same for the different lipids (Fig. 11). This indicates that although all the lipids are in the so-called "fluid state", at molecular level there are important differences in the CH_2 region. The relative increase in isolated populations of CH_2 residues is concomitant to the increase in the slope in the plots of $\Delta\Pi$ vs excess of water according to equations [8] or [9]. From DMPC to DOPC the isolated population grows from the gel to liquid crystalline state is 2.04 for an increase in the slope of 1.32. Thus, the increase in the slope is a measure of the water regions present in the membrane phase according to the acyl chain composition.

Parallel to the increase in isolated populations a change in the state of water in terms of hydrogen bonding populations is observed (Fig. 12). An increase in the hydrogen bonded water populations appears when water domains surround CH_2 groups or water domains are created around CH_2 groups.

The observation that in fully hydrated lipids, bands at low frequencies drastically increase maintaining the band centered at 3400 cm^{-1} observed in solid suggests that water molecules are adsorbed to a rigid matrix. In contrast, when acyl chains melt the pattern of solid disappears and the spectra are qualitatively different. However, it should be noticed that the band in the gel state is shifted to higher frequencies, indicating a decrease in water bonding.

What types of H bonds are formed in water according to the different frequency bands observed? As reported elsewhere by Giovanbattista et al. [86] and Arsov et al. [54,102], the boundary structure greatly influences the structure and dynamics of the water. Specifically, the hydrophobic apolar surfaces slow down the dynamics [102]. In small systems, with dimensions of 3–8 Å, the dynamics is slowed significantly, and the velocity autocorrelation function is similar to that of solid ice, i.e. low frequency populations [103,104]. However, recently a new set of information suggests that ice like formation is unlikely near the surfaces [105].

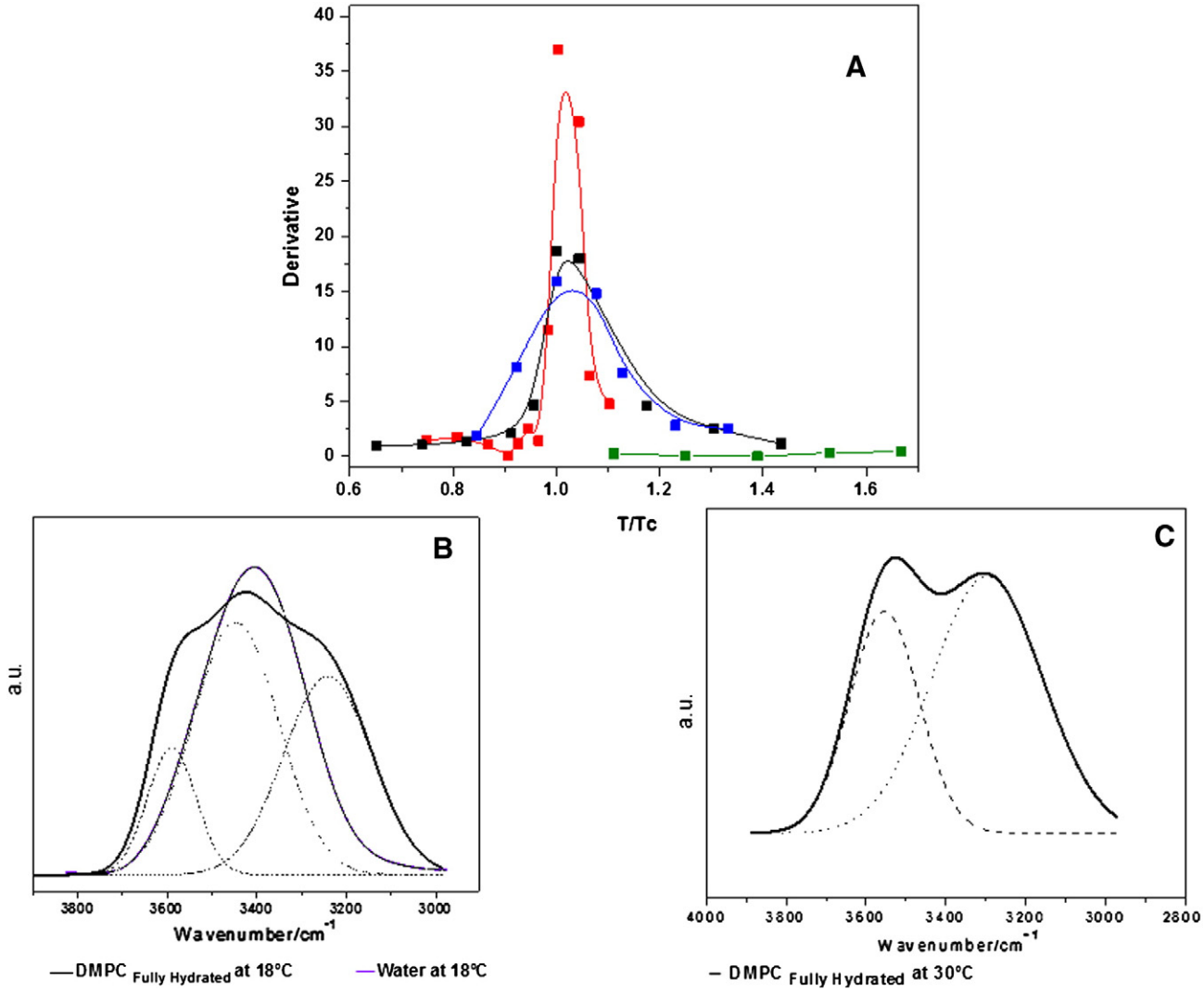

Fig. 12. A) Distribution of connected and isolated CH_2 populations around the reduced temperature and the water species linked to them. B) in the gel state, C) in the liquid crystalline state.

Histograms of the spatial dependence of the hydrogen-bond lifetimes show confinement or local template environmental ordering, and one can infer that the dynamics are significantly slower near the structured hydrophilic boundary. Local environment indeed affects the structure and dynamics of water. The magnitude of the effects of confinement greatly depends on the number of hydrogen bonds available per water molecule as well as lifetime of nearby hydrogen bonds.

From the comparison of the results obtained in the CH_2 region with those of the water bands it is concluded that in the gel state, CH_2 contact (giving place to low frequencies) are concomitant to low H-bonded water populations, i.e. low water clusters. Water shows a strengthening of the "local tetrahedral" structure as confined template environment is formed due to thermal fluctuations of the acyl chains. For smaller systems, local order is prevalently dominant, and hence, frequency band of the water populations shifts to higher values while the larger systems tend toward bulk-like dynamics.

Above the phase transition, CH_2 frequency increases denoting isolated populations with an increase in H-bonding in water molecules. Thus, water domains at least partially organized by H-bonding are formed in between acyl chains in the liquid crystalline state.

Above the phase transition, the appearance of strong H-bonds between water molecules is consistent with the reinforcement of water structure in the presence of nonpolar residues such as the CH_2 residues. In conclusion, the increase in isolated CH_2 populations is congruent with the small nanoenvironments of water structure. This is compatible with the formation of water clustering in between the lipid acyl chains when the bilayer is in the liquid crystalline state [55,106] and also with the appearance of hydrophobic defects [62,63].

It is clear that cooperativity units derived from DSC are usually identified with the rotational isomers of the CH_2. In this regard, the high order parameter is given by the highest CH_2 connected population. Similarly, low order is concomitant with an increase isolated CH_2. In this condition, the connected and isolated populations of methylene groups are not similar for different lipids although they are in the same phase state. Thus, although it is frequently identified that at the phase transition a transition from a solid crystalline to a liquid crystalline (more frequently denoted as fluid) state occurs, the microscopic configurations may be quite different depending on the lipid specie.

Another important point is to take into account that cooperativity may also include a contribution related to water reorganization, concomitant with the changes in the CH_2 populations. In this regard, FTIR data indicate that the liquid crystalline state is characterized by a low entropy phase due to water clustering in low density networks due to an increase in H-bonds.

The fine structure of the different water populations derived from FTIR data is a matter of discussion [54,55]. It is reasonable to think that the bandwidth is due to water molecules with zero, one, two, three and four hydrogen bonds. As H-bonds are also cooperative, its energy depends on the coordination number of the water molecules giving the broad line spectra. Therefore, fluid states, although macroscopically similar may be composed of different microscopic components due to different water intercrossing and hence different free energy content.

In this regard, it has been recognized that water activity is an intensive parameter of the thermodynamic state of the lipid system. Water molecules present within the hydrocarbon region of the phospholipid membranes interact with phospholipid molecules through their chemical potentials (Gibbs–Duhem relation) implying the conformational state of the acyl chains [37,116,117]. This might be the reason for which similar structural compounds interact differently with fluid membranes of different lipid components.

Water molecules in confined pools of few nanometers in diameter or at interfaces undergo hydrogen bond structural dynamics that differ drastically from the dynamics they undergo in bulk water. Orientational motions of water require hydrogen bond network rearrangement [107]. It has also been suggested that reorientation of the O – H vector and hydrogen bond time correlation is less influenced by hydrophobic groups than hydrophilic groups [108,109]. This is somehow related with the idea that interfacial effects may dominate the hydration forces linked to interfacial structural messages [67].

Finally some considerations about H bonds in water and in CH_2 matrix should be done. H bonds are conventionally defined as the intermolecular interaction of X – H—X were X and Y are atoms of moderate and strong electronegativity [110]. Water may be in contact with the carbonyl groups forming the – C═O – H association or with the methylene groups forming the CH – OH. It has been argued that the last one is rather a contact or and interaction rather than a bond. This last case is classified as a non-conventional H-bond since the donor atom is not oxygen [111,112].

Recently, reported rotational spectroscopic studies on small dimers and oligomers bound by weak hydrogen bonds show that the driving forces, the spatial arrangement and the dynamical features displayed are very different from those involved in stronger and conventional hydrogen bonds [113]. The very small binding energies (similar to those of van der Waals interactions) imply that networks of weak hydrogen bonds often increase the stabilization of the dimer. Even in the presence of multiple bonds the partner molecules show a high degree of internal freedom within the complex. Several examples of molecular adducts bound by weak hydrogen bonds formed in free jet expansions and recently characterized by rotational spectroscopy. They include weakly bound complexes of weak donors with strong acceptors (C – H O, N, S – H O, N), and strong donors (O – H, N – H) with weak acceptors such as the halogen atoms [114].

The hydration of some CH groups as in ethers produces a shift to blue and a decrease in the intensity, meaning a decrease in H bonding [115]. This is in complete agreement with our analysis of water in the fluid state.

9. Conclusions

The electrical, thermodynamic and structural properties of lipid membranes described in this review are consistent with a model in which water is organized at the interphases. The dynamical picture of the membrane denotes that it cannot be considered as a constant dielectric slab. Water does penetrate according to the variation of the lateral surface pressure at critical areas.

Near the phase transition, the lipid membrane expansion causes an increase in capacitance, suggesting water penetration and peptide or protein insertion.

This last process is related with the water activity at the interphase and has different structural components according the lipid.

Head groups protruding toward the water phase organize water in different dynamic and structural patterns. In this sense, water organization is imposed by the lipid composition.

The lability of the water domains is given by the thermodynamics of the surface water arrangements. The free energy changes produced by peptide or protein insertion are related to changes in the chemical potential of surface water.

Lability is related to the domains formed according to cholesterol level and unsaturation when the surface pressure is below that corresponding to the saturation pressure, i.e. when the hydration shells of the lipids are in contact.

The excess of water beyond the hydration shell is thermodynamically labile and depends on the cholesterol level and the unsaturation saturation ratio.

The water domains depend on the phase state of the lipids, specifically near the phase transitions. Different water populations in terms of H bonding species are found below and above the phase transition and act as precursors for the clusters of confined water domains.

Changes in the kind of water populations are concomitant with the shift of methylene vibrational mode frequencies to higher values.

The increase in isolated populations of methylenes is congruent with the formation of highly ordered water cluster bonded by hydrogen bonds.

This is consistent with the formation of water pockets in nano-environments that accumulates free energy. The low entropy of these water arrangements, compensated by the disorder in the acyl chains, can be the thermodynamic driving force for peptide insertion into membranes.

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