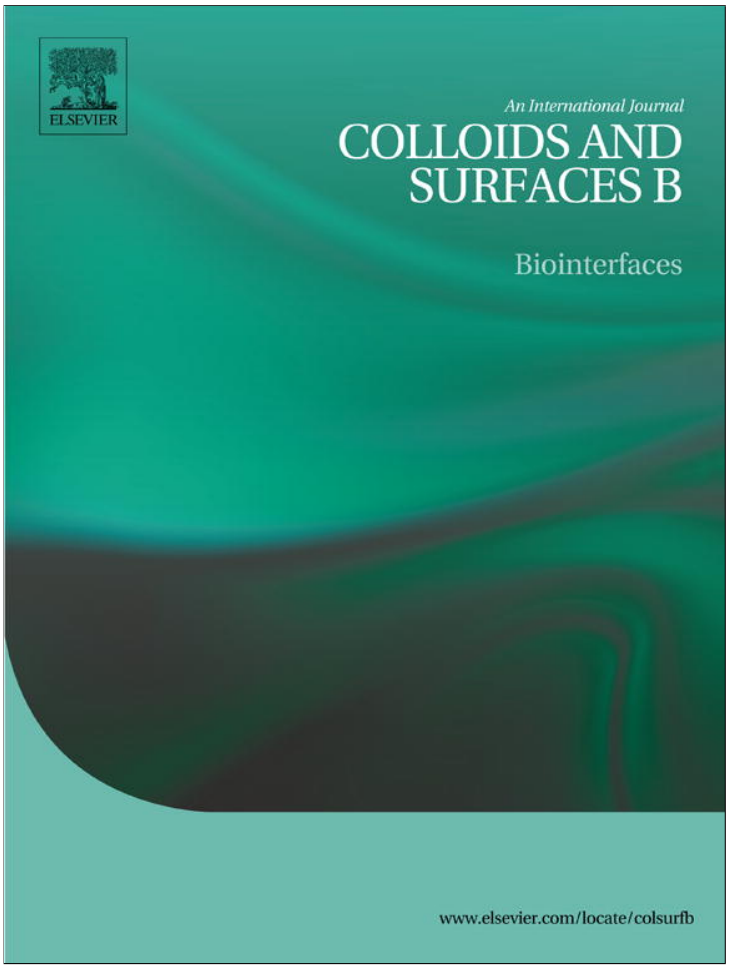

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Water defects induced by expansion and electrical fields in DMPC and DMPE monolayers: Contribution of hydration and confined water

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ABSTRACT

The values of capacitance of dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylethanolamine (DMPE) monolayers on Hg, derived from cyclic voltammetry studies indicate that when the lipids are near the phase transition temperature fractures are formed at a critical area beyond that corresponding to the hydration shell of the lipids in the liquid expanded state. Similar fractures are inferred to be formed when an electric field is applied at constant area, at a breaking potential which is a function of the lipid species. These voltage values denote that energy involved in the transition induced by the electrical field is much higher for DMPE than for DMPC at low areas. This can be explained by the higher intermolecular lateral interactions by H-bonds between the ethanolamine and phosphate groups. However, at larger areas, the energy values for DMPC are as high as for DMPE which is understood to be due to the higher hydration of phosphocholine head groups. This finding gives a new insight in relation to the dynamics of the lipid head groups at the membrane interphase region in terms of the states of water between the lipids. This is congruent with previous results evaluated with the well known $\Delta\Pi$ vs. surface pressure plots in monolayers of the same lipids at air-water interfaces.

1. Introduction

The organization of biological membranes in in-plane microdomains is now believed to play a key role in the development and regulation of membrane functions [1–3]. The characterization of these domains suggests that the lateral phase separation of lipid molecules is critical for the understanding of membrane response to environmental stimuli [4,5]. Earliest studies based on the use of freeze-fracture electron microscopy and electron diffraction described domains in liposomes made of various phase-separated phospholipid components [6]. Many of them have focused on the non equilibrium dynamic ordering processes and the topology of coexisting liquid crystalline/gel phases in phosphatidylcholine mixtures. These topological properties imply lateral contacts between domains and packing defects where the penetration of water beyond the polar head group interphase can occur. However, how water contributes to this domain picture is not well known. In this regard, a relevant question is to understand how water microdomains can be formed buried in the lipids in response to external variables.

Water in membranes is an old problem in membrane biophysics but little has been rationalized in terms of surface physical chemical properties. The presence of water was recognized in several studies as contributing to the bilayer permeability barrier for non electrolytes [7], as the origin of the repulsive hydration forces between membrane surfaces [8] and as a substantial part of the surface membrane potential (dipole polarization and potential) [8–10]. In addition, water has been postulated to penetrate deeply into the membrane interior based on the changes in the dielectric properties of bilayers [11,12].

However, direct evidences of water stabilization as microdomains in the lipid matrix have been controversial and speculative. 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 [13–15].

According to Wimley and White [16], the partition free energy is highly positive for arginines. For this reason, the penetration of arginine-peptides in lipid membranes has been explained by considering the formation of water pockets or defects [17]. In this regard, recent studies in lipid monolayers at different surface pressures indicate that protein interactions can only occur when

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the surface pressure is below that corresponding to the area of contact of the hydrated head groups of the lipids in the liquid expanded state [18,19]. Thus, water pockets or defects are a function of the lateral pressure of the monolayer. The aim of this work is to determine by means of capacitance measurements the critical points at which those breaks or defects can be formed. Pockets filled with water are postulated to be formed by membrane deformations probably due to changes in the polar head group conformation. How these defects can be formed in relation to membrane deformation, such as expansion–contraction processes and membrane phase state, is not clear neither structurally nor energetically [20]. The boundary structures that may affect the water properties are within a dimension of 3–8 Å [21]. Thus, water properties significant for protein insertion could be modulated by changes at a distance within a few water diameters, implying that they may be formed by intramolecular rotations and not necessarily by a bulk volume of water between the lipids [21].

If water content can be affected by the packing in the different phase states it may be possible that it may also affect the cohesion state of the lipid interphase upon isothermal expansion. Due to the striking differences in the hydration between phosphatidylcholines and phosphatidylethanolamines, different responses should be expected in terms of location and properties of water molecules in these two lipids. One question is whether it is possible to find restricted water domains between the lipids beyond the hydration shell that may grow with expansion. The other is how these micro domains are related to the head group structure and orientation.

A method less familiar to membrane biophysics is cyclic voltammetry applied to lipid monolayers spread on Hg. This technique was studied with electrochemical criteria and considerable information has been published since then [22]. Cyclic voltammetry is a valuable tool to get new insights in relation to membrane water content and membrane dynamics by means of the evaluation of capacitance data as a function of area and temperature.

In terms of monolayers formed on a metal, it is reasonable to think that if changes in the packing may affect water penetration this should give an electrical signal in terms of capacitive current. If this is the case, it is expected that the analysis of the voltammograms could give information about the structural changes involved in water path formation.

Most studies of cyclic voltammetry of lipid monolayers on Hg electrodes have been done using a lipid in the fluid state, DOPC [22]. An increase in the capacitance at different electrode potentials indicates the access of the electrolyte solution to the Hg surface. The origin and the mechanisms of formation of these water paths are controversial. In general, they have been ascribed to a reorganization of the lipids on the surface such as pores in the monolayer, interaction between the head groups and the charged electrode surface, formation of direct or inversed micelles on the electrode surface and detachment of the lipids from the charged surface. These different rearrangements would result in different orientations of the lipids (perpendicular or parallel to the electrode surface) and the formation of bare regions in the Hg surface in direct contact with the aqueous solvent, all of them with different energetic requirements. In order to use this methodology for membrane studies, it is necessary to demonstrate that lipids on the electrode are maintained in a conformation that is comparable to other experimental lipid membrane models such as monolayers on air–water interfaces and bilayers.

The previous observation that the peak potential of the central region of DMPC voltammograms decreases abruptly at the temperature corresponding to the phase transition of the lipid, i.e. 24–25 °C was the first direct evidence that lipids on Hg are organized as in a monolayer and in consequence it could be satisfactorily considered as an adequate experimental model for lipid membrane biophysics [23]. In this context, we have reexamined the

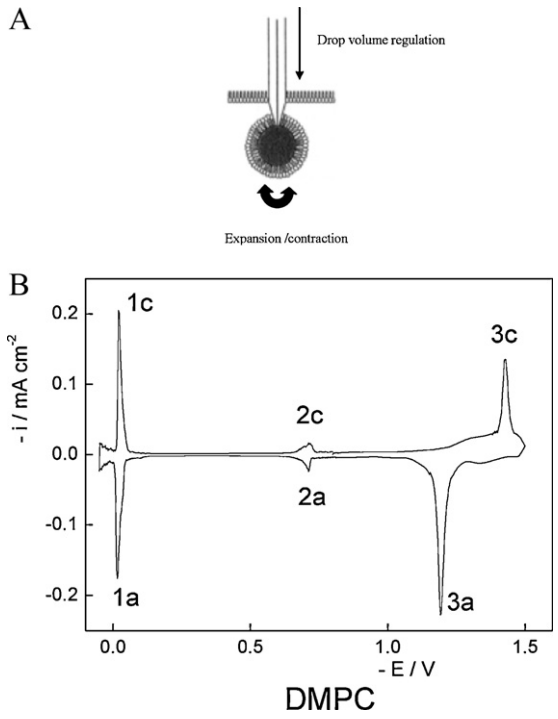

Fig. 1. (A) Schematic representation of the expansion–contraction of a lipid monolayer on the surface of a Hg droplet. The micrometric screw allows to change the volume drop and hence the area of the monolayer with respect to the area of transference. (B) Voltammograms of DMPC lipids attached on the surface of a Hg hanging drop 1c, 2c, 3c cathodic sweep peaks. 1a, 2a and 3a anodic sweep peaks. 1 and 3 represent the peak at the anodic and cathodic regions and 2 to the central region. $T = 23\,^{\circ}C$, $A = 0.019\,cm^2$.

voltammetric response of lipids on Hg in order to reconsider at what ranges of the voltammograms the lipids conform a monolayer, in which regions they are forced to abandon it and which is the effect of the acyl chain state and polar head groups on that behavior. For this purpose, we have studied the voltammetric response at different temperatures and at different surface pressures.

The specific capacitance ($C_m = C/A$) of lipids organized as a monolayer spread on a surface is given by

$$C_m = \frac{\varepsilon\varepsilon_0}{d_m} \quad (1)$$

where ε_0 is the permittivity in vacuum; ε is the permittivity and d_m is the monolayer hydrocarbon thickness.

Hence, changes in area would affect thickness thus changing the monolayer capacitance. An estimation of the capacitance can be done in monolayers formed on a Hg drop by means of cyclic voltammetry at different areas by changing the volume of the drop (Fig. 1A) [23]. For this reason, we have measured the changes in capacitance of lipid monolayers on Hg as a way to detect the formation of domains by expansion or by applying an external electrical potential. In regard to the role of water, it is of interest to inspect the capacitance changes in relation to area in DMPC and DMPE monolayers which, as said above, have different degrees of hydration.

2. Materials and methods

Dimyristoylphosphatidylcholine (DMPC), dimyristoylphosphatidylethanolamine (DMPE) were obtained from Avanti Polar Lipids Inc., and used to prepare stock solutions of 2 mg/ml in mixtures pentane/chloroform (Merck, PA). Aqueous solutions were prepared with water from a Milli-Q purification system (Millipore) (resistivity > 18 MΩ). The pH of the support electrolyte was maintained at 7 by a 0.1 M KH_2PO_4/0.1 M K_2HPO_4 buffer

solution (Merck, PA). Mercury for polarographic analysis (Merck) was used to fill the capillary electrodes.

The metal surface was generated by means of a hanging mercury drop electrode (HMDE). It is assembled on a vertical motion system, which allows the droplet to run slowly through the solution surface inside the electrochemical cell. A micrometric screw adapted to the capillary allows droplet size adjustment, and consequently, droplet area regulation (Fig. 1). A three-electrode cell was employed; the counter electrode was a Pt gauze of 1 cm^2 area and the reference electrode was an Ag/AgCl (3.5 M, AgCl saturated) electrode. The HMDE penetrates the cell through a silicon rubber septum, which allows its vertical displacement inside the cell. The cell was jacketed and the temperature of the circulating water was controlled with a Lauda thermostat (±0.1 °C) at the work temperature (23 °C or 48 °C). Buffer solutions were degassed with nitrogen and kept under a constant flow of the gas during measurements. The purity of the solvents was checked by measuring the double-layer capacitance of the mercury surface, after being lowered through the aqueous solution surface on which only the solvent had been spread. No difference between Hg drops extruded directly in the bulk of the solution and those previously made above the surface and then transferred across the air–solution interfacial plane was observed.

Measured aliquots of the lipid solution in the organic solvent were added to the air-buffer surface and let to spread. Subsequent aliquots were added, after allowing solvent evaporation, until a surface pressure of 47.8 mN/m for DMPC and 45.0 mN/m for DMPE were obtained, respectively [24,25], at 23 °C. At this temperature, both lipids are in the liquid condensed state. In these conditions, the monolayer self-assembled at the air/solution surface is saturated and the resulting area per lipid corresponds to those reported in literature for each lipid state (56.7 $Å^2$ for DMPC and 54 $Å^2$ for DMPE) [24,25]. In this condition, the mercury drop was passed once through the monolayer. Sequential potential sweeps showed constant and reproducible potentiodynamic profiles giving capacitance values typical of phospholipid monolayers, ca. 2 μF cm^{-2}. This was taken as an indication that lipids adsorbed on the Hg surface remained as a monolayer and, neither additional lipid molecules were desorbed nor other aggregates were formed during the process [23,25].

2.1. Cyclic voltammetry

Complete voltammograms were obtained by potential cyclic linear sweeps between 0.1 V and −1.5 V against Ag/AgCl applied to the Hg/phospholipid/solution interface by means of a PAR 273A potentiostat, at a scan rate of 1 V/s (Fig. 1B). Higher scan rates were tested and it was shown that slow scan rates allow a better resolution of the peaks. In these conditions, consecutive voltammograms of DMPC and DMPE were obtained after the monolayer formation on the Hg droplet at constant temperature and mass of lipid adsorbed. The measured voltammograms were highly reproducible within a resolution of ±5 mV.

The error in differential capacitance is given by the amplitude of the noise superimposed on the curves C vs. E. The error in electrode area was estimated from the magnitude of the scale divisions and the number of turns of the micrometric screw of the hanging mercury drop electrode.

The reported data are the results of at least three different monolayer batches for each lipid and conditions.

The middle part of the voltammograms presents two flat regions: one previous and another after the peak potential centered at around −0.71 and −0.85 V for DMPC and DMPE, respectively. Values of capacitance can be calculated from the differences of the flat regions obtained in the complete voltammetry sweep below and after this central peak (see Fig. 2A). The Hg drop area at which the lipid films were transferred from the gas/solution interface at the

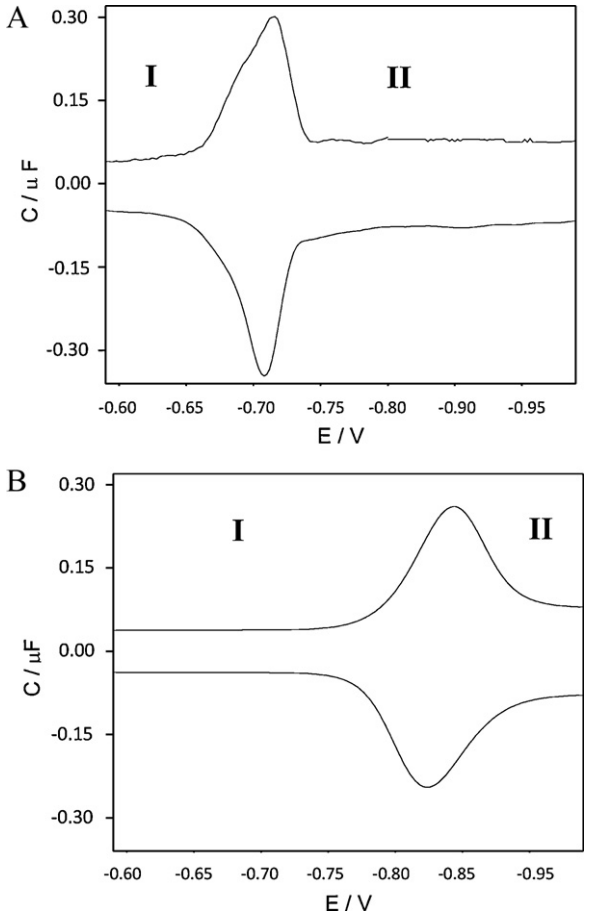

Fig. 2. Amplified view of the central region of voltammograms of Fig. 1 for DMPC (A) and DMPE (B). Capacitance of DMPC (A) and DMPE (B) monolayers on Hg before (region I) and after the peak potential (region II) at the area of transference 0.019 cm^2. T = 23 °C.

surface pressure given above was 0.019 cm^2. In a previous paper, the transition temperature of the lipid stabilized on Hg, after the transference, was determined by measuring the potential peak as a function of temperature at constant area. As reported, DMPC monolayers at 23 °C can be considered to be in the liquid condensed state [26], being the area per molecule 56.7 $Å^2$. In this condition, monolayer expansion was achieved in the range of 0.019–0.037 cm^2 for all lipids by increasing the volume drop. Additionally, in the case of DMPC, monolayer compression in the range between 0.019 cm^2 and 0.012 cm^2 were achieved by decreasing the drop volume. In all cases, after reaching the corresponding area consecutive reproducible voltammograms were obtained.

2.2. Calculation of energy

The capacitance peak observed in the center of the voltammogram of Fig. 2 reaches its maximum at around −0.71 V for DMPC and −0.85 V for DMPE. These peak potentials (E_p) are related to the energy required to trigger the reorganization processes at the interphase (W_p) by

$$W_p = \int_{E_{pzc}}^{E_p} (E - E_{pzc})C(E)dE \quad (2)$$

where E is the applied potential, E_p is the peak potential value, E_{pzc} is the zero charge potential of the metal in the presence of the adsorbed lipid film and C is the capacitance value before the peak.

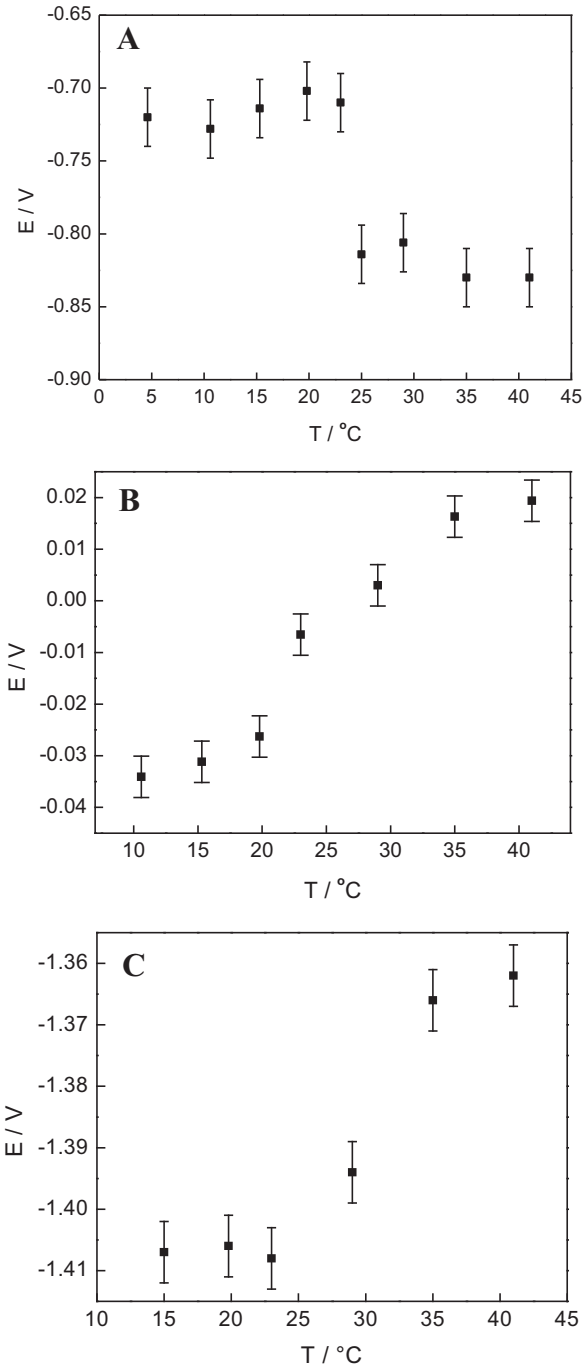

Fig. 3. Effect of temperature on peak potentials of central (A), anodic (B) and cathodic peaks (C) of DMPC voltammograms of Fig. 2. Electrode area = 0.019 cm^2. Error bars (±5 mV) corresponds to the uncertainty in the appreciation of peak potential values on the registered voltammograms.

Thus, W_p is proportional to the difference between the applied potential and the zero charge potential, E_{pzc}, that is

$$W_p \propto \left| E_p - E_{pzc} \right| \quad (3)$$

The zero charge potential of the system Hg/phospholipid/solution has been determined for phosphatidylcholines, being E_{pzc} = −0.243 V against Ag|AgCl (KCl 3.5 M) [23,26].

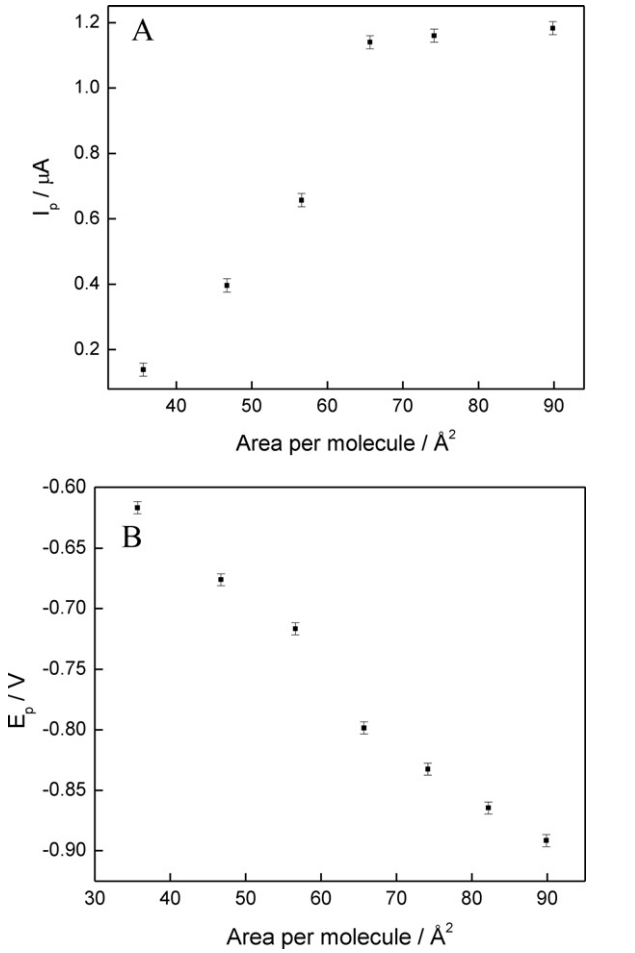

Fig. 4. (A) Central peak current of DMPC vs. area per molecule at 23 °C. Error bars (±0.01 μA) corresponds to the constant error characteristic of the current scale of the potentiostat used in the measurements. (B) Central peak potential of DMPC vs. area per molecule at 23 °C. Error bars (±5 mV) corresponds to the uncertainty in the appreciation of peak potential values on the registered voltammograms.

3. Results

A typical voltammogram of DMPC is shown in Fig. 1B. A single capacitance peak in the region of −0.71 V (denoted as 2c and 2a) is observed. This region is amplified in Fig. 2 for DMPC (A) and DMPE (B). In this last case, the peak is centered at −0.85 V. The capacitance before the peak (denoted as I) is lower than the capacitance after the peak (denoted as II) and the peak voltage is related to the energy involved in the changes of organization for a given lipid species. For DMPE (panel B), the peak is shifted to more negative potentials in comparison to DMPC (panel A). In addition, the specific capacitance after the peak is approximately 4 μF cm^{-2} for dimyristoylphosphatidylethanolamines and 6 μF cm^{-2} for dimyristoylphosphatidylcholines, respectively, indicating that polar head group nature plays a role in the process induced by the electrical field.

The shifts with temperature of the peaks observed at central (A) anodic (B) and cathodic (C) regions of the voltammograms of Fig. 1B for DMPC are shown in Fig. 3. The potential at the anodic and cathodic extremes displaces to less negative or positive potentials (Fig. 3B and C) in comparison to the previously reported shift of the central peak to negative values, which is reproduced in panel A for comparison [23]. This denotes that the application of the field in different potential regions results in different final reorganization of the lipids.

Table 1
Specific capacitance before and after the central peak for different DMPC/DMPE ratios.

$X_{DMPE/DMPC}$	E_p (V)	$\bar{C}_{before}$ (μF cm^{-2})	$\bar{C}_{after}$ (μF cm^{-2})
0	0.712 ± 0.005	≅2	≅11.6
0.1	0.796 ± 0.005	≅2	≅10.4
0.5	0.822 ± 0.005	≅2	≅7.3
0.75	0.851 ± 0.005	≅2	≅6.0
1.0	0.870 ± 0.005	≅2	≅4.0

The variation of the central peak at the phase transition for DMPC goes from −0.72 V in the liquid condensed state to −0.85 V in the liquid expanded above the transition temperature. In this range of capacitance, the values below and above the phase transition are approximately twice that reported for solvent-free black lipid membranes (BLM) [27,28]. Thus, it is reasonable to conclude that lipids are maintained in a monolayer. This is not the case in the anodic and cathodic regions. At these potentials, structural changes involving other ensembles of lipids, in addition to monolayers, such as micelles are probably present. For this reason, the peaks at the anodic and cathodic extremes of the voltammograms are beyond the purpose of this study.

Therefore, we focused only on the analysis of the behavior of the central peak, for DMPE and DMPC as a function of the area for lipids in different states.

As shown in Fig. 4, the intensity of the central peak increases continuously with the electrode area to reach a plateau at ca. 0.022 cm^2, which corresponds to an area per molecule of about 67 Å^2 parallel to the shift of the potential peak to more negative values (Fig. 4B). After the isothermal expansion to the highest area at 23 °C (Fig. 4B) the central peak potential of DMPC reaches the same value as that found by increasing the temperature above the phase transition at constant area (see Fig. 3A values above 24 °C) (E_p = −0.83 V at 74 Å^2/molecule). This means that a similar lipid state can be achieved by isothermal expansion of the liquid condensed state or by a thermotropic transition.

The specific capacitance calculated from the capacitance value in Fig. 3 before the peak (region I) within the area range between 0.012 and 0.022 cm^2 is shown for different DMPC/DMPE ratio in Table 1.

The changes in the area of the droplet allow to change the area per molecule. The area of the droplet corresponding to the transference of a DMPC monolayer from the air–water interface is around 0.019 cm^2. In this condition, the area per lipid is 56.7 Å^2 at 23 °C that corresponds to the excluded area of hydrated lipids in a saturated monolayer at the air–water interface. The increase or the decrease of the volume droplet allows to expand or to compress the monolayer, respectively changing the area per molecule beyond the hydration shell.

In the area range between 0.012 and 0.022 cm^2, that is an area per molecule for DMPC below 64 Å^2 or 72 Å^2 for DMPE at 48 °C, the differential capacitance at E = −0.4 V, remains virtually constant within the experimental error (Fig. 5). This observation suggests that compression of the electrode at potentials below the central peak potential do not change significantly the dielectric monolayer properties.

The relevant data obtained in Figs. 4 and 5 is that intensity and capacitance show a critical break at an area that depends on the phase state and the type of lipid. After the break, the intensity shows a saturation value and the capacitance varies linearly with the area with a slope that corresponds to the specific capacitance of the free-lipid Hg surface, meaning that the expansion above 0.022 cm^2, i.e. slightly above the area per lipid at saturation, the electrolyte has access to the metal. The specific capacitance of the interface Hg|buffer 0.1 M KH_2PO_4/0.1 M K_2HPO_4 depends on the potential. At E = −0.4 V its value is about 26 μF cm^{-2}. At this potential, the

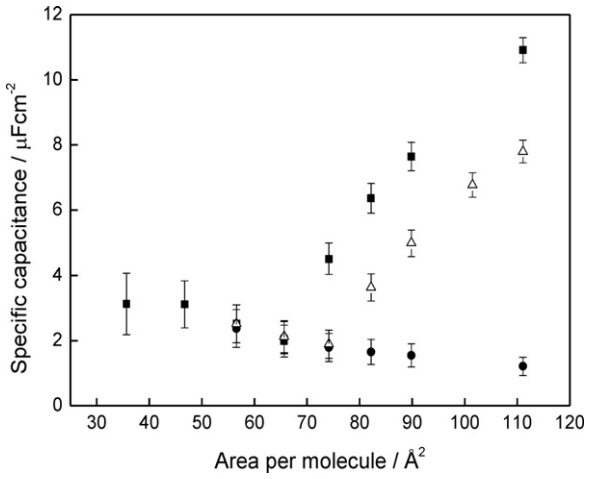

Fig. 5. Specific capacitance of DMPC at 23 °C (■), DMPE at 23 °C (●) and DMPE at 48 °C (△) as a function of the area per molecule. E = −0.4 V for the three cases. Error bars amplitudes for specific capacitance $\bar{C} = C/A$ were evaluated from the errors in differential capacitance (ΔC = ±0.01 μF) and electrode area ΔA = ±4.10^{-4} cm^2 (for details see Section 2).

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.

At 23 °C, the break point is observed for DMPC, but it is not in DMPE although both lipids are in the liquid condensed state at that temperature (Fig. 5). However, it must be noticed that DMPC at 23 °C is very near the phase transition (T_m = 24 °C). When the same expansion is done at 48 °C a break is also found for DMPE (T_m = 52 °C). This denotes that the break by expansion of the monolayer can only be achieved near the phase transition, a condition in which defects in packing can be found.

Finally, in Fig. 6 we have plotted the difference between the capacitance values before and after the peak (ΔC) (values at region I and II in Fig. 3) as a function of the area per lipid for DMPC (A) and DMPE (B). It is observed that in both lipids the capacitance change increases with the area per lipid, being all these values below those corresponding to the break point shown in Fig. 5. That is, the change in capacitance can be ascribed to the perturbation of the monolayer by the field input at constant area.

The differences of the capacitances are compared in the same plots with the energy input calculated from the peak potential as described in Section 2. The energy input required for DMPE is higher than those for DMPC. In both cases, the energy increases with the area increase, being more noticeable in DMPC.

4. Discussion

Previous results have shown that the peak in the central region of Fig. 2 shifts abruptly at the phase transition temperature of DMPC (24 °C) from ca. −0.72 V to −0.83 V [23]. The shift of the potential at the transition temperature to more negative values means that the process of lipid arrangement with the field requires more energy after the phase transition. A similar value of potential is achieved by the isothermal expansion of DMPC at 23 °C (Fig. 4B). In both cases, lipid hydration has increased.

The shift to negative values of the potential is also observed when the area increases and when DMPC is replaced by DMPE. In the case of DMPE, it is reasonable that the energy required is higher due to the increased lateral lipid–lipid interaction by H-bonds. The

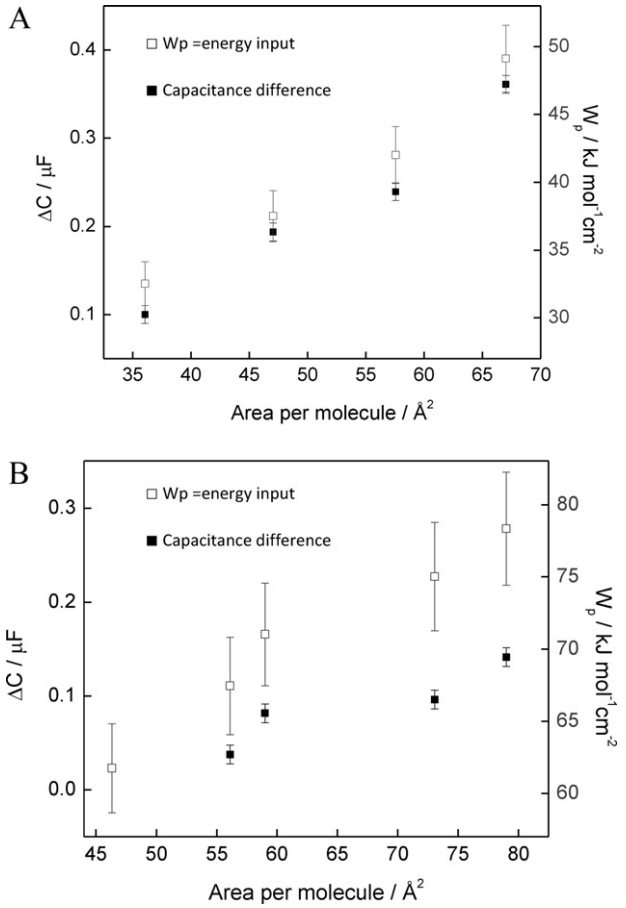

Fig. 6. (■) Difference between the differential capacitance values after (region II) and before the peak (region I) taken from Fig. 2 below the critical area. (□) Energy required by the interfacial rearrangement process that gives place to the central peak for DMPC (A) and for DMPE (B), as a function of the area per lipid. $T = 23\,^{\circ}C$. As was explained in the legend at Fig. 5A, the error bars in the difference of differential capacitances have amplitudes in the order of 0.01 μF. The amplitudes of error bars in peak energy (W_p) were calculated from the expression (2) using $\Delta A = \pm 4 \times 10^{-4}$ cm^2.

shift to negative values in DMPC with temperature and with area increase deserves some discussion.

The capacitance variation with the total electrode area (A) can be described by

$$C = \bar{C}_{LE}A_{LE} + \bar{C}_0 A_{free} = (\bar{C}_{LE} - \bar{C}_0)A_{LE} + \bar{C}_0 A \quad (4)$$

where $A_{free} = A - A_{LE}$.

A_{LE} corresponds to the area occupied by lipids and A_{free}, the free-lipid electrode area exposed to water.

Thus, the slope of C vs. A is $\overline{C}_0$, which is the capacitance of the Hg/electrolyte interface.

Based on this equation, the specific capacitance is given by

$$\bar{C} = (\bar{C}_{LE} - \bar{C}_0)\left(\frac{A_{LE}}{A}\right) + \bar{C}_0.$$

When $(A_{LE}/A) \to 0$, i.e. the degree of coverage decreases, $\overline{C}$ goes asymptotically to $\bar{C}_0$.

A similar behavior of DMPC and DMPE as a function of surface pressure was found when the perturbation in the surface pressure induced at different initial surface pressure by penetrant solutes in the subphase. In this case, the data plotted as $\Delta\Pi$ vs. Π curves indicates that the critical pressure at which no perturbation occurs is lower for DMPE than for DMPC at 23 °C [19]. This picture is congruent with the interpretation that perturbants need water beyond the hydration shell of the phospholipid head groups to affect the monolayer.

Thus, the relevant point to discuss in relation of monolayer cohesion is the critical point denoted in the break of the curves in Fig. 5 because it denotes the condition at which water lipid ratio modulates surface free energy. The first measurable point after the break in Fig. 5 corresponds to 12% area increase with respect to the initial area. 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 reported elsewhere by impedance measures [11].

It must be noticed that the break in Fig. 5 is observed when the surface is expanded at 23 °C, a temperature near the transition temperature for DMPC which is 24 °C. The absence of break for DMPE at 23 °C is congruent with the strong lateral interaction in comparison with DMPC by the intermolecular H-bonds. However, when temperature is 48 °C (near the phase transition of 52 °C), the break shown by DMPE is similar to that found with DMPC at 23 °C, although slightly displayed to higher areas (0.022 cm^2 for DMPC and 0.025 cm^2 for DMPE). This response of the lipids in the adjacency of the phase transition is congruent with the suggestion that they are organized as a monolayer in that condition. If it is accepted that monolayers probably have defects of packing near the phase transition temperature [29,30], the small increase in area would promote the appearance of spaces between lipids through which water may have access to the metal surface. The formation of fractures due to the presence of defects near the phase transition temperature would favor stabilization of water microenvironments.

The inspection of Fig. 5 shows that for an increment of 4.3% in area expansion above the critical point, the minimum capacitance value above the experimental error is 0.01 μF. The resulting area per lipid would be $A_w = 2 \times 10^{12}$ Å^2 and the water spaces increases in 6%, with respect to the initial area. In this regard, it is interesting to recall that bilayers in liposomes become leaky to K^+ ions when a 4% of the liposome area is overcome [31]. The increase in intensity with area is congruent with a leaky monolayer after the break in which water penetrates due to the dielectric barrier fall down.

The observation that the capacitance increase produced by the electrical input is a function of the area per lipid can be analyzed as the effect of a perturbation on the lipid interface (Fig. 6). As described before, the capacitance values before the peak are similar for both lipids. Hence, the capacitance difference is a measure of the perturbation given by a different final state attained by each lipid at each area value. Thus, at similar area per lipid, the perturbation is higher in PC than in PE.

As the total area is maintained constant, the structural transition giving place to the capacitance increase can be interpreted as a consequence of the rotation of the polar head group against lateral interactions. This rotation imposed by the field sign is opposed by the hydration forces toward the metal surface forming water spaces. The rotation of the whole molecule is unlikely due to lateral packing restrictions. NMR spectroscopy [32,33] and dielectric relaxation [34,35] results have shown that polar heads may reorient by the rotation of the phosphocholine or phosphoethanolamine groups around the P—O ester bond axes. In absence of electrical fields, the polar heads lay almost parallel to the membrane plane [32,36,37]. An angle of 27° has been reported for the orientation of the head group of DMPC [38]. On this ground, the input of the electrical field may induce the rotation of the positive end of the polar head groups toward the metal with the concomitant increase of water spaces in the interphase.

The required energy input grows with the area per lipid for DMPC and DMPE being much higher for DMPE. It is known that PC's polar head groups are more hydrated than PEs. In the liquid condensed state, the average number of water molecules per lipid is higher for DMPC than for DMPE [39,40]. In monolayers of PEs, polar heads of adjacent molecules interact laterally by hydrogen bonds between $(NH_3)^+$ and $(PO_4)^-$ groups while in PCs, lateral interactions between polar head groups of neighboring molecules take place through indirect H-bonds mediated by a water molecule (water bridge), which are more labile than direct H-bonds [41]. As a result, DMPE membranes have a much lower hydration than DMPCs (4 moles per PEs in comparison to 20 in PCs in the fluid state), thus reducing the area per lipid [40,41].

On this base, it is reasonable to expect that the energy required for the rearrangement would be larger for DMPE than for DMPC, as observed in Fig. 6. However, if the lateral interaction would be the only force involved in the process, the energy should decrease with expansion contrary to the experimental findings described in Fig. 6. The increase is much more noticeable in DMPC than in DMPE. A possibility is to consider that, along with the monolayer expansion; the decrease in lateral interaction gives place to an increase in the water per lipid in the monolayer. Taking into account that the peak potential shifts to more negative values at the phase transition [23] (Fig. 3A) and with monolayer expansion (Fig. 4B) it is reasonable to think that capacitance changes takes place by head group rotation against hydration.

The capacitance increases with the area per lipid in the same direction as the energy requirement, i.e. higher area per lipid gives higher capacitance changes at more energy cost indicating that head group rotation is hindered with the area increase. This energy cost may be ascribed to the increase of water molecules beyond the hydration layer and the concomitant work of rotation against dehydration. As the hydration is higher in DMPC than in DMPE the energy requirement increases due to the increase in the drag forces for head group orientation against its hydration shell. This requires the rupture of hydrogen bonds of waters surrounding the polar heads reflecting the importance of hydration in the interfacial processes.

It is reasonable to consider that the variation of the capacitance, either induced by area changes at constant field or by the field at constant area is related to the surface tension of the lipid ensemble at the interphase. In the present study, the increase in area brings the lipid ensemble to a state with different dielectric properties in comparison to the non expanded monolayer, as derived from the capacitance changes. The opening of spaces involves energy for the creation of one hole of 67 $Å^2$ per eight lipids. This hole is equivalent to 10 water molecules occupying the area of a lipid, that is, less than two water molecules per lipid.

This is a similar picture as that derived from studies of surface pressure in air–water monolayers. The fact that proteins or peptides can only interact with lipid monolayers of different composition if they are below the surface pressure corresponding to an area per lipid larger than that given by the contact of the hydration shells, i.e. larger than 67 $Å^2$, is congruent with the description of the change observed in the monolayer capacitance. In both cases, the lipid interface can be described as a bidimensional solution of hydrated polar head groups imbibed in water [42]. Thus, the presence of water beyond the hydration shell, i.e. that corresponding to the 67 Å per lipid affects the surface free energy driving the protein adsorption and confers a different dielectric property to the monolayer. This water seems to be confined beyond the hydration layers of phosphate and carbonyl groups and appears to have thermodynamic and dynamical properties determining protein, peptide and aminoacid insertion [18,24,25]. These water spaces can be considered as the water accessible regions between the first hydrocarbon methylenes as described elsewhere [43].

5. Conclusions

The take home lesson inferred from the present results and analysis is that water regions can be formed by expansion of the interphase when lipids are near the phase transition temperature.

Those regions can be formed also by distortions or reorientations of the polar head groups when the monolayer is perturbed by the electrical field at constant area. In this case, the exchange of water molecules is energetically much more relevant than the lateral interaction meaning that water defects have a cost of energy.

The present results also give support to the idea that membrane deformation produced by head group reorientation may produce water pockets were polar aminoacids can stabilize. In this work, the energy involved in that process is mainly determined by the hydration of the lipid head group rather than by the lateral interaction. Although this was determined by applying an electrical field across the monolayer, the influence of this reorientation on the mechanisms of interaction of charged groups of proteins cannot be discarded.

This paper is the first one in considering the importance of defects near the phase transition as possible nuclei for water path formation by expansion. As the area change required for this phenomenon to occur is the entrance of few water molecules beyond the hydration shell of the lipids, it may be related to the decrease in surface pressure necessary to favor the insertion of peptides and protein in monolayers as observed in $\Delta\Pi$ vs. Π curves. Further studies should clarify the cohesion of these few water molecules in a confined space and its structural properties.

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