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Supported planar bilayers from hexagonal phases

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Abstract

In this work the presence of inverted hexagonal phases H_{II} of 1-palmitoy-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) and cardiolipin (CL) (0.8:0.2, mol/mol) in the presence of Ca^{2+} were observed via ^{31}P-NMR spectroscopy. When suspensions of the same composition were extended onto mica, H_{II} phases transformed into structures which features are those of supported planar bilayers (SPBs). When characterized by atomic force microscopy (AFM), the SPBs revealed the existence of two laterally segregated domains (the interdomain height being ~1 nm). Cytochrome *c* (cyt *c*), which binds preferentially to acidic phospholipids like CL, was used to demonstrate the nature of the domains. We used 1-anilinonaphtalen-8-sulfonate (ANS) to demonstrate that in the presence of cyt *c*, the fluorescence of ANS decreased significantly in lamellar phases. Conversely, the ANS binding to H_{II} phases was negligible. When cyt *c* was injected into AFM fluid imaging cells, where SPBs of POPE:CL had previously formed poorly defined structures, protein aggregates (~100 nm diameter) were ostensibly observed only on the upper domains, which suggests not only that they are mainly formed by CL, but also provides evidence of bilayer formation from H_{II} phases. Furthermore, a model for the nanostructure of the SPBs is herein proposed.

Keywords: Liposome; Supported planar bilayers (SPBs); Hexagonal phase (H_{II}); ^{31}P-NMR; ANS fluorescence; AFM; Cytochrome *c* (cyt *c*)

1. Introduction

It is well known that phosphatidylethanolamines (PEs) and cardiolipin (CL), among other phospholipids, can adopt inverted hexagonal phases H_{II} in suspension under specific conditions [1]. However, the predominance of these kinds of phospholipids in the mitochondria or in the *Escherichia coli* cytoplasmic membrane does not result in hexagonal but in bilayer structures [2,3]. On the other hand, the formation of supported planar bilayers (SPBs) [4] from liposomes has emerged as an appropriate model to investigate the physicochemical properties of biomembranes by means of atomic force microscopy (AFM). SPBs represent an adequate model for investigating membrane properties such as phospholipid domains, protein insertion, and processes like cell adhesion or interactions of active molecules under biomimetical conditions [3,5–8]. SPBs are currently obtained by the so-called vesicle-fusion technique [9], which consists on the deposition of liposomes onto a convenient solid support and the latter's transformation into a planar bilayer after rupture and spreading [4,5,10,11]. In fact, an intriguing question is whether SPBs could be obtained from preparations of phospholipids in non-lamellar phases.

Of particular interest are the mixtures of PE and CL, due to their implications in many bioenergetic processes [12]. Although the occurrence of non-lamellar phases in vivo is doubtful, it is noteworthy that when cytochrome (cyt *c*), primarily associate with CL, it is released from the mitochondria at the onset of apoptosis [13]. Moreover, it has been demonstrated that the release mechanism depends on the presence of Ca^{2+} [14]. It is not known, however, how

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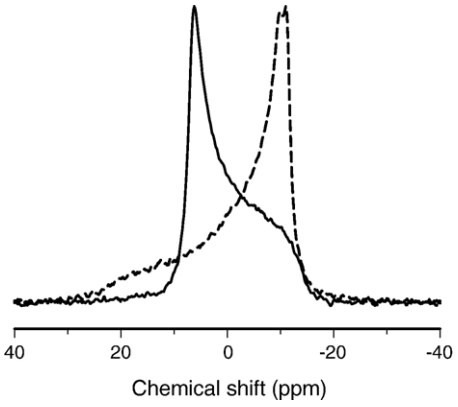

Fig. 1. Solid-state ^{31}P-NMR spectra at 25 °C corresponding to POPE:CL (0.8:0.2, mol/mol) dispersions in Ca^{2+} free 50 mM Tris–HCl, pH 7.40, 150 mM NaCl buffer (dashed line), and in 20 mM $CaCl_2$ added buffer (continuous line). Spectra were normalized to the same height. All chemical shift values are quoted in ppm with reference to external 85% phosphoric acid in H_2O as a reference (0 ppm), positive values referring to low-field shifts.

phospholipid phases might provide the adequate interfacial structure for cyt *c* binding under such conditions.

We have shown that SPBs can be formed from liposomes of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) and CL (0.8:0.2) mol/mol [15]. On the other hand, it is worth noting that such liposomes are normally incubated in the presence of divalent cations when the suspensions are deposited onto mica. As discussed elsewhere [5,10], Ca^{2+} and Mg^{2+} ions appear to facilitate the extension and fusion of liposomes onto the negatively charge borne by the mica. Therefore, the existence of non-lamellar phases, albeit in two dimensions, should be considered.

In this regard, the main purposes of this study are to investigate whether: (i) SPBs of POPE:CL (0.8:0.2, mol/mol) could be effectively formed from H_{II} suspensions of the same composition; and (ii) if lamellar or H_{II}, phases may bind cyt *c*. By combining our AFM observations of SPBs and binding experiments in solution, using 1-anilinonaphtalene-8-sulfonate (ANS), we have investigated whether CL forms laterally segregated domains onto mica.

2. Materials and methods

2.1. Materials

1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) and 99% pure cardiolipin (CL) were purchased from Avanti Polar Lipids (Alabaster, AL, USA) and used without further purification. 1-Anilinonaphtalene-8-sulfonate (ANS) was obtained from Molecular Probes (Eugene, OR). Horse heart

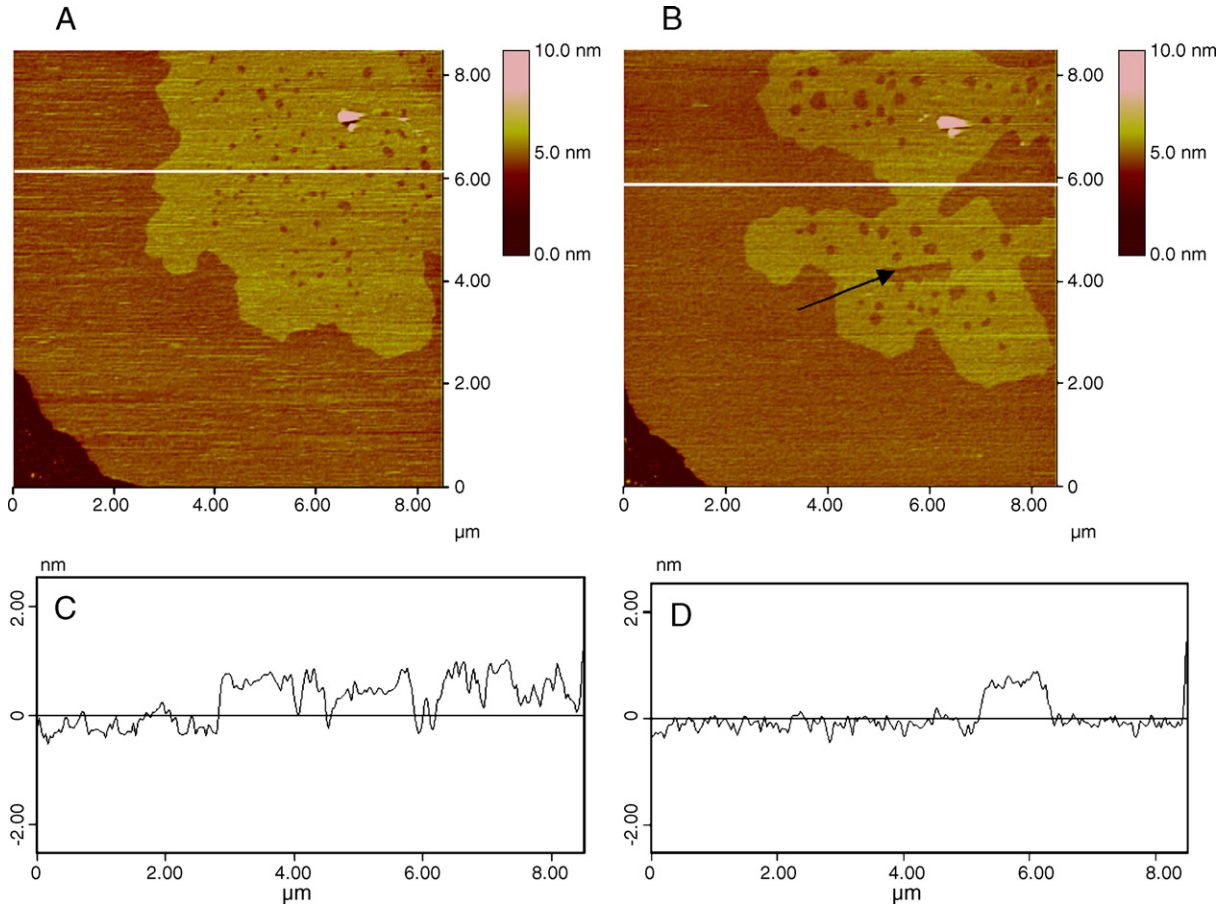

Fig. 2. (A) Topographic image of planar structures obtained by spreading POPE:CL (0.8:0.2, mol:mol) suspensions in 50 mM Tris–HCl, pH 7.40, 150 mM NaCl, 20 mM $CaCl_2$ after being washed with Ca^{2+} free buffer; (B) the same image scanned repeatedly by the AFM probe at a higher force (30 nN); (C and D) are the height profile analyses along the white lines shown in panels A and B, respectively.

cytochrome *c* was purchased from Sigma Chemical Co. (Madrid, Spain). Mica was obtained from Asheville-Schoonmaker Mica Co., VA, USA). Two different buffer solutions were used: (A) 50 mM Tris–HCl, pH 7.40, 150 mM NaCl and (B) 50 mM Tris–HCl, pH 7.40, 150 mM NaCl, 20 mM $CaCl_2$.

2.2. ^{31}P-nuclear magnetic resonance measurements

Chloroform/methanol (50:50, v/v) stock solutions of the desired mixture of POPE and CL were evaporated to dryness in a conical tube using a rotavapor. The resulting thin lipid film was then kept under high vacuum overnight to ensure the absence of organic solvent traces. Suspensions for ^{31}P-Nuclear Magnetic Resonance (^{31}P-NMR) spectroscopy were obtained by hydration in excess of either buffer A or B to a final concentration of 2.2 mM. The resulting suspension was then pelleted by ultracentrifugation at 115,000 × *g* for 1 h at 5 °C. The hydrated pellet was then resuspended in 300 μL of supernatant and placed in a conventional 5 mm NMR tube. A capillary tube containing $^{2}H_2O$ was added for field-frequency stabilization.

^{31}P-NMR spectra were recorded as detailed elsewhere [16] on a Bruker ARX-400 spectrometer (Bruker Española, S.A., Madrid), operating at 161.98 MHz using a 90° pulse sequence, with proton-decoupling conducted during signal sampling by means of a Waltz-16 composite pulse sequence [17,18]. The single pulse sequence was used instead of the phase-cycled Hahn echo pulse sequence [19] to obtain spectra with higher signal-to-noise ratios [20]. Each spectrum was the result of accumulating 4096 sampled scans using 2048 complex data points, with a 90° pulse of 16 μs (B_{eff}=19.5 kHz), an interpulse delay of 2.1 s, and a spectral width of 50 kHz. An exponential multiplication resulting in a line broadening of 50 Hz was applied before Fourier transformation to improve the signal-to-noise ratio. Spectra were processed on a personal computer running the TopSpin v. 1.3 software (Bruker Biospin GmbH, Germany) on Debian GNU/Linux v. 3.1. All chemical shift values are quoted in parts per million (ppm) with reference to pure external 85% phosphoric acid in H_2O as a reference (0 ppm), positive values referring to low-field shifts.

2.3. Fluorescence measurements

As described elsewhere for fluorescence measurements [3] the lipids suspensions obtained either in buffer A or B (liposomes or H_{II} phases, respectively) were filtered through polycarbonate membranes (400 nm nominal diameter) using an Extruder device (Lipex Biomembranes Inc., BC) and diluted with appropriate buffer to a final concentration of 100 μM total phospholipids. Fluorescence measurements were carried out using an SLM-Aminco 8100 spectrofluorometer at 21 °C. Under continuous stirring ANS was added from a stock solution in ethanol (5 mM) to the cuvette to the desired final concentration (0–160 μM). The measurements were done using excitation and emission wavelengths of 380 and 480 nm, respectively. The excitation and emission slits

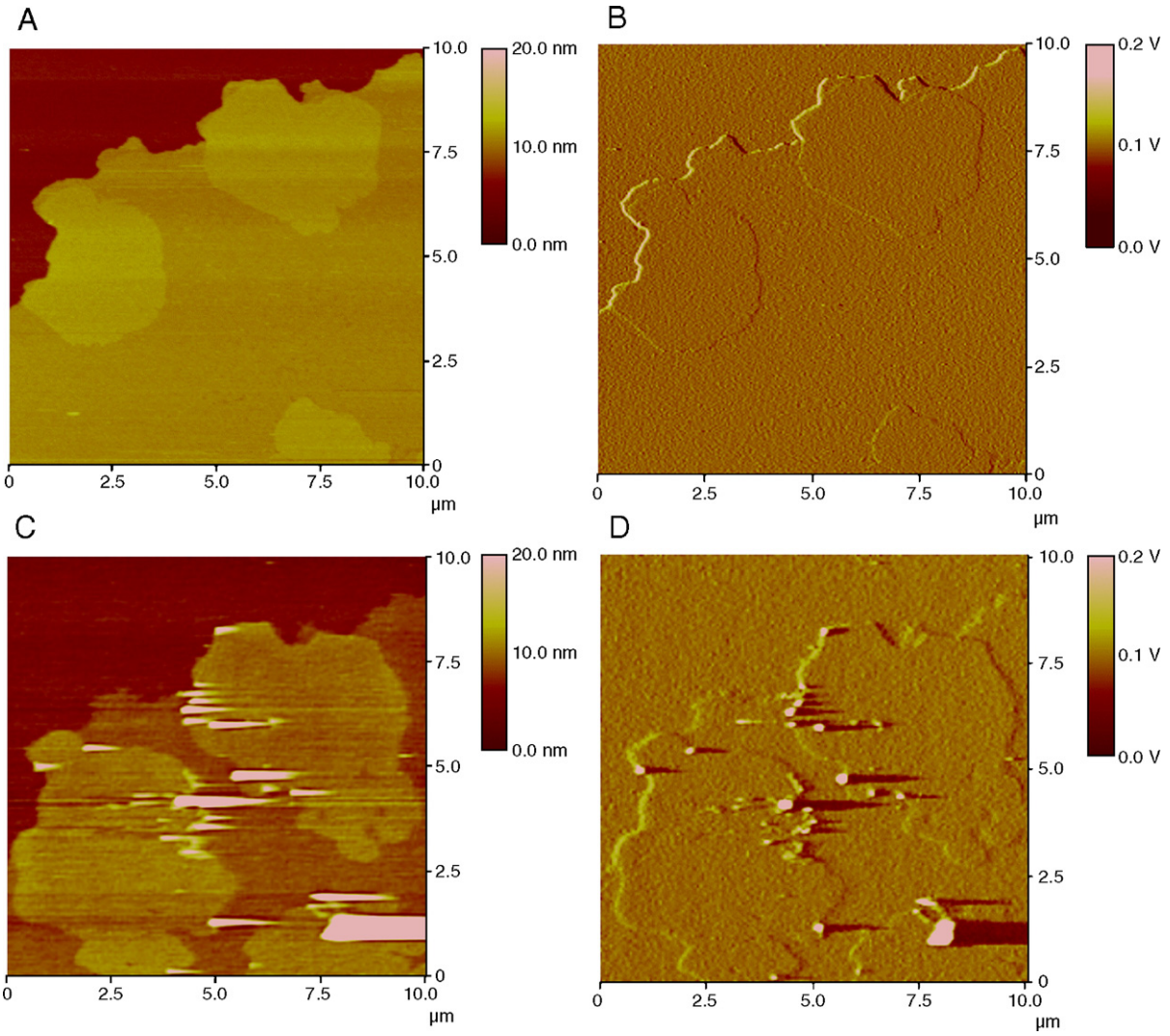

Fig. 3. (A) Topographic image of POPE:CL (0.8:0.2, mol:mol) SPBs at 24 °C; (B) deflection image of panel A; (C) topographic image of the SPB after the addition of cyt *c* (1 μM); (D) Deflection image of panel C.

were 8 and 8 nm and 4 and 4 nm, respectively. Cyt *c* was added to the cuvette to a final lipid-to-protein molar ratio of ~1 and incubated for 30 min at 24 °C.

2.4. Supported planar bilayers formation and AFM observations

The experimental procedures were adapted form previous works [3,7,15]. Briefly, 50 μL of the H_{II} phase suspensions (in buffer A) were pippeted onto ~0.5 cm^2 freshly cleaved mica sheet. After allowing the vesicles to adsorb at room temperature (24 °C) for 30 min the surface was gently washed with Ca^{2+} free buffer (buffer B) and the tip immediately immersed in the buffer. For in situ AFM measurements, cyt *c* was added into the fluid cell at a final concentration of 5 μM. All images were acquired in tapping mode with a Multimode Digital Instruments (Santa Barbara, CA) microscope controlled by a Nanoscope IV electronics fitted with a 15×15 μm scanner ("J"-scanner). Standard V-shaped Si_3N_4 tips, with a nominal force constant of 0.08 N m^{-1} (Olympus, Germany), were used. In order to minimize the forces exerted on the sample, the vibration amplitude of the tip was reduced as much as possible and amplitude setpoint was set as high as possible without losing contact with the surface of the sample. The scan rate was set to 1.5 Hz. Before every sample, the AFM liquid cell was washed with ethanol and ultra pure water (Milli Q reverse osmosis system), and allowed to dry in an N_2 stream. Mica discs (green mica) were cleaved with scotch and glued onto a Teflon disc by a water-insoluble epoxy. These Teflon discs were glued onto a steel disc and then mounted onto the piezoelectric scanner. All images were scanned under aqueous solution and processed with the Nanoscope IV software.

3. Results and discussion

In Fig. 1, the ^{31}P-NMR powder pattern spectra of the POPE:CL (0.8:0.2, mol/mol) systems at 25 °C in the buffer in the presence (continuous line) and absence (dashed line) of 20 mM $CaCl_2$ shows the typical features for the H_{II} and lamellar organizations, respectively. These results are in accordance with the fact that PEs, as well as CL, have a strong tendency to adopt H_{II} phases in the presence of Ca^{2+}. The existence of hexagonal phases of POPE:CL at 25 °C is in concordance with two facts: (i) POPE exhibits a typical bilayer-to-hexagonal phase transition between 66 and 74 °C, which in turn decreases in the presence of Ca^{2+} [21]; and (ii) CL adopts an H_{II} phase in the presence of Ca^{2+} [22]. Indeed, the POPE:CL system exhibits the coexistence of lamellar and H_{II} phases between 5 and 18 °C, H_{II} phase being predominant above 18 °C remaining so up to 70 °C in the presence of Ca^{2+} (^{31}P-NMR data not shown).

When POPE:CL lipid suspensions in Ca^{2+} containing buffer (i.e., in the H_{II} phase) (Fig. 1) were deposited onto mica, planar bilayer-like structures [4] with two domains were observed (Fig. 2A). The absence of distinctive geometrical facets as those reported for cubic and hexagonal phases of other compositions [23] indicate that the H_{II} phase of the POPE:CL system is not retained after adsorption onto the mica surface. Bilayer thickness can be inferred by measuring the step height between the top of the layer and the uncovered mica (red color in the lower-left corner). Thus, while the height of the lower domain, 4.64±0.16 nm (n=25), fell within the range expected for bilayer thickness [7] the upper domain appeared as an upper layer, measuring ~1.00±0.08 nm (n=50) above the lower domain. In addition, the thickness of the upper layer coincided with the depth of numerous defects (holes) observed. When this SPB was repeatedly scanned at a force of ~30 nN for 20 s (Fig. 2B), the upper layer became unstable and lipid molecules could be swept away. As can be observed by following the cross-sectional analysis along the white line drawn in Fig. 2C and D, this observation is consistent with the fact that the surface overlaying the lower domain decreased. Furthermore, the height of the upper domain was confirmed by measuring the depth of the small scratched area (indicated by the black arrow in Fig. 2B). As expected, the resulting measurement (~0.94±0.09 nm) coincided with the interdomain height reported above.

To investigate the nature of the upper domain, cyt *c* was injected into the AFM fluid imaging cell where the POPE:CL system was previously formed. Similar to Fig. 2, two domains can be easily distinguished by differences in the color scale of the topography image (Fig. 3A), as well as by the deflection images (Fig. 3B). When cyt *c* (positively charged at this pH) [24] was injected, small bright spots with a higher height emerged predominantly at the edges of the upper domain (Fig. 3C). Such behaviour has been observed for an alkaline phosphatase [25] which has been related to a decrease in the interfacial tension at the phase boundary. Besides, there is a lateral grow of the upper domain. This could be attributed either

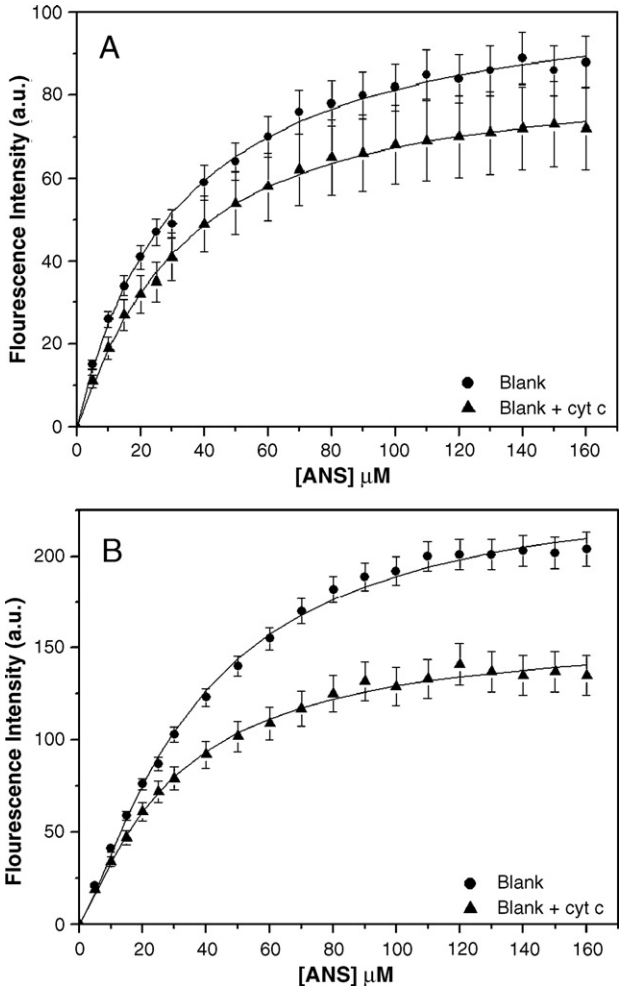

Fig. 4. Fluorescence intensity of ANS bound to POPE:CL (0.8:0.2, mol/mol) of the H_{II} phase (A) and liposomes (B) as a function of the free ANS concentration, in the absence (●) and presence of cyt *c* (▲). The total lipid concentration was 50 μM.

to a partial insertion of cyt *c* into the bilayer or to the thermal response of the phospholipids as a consequence of the continuous action of the laser. To assist with visualization, the corresponding deflection image (Fig. 3D) is presented. Here, poorly defined structures, presumably protein aggregates (~100 nm diameter), can be observed on the upper domain. In agreement with these observations, circular aggregates of cyt *c* have been observed on acid lipid bilayers, although of larger diameter (>400 nm) [26] than those observed here. However, the observation of single entities of cyt *c* appeared to depend on the balance between electrostatic and hydrophobic forces [27,28].

ANS is a fluorescent molecule extensively used for monitoring changes in the hydrophilic phosphate moiety of phospholipids [29,30]. Typically, the fluorescence intensity increases as the label is added to phospholipid suspensions until a plateau is reached at high probe concentrations [31]. These behaviours are illustrated by the Langmuir-like isotherms [16] obtained by labelling the POPE:CL systems with ANS in the presence (Fig. 4A) and absence (Fig. 4B) of Ca^{2+}. It is well known that cyt *c* associates electrostatically with acidic phospholipids at neutral pH [32]. On the other hand, at high CL contents (>20%) the protein becomes partially inserted into the bilayer [33]. Hence that ANS can be displaced from suspensions of POPE:CL in presence of cyt *c*. Consequently, the fluorescence of ANS decreased in both the H_{II} phase (Fig. 4A) and liposomes (Fig. 4B) as a result of the competition between cyt *c* and ANS for the same binding sites. Fluorescence intensity values for H_{II} phases in the presence of cyt *c* (Fig. 4A) revealed that cyt *c* binding was practically negligible because the values in the presence of protein fell within the same standard deviation values as obtained in its absence. In addition, the fluorescence intensities exhibited were higher for the lamellar than for the H_{II} phases. This behaviour is probably due to the fact that the anionic phospholipid-buffer interfaces are less exposed (negative curvature) compared with that of liposomes (positive curvature). However, the accessibility of cyt *c* for the polar head regions, which should diffuse through apolar regions to access the binding site, was clearly reduced in the case of the H_{II} phases. Although indirectly, this confirms the lamellar nature of the supported structures onto which cyt *c* binds (Fig. 3).

All of these findings led us to propose a model for interpreting those structures observed through the AFM (Fig. 5). Basically, POPE:CL is in a hexagonal phase when deposited onto the substrate (Fig. 5A). The presence of Ca^{2+} induces, in several intermediate steps similar to those depicted in Fig. 5B and C, the formation of planar structures in two levels or domains (Figs. 2 and 3). Although the composition of each domain is difficult to ascertain, we have previously reported [15] that there is a decrease only in the thickness of the lower domain but not in the upper domain by increasing the temperature. Therefore, it could be assumed that the lower domains are POPE-enriched bilayers. This is further substantiated by two facts: (i) the height of these domains was consistent with the PE bilayers' height [11]; and (ii) the thermotropic gel-to-fluid phase transition was observed around

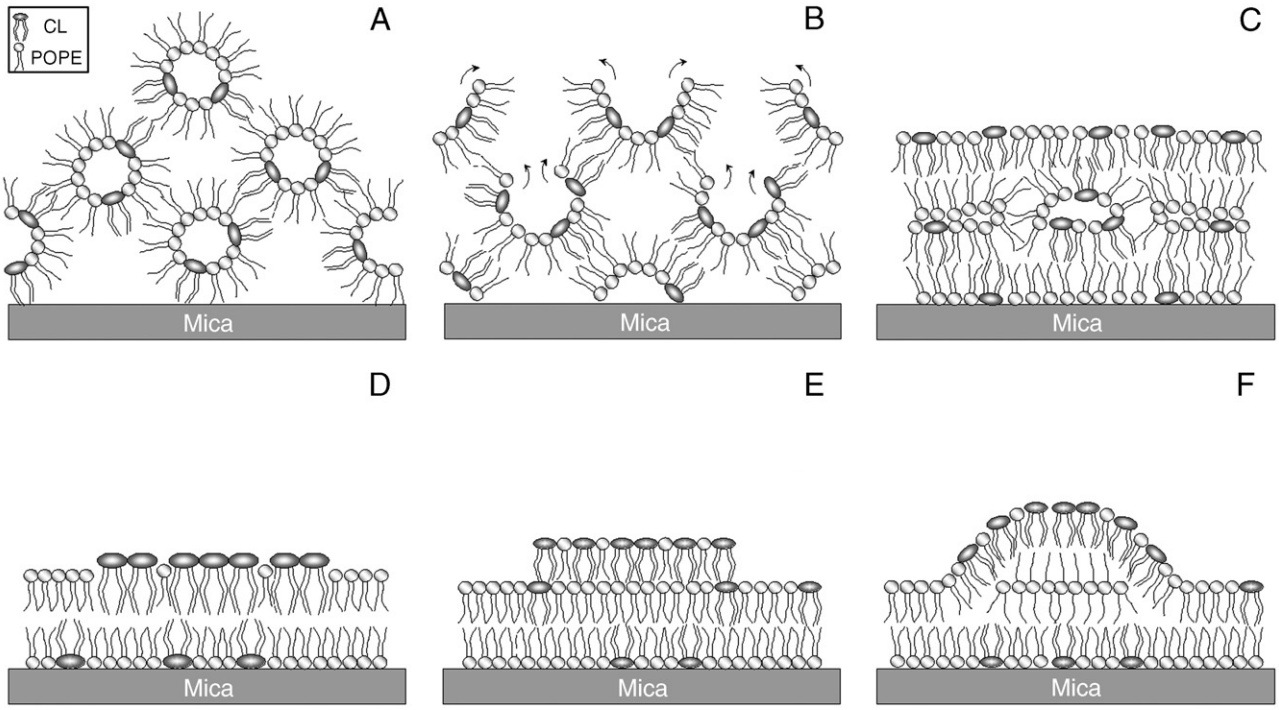

Fig. 5. Some of the events thought to occur during the formation of planar structures on mica: (A) POPE:CL (8:2, mol/mol) hexagonal (H_{II}) phase adsorption; (B) fusion onto mica; (C) H_{II} to lamellar phase transition showing an inverted micelle; (D) supported planar bilayer showing laterally segregated CL; (E) a hypothetical trilayer; and (F) a planar bilayer with the extended CL conformation.

24 °C [15]. Interpreting the upper domain that binds cyt *c* is a bit more difficult. As cyt *c* is anionic phospholipid-specific in nature, and taking into account the ability of Ca^{2+} to induce lateral phase separation of mixtures containing negatively charged phospholipids [10], it seems reasonable to assume that the upper domain should be mostly formed by CL (Fig. 5D). It is more difficult, however, to provide a reasonable explanation for the height of this domain, (within the range of the height of a phospholipid monolayer). Nevertheless, an overestimation of the height as a result of tip repulsion could not be excluded. Other potential scenarios to explain the occurrence of these domains include: (i) one possessing an upper CL-enriched monolayer adsorbed onto the bilayers (Fig. 5E); or (ii) one with CL molecules in "extended" conformation [34] resulting in the upper domains (Fig. 5F). Although possible, the existence of a monolayer with the acyl chain adsorbed on top of the polar head groups is unlikely based on the nature of the intermolecular forces that would be involved. On the other hand, an extended conformation would result as an intermediate step in the transformational process of H_{II} phases into bilayers when deposited onto mica. Thus, assuming that by the action of the tip, the molecules that are swept away (Fig. 2B) might be mainly of CL, the structural model shown in Fig. 2D could be the most likely. Other approaches and techniques will be undertaken for our group in the near future to demonstrate this hypothesis.

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