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Atomic force microscopy and force spectroscopy study of Langmuir–Blodgett films formed by heteroacid phospholipids of biological interest

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Abstract

Langmuir–Blodgett (LB) films of two heteroacid phospholipids of biological interest 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), as well as a mixed monolayer with $\chi_{POPC}=0.4$, were transferred onto mica in order to investigate by a combination of atomic force microscopy (AFM) and force spectroscopy (FS) their height, and particularly, their nanomechanical properties. AFM images of such monolayers extracted at 30 mN m^{-1} revealed a smooth and defect-free topography except for the POPE monolayer. Since scratching such soft monolayers in contact mode was proved unsuccessful, their molecular height was measured by means of the width of the jump present in the respective force–extension curves. While for pure POPC a small jump occurs near zero force, for the mixed monolayer with $\chi_{POPC}=0.4$ the jump occurs at ~800 pN. Widths of ~2 nm could be established for POPC and $\chi_{POPC}=0.4$, but not for POPE monolayer at this extracting pressure. Such different mechanical stability allowed us to directly measure the threshold area/lipid range value needed to induce mechanical stability to the monolayers. AFM imaging and FS were next applied to get further structural and mechanical insight into the POPE phase transition (LC–LC′) occurring at pressures >36.5 mN m^{-1}. This phase transition was intimately related to a sudden decrease in the area/molecule value, resulting in a jump in the force curve occurring at high force (~1.72 nN). FS reveals to be the unique experimental technique able to unveil structural and nanomechanical properties for such soft phospholipid monolayers. The biological implications of the nanomechanical properties of the systems under investigation are discussed considering that the annular phospholipids region of some transmembrane proteins is enriched in POPE.

Keywords: Atomic force Microscopy (AFM); Force spectroscopy (FS); Langmuir–Blodgett film (LBs)

1. Introduction

The physicochemical properties of biological membranes are crucial for understanding the membrane function and structure. Of particular interest is the influence of physical properties of natural occurring phospholipids on the activity and the structure *in situ* of transmembrane proteins [1]. Lactose permease (LacY) of *Escherichia coli*, for instance, a protein which is often taken as a paradigm for membrane secondary transport, depends on phosphatidylethanolamine (PE) for *in vivo* function [2] and correct folding [3]. On the other hand lipids have been found in transmembrane protein crystals forming specific complexes [4]. Such is the case of three-dimensional crystals of LacY, obtained in presence of *E. coli* lipid extracts with high proportions of PEs [5] and two-dimensional crystals obtained in presence of heteroacid phospholipids as 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocoline (POPC) [6–8]. In addition, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) appears to

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be related to the formation of phospholipid domains when LacY is reconstituted into proteoliposomes [9,10]. Within this framework of protein–lipid interface interactions, the influence of the phospholipids on the function of potassium channels becomes relevant. It is worth mentioning that the homotetrameric potassium channel KcsA from *Streptomyces lividans*: (i) requires anionic lipids for its activity [11]; and (ii) has been crystallized in presence of membrane phospholipids [12] and thereafter]. As has been discussed elsewhere, PE is important for efficient membrane association and tetramerization of KcsA [13]. On the other hand the voltage-dependent function of the potassium channel KvAP from *Aeropyrum pernix* remains intact when reconstituted in membranes containing phospholipids with anionic phosphate groups such as POPE or POPC [14]. These experimental observations indicate that the phospholipids in the immediate vicinity of the transmembrane proteins, the so-called annular lipid region, should be phospholipids that may easily adapt to the highly irregular surface of the integral proteins [15]. To accomplish such physicochemical requirements, the annular phospholipids should be highly compressible, in fluid phase and with a definite shape in order to provide an adequate thickness to match the hydrophobic membrane-spanning region of the protein. Most remarkably, there are evidences that the presence of non-bilayer phospholipids (i.e. PEs or cardiolipin) is essential for peripheral and integral membrane proteins [2,3,13,16]. It has been proposed that these phospholipids might modulate the protein function since they exert different lateral pressure at different depth of the bilayer [17]. Hence, the study of the physicochemical properties of these heteroacid phospholipids becomes relevant for a better understanding of membrane physiology and activity.

In a previous work, the Langmuir–Blodgett (LB) technique has been used to obtain information of the mixing properties of POPE:POPC binary monolayers through thermodynamic analysis [18]. Atomic force microscopy (AFM) of LB films may provide new topographic information [19,20]. Besides, the nanomechanical properties of the monolayers of interest at a desired lateral surface pressure could be obtained when working in force spectroscopy (FS) mode [21]. Indeed, FS mode has allowed researchers to gather additional information about the mechanics of different substrates using nanometric and subnanonewton resolution. In fact, FS has been applied to the understanding of nanomechanical properties of a vast number of well-defined molecular systems. These include indenting hard surfaces [22,23] or cells [24] by an AFM tip, measuring the interactive forces arising between a chemically functionalized tip and a chemically derived surface (known as chemical force microscopy) [25], studying the solvation forces of liquids in the proximity of flat surfaces [26], measuring the entropic forces that play a role in the stretching simple polymers [27] or the unfolding polyproteins [28,29]. Extensive research has been lately conducted on lipid bilayers with AFM, which revealed important information regarding their topography [30–34] and their nanochanical properties through force–extension curves [35–38]. These latter studies revealed the existence of a jump in the approaching force curve, which is interpreted as the membrane breakthrough or membrane failure, which results from the AFM tip penetrating the membrane [30,35,36]. The force at which this jump occurs is the maximum force that the membrane is able to withstand without breaking. Since the width of the jump in the force–distance curves mentioned above is intrinsically related to the monolayer depth, in this case FS allows the researchers to extract valuable structural and mechanical information that could not have been otherwise experimentally obtained.

The goal of this paper is two-fold. On the one hand, we aim to get further insight into the topographic characterization of the POPC and POPE LB monolayers by means of AFM. On the other hand, we aim to gain information on the mechanical properties of such LBs using the FS mode through the force–distance curves. In particular, we are interested in studying the nanomechanical stability of the studied monolayers, which is a straightforward reflection of their molecular organization and in the measurement of their thickness. Particular attention has been put into the distinctive phase transition that POPE undergoes at 36 mN m^{-1} [39].

2. Materials and methods

2.1. Preparation of Langmuir–Blodgett films

1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE), and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), specified as 99% pure, were purchased from Avanti Polar Lipids (Alabaster, AL, USA) and used without further purification. The subphase buffer for preparing the LB films was a 50 mM Tris–HCl buffer (pH 7.40) containing 150 mM NaCl, prepared in Ultrapure water (Milli Q® reverse osmosis system, 18.3 MΩ cm resistivity). Chloroform and methanol, HPLC grade were purchased from SIGMA (St. Louis, MO, USA).

Details of these experiments with monolayers have been described elsewhere. Briefly, the lipids were dissolved in chloroform–methanol (3:1, v/v) to a final concentration of 1 mg mL^{-1}. LB monolayers for POPE, POPC and POPE:POPC (0.6:0.4, mol:mol) were prepared using a 312 DMC Langmuir–Blodgett trough manufactured by NIMA Technology Ltd. (Coventry, England). The trough was placed onto a vibration-isolated table (Newport, Irvine, CA, USA) and enclosed in an environmental chamber. The subphase was filtered with a Kitasato system (450 nm pore diameter) before use. The resolution of surface pressure measurement was ±0.1 mN m^{-1}. In all experiments the temperature was controlled at 24.0±0.2 °C by an external circulating water bath. Before each experiment, the trough was washed with chloroform and rinsed thoroughly with purified water. The cleanliness of the trough and subphase was ensured before each run by cycling the full range of the trough area and aspirating the air–water surface, while at the minimal surface area, to zero surface pressure.

The corresponding aliquots of lipid were spread, drop-by-drop, onto subphase solution with a Hamilton microsyringe. A period of 15 min was required to allow the solvent to evaporate before the experiment was started. The compression barrier speed, vis-à-vis the final surface pressure, was 5 cm^2 min^{-1}. LB films were transferred onto freshly cleaved mica, lifting the substrate, at a constant rate of 1 mm min^{-1}. The transfer ratios were evaluated and proved near the unity, indicating that the mica was almost covered with the monolayer.

2.2. Atomic force microscopy imaging

AFM measurements were made at a constant temperature of 24 °C with a Nanoscope IV from Digital Instruments, Santa Barbara, CA, equipped with a 50 μm piezoelectric scanner. All the images were taken in air in contact mode with a silicon nitride cantilever and a nominal spring constant of 80 pN nm^{-1}. The applied force was kept as low as possible to minimize monolayer damaging.

All the images were processed using Digital Instrument (Nanoscope 6.12r1) software.

2.3. Force spectroscopy

FS was performed with a Molecular Force Probe1-D, (Asylum Research, Santa Barbara, CA). Force plots were acquired using V-shaped Si_3N_4 tips (OMCL TR400PSA, Olympus) with a nominal spring constant of 80 pN nm^{-1}. Individual spring constants were calibrated using the equipartition theorem after having correctly determined the photodetector optical sensitivity (V/nm) by measuring it at high voltages after several minutes of performing force plots to avoid hysteresis. Applied forces F are given by $F=k_c\times\Delta$, where Δ stands for the cantilever deflection. The surface deformation is given as penetration (δ), evaluated as $\delta=z-\Delta$, where z represents the piezo-scanner displacement. Hundreds of force versus tip-sample distance were acquired in at least 10 different spots of the same sample. The tip-sample approaching velocity was set for all force curves at 600–1000 nm s^{-1}.

3. Results and discussion

Fig. 1 displays the surface pressure–area isotherms of the pure POPE, POPC and the mixed monolayer with $\chi_{POPC}=0.4$, the more stable mixture accordingly a surface thermodynamic analysis elsewhere published [18]. The features of these isotherms were consistent with others in the literature [40]. The monolayer of pure POPC was always in the liquid expanded (LE) phase and showed a collapse surface pressure of 46 mN m^{-1}. In turn, the monolayer of POPE is in LE up to 36.0 mN m^{-1} where it exhibits a characteristic transition that according to the literature [39] occurs between two liquid condensed phases (namely LC and LC′) [41]. The collapse was observed at 50.7 mN m^{-1}. The mixed monolayer, with a $\chi_{POPC}=0.4$, presents also the LC–LC′ phase transition, but now at surface pressure of 44.7 mN m^{-1} and a collapse surface pressure at 48.7 mN m^{-1}.

The variation of compression modulus (C_s) values with π for the three monolayers is shown in the inset of Fig. 1. As it can be seen, below ~7 mN m^{-1} the POPE monolayer always displays greater C_s values than the POPC and the mixed monolayer. This

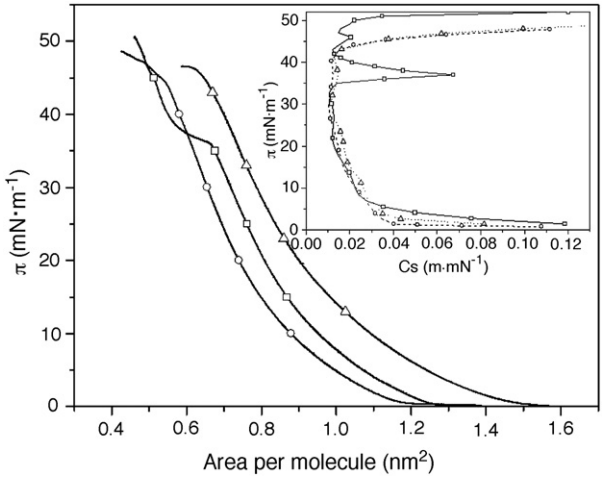

Fig. 1. Surface–pressure area isotherms of pure and mixed monolayers at the air–water interface. (Δ) POPC, (□) POPE, (○) $\chi_{POPC}=0.4$. INSET: Compressibility modulus of the three monolayers. (Δ) POPC, (□) POPE, (○) $\chi_{POPC}=0.4$.

demonstrates that pure POPE monolayers have more compressibility at low surface pressures. From 7 to 25 mN m^{-1}, the C_s values were always greater for the POPC than for the POPE monolayer. This fact should be attributed to the different size of the PE and PC headgroups [42,43]. Furthermore, above 25 mN m^{-1} the three monolayers showed similar mechanical compressibility as reflected by the C_s values. POPE monolayer showed a first-order transition at 36.0 mN m^{-1} and a second order transition at 45.5 mN m^{-1}. The first transition supports the existence of two states, LC and LC′, which simply would reflect, as in other systems [44], a different molecular ordering. Such subtle property would be related with the ability of POPE to contribute to the adequate lateral pressure profile around the hydrophobic domain of transmembrane proteins [13,17].

To perform a topographical characterization, POPC and POPE monolayers were transferred onto a mica substrate at surface pressures of 30 mN m^{-1} and imaged with AFM in contact mode. Based on theoretical considerations [45,46] 30 mN m^{-1} is currently assimilated to the lateral surface pressure of a bilayer but it is reasonable to assume that large variations may occur under physiological conditions. The AFM images of POPC, POPE and the mixed monolayer with $\chi_{POPC}=0.4$ are shown in Fig. 2. With the only exception of the LB of POPE transferred at 30 mN m^{-1} (Fig. 2B), the AFM images provided continuous and smooth monolayers with average surface roughnesses (*Ra*) ranging from 0.05 to 0.14 nm. These values are in the range of others elsewhere reported [47]. In turn, the LB image of the $\chi_{POPC}=0.4$ monolayer (Fig. 2C) provided visual evidence that POPE and POPC mix ideally at this molar fraction [18] since no lateral phase separation is observed.

The hydrophobic thickness values for transmembrane proteins estimated from three-dimensional crystal structures at high resolution falls within the range of ~2.3 to 3.5 nm [1]. Particularly, LacY with 6 nm along the membrane normal, that is ~2.7 to 3 nm embedded within the membrane [48] would show preference for phospholipids with adequate length to match this hydrophobic surface. Those would be according to excimer–monomer fluorescence measurements carried out with liposome containing pyrene–phospholipid derivatives [9], molecules that according to AFM measurements would have an effective length ~3 nm [49,50]. This will result in a ~6 nm bilayer thickness that seems to mismatch with the currently reported size of Lac Y [48]. Therefore, determining the thickness values for phospholipids as POPC or POPE, both relevant in structural [6,7] and physiology [2,3] of LacY and probably for KvAP [12,14], may provide indirect support for its presence at the annular region.

The thickness values of supported monolayers are often obtained either by measuring the depth of the defects [30] or by scratching the surface and measuring the step height between the top of the monolayer and the uncovered mica [51]. Thus, the depth of the POPE monolayer at 30 mN m^{-1} (Fig. 2B) could be established in 1.16 nm by means of the numerous holes observed. Since the rest of the LBs were defect-free, we attempted to establish their height by scratching the sample. This revealed, however, as an unsuccessful method for both,

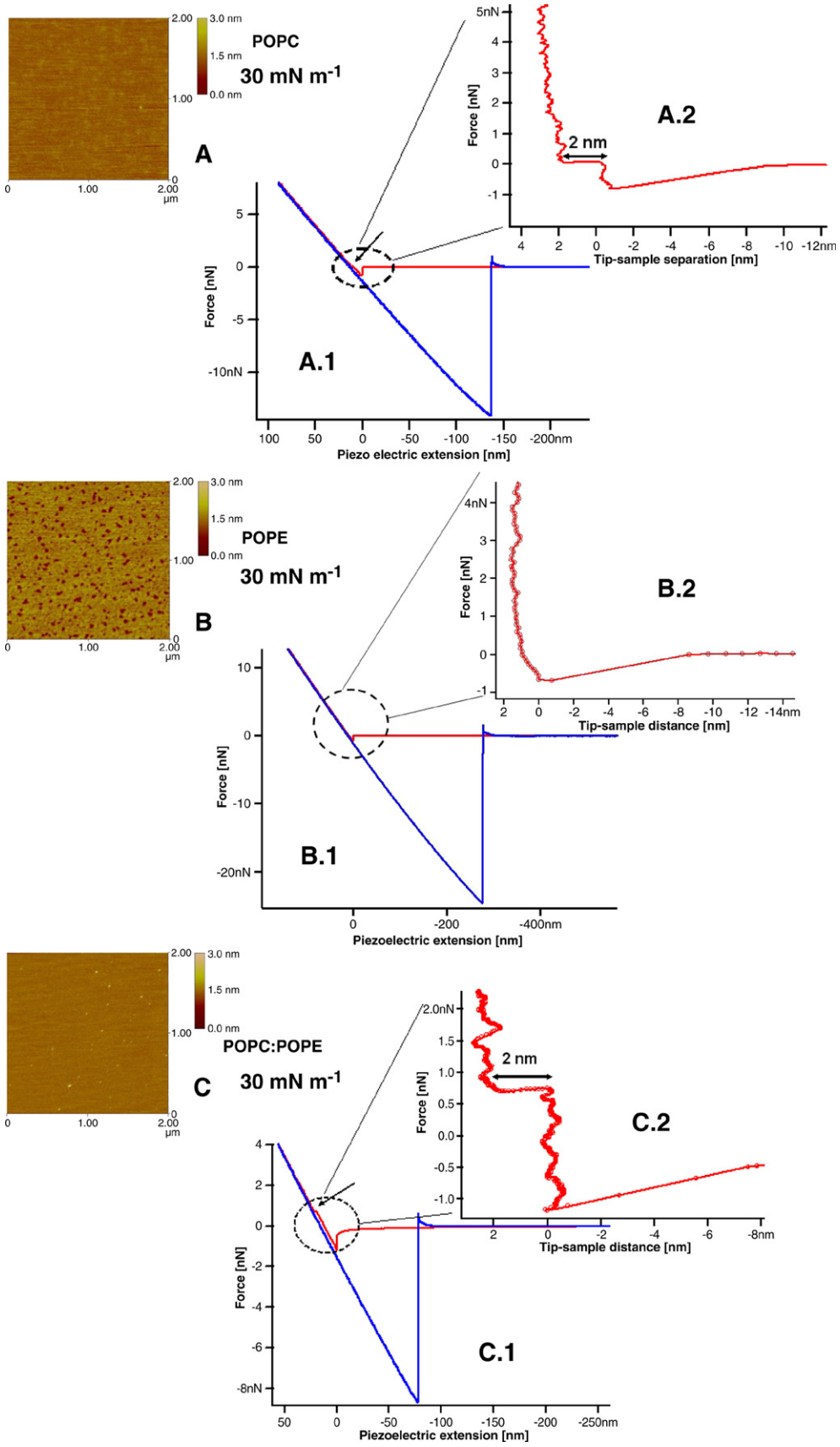

Fig. 2. 2 μm^2 AFM contact mode images of POPC (A), POPE (B) and POPC:POPE ($\chi_{POPC}=0.4$) (C) monolayers extracted at 30 mN m^{-1}. Force–piezoelectric extension curves on these POPC (A.1), POPE (B.1) and POPC:POPE ($\chi_{POPC}=0.4$) (C.1) monolayers. Inset: force vs. tip-sample separation approaching curves derived from the previous force vs. piezoelectric extension curves for POPC (A.2), POPE (B.2) and POPC:POPE ($\chi_{POPC}=0.4$) (C.2), underscoring the low force contact region in which the jump or monolayer indentation can be observed.

pure POPC and $\chi_{POPC}=0.4$, monolayers (results not shown). The behavior was similar even using softer tips. For this reason we suspect that because of their diffusion coefficients, the phospholipid molecules tend to respread immediately filling up the area uncovered or, alternatively, in contact mode the tip indents into the monolayer [32]. Unexpectedly, as it is shown in Fig. 3 for the POPE monolayer at 30 mN m^{-1}, the LB could be scratched, in principle, to allow the measurement of the height. According to the height profile analysis (Fig. 3B) the thickness was ~0.48 nm, clearly inconsistent with the height of a phospholipids monolayer and in disagreement with the value obtained by measuring the depth of the hole. This could be attributed to the fact that the molecules are nearly lying down onto mica by the action of the tip as it has been reported for lipids in mobile phase [52]. In addition, it is very possible that phospholipid molecules become adsorpted onto the tip during the scanning process [53] which explains the value of $Ra=0.05$ of the scratched area. Hence, an alternative experimental way able to accurately measure the monolayer height at the nanometer scale was required. We therefore decided to conduct FS experiments in order to test the mechanical stability of such monolayers, which decisively provides with valuable structural information such as the monolayer height [37,54].

The force plots performed on different monolayers extracted at 30 mN m^{-1} reveal clear differences in their mechanical stability. Whereas a small jump in the approaching force curve occurs at very low forces for POPC (Fig. 2A.1) or does not occur on average for POPE (Fig. 2B.1) (only in 7 cases out of 4453 attempts did we observe a breakthrough at low forces; $x=501\pm210$ pN, $n=7$), for the monolayer with $\chi_{POPC}=0.4$ (Fig. 2C.1) the jump or discontinuity in the force curve takes place repeatedly at higher yield forces. The distribution of forces at which the jump takes place is shown in the histogram presented in the Supporting Information. The Gaussian fit to the histogram yields $x=777\pm81$ pN, $n=488$. Note that negative values observed in Fig. 2A.1 for POPC and also in the tail of the force histogram for $\chi_{POPC}=0.4$ shown in the Supporting Information correspond to yield threshold values occurring at lower cantilever deflection than the zero force lever deflection, due to the high jump-into contact value occurring between the cantilever tip and the surface under air conditions. Remarkably, when such jump-in events are observed, their width is a direct, accurate signature of the monolayer height, which in this case measures ~2 nm (Figs. 2A.2 and C.2). This value fits reasonably well within the average height of phospholipid monolayers elsewhere published [20,55]. Therefore, according to the experimental data here presented, it is reasonable to assume that when LacY are reconstituted into liposomes of such composition, an adequate match between the hydrophobic protein surface and the phospholipids may occur.

Strikingly, no jump or breakthrough was observed on average in the POPE monolayer extracted at 30 mN m^{-1} (Fig. 2B.2). Therefore, the monolayer height value could not be obtained using FS. This result, however, was not totally unexpected because: (i) very low yield threshold values (~1.63 nN) have been reported for POPE bilayers [37]; and (ii) because of the extreme soft nature of unsaturated PE monolayers [20,30,56]. As it has been above mentioned (Fig. 2A.1) one can observe in the case of POPC that the jump occurs at a negative force values, which implies that the POPC monolayer is punctured since the cantilever is in physical contact with the monolayer. Consequently, if lower values than those observed for POPC may be expected for the POPE monolayer, it is not surprising that the jump could not be measured in this case (Fig. 2B.1). This implies that when acquiring AFM images of samples as those studied in this work the tip may be partially indenting the surface. This interpretation is in agreement with force determinations carried out with unsaturated homoacid phospholipids as DOPC and DOPE [30,56]. On the other hand, taking into account that the acyl chains are the same, the different behavior should be exclusively attributed to the chemical differences between PC and PE headgroups [42,43]. As a consequence, the nanomechanical response of the studied phospholipids at 30 mN m^{-1} can be directly related to their molecular packing, i.e., to their area per molecule value obtained from the isotherms shown in

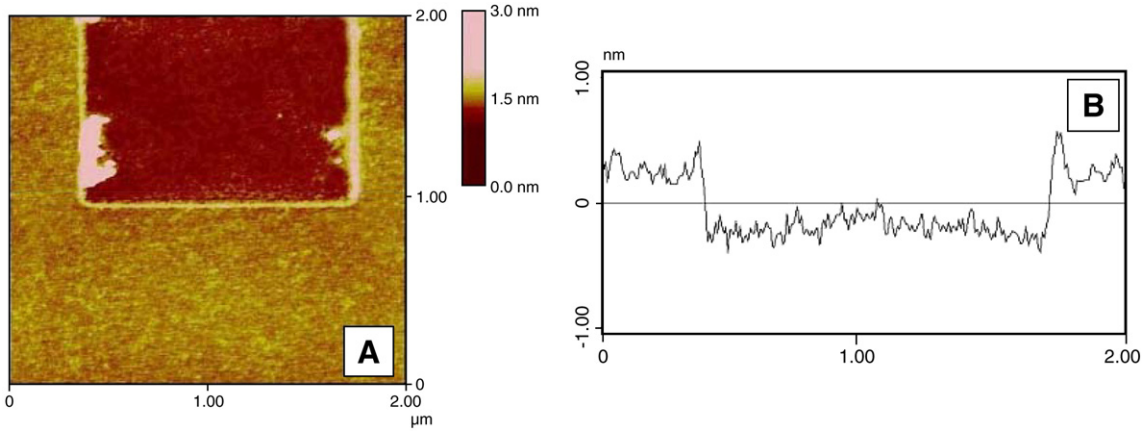

Fig. 3. Example of the monolayer thickness determination method by using AFM contact mode. AFM topography image of a POPE monolayer transferred at 30 mN m^{-1} (A). The monolayers thickness can be evaluated by scratching the surface. The height profile analysis (B) evidenced, however, that the AFM tip drift the phospholipid towards to the edges and that the mica is not totally flat due to the re-spreading of material over the substrate. The thickness of the monolayer obtained by measuring the step height between the monolayer and the substrate were 0.42 ± 0.07 nm, which is clearly below the values expected for a monolayer.

Fig. 1. POPC and POPE monolayers show a high area per molecule value of 0.82 nm^2 and 0.74 nm^2, respectively, and they do not feature any jump or breakthrough (or it occurs at very low forces in the few cases that it has been measured). In contrast, the isotherm corresponding to the $\chi_{POPC}=0.4$ monolayer reveals an area per molecule value of $\sim$0.65 nm^2 at 30 mN m^{-1} and a series of repetitive jumps at relatively high forces (about 800 pN) in the force curves are observed. These observations are a clear signature that: (i) monolayers keep their extraction pressure once transferred onto the mica substrate and, more important, (ii) a threshold area/molecule value between about 0.65–0.74 nm^2 is required to give mechanically stability to the monolayers.

As expected, at surface pressures lower than 30 mN m^{-1} no breakthroughs were observed neither in POPE nor in POPC or $\chi_{POPC}=0.4$ monolayers. Hence, a thorough correlation between the surface pressure of the monolayers and the breakthrough force within a wide range of extracting pressures is experimentally difficult to be tested in this case due to the soft properties of such biological relevant phospholipid monolayers. However, such correlation is faithful to be tested for stiffer phospholipids (i. e. PC phospholipids) or for even harder LBs [57].

As mentioned before, however, the very distinctive feature of POPE and OPPE isotherms is the so called LC–LC′ transition that occurs at $\sim$36.5 mN m^{-1} [39]. Fig. 4A shows a contact

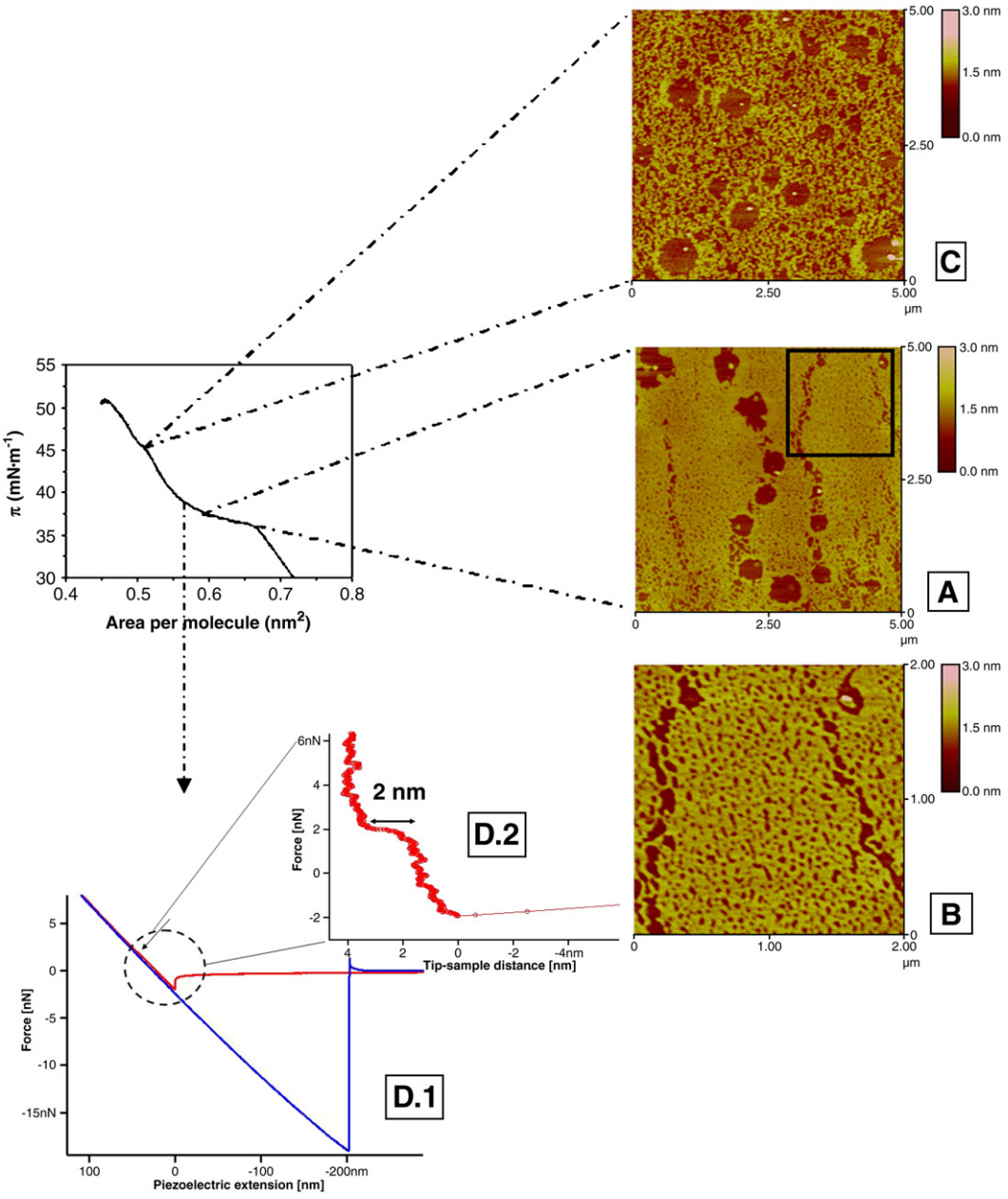

Fig. 4. π–A isotherm of the POPE monolayer with two AFM images: LB films at 36.5 mN m^{-1} (A) and its magnification (B); and at 45 mN m^{-1} (C). Force vs. piezo displacement curve on monolayer extracted at 38 mN m^{-1} (D1) and force vs. tip-sample approaching curves (D2) derived from the previous force vs. piezoelectric extension curves, underscoring the low force contact region in which the jump or monolayer indentation can be observed.

mode AFM image of a POPE monolayer extracted at 36.5 mN m^{-1} which corresponds to the value of the first-order transition observed (inset Fig. 1). The topography of the monolayer shows here a more complex structure with a $Ra=0.16$ in which the defects observed at 30 mN m^{-1} appear to increase in size (see a magnification of the monolayer at 36.5 mN m^{-1} in Fig. 4B). Clearly, the LB films transferred at 36.5 mN m^{-1} are not homogeneous and numerous domains, predominantly round in shape with diameters ranging from 500±100 nm ($n=10$) to 87±18 nm ($n=30$), are observed. At first sight, just judging by the color scale, it is an appealing possibility that we would be observing some stage of the LC–LC′ phase separation. Otherwise, these domains would rather be interpreted as vacancies produced in the process of transference of the monolayer to the mica substrate. Indeed, although values as low as ~1.8 nm have been reported, for instance for DOPE monolayers at 40 mN m^{-1} [30,56], the depth of the domains in Fig. 4, 0.80±0.06 nm ($n=30$), may not account for the step height difference between two LC phases.

At this point it is clear that the topography images cannot provide any information to discriminate between both phases. The scratching method (see supporting information) reveals that the domains observed in Fig. 4 are real vacancies and cannot be attributed to the LC–LC′ phospholipid phase transition. Furthermore, the occurrence of such defects is not occasional but repetitive and surface pressure dependent. Thus, the small holes observed at 30 mN m^{-1} (Fig. 2B) grow in number and size at 36.5 mN m^{-1} (Fig. 4B) and even more at 45 mN m^{-1} (Fig. 4C). Hence, we suspect that during the LC–LC′ transition, the molecular tilt changes the interaction from the next-nearest neighbors to the nearest [41] resulting both, in the compaction of the monolayer and in the formation of free spaces that we observe as defects. It cannot be excluded that the holes will be formed as a result of the transformation into some kind of hexagonal-like phase since it occurs under severe dehydration in solution [58]. However, this will be speculative under the basis of the simple topography analysis and the negative results of the digital Fourier transformation performed on the AFM images.

Finally, to evaluate whether the LC–LC′ transition has any effect on the mechanical properties of the monolayer, we have performed FS on LBs of POPE extracted at 38 mN m^{-1} (Fig. 4D). Remarkably, while no breakthroughs of any kind were observed at 30 mN m^{-1} (Fig. 2B.1) the force curves obtained on the monolayer extracted at 38 mN m^{-1} exhibited a repetitive, clear jump in the approaching curve at significant higher values. The histogram shown in Fig. 5 shows the distribution of the measured yield threshold forces. Gaussian fit to the histogram yields $x=1.72\pm0.99$ nN, $n=151$. The high yield threshold force value measured in this case is a strong evidence of the molecular rearrangement induced by the phase transition. It seems then clear that the sudden reduction in the area/molecule value (the plateau region in Fig. 1) results in a more compact and packed molecular structure. This gives rise to an important increase in the force that the monolayer is able to withstand before breaking. Interestingly, this yield threshold force value compares well with the one obtained for the POPE bilayers,

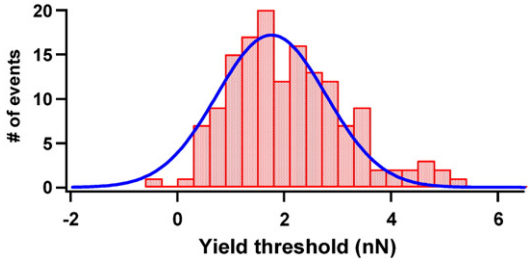

Fig. 5. Histogram corresponding to the yield threshold force value for POPE monolayers extracted at 36.5 mN m^{-1}. $x=1.72\pm0.99$ nN ($n=151$) assuming the Gaussian fitting of the data.

which in principle one should expect to be much higher. This observation points towards the conclusion that POPE lipid bilayers naturally assemble with a lateral pressure value much lower than 38 mN m^{-1}. Therefore, the jump in the force curve can be now regarded as a fingerprint of the phase transition induced by external pressure, similar to what it has been recently observed for variable temperature experiments on lipid bilayers, where the phase transition was intimately correlated with a change in the (nano)mechanical stability of the studied bilayers [38]. Interestingly, these results can be again directly related to the molecular packing of the phospholipids on the surface (i.e. the area/molecule value) as revealed by the extracting isotherms shown in Fig. 1. While the POPE monolayer does not show mechanical stability at 30 mN m^{-1} (area/molecule ~0.74 nm^2), it shows a high mechanical stability (yield threshold=1.72 nN, Fig. 5) when extracted at 36.5 mN m^{-1} (area/molecule ~0.58 nm^2). According to Fig. 1, during the phase transition occurring at 36.5 mN m^{-1}, the POPE isotherm crosses $\chi_{POPC}=0.4$ isotherm while inducing a mechanical stability in the monolayer. Again, the 'threshold' area/molecule value falls within the range 0.58–0.70 nm^2, in excellent agreement with the range value observed for the monolayers extracted at 30 mN m^{-1}. These measurements allow us again to establish the monolayer width in ~2 nm (inset Fig. 4.D). This value, however, is greater than the obtained from topographic measurements (Fig. 2) but compares well with the values obtained at 30 mN m^{-1} for POPC and the $\chi_{POPC}=0.4$ monoloyers.

In summary, the heteroacid phospholipids under study: (i) are in fluid phase at physiological temperatures; (ii) are easily compressible, that is, they can undergo significant distortion to adapt to the highly irregular surface of integral proteins; and (iii) the heights estimated from FS matches reasonable well with the hydrophobic length of an average transmembrane protein. Therefore, we infer that, assuming that POPE is the major component of the annular phospholipids of LacY [2,10,16] the lateral pressure in natural occurring membranes could be higher than 36 mN m^{-1}.

On the other hand, this work highlights technical issues that have to be borne in mind when dealing with soft phospholipid monolayers. In particular, the thickness measurements obtained from AFM images for soft samples can, in general, be somewhat inaccurate or just impossible due to the experimental constraints and, especially, to the low mechanical resistance of

the studied substrates. This fact is particularly relevant in those cases in which the jump-to-contact force (enhanced when measurements are conducted in air) exceeds the mechanical stability of the monolayer. There, once the tip is sufficiently close to the sample as to experience the snap-in (or jump-to-contact) force, it falls into physical contact with the surface. Upon further extending the piezo, the cantilever starts exerting a pressure on the monolayer until it breaks, giving rise to the above-mentioned jump or breakthrough. If the force required to break the monolayer is lower than the jump-to-contact force, then the yield threshold force occurs at 'negative forces', which implies that when performing AFM images in contact mode the surface may be damaged during the entire scan. This provides explanation for the unexpected lower values of height obtained by scratching the samples.

Thus, FS through force–extension curves reveals to be a suitable experimental tool that allows us to unveil additional structural information at the nanometer scale, such as monolayer height, which cannot be obtained by contact mode AFM images. More interestingly, FS provides valuable information about the mechanical properties of the studied soft monolayers, which is a clear fingerprint of the monolayer molecular organization. Indeed, this technique allows us to experimentally measure the threshold area/lipid value needed to induce mechanical stability to the monolayer. Such area/molecule value is drastically modified when pressure-induced phase transitions occur, thereby greatly modifying the nanomechanical response of the overall monolayer.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbamem.2007.02.004.

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