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ATOMIC FORCE MICROSCOPY STUDIES OF LANGMUIR-BLODGETT FILMS. 4. THE INFLUENCE OF ALUMINUM SUBSTRATE ON DIPALMITOYL PHOSPHATIDYLCHOLINE NANOLAYERS

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ABSTRACT. The dipalmitoyl phosphatidylcholine (DPPC) nanolayers in the absence and in the presence of procaine (P), in concentration of 10^{-3} mole dm^{-3} in the aqueous phase of pH 5.6, have been investigated using Langmuir-Blodgett (LB) technique and atomic force microscopy (AFM). The LB films were vertically transferred from DPPC Langmuir nanolayers to a substrate made of glass covered by aluminum thin layer, at two controlled surface pressures, namely at a low surface pressure (of about 8 mN/m) corresponding to the main two-dimensional phase transition from expanded liquid (EL) to condensed liquid (CL) for pure DPPC nanolayers, and at a high surface pressure (around 70 mN/m) corresponding to the over compressed (advanced collapse) state of DPPC nanolayers. The structure of pure DPPC films show regular rounded condensed domains with densely packed DPPC molecules with polar groups on the solid substrate and methyl ends of the chains sticking out from the surface. Depending on the surface pressure the condensed domains coexist with less ordered features (primarily expanded liquid domains) at the main phase transition or with three layered domains under over compression at advanced collapse. In the presence of P, the stability of DPPC films is highly increased. This effect is shown by the increased collapse pressure of mixed DPPC and P nanolayers at the air/water interface. The nano-structures visualized by AFM observations on LB mixed films of DPPC and P reveal some specific molecular interactions between these biologically relevant compounds through hydrogen bonds and electrostatic attractions. In addition a strong adhesion between LB films and aluminum substrate was found which might further stabilize the LB films.

INTRODUCTION

Various model membranes have been used in science including monolayers, also called nanolayers [1, 2], lipid vesicles or liposomes [3] and Langmuir-Blodgett (LB) films [4-8]. These models are of substantial importance in many industrial, environmental and biological processes, such as wastewater treatment, biocompatibility and molecular recognition as well as in the investigation of structure and properties of biological membranes [1, 9].

To obtain a better understanding of model membranes it is essential to find a suitable model system, such as phospholipid nanolayers. These nanolayers are very interesting in this respect and have attracted considerable interest during the past few decades [10-16] both for fundamental research and biological applications. In particular, dipalmitoyl phosphatidylcholine (DPPC) nanolayers were studied by different

techniques including Langmuir technique [10, 11], Langmuir-Blodgett technique coupled with fluorescence microscopy [12] and, recently, with atomic force microscopy [13, 14]. The obtained data lead to the molecular orientation of DPPC molecules within nanolayers at fluid interfaces.

In this study, we use AFM and LB technique to investigate the main two-dimensional phase transition and the collapse mechanism within DPPC nanolayers in the absence and in the presence of procaine (P). The substrate for LB films is a glass covered by a thin aluminum layer. The high resolution of AFM gives valuable information about the size of DPPC molecules and their lateral arrangement. These data allow for a detailed description of the influence of aluminum and of procaine on the surface behavior of DPPC nanolayers.

EXPERIMENTAL SECTION

Chemicals and materials

Dipalmitoyl phosphatidylcholine (DPPC) and procaine hydrochloride (P) were purchased from Sigma. The n-hexane was purchased from Merck. All chemicals were used without further purification. Our investigations have been performed on aqueous subphases of pH 5.6, where P molecules are positively charged [16-19]. All aqueous subphases were prepared with purified water of at least 18 Mohm cm obtained using an Elga system. Under these conditions DPPC forms a stable Langmuir nanolayer at the air/aqueous solution interface. The glass covered by aluminum thin layer, with size of 25x25 mm^2 and 0.1 mm thick, were used for the LB film fabrication.

LB sample preparation.

The Langmuir nanolayers of DPPC in the absence and in the presence of P at the air/water interface were studied by compression isotherms, which were experimentally obtained as described elsewhere [16-20]. In particular, DPPC was dissolved in n-hexane at a concentration of 1 mg/ml and spread on aqueous solutions of pH 5.6, at 20 ^oC, in a teflon trough. After waiting time for 5 to 10 minutes to allow the solvent to be fully evaporated, the DPPC nanolayer was manually compressed at a speed of 10 cm^2/min to a chosen lateral surface pressure, e. g. corresponding to the main phase transition or for the advanced collapsed state.

The method of engineering LB films, known as Langmuir-Blodgett (LB) method, consists of vertically passing the hydrophilic solid substrate upwards to the air phase [8, 14, 21], at a controlled speed from aqueous phase through the Langmuir nanolayer, held at a constant chosen surface pressure.

In the present study we used the LB vertical deposition to engineer the LB samples of DPPC in the absence and the presence of P (0.001 mole dm^{-3} in aqueous phase) on glass covered with thin aluminum layer. At least three LB films were prepared for every independent sample, under controlled conditions.

During the vertical transfer of LB film, the area of the Langmuir nanolayer is decreased due to the transfer of DPPC amphiphilic molecules from air/water interface to the solid substrate. Usually, a transfer ratio is determined as the ratio of

the decreased Langmuir monolayer area divided by the total surface area of the substrate. The experimental transfer ratio was found comparable with that one for the transfer of DPPC on glass [14] and it was larger than 0.98. The transfer ratio indicates a good LB film deposition and consequently, a replica of Langmuir monolayer is obtained on the solid substrate. Then, AFM measurements were made in tapping mode for each LB sample.

Atomic force microscopy (AFM) tapping mode.

Investigation of the surface morphology and domain structure of the LB samples of DPPC in the absence and presence of P was conducted in tapping mode on a research AFM system with a 90 x 90 (x-y) μm scanner described elsewhere [14, 22] and on a scanning probe microscope (JSPM-4210) with a 10 x 10 (x-y) μm scanner, recently purchased by us from JEOL, Japan. The obtained AFM images were similar of high resolution. Standard cantilevers, non-contact conical shaped tips of silicon nitride coated with aluminum, were used. The tip was on a cantilever with a resonant frequency in the range of 200 - 300 kHz and with a spring constant of 17.5 N/m.

AFM observations were repeated on different areas from 30 x 30 μm^2 to 2 x 2 μm^2 of the same LB film. The images were obtained from at least five macroscopically separated areas on each LB sample. All images were processed using the standard procedures for AFM. Dimensions of the domains were measured directly from AFM topographic images and the thickness variations were estimated from vertical linear cross sections and height distributions on AFM images [23, 24]. Cross section analysis provides estimations of the size of nanolayer domains and various collapse phase structures. Therefore, a detailed morphological analysis of LB film can be reached.

AFM images consists of multiple scans displaced laterally from each other in y direction with 256 x 256 pixels [22]. Low pass filtering was performed to remove the statistical noise without to loose the features of the sample. All AFM experiments were carried out under ambient laboratory conditions (about 20 ^{o}C) as previously reported [14, 23, 24]. The dark regions in AFM topography indicate the substrate surface.

Through AFM image analysis, it was found that the interaction between LB film and aluminum surface was an important factor for the adhesion of LB film with the substrate and for the LB film stability.

RESULTS AND DISCUSSION

Phase behavior of Langmuir nanolayers.

The compression isotherm is obtained (Fig.1, curve 1) by compression of Langmuir nanolayers of pure DPPC, spread at the air/water interface, at 20 ^{o}C. The isotherm presents sharp breaks in curve slopes which illustrate phase transitions under known experimental conditions. Previously, we have already demonstrated that the DPPC film exhibits a variety of characteristic phases [10], like expanded liquid (EL) and condensed liquid (CL), and collapsed multilayer phases at advanced monolayer collapse under over compressed experimental conditions [14].

As a general behavior, one may observe that the isotherm (Fig.1, curve 1) contains a well defined two-dimensional phase transition from EL to CL at about 8 mN/m. This main phase transition was also identified by surface compressibility measurements [10] and shows that the two surface phases coexist in DPPC nanolayers.

The linear portion at high surface pressures corresponds to the CL state, followed by an intermediary liquid corresponding to intermediate surface pressures (between 8 and 20 mN/m) and by an EL under 8 mN/m.

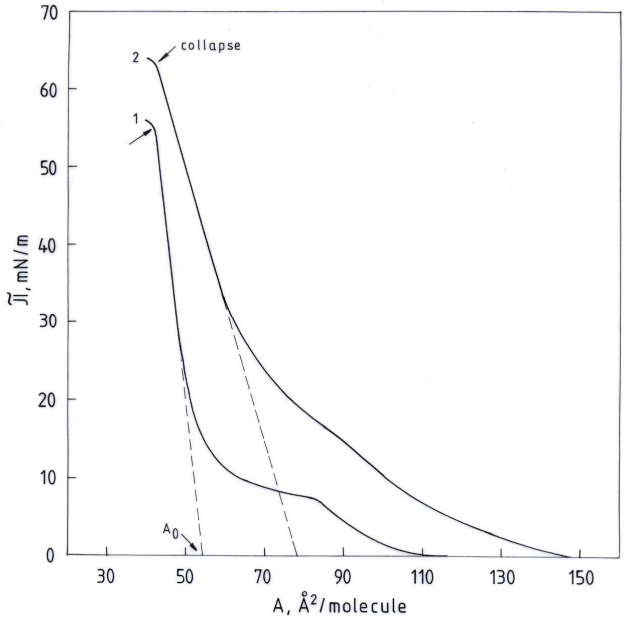

Fig. 1. Surface pressure versus mean molecular area of pure DPPC nanolayer (curve 1) at the air/water of pH 5.6 interface and for DPPC nanolayer in the presence of P (10^{-3} mole/dm^3, curve 2), at 20 ^oC. Dashed lines indicate the limiting areas for DPPC molecule in CL state (A_0). Arrows at high surface pressures indicate the incipient collapse of DPPC nanolayers.

In the presence of procaine (10^{-3} mole dm^{-3}; Fig. 1, curve 2) the compression isotherm is moved to larger molecular areas of DPPC showing the expansion of DPPC nanolayers. It appears that for this P concentration the main phase transition, existing in pure DPPC nanolayers, is almost vanished. From these compression isotherms, the surface characteristics of DPPC nanolayers were determined and they are given in Table 1.

The A_0 is the limiting molecular area for the CL state of DPPC monolayer in the absence or in the presence of procaine. These values are obtained by extrapolation to π equals zero of the linear portion of the isotherm recorded at high lateral surface pressures (Fig. 1).

The highest surface pressure to which a Langmuir monolayer can be compressed at the air/water interface [25-29], without the formation of a detectable bulk collapsed phase, represents the collapse pressure (π_c) and corresponds to the sudden slope change observed on the isotherms (indicated by arrows at high surface pressures, on Fig.1). The corresponding mean molecular areas are the collapse areas (A_c) and they are also given in Table 1.

Table 1.

Surface characteristics of DPPC nanolayers in the absence and in the presence of P (10^{-3} mole dm^{-3} in aqueous subphases of pH 5.6). The mean area values (A_0, and A_c) are given in nm^2/molecule of DPPC. The π_c values correspond to the monolayer collapse.

Monolayer	A_0 (nm^2)	A_c (nm^2)	π_c (mN/m) incipient	(advanced)
DPPC	0.54	0.42	55	(70)
DPPC and P	0.78	0.42	63	(70)

In CL state the DPPC molecules are densely packed and A_c values are close to the packing area of hydrocarbon chains in crystalline lipids at 20 ^oC, e. g. 38 $Å^2$ per pair of chains [30], and with the values found earlier for DPPC from geometric models [10].

It is important to emphasize the increased stability of DPPC nanolayers in presence of procaine as indicated by the enhanced collapse pressure of mixed DPPC and procaine nanolayers. The procaine effect on DPPC nanolayers reflects stronger specific interactions between these biocompounds, due to their ability to make stable self assembled supramolecular associations [31] primarily through hydrogen bonds.

To better understand the phase behavior of pure DPPC nanolayers and of mixed nanolayers of DPPC and P we have used AFM investigations on these nanolayers transferred on glass covered by aluminum thin layer using vertical LB deposition, under well controlled experimental conditions.

AFM images and analysis

LB FILMS OF DPPC

The morphology of LB films of pure DPPC at both the phase transition from EL to CL, for a lateral surface pressure of 8 mN/m (Fig. 2), and the advanced collapse state of 70 mN/m (Figs. 3 and 4), where another phase transition occurs from two-dimensional nanolayer to a collapsed bulk phase (Table 1), was examined by AFM tapping mode. The AFM allows simultaneous acquisition of both topographic data (topographic image) and material-properties data (phase image).

The topographic images show the morphology of LB samples which is obtained by monitoring the cantilever's oscillation amplitude changes in response to tip and sample spacing. Simultaneously, the phase image is obtained by monitoring of the phase lag between the signal that drives the cantilever to oscillate and the cantilever oscillation output signal. Changes in the phase lag reflect modifications in surface properties of LB sample, such as elasticity, friction and adhesion.

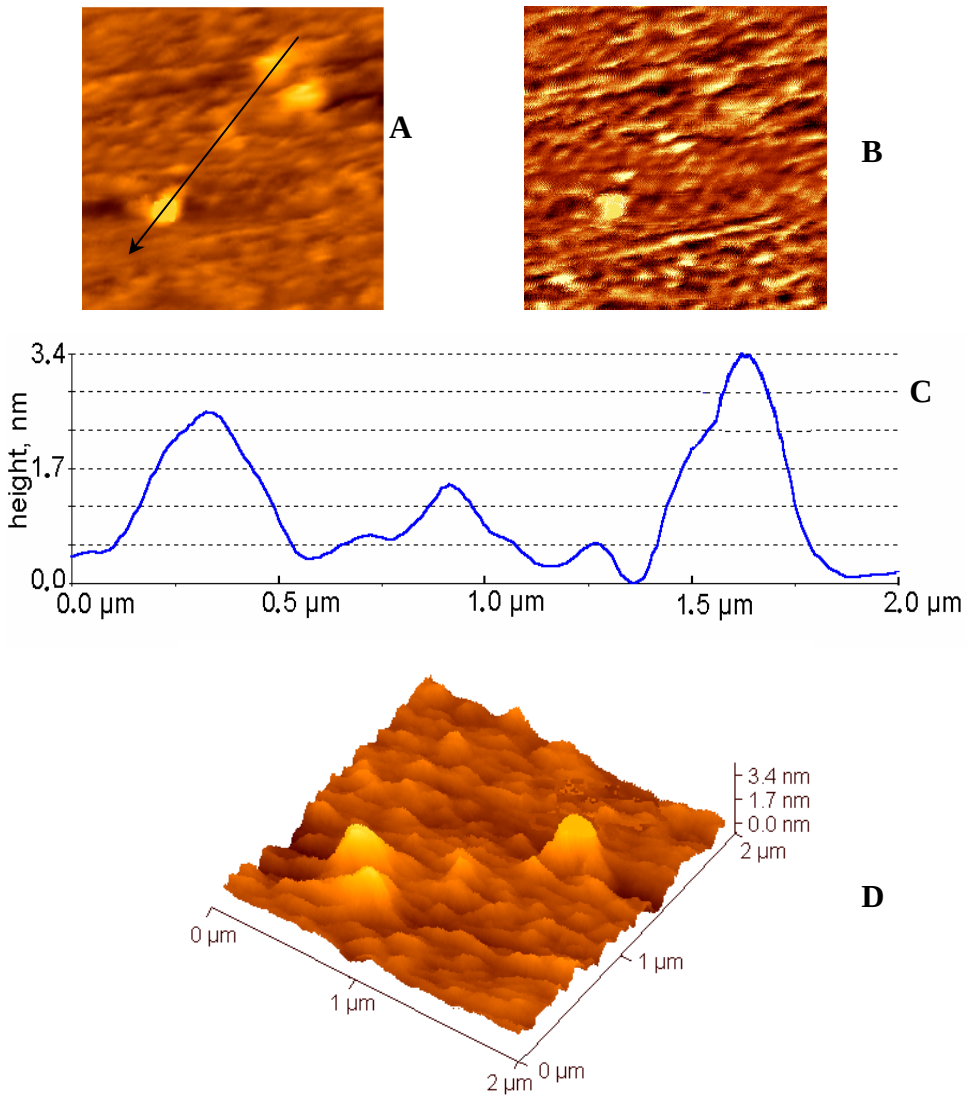

Fig. 2. Two-dimensional (2D) topographic (panel A) and phase (panel B) AFM images of LB films of pure DPPC transferred on aluminum substrate at main phase transition of 8 mN/m (Fig. 1, curve 1); scanned area 2 x 2 μm^2. Panel C represents the cross section profile along the arrow in Fig. 2A. The 3D view (panel D) of 2D topographic image (Fig. 2A).

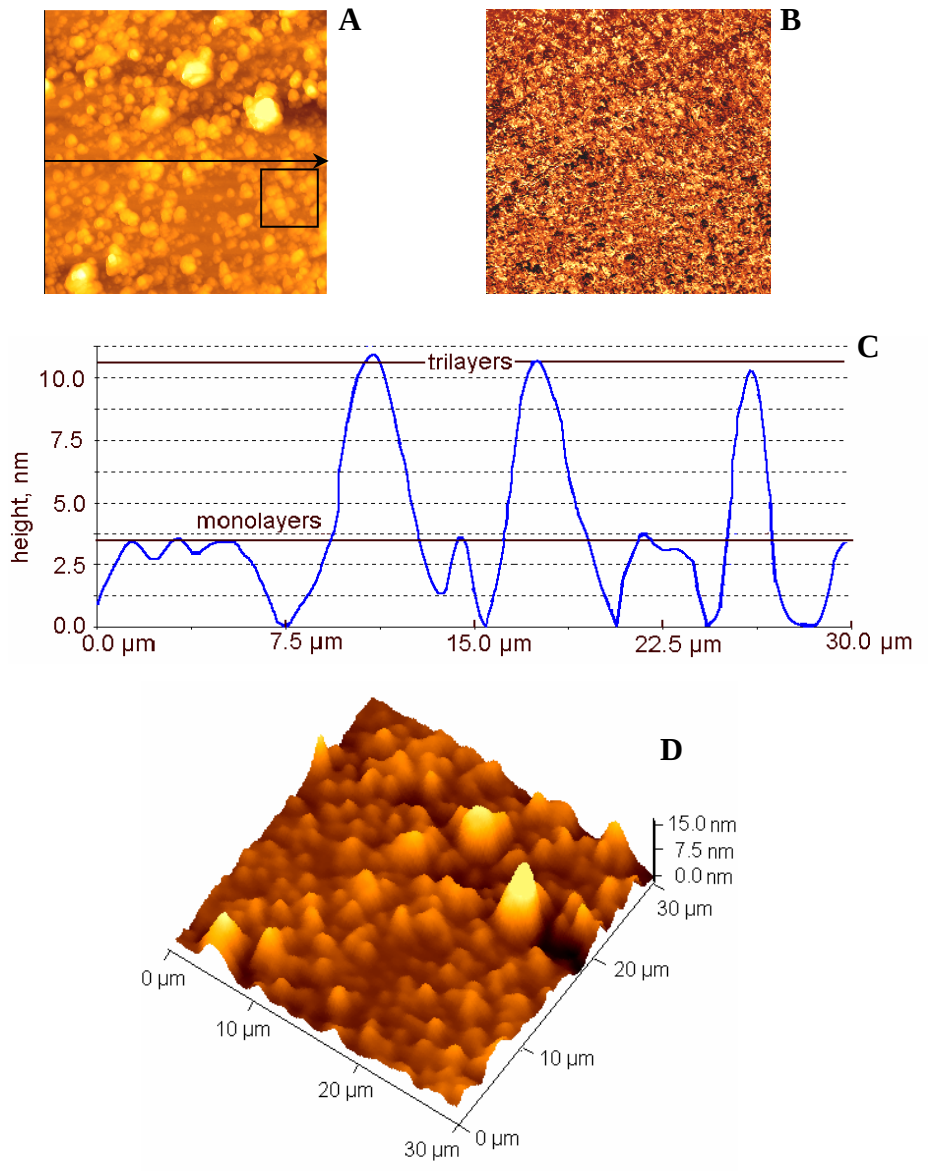

Fig. 3. 2D-topographic (panel A) and phase (panel B) AFM images of LB films of pure DPPC at advanced collapse state of 70 mN/m; scanned area 30 x 30 μm^2. Panel C represents the cross section profile along the arrow in Fig. 3A. The 3D view (panel D) of 2D topographic image (Fig. 3A); marked area indicates clusters of triple layer domains.

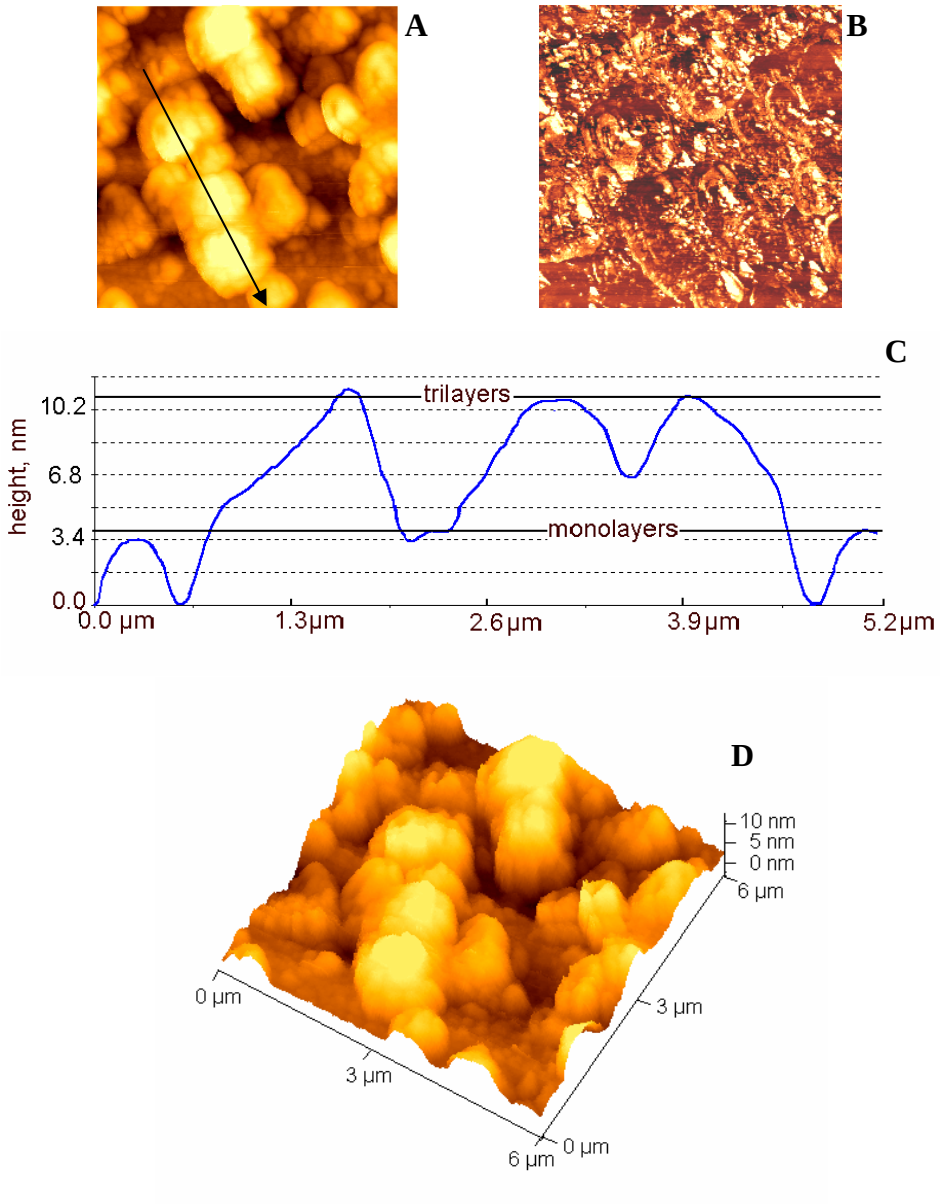

Fig. 4. 2D topographic (panel A) and phase (panel B) AFM images of LB films of pure DPPC; scanned area 6 x 6 μm^2; panel C represents the cross section profile along the arrow in Fig. 4A; 3D view (panel D) of 2D topographic image (Fig. 4A). Experimental conditions as for the marked area in Fig. 3A.

Fig. 2 shows the topographic image (panel A) and phase image (panel B) representing the morphology and surface properties, respectively, of LB film of DPPC at the main phase transition from EL to CL state. The topographic and phase images appear to be complementary to one another. They show clearly the coexistence of ordered CL and less ordered EL domains indicating that the pure DPPC film is not homogeneous at the main two phase transition as also evidenced by epifluorescent microscopy [12].

The topographical profile along the arrow, indicated in Fig. 2A, is shown in Fig. 2C. It can be seen that some condensed domains are found with the height up to about 3.4 nm, corresponding to a single molecular layer, when the DPPC molecules stand with hydrocarbon chains almost perpendicular and polar groups parallel to the aluminum substrate. This surface configuration corresponds to CL domains because the size of the DPPC molecule with the hydrocarbon chain extended is about 36 Å and it corresponds to calculated value using molecular geometric models [10]. In addition to these features the rest of the LB film is covered by nano-aggregates and their heights are less than 25 Å or equal to those of some islands comprised between 5 Å and 15 Å, corresponding to the EL domains. Molecules inside the EL aggregates may not orient straight on the aluminum surface in a single layer.

The images of the DPPC film at advanced collapse state (Fig. 3) correspond to a two-dimensional CL in equilibrium with collapsed fragments. At high resolution (Fig. 4) they show a film structure with a regular distribution of highly condensed domains mostly round in shape. These arrangements may include the interaction among DPPC molecules and between DPPC molecules and aluminum substrate. The collective effect of these interactions might lead to the formation of larger ordered structures, as seen in 2D-topographies (panels A), phase images (panels B), cross sections (panels C), and in 3D- topographies (panels D) of Figs. 3 and 4. The average size of CL domains varies from 0.5 μm up to 5 μm and for the triple layer structures from 2 μm up to 3 μm, as given in Figs. 3C and 4C, respectively.

The roughness of the LB film surface in the region of CL domains, defined as the root mean square (rms) of the height differences on the surface [5], is approximately 3 Å. This value is typical for lipid nanolayers and can be measured directly by atomic force microscopy. This is less than the roughness (7 Å) of glass covered by aluminum thin layer. Apparently, the LB film coating smoothens the aluminum surface. This effect might be probably explained by incorporation of water molecules between hydrophilic strongly hydrated DPPC head groups and the substrate surface. A decrease in surface corrugation caused by LB film transfer is consistent with other similar results reported using quartz [5] or glass [14] as substrates for LB samples.

At advanced collapse, the DPPC film structure consists of CL domains aggregated in rounded forms with bridges among them. The highest thickness of the DPPC film remains almost unchanged at 36 ± 2Å as shown in cross section profile (Figs. 3C and 4C). This thickness corresponds to the height of DPPC molecule (36 Å) in extended conformation [10] of both hydrocarbon chains (19 Å) and the head phosphatidylcholine polar group in its all-trans conformation (17 Å) vertically oriented on the LB substrate. This value can be considered as an

important parameter relevant for the thickness of a DPPC nanolayer relatively well packed at collapse in condensed domains. Figs. 3A and 4A show also the presence of many three layered fragments (up to about 11 nm height, panel C, of Figs. 3 and 4) and some other colloidal particles (up to 15 nm height, Fig. 3D) around the condensed domains. By increasing the lateral surface pressure the width of CL domains increases from about 0.3 μm (Fig. 2C) to almost 5 μm (Fig. 3C) indicating an increased stability of CL domains at advanced collapsed state.

MIXED LB FILMS OF DPPC AND P

The surface structure and properties of mixed LB films of DPPC and P, vertically transferred from Langmuir nanolayers (Fig. 1, curve 2) on glass covered by aluminum thin layer, at the two surface lateral pressures as chosen in the case of pure DPPC films, are investigated using AFM observations. Accordingly, the AFM images are given for mixed LB films of DPPC and P at 8 mN/m (Fig. 5) and at advanced collapsed state of 70 mN/m (Fig. 6, Table 1).

Fig. 5 shows the topographic image (panel A) and phase image (panel B) representing the morphologies and surface properties of mixed LB films of DPPC and P at 8 mN/m. Topographic image corresponds to a mosaic mixture of CL with the height up to 34 Å and EL with the height between 10 and 22 Å, as shown in panel C of Fig. 5. The topographic image show a LB film structure with an irregular distribution of some highly condensed domains mostly rounded in shape (Fig. 5D).

Comparing the AFM observations for DPPC pure film (Fig. 2D) with those for mixed DPPC and P (Fig. 5D) it is to be noted that the presence of procaine facilitates the condensed domains formation at low surface pressure as 8 mN/m.

With the increasing lateral surface pressure to the advanced collapse state of mixed LB films of DPPC and P at 70 mN/m the number of condensed domains and of three layered fragments is increased (Fig. 6). These features are in equilibrium with several large domains of higher order, named multilayered colloidal particles with the height up to 20 nm (Fig. 6D).

The main feature observed in these images and in the cross section profile of Fig. 6C is that the DPPC molecules self-assemble in bright trilayers with the height up to 11 nm in equilibrium with condensed monolayer domains on aluminum surface and probably procaine penetrates within the DPPC domains and among the domains reducing the line tension, as demonstrated for DPPC and P in epifluorescent microscopy [12].

The analysis of phase images (Figs. 5B and 6B) reveals differences among the mixed LB films of DPPC and P as function of the lateral surface pressures. In all cases a good adhesion of LB film with the aluminum mirror is found. The width of CL domains in Figs. 5C and 6C, is up to 0.3 μm and corresponds to supramolecular aggregates, probably, made up from well oriented DPPC and P molecules through hydrogen bonds. The width of triple layers domains is between 0.2 and 0.3 μm (Fig. 6C).

The correlation between topographical and phase images of mixed DPPC and P, given in Figs. 5 and 6, and the corresponding Figs. 2, 3 and 4 for pure DPPC films suggests that the mixed aggregates of DPPC and P are smaller than those formed by pure DPPC in agreement with the P effect on stearic acid (SA) films [24].

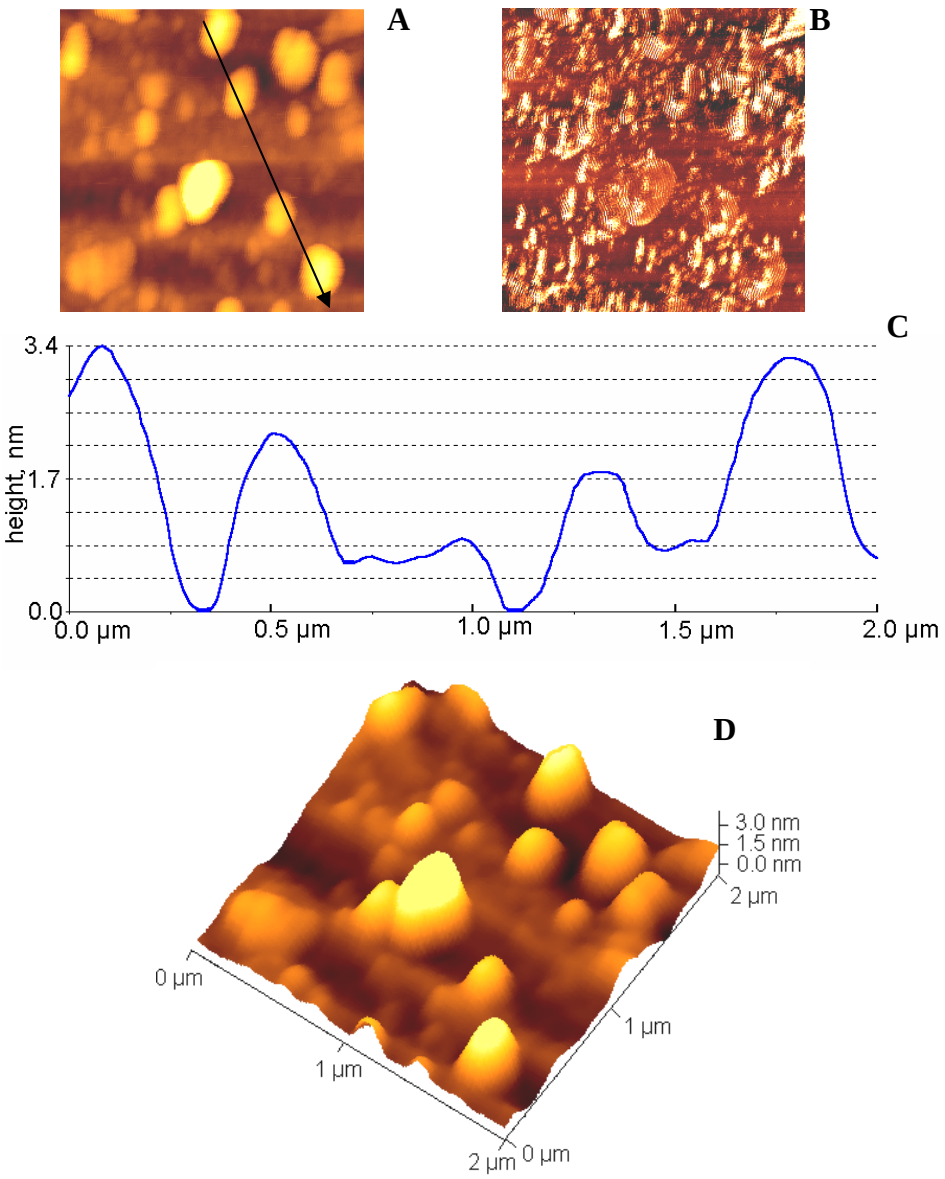

Fig. 5. 2D topographic (panel A) and phase image (panel B) AFM images of LB films of DPPC in presence of P (10^{-3} mole dm^{-3} in aqueous subphase of pH 5.6) transferred at 8 mN/m, see Fig. 1 (curve 2); scanned area 2 x 2 μm^2. Panel C represents the cross section profile along the arrow in Fig. 5A. The 3D view (panel D) of 2D topographic image (Fig. 5A).

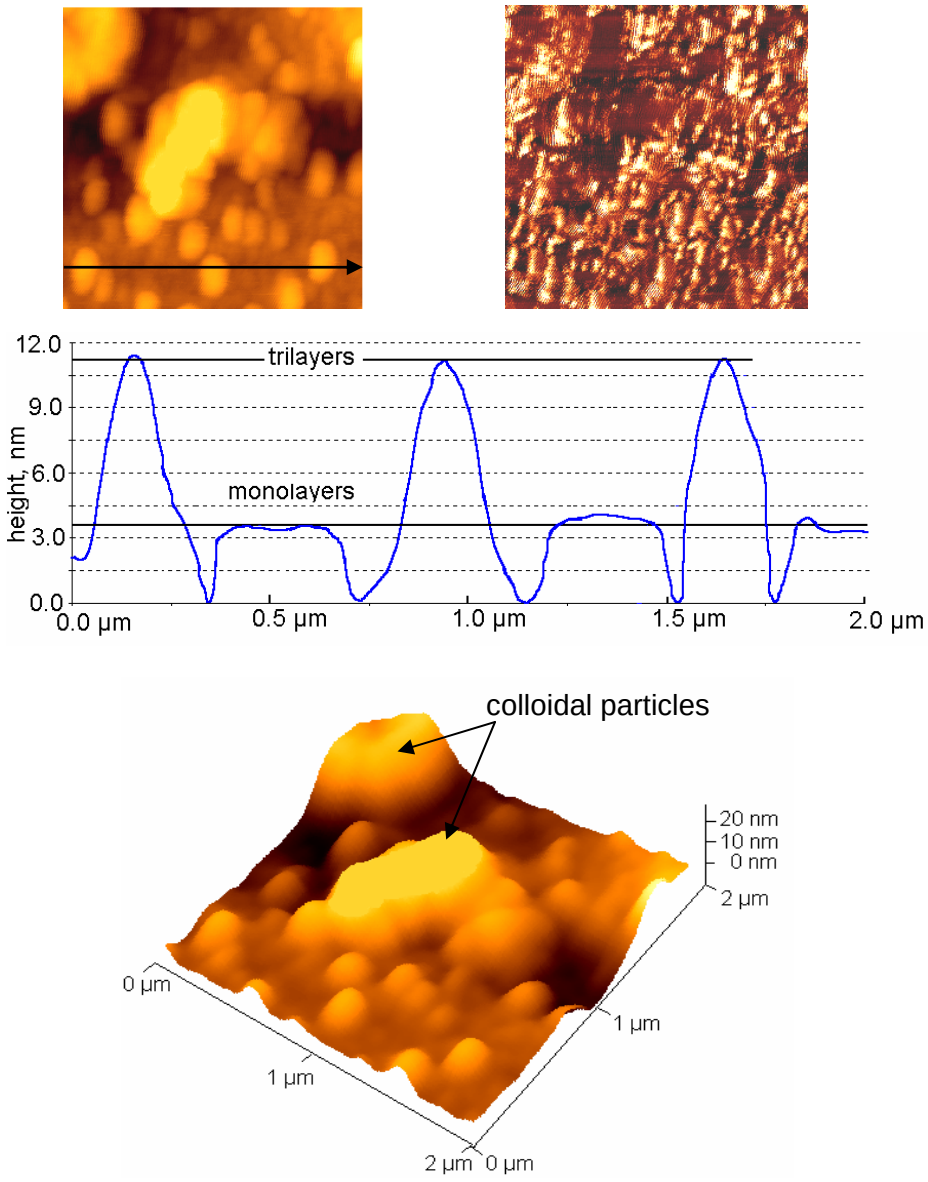

Fig. 6. 2D topographic (panel A) and phase (B) AFM images of mixed LB films of DPPC and P transferred at advanced collapse state (lateral surface pressure of 70 mN/m). Panel C indicates the cross section profile along the arrow in Fig. 6A. The 3D view (panel D) of 2D topographic image (panel A).

At advanced collapsed state, the total thickness of the CL domains remains almost unchanged at 36 ± 2 Å for the over compression at 70 mN/m for both LB films of pure DPPC (Figs. 3 and 4) and for the mixture of DPPC and P (Fig. 6). The measured thickness using AFM is again in good agreement with experimental and calculated value of 36 Å for the molecular length of DPPC [10].

The experimental collapsed structures of LB films visualized by AFM images (Figs 3, 4 and 6) and their section profiles can be explained quite suggestively by the collapse model of nucleation and subsequently growth of nuclei [26-29]. Several types of domains were observed, some are disk like and some are with rounded shape depending on the molecular interactions among molecules, their molecular shape and the interactions with the solid substrate. Sometimes, there are also irregularly shaped aggregates clustered lying beside or above the CL domains as observed in AFM images in the present study.

Further, the AFM images of DPPC and P revealed their association into less aggregated particles at advanced collapse (Fig. 6) on aluminum substrate. This effect of P on DPPC films is comparable with its effect on fatty acid monolayers transferred on glass substrate [24], but the stability of LB films is enhanced on aluminum substrate. Thus, we suggest that aluminum mirror is an effective substrate for DPPC to form stable LB films both in the absence and in the presence of P.

Usually, a high stability of LB films indicates also a high stability of their Langmuir nanolayers at air/water interface. Nevertheless, the high increased stability of DPPC nanolayer in the presence of P in the aqueous solution is strongly supported by its recorded high incipient collapse pressure (Table 1).

To describe the increased stability of Langmuir nanolayers we suggest that the electrostatic interaction between the zwitterions of polar head groups of DPPC and protonated amino terminal of P molecules [17] is an important factor in stabilizing the long-range structure in addition to the interaction between the hydrophobic tail groups of DPPC oriented in the air phase. Also P molecules oriented at the air/water interface can make surface complexes with DPPC molecules through hydrogen bonds facilitating the order in two dimensions. These complexes can have a physiologic role in vivo as well as in the molecular mechanism of anesthesia.

CONCLUSION

The present study demonstrates that AFM coupled with the LB technique gives valuable information on the film structure of the pure DPPC and its binary mixtures with P.

In Langmuir nanolayers of DPPC and P at the air/water interface, the stability of mixed films is highly increased as compared with pure DPPC nanolayers. The increased stability is strongly reflected in the high collapse pressure of DPPC nanolayer in the presence of P.

The Langmuir nanolayers of pure DPPC and of its binary mixtures with P were transferred from the air/water interface on aluminum substrate at two surface lateral pressures, namely, at 8 mN/m, which is characteristic for the main phase transition from EL to CL of pure DPPC nanolayers, and at 70 mN/m chosen for the advanced collapse state of Langmuir nanolayers. Then, the surface of their LB films was for the first time visualized using AFM observations on LB films.

The analysis of AFM images shows differences among the LB film surfaces of pure DPPC and mixed DPPC and P, suggesting that there is also a modification of the surface composition when single component and mixed films are compared.

The experimental collapsed structures of LB films visualized by AFM images and section profiles can be explained quite suggestively by the collapse model of nucleation and subsequently growth of nuclei. The AFM images of DPPC and P revealed their association into less aggregated particles at advanced collapse than in the case of pure DPPC film.

Our studies have also shown a long term stability of pure DPPC films and mixed DPPC and P films, transferred on aluminum substrate. This high stability may include the interaction among DPPC molecules and between DPPC and P molecules in addition to the interaction between the underlying aluminum substrate and the film forming molecules.

The LB films were measured several days after the transfer and several months up to one year after the transfer. The high stability of both LB films and Langmuir nanolayers would be useful in various applications for medicine, pharmacy and biology.

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