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Interaction of *N*-myristoylethanolamine with cholesterol investigated in a Langmuir film at the air–water interface

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ABSTRACT

The dramatic increase in the content of *N*-acylethanolamines (NAEs) having different acyl chains in various tissues when subjected to stress has resulted in significant interest in investigations on these molecules. Previous studies suggested that *N*-myristoylethanolamine (NMEA) and cholesterol interact to form a 1:1 (mol/mol) complex. In studies reported here, pressure-area isotherms of Langmuir films at the air–water interface have shown that at low fractions of cholesterol, the average area per molecule is lower than that predicted for ideal mixing, whereas at high cholesterol content the observed molecular area is higher, with a cross-over point at the equimolar composition. A plausible model that can explain these observations is the following: addition of small amounts of cholesterol to NMEA results in a reorientation of the NMEA molecules from the tilted disposition in the crystalline state to the vertical and stabilization of the intermolecular interactions, leading to the formation of a compact monolayer film, whereas at the other end of the composition diagram, addition of small amounts of NMEA to cholesterol leads to a tilting of the cholesterol molecules resulting in an increase in the average area per molecule. In Brewster angle microscopy experiments, a stable and bright homogeneous condensed phase was observed at a relatively low applied pressure of 2 $mN.m^{-1}$ for the NMEA:Chol. (1:1, mol/mol) mixture, whereas all other samples required significantly higher pressures (>10 $mN.m^{-1}$) to form a homogeneous condensed phase. These observations are consistent with the formation of a 1:1 stoichiometric complex between NMEA and cholesterol and suggest that increase in the content of NAEs under stress may modulate the composition and dynamics of lipid rafts in biological membranes, resulting in alterations in signaling events involving them, which may be relevant to the putative cytoprotective and stress-combating ability of NAEs.

1. Introduction

N-Acylethanolamines and their precursors, *N*-acylphosphatidylethanolamines (NAPEs) are widely distributed in nature and appear to be ubiquitous. Their content increases dramatically in different organisms when they are subjected to different types of stress, such as an injury in animals or dehydration in plants [1–3]. These observations led to the speculation that the increased production of NAEs and NAPEs under stress conditions may form part of the stress-combating response of the parent organism [1,2,4]. NAEs also exhibit anti-inflammatory, antibacterial and antiviral properties, which are of considerable application potential [1]. Further, both NAEs and NAPEs are likely to be useful in developing liposomal formulations for drug delivery applications because compounds belonging to both these classes have been shown to stabilize the bilayer structure [5–7].

In view of the interesting biological and medicinal properties of NAEs and NAPEs, as well as their putative role in combating stress, it is important to investigate their biosynthesis, catabolism and biophysical properties such as 3-dimensional structure and interaction with membrane lipids and proteins, in order to develop structure–function correlations and to rationalize their role in the parent organisms. The metabolism of NAEs and NAPEs has been investigated in considerable detail and several of the important enzymes involved in the metabolism of NAPEs and NAEs have been purified, characterized and cloned [8–10].

A number of biophysical studies have also been carried out on NAEs and NAPEs [11]. In studies from this laboratory the phase transitions of a homologous series of NAEs were characterized by DSC, which suggested that they exhibit structural polymorphism in the solid state [12,13]. The molecular packing and intermolecular interactions of one polymorph of NMEA was studied by single-crystal X-ray diffraction [14]. More recently we reported the crystal structures of two different polymorphs of *N*-palmitoylethanolamine (NPEA)–which firmly established structural polymorphism in NAEs in the solid state–and provided a possible mechanism for the interconversion between

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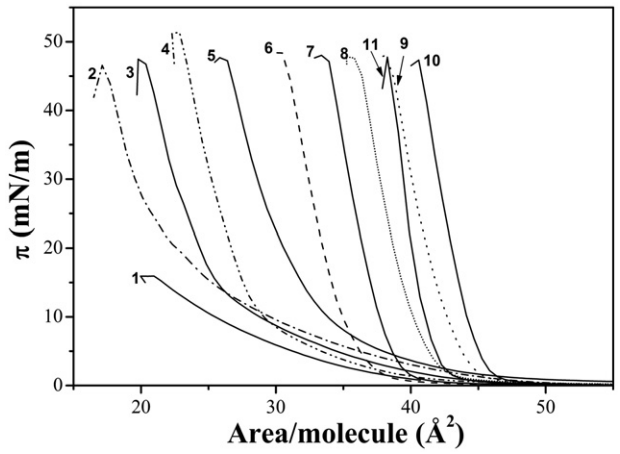

Fig. 1. π-A isotherms of NMEA-cholesterol mixtures. The different isotherms shown correspond to 1) pure NMEA, 11) pure cholesterol and mixtures of NMEA and cholesterol in the ratios indicated: 2) 9:1, 3) 8:2, 4) 7:3, 5) 6:4, 6) 5:5, 7) 4:6, 8) 3:7, 9) 2:8, 10) 1:9.

them [15]. In all these structures NAE molecules pack in a bilayer format, analogous to that found in phospholipid membranes [14,15].

At least some of the biological and pharmacological effects of NAEs are expected to be mediated by their interaction with other membrane constituents, or will be affected by such interactions. Therefore, it is essential to investigate the interaction of NAEs with major membrane lipids such as phospholipids and cholesterol in order to understand how they function in eliciting the various biological and pharmacological effects attributed to them. In one study, the interaction of NPEA with DPPC was investigated by DSC, ^{31}P-NMR and small-angle X-ray scattering experiments [16]. In a recent study, the interaction of two NAEs (NMEA and NPEA) with diacyl PEs of matched acyl chain-lengths (DMPE and DPPE) were investigated by DSC, spin-label ESR and ^{31}P-NMR spectroscopy [17]. In yet another study, DSC and FAB-MS experiments, complemented by computational modeling studies have provided strong evidence for the formation of a 1:1 (mol/mol) complex between NMEA and cholesterol [18]. Recent DSC experiments suggest that other NAEs such as NPEA and N-stearoylethanolamine (NSEA) also form 1:1 (mol/mol) complexes with cholesterol [19]. In the present work we have investigated the interaction between NMEA and cholesterol further using a monolayer approach. The interfacial packing properties of NMEA and its mixtures with cholesterol at different compositions in Langmuir films at the air–water interface have been characterized by pressure-area isotherms and Brewster angle microscopy at ambient temperature. The results obtained provide new insights into the molecular level interactions and reveal novel aspects of the supramolecular chemistry. Further, the stoichiometric complexation between NMEA and cholesterol suggests that stress-induced increase in the content of NAEs can modulate the composition and dynamics of lipid rafts in cell membranes, which in turn can lead to alterations in signaling events involving raft components.

2. Materials and methods

2.1. Materials

N-Myristoylethanolamine was synthesized and characterized as described earlier [12]. Cholesterol was purchased from Avanti Polar Lipids (Alabaster, AL, USA).

2.2. Sample preparation for monolayer studies

Stock solutions of NMEA and cholesterol were prepared in chloroform (Uvasol grade, E. Merck). Appropriate volumes of the stock solutions were mixed to yield desired mol ratio of the two components. The mixed solution was spread on the surface of water in the Langmuir trough so that it will form a thin monolayer after evaporation of the solvent. Hamilton microsyringes were used to ensure accurate pipetting of the lipid solutions in the preparation of monolayers.

2.3. Pressure-area isotherms

Pressure-area (π-A) isotherms were recorded on a Nima Model 611M Langmuir–Blodgett trough using a Wilhelmy plate for pressure sensing. All experiments were carried out at 25 (±1) °C. Water for the subphase was purified by double distillation followed by processing in a Milli-Q academic system (Millipore, Bedford, MA); resistivity = 18 MΩ.cm, pH = 5.5. After spreading the amphiphile solution, a 15 min wait period was given before recording the π-A isotherm. All isotherms were recorded using a barrier speed of 50 cm^2/min. Stability of the Langmuir films was verified by performing isocycles (compression–expansion cycles), which gave reproducible isotherms. All π-A isotherm experiments were repeated on fresh subphases 3 times to ensure reproducibility.

2.4. Brewster angle microscopy

Morphology of the Langmuir films at air–water interface was observed by a Brewster angle microscope. BAM images of the monolayers were recorded using a Nanofilm Model BAM2Plus microscope equipped with a frequency doubled Nd:YAG laser (20 mW power) at a wavelength of 520 nm. The air–water interface was illuminated at the Brewster incidence angle (~53°) and the monolayer film was examined at different stages of compression. The reflected beam was received by a microscope and analyzed by a polarization analyzer, and the signal was recorded by a CCD video camera with a 20×-magnification lens to develop an image of the monolayer. The image size collected from BAM experiment was 220×273 μm. Length scales of the images were corrected for the angle of incidence of the beam.

3. Results and discussion

Langmuir monolayers of NMEA, cholesterol and their mixtures ranging from 1:9 to 9:1, formed at the air–water interface have been investigated in the present study. Pressure-area isotherms of the mixtures with different compositions are shown in Fig. 1. The monolayer formed at the air–water interface by pure NMEA is not stable and collapses when the surface pressure reaches ca. 16 mN.m^{-1}; the corresponding molecular area is 21 Å^2 (curve 1). On the other hand,

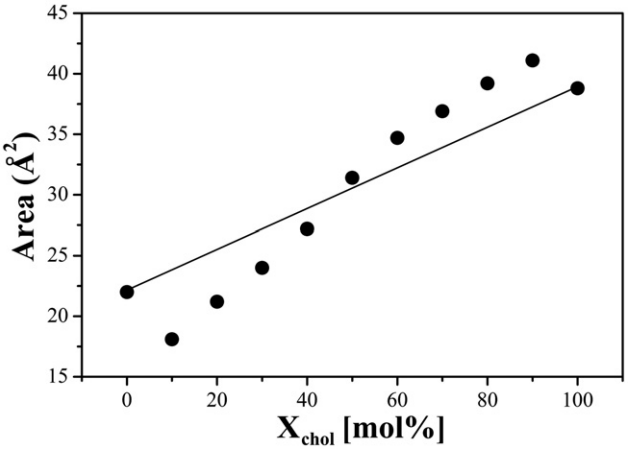

Fig. 2. Dependence of average area per molecule on the mol fraction of cholesterol in NMEA-cholesterol mixtures. The straight line indicates the average area for ideal mixing.

cholesterol forms a very stable monolayer which does not collapse at least up to 47.5 mN.m^{-1} (curve 11). Addition of even small proportions of cholesterol to NMEA has a marked effect on the monolayer; addition of as little as 5 mol% of cholesterol resulted in a significant stabilization of the monolayer formed by NMEA and at 10 mol% of the sterol the collapse pressure increased to about 45.5 mN.m^{-1} (curve 2). All the other mixtures with up to 90 mol% cholesterol yielded highly stable monolayers, with collapse pressure being in the neighborhood of 50 mN.m^{-1} (Fig. 1).

In order to investigate the effect of cholesterol on the chain ordering of NMEA, the mean molecular area at 40 mN.m^{-1} was plotted as a function of cholesterol fraction in the mixture (Fig. 2). The isotherms indicate fairly condensed phases at this pressure, in all cases except those with very low fractions of cholesterol. Since NMEA alone does not form a stable monolayer, it was not possible to utilise its isotherm data in this exercise. Therefore, the molecular area of NMEA determined from its crystal structure [14] was employed. Since the molecules are expected to be rather tightly packed at a pressure of 40 mN.m^{-1}, and the molecular areas obtained at this surface pressure should provide a good approximation to the value relevant to the close packed solid state, it is reasonable to relate the monolayer data to the molecular area of NMEA obtained from its crystal structure. Consistent with this notion, the area per molecule of cholesterol at 40 mN.m^{-1} (38.8 Å^2), obtained from the π-A isotherm shown in Fig. 1 is in close

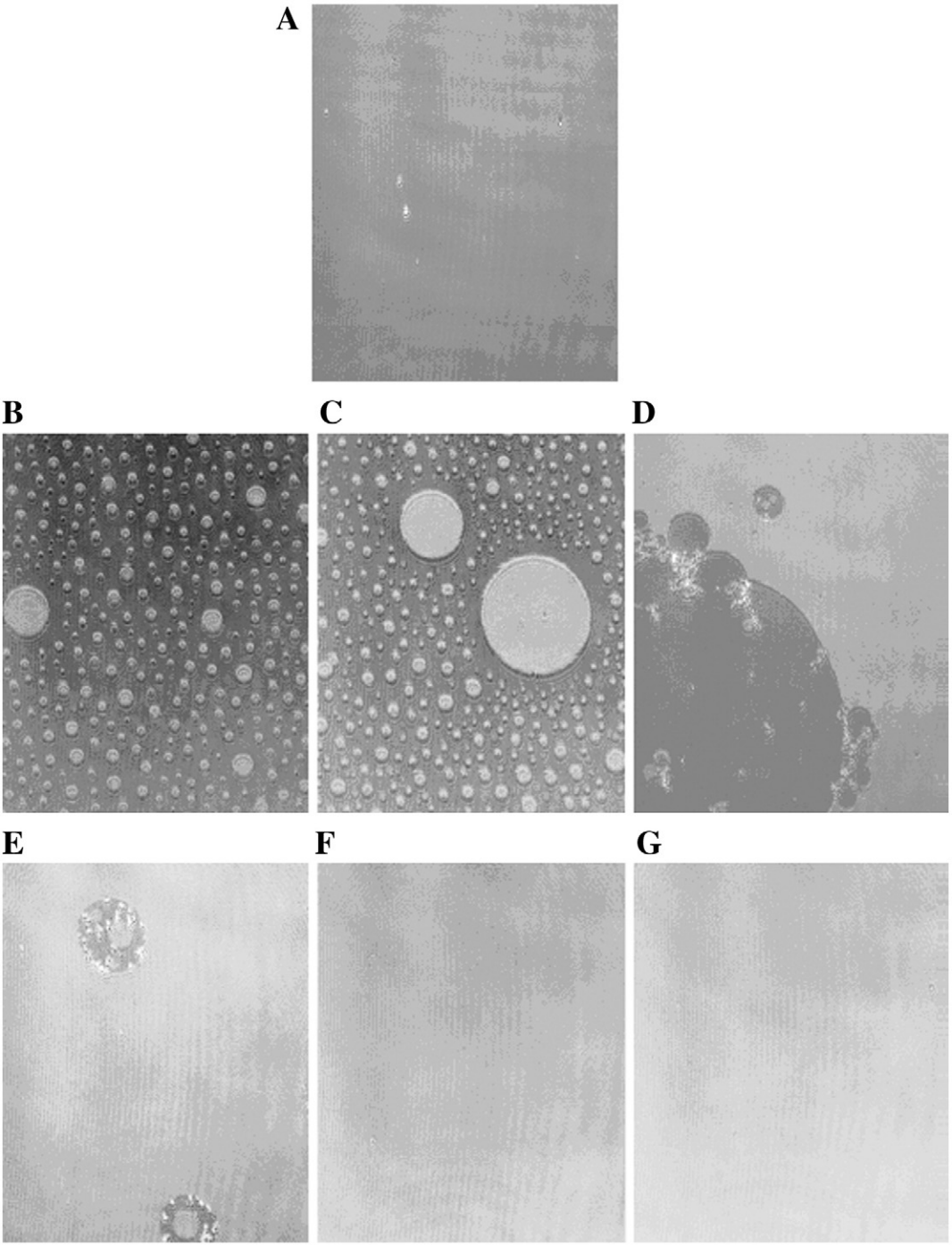

Fig. 3. BAM images of NMEA (A) and cholesterol (B–G). The surface pressures at which the images were recorded are: A) 13.5, (B) 0, (C) 0.4, (D) 5, (E) 10, (F) 15, and (G) 40 mN.m^{-1}. The size of each image is 220 μm (horizontal)×273 μm (vertical).

agreement with the values of 38±0.6 Å^2 reported in several monolayer studies [20–22] and is only slightly higher than the value of 36.2 Å^2, determined from single-crystal X-ray diffraction studies [23]. The solid line in Fig. 2 represents the ideal average value predicted from the molecular areas of pure NMEA from its crystal structure and that of pure cholesterol from its isotherm at 40 mN.m^{-1}. Interestingly, the area per molecule for mixtures with cholesterol contents below 50 mol% is lower than that expected from ideal mixing, whereas above 50 mol% cholesterol, the average molecular area is larger than the value expected from ideal mixing (Fig. 2). The cross-over occurs at 50 mol% cholesterol.

The above observations are quite interesting and although it is not possible to rationalize them in a definitive manner, a logical model can be visualized as follows. The crystal structure of NMEA shows that the molecules have a bent conformation as a result of the gauche structure at the C4–C5 bond. If it is assumed that a similar structure could exist in the monolayer, then the molecular area in the monolayer at collapse pressure should be comparable to that observed in the crystal. The area per molecule of 21 Å^2 obtained (at 16 mN.m^{-1}) from the monolayer measurements is quite close to the value of 21.95 Å^2 determined from the crystal structure of NMEA [14] and supports the above interpretation. Addition of cholesterol at small proportions decreases the area per molecule, despite the fact that the area per molecule for pure cholesterol is significantly higher than that of NMEA. This can be interpreted as resulting from the straightening of the tilted acyl chains of NMEA molecules, besides close-packing due to favorable interactions between NMEA and cholesterol. Consistent with this notion, the area per molecule of ~18 Å^2 observed in the presence of 10 mol%, which is the lowest area per molecule observed among the different mixtures, is in close agreement with the cross-sectional area of 19 Å^2 of a saturated hydrocarbon chain [24]. At the other end of the composition range, addition of NMEA to cholesterol appears to lead to the tilting of the orientation of the cholesterol molecules resulting in an increase in the area per molecule, as compared to that of pure cholesterol. The two effects observed at either end of the composition range cancel each other towards the middle causing the ideal area per molecule to be observed near 1:1 composition.

The change occurring at the equimolar composition is also reflected in the shape of the π-A isotherms (Fig. 1). As long as free NMEA is present, the isotherms show a slow rise with compression indicative of a liquid-like character (see curves 2–5). However, once all the NMEA are in the complexed state, *i.e.* from the 1:1 composition onwards, there is a drastic change in the shape of the isotherm indicating transition to a solid condensed phase at lower pressures (curves 6–10). These observations are consistent with a strong 1:1 stoichiometric complexation between NMEA and cholesterol. At higher compositions the isotherms continue to behave similarly because of the higher percentage of cholesterol. These observations are consistent with previous studies using mass spectrometry, calorimetry and computational modeling, which showed that NMEA and cholesterol form a 1:1 (mol/mol) complex [18].

Brewster angle microscopy is a convenient technique for investigating the morphology of Langmuir films at the air–water interface, exploiting the changes in the refractive index accompanying the

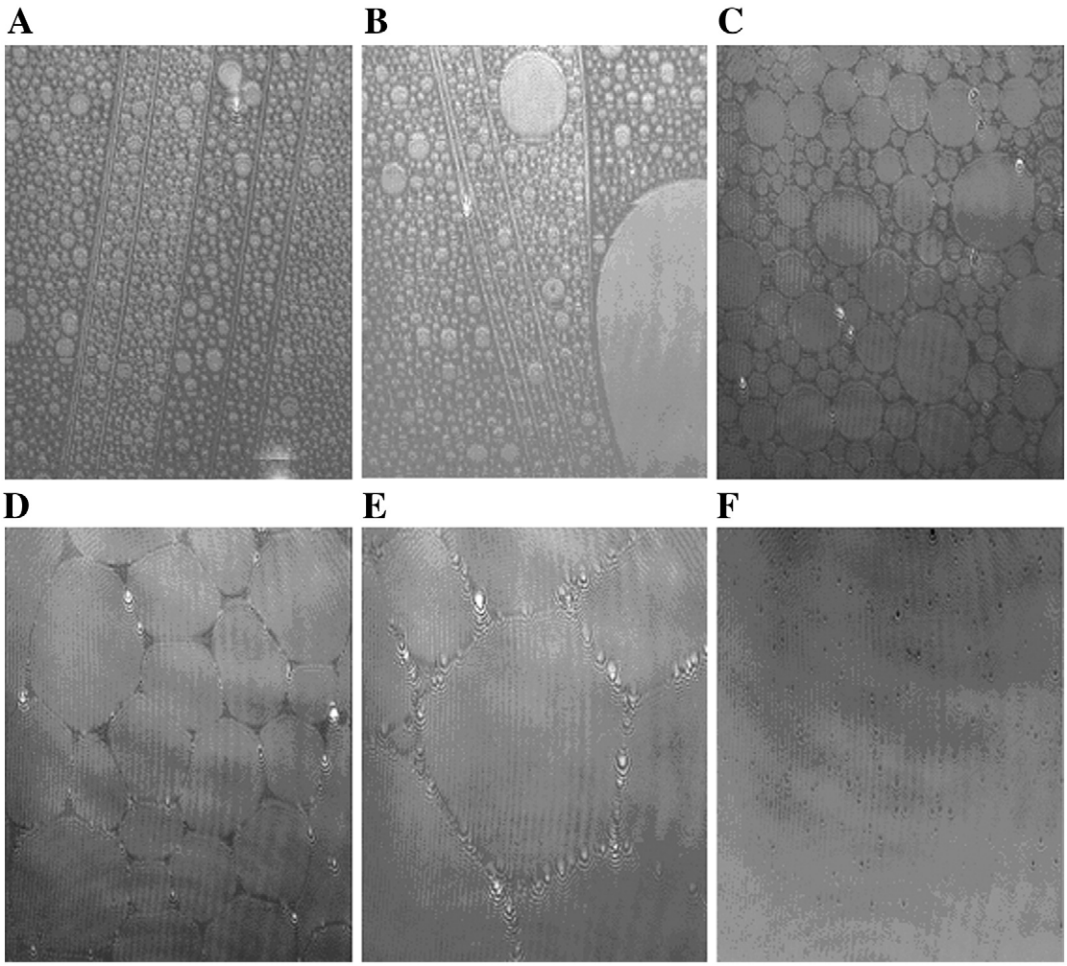

Fig. 4. BAM images of NMEA:Chol (7:3, mol/mol) at surface pressures: (A) 0, (B) 1, (C) 10, (D) 15, (E) 20, and (F) 25 mN.m^{-1}. The size of each image is 220 μm (horizontal)×273 μm (vertical).

formation of the film and its structural changes [25]. The monolayers formed in a Langmuir trough can be investigated by recording BAM images at different surface pressures. Local differences in the monolayer refractive index due to differences in molecular density or packing result in differences in the brightness in BAM images [26]. A dark image is ascribed to a low density monolayer, as in liquid expanded (LE) phase, while a bright image is attributed to a high density of molecules at the interface e.g., a liquid condensed (LC) or a solid phase.

As the pressure-area isotherms showed interesting trends with varying compositions of NMEA and cholesterol in the mixture (Fig. 1), we have performed BAM experiments to investigate changes in the surface morphology of the monolayers as a function of the applied pressure. BAM images of pure NMEA at 13.5 $mN.m^{-1}$ and pure cholesterol at different applied pressures are given in Fig. 3. Even though the image in Fig. 3 A of pure NMEA is provided at a pressure close to its collapse pressure, the image shows a relatively low brightness, implying that the molecules are not closely packed. This is consistent with the results obtained from its π-A isotherm, where NMEA did not form a stable monolayer.

In contrast to NMEA, cholesterol forms a rather stable monolayer. BAM images of cholesterol shown in Fig. 3 (images B–G) indicate a clear progression from a predominantly liquid expanded phase to a solid condensed phase. Image B (π=0 $mN.m^{-1}$) contains mostly small, bright domains of LC phase in a rather dark LE background. Increasing the pressure to 0.4 $mN.m^{-1}$ leads to the formation of larger domains of LC phase (image C) which expand further at 5 $mN.m^{-1}$ (image D). At higher pressures the monolayer consists of an essentially homogenous condensed phase, with gradually increasing brightness (images E, F and G).

BAM images corresponding to NMEA:Chol. (7:3, mol/mol) mixture are shown in Fig. 4. Even in the absence of any applied pressure (0 $mN.m^{-1}$, image A) the monolayer shows small domains of intermediate brightness in a continuous dark background. At 1 $mN.m^{-1}$ some of the smaller domains coalesce resulting in the coexistence of small and large domains (image B). Increasing the surface pressure to 10 $mN.m^{-1}$ results in a closer packing of small and large domains (image C), while further increase to 15 and 20 $mN.m^{-1}$ led to an increase in the average size of the domains which are even more closely packed (images D and E). At 25 $mN.m^{-1}$ the domains (image F) coalesce to form a nearly continuous liquid condensed phase of intermediate brightness.

Fig. 5 shows BAM images of monolayers formed by NMEA:Chol. (1:1, mol/mol) mixture at different pressures. Interestingly, even when the barrier is fully open and the pressure is 0 $mN.m^{-1}$, the monolayer shows several bright and relatively large domains, in addition to smaller domains (image A). Even at the nominal surface pressure of 1 $mN.m^{-1}$, formation of rather tightly packed bright domains is observed. The domains possess well-defined shapes, suggesting highly ordered lattice structures within each one. When the pressure increases to 2 $mN.m^{-1}$, the domains coalesce to form a stable monolayer (image C), the brightness of which increases with further rise in pressure (images D–F).

The BAM images shown in Fig. 6 correspond to NMEA:Chol. (3:7, mol/mol) mixture where cholesterol is the excess component. At zero

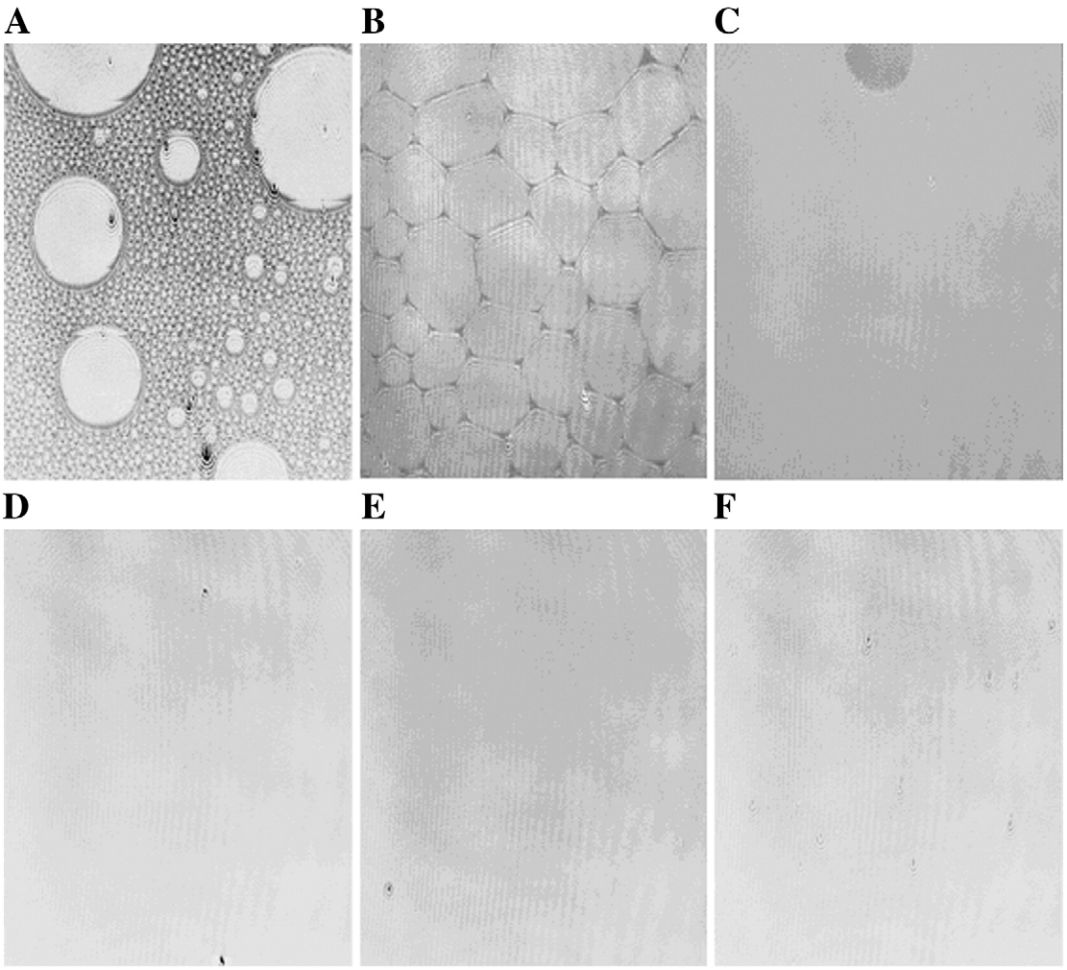

Fig. 5. BAM images of NMEA:Chol (1:1, mol/mol) at surface pressures: (A) 0, (B) 1, (C) 2, (D) 4, (E) 20, and (F) 39 $mN.m^{-1}$. The size of each image is 220 μm (horizontal)×273 μm (vertical).

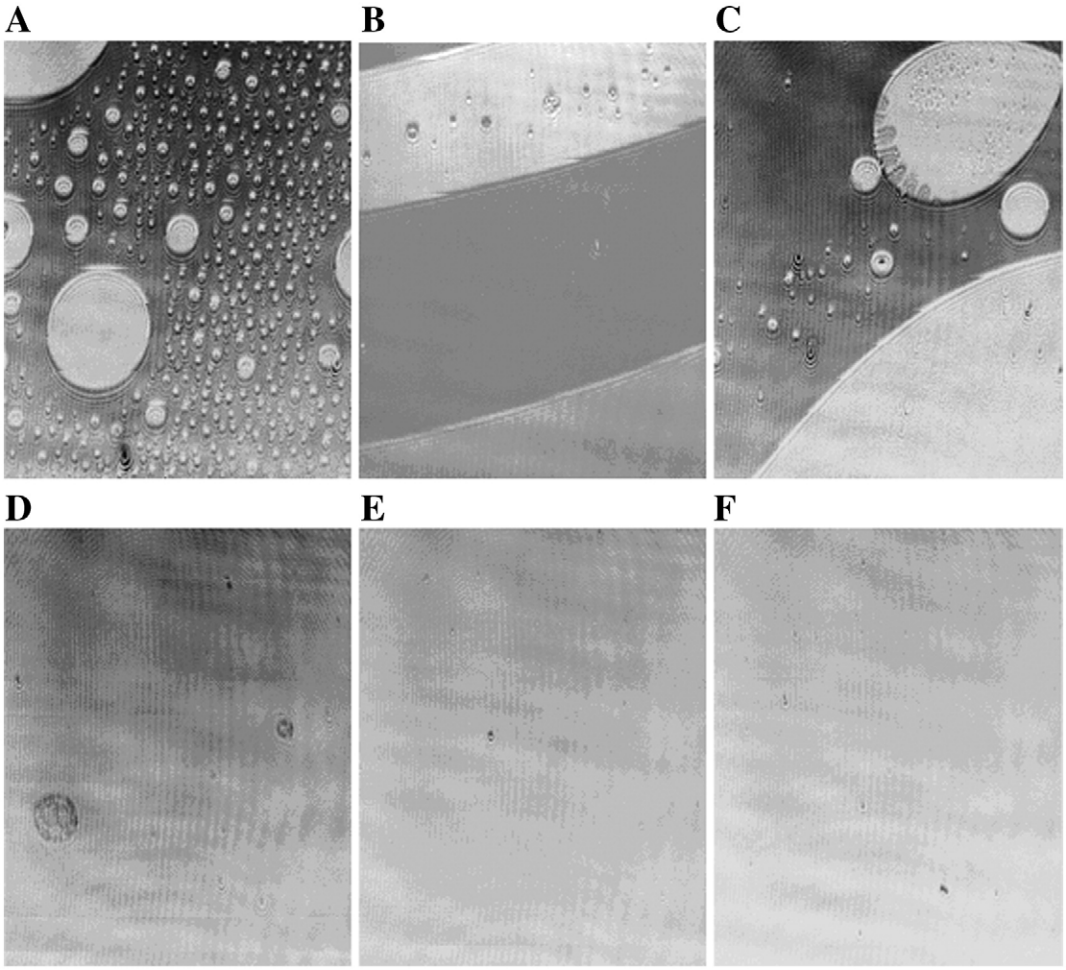

Fig. 6. BAM images of NMEA:Chol (3:7, mol/mol) at surface pressures: (A) 0, (B) 1, (C) 5, (D) 10, (E) 20, and (F) 35 mN.m^{-1}. The size of each image is 220 μm (horizontal)×273 μm (vertical).

applied pressure (image A) rather bright domains of different size are seen against a dark background. Increase in the pressure to 5 mN.m^{-1} results in the coalescence of the domains forming larger regions of brightness (images B and C). Further increase in pressure leads to a complete coalescence of the domains, forming a stable monolayer and its brightness increases with increasing applied pressure (images D–F), indicating the formation of a homogeneous condensed phase. However, the brightness of the homogeneous phase is significantly lower than that obtained with the NMEA:cholesterol (1:1, mol/mol) sample (see Fig. 5).

The above observations from Brewster angle microscopy are consistent with the formation of a 1:1 (mol/mol) complex between NMEA and cholesterol. Most interestingly, the NMEA:cholesterol (1:1, mol/mol) sample forms a homogeneous liquid condensed monolayer at a surface pressure of 1 mN.m^{-1}, whereas all other samples require considerably higher pressures (ca 10 mN.m^{-1} or higher) to form similar uniform LC phases. This shows that NMEA and cholesterol interact to form a 1:1 (mol/mol) complex and the heterodimers assemble into tightly packed ordered structures in the monolayers at the air–water interface. These results are in complete agreement with the conclusions drawn from previous DSC, FAB-MS and computational modeling studies regarding the formation of a 1:1 stoichiometric complex between NMEA and cholesterol.

In view of its ubiquitous nature and its role in the formation of membrane rafts the interaction of cholesterol with different phospholipids and sphingolipids has been investigated extensively [27,28]. One of the important results obtained from these studies is the identification of novel "condensed complexes" between cholesterol and phospholipids, which have been implicated in the formation of lipid rafts [29,30]. The stoichiometric (1:1; mol/mol) complexation between NAEs such as NMEA, NPEA and NSEA and cholesterol demonstrated in this study and our previous studies [18,19], suggests that the increase in the content of *N*-acylethanolamines under conditions of stress can alter the composition and dynamics of rafts by competing with the above lipids to interact with cholesterol. This can, in turn, result in changes in the signaling events associated with raft components. The putative cytoprotective and stress-combating actions of *N*-acylethanolamines may involve such modulation of raft characteristics.

In summary, in the present study, the interaction between *N*-myristoylethanolamine and cholesterol has been investigated in Langmuir monolayers at the air–water interface, using π-A isotherms and Brewster angle microscopy. Addition of cholesterol at low mole fractions was found to not only stabilize the monolayer formed by NMEA but also to decrease the average area per molecule, which could possibly result from a straightening of the acyl chain of NMEA upon complexation. BAM studies provide clear evidence for the ordered and close packed assembly in Langmuir monolayers of the NMEA:cholesterol (1:1; mol/mol) mixture. These observations provide further support to the formation of a 1:1 molar complex and also reveal further insights into the nature of the nanoscopic assembly of the two-dimensional complex.

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