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Effect of SP-C on surface potential distribution in pulmonary surfactant: Atomic force microscopy and Kelvin probe force microscopy study

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ABSTRACT

The air–lung interface is covered by a molecular film of pulmonary surfactant (PS). The major function of the film is to reduce the surface tension of the lung's air–liquid interface, providing stability to the alveolar structure and reducing the work of breathing. Earlier we have shown that function of bovine lipid extract surfactant (BLES) is related to the specific molecular architecture of surfactant films. Defined molecular arrangement of the lipids and proteins of the surfactant film also give rise to a local highly variable electrical surface potential of the interface. In this work we investigated a simple model of artificial lung surfactant consisting of DPPC, eggPG, and surfactant protein C (SP-C).

Effects of surface compression and the presence of SP-C on the monolayer structure and surface potential distribution were investigated using atomic force microscopy (AFM) and Kelvin probe force microscopy (KPFM). We show that topography and locally variable surface potential of DPPC–eggPG lipid mixture are similar to those of pulmonary surfactant BLES in the presence of SP-C and differ in surface potential when SP-C is absent.

1. Introduction

The lung interface is covered by a molecular film of pulmonary surfactant (PS), whose function is to reduce the surface tension of the alveolar air–liquid interface, to provide stability to the alveolar structure, and to reduce the work of breathing. The structure–function relationship of PS has been studied for more than four decades in the context of respiratory distress syndrome (RDS) in premature babies lacking this substance, in adult acute respiratory distress syndrome (ARDS), and deteriorated surfactant in the case of asthma.

Pulmonary surfactant is a phosopholipid-based film incorporating specific proteins (surfactant proteins A, B, C, and D) in the alveoli of mammalian lungs [1,2]. The primary purpose of surfactant is to lower the surface tension of this interface to near zero values [3]. Experimental measurements indicate that the equilibrium surface tension is at maximum 23 mN/m at the beginning of expiration [4]. Low surface tension is necessary to allow minimal effort while breathing and to prevent alveolar collapse [5]. Surfactant is secreted by exocytosis from the lamellar bodies which are formed by alveolar type II cells in the lung epithelium.

The primary lipids (70–80%) in pulmonary surfactant are phosphatidylcholine lipids (PC). 50–70% of all PC lipids are saturated lipids [6], mostly dipalmitoylphosphatidylcholine (DPPC) type lipids. DPPC is capable of reducing surface tensions to values approaching zero. However DPPC alone fails as an effective surfactant. This failure is because DPPC is slow to re-spread when compression is relieved, and it is slow to absorb from an aqueous suspension [7]. A DPPC monolayer is oriented with the fatty acid tails pointed up (towards the air interface). The polar headgroups are in the subphase with the fatty acid tails tilted between 21.5° and 29° depending on humidity [8]. In order for lung surfactant to maintain its biophysical properties, the lipids are required to allow the surfactant surface tension to reduce to very low values, as well as re-spread rapidly during the respiratory cycle. The individual components of surfactant are each good at lowering surface tension (DPPC) or fluidizing the monolayer (PG, PC, proteins). However none of the individual components exhibit both properties [9].

Lack of surfactant or dysfunctional surfactant is associated with high surface tension, which strongly impairs lung function.

Abbreviation: DPPC, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine; eggPG, L-a-phosphatidylglycerol; SP-C, surfactant protein C; BLES, bovine lipid extract surfactant; AFM, atomic force microscopy; KPFM, Kelvin probe force microscopy; PS, pulmonary surfactant

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Preterm infants do not have sufficient mature surfactant and as a result develop neonatal respiratory distress syndrome (NRDS). These neonates are treated by intra-tracheal administration of animal extract surfactant, spread from a bolus of a highly concentrated aqueous suspension. In spite of the great success of this treatment, RDS remains one of the leading causes of the neonatal morbidity and mortality.

Although animal extracts are effective for RDS patients, they involve the risk of animal infections, are associated with a costly purification procedure, and are difficult to produce uniformity among batches. Therefore, the preparations of pure synthetic surfactants are highly desired as a clinical surfactant replacement therapy for RDS. Moreover, exogenous surfactant administration has not proven efficacious in ARDS in all clinical studies carried out to date. Understanding the molecular mechanism of PS function is important for the development of synthetic surfactant formulations for NRDS and hopefully for ARDS. Extensive research has already been conducted on natural PS, and various synthetic lipid films [10–12]. However, comparatively little research has been conducted on mixtures of lipids and peptides which can functionally mimic natural PS and might potentially be useful for treatment of ARDS.

We studied lipid mixtures consisting of DPPC–eggPG and SP-C in comparison with natural bovine lung extract surfactant (BLES). BLES is an animal extract surfactant which readily adsorbs to the air–water interface and successfully reduces surface tension to near zero during compression cycles [13]. Our previous study with atomic force microscopy showed that functional surfactant forms multilayer structures which vary in height and surface potential [14,15].

Amrein et al. [16] demonstrated that similar multilayer formations are present when a mixture of DPPC, DPPG, and SP-C are compressed. The surface tension can reach near zero surface tension for this mixture. According to the molecular model proposed earlier by Amrein et al., the multilamellar structures are stabilized by the SP-C molecules incorporated with their hydrophobic α-helix in one bilayer, and by stretching their palmitoyl groups into a neighboring bilayer or the surface active monolayer.

Our previous research on BLES has shown that the presence of cholesterol adversely affects both the structure and the function of the BLES surfactant [15,17]. This loss of function is caused by the failure of the monolayer to form multilayers upon compression, making the achievement of higher surface tensions impossible. Furthermore, it was shown that cholesterol molecules produce significant changes in localized surface potential, and also alters adhesion properties of BLES films which affect the structure and function of surfactant films [14].

This paper focuses on a model surfactant mixture composed of DPPC, eggPG, and SP-C. We investigated the structure of this model surfactant mixture in comparison to BLES natural surfactant. Using atomic force microscopy (AFM) and Kelvin probe force microscopy (KPFM) we showed the presence of multilayered structures and resolve the local surface potential for these films, formed by Langmuir–Blodgett deposition. We also demonstrated that the presence of SP-C contributes to the surface potential distribution of the film.

2. Materials and methods

2.1. Sample preparation

DPPG, eggPG, and cholesterol were purchased from Avanti Polar Lipids Inc., Alabaster, AL, USA. SP-C was obtained from Dr. Jan Johansson in Uppsula, Sweden. Lipid solutions were made with a concentration of 25 mg/mL in chloroform, containing DPPC/eggPG in a ratio of 80:20 mol%.

SP-C (5 mg/mL) was mixed in aqueous buffer (pH 7) to make a mixture containing DPPC, eggPG, and SP-C in a ratio of 80:20:0.4 mol%. DPPC, eggPG solutions were mixed in chloroform and evaporated using liquid nitrogen. SP-C solution was added to the precipitate and the corresponding solution was again evaporated under liquid nitrogen. Buffer was added to make a 27 mg/mL solution. Lipid solutions were spread with a Langmuir–Blodgett trough compressed at 47–50 mN/m, and lipid films were deposited on mica support for imaging.

2.2. Imaging

Atomic force microscope Nanowizard II (JPK Instruments AG, Berlin) was used in this study. For imaging mica slides with supported lipid and BLES films (prepared by a Langmuir–Blodgett trough) were placed onto electrically conductive tape purchased from Ted Pella. AFM topography and KPFM images were collected in air using NanoWorld cantilevers with spring constant 42 N/m. KPFM measurements were recorded using the hover mode technique where the topography was measured on the first pass, and the surface potential was measured during the return path. During the retrace, the tip was offset 50 nm from the measured topography of the trace path, and a 5 V AC voltage was applied to the tip. A DC bias was applied to the cantilever such that the electrostatic interactions between the tip and sample were nullified, which corresponds to the surface potential under the tip.

3. Results and discussions

Successful formulation of artificial surfactant depends on the detailed understanding of the molecular mechanism of surfactant function, and the contribution of various components present in natural surfactant. The difficulties in studying this mechanism include complex structure of natural surfactants and inability to reproduce in vitro the environment and processes in the lung. In this work we investigated model lipid–protein mixtures in comparison with natural lung surfactant BLES to elucidate how SP-C affects the structure and function of pulmonary surfactant, and in particular the electrostatic interactions in PS using a combination of AFM and KPFM imaging.

Phospholipids are major constituents of PS, which are responsible for surface tension reduction. Phosphatidylcholines (PC) represent 80% of its mass. Half of the PC is the saturated dipalmitoylphosphatidylcholine (DPPC); 5–10% by mass is the negatively charged phosphatidylglycerol (PG) [18]. In a pure film, DPPC permits surface tension reduction to near zero values upon compression. A significant amount of phosphatidylglycerols suggests an important role of negatively charged phospholipids [19]. eggPG has an unsaturated acyl chain and has been shown to improve the adsorption of surfactant to the interface.

Natural Pulmonary surfactant BLES contains two hydrophobic surfactant-associated proteins (SP-B, SP-C). Mixed films that also contain SP-C can sustain a very low surface tension equally well as pure DPPC films. The proteins SP-C and -B efficiently recruit the lipids to the interface and promote more effective film organization. They are also responsible for the cohesiveness and mechanical strength of the film [16,19–24].

It has been shown that artificial or natural pulmonary surfactants that are functional (i.e. capable of reducing the surface tension to near zero) show characteristic pattern of monolayer and multilayer patches on top of the monolayer [15,20]. We found that multilayer patches were formed when DPPC/eggPG mixed

film was imaged, Fig. 1A, as well as in the presence of SP-C in the mixture, Fig. 1B. This multilayer organization is similar to what we observed in BLES films at the same compression (Fig. 1C). In BLES films we observed both large flat bilayers and smaller size higher multilayers. The height within multilayers increases in increments of about 5 nm or multiples thereof. This means that we have multiple bilayer patches attached to the surface of the monolayer. It has been suggested that this monolayer–bilayer conversion is a well-defined process with the involvement of SP-C [15].

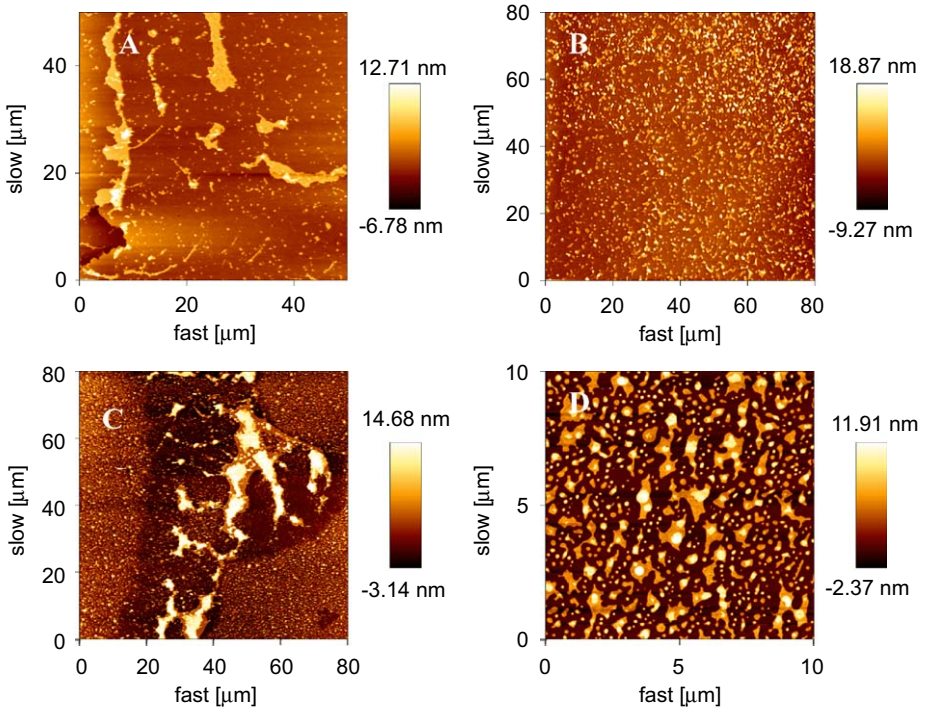

Fig. 1. AFM topography images of DPPC–eggPG (A) and DPPC–eggPG–SP-C (B) lipid films and BLES (C, D) films, compressed at 48 mN/m, deposited on mica by Langmuir–Blodgett technique and imaged in air. Fig. 1D is smaller scan area from (C), showing small multilayer patches of BLES film.

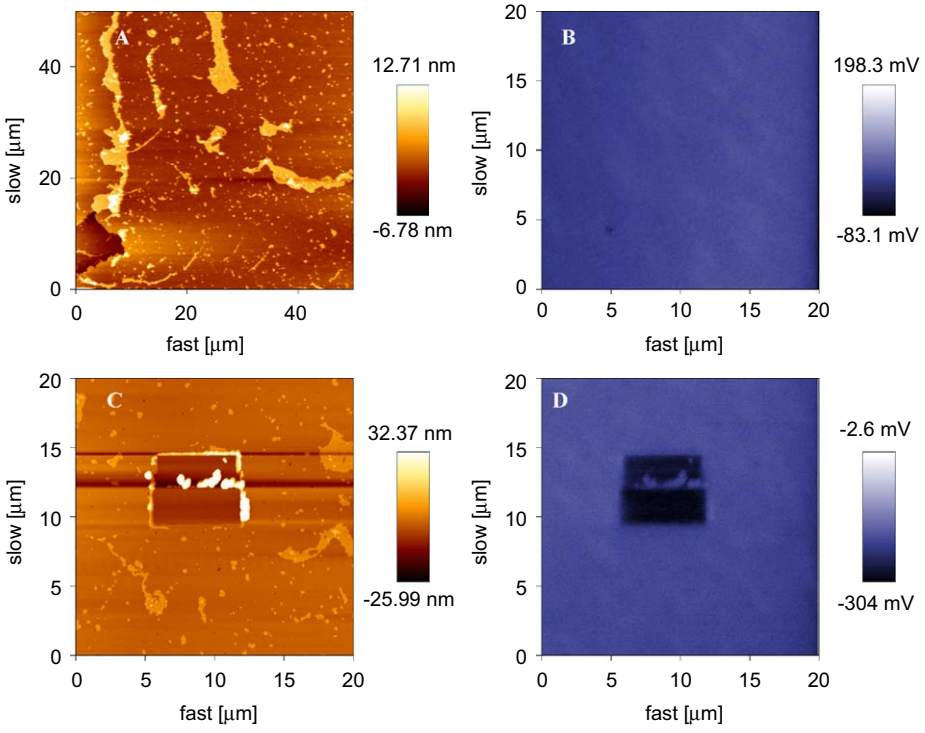

Fig. 2. AFM topography image (A) and KPFM image (B) of DPPC/eggPG lipid films. Lipid films were compressed at 48 mN/m, deposited on mica by Langmuir–Blodgett technique and imaged in air. Lower row of images show the result of scratching a hole in the DPPC/eggPG lipid film with AFM tip: (C) AFM topography image and (D) KPFM image.

Atomic force microscopy images show that multilayer structures are present in both DPPC/eggPG lipid mixture and DPPC/eggPG/SP-C mixture, Fig. 1A and B. In the case of just lipid mixture the bilayer patches are more flat and rare in appearance, Fig. 1 A. In the case of lipid–protein mixture, the bilayer patches are smaller in x and y directions but higher in the z direction. In BLES films we observed both large and flat multilayers which are more densely packed with small protrusions.

Various bilayer stacks, observed in BLES are required for the mechanical stability of the functional surfactant films. In order for the bilayer stacks to be mechanically reinforcing, they must be cross-linked to the monolayer. Otherwise, they will separate from- or glide over the monolayer area reduction and have no mechanical effect. As suggested earlier, SP-C is likely acting as the cross-linker (e.g. 9, 21, 34). Therefore SP-C should be present in high multilayer structures [15,20,25].

In addition to AFM imaging we collected the surface potential maps on BLES, lipid, and lipid–protein mixed films using Kelvin probe force microscopy. KPFM is a non-contact scanning probe technique capable of mapping the local surface potential or surface charge distribution with high spatial resolution. In KPFM, also known as surface potential microscopy, the electrostatic interaction between the tip and sample is minimized by application of an appropriate bias voltage during imaging. With KPFM, the work function or surface potential can be resolved with lateral resolution at nm scale [26–29]. To date, [30–32] KPFM on organic films has been successfully used to study amphiphilic molecules, and a biological membrane [33]. KPFM is designed to map the surface potential of an interface to the air or vacuum.

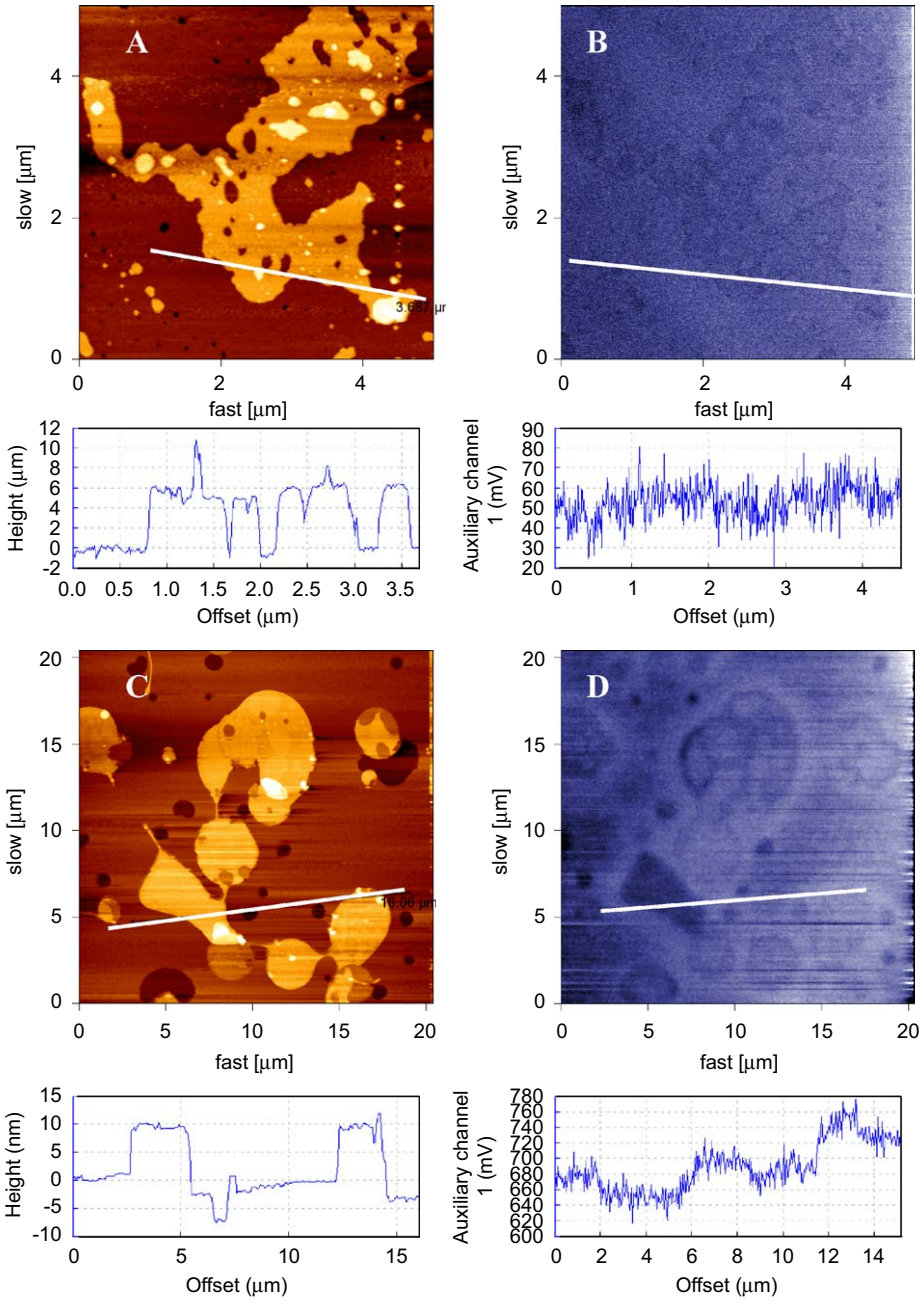

Fig. 3. DPPC/eggPG lipid film without SP-C: AFM topography image (A) and KPFM image (B), DPPC/eggPG/SP-C film: AFM topography image (C) and KPFM image (D). Cross-section plots are shown below the images. Lipid films were deposited on mica by Langmuir–Blodgett technique at the compression of 48 mN/m and imaged in air.

KPFM is therefore particularly well suited to investigate pulmonary surfactant films as well as lipid–protein mixtures.

Lipid films at the air–water interface exhibit an electrical surface potential defined by the molecular dipoles in the direction perpendicular to the interface μ, the dielectric constant ε and the packing density or area covered by each molecule A. For phospholipids, contributions have been ascribed to various regions of the molecules, each in its own dielectric environment; numbered 1, 2, 3, corresponding to the head-group region, the aliphatic tail and the terminal methyl group [34].

$$V = \frac{1}{A\varepsilon_0}\left[\frac{\mu_1}{\varepsilon_1} + \frac{\mu_2}{\varepsilon_2} + \frac{\mu_3}{\varepsilon_3}\right]$$

We showed earlier that in BLES films specific molecular arrangement of multilayers give rise to the locally variable surface potential. The surface potential maps of functional pulmonary surfactant reveal that the surface potential of multilayers is more positive than that observed on monolayer areas [16,17]. It is expected that a strong surface potential arises from surfactant-associated protein C (SP-C). SP-C is a small (molecular mass ~4 kDa) hydrophobic protein with a membrane spanning α-helical segment [35,36].

Two palmitoyl groups are covalently linked to the two cysteine residues located in the N-terminal region. It has been suggested that SP-C promotes the formation of lipid bilayer patches and resides in these multilamellar regions of the surfactant films [37]. The arrangement of SP-C in the lamellar region is with its helical axis perpendicular to the interface was proposed by Amrein [16]. SP-C possesses a strong molecular dipole moment in the direction of its molecular axis due to its α-helical secondary structure, and because of the positive charge at the N-terminal. If the N-terminal points towards the air, it should effectively induce a more positive surface potential in the multilayered areas of the film and thus explain the more positive surface potential found on the multilayered regions (Fig. 4 and [14]).

Due to a small percentage of SP-C in the surfactant film, some regions of the film may consist of lipid bilayers or multilayers devoid of protein. For example, Fig. 4A shows AFM topography of multilayers of similar height, and Fig. 4B shows electric surface potential of these multilayers, which differ considerably in Kelvin signal. In order to understand this we should consider that bilayers devoid of protein should be electrically "transparent" as the molecular dipoles from the lower and the upper leaflet are of opposite directions, and as a result their contributions to the surface potential cancel out. It has been also shown earlier by scanning near-field optical microscopy that multilayered regions with and without SP-C may indeed co-exist [14].

To prove this concept we investigated the DPPC/eggPG film by both AFM and KPFM. Fig. 2 shows AFM topography (A) and KPFM (B) of DPPC–eggPG lipid only film. Even though the topography image shows large flat bilayer patches, the surface potential for these patches is not different from the monolayer. It is interesting that when we removed the monolayer from the mica substrate by scratching the lipid surface with AFM tip, Fig. 2C, a change in Kelvin signal is clearly seen, showing contrast between mica substrate and lipid film, Fig. 2D, highlighting the potential of the lipid film itself. This means that multilayers of DPPC/eggPG film devoid of protein do not differ or differ very little from the monolayer in terms of electrical surface potential. This is consistent with the idea that molecular dipole moments of lipids are cancelled when lipids are arranged in bilayers or multilayers.

We observed an enhanced Kelvin contrast when we introduced SP-C protein to the mixture of DPPC/eggPG. Fig. 3 upper row shows DPPC/eggPG film void of protein, A—AFM topography, B—Kelvin signal, and lower raw shows DPPC/eggPG/SP-C film, D—AFM, C—Kelvin signal. At higher resolution we can see that very little contrast in the Kelvin signal is visible for lipid patches on the DPPC/eggPG film void of protein, Fig. 3B. The difference in surface potential is only a few mV. When SP-C is present in similar bilayer stacks, Fig. 3D. The Kelvin signal has a much higher

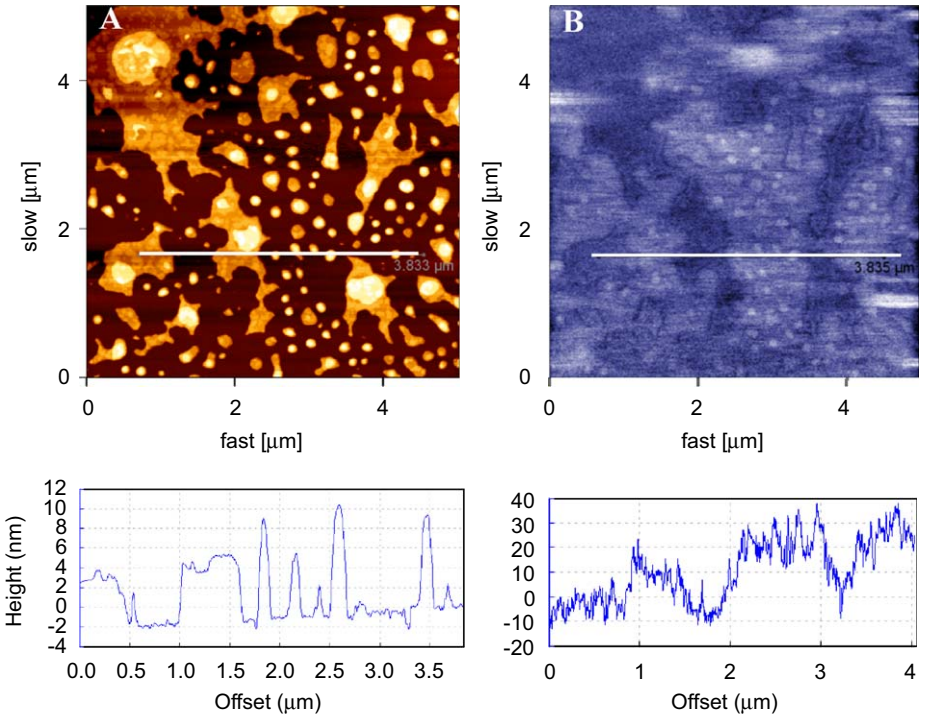

Fig. 4. AFM topography images (A) and KPFM image (B) of BLES film on mica. BLES films were deposited on mica by Langmuir–Blodgett technique at the compression of 47 mN/m dried and imaged in air.

contrast, Fig. 3D, the difference in surface potential is around 50–100 mV.

The absence or reduced contrast in the Kelvin signal between multilayer and monolayer in lipid films without the presence of SP-C (Fig. 3) proves the importance of SP-C in multilyer formation and its contribution into the more positive surface potential observed on multilayered structures in BLES surfactant film (Fig. 4).

4. Conclusions

In summary, we investigated DPPC–eggPG and DPPC–eggPG/SP-C films as a simple model for surfactant formulation replacement and compared these with BLES surfactant. We found that both model films show multilayer formation upon compression, which look similar in AFM topography mode, but differ considerably in surface potential maps. The presence of SP-C in the DPPC–eggPG mixture contributes significantly to the surface potential of multilayers formed upon compression. Therefore, DPPC–eggPG/SP-C mixture is more close to BLES surfactant and possesses similar electric surface potential among other features.

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