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Structures of surfactant films: a scanning force microscopy study

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Structures of surfactant films: a scanning force microscopy study. R. Grunder, P. Gehr, H. Bachofen, S. Schürch, H. Siegenthaler. © ERS Journals Ltd 1999.

ABSTRACT: The alveolar lining layer is thought to consist of a continuous duplex layer, *i.e.*, an aqueous hypophase covered by a thin surfactant film which is a monolayer with dipalmitoyl-phosphatidylcholine (DPPC) as its most important component. Findings obtained by electron microscopy and results from *in vitro* experiments suggest, however, that the structure and hence the structure-function relations of surfactant films are more complex. In order to better define their structures films of surfactants were studied by scanning force microscopy.

Four different surfactants were spread on a Langmuir-Wilhelmy balance, and then transferred onto a solid mica plate by the Langmuir-Blodgett technique, under various states of film compression. Imaging of the films by scanning force microscopy was performed in the contact (repulsive) mode in air.

The scanning force micrographs revealed that surfactant films are not homogeneous, but rather undergo phase transitions depending on the surface pressures. Even at comparable surface pressures different surfactants show quite different surface patterns. Differences in surface structure can even be observed in films containing surfactant proteins (SP)-B and SP-C.

These observations give further evidence that the widely accepted hypothesis of a regular monolayer of phospholipids governing the surface tension probably does not hold true, but that the structure-function relationship of surface active surfactant films is even more complex than hitherto thought.

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According to a widely accepted hypothesis the alveolar lining layer has the following structural properties: it is a continuous duplex layer consisting of an aqueous hypophase covered by a thin surfactant film, and the surfactant film is a monolayer with dipalmitoyl-phosphatidylcholine (DPPC) as its most important component [1–4].

There are numerous studies of the structure of the lining layer, all of which are fragmentary. Indeed, the sample preparation and preservation of this delicate structure for microscopy is extremely difficult; there is no standard to distinguish between facts and artefacts [5], and it is not easy to conceive of how monolayers of saturated phospholipids can be preserved and visualized by the usual fixatives [6]. As to the first condition, *i.e.*, the existence and continuity of a duplex layer, GIL and WEIBEL [7] were the first to convincingly demonstrate a two-phase lining layer by transmission electron microscopy. However, the film was often fragmented and disposed in patches, and could not be detected on flat parts of the alveolar walls. Although studies of freeze-fractured preparations suggested its continuity [8], the exact structure of the lining layer has remained a matter of debate. More recently, BASTACKY *et al.* [9] have clearly demonstrated the continuity of the lining layer by low-temperature electron microscopy, and improved tissue preparations have also resulted in a better visualization of the film such that all of the evidence now available suggests a continuum of the duplex layer.

With regard to the second postulate, that the film is a monolayer, the structural evidence is rather scanty. Already the micrographs of GIL and WEIBEL [7] as well as more recent high-power magnifications of the film, have revealed a rather polymorphous structure. At some sites, the surface film appears to be an amorphous material, at other sites, triple layers or even multilayers can be recognized, and quite generally the film appears to be thicker than a monolayer of phospholipids. Certainly, the latter finding might be an artefact caused by the heavy metals used as staining material. The same features can also be observed with surfactant films formed at the surface of captive bubbles *in vitro* [10].

In addition to the studies of surfactant films by electron microscopy, such films have also been investigated with fluorescence light microscopy by incorporating small amounts of fluorescent dye into the surfactant layer [11–13]. Films from both surfactant lipids and pulmonary extracts showed that the films undergo a phase separation at relatively high surface tensions of about 50 $mN \cdot m^{-1}$ (surface pressure of 10–15 $mN \cdot m^{-1}$). This became apparent by the appearance of two kinds of domains distinguishable by

the intensity of fluorescence light emitted by the dyes. Dark domains in the form of round patches of varying size appeared in a bright background [11, 12]. This phenomenon was explained by a less dense packing of the lipids (the liquid expanded (LE) phase) in the bright domains with respect to the dark domains (the liquid condensed (LC) phase).

This introductory summary shows that alternative techniques are required to tackle the problem of imaging surfactant films. More recently, scanning force microscopy (SFM) has become a promising tool for investigating the topography of surface films. SFM offers the advantage that neither fixation nor staining is required for imaging organic films, such that potential artefacts can be eliminated. With regard to lung surfactant films, the group of AMREIN and coworkers [14, 15] has produced quite recently the first and most revealing micrographs of particular films of different compositions. In the present study, similar techniques have been applied in order to address the following questions: 1) what are the structural differences between films from pure phospholipids and those containing phospholipids and the surfactant proteins (SP)-B and SP-C?; 2) does the film topography as revealed by SFM support the concept that the amphiphilic molecules are arranged as a monolayer, or is there evidence for multilayers as suggested by transmission electron micrographs?

Material and methods

Surfactants

Four different surfactants were used to form Langmuir-Blodgett (LB) films for analysis by SFM: 1) pure DPCC at a concentration of 1 mg·mL^{-1} (Fluka Chemicals, Buchs, Switzerland); 2) a mixture of DPPC and L-α-phosphatidylcholine (PC; extracted from yolks of eggs; Fluka Chemicals) in a ratio of 2:1 and at a total phospholipid concentration of 1 mg·mL^{-1}; 3) Curosurf®, a lipid extract surfactant isolated from minced pig lungs, which is widely used for the treatment of respiratory distress syndrome. Curosurf® contains ~99% polar phospholipids (30–35% DPPC) and 1% hydrophobic, SPs (SP-B and SP-C) in an approximate molar ratio of 1:2 (a gift from the Laboratoires Serono, Zug, Switzerland); 4) lipid extract surfactant (bovine lungs) containing 7.3% SP-B and SP-C and 93% phospholipids (a gift from F. Possmayer, Depts of Biochemistry and Obstetrics and Gynecology, University of Western Ontario, Canada). The high protein concentration was achieved by partial extraction of the phospholipids. Films were formed from this stock solution and from a diluted mixture with a protein concentration of 1% for a better comparison with Curosurf® films. Pure DPPC was used as the diluent. In both cases the total phospholipid concentration was 1 mg·mL^{-1}. In detail, the bovine surfactant extract was dissolved (not sonicated) in chloroform:methanol 9:1 by volume. DPPC was also dissolved in chloroform: methanol 9:1 by volume, and added to the above stock solution to adjust the surfactant protein content to 1%. Films of the stock and diluted solutions (equal total phospholipid concentration of 1 mg·mL^{-1}) were formed by spreading the solutions dropwise (20 μL) onto the aqueous subphase in the surface balance.

Langmuir-Blodgett films

LB films were deposited on freshly cleaved mica substrates. The hydrophilic nature of clean mica allows for the formation of an LB film with the polar heads of the phospholipid molecules associated closely with the mica surface, and with the acyl chains oriented towards the air phase. Monolayers were prepared on a commercially available Langmuir-Wilhelmy balance (Riegler and Kirstein, Ultrathin Organic Film Technology, Wiesbaden, Germany). Milli-Q-water (Milli-Q Plus System; Milipore Corp., Bedford, MA, USA) was used as the subphase. The monolayer after its formation by spreading on the water surface was compressed at a constant speed of 0.32 cm^2·s^{-1} to the desired surface pressure. The temperature was 21±1°C. The film was transferred onto the hydrophilic mica plates, which had been submerged in the aqueous subphase before film formation, by pulling them vertically through the air-water film, with a deposition speed of 5 mm·min^{-1}. The film pressure was kept constant during the film transfer to the mica plate by a feed-back system incorporated into the surface balance. The mica plates were glued onto a magnetic disc, leaving one side of the mica plate with the LB film intact. The prepared samples were mounted in the SFM. The freshly formed LB films were allowed to dry in ambient air at 21±1°C in order to minimize the thickness of the water layer between the surfactant molecules and the mica substrate. This drying phase was important, as LB films imaged by SFM proved to be unstable if investigated immediately after their formation.

Scanning force microscopy

SFM-imaging was performed in the contact (repulsive) mode in air using a commercial system (Universal Ambient System; Park Scientific Instruments, Sunnyvale, CA, USA). All films were examined by SFM within the same day after deposition. The Si3N4 triangular cantilevers used for imaging were 2 μm thick, 85 μm long, and had 28 μm wide legs (Park Scientific Instruments). The force constant of this type of cantilever was 0.17 N·cm^{-1}. The scanning speed used was in the range of 3–20 μm·s^{-1} depending on the scan size.

Results

DPPC-films

Pure DPPC films were transferred onto mica by the Langmuir-Blodgett technique at surface pressures of 4.5, 6.5, 10 and 20 mN·m^{-1}. Figure 1 shows a typical DPPC film isotherm registered by the Langmuir-Wilhelmy balance, and the sampling sites of the films shown in the SFM images of figures 2 and 3. At the low surface pressure of 4.5 mN·m^{-1} pure DPPC films have a heterogeneous structure. There are irregularly distributed domains of 3.5–8 μm width which are 1.5 nm higher than the surrounding phase. Between these probably LC domains a scatter of small granular structures of the same height can be seen. With increasing surface pressure the films become more organized, and at a surface pressure of 20 mN·m^{-1} they are almost homogeneous (fig. 3), *i.e.*, they

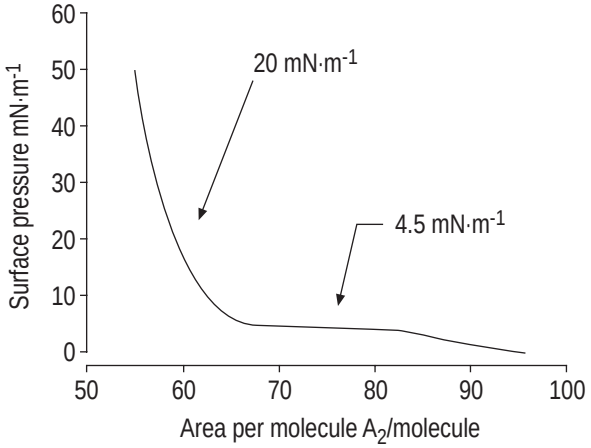

Fig. 1. – Surface pressure area diagram of a pure dipalmitoyl-phosphatidylcholine (DPPC) film. The arrows indicate the site of Langmuir-Blodgett transfer of the films shown in figs. 2 and 3. The DPPC isotherm was performed at 20°C.

are in a predominantly liquid condensed state in accordance with the extremely low compressibility of DPPC films at this pressure range (fig. 1). Small holes of LE phase with a depth of about 0.9 nm can still be observed.

Films of dipalmitoyl-phosphatidylcholine/L-α-phosphatidylcholine mixture

Mixed films of DPPC and (egg) PC reveal a quite different pattern. Although the films were transferred onto mica at a higher surface pressure (40 $mN \cdot m^{-1}$), their structures are far from homogeneous (fig. 4a). Large polygonal LC-domains, with a surface area of 36±24 μm^2 (mean ±SD), which cover almost 60% of the total surface area, are surrounded by countless granular structures. The relation between the former and the latter might reflect a "quantum growth mechanism" of LC-domains, *i.e.*, a growth by continuous mergers with small domains (fig. 4b).

Curosurf® films

Although Curosurf® is a lipid extract surfactant from pig lungs and hence a complex mixture of different phospholipids and SP-B and SP-C, the films reveal a quite similar pattern as the fully synthetic DPPC/PC films as the diameters of the LC domains are ~6 μm for both kinds of films (compare fig. 5 with fig. 4). Against all expectations, a direct imprint of the surface-associated proteins was not found on the surface topography of Curosurf® films. However, one quantitative difference is notable in that the LC-domains of Curosurf® films cover a mere 17±5% of the film surface as compared with 56±7% measured in DPPC/PC films, and this at identical surface pressures of 40 $mN \cdot m^{-1}$.

Comparison of surfactants with different concentrations of surfactant protein-B and C

As described in the *Materials and methods* section, surfactants with protein contents of 1% and 7.3% were

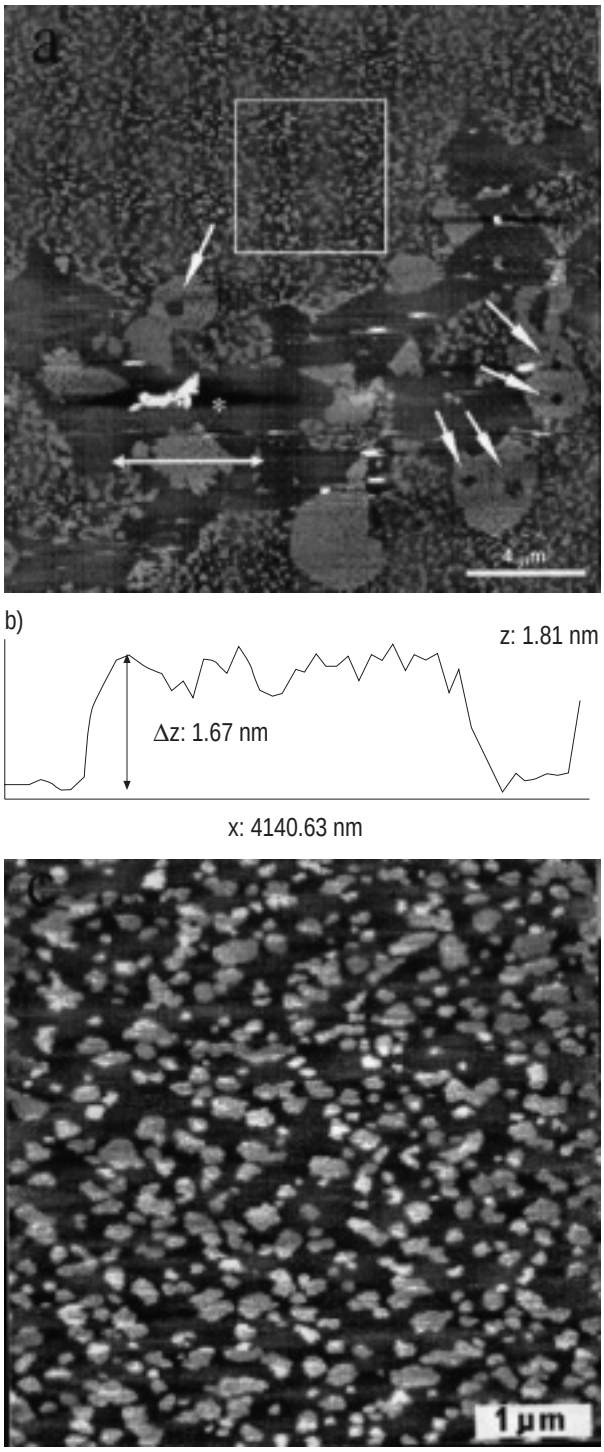

Fig. 2. – a) Scanning force microscopy (SFM) contact mode images of a dipalmitoyl-phosphatidylcholine (DPPC) film deposited onto a mica substrate at a surface of 4.5 $mN \cdot m^{-1}$. Arrows point to liquid condensed domains. Horizontal double arrow crosses the liquid condensed domain whose height is shown in b). * denotes an artefact. c) Higher magnification of inset of figure 2a. The liquid expanded phase contains countless corpuscles with the same height as the liquid condensed domains in figure 2a.

prepared to further examine possible influences of SP-B and SP-C on film structures. The total phospholipid concentrations were kept equal in both mixtures, and both films were compressed to the same surface pressure (40

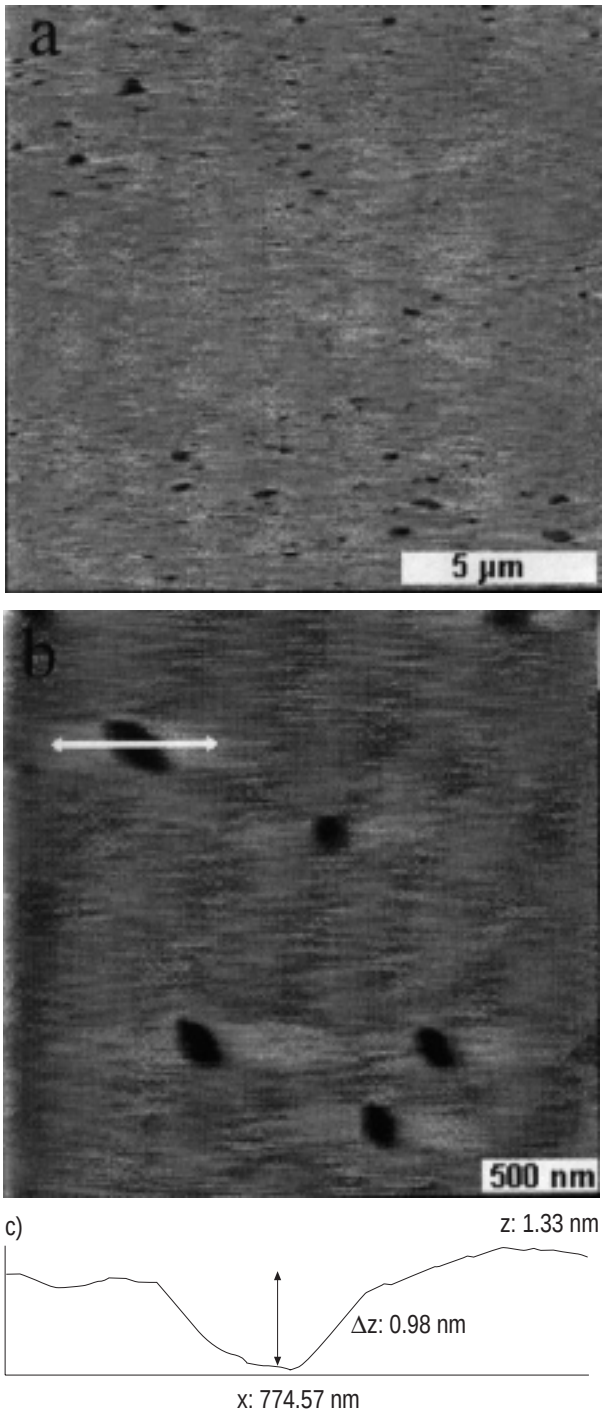

Fig. 3. – a) Scanning force microscopy (SFM) contact mode images of a dipalmitoyl-phosphatidylcholine (DPPC) film deposited onto mica at a surface pressure of 20 $mN \cdot m^{-1}$. b) Higher magnification of the same, rather homogenous film with small holes only, probably remnants of the liquid expanded phase. Horizontal double arrows crosses hole whose depth is shown in c).

$mN \cdot m^{-1}$). In comparison with Curosurf® films, which also contain SP-B and SP-C at a concentration of ~1%, these films show quite particular features (fig. 6). The most conspicuous findings are rather irregular, tapering phases which, in part, form web-like structures. In films with the higher protein content these webs appear to be finer and more coherent. At low protein concentrations, on the other hand, coarse and more fragmentary pieces are predominant. Interestingly, within the meshwork the film seems rather homogeneous, and phase separations as those observed in other phospholipid films (figs. 2–5) cannot be recognized. The heights of the web-like structures varies 1–2 nm, *i.e.*, the steps are not high enough to postulate bilayer or multilayer formations of the film consisting of stack bilayers with a repeating distance of 4–5 nm, the usual distance between two layers in lipid bilayers.

Discussion

The present experiments demonstrate that the structures themselves, and more so the structure-function relation of surfactant films are extremely complex. Evidently, the hypothesis of a surface film being a homogeneous monolayer of phospholipids has to be revised. This follows from the SFM studies as well as from the investigations with electron and fluorescence light microscopy.

As to the methods, some particularities are worth mentioning. In contrast to electron microscopy, SFM does not require chemical fixation and staining of films and hence is not afflicted by the usual artefacts. Possibly, this advantage has to be paid for by alternative artefacts. Firstly, it cannot be excluded that the film structure are different in different environments and on differing substrates (Wilhelmy balance, captive bubbles, alveoli, *etc.*) although the films exhibit a similar surface activity. Secondly, the topography of films as characterized by height differences, might not truly reflect the molecular arrangement, but might be influenced by force interactions between molecules and the probe tip of the SFM. And finally and most importantly, surfactant films cannot be examined by SFM *in situ*, or on an aqueous subphase, respectively, but have to be transferred onto a mica substrate. However, the LB technique is a well established method for film preservation, and VON NAHMEN *et al.* [13] have shown by fluorescence light microscopy that LB transfers of surfactant films are quite reliable, in that identical patterns are observed in films supported on an aqueous substrate and in those transferred by the LB technique.

Pure DPPC films may be considered a simple model for pulmonary surfactant films which reveals a clearcut phase separation, and this is in agreement with recently published results obtained by SFM and by fluorescence light microscopy [11, 13]. At low surface pressures both LE- and LC-phases coexist in a rather irregular pattern. Already at moderate surface pressure (*i.e.* relatively high surface tensions as compared with those prevailing in the lung) DPPC films become homogeneous with an almost exclusive predominance of the LC-phase. This observation may explain the extreme low compressibility of pure DPPC films at surface pressures higher than 20 $mN \cdot m^{-1}$ [10, 16].

The addition of unsaturated phospholipids appears to profoundly affect the phase separation, *i.e.* the formation of LE- and LC-domains. Even at relatively high surface pressures of 40 $mN \cdot m^{-1}$ mixed DPPC/PC and Curosurf® films show an unequivocal phase separation and ongoing phase transition (figs. 4 and 5). The presence of both large LC-domains and the small granular structures with comparable height are of interest: the latter might be nuclei of crystalline phases which grow on further film

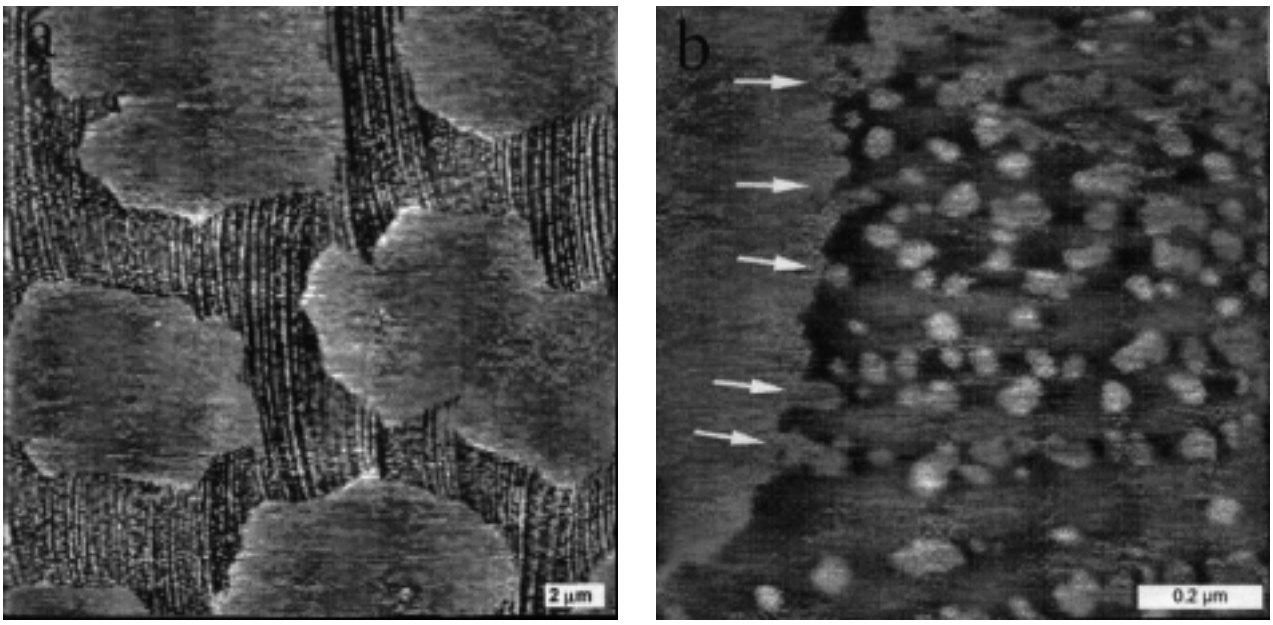

Fig. 4. – a) Film formed by a mixture of dipalmitoyl-phosphatidylcholine (DPPC)/L-α-phosphatidylcholine (PC) and transferred onto mica at a surface pressure of 40 $mN \cdot m^{-1}$. Note the large polygonal liquid condensed phase (LC)-domains surrounded by a liquid expanded phase (LE) phase and granular matter. b) Higher magnifications suggest that the granules are nuclei of LC-domains which in part merge with pre-existing domains (arrows).

compression by merging with each other [17, 18], whereby "squeeze-out" mechanisms may play a role [11].

The comparison of DPPC/PC with Curosurf® films, both of which were compressed to the same surface pressure of 40 $mN \cdot m^{-1}$, gave additional evidence that the area fraction of LC-domains is not an index of the structure-function relationship of surfactant films. Indeed, the relative area of the LC-domains is much smaller in Curosurf® than in DPPC/PC films. Curosurf® films, after their formation by adsorption to the equilibrium surface tension of 23-25 $mN \cdot m^{-1}$ (surface pressure of ~48 $mN \cdot m^{-1}$) need to be compressed only by approximately 28% to reach near zero surface tensions [16]. In contrast, mixed films of DPPC and unsaturated phospholipids, similar to those studied here, require an area compression of >70% to achieve near zero surface tension from 25 $mN \cdot m^{-1}$, that is they are much more compressible than the Curosurf® films. As the relative area of the LC- domains in the mixed phosholipid films is 56%, whereas that in Curosurf® films was only 17% it would be expected that the Curosurf® films would be more compressible. The difference might be explained by an effect of SP-B and SP-C in Curosurf®. This hypothesis is supported by the experiments of NAG *et al.* [19] who showed that the addition of SP-C to phospholipids modifies the transition of LE- to LC-phases. Enigmatic, however, are the differences in surface topography between porcine lipid extract (Curosurf®) films containing SP-B and SP-C on the one hand, and bovine lipid extract surfactant films with different concentrations of SP-B and SP-C. In the former, the surface structure is quite similar to those consisting of pure phospholipids, and there is no direct imprint of the hydrophobic proteins. In the latter, conspicuous web-like phases are formed, but the phase separation typical for pure phospholipid films is absent. This latter observation is in good agreement with findings obtained by fluorescence microscopy [11, 13, 14] and also by SFM [14, 15] on phospholipid films

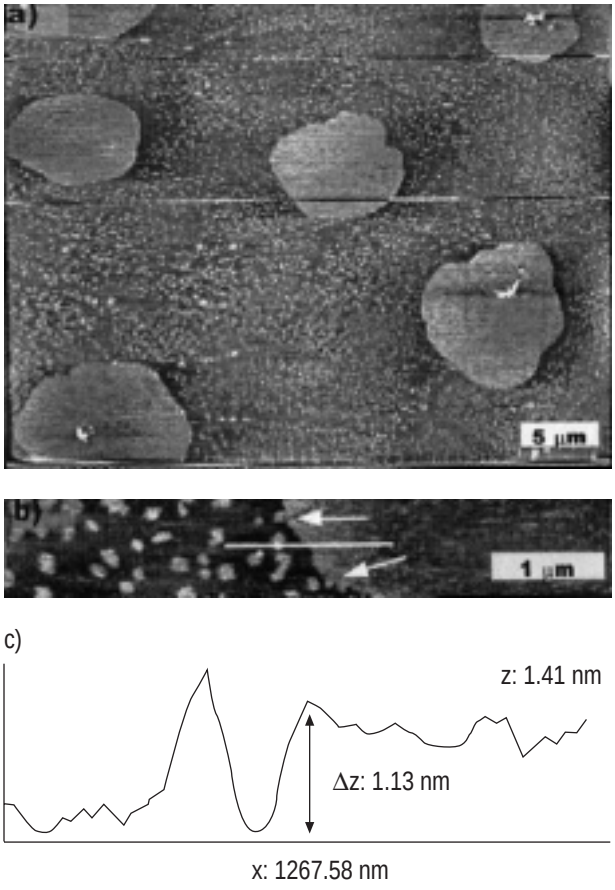

Fig. 5. – a) Curosurf®-film at a surface pressure of 40 $mN \cdot m^{-1}$. b) At higher magnification similar granules can be seen in the liquid expanded (LE) phase as in dipalmitoyl-phosphatidylcholine (DPPC)/L-α-phosphatidylcholine (PC) films. The horizontal bar in b) covers structures whose vertical dimensions are shown in c).

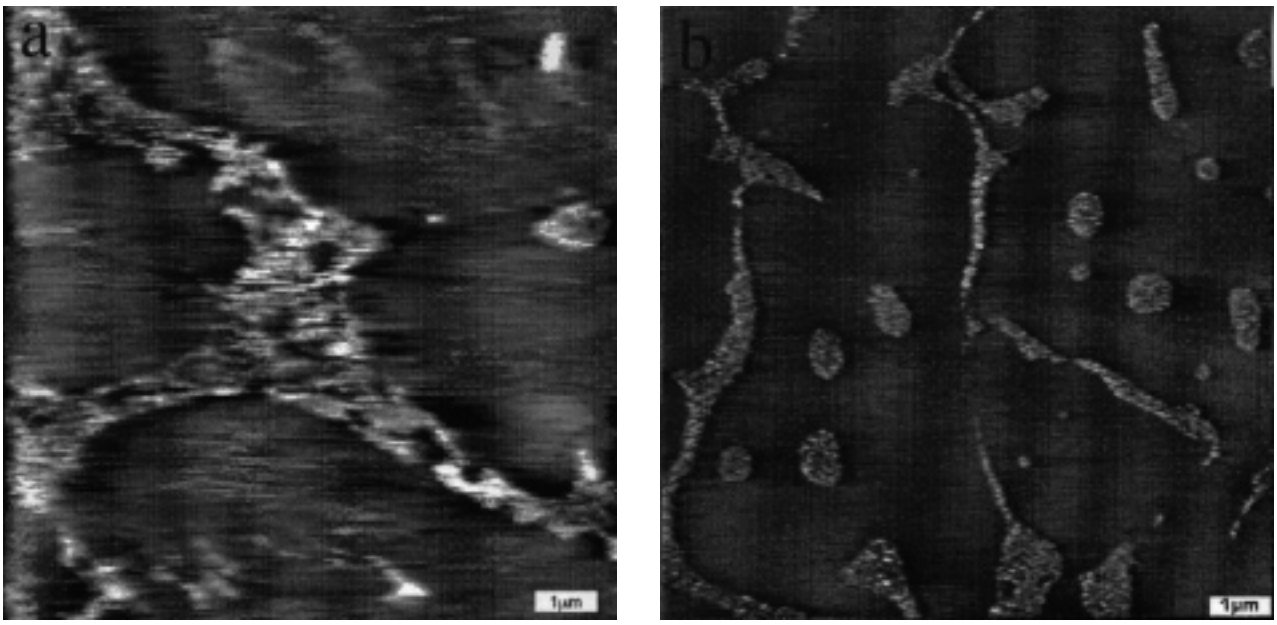

Fig. 6. – Lipid extract surfactant films containing; a) 1% surfactant protein (SP)-B and SP-C; and b) 7.3% SP-B and SP-C at a surface pressure of 40 $mN \cdot m^{-1}$. The web-like protrusions are courser and more fragmented at low than at high protein concentrations.

which have been enriched with SP-C. It could be argued that the difference is due to the different processing of the surfactant material, and it is conceivable that the proteins are more homogeneously distributed in Curosurf® than in material first concentrated and then diluted. As an alternative, the differences in the phospholipid profiles between these surfactants which certainly have a forming effect (compare figs. 3 and 4) must be considered. However, the hypothesis that the web-like pattern of the bovine lipid extract surfactants reflects an effect of SP-B and SP-C appears to be supported not only by previous experiments [14, 15], but also by the different structural patterns of pure DPPC films and the film shown in figure 6b whose DPPC fraction of phospholipids is ~90%. Since both Curosurf® and bovine lipid extract surfactant films exhibit quite similar surface activities, the structural difference points to a further problem in the endeavour to define the structure-function relation of surfactant films.

Obviously, the present experiments have not been conclusive with regard to the three-dimensional arrangement of film molecules. Steps in height on the films consistent with bilayer, or multilayer formation, respectively, could not be observed. The lack of evidence does not exclude the existence of multilayers under particular conditions. It has to be pointed out that the authors could not transfer films on mica which were compressed to such high surface pressures as they prevail in lungs (*i.e.* equivalent to surface tensions of close to zero). The maximum surface pressures achieved in the present study were approximately 40 $mN \cdot m^{-1}$, *i.e.*, considerably below the expected plateau level of compression. Quite recently, the group of AMREIN and coworkers [15] was successful in imaging films of SP-C containing surfactants compressed at the plateau level of ~50 $mN \cdot m^{-1}$, and they could demonstrate by SFM film, protrusions of 28 and 77 nm in height, which are compatible with the formation of multilayers, and this in agreement with findings previously obtained by electron microscopy [10].

In conclusion, images of surfactant films obtained by scanning force microscopy give further evidence that the traditional and widely accepted hypothesis of a homogeneous monolayer governing the surface tension probably does not hold true: the structure-function relationship of the surface layer appears to be much more complex. The addition of the surfactant proteins-B and -C to surfactant phosholipids also appears to contribute to the complexity of surface films in that web-like protrusions are seen in the scanning force microscopy images. These protrusions are well distinguishable from the large polygonal liquid condensed-domains characteristic of pure phospholipid films. Probably due to insufficient film compressions, however, the authors were not able to demonstrate protrusions with consistent heights with the formation of film bilayers, or multilayers, respectively. Future experiments have to be directed toward a better definition of this relationship. A better knowledge with regard to film architecture is probably relevant for the understanding of its surface activity and resistance to mechanical disturbances, and also of the inhibitory action of inflammatory products and even blood proteins [20–23].

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