

Structures of pulmonary surfactant films adsorbed to an air–liquid interface in vitro

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Abstract

Phospholipid films can be preserved in vitro when adsorbed to a solidifiable hypophase. Suspensions of natural surfactant, lipid extract surfactants, and artificial surfactants were added to a sodium alginate solution and filled into a captive bubble surfactometer (CBS). Surfactant film was formed by adsorption to the bubble of the CBS for functional tests. There were no discernible differences in adsorption, film compressibility or minimal surface tension on quasi-static or dynamic compression for films formed in the presence or absence of alginate in the subphase of the bubble. The hypophase-film complex was solidified by adding calcium ions to the suspension with the alginate. The preparations were stained with osmium tetroxide and uranyl acetate for transmission electron microscopy. The most noteworthy findings are: (1) Surfactants do adsorb to the surface of the bubble and form osmiophilic lining layers. Pure DPPC films could not be visualized. (2) A distinct structure of a particular surfactant film depends on the composition and the concentration of surfactant in the bulk phase, and on whether or not the films are compressed after their formation. The films appear heterogeneous, and frequent vesicular and multi-lamellar film segments are seen associated with the interfacial films. These features are seen already upon film formation by adsorption, but multi-lamellar segments are more frequent after film compression. (3) The rate of film formation, its compressibility, and the minimum surface tension achieved on film compression appear to be related to the film structure formed on adsorption, which in turn is related to the concentration of the surfactant suspension from which the film is formed. The osmiophilic surface associated surfactant material seen is likely important for the surface properties and the mechanical stability of the surfactant film at the air–fluid interface.

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1. Introduction

The structure–function relationship of surfactant films covering the alveolar air–liquid interface is still ill defined. Weibel and Gil [1,2] were the first to convincingly demonstrate an osmiophilic lining layer in alveoli by transmission electron microscopy. However, the film was fragmented and disposed in patches, and in contradiction to the physiological hypothesis of a continuous monolayer, the morphology of the surface film was rather inhomogeneous in that lamellar structures with stacks

of three to six osmiophilic layers were observed. By using freezing techniques, the postulated continuity of the alveolar lining could be shown [3,4], but without revealing any details of its fine structure. Observations made with fluorescence light [5,6], and atomic force microscopy [6–8] showed that the surface texture of adsorbed films is not homogeneous, but that there are large differences depending on the composition of the material in the suspension (i.e., before adsorption), and the degree of film compression.

The objective of this study was to better characterize the structure–function relation of surfactant films in vitro by electron microscopy, and this under well-controlled conditions. To this aim, a major obstacle had to be overcome, since it has been shown that surfactant films cannot be fixed by the usual fixatives, i.e., glutaraldehyde (GA), osmium tetroxide (OsO₄)

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and uranyl acetate (UA); and if adsorbed to an aqueous hypophase, cannot be preserved for further processing by electron microscopy [2]. Some observations made in fixed lungs suggested a way to overcome this difficulty. Indeed, in normal lungs surfactant films can best be seen at sites with an appreciable, proteinaceous hypophase, i.e., in alveolar corners, and the most convincing images of coherent films can be visualized in lungs with discrete, proteinaceous alveolar edema whose albumin concentration was high enough to be fixed and solidified by GA [9,10]. This finding suggested that the preservation of films may be feasible by an “en bloc” solidification of film and hypophase with which the film is closely associated by its interaction through the polar heads of the phospholipids.

A solidifiable hypophase had to be found on which surfactant films can be spread or adsorbed, and which does not interfere with the function of the surfactant. Numerous attempts have been made in this direction. Suspensions of surfactant in a sodium alginate solution, which can be solidified by adding calcium ions [11] appeared to be most promising. Within a captive bubble surfactometer [12,13] a surfactant film can be adsorbed to the surface of an air bubble, the surface activity of the film tested, and then the alginate–film complex solidified by adding calcium ions, fixed and stained with GA, OsO₄, and UA for further processing for electron microscopy.

By means of this technique, we have addressed several issues pertaining to the structure–function relationship of surfactant films, as differences in structure were found in suspensions of phospholipid mixtures and lipid–protein recombinants contained in natural surfactant [14,15]. In addition, clear evidence was presented that the molecular organisation of surfactant material was modified by surfactant associated proteins [16,17].

Thus, we have attempted to resolve the following questions: (1) Are the differences in composition of different exogenous lung surfactants reflected in differing structures of adsorbed films? (2) Is the film structure dependent on the concentration of the surfactant in the hypophase, as higher surfactant concentrations in the hypophase ensure better surface activity of the film with respect to rate of adsorption, compressibility, and stability [10,13]? Another question (3) pertains to the alteration of the structure of the film caused by its compression and decompression. Can mechanisms such as the selective removal of non-DPPC lipids (“squeezing-out”), of surfactant material on compression [18–20] be visualized by electron microscopy? (4) Can islands of solid phase coexisting with continuous multilayer “reservoirs” of fluid phase adjacent to the air–water interface seen by atomic force microscopy (AFM) [21], be visualized by electron microscopy? There is evidence that fully saturated phospholipids such as DPPC cannot be stained with OsO₄ and UA (this issue is still contested, though [22–24]). The problem is fundamental for the interpretation of the findings. In order to obtain more insight into the formation of films, additional experiments were carried out with synthetic surfactants, without any proteins added, one consisting of a mixture of DPPC and saturated phospholipids (DPPC/egg PG), the other consisting of a mixture of DPPC, hexadecanol, and tyloxapol (Exosurf®), but without unsaturated phospholipids.

2. Methods

2.1. Instrument

The captive bubble surfactometer served as the basic instrument for the study of the structure–function relationship of surfactant films. This device has been described in detail elsewhere [12,13]. It allows a reliable and reproducible estimation of the surface tension of surfactant films, and, by procedures described below, a solidification of the hypophase–surface lining complex for fixation and further processing for electron microscopy.

2.2. Hypophase

As a solidifiable hypophase a 1.2% sodium alginate solution was chosen (Fluka; alginic acid sodium salt, solved in 0.9 NaCl+2.5 mM CaCl₂). Sodium alginate is a natural biopolymer produced from marine algae. Chemically it is a linear copolymer of beta-D-mannuronate and alpha-L-guluronate arranged with 1, 4 linkage [25]. The solution was stirred for 2 h at 37 °C. Preliminary experiments have shown that (1) this solution is transparent enough to accurately measure size and configuration of the captive bubble to determine surface tensions; (2) surfactants can easily be brought into a homogeneous suspension within the alginate solution; (3) this hypophase does not interfere with the surface activity of surfactants: equilibrium tensions after film adsorption, minimum surface tension upon film compression, and film area compression required to achieve near zero minimum surface tension were equal to results obtained with usual salt solutions as hypophase (see results below).

2.3. Surfactants

The following surfactants were used for the electron microscopy: (1) Pure DPPC (Fluka), at a concentration of 1 mg/ml in ethanol; (2) a mixture of DPPC and phosphatidylglycerol (PG; extracted from egg yolk lecithin; Fluka) in a ratio of 7:3 by mass, and at a total phospholipid concentration of 1 mg/ml; (3) Exosurf® (Wellcome), consisting of 85% DPPC, 9% hexadecanol, and 6% tyloxapol, also at a concentration of 1 mg/ml; (4) Curosurf® (Chiesi Farmaceutici, Parma, Italy), produced from minced pig lungs, containing 99% polar phospholipids (about 40% DPPC) and 1% SP-B and SP-C [26]. (5) bovine lipid extract surfactant (BLES) obtained by organic extraction of bovine lung material. It contains all of the phospholipids of natural surfactant (99%), about 40% DPPC, and about 1% of SP-B and SP-C (BLES Biochemicals Ltd., London, Ontario, Canada); and (6) natural bovine surfactant obtained from alveolar lavage material of bovine lungs (a gift from Dr. F. Possmayer, Departments of Biochemistry and Obstetrics and Gynaecology, University of Western Ontario, Canada). The preparation contains about 70% phospholipids, and 10% proteins (i.e., about 8% SP-A, 1% SP-D, and 1–1.5% SP-B and SP-C) [27]. In order to obtain homogeneous surfactant suspensions appropriate amounts of surfactants were added to the alginate solution and stirred at 37 °C; for each experiments fresh mixtures were prepared. BLES preparations in the alginate solutions were 1 mg/ml and 5 mg/ml of total phospholipids (PL). The preparations with Curosurf were 0.3, and 1.0 mg/ml of PL. The concentrations of natural surfactant, DPPC/eggPG, egg PG, and Exosurf were 1 mg/ml of PL. Since pure DPPC is difficult to suspend in an aqueous solution, a modification was required to create DPPC films. DPPC was solved in ethanol (2 mg/ml), and with a fine needle a droplet of the solution was deposited onto the surface of the air bubble for the formation of the film by spreading to produce an equilibrium surface tension of approximately 25 mN/m.

2.4. Procedure

For the combined structure–function studies of the surfactant lining layer at the air–liquid interface in the presence of alginate in the subphase of the captive bubble, a recently described modification of the surfactometer technique was necessary [28]. Instead of directly filling the chamber of the surfactometer with the surfactant suspension, the alginate–surfactant mixture together with a small air bubble were introduced into a short piece of dialysis tubing consisting of a molecular porous membrane. The tubing (Spectra/Por® 4MWCO: 14.00; the Spectrum Companies, Gardena, CA) was tied up at both

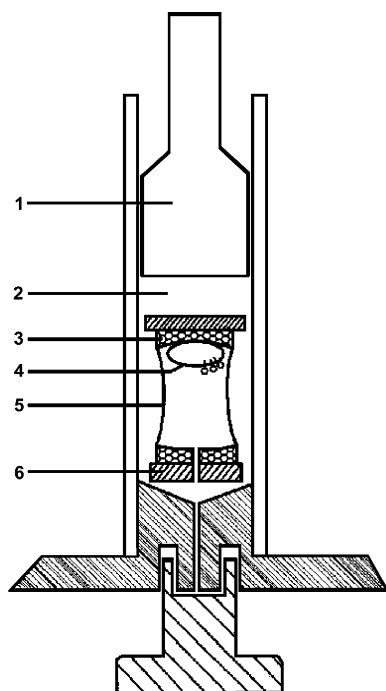


Fig. 1. Modification of the captive bubble surfactometer to solidify the bubble-hypophase complex for further processing: (1) pressure tight piston; (2) chamber filled with 50 mM CaCl_2 ; (3) 1% agarose gel; (4) air bubble with adsorbed surfactant film inside alginate-surfactant mixture (see text); (5) dialysis tubing; (6) support for dialysis tubing and captive bubble set-up.

ends. It is fully transparent, and allows perfect visualization of the bubble size and shape, and hence the measurement of surface tension. The inner top end of the tubing was fitted with a cap of a 1% agarose gel to ensure a smooth and fully hydrophilic contact with the bubble. The filled piece was introduced vertically into the chamber; its volume was about half of that of the chamber. The remaining chamber volume was filled with a solution of 50 mM CaCl_2 (Fig. 1). Since the diffusion of calcium ions through the tubing wall and the resulting solidification of the alginate are slow processes, there was enough time for unrestrained film adsorption (5 min), and film compression (three quasi-static compression-decompression cycles after adsorption, followed by 20 dynamic cycles at 20 cycles per min). Criteria for a normal surface activity and hence for further processing were equilibrium tensions of about 30 mN/m, and minimum surface tensions of less than 5 mN/m upon quasi-static and dynamic cycling. Since the solidification of the alginate-bubble complex had to occur at ambient pressure, creation of bubbles with compressed films required a particular procedure. After filling the chamber and the introduction of a small bubble of 2–3 mm in diameter, the size of the bubble was increased by lowering the pressure inside the chamber. After film formation by adsorption, the negative pressure was slowly released resulting in a decrease of volume and the surface of the bubble, and thus in a compression of the film. Following these functional adjustments of the films, 2 h were allowed for the solidification of the alginate. During the entire procedure, i.e., from preparation of the suspension, the filling of the surfactometer until the solidification of the alginate, the temperature was kept constant at 37 °C.

At first, large series of experiments were done with functionally and biochemically well-defined surfactants (1 mg/ml of PL), added as suspensions to the alginate solution.

2.5. Processing and staining/fixation

With a razor blade a small piece of the solidified alginate cylinder which contained the air bubble was cut away for sequential fixation/staining with glutaraldehyde (GA; 2.5% in 0.1M sodium cacodylate), osmium tetroxide (OsO_4 ; 2%, also in sodium cacodylate), and uranyl acetate (UA; 0.5% in maleate buffer). The sections were not stained. To all of these solutions 7 mM CaCl_2 had to be added to prevent the liquefaction of the alginate. These

fixatives were chosen to compare the findings with those obtained previously in vitro [14,15] and in situ experiments [1,2]. After staining/fixation, the blocks containing the bubbles were cut into two pieces, dehydrated in alcohol, and embedded in Epon. Thin sections for transmission electron microscopy (Philips 400 EM) were done such that each section showed a part of the free bubble surface. The goniometer was not used as it did not improve the imaging.

2.6. Surface activity tests in the CBS

The surface activity of the substances was determined by using a laboratory-built fully computer controlled CBS evolved from the apparatus described earlier [13]. The chamber was filled with a solution of 140 mM NaCl, 10 mM HEPES, and 2.5 mM CaCl_2 , and either with 1.5%, by mass, of sodium alginate or without it for the controls, pH 6.9. The temperature was maintained between 37.0° and 37.5 °C. An air bubble of approximately 7 mm in diameter was introduced into the chamber, and the surface tension over time was monitored for 5 min. After the adsorption period of 5 min, quasi-static cycling commenced. In the quasi-static portion of the experiment, the bubble size was first reduced and then enlarged in a stepwise fashion by altering the internal volume of the chamber. Each step had two components: a 3-s change in volume followed by a 4-s delay where the chamber volume remained unchanged and the film was allowed to “relax”. Compression was stopped when minimum surface tension was obtained, as seen when the bubble height no longer decreases upon volume reduction, but only the diameter: there was an intercycle delay between each of the four quasi-static cycles and further a 1-min delay between the quasi-static and 20 dynamic cycles, which followed the quasi-static cycles. In the dynamic cycles, the bubble size was smoothly varied over the same range as the quasi-static cycles for 20 cycles at a rate of 20 cycles/min. Bubble volume, interfacial area, and surface tension were calculated using height and diameter of the bubble as described by Schoel et al. [29]. In addition to adsorption and minimum surface tension, the film area compressions required to achieve the minimum surface tension from the equilibrium tension, was calculated, to assess film compressibility [13,30].

The surfactants were tested at the concentrations of total phospholipids as stated above. Four independent experiments were performed for each surfactant and each concentration. The surface tensions measured during adsorption, minimum surface tension and film area compression required to obtain near zero minimum surface tension, between a particular surfactant without alginate (control) and the corresponding sample with alginate were analyzed by using the paired two-tailed *t*-test.

3. Results

3.1. Control experiments

Fig. 2 shows the images from air bubbles within surfactant free, solidified alginate. Obviously, some reticular material can be

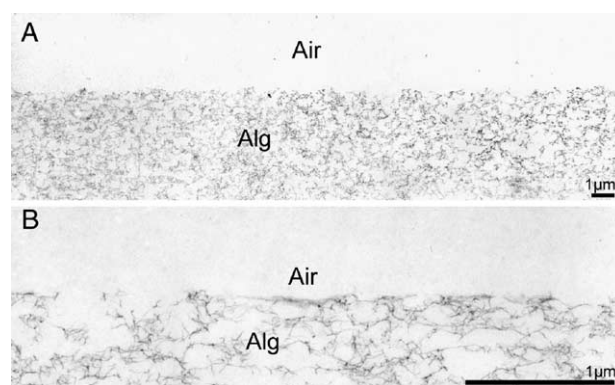


Fig. 2. Control experiments. (A) Low magnification of an air-alginate interface of a captive bubble without surfactant. The interface is formed by loose reticular material of alginate. (B) At high magnifications, layer-like segments may appear which are formed by realigned reticular material of alginate.

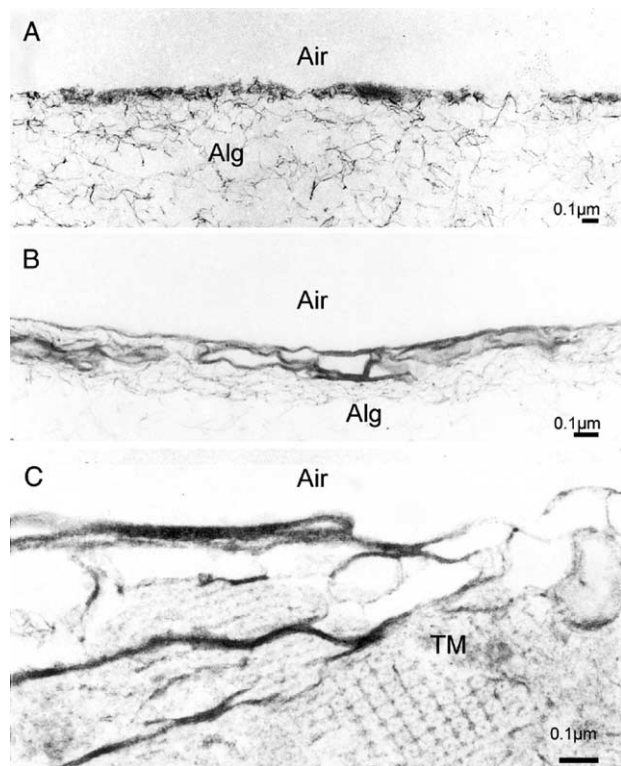


Fig. 3. Surface lining layer of natural surfactant (phospholipids 1 mg/ml) containing SP-A, SP-B, and SP-C. (A) After surfactant adsorption (surface tension about 25 mN/m) a faint and fuzzy osmiophilic band is formed at most sites of the surface. (B) On film compression (surface tension less than 5 mN/m), a distinct film appears, and also the surface-associated structures are better contrasted. (C) On film compression. At sites with large associated structures beneath the surface the surface lining layer is usually multi-laminated (TM=tubular myelin).

stained by the fixatives (OsO_4 , uranyl acetate), allowing an easy recognition of the air–liquid interface. As expected, an osmiophilic lining layer could not be detected. At high magnifications (Fig. 2B) some linear osmiophilic specks are visible at the interface. A closer look reveals that these structures consist of reticular material of alginate aligned to the surface of the bubble.

3.2. Native and lipid extracted surfactants from bovine and porcine lungs

Experiments with the captive bubble surfactometer indicate that the suspended material is adsorbed to the air–liquid interface, in that equilibrium surface tensions of less than 30 mN/m, and minimum surface tensions close to zero upon quasi-static or dynamic compressions are obtained. In addition, the films of these natural materials reveal a low compressibility. The micrographs appear to verify the hypothesis that the suspended material can be adsorbed to the interface to form continuous surface lining layers as observed in lungs fixed by vascular perfusion. However, the layers appear rather inhomogeneous within the same bubble (i.e., within the same experiment), and there are differences in film structure depending on the type of surfactant, on the concentrations of the surfactants in the bulk suspensions, and on whether the films were adsorbed only or compressed afterwards.

3.2.1. Native surfactant from bovine lungs

At a concentration of 1 mg/ml of phospholipids in the bulk suspension, natural surfactant containing SP-A, SP-D, SP-B, and SP-C forms on adsorption at most sites a rather fuzzy osmiophilic band of low contrast, which appears to be interfused with reticular material of alginate (Fig. 3A). At some sites the surface associated surfactant material (reservoirs) in form of vesicular structures or multilayers are observed adjacent to the surface film. More distinct pieces of film are seen above the relatively large structures of the reservoirs. On compression, however, clear-cut lining layers became visible (Fig. 3B). At locations with considerable reservoirs, occasionally associated with tubular myelin, bi-, and multilayers are predominant (Fig. 3C).

3.2.2. Lipid extract surfactant from bovine lungs (BLES)

Similar patterns of adsorbed films are formed by BLES suspensions. Again, at a concentration of 1 mg/ml, the adsorbed films are mostly fuzzy, and of low contrast (Fig. 4A). At some places, a continuous film is missing. Instead, the interface is lined by strings of surface associated material in form of small osmiophilic vesicles (Fig. 4B). Compressed films are well contrasted. At a higher bulk concentrations of 5 mg/ml of phospholipids, copious surface associated surfactant reservoirs and distinct, well-contrasted osmiophilic films became visible (Fig. 5A). Films adjacent to large multi-vesicular reservoirs showed multilayers of various thickness and contrast (Fig. 5B).

3.2.3. Curosurf®

Curosurf is a lipid extract surfactant of minced porcine lungs: We carried out a very large number of experiments with Curosurf, a surfactant widely used for the treatment of the respiratory distress syndrome of neonates. Suspension of low

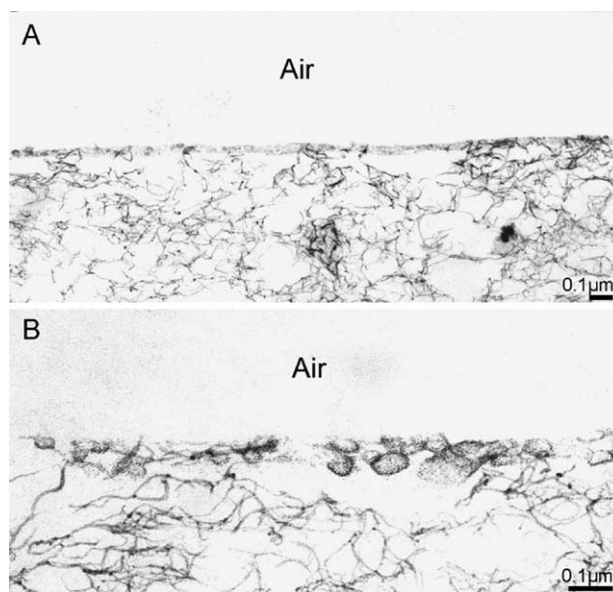


Fig. 4. Surface lining layers of BLES (1 mg/ml) after adsorption. (A) As with natural surfactant, a faint continuous band is formed. (B) At some sites, a continuous film is missing. Instead, the interface is lined by strings of small osmiophilic vesicles.

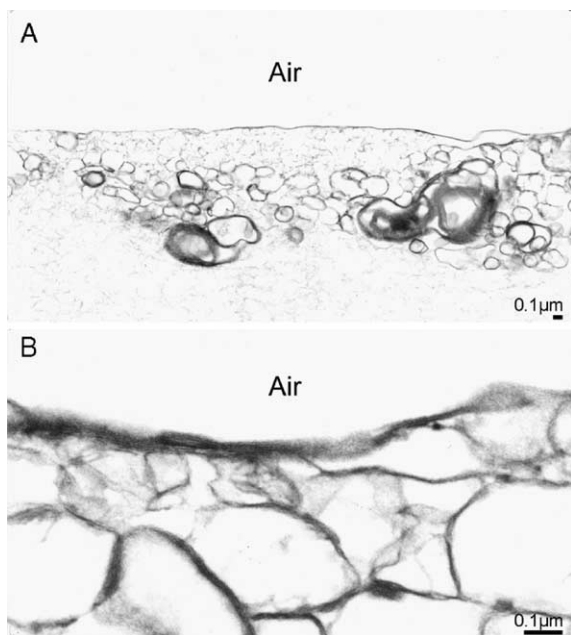


Fig. 5. (A) After adsorption. BLES at higher concentrations in the bulk phase (5 mg/ml) forms distinct osmiophilic films with copious surface associated structures (reservoir material). (B) On compression, multi-lamellar segments covered the surface close to reservoirs.

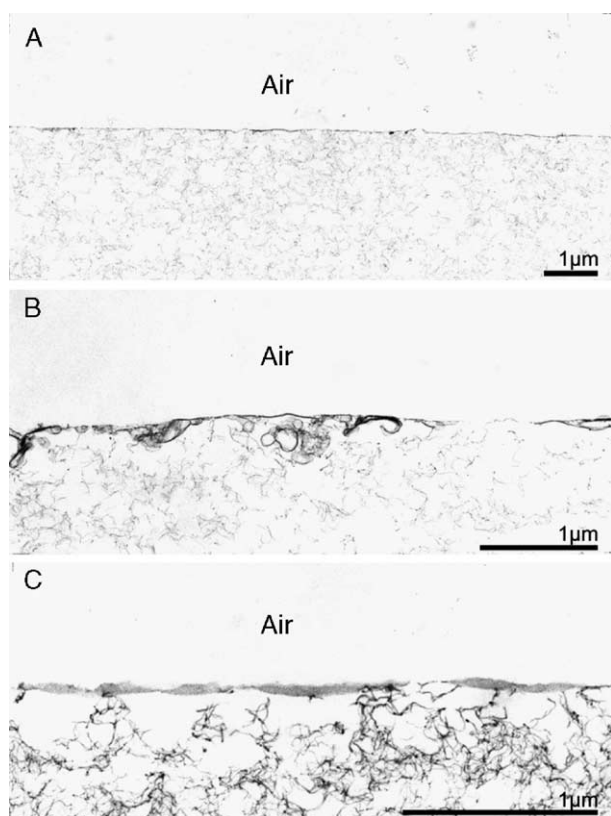


Fig. 6. After adsorption. Appearances of Curosurf films at low concentrations of phospholipids of 0.3 mg/ml. (A) At some sites extremely thin but well-contrasted films were visible. (B) At other sites the film was accompanied by small vesicular structures, which appear to become inserted into the surface lining layer. (C) Rarely, band formations could be observed, possibly the result of oblique sections through the film.

phospholipid concentrations (0.3 mg/ml) produce films with surface tensions of approximately 30 mN/m on adsorption for 5 min, and minimum surface tensions after four consecutive quasi-static compressions of approximately 10 mN/m. The cycles show a relatively large plateau at 20–23 mN/m, indicating a region of high compressibility in that range (Fig. 13 top). The micrographs show a thin osmiophilic film (Fig. 6A) with relatively few surface associated vesicular structures (Fig. 6B). However, upon dynamic cycling at 20 cycles per min (cpm), near zero (~ 2 mN/m) minimum surface tensions are achieved with area compressions of $\sim 28\%$ from 25 mN/m (Fig. 13 bottom). At a higher concentration of 1.0 mg/ml of PL, the surface tension on adsorption after 5 min is ~ 24 mN/m, indicating equilibrium. Minimum surface tensions of ~ 1 mN/m are obtained already on the first quasi-static compression, and the area compression required to reach that minimum surface tension was $\sim 15\%$ (Fig. 12 top), indicating a very low film compressibility [30]. The micrographs of Curosurf films formed from suspensions at 1.0 mg/ml show substantially more surface associated vesicular structures and multilayers (Fig. 7A, B, C) compared to those from the lower concentrations (Fig. 6). Curosurf exhibits comparable functional features as natural surfactant and BLES. However, with regard to the structural

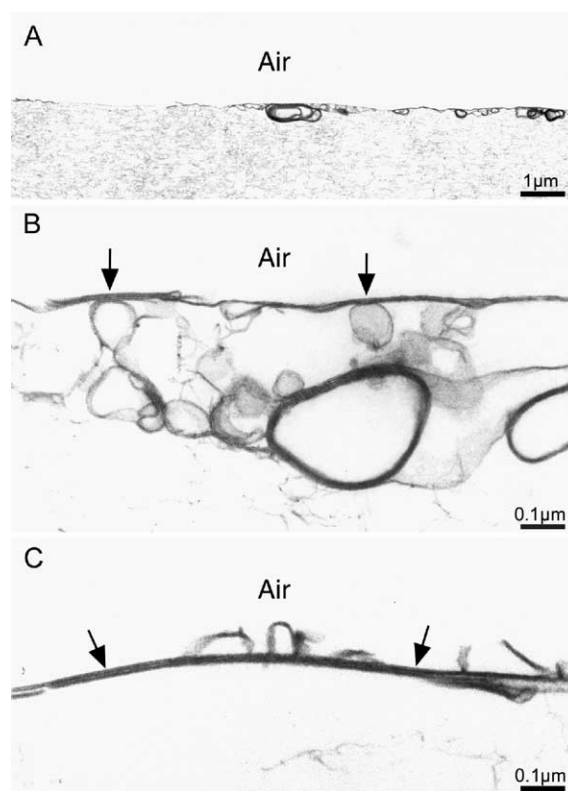


Fig. 7. After adsorption. Curosurf films at higher concentrations of phospholipids in the bulk phase (1 mg/ml). (A) The thin films are accompanied by numerous surface associated structures. (B) Above these structures, conspicuous multi-lamellar segments are formed at the surface. (C) Occasionally splintered multilayers probably reflect cutting artefacts, which suggest a low cohesion between the layers. (B) Reprinted from *Biochimica et Biophysica Acta*, Vol. 1408: 180–202, 1998. S. Schürch, F.H.Y. Green, H. Bachofen, "Formation and structure of surface films: captive bubble surfactometry". With permission from Elsevier.

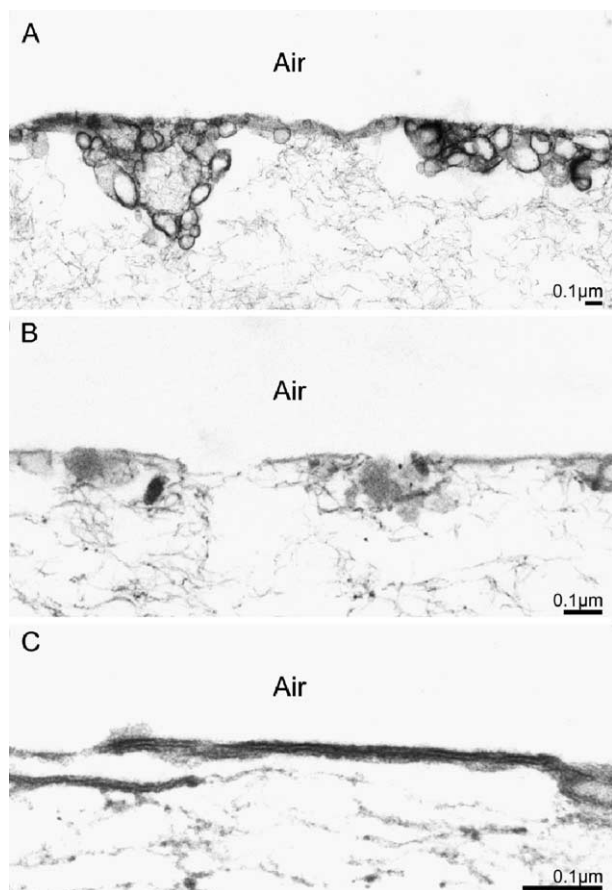


Fig. 8. Heterogeneous appearance of compressed Curosurf films. (A) Accumulations of clusters of microvesicles, and (B) of fuzzy osmophilic scraps beneath the surface lining layer. (C) Occasionally, multilayers are pressed against each other, creating structures which resemble pack-ice.

appearance, Curosurf forms the best surface films for the observation by electron microscopy. Even suspensions with minimal concentrations of 0.3 mg/ml produce continuous and distinct films, albeit the morphology of the films is not uniform. Without an association to multilayer structures (reservoirs), wide surface segments may be covered with a very thin film of high contrast (Fig. 6A). At other sites, there are irregularities due to the surface associated reservoir material, the vesicles of which appear to unfold and to integrate into the lining layer (Fig. 6B). Occasionally, faint and fuzzy bands can be observed (Fig. 6C), similar to those created by natural surfactant or BLES.

Thus, at higher phospholipid concentrations of the suspensions, 1 and 5 mg/ml, for Curosurf and BLES, respectively, the morphology is dominated by the accumulations of surface associated material or reservoirs (Figs. 5A, B and 7A, B), and the formation of conspicuous multilayers on the adjacent surfaces (Fig. 7B). Occasionally, the multilayers appear splintered: structures such as those shown in Fig. 7C are probably cutting artefacts which indicate the presence of a true multilayer system. On compression, Curosurf films show a different structural pattern than films formed by natural surfactant or BLES. The compressed lining layer assumes a rather heterogeneous appearance, and this in particular by a conspicuous accumulation of osmophilic material immediately beneath the surface lining layer. At some

sites, this material consists of clusters of microvesicles (Fig. 8A), at other sites of floccular, fuzzy scraps (Fig. 8B), and occasionally of distinct layers and multilayers pressed together and thus resembling pack-ice (Fig. 8C).

3.3. Synthetic surfactants, DPPC, DPPC/eggPG and Exosurf

3.3.1. DPPC

DPPC is unquestionably the most important component of lung surfactant, but it is difficult to form films from suspensions at 37 °C. We dissolved the DPPC, 2 mg/ml, in chloroform/methanol, 1:1 by volume, and formed the films by spreading the solution onto the surface of the bubble to produce an equilibrium surface tension of ~25 mN/m. Thus, with regard to DPPC our method described above, had to be modified. In the captive bubble surfactometer filled with alginate solution, these pure DPPC films showed a minimum surface tension close to zero and a low compressibility. Fig. 9A–C show, that with the staining agents used in these experiments we were not able to clearly visualize the DPPC films. After absorption to the bubble surface, the micrographs (Fig. 9A, B) were not different from those with alginate only, that is without any DPPC (Fig. 2A, B). On compression, an apparent faint and fuzzy lining layer could be observed at some sites. However, these “film fragments” are neither sharp nor clear-cut, and it cannot unequivocally be decided whether they are formed by phospholipids blackened by the staining agents, or rather, by realigned reticular material of alginate.

3.3.2. DPPC/egg PG and Exosurf®

Remarkably, these simple, but ingenious mixtures allow film formation at an air–liquid interface without the aid of

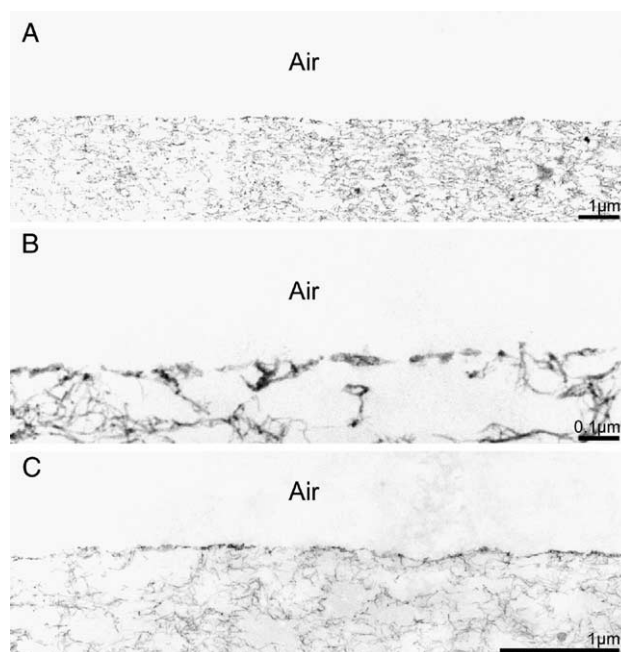


Fig. 9. Films of pure DPPC could not be visualized, neither after film formation by solvent spreading (A and B), nor on compression (C). However, there was a conspicuous realignment of reticular material of alginate at the surface.

surfactant associated proteins. Both, DPPC/egg PG (ALEC [31]) and Exosurf (the latter has been successfully used for the treatment of the respiratory distress syndrome of neonates) have been shown to exhibit excellent surface activity, although their equilibrium surface tensions on adsorption are slightly higher, and their film compressibility considerably higher than those of surfactants containing proteins [26,27]. DPPC/egg PG: at a ratio of 7 to 3 by mass, and at a total phospholipid concentration of 1mg/ml, formed distinct osmiophilic lining layers at the interface (Fig. 10C), which occasionally were associated with linear material and uni- and bilamellar vesicles beneath the surface. In view of the invisibility of the highly surface active DPPC films, this remarkable finding prompted us to test the structure–function relation of egg PG alone. Egg PG suspensions (concentration 1mg/ml) reduced the equilibrium tension of the bubble surface to about 35 mN/m. However, by compression of the surface area, the surface tension could not be reduced to values below approximately 25 mN/m. Thus, PG alone does not exhibit the surface activity of normal pulmonary surfactant. Regardless of this functional deficit, PG forms distinctive osmiophilic lining layers (Fig. 10A). At sites of reservoirs material, which may assume a vesicular pattern, film segments are rather thick indicating multi-lamellar structures (Fig. 10B). At large, pure PG films with limited surface activity appear to exhibit more osmiophilic material at

the air–liquid interface (Fig. 10A, B) than the highly surface active DPPC/egg PG films (Fig. 10C).

3.3.3. Exosurf

Although Exosurf does not contain unsaturated phospholipids, an osmiophilic film can be observed at the interface, which appears to be more fragmentary than that formed by surfactants containing unsaturated phospholipids. However at some sites fine and distinct, though frequently fringed lining layers are present (Fig. 11A). At high magnifications, interspersed multi-lamellar structures can occasionally be seen (Fig. 11B). In other regions, the layers consist of faint band formations, or are not visible. Surface associated structures (reservoirs) are rather rare; they consist of diffuse osmiophilic patches, and occasionally of crystalline structures (Fig. 11C), as described by Bernhard et al. [32]. Between adsorbed and compressed films distinctive differences in film structure cannot be detected. In contrast to egg PG, the carrier substances of DPPC in Exosurf, tyloxapol and hexadecanol, which can be easily dissolved in aqueous liquids, do not form osmiophilic films at the interface.

3.4. Surface activity tests

3.4.1. Adsorption

There were no discernible differences in surface tension between the control samples and those with 1.2% sodium alginate ($P>0.1$), except for Exosurf, at all time intervals during the entire adsorption period of 5 min, determined at 5, 10, 30 s and 1 and 5 min. For Exosurf at and after 30 s the surface tension values due to adsorption were lower in the presence of alginate than for the controls. The final values after 5 min were: (25.7 ± 2.3) mN/m and (33.4 ± 2.0) mN/m, mean \pm S.D., $n=4$, $P<0.01$, for the samples with alginate vs. the controls, respectively.

3.4.2. Minimum surface tension and film area compressions

There were no discernible differences between the control samples and those with 1.2% sodium alginate on quasi-static and dynamic cycling ($P>0.1$). All of the minimum surface tensions were 5 mN/m or below that.

3.4.3. Film area compression

There were no discernible differences in the film area compressions required to reach the minimum surface tensions from 25 mN/m between the control samples and those with 1.2% sodium alginate ($P>0.1$).

Fig. 12 top, shows the quasi-static cycle nos. 1, 2 and 4 of a typical experiment of Curosurf at a concentration of 1mg/ml of total phospholipids (PL), in the presence of alginate, performed after film formation by adsorption in the captive surfactometer (CBS). Minimum surface tensions of 1 mN/m are obtained upon a film area compression of approximately 16% from 25 mN/m. Fig. 12, bottom, shows a series of three dynamic cycles at 20 cpm, centred on cycle number 10. Minimum surface tension was less than 1mN/m upon a film area compression of 14% from 25 mN/m.

At the lower concentration of 0.3 mg/ml of PL, in the presence of alginate, (Fig. 13 top), the quasi-static cycles 1,2,

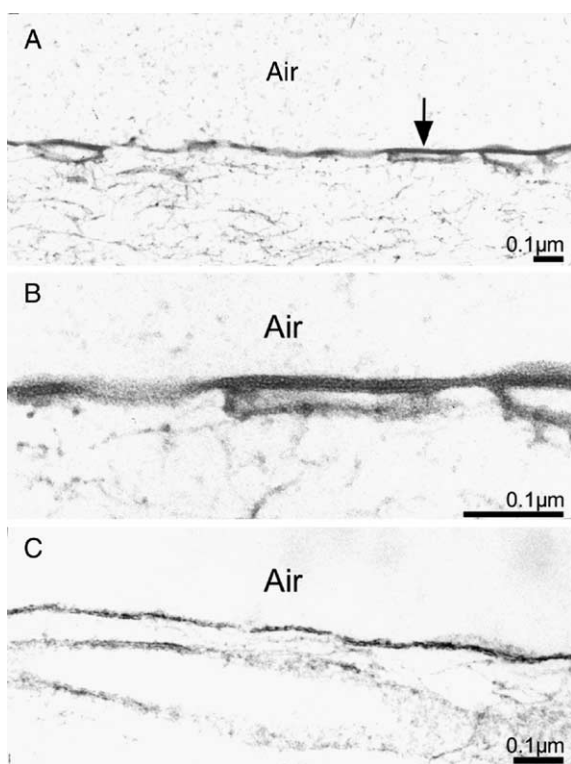


Fig. 10. After adsorption. Lining layers of egg PG and DPPC/egg PG. (A) Although low minimum surface tensions cannot be obtained on film compression, egg PG forms well-contrasted films. (B) At higher magnifications (region indicated by arrow in A) frequently multi-lamellar film fragments can be observed. (C) DPPC/egg PG films are well contrasted, but appear to be somewhat granulated.

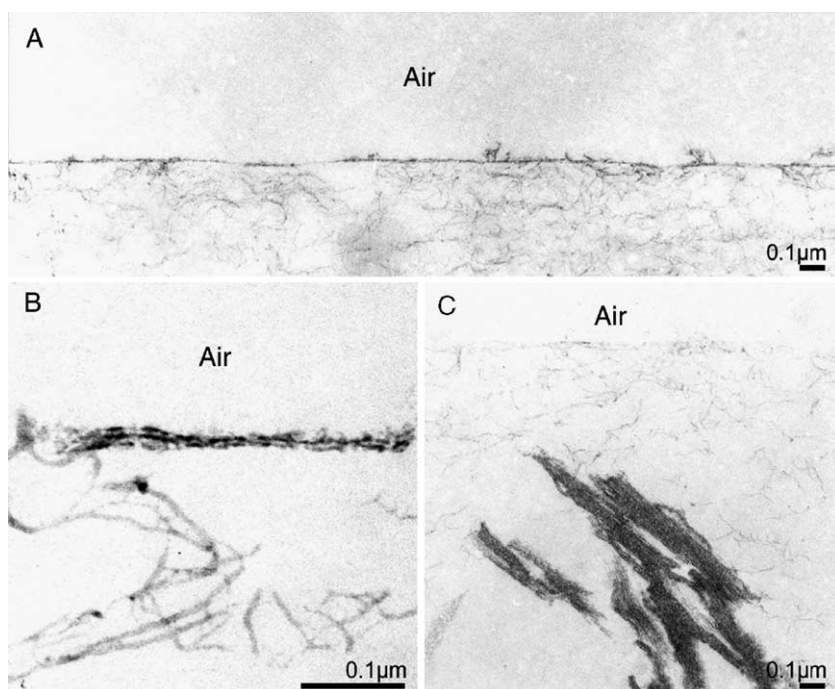


Fig. 11. After adsorption. (A) Exosurf forms at some sites well-contrasted thin but often fringed films. (B) At high magnification granulated multi-layers can be detected. (C) Rare surface associated structures (reservoirs) are either patchy, or consist of osmophilic crystalline structures.

and 4 show a large plateau at the surface tension of 20–23 mN/m, and a minimum surface tension between 10 and 14 mN/m. The film area compressions required to reach the minimum surface tension from 25 mN/m is approximately 40%.

Fig. 13 bottom shows the dynamic cycles number 9, 10, and 11, of 20 consecutive dynamic cycles of a Curosurf film formed from a suspension of 0.3/ml of PL, in the presence of alginate. The dynamic cycles were conducted after completion of the 4 quasi-static cycles shown in Fig. 13 top. In contrast to the quasi-static cycles, near zero minimum surface tensions were achieved, upon a film area reduction of about 15% from 25 mN/m.

Fig. 14 shows 4 typical dynamic curves (20cpm) of BLES, 1mg/ml of PL, centred on number 10, of 20 consecutive cycles, in the presence of alginate (top), and without alginate (bottom). Although the curves appear slightly different in shape, there were no discernible differences between the samples with and without alginate, in minimum surface tension and film area compressions required to achieve minimum surface tension from 25 mN/m.

4. Discussion

In mammalian lungs, surfactant material is synthesized in type II epithelial cells, secreted into an aqueous hypophase, and then adsorbed to the alveolar air–liquid interface. This process of film formation has been studied in model systems *in vitro* for various purposes, e.g. [21,33–35], and it has been shown that the surface properties of such films are in line with those studied *in situ* in excised lungs [36,37]. Early studies by Weibel and his co-workers have already demon-

strated multilamellar aspects of the alveolar film [1–3]. More recently, by using improved fixation techniques and electron microscopy, large sections of the pulmonary film were shown to be multilaminated with associated vesicular structures [10,38]. Evidently, similar film formations take place in the captive bubble surfactometer filled with suspensions containing alginate and surfactants. After the solidification of the hypophase, the surfactant lining layers can be preserved for electron microscopy.

Prior to a detailed discussion of the findings, a critical review of the technique and the experiments appear appropriate. The major problem is to preserve and stain surfactant films for transmission electron microscopy. There is good evidence that surfactant films, formed either by adsorption from an aqueous suspension or by spreading from a solution in solvents such as chloroform/methanol, on top of an aqueous and non-fixable hypophase, cannot be preserved [28]. For the preservation, the polar heads of the phospholipids of the film must be closely associated with a solidifiable hypophase. Already, Gil and Weibel [1] observed that films in lungs fixed with a perfusate of high osmolarity (500 mosM) could be fixed more easily and properly imaged. By using such perfusates, a certain concentration of proteins is introduced into the hypophase, and thus, its fixation is facilitated. Additional evidence for this hypothesis was obtained in lungs with proteinaceous pulmonary edema, with which the most convincing images of coherent osmophilic lining layers could be visualized [39,40]. Thus, in our first attempt to image surfactant layers *in vitro*, 3% albumin solution was used as a bulk liquid for the surfactant suspensions and as hypophase for the bubble. This suspension was then fixed with glutaraldehyde. This attempt was not successful as the turbidity of the solution precluded

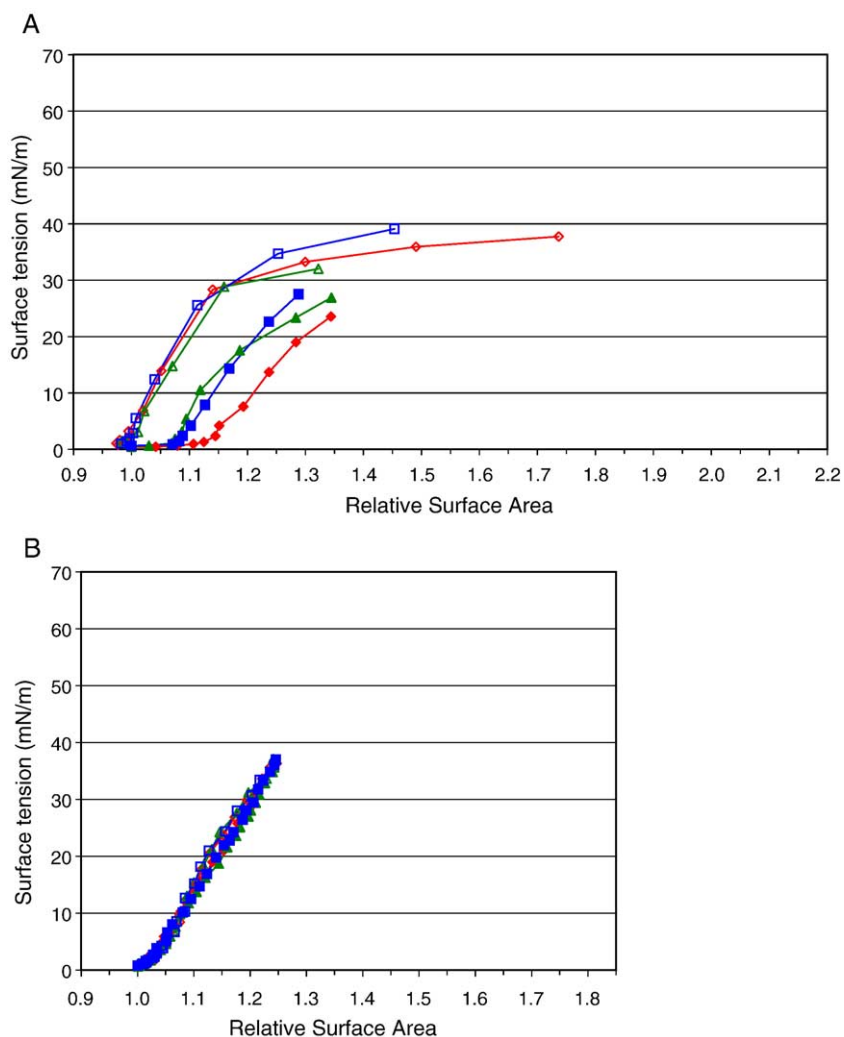


Fig. 12. Captive bubble, surface tension vs. area relations of Curosurf films, at a concentration of 1 mg/ml of phospholipids (PL), in the presence of 1.5% alginate. (A) Consecutive quasi static cycles no 1, 2 and 4. (B) 3 consecutive dynamic cycles at 20 cycles per min (cpm), centred on number 10.

reliable surface tension measurements. In addition, the fixation of the “albumin sausage” within the dialysis tubing was inhomogeneous, resulting in irregular distortions of the bubble and its surface.

The use of a sodium alginate solution, which can be solidified by the addition of calcium ions, appeared to be a useful alternative. Solidified alginate has a discrete reticular structure, such that the air–liquid interface can be detected by electron microscopy. The paramount question was whether or not the alginate solution interferes with the surface activity of the surfactants to be tested. In comparison to a pure saline solution with an interfacial surface tension of about 70 mN/m, the surface tension of the alginate solutions used in these experiments is only about 50 mN/m. However, after the addition of surfactant suspensions to the alginate, essentially the same results with regard to equilibrium tensions, minimum surface tensions, and film area compressions required to achieve near zero minimum surface tensions were obtained as with the standard carrier solution (0.9% NaCl+2.5 mM CaCl_2 +10 mM HEPES, pH 6.9)(controls). The results from these control experiments demonstrate that alginate does not

impair the surface activity of surfactants and hence it is unlikely that the structure of the surface lining layers is modified due to the presence of alginate in the surfactant suspensions. It is interesting to note that film formation with Exosurf, a surfactant that does not contain any surfactant proteins seem to be enhanced by the presence of alginate, whereas film formation of DPPC/egg PG is not affected.

A further question pertains to the characteristics of the fixatives, or the staining agents, OsO_4 , glutaraldehyde and uranyl acetate, with respect to their interference with the surfactant surface activity [28]. In view of the ongoing discussion about the “best” staining agents for phospholipids, numerous preliminary experiments were conducted. The results showed that in our hands, and for the particular experimental model, the sequential fixation and staining with OsO_4 and uranyl acetate yielded the most reproducible results with a minimum of artefacts such as structural derangements caused by patchy precipitations of staining material. Manifold queries with regard to artefacts are justified, most of which are difficult to answer. Even in the presence of a solidified hypophase, surfactant films are extremely flimsy structures. Any step in the

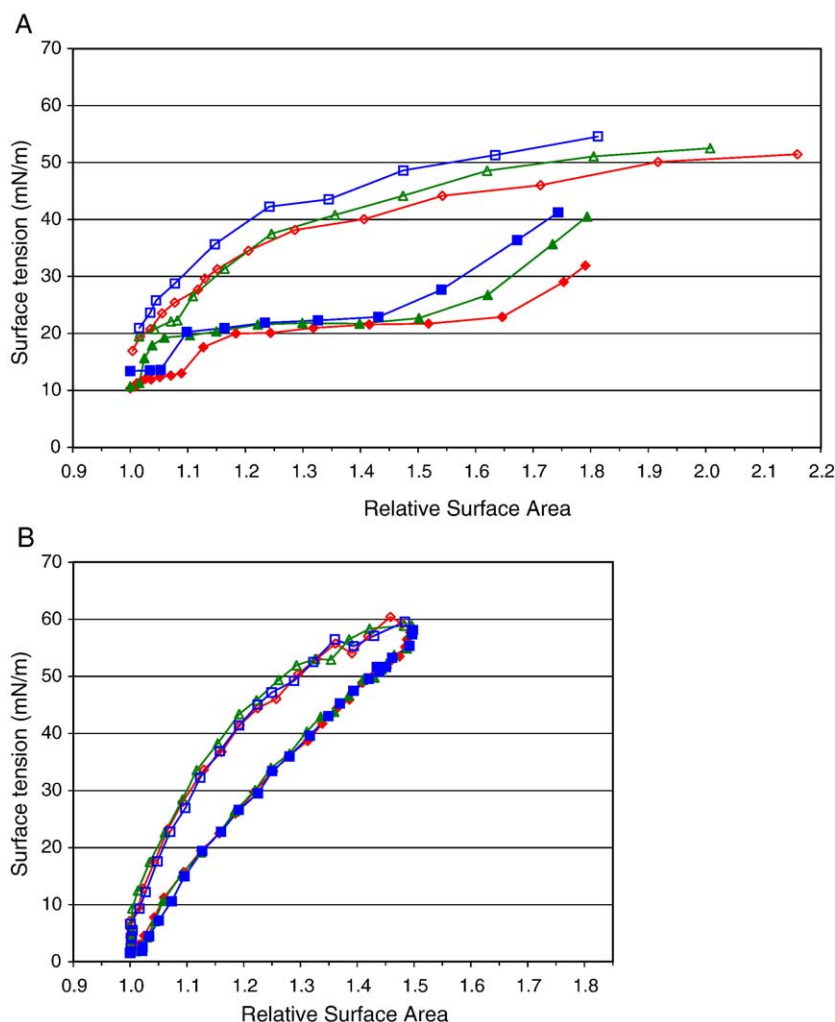


Fig. 13. Captive bubble, surface tension vs. area relations of Curosurf films, at a concentration of 0.3 mg/ml PL, in the presence of 1.5% alginate. (A) Consecutive quasi static cycles no 1, 2 and 4. (B) 3 consecutive dynamic cycles (20 cpm) centred on no 10. Note: Near zero surface tension only on dynamic cycling.

complex processing of the preparations (fixation, dehydration, embedding, cutting, etc.) can induce structural alterations. Most intriguing is the fact that in spite of normal surface tension values obtained in the presence of alginate, the films as seen by electron microscopy are frequently broken up, or are even missing at some sites. If there are associated ruptures and distortions of the underlying reticular material, the deficits can easily be explained by cutting artefacts. Alternatively, the visibility of the films decisively depends on whether the section is orthogonal or oblique to the surface of the bubble. Moreover, atomic force micrographs [6–8,21], and also fluorescence micrographs of surfactant films [5,6] revealed that the arrangement of phospholipids is not homogeneous, but partitioned into particular domains, i.e., condensed phases of protein depleted, but DPPC enriched areas, and domains which contain lipids other than DPPC and SP-C. From our results follows that domains which mostly contain fully saturated surface lipids cannot be contrasted by osmium, as will be discussed below. However, as the structural analyses in all experiments were comparable, the results would have been affected the same way by processing methods.

Structural alternations of the surfactant films during the 2 h period for the solidification of the alginate solution and during further processing cannot be completely excluded. However, the following facts speak against the generation of major artefacts in film structure by sodium alginate and during its solidification: The structures of surfactant films in lungs with proteinaceous pulmonary edema [9,10], and in lungs fixed by non-aqueous fixation techniques(38) resemble closely those of films from natural surfactant adsorbed to the bubble surfaces in alginate.

There are numerous excellent studies of the ultrastructure of different surfactants, both natural and synthetic, enclosed in pellets made from suspensions [14,15]. These studies clearly show that the molecular organisation of the surfactant lipids depend on the type of lipids, the presence of surfactant associated proteins, and environmental factors such as calcium ions. Essentially we can confirm these findings with respect to the structures of the surface associated surfactant material (the surfactant reservoirs). For example, tubular myelin could only be observed in native surfactant containing SP-A. Regardless of the structures of reservoir material, the structure of the

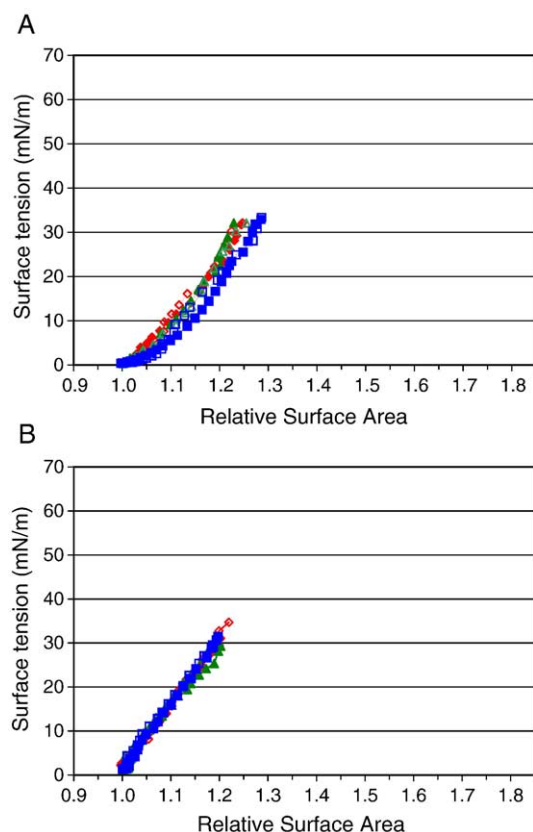


Fig. 14. Captive bubble, surface tension vs. area relations of bovine lipid extract surfactant (BLES), at 1 mg/ml of PL. Dynamic cycles at 20 cpm. (A) In the presence of 1.5% alginate. (B) Control, without alginate. Note: Near zero minimum surface tensions for both experiments and approximately the same film area compression of 14% to achieve minimum surface tension from 25 mN/m.

surface films appears to be much more uniform, although some differences are noticeable.

Natural surfactant and lipid extract surfactants exhibit about the same features regarding the surface activity investigated with the captive bubble. Notwithstanding the functional similarities, the adsorbed films of native surfactant and BLES are rather faint in comparison with the distinct films of Curosurf (Figs. 3, 4, 7). In view of the large number of experiments and microscopic sections, the probability is extremely low that oblique sections were obtained only from native surfactant and BLES preparations. More probable explanations are either the higher concentration of unsaturated phospholipids of Curosurf in relation to the amount of DPPC [26], or else a more efficient transport of phospholipid material of Curosurf to the surface [30]. The latter hypothesis is supported by the observation that film adsorption is improved by an increase of the surfactant concentration in the bulk phase (Figs. 5, 12, 13). Enigmatic is the observation of differences of the effect of film compression. On compression, films of native surfactant and BLES become quite distinct and well contrasted with osmium. Compressed Curosurf films, on the other hand, become more heterogeneous (Fig. 8), and are often accompanied by conspicuous accumulations of osmiophilic material in the adjacent hypophase. Vesicular structures of lipid bilayers or

multilayers or stacks of multilayers appear associated with the surfactant film and may form on compression in the plateau region between 20 and 23 mN/m where the film compressibility is relatively high [30], as also seen in Fig. 13 top. However, the distinct molecular architecture consisting of planar regions, that may have associated vesicular structures, interrupted by areas with stacks of multilayers may also be generated upon film formation by adsorption to approximately 23 mN/m, that is, if the surfactant concentration is sufficiently high (e.g., Figs. 5A, 7A–C, 12). Multilayer formation due to over-compression at minimum surface tension (film collapse) cannot completely be ruled out, although the compressions have been done carefully, and the features were present in all experiments (Fig. 5B).

The controversial question of whether the surfactant film is a monolayer or a multilayer has been recently discussed in detail. We have previously shown by electron microscopy that the alveolar film in situ frequently appears multilaminated, not only in alveolar corners and crevices, but also at the thin air–blood barrier above the capillaries. Disk-like structures or multilamellar vesicles appear attached or partially integrated into the planar multilayered film [10,38]. Studies with the captive bubble have shown that there is “surplus” material in excess of a monolayer at the air–liquid interface upon de novo adsorption of surfactant derived from natural sources [41]. In the present study, we also show that multi-lamellar film fragments and surface associated vesicular structures are generated upon adsorption in systems consisting of synthetic surfactants such as Exosurf (Fig. 11), DPPC/ egg PG, or egg PG (Fig. 10). Furthermore, in addition to the “reservoir” formed upon de novo adsorption, additional surface associated multilamellar structures appear to be generated upon film compression. The formation upon adsorption of multilayer “reservoirs” adjacent to the air–water interface were also shown recently by Brewster angle microscopy on surface films from human broncho-alveolar lavage fluid [42], and on films from bovine lipid extract surfactant (Survanta) by AFM; multilayer reservoirs adjacent to the air–liquid interface were shown to coexist with monolayer islands of solid phase [21]. This is in line with our observation, although we were not able to image monolayer islands by electron microscopy. The multilayer structures associated with the air–fluid interface are seen upon film adsorption, if the surfactant film is formed from an aqueous surfactant suspension. This is in contrast to the films formed from surfactant solutions in solvents such as chloroform/methanol by spreading at the air–water interface. For solvent-spread films, multilayers seem to be formed upon compression only, e.g. [43]. Workers using these systems, describe the surfactant film as a monolayer with coexisting semi-crystalline, or solid phases with disordered fluid, or liquid-expanded phases. Film transformation upon compression and the formation by monolayer collapse of bilayer disks associated with the monolayer, is analyzed by a thermodynamic model as shown in a series of articles by Hall and his associates, e.g., [44,45,46]. Furthermore, it appears that solvent-spread films need to be compressed continuously at a certain minimum speed to achieve near zero minimum surface

tensions, that is, their collapse is kinetically determined [47,48]. This is in contrast to the present lipid extract films which reach near zero surface tension upon quasi-static compressions of 20–30% from the equilibrium surface tension of ~ 25 mN/m, provided the total PL concentration is at or above 1 mg/ml (Fig. 12 top). However, Curosurf films at the relatively low concentration of 0.3 mg/ml show a plateau of high compressibility between 20 and 25 mN/m (Fig. 13 top) and only achieve near zero minimum surface tensions upon dynamic cycling (Fig. 13 bottom). The behaviour of such films appears to be consistent with that observed by Hall and his co-workers [47,48]. The micrographs of the low concentration Curosurf films demonstrate a relatively thin osmiophilic layer with fewer clear multilayer sections and associated vesicular structures compared to the films from PL concentrations of at least 1 mg/ml. The presence of alginate in the subphase has no substantial influence on the film surface properties, see “Surface activity tests” and Fig. 14).

It appears that surfactant films formed from an aqueous suspension, as shown in the present work, are more than a monolayer, and the film architecture with multilayer structures associated with the interface is likely important for the mechanical properties of the film, including stability, compressibility and viscosity [21,38]. The present, systematic experiments show clear-cut multilaminated film segments, and these in particular adjacent to surface associated surfactant material, or “reservoirs”. A vexing observation is the occasional formation of apparent bilayers or multilayers with an even number of laminae. One might speculate that the existence of this odd surface structure is due to the missing top layer facing the air because of its insufficient stabilization or staining.

Bubbles lined with pure DPPC showed the well-defined surface activity of DPPC-films in the surfactometer. These functioning films, however, could not be contrasted with osmium and uranyl acetate, and thus remained “invisible”. The controversy is still open of whether fully saturated phospholipids bind osmium [22–24]. In any case, in the present model we did not succeed to image DPPC films. However, films formed from the synthetic surfactants DPPC/egg PG [31] and Exosurf (devised by John Clements), could be imaged by our methods. This is likely due to the presence of unsaturated phospholipids in the former and perhaps tyloxapol in the latter preparation. As to the DPPC/egg PG films, the results of additional experiments are revealing. Suspensions of pure PG form as good, or even more distinct films (Fig. 10A, B) than suspensions of the mixture DPPC/egg PG (Fig. 10C), and this in spite of the fact that with PG films when compressed, near zero minimum surface tensions cannot be obtained with the captive bubble surfactometer. It thus appears that the visible part of DPPC/egg PG films consists of PG, and that PG serves as a carrier only to bring the not stainable but functionally important component of the film, DPPC, to the air–liquid interface for adsorption and spreading. In fact, it has been shown that acidic phospholipids enhance the adsorption of DPPC [49]). It is interesting to note that there are multilaminated structures associated with the surface film formed

from PG alone. These structures might also be related to the surface associated reservoir which seem to form even when the surfactant associated proteins are absent.

The formation of osmiophilic Exosurf films appears to be even more peculiar. Although pure DPPC could not be contrasted in our model, and Exosurf does not contain unsaturated phospholipids, quite distinct fragments of films could be detected. The carrier substances for DPPC in these artificial surfactants, PG and hexadecanol/tyloxapol, differ in two aspects from each other. On the one hand, hexadecanol/tyloxapol at a concentration contained in Exosurf, do not form osmiophilic surface films, although tyloxapol is osmiophilic. On the other hand, hexadecanol/tyloxapol exhibits a considerable surface activity. When admixed to alginate solutions the interfacial surface tension can easily and reproducibly be lowered to below 5 mN/m on compression of the bubble surface. Also remarkable are (rather rare) surface-associated accumulations of osmiophilic material resembling disordered piles of crystalline matter [32]. Evidently, DPPC and the additives contained in Exosurf form complexes, which integrate into the surface, and which can be stained with osmium and uranyl acetate.

Taken together, the results of the manifold experiments once more illustrate the enormous complexity of the structure–function relationship of surfactant films. Notwithstanding, some findings appear noteworthy and give a partial answer on the initial questions: (1) As postulated from functional evidence, surfactants suspended in a liquid bulk phase do adsorb to the bubble surface within a captive bubble surfactometer and form lining layers which can be imaged by electron microscopy; this with the exception of pure DPPC layers. In contrast to the distinct structures of surfactant material of different composition suspended in pellets [14,15], the anatomy of surface films is rather uniform. (2) The distinctiveness of the surface films, as revealed by electron microscopy, appears to depend on the composition of the surfactant material, on the concentrations of surfactant in the bulk phase, and on whether the films are compressed or not. (3) There appears to exist a relationship between film structure and surface active properties as demonstrated with Curosurf films at low concentrations and higher concentrations (Figs. 6, 7, 12, and 13). We observed equivalent features on films from BLES (not shown). However, a clear relationship between a particular lipid extract surfactant and their films as visualized by transmission electron microscopy has not been established.

For a comparison of the results obtained by electron microscopy with those obtained by fluorescence light and atomic force microscopy, one has to consider a basic difference between the imaging techniques. The latter reveal a two-dimensional view of the surface, and make it feasible to image the highly differentiated molecular organisation of the uppermost layer of surfactant films [5–8,21]. In contrast, electron micrographs show a two-dimensional view of orthograde sections through the lining layer together with the surface associated material “reservoirs”, which may assume quite different structures, i.e., vesicular material, lattices, and multi-

layers. However, particular surface phenomenon such as liquid-expanded and liquid-condensed domains of the uppermost monolayer cannot be imaged by electron microscopy, since the film segments with relatively high amounts of saturated phospholipids likely cannot be contrasted, and because the power of resolution is limited. As pointed out above, a well-functioning surfactant film is more than a pure DPPC monolayer, in that the surface associated structures or reservoirs appear to ensure a coherent lining layer and hence mechanical stability of the film [21,38]. The different methods are complementary and enhance the understanding of the structure–function relation of surfactant films.

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