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PO Box 117
221 00 Lund
+46 46-222 00 00

Human SP-A and a pharmacy-grade porcine lung surfactant extract can be reconstituted into tubular myelin – a comparative structural study of alveolar surfactants using cryo-transmission electron microscopy

Marcus Larsson[1,2], J. Freek van Iwaarden[3], Jack J. Haitsma[4], Burkhard Lachmann[4] and Per Wollmer[1]

[1]*Department of Clinical Physiology,* [2]*Department of Physical Chemistry 1, Lund University, Lund, Sweden,* [3]*Laboratory of Paediatrics and* [4]*Department of Anaesthesiology, Erasmus Medical Centre Rotterdam, Rotterdam, The Netherlands*

Summary

Correspondence

Marcus Larsson, Department of Clinical Physiology, Lund University, S-205 02 Malmö, Sweden
Tel.: +46 70 5678757,
Fax: +46 40 336620
E-mail: m.larsson@klchem.se

Cryo-transmission electron microscopy (cryo-TEM) is a rather artefact-free method, well suited to study the alveolar surfactant system. A pharmacy grade porcine lung surfactant extract (HL-10) was mixed with human SP-A and Ringer's solution (for calcium ions), and it was shown by cryo-TEM that the tubular myelin (TM) type of structure was reconstituted. These aggregates were associated to liposomal aggregates, and resulted in macroscopic phase-separation. This phase showed a weak birefringence in the polarising microscope, which is characteristic for a liquid-crystalline type of structure. TM from rabbit lung lavage was also examined, and showed the same periodic arrangement of bilayers as alveolar surface layer from freshly cut rabbit lungs deposited directly on the cryo-TEM grids. The distance between the bilayers of TM was 40–50 nm, and an electron dense material, assumed to be SP-A, was sometimes seen to occur periodically along the bilayers, oriented perpendicularly to the tubuli. The results are consistent with the surface-phase model of the alveolar lining.

Introduction

In earlier ultrastructural studies of the alveolar surface layer by cryo-transmission electron microscopy (cryo–TEM), the surface layer from a freshly opened rabbit lung could be deposited directly on EM grids. Homogeneous films thin enough for vitrification and cryo-TEM examination were formed, and a coherent bilayer organization consistent with that of tubular myelin (TM) was revealed (Larsson *et al.*, 1999, 2002b), and a surface-phase model of the alveolar lining was proposed.

The aim of this study was to examine the reconstitution of a pharmacy-grade surfactant extract, with human SP-A, using cryo-TEM to study the structural transformations. When lamellar bodies (LBs) secreted by epithelial Type II cells interact with the hydrophilic protein SP-A in the presence of calcium at the alveolar surface, the complex bilayer structure known as TM is formed. As reference material for cryo-TEM analysis of TM structures, we also analysed TM isolated from lung lavage. Furthermore, we extended this ultrastructural comparison to directly deposited alveolar surface material.

The reconstitution experiments were designed so as to provide information relevant to surfactant substitution therapy.

Such therapy, aiming towards restoring the alveolar function, utilizes either synthetic lipid/lipid analogue formulations or mammalian lung surfactant extracts. The extraction procedures result in a mixture of phospholipids and the hydrophobic proteins SP-B and SP-C. It is not known whether such extracts, with low concentrations of SP-B and SP-C compared to the physiological situation, can reconstitute the native alveolar surface structure. Although reconstitution of TM has been reported earlier, cf. Suzuki *et al.* (1989); the experimental conditions have been far from those at surfactant administration *in vivo*.

Polarizing microscopy was also used in order to provide additional information on macroscopic phase behaviour, based on the birefringence of liquid-crystalline phases.

Methods

Deposition of the alveolar surface layer

Four brown rabbits were killed by intravenous injection of pentobarbital sodium. The chest was opened and a catheter inserted through the right ventricle into the pulmonary artery.

The left ventricle was opened, and the lungs were perfused with Ringer's solution until the perfusate appeared clear. The lungs were removed and approximately 30 min after sacrifice, the lungs were cut in the periphery and samples for cryo-TEM and polarizing microscopy were prepared as described below.

Lung lavage

Two brown rabbits were killed by an intravenous injection of pentobarbital sodium. After tracheotomy the lungs of each rabbit were lavaged twice with 30 ml Ringer's solution per kilogram body weight.

Cell debris was first spun down and removed (1100 rpm using a table centrifuge for 15 min). The supernatant was then centrifuged at 15 000 *g* for 15 min. The pellet of enriched lung surfactant (large aggregate/TM fraction) was re-dispersed in minute amounts of Ringer's solution.

Human SP-A was isolated from the alveolar lavages of human alveolar proteinosis patients (van Iwaarden et al., 1995).

Reconstitution experiments

The reconstitution experiments were performed with a porcine lung surfactant extract, HL-10 (HL-10; Leo Pharmaceutical Products, Ballerup Denmark, and Halas Pharma GmbH, Oldenburg, Germany; Gommers et al., 1993). Besides phospholipids, the extract contained 1–2 wt% of the hydrophobic proteins SP-B and SP-C and about 2 wt% neutral lipids: cholesterol, free fatty acids and fatty acid glycerides.

The reconstitution experiment was repeated three times. To 1 ml of a solution of 1·4 mg of SP-A in Tris–HCl buffer was added 56 ± 1 mg of HL-10 powder. Mixing was achieved by syringe passages for half a minute. Then, 2 ml of Ringer's solution was added and the mixture was passed through a syringe for another minute. A dispersion, which visually appeared homogeneous, was obtained and kept at 37°C for 40 min. The sample was stored at 8°C for observation. After 4 h a clear phase separation was observed. After 7 days, to allow sample stabilization, cryo-TEM was performed, and polarization microscopy was performed after 8 days.

The concentration of SP-B in HL-10 was assumed to be about 0·5 wt% calculated on the dry lipid content and the concentration of SP-A was 2·5% calculated on the dry lipid content.

In addition, we also used 1·25% of SP-A in relation to HL-10. The kinetics of phase separation behaviour was much slower than at a concentration of 2·5% SP-A.

Cryo-TEM and polarizing microscopy

The cryo-TEM grids were allowed to touch the surface of the freshly cut lung for about 1 s and immediately thereafter plunged into liquid ethane (−180°C). The lavage and the reconstituted samples were spread on the grid in minute amounts (5 µl) using a pipette and thereafter vitrified. The samples were viewed in a Philips Bio-twin 120 cryo with a La2B6 filament. The cryo-samples were at all times kept below −160°C.

Optical microscopy samples were prepared by putting a minute droplet of lavage-pellet material or reconstituted TM onto a microscopy slide. The samples were observed in a Leitz microscope at 25°C. The photographs were recorded with a Sony CCD camera connected to a Sony printer.

Results

When vitrifying large, viscous structures, like TM aggregates, the probability of crystalline ice formation (totally occluding the view) increases with the thickness of the film.

Reconstitution of TM

Phase separation takes place successively after HL-10/SP-A mixing, as could be followed visually. The two phases, which have separated after about 4 h, are shown in Fig. 1. The upper phase is a clear water solution, and the lower phase is semi-liquid and non-transparent, showing birefringence in the polarizing microscope. It is thus a liquid-crystalline phase. Estimation from the volume of this phase corresponds to water content in the range 90–95%.

The phase behaviour of aqueous dispersions of the HL-10 extract before the addition of SP-A is quite different. A sample of HL-10 in Ringer's solution is weakly turbid, with a continuous increase in turbidity towards the bottom without any phase separation. The lamellar liquid-crystalline phase that this HL-10 extract (without SP-A) gives with water has a water content of about 50% (w/w; Larsson et al., 2002a).
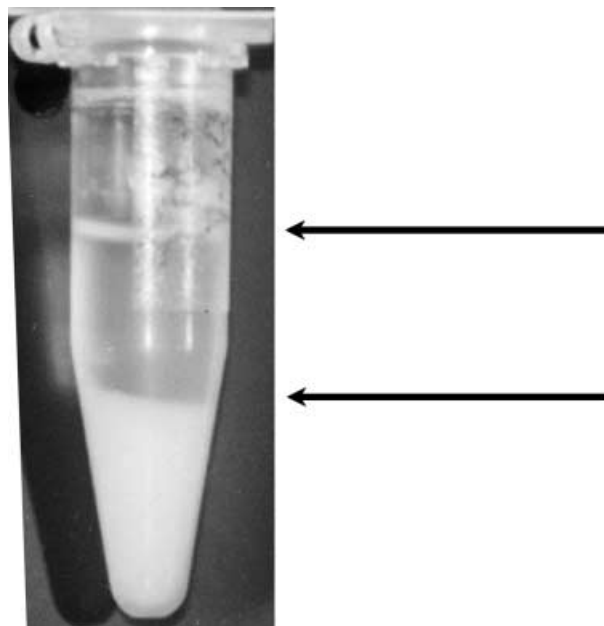

Figure 1 A test-tube with reconstituted TM from SP-A interaction with HL-10, showing phase separation at 4 h.

Cryo-TEM textures observed in the liquid-crystalline phase reconstituted with SP-A are shown in Figs 2 and 3, with textures in agreement with the tubular character of the bilayer structure known as TM. The observed bilayer spacing (about 40 nm) and the distribution of electron-dense material along the bilayers are consistent with cryo-TEM observations of directly deposited and lavage material textures (discussed below). In some images, LBs are seen.

Typical textures observed in the polarizing microscope are shown in Figs 4–6. A remarkable feature is the existence of
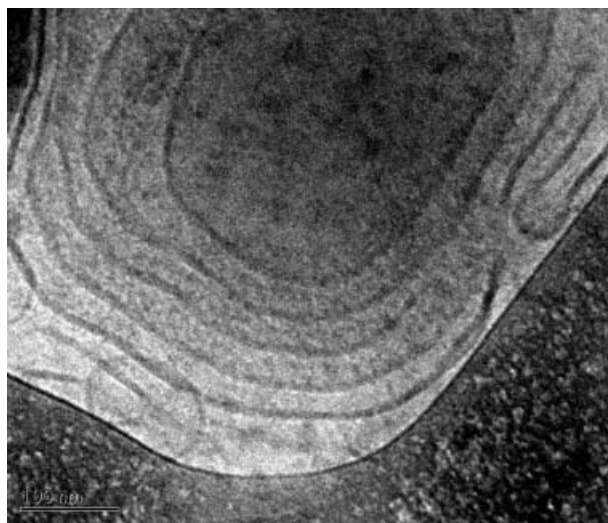

Figure 4 A low-magnification view in the polarizing microscope of the reconstituted TM phase, showing a homogeneous birefringence with orientation effects in lamellae between large air bubbles.

Figure 2 Cryo-TEM of a sample of HL-10 + SP-A showing regularly spaced bilayers. Protein deposits can be seen along and perpendicular to the tubuli.
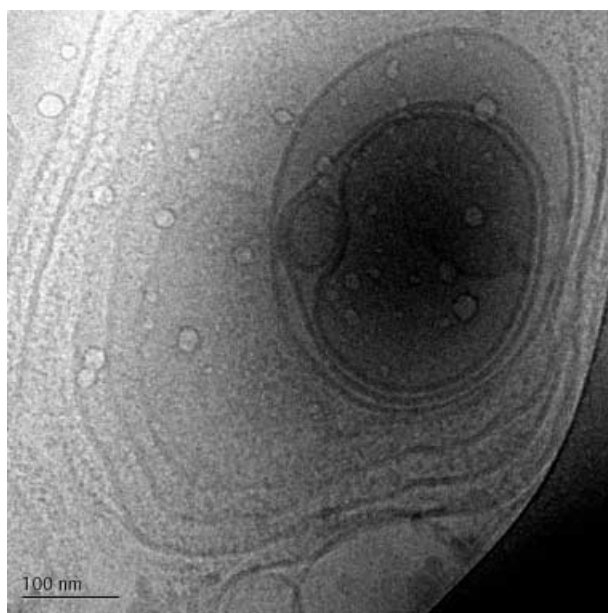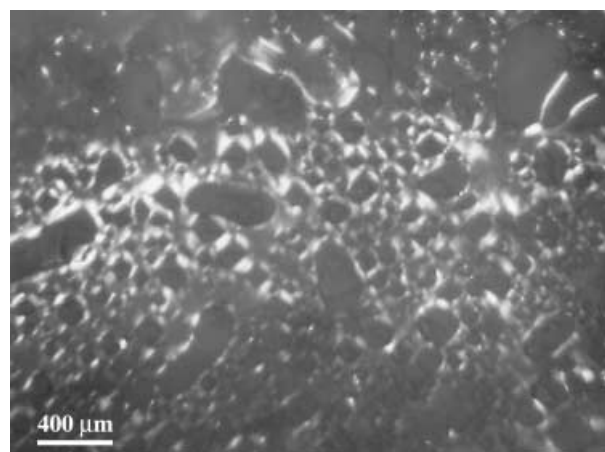

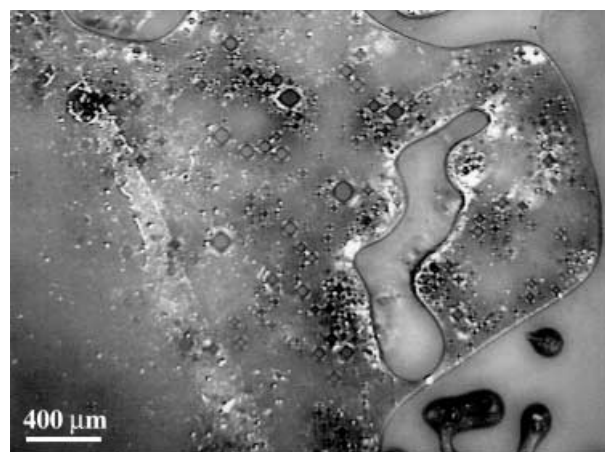**Figure 5** A sample of reconstituted TM viewed in the polarizing microscope with the polarizer and analyser directions deviating from perpendicular directions.

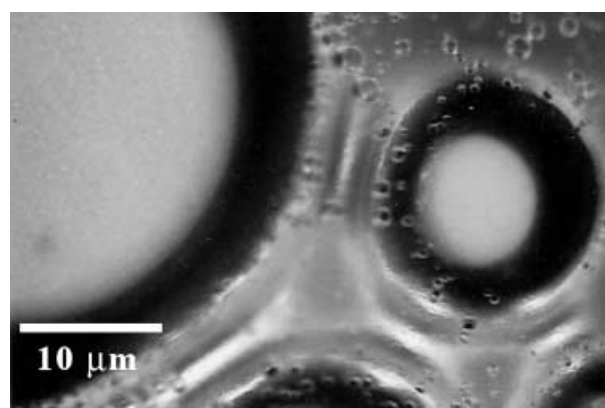**Figure 3** Cryo-TEM of HL-10 + SP-A. A liposomal-type of bilayer arrangement is also seen, located at the surface towards the TM-type of bilayers.

Figure 6 Another region from the sample shown in Fig. 3 viewed in the polarizing microscope.
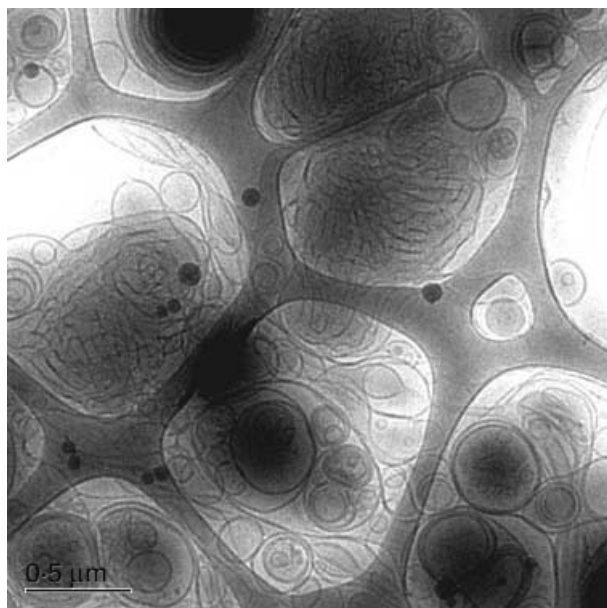
Figure 7 A cryo-TEM overview of a lung lavage sample. A TM-aggregate can be seen in the centre and a LB above. Many vesicles occur and the bilayer sometimes continues from one grid opening to the next (centre of image).

Figure 8 A cryo-TEM of a lung lavage sample showing a vesicle fusing with a TM region.

square-shaped air cells with birefringent edges. Furthermore these cells tend to have parallel sides (Figs 4 and 5).

Lung lavage

A typical overview of a cryo-TEM sample from the lavage is shown in Fig. 7. Numerous vesicles are seen, most of them unilamellar. There is one grid opening filled with a TM aggregate, which is linked to adjacent vesicles. At a few places it can be seen that the bilayers also cover the grid. The size of one of the multilayer vesicles (LBs), which is assumed to be spherical, is about 0·5 μm. The darkness indicates that this LB is much thicker than the rest of the sample.

A semi-spherical vesicle that fuses with a bilayer around a TM region is shown in Fig. 8. The bilayer arrangement both along and perpendicular to the tubuli can be seen.

Direct deposition of alveolar surface layer

A cryo-TEM texture of a TM-region of a deposited surface layer from rabbit alveoli is shown in Fig. 9. Regularly spaced bilayers are observed, which is in agreement with earlier reported cryo-TEM results (cf. Larsson et al., 1999, 2002b). The spacing is in the range 40–50 nm, and varies somewhat with bilayer curvature. This texture is consistent with earlier observations of TM. In a few cases, a periodic pattern of electron-dense material was seen oriented perpendicularly to the tubuli.

Besides the TM-type of texture, we also observed spherical-concentric multilayer particles, with textures consistent with LBs. The outer bilayers of the LBs were sometimes continuous with bilayers of TM textures.
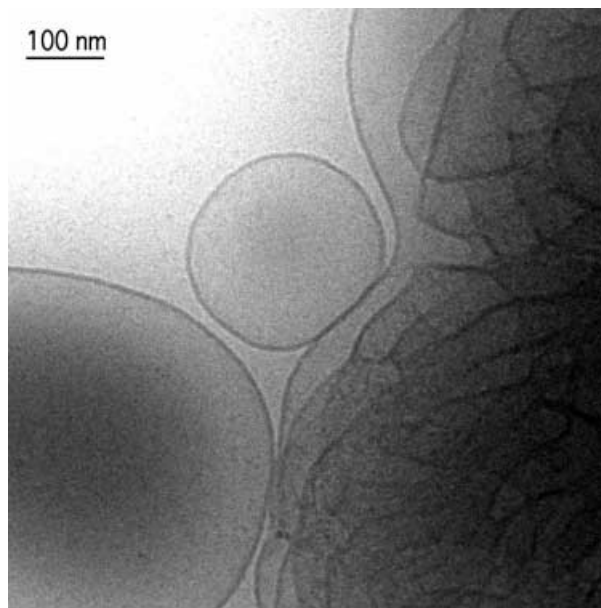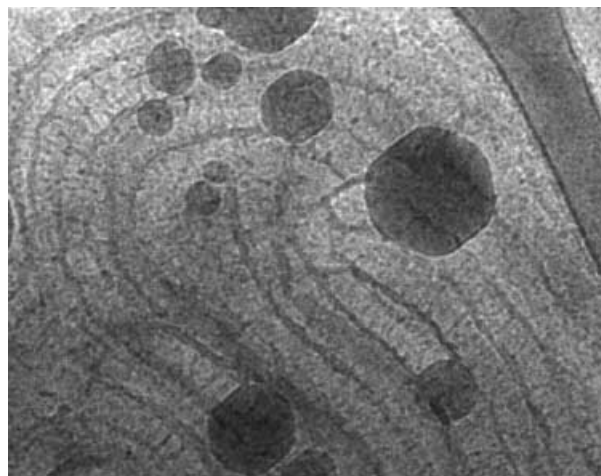

Figure 9 Cryo-TEM texture of an alveolar surface layer sample directly deposited on the grid showing a periodic pattern of electron dense material perpendicular to and along the tubuli. Dark large 'circles' are crystalline ice.

Discussion

Recently, the traditional monolayer model of lung surfactant has been challenged by a surface-phase model (Larsson et al., 1999, 2002b). A remarkable, macroscopic phase separation occurs when human SP-A interacts with a lung surfactant extract, HL-10. The phase separation occurs rapidly. Within 4 h, macroscopic steady state has been reached. Liposomal dispersions, like HL-10 in water (Larsson et al., 2002a), never show such a well-defined phase separation. The cryo-TEM textures of the reconstituted samples, shown in Figs 2 and 3, show that this

phase consists of a LB-TM continuum. Observations in the polarizing microscope of the reconstituted HL-10/SP-A system shows that the phase that separates is liquid-crystalline. The square-shaped air cells seen in Figs 4–6 probably reflect the tetragonal inner structure of this phase.

The textures from all three types of samples in this study contain regions that are consistent with earlier reports of the TM structure. The observed parallel and equidistant bilayers correspond to the texture that should be expected when the tubuli of TM are oriented along the grid surface. The observed spacing in the range 40–50 nm are also in agreement with earlier reported EM textures (Nichols, 1976; Williams, 1977; Hasset et al., 1980; Young et al., 1992; Nag et al., 1999). Besides the lateral periodic repetition of the bilayers, a periodic repetition of electron-dense material was sometimes seen along the bilayers as linear sections perpendicular to the bilayer (Fig. 9). The spacing is somewhat irregular, probably due to high bilayer curvature, further discussed below.

Williams (1977) in her pioneering study of TM formation observed periodicity along the 'transient' bilayers when LBs transform into TM. Particles were seen along one side of the bilayer with a diameter of about 8 nm, and a similar distance was reported to occur between particles.

Hasset et al. (1980) also described an electron-dense material periodically arranged along the TM tubuli. The distance between rod-shaped particles perpendicularly oriented along the bilayer was reported to be 14–16 nm. They also observed protein molecules along the diagonal directions in the square bilayer pattern of cross-sections of TM, and concluded that this protein was the same as that seen along the bilayers, i.e. SP-A. There is other evidence indicating that SP-A is localized in this mode; textures of ×-shaped ('cross-hatched') electron-dense linear formations in the tubular cross-sections have been observed, cf. Nag et al. (1999). It seems likely, therefore, that the electron dense material along the tubuli shown in Fig. 9 is SP-A.

Our cryo-TEM results reflect the structure at 37°C in its hydrated state, i.e. at physiological conditions. In the previously mentioned studies (Nichols, 1976; Williams 1977; Young et al., 1992), chemical fixation was performed at room temperature, typically followed by post-fixation in osmium tetraoxide at 4°C and dehydration. At such fixation conditions, the hydrocarbon chain core of the lipid bilayer should crystallize (at least partly) according to a recent X-ray scattering/diffraction study (Larsson et al., 2003). Crystallinity of bilayers will reduce bilayer curvature. This might be the explanation of the more regular square bilayer patterns, regarded as the characteristic structural feature of TM, seen in earlier EM work compared to those reported here. Also, the protein structures and distribution should be expected to be influenced by the above-mentioned differences in preparation procedures.

Suzuki et al. (1989) in their reconstitution of TM used synthetic phospholipids and SP-B (termed SP-15 in their paper) at incubation in a SP-A solution (termed SP-35). We have used a different preparation procedure in order to be closer to the therapeutic situation, when lung surfactant extracts, such as HL-10, are delivered to the alveolar surface. In our study, the protein concentration in the bilayer was more than one order of magnitude lower, and the relative amount of SP-A was also much lower. SP-A is secreted in a different pathway from LBs. There is a possibility that in some clinical ARDS-like situations, there could be a lack of LB-material but sufficient amounts of SP-A. Under these conditions the administration of surfactant extracts such as HL-10 could lead to the formation of a tetragonal surface-phase at the alveolar surface.

Acknowledgments

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