

Contents lists available at [ScienceDirect](http://ScienceDirect.com)

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Physiological variables affecting surface film formation by native lamellar body-like pulmonary surfactant particles



Nina Hobi ^{a,c,*}, Gerlinde Siber ^a, Virginia Bouzas ^b, Andrea Ravasio ^a, Jesus Pérez-Gil ^b, Thomas Haller ^a

^a Department of Physiology and Medical Physics, Division of Physiology, Innsbruck Medical University, 6020 Innsbruck, Austria

^b Department of Physiology and Medical Physics, Division of Physiology, Medical University Innsbruck, Fritz-Preglstr. 3 6020 Innsbruck, Austria

^c Institute of General Physiology, University of Ulm, 89081 Ulm, Germany

ARTICLE INFO

Article history:

Received 21 November 2013

Received in revised form 14 February 2014

Accepted 19 February 2014

Available online 27 February 2014

Keywords:

Lamellar body-like surfactant particles
Physiological milieu in alveolar lining fluid
Adsorption at the air–liquid interface
Phospholipids
Lung diseases
Surfactant therapies

ABSTRACT

Pulmonary surfactant (PS) is a surface active complex of lipids and proteins that prevents the alveolar structures from collapsing and reduces the work of breathing by lowering the surface tension at the alveolar air–liquid interface (A_{LI}). Surfactant is synthesized by the alveolar type II (AT II) cells, and it is stored in specialized organelles, the lamellar bodies (LBs), as tightly packed lipid bilayers. Upon secretion into the alveolar lining fluid, a large fraction of these particles retain most of their packed lamellar structure, giving rise to the term lamellar body like-particles (LBPs). Due to their stability in aqueous media, freshly secreted LBPs can be harvested from AT II cell preparations. However, when LBPs get in contact with an A_{LI} , they quickly and spontaneously adsorb into a highly organized surface film. In the present study we investigated the adsorptive capacity of LBPs at an A_{LI} under relevant physiological parameters that characterize the alveolar environment in homeostatic or in pathological conditions. Adsorption of LBPs at an A_{LI} is highly sensitive to pH, temperature and albumin concentration and to a relatively lesser extent to changes in osmolarity or Ca^{2+} concentrations in the physiological range. Furthermore, proteolysis of LBPs significantly decreases their adsorptive capacity confirming the important role of surfactant proteins in the formation of surface active films.

© 2014 Elsevier B.V. All rights reserved.

1. Introduction

Due to mammalian lung's specific design, containing millions of small alveolar chambers, pulmonary surfactant (PS) is crucial to stabilize this structure and to support its genuine respiratory functions. PS is synthesized and secreted by the alveolar type II (AT II) cells [19]. Released surfactant material is quickly adsorbed at the air–liquid interface (A_{LI}) in order to lower surface tension of the alveolar lining fluid (ALF) and, according to recent findings, to favor oxygen diffusion [42,44,45]. Thereby, the integrity of the lung and the respiratory interface is maintained and an alveolar collapse avoided.

PS is a lipid–protein complex composed of 80% phospholipids (half of which is disaturated dipalmitoylphosphatidylcholine; DPPC), 10% neutral lipids (mainly cholesterol) and approximately 10% surfactant proteins [19,64]. After the synthesis and proper processing of the different protein and lipid components through the endoplasmic reticulum and the Golgi apparatus, surfactant complexes are finally assembled and stored in characteristic subcellular compartments known as lamellar bodies (LBs). LBs are lysosome-derived secretory organelles with a diameter of up to $\sim 2 \mu\text{m}$ (in humans and rodents) [41,54,64], and constitute $\sim 20\%$ of the cytosolic AT II cell volume [22,54]. In several studies it has been shown that PS, during and after release, maintains much of its packed structure unless contacting the A_{LI} . Consequently,

these surfactant particulate entities were termed LB like-particles (LBPs) to underscore this morphological similarity with the intracellular surfactant storage form (LBs). LBPs exhibit a spontaneous disintegration upon contact with the A_{LI} , leading to an immediate spreading of material and a decline of surface tension to values below 30 mN/m followed by a further reduction in the lung close to 1–2 mN/m, probably due to film refinement during film compression [25,50,58].

Changes in the lung environment caused by physiological stress factors and during various pathophysiological conditions, e.g. inflammatory processes, respiratory/metabolic alkalosis or acidosis, hypo- or hyperthermia, airway drying or osmotic stress caused by edema formation, hyperventilation or swallowing of noxious fluids, may all alter the surfactant's functional properties. Naturally, we assume that most of these changes will lead to impairment or loss of surfactant function rather than to its improvement. Those impaired functions will entail, by definition, a high surface tension at the A_{LI} and, further on, may lead to a reduction in the lung compliance and functional residual capacity [3,23]. They even may cause hypoxemia and hypercapnia and may contribute to numerous serious lung syndromes involving diffuse alveolar damage (DAD), ventilation induced lung injury (VILI), acute lung injury (ALI) and other critical acute respiratory distress syndromes (ARDS) that are often observed in mechanically ventilated patients and newborn babies [22,23]. However, a systematic investigation of all physiological parameters that may be subject to changes is lacking,

* Corresponding author.

mainly because of intrinsic limitations in the application of standard biophysical measurements to a large amount of samples and variables.

One well known cause of surfactant inactivation is the presence of plasma proteins, which can leak from the lung capillaries into the alveolus and compete with surfactant phospholipids at the A_{LI} . For instance, in ARDS, albumin concentrations may reach up to 100 mg/ml, with a mean value of 25 mg/ml [31], whereas in vitro and in vivo studies confirmed that even much lower protein concentrations may cause severe interruption of the pressure–volume (P–V) mechanics in rat lungs [27,62,65]. Inhibition by plasma proteins has been sought to be reverted or at least partially ameliorated by administration of various exogenous surfactant preparations, though the outcome of these investigations did not unambiguously lead a clear result [23].

This exogenous surfactant material is prepared from natural surfactant isolated from animal lungs by a series of lipid extraction steps with organic solvents. Through this harsh purification procedure hydrophilic proteins like SP-A and SP-D are removed and the obtained material contains only lipids and small amounts of the hydrophobic proteins SP-B and SP-C. For example Curosurf, a widely used exogenous surfactant extracted from minced porcine lungs, contains a significantly lower concentration of DPPC and biophysically active proteins like surfactant proteins B and C (SP-B, SP-C) than native surfactant, and no SP-A [9]. Such, ‘lipid extract’ is unable to generate tubular myelin, a membrane-based lattice-like surfactant structure, which has been proposed to act as intermediate in alveolar surfactant turnover [56]. However, Curosurf adsorbs rapidly to an A_{LI} , effectively spreads in the airspaces, is physiologically functioning in vitro and in vivo [21,52], and its administration to newborns suffering from IRDS significantly decreased their mortality [9,52]. In the same line, knock-out mice models lacking expression of the SP-A gene lack tubular myelin but breath normally [32], suggesting that tubular myelin is not a necessary intermediate but possibly a reservoir of surface active material [44].

The successful response to surfactant therapy depends primarily on the timing of treatment in relation to the clinical course and the mode of delivery. Secondary, the quality of the exogenous material is crucial for a successful application. This quality mainly depends on the chemical composition of the surfactant but might be influenced by the physiological state of the patients lung, (e.g. hypo- or hyperthermia during anesthesia or fever, increase of serum due to lung injury, exogenous/endogenous airways acidification, etc.) as well. Several attempts have been made to improve the known surfactant replacement therapies, however, regarding the interfacial adsorption, exogenous surfactants currently in clinical trials have been proven to be less efficient than endogenous surfactant, particularly in the presence of inhibitory compounds [10,11,62]. Reasons for this reduced functionality are still unclear, but are likely related with differences in composition and structure. In vitro and in vivo studies emphasize the importance of standardized protocols for the assessment of therapeutic surfactants, and suggest that surfactant function strongly depends on the physiological environment [9].

In this paper we analyzed how the adsorptive behavior of LBPs that have been collected from cell cultures of primary rat AT II cells is affected by physical and chemical parameters like Ca^{2+} concentration, osmotic strength, pH, temperature and protein concentration. LBPs, in contrast to extracted surfactant, are likely to contain the full complement of various proteins and lipid species as assembled and secreted by the pneumocytes. In particular, Western blot analysis demonstrated the presence of SP-A, SP-B and SP-C in LBPs collected from pure AT II cell cultures [50]. The application of a mid-high throughput fluorescence-based assay developed in our laboratories has proven to be particularly useful to assess a relatively large number of ultra-small sample volumes as it is the case for LBPs, which can be collected from the cells in a μ g amount only and preclude, due to this quantitative limitation, a multi-parametric analysis by e.g. captive bubble surfactometry. Although this assay does not allow assessing the behavior of surfactant films during repeated compression–expansion cycles, it permits, however, a detailed

assessment of the formation and stability of the surface associated surfactant reservoir. The study advances to provide a widened understanding of the biophysical mechanisms of LBPs adsorption process at the A_{LI} , and how this process is governed by factors in the ALF, whose composition and physical state is not precisely known. This knowledge, beside its relevance in lung physiology and pathophysiology, may also have some implications in the design and application of new surfactant compositions and surfactant therapies.

2. Material and methods

2.1. Reagents

Phospholipase D from streptomyces chromofuscus, Brilliant Black (BB), salts and chemicals were purchased from Sigma (Sigma-Aldrich, Germany). High purity bovine serum albumin (BSA, albumin bovine Fraction V receptor grade lyophilized) was purchased from SERVA (SERVA Electrophoresis, Germany), BODIPY-PC 2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoyl)-1-hexadecanoyl-sn-glycero-3-phosphocholine, and Amplex Red Phospholipase D Kit from Invitrogen (Invitrogen, Austria).

2.2. Test solutions and conditions

Control solution (ctrl): For the control conditions we used standard Ringer Solution containing (in mM): 140 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, pH 7.4. **pH solutions:** The pH of the sub-phases were adjusted by titrating NaOH and HCL into the control solution. The pH values were measured before every experiment with a digital pH meter (WTW Austria). **Calcium solutions:** The control solution was adjusted to the indicated $CaCl_2$ concentrations (0–5 mM), by addition of $CaCl_2$. The zero Ca^{2+} solution was made by using 0 mM $CaCl_2$ + 2 mM EGTA, pH 7.4. **Hyper-hypotonic solutions (in mM):** ~600 mOsm solution = 240 NaCl, 10 KCl, 2 $MgCl_2$, 4 $CaCl_2$, 20 HEPES; ~300 mOsm solution = ctrl solution; ~150 mOsm solution = 70 NaCl, 2.5 KCl, 0.5 $MgCl_2$, 1 $CaCl_2$, 5 HEPES; and ~20 mOsm solution = 10 NaCl, 0.357 KCl, 0.071 $MgCl_2$, 0.142 $CaCl_2$, 0.714 HEPES, pH 7.4. Final osmolarity was checked with an osmometer (Wescor, United States). **Temperature control:** Experimental solutions and 96-wells were pre-heated/cooled and multiplate reader settings were adjusted to the indicated temperatures (15 °C, 25 °C, 30 °C, 37 °C, 42 °C). Each T-point was measured individually in a single experiment. **Protein solutions:** BSA was diluted in the control solution to the indicated concentrations (0–2.5 mg/ml BSA).

2.3. Surfactant preparations

LBPs were harvested from the supernatants of purified rat AT II cells grown on petri dishes (\emptyset 10 cm) in high density. AT II cells were isolated from Sprague–Dawley rats as described elsewhere [24]. After two days in culture, these cells were washed two times with PBS and these cells were stimulated for 4 h at 37 °C in 4 ml bath solution containing ATP (100 μ M), PMA (500 nM) and Ionomycin (1 μ M) supplemented with antibiotics as previously described. After stimulation, supernatants containing exocytosed surfactant rich in LBPs were collected and stored at –20 °C until use. As shown by fluorescence microscopy in Fig. 1a, LBPs are present as a suspension of variably sized, partially unfolded but globular structures throughout, consistent with recent TEM analysis [50]. According to earlier investigations by our group, the size and compactness of these particles remain essentially stable over time [25]. Tubular myelin, a surfactant intermediate with a still undefined role [44] was not detectable in our preparations, neither by light microscopy nor by TEM [25,50].

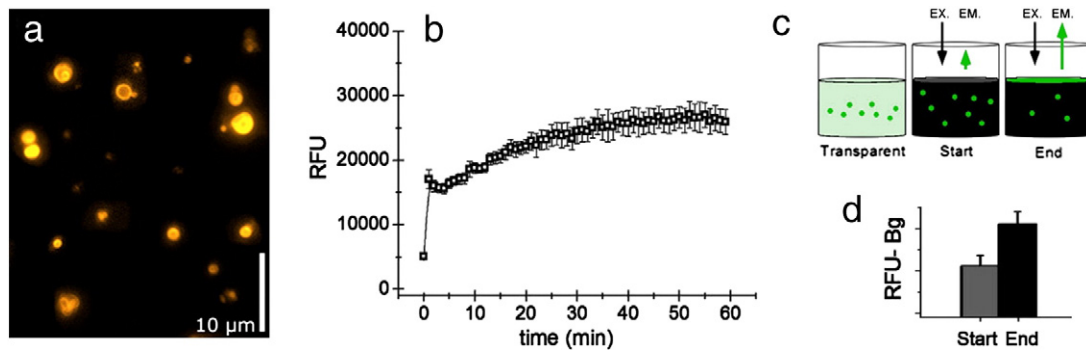


Fig. 1. Measurement of interfacial adsorption and accumulation of LBPs. a) Suspension of LBPs viewed by a lipophilic fluorescent probe (FM 1–43, 470 nm EX., >510 nm EM.). LBPs appear as compact or partially unraveled globular particles. b) Typical raw fluorescence data as obtained by the adsorption assay (details see text). Time zero corresponds to background measurement (wells with BB before addition of Bodipy labeled LBPs). c) Explanation of measurement principle. Transparent: wells contain Bodipy (light green) + LBPs (dark green circles) w/o BB. This control was necessary to test and to eventually correct for unspecific effects (e.g. various buffer pH values) on the intrinsic fluorescence intensity of Bodipy. Start: corresponds to the second time point in b, directly after addition of Bodipy labeled LBPs. End: Plateau phase in b with stable surface associated film (green). EX, excitation, and EM, emission (both from top). d) Data representation. Background corrected RFU values (RFU-Bg) are shown for the start and endpoints, respectively.

2.4. LBPs quantification

Phospholipid concentration of the supernatants was measured by a lipid chloroform/methanol extraction protocol followed by choline determination using a coupled enzymatic reaction according to published references [35], with slight modifications: For the lipid extraction we used 250 µl sample (LBPs) and added 940 µl chloroform/methanol (1:2) solution, followed by a strong vortex step (1 min). Afterwards, 310 µl chloroform was added and samples were subsequently mixed by short vortexing. Finally, 310 µl of distilled water was added and again intensely mixed (vortexing). Samples were centrifuged for 10 min at 13,000 rpm, and subsequently the lower organic phase containing the lipids was extracted and filled in fresh vials. Samples were dried overnight in the hood. Next day, α -lecithin standards and dried samples were rediluted in reaction buffer of the Amplex Red Phospholipase D Assay Kit (Invitrogen) and thereafter 100 µl of the diluted samples/standards was mixed with 100 µl reaction mix containing: phospholipase D 1 U/ml, choline oxidase 0.2 U/ml, horseradish peroxidase 2 U/ml, and Amplex Red 100 µM. Samples were measured in a 96-well plate in a multiplate reader (TECAN M200pro, Tecan, Switzerland) by using 590 nm \pm 20, 540 \pm 9 nm for excitation and emission, respectively.

2.5. Surfactant labeling

BODIPY-PC was dissolved in DMSO to yield a concentration of 1 mg/ml. LBPs were stained by incubation with BODIPY-PC at 37 °C for 2 h to obtain a final molar ratio of 4% (dye/surfactant) [48].

2.6. Adsorption kinetics

Experiments were performed using an assay system specifically designed to evaluate interfacial adsorption of LBPs. Moreover, it has been used to compare adsorption of LBPs with standard surfactant materials like Curosurf and native surfactant purified from porcine lungs. It provides a direct readout of the amount of surfactant reaching the interface but also of the material, which stably associates with the forming interfacial film. This method is described in detail in a previous publication [48]. Briefly, the wells of a 96-well microplate (sterile, flat, transparent Cat.# 655185, Greiner, Germany) were filled with 100 µl of a solution containing 5 mg/ml Brilliant Black as a photoquencher, adjusted to the conditions of interest (pH, Ca^{2+} , osmotic strength, temperature and albumin concentration; Fig. 1).

The plate, without lid, was inserted into the microplate reader (TECAN GENios Plus, Switzerland) and all measurements were done using 485 \pm 9 nm and 540 \pm 9 nm with following standard settings:

number of flashes 3, lag time 0 µs, and integrations time 1000 µs for excitation and emission, respectively, on the top reading mode (i.e. fluorescence excitation and emission is performed from the top of the wells). Firstly, unspecific effects of the test variables (pH, Ca^{2+} , osmotic strength, temperature, albumin concentration) on the intrinsic BODIPY-PC fluorescence were evaluated by measuring, in a separate plate row, the 4% BODIPY-PC loaded LBPs in the respective transparent bath solution (Fig. 1c, transparent). Secondly, one background measurement (Brilliant Black only), was obtained (Fig. 1b, time 0). Thirdly, the 96-well plate was moved out and 0.5 µg of 4% BODIPY-PC (dye/surfactant) labeled surfactant distributed within the well by orbital shaking, and was finally irreversibly (because resistant to intermittent orbital shaking) adsorbed into the ALF, where the fluorescence signal was detected and quantified kinetically. Data were normalized with respect to the transparent values and by subtracting the background.

2.7. Data analysis and data presentation

MS Excel and Origin8 were used for statistics, curve fitting and graph design. Unless otherwise stated, all data are presented as mean \pm SEM. RFU values tested in physiological conditions (blue curves/bars) were set as control values. Significance was tested using a student's *t*-test. Significant different values were indicated with **P* < 0.05, ***P* < 0.01 and ****P* < 0.001.

3. Results

3.1. Effect of pH on the interfacial adsorption and accumulation of LBPs

The pH in the ALF of healthy lungs is presumably the same as in the capillaries surrounding the alveoli, between the values of venous (pH 7.36) and arterial (pH 7.4) blood. In airways, it is reported that abnormalities can occur during exo- and endogenous airways acidification/alkalinization (e.g. pollution, cystic fibrosis, COPD) irrespective of their etiology [36]. These critical factors may affect the pH in the ALF, and thus, they may alter the electrostatic charges and surface potential of the ALF. However, there are only few reports of direct pH measurements in the ALF, all demonstrating slightly acidic conditions: e.g. pH 6.9 [38,39,43] but no physiological explanation thereof has been given up to now. As shown in Fig. 2, acidic conditions completely blocked the formation of an interfacial surfactant layer. To exclude a false negative result, we carefully tested whether BODIPY-PC fluorescence is sensitive to low pH, but this was not the case (data not shown). In

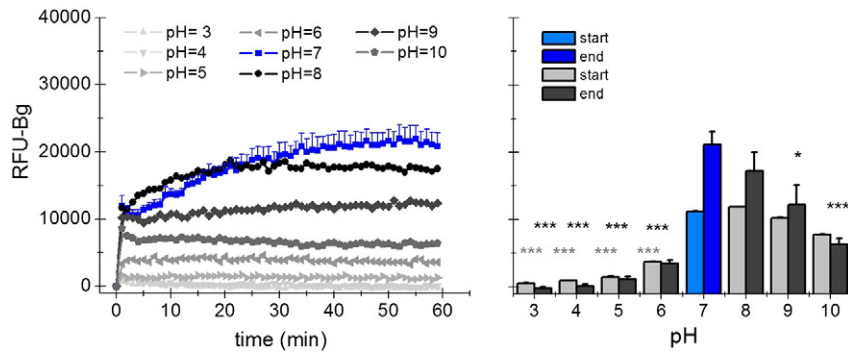


Fig. 2. Effect of pH (pH 3–10) on LBPs. Left: Adsorption kinetics was measured for 60 min, the ctrl condition is indicated in blue. Right: Startpoints (light gray bars) and endpoints (dark gray bars) are shown. Maximal adsorption occurred at pH 7, acidic (pH ≤ 6) and alkaline conditions (pH ≥ 9) significantly blocked LBPs adsorption.

contrast, maximum surface activity was achieved in the alkaline regime, starting abruptly at pH of 7 with a following decrease that was significant at pH ≥ 9. In a following series of experiments (Fig. 3), we tested pH values that are close within the physiological range (pH 6.2–7.8). Slightly acidic solutions (pH 6.0–6.6) significantly reduced surfactant adsorption, whereas pH values of 7.0, 7.2 (n.s.), 7.8, and 8.0 promoted it. As shown in Fig. 3, the pH influenced surfactant adsorption in a biphasic manner, suggesting that the acid–base status might have high significance in lung function.

3.2. Effect of calcium

The physiological Ca²⁺ concentration in the alveoli is reported to be 1.5 mM [9,40], and thus, almost identical to plasma values (free Ca²⁺ concentration of about 1.25 mM). As for the pH, no data exist on the possible range of concentrations in health and disease. Therefore, we used Ca²⁺/EGTA buffered solutions between 0 and 5 mM. According to our results, adsorption of LBPs was highest at a Ca²⁺ concentration of 0.5 mM. By estimating initial adsorption rates as the slope between the time points 1 and 3 min, we found that at 0.5 mM Ca²⁺ LBPs had a faster adsorption rate in the beginning and reached a plateau phase at 25 min. High calcium concentrations (5 mM) attenuated the adsorption kinetics. At this concentration the adsorption rate was significantly decreased, reaching a plateau at 25 min, which was followed by a signal reduction, probably due to partial desorption of surfactant material. Notably, a Ca²⁺ concentration of zero had no significant effect on the spreading rate of LBPs (Fig. 4).

3.3. Effect of osmotic strength

The actual osmolarity of the ALF in healthy and diseased lungs is subject to controversy [18,51,63]. A reasonable assumption for ALF

osmolarity would be ~300 mOsm, about the same value than in blood if passively regulated by Starling factors. However, due to the fact that the alveolar epithelium is both, a secretory and resorptive tissue, large deviations from this value could be expected as well. In addition, very hypotonic and hypertonic conditions may occur during drowning of fresh water and saltwater, for example. Here, we prepared solutions between 0 and 600 mOsm by adjusting the NaCl concentration. As control we used a physiological saline with 300 mOsm. As can be seen in Fig. 5, surfactant material adsorbs rapidly at the interface at low ionic strength, while a reduction in the adsorption rate was observed at the highest osmolarity values tested. Maximal adsorption occurred at 20 mOsm, and a minimum at 300 mOsm. At 20 mOsm we calculated a steep, initial and almost linear slope up to 15 min that was followed by a slight constant increase till the end of the measurement (Fig. 5).

3.4. Thermotropic effects

Pulmonary surfactant function is very sensitive to temperature, which has been interpreted as a consequence of optimization of the composition of surfactant membranes close to a critical transition. Surfactant would thus simultaneously optimize a dynamic behavior required for proper adsorption and re-spreading along the breathing cycles and enough stability to sustain maximal pressures at the end of exhalation [44,46]. Numerous studies have confirmed the existence of physiological mechanisms to ensure proper matching between surfactant composition, structure and physiological temperature. For instance, the group of Orgeig [59] analyzed native surfactants from heterothermic animals to demonstrate that surfactant composition shifts to adapt to lower body temperatures, probably to compensate the loss of function. Whether such a temperature–function coupling is also pertinent in freshly secreted LBPs collected from rats (a homeothermic mammal) has not been determined yet. Temperature effects on the interfacial

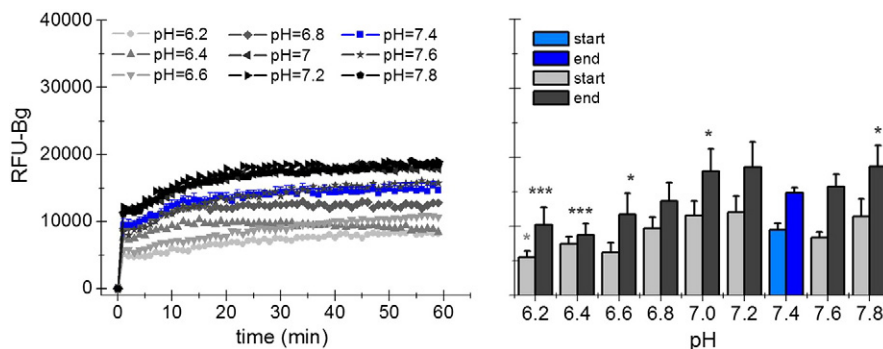


Fig. 3. Effect of pH (pH 6.2–7.8) on LBPs. Left: Adsorption kinetics was measured for 60 min, the ctrl condition is indicated in blue. Right: Startpoints (light gray bars) and endpoints (dark gray bars) are shown. Maximal adsorption was occurred at pH 7.0 and 7.8, acidic conditions (pH 6.2 and 6.4) significantly reduced LBPs adsorption.

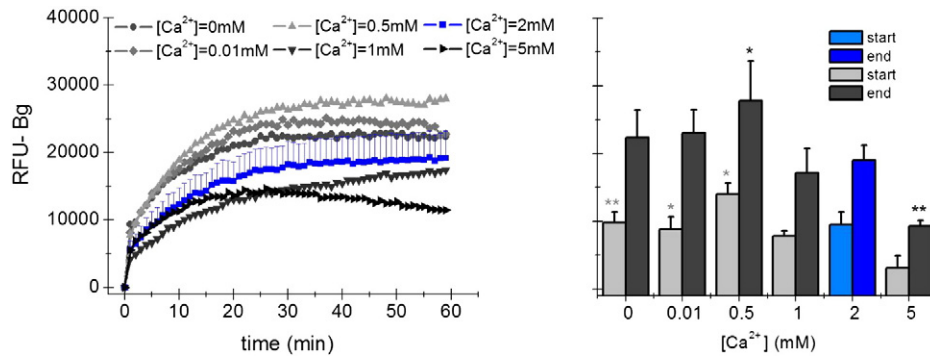


Fig. 4. Effect of Ca^{2+} concentration (0–5 mM). Left: Adsorption kinetics was measured for 60 min, the ctrl condition is indicated in blue. Right: Startpoints (light gray bars) and endpoints (dark gray bars) are shown. Maximal adsorption was occurred at 0.5 mM Ca^{2+} , highest Ca^{2+} concentration (5 mM) significantly reduced LBPs adsorption.

accumulation of LBPs were measured within individual wells, at the indicated T points (15 °C, 25 °C, 30 °C 37 °C and 42 °C) and not unexpectedly, temperatures below the body temperature significantly diminished surfactant adsorption rates and the same accounts for the initial adsorption rates at 42 °C (Fig. 6).

3.5. Effect of the presence of proteins

In ARDS, where the integrity of the alveolar-capillary permeability barrier is impaired, albumin concentrations in the alveolar fluid may reach 100 mg/ml, with an average of 25 mg/ml as reported by Ishizaka and coworkers [29]. We therefore tested the effect of the presence of albumin on the interfacial adsorption and accumulation of material from LBPs. The concentrations of albumin tested here are significantly lower than those typically found in ARDS patients, since we found that they are still sufficient to inhibit adsorption of LBPs. Our results revealed a dose-dependent decrease in surface activity and a complete block at 2.5 mg/ml albumin (Fig. 7). These results are entirely in agreement with many other reports using various other surfactant materials [12,27,62].

3.6. Stability of LPBs

LBPs contain several different proteins, whereas the hydrophobic surfactant proteins SP-B and, to a lesser extent, SP-C play crucial roles in surface film formation and stability during film expansion and compression [44,46]. Furthermore, we hypothesized that the strong pH-dependency in LBP adsorption (Figs. 2 and 3) might reflect the contribution of pH-sensitive protein conformations or protein–lipid interactions that are important for the adsorption of surfactant at the A_{Lr} . Consequently, any modification of the proper conformation of these proteins should

have a demonstrable effect. Thus, we used three different methods to disrupt protein integrity in LBPs: trypsin proteolysis, mild urea treatment [6] and mild sonification.

LBPs treated with 1% trypsin indeed showed a complete block in adsorption, whereas 1 M urea leads to an increased film formation by LBPs. However pre-exposure of LBPs to cavitation-induced mechanical forces (sonification with an ultrasonic bath from Elma, T420, with 70 peak/W high frequency) had no effect on their adsorption kinetics (Fig. 8).

4. Discussion

The generally accepted view of the ALF is that of an exceedingly thin, but most likely continuous layer of water, ranging in thickness from several nanometers to micrometers with a tendency to be the thickest in the septal corners [17]. Owing to the difficulties encountered in analyzing such a thin and dynamic layer and to sample it independently from airway fluids, its physical state and chemical composition are not exactly known. Taking together the few available studies, the fluid volume of the hypophase is estimated to be ~0.1–5 ml/kg for humans, with a lipid concentration of ~50 mg/ml [60] a Ca^{2+} concentration of ~1.5 mM and a pH of ~6.9 in healthy lungs [9,34,39]. An even greater lack of information exists with respect to the possible deviations of those parameters in pathophysiological situations and various lung diseases. Due to that, most in vitro studies on biophysical surfactant properties have been performed within a small set of standardized conditions, in spite that alterations in the hypophase could have drastic impacts on the surfactant film formation and its performance at the A_{Lr} . Here, we explored the potential effect of changes in the ALF during diseased and healthy conditions on the dispersion, film formation and accumulation of surfactant material as it is secreted by pneumocytes.

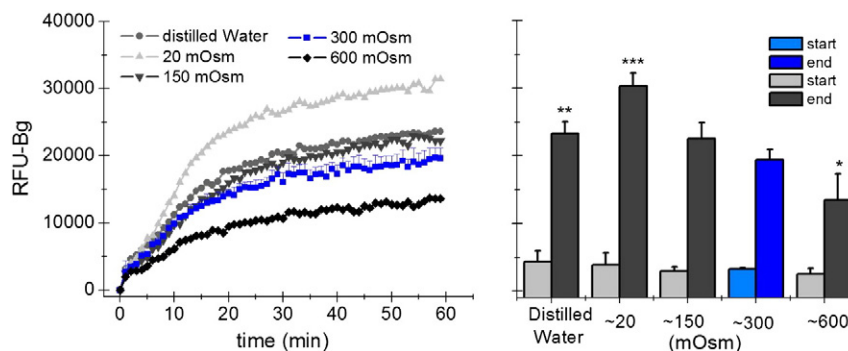


Fig. 5. Effect of osmotic strength (0–300 mOsm). Left: Adsorption kinetics was measured for 60 min, the ctrl condition is indicated in blue. Right: Startpoints (light gray bars) and endpoints (dark gray bars) are shown. Maximal adsorption occurred at 20 mOsm, and a minimum at 300 mOsm.

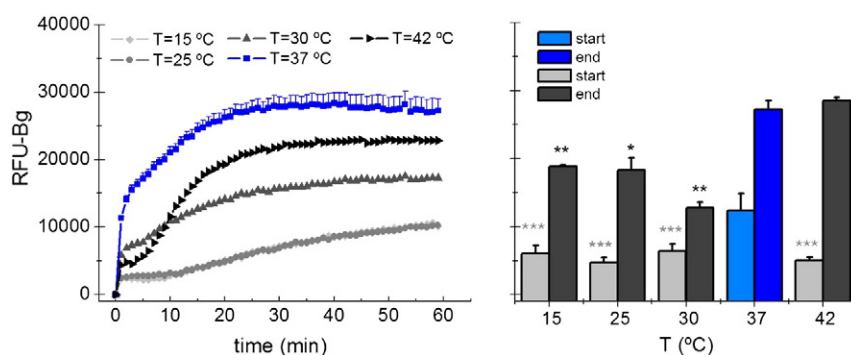


Fig. 6. Thermotropic effect. Left: Adsorption kinetics was measured for 60 min, the ctrl condition is indicated in blue. Right: Startpoints (light gray bars) and endpoints (dark gray bars) are shown. Maximal adsorption occurred at 37 °C (body temperature), temperatures below body temperature diminished surfactant adsorption rates.

Recently, two studies reported a unique adsorption behavior of freshly collected LBPs and demonstrated that native LBPs form highly dynamic interfacial films with defined 2- and 3-D complexities [50]. These results suggest that surfactant may be transferred to the A_{LI} in the same compact form as it is released by the cells. This model gains additional plausibility considering the fact that the mean size of LBPs (1–3 μm) even surmounts the average thickness of the ALF (0.1 μm ; [5]), which means that LBPs may adsorb to the A_{LI} almost at the instance of extrusion through the exocytotic fusion pore. Despite the fact that the distance between the site of cellular release and the A_{LI} is ultimately small, and the transit time probably ultimately fast, these particles are exposed to the particular environmental conditions prevailing in the ALF, which effects have been simulated in this study.

In vivo, pulmonary surfactant presents in manifold other structural entities lacking a morphological similarity with compact LBPs, e.g. tubular myelin. Based on several lines of evidence, it can be concluded that these structures are metabolically related to LBPs. However, it remains an open question, whether tubular myelin is actually an extracellular reservoir of surface material preceding adsorption or formed secondarily as a consequence of dynamic film expansion and compression, or by interaction with surfactant proteins already present in the A_{LI} , notably SP-A (discussed in [17]). Hence, we concentrated our study at those structures (LBPs), which are definitely as close as possible at the start of the sequence of transformation processes, directly after release out of the cells. In contrast to conventional methods used for evaluating surface properties of pulmonary surfactant preparations, such as captive bubble surfactometry or Langmuir balances, our method enables a direct readout of the amount of surfactant adsorbing at the interface, including the functionally important amount of material firmly associated with the interfacial film, in a medium-high sample throughput manner. However, our assay has limitations, because neither direct

measurements of surface tension nor modulations of the surface area are possible.

4.1. Effect of pH

Little is known about the acid–base status of the ALF, probably due to the metrological inaccessibility of the hyperthin and dynamic fluid layer [22,36,39]. Here, we describe a strong inhibitory effect of an acidic milieu on LBPs adsorption. In an earlier study using a captive bubble surfactometer, natural porcine surfactants containing surfactant proteins showed, in contrast to artificial products, a relatively stable activity over a broad pH-range [2]. Another study reported that the ability of porcine native surfactant, and of preparations reconstituted from its organic extract, to form surface active films and reduce surface tension to equilibrium, was impaired at alkaline but not at acidic pH [13]. It is important to note that the fluorescent method used in the present experiments detects not only the interfacial adsorption of the limited amount of material required to reduce surface tension to near equilibrium (~25 mN/m) but also the accumulation of an excess of material to likely form a surface-associated reservoir. It could be possible that the ultimate transfer of surface active lipids from the LBPs to the interface is not so much altered at acidic pH, but the ability of LBPs to stably maintain an association with the surface film. This should be explored further in future work, because it is thought that the ability of an operative surfactant to form dense surface multilayers is important to sustain interfacial stability along successive compression–expansion cycles [8,46,55,57]. The relative impairment of interfacial adsorption at alkaline pH has been previously attributed to the possible neutralization of positive charges in SP-B and SP-C and the consequent loss of electrostatic interactions with anionic lipids [13]. This could be also the reason for the impairment of surface activity of LBPs. The dramatic loss of surface

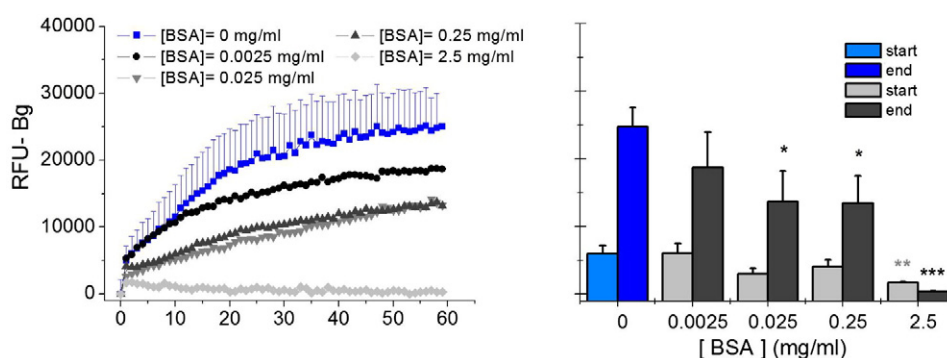


Fig. 7. Effect of albumin protein concentration (0–2.5 mg/ml). Left: Adsorption kinetics was measured for 60 min, the ctrl condition is indicated in blue. Right: Startpoints (light gray bars) and endpoints (dark gray bars) are shown. Albumin concentration inhibited LBPs adsorption rate in a dose-dependent manner.

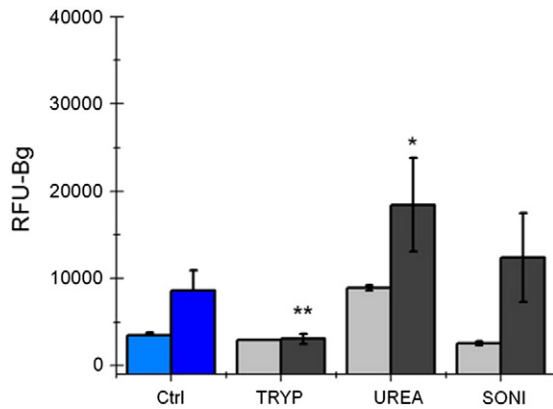


Fig. 8. Stability of LBPs. Adsorption of LBPs pretreated with 1% trypsin, 1 M urea and sonication (70 peak/W) respectively. Proteolysis of LBPs significantly decreases their adsorptive capacity.

adsorption capabilities of LBPs at acidic pH could be related with the particular structure of these assemblies, once they are secreted to the extracellular milieu. It has been proposed that a progressive acidification of pH could be important for the maturation and assembly of surfactant membranes in the LBs, and that the neutralization of pH upon secretion of LBs might convert LBPs into an adsorption competent state [46,53]. Acidification of LBP environment could therefore revert some of these possible pH-triggered changes, making LBPs less prone to unpack and spread upon adsorption at the interface. Our results thus underline the crucial role of pH in the ALF and lay emphasis on the critical monitoring of this parameter when evaluating surfactant function. Further, these results may have implications to understand the possible involvement of surfactant inactivation in patients suffering of acid induced lung injury.

4.2. Effect of Ca^{2+}

During the first breath, the Ca^{2+} concentration in ALF increases rapidly [16,37]. This extracellular Ca^{2+} rise seems to be the main source for increased lung surfactant secretion [20,26,49]. Further, it is reported that Ca^{2+} is required for tubular myelin assembly and stabilization in the subphase, and enhances surfactant film formation at the A_{LI} by interacting with the surfactant proteins and phospholipids [7,61]. Here, we demonstrate that adsorption kinetics of LBPs is unaffected by Ca^{2+} concentrations between 0 and 2 mM, while we observed an inhibitory effect at unphysiologically high concentrations (5 mM). This result is in agreement with earlier studies using the captive bubble, which revealed that functionality of native porcine surfactant is relatively independent on subphase Ca^{2+} concentration [9]. Thus, we propose that a rapid Ca^{2+} rise after birth has its main physiological relevance in the stimulation of surfactant secretion rather than in promoting adsorption efficiency.

4.3. Effect of osmolarity

Several studies described the epithelial lining fluid solute concentration in healthy conditions and different lung diseases like COPD, cystic fibrosis, and bronchial asthma [1,18,30,47]. For example in COPD patients epithelial lining fluid was isotonic (cations concentration ~150 mM), and not significantly different from normal subjects [18]. In contrast, Quinton et al. reported a hypotonic condition in the lungs from healthy patients and an isotonic lining fluid in patients with cystic fibrosis [47]. It is also reported that, in cystic fibrosis, hypertonic saline increases the volume of airway surface liquid, restores mucus clearance, and improves lung function. However, no data exist on surfactant function under varying osmotic conditions. Furthermore, whether such osmotic changes might actually occur in the distal lung during e.g.

lung disease induced hyperventilation and associated evaporative water loss is still under dispute (see discussion in [15]). In this study on adsorption of LBPs, we indeed observed a strong osmotic effect, as hypotonic conditions promoted surfactant film formation, whereas hypertonic conditions had the opposite effect. We speculate that high ionic strength could impair critical lipid–protein or protein–protein electrostatic interactions that could be of importance to sustain LBP adsorption and transformation at the interface.

4.4. Effect of temperature

Body temperature in humans, as in most homeothermic mammals, is tightly regulated between 36.5 and 37.5 °C, but it might change significantly during high or severe hypothermia. Values of 16 °C body temperature have been documented in accidentally hypothermic people with successful resuscitation. During such condition, also the temperature in ALF changes significantly, which might cause an impairment of surfactant function [23]. Several studies already measured temperature effect on surfactant activity [12,59]. As expected, normal body temperature (37 °C) favors LBPs adsorption, whereas cooling leads to a significant reduction. It has been proposed that surfactant composition, including practically equivalent proportions of saturated and unsaturated phospholipids, may have evolved to sustain a coexistence of ordered and disordered phases at physiological temperatures. These coexisting phases would permit surfactant to simultaneously sustain two apparently contradictory properties: a highly dynamic character, necessary for very rapid adsorption and re-spreading at the interface, and enough stability during compression at exhalation [4,14,44]. Adaptations to seasonal low body temperature in hibernating animals actually include changes in lipid composition to reduce the level of phospholipid saturation in order to maintain rapid enough interfacial adsorption [59]. A reduced accumulation of LBP material at low temperature could then have to do with a reduced fluidity of the most dynamic phases.

4.5. Protein inhibition and LBs stability

Inactivation of surfactant by serum leakage from the capillaries during lung edema or acute lung injury is well documented in clinical and biophysical studies [28,33,62,65]. Here, we have shown that a small amount of BSA can also lead to a dramatic reduction of the capabilities of LBPs to adsorb at the A_{LI} , confirming that leakage of serum into the alveolar spaces may also compromise the transfer of freshly secreted surfactant into a surface active film. The inhibitory action of serum proteins on the adsorption of different natural and clinical surfactants has usually been interpreted as a consequence of the establishment of a steric and electrostatic barrier between the surfactant and the interface [62,65]. It remains to be established, whether inhibition by serum of LBP adsorption is also due to a generic competitive effect towards reaching the interface, or whether some of the components leaked into serum could inhibit more specifically the machinery in charge of “opening” and unraveling LBPs. To obtain additional information about possible differences in the adsorption “machineries” of LBPs and other surfactants, and their potential susceptibility to inactivating environmental factors, we observed LBPs in respect to their sensitivity to agents potentially affecting protein integrity. Trypsination and thus full cleavage of the surfactant material completely blocked accumulation of LBP material at the A_{LI} , whereas urea-promoted soft denaturation even speeds up the adsorption rate. These findings support the general hypothesis that surfactant proteins are crucial and irreplaceable in surfactant film formation, even if they are available in low fraction like in Curosurf (containing ~3.2 µg SP-B/µmol phospholipid and ~10.1 µg SP-C/µmol phospholipid [9]). Surfactant proteins are therefore absolutely required for triggering and promoting sufficient interfacial surfactant accumulation.

In summary, our results show that adsorption of LBPs and formation of stably surface associated films is highly sensitive to pH, temperature

and protein concentration. The systematic analysis by this sensitive method of the capability of samples taken from patients to adsorb and accumulate at the interface could therefore provide clues on the potential contribution of surfactant impaired performance to different pathologies. These findings, besides their relevance in lung physiology and pathophysiology, may also have some implications in the design and application of new surfactant compositions and surfactant therapies.

Contributions

NH, TH, JPG and AR designed the study. VB, NH, AR and GS performed the experiments, analyzed the data and created the graphs. NH, TH, JPG wrote the manuscript.

Ethical principle

This study was reviewed and approved by the Austrian Government, and cell preparations were conducted in conformity with the Austrian rules for animal care and testing.

Competing financial interests

The authors declare no competing financial interests.

Acknowledgments

This work was supported by the Austrian Science Fund P 204720 and the Tyrolean Science Fund, the Spanish Ministry of Economy and Competitively (Grants BIO2012-30733, CSD2007-00010) and the Regional Government of Madrid (Grant S2009MAT-1507). Co-author Andrea Ravasio is currently working at the Mechanobiology Institute, Singapore National University, Singapore 11741.

References

- [1] T.M. Adamson, R.D. Boyd, H.S. Platt, L.B. Strang, Composition of alveolar liquid in the foetal lamb, *J. Physiol.* 204 (1969) 159–168.
- [2] J.D. Amirkhanian, T.A. Merritt, The influence of pH on surface properties of lung surfactants, *Lung* 173 (1995) 243–254.
- [3] H. Bachofen, S. Schurch, Alveolar surface forces and lung architecture, *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 129 (2001) 183–193.
- [4] L.A. Bagatolli, To see or not to see: lateral organization of biological membranes and fluorescence microscopy, *Biochim. Biophys. Acta* 1758 (2006) 1541–1556.
- [5] J. Bastacky, C.Y. Lee, J. Goerke, H. Koushafar, D. Yager, L. Kenaga, T.P. Speed, Y. Chen, J.A. Clements, Alveolar lining layer is thin and continuous: low-temperature scanning electron microscopy of rat lung, *J. Appl. Physiol.* 79 (1995) 1615–1628.
- [6] B.J. Bennion, V. Daggett, The molecular basis for the chemical denaturation of proteins by urea, *Proc. Natl. Acad. Sci. U. S. A.* 100 (2003) 5142–5147.
- [7] B.J. Benson, M.C. Williams, K. Sueishi, J. Goerke, T. Sargeant, Role of calcium ions the structure and function of pulmonary surfactant, *Biochim. Biophys. Acta* 793 (1984) 18–27.
- [8] J. Bernardino de la Serna, R. Vargas, V. Picardi, A. Cruz, R. Arranz, J.M. Valpuesta, L. Mateu, J. Perez-Gil, Segregated ordered lipid phases and protein-promoted membrane cohesivity are required for pulmonary surfactant films to stabilize and protect the respiratory surface, *Faraday Discuss.* 161 (2013) 535–548.
- [9] W. Bernhard, J. Mottaghian, A. Gebert, G.A. Rau, H. von der Hardt, C.F. Poets, Commercial versus native surfactants. Surface activity, molecular components, and the effect of calcium, *Am. J. Respir. Crit. Care Med.* 162 (2000) 1524–1533.
- [10] O. Blanco, A. Cruz, O.L. Ospina, E. Lopez-Rodriguez, L. Vazquez, J. Perez-Gil, Interfacial behavior and structural properties of a clinical lung surfactant from porcine source, *Biochim. Biophys. Acta* 1818 (2012) 2756–2766.
- [11] O. Blanco, J. Perez-Gil, Biochemical and pharmacological differences between preparations of exogenous natural surfactant used to treat respiratory distress syndrome: role of the different components in an efficient pulmonary surfactant, *Eur. J. Pharmacol.* 568 (2007) 1–15.
- [12] R. Bruni, B.R. Fan, R. David-Cu, H.W. Tausch, F.J. Walther, Inactivation of surfactant in rat lungs, *Pediatr. Res.* 39 (1996) 236–240.
- [13] L. Camacho, A. Cruz, R. Castro, C. Casals, J. Perez-Gil, Effect of pH on the interfacial adsorption activity of pulmonary surfactant, *Colloids Surf. B: Biointerfaces* 5 (1996) 271–277.
- [14] C. Casals, O. Canadas, Role of lipid ordered/disordered phase coexistence in pulmonary surfactant function, *Biochim. Biophys. Acta* 1818 (2012) 2550–2562.
- [15] M.S. Davis, E. Daviskas, S.D. Anderson, Airway surface fluid desiccation during isocapnic hyperpnea, *J. Appl. Physiol.* 94 (2003) 2545–2546.
- [16] P. Dietl, M. Frick, N. Mair, C. Bertocchi, T. Haller, Pulmonary consequences of a deep breath revisited, *Biol. Neonate* 85 (2004) 299–304.
- [17] P. Dietl, T. Haller, Exocytosis of lung surfactant: from the secretory vesicle to the air-liquid interface, *Annu. Rev. Physiol.* 67 (2005) 595–621.
- [18] R.M. Effros, B. Peterson, R. Casaburi, J. Su, M. Dunning, J. Torday, J. Biller, R. Shaker, Epithelial lining fluid solute concentrations in chronic obstructive lung disease patients and normal subjects, *J. Appl. Physiol.* 99 (2005) 1286–1292.
- [19] H. Fehrenbach, Alveolar epithelial type II cell: defender of the alveolus revisited, *Respir. Res.* 2 (2001) 33–46.
- [20] M. Frick, C. Bertocchi, P. Jennings, T. Haller, N. Mair, W. Singer, W. Pfaller, M. Ritsch-Marte, P. Dietl, Ca²⁺ entry is essential for cell strain-induced lamellar body fusion in isolated rat type II pneumocytes, *Am. J. Physiol. Lung Cell. Mol. Physiol.* 286 (2004) L210–L220.
- [21] T. Fujiwara, H. Maeta, S. Chida, T. Morita, Y. Watabe, T. Abe, Artificial surfactant therapy in hyaline-membrane disease, *Lancet* 1 (1980) 55–59.
- [22] J. Goerke, Pulmonary surfactant: functions and molecular composition, *Biochim. Biophys. Acta* 1408 (1998) 79–89.
- [23] M. Griese, Pulmonary surfactant in health and human lung diseases: state of the art, *Eur. Respir. J.* 13 (1999) 1455–1476.
- [24] T. Haller, K. Auktor, M. Frick, N. Mair, P. Dietl, Threshold calcium levels for lamellar body exocytosis in type II pneumocytes, *Am. J. Physiol.* 277 (1999) L893–L900.
- [25] T. Haller, P. Dietl, H. Stockner, M. Frick, N. Mair, I. Tinhofer, A. Ritsch, G. Enhorning, G. Putz, Tracing surfactant transformation from cellular release to insertion into an air-liquid interface, *Am. J. Physiol. Lung Cell. Mol. Physiol.* 286 (2004) L1009–L1015.
- [26] N. Hobi, A. Ravasio, T. Haller, Interfacial stress affects rat alveolar type II cell signaling and gene expression, *Am. J. Physiol. Lung Cell. Mol. Physiol.* 303 (2012) L117–L129.
- [27] B.A. Holm, G. Enhorning, R.H. Notter, A biophysical mechanism by which plasma proteins inhibit lung surfactant activity, *Chem. Phys. Lipids* 49 (1988) 49–55.
- [28] B.A. Holm, R.H. Notter, J.N. Finkelstein, Surface property changes from interactions of albumin with natural lung surfactant and extracted lung lipids, *Chem. Phys. Lipids* 38 (1985) 287–298.
- [29] A. Ishizaka, T. Matsuda, K.H. Albertine, H. Koh, S. Tasaka, N. Hasegawa, N. Kohno, T. Kotani, H. Morisaki, J. Takeda, M. Nakamura, X. Fang, T.R. Martin, M.A. Matthay, S. Hashimoto, Elevation of KL-6, a lung epithelial cell marker, in plasma and epithelial lining fluid in acute respiratory distress syndrome, *Am. J. Physiol. Lung Cell. Mol. Physiol.* 286 (2004) L1088–L1094.
- [30] S. Jayaraman, Y. Song, A.S. Verkman, Airway surface liquid osmolality measured using fluorophore-encapsulated liposomes, *J. Gen. Physiol.* 117 (2001) 423–430.
- [31] T. Kobayashi, K. Ohta, K. Tashiro, K. Nishizuka, W.M. Chen, S. Ohmura, K. Yamamoto, Dextran restores albumin-inhibited surface activity of pulmonary surfactant extract, *J. Appl. Physiol.* 86 (1999) 1778–1784.
- [32] T.R. Korfhagen, M.D. Bruno, G.F. Ross, K.M. Huelsman, M. Ikegami, A.H. Jobe, S.E. Wert, B.R. Stripp, R.E. Morris, S.W. Glasser, C.J. Bachurski, H.S. Iwamoto, J.A. Whitsett, Altered surfactant function and structure in SP-A gene targeted mice, *Proc. Natl. Acad. Sci. U. S. A.* 93 (1996) 9594–9599.
- [33] E. Lopez-Rodriguez, O.L. Ospina, M. Echaide, H.W. Tausch, J. Perez-Gil, Exposure to polymers reverses inhibition of pulmonary surfactant by serum, meconium, or cholesterol in the captive bubble surfactometer, *Biophys. J.* 103 (2012) 1451–1459.
- [34] R.L. Lubman, S.I. Danto, E.D. Crandall, Evidence for active H⁺ secretion by rat alveolar epithelial cells, *Am. J. Physiol.* 257 (1989) L438–L445.
- [35] M.N. Nanjee, A.K. Gebre, N.E. Miller, Enzymatic fluorometric procedure for phospholipid quantification with an automated microtiter plate fluorometer, *Clin. Chem.* 37 (1991) 868–874.
- [36] A.W. Ng, A. Bidani, T.A. Heming, Innate host defense of the lung: effects of lung-lining fluid pH, *Lung* 182 (2004) 297–317.
- [37] T.E. Nicholas, J.H. Power, H.A. Barr, The pulmonary consequences of a deep breath, *Respir. Physiol.* 49 (1982) 315–324.
- [38] D.W. Nielson, Electrolyte composition of pulmonary alveolar subphase in anesthetized rabbits, *J. Appl. Physiol.* 60 (1986) 972–979.
- [39] D.W. Nielson, J. Goerke, J.A. Clements, Alveolar subphase pH in the lungs of anesthetized rabbits, *Proc. Natl. Acad. Sci. U. S. A.* 78 (1981) 7119–7123.
- [40] D.W. Nielson, M.B. Lewis, Calcium increases in pulmonary alveolar fluid in lambs at birth, *Pediatr. Res.* 24 (1988) 322–325.
- [41] M. Ochs, The closer we look the more we see? Quantitative microscopic analysis of the pulmonary surfactant system, *Cell. Physiol. Biochem.* 25 (2010) 27–40.
- [42] B. Olmeda, L. Villen, A. Cruz, G. Orellana, J. Perez-Gil, Pulmonary surfactant layers accelerate O₂ diffusion through the air-water interface, *Biochim. Biophys. Acta* 1798 (2010) 1281–1284.
- [43] R.E. Oliver, L.B. Strang, Ion fluxes across the pulmonary epithelium and the secretion of lung liquid in the foetal lamb, *J. Physiol.* 241 (1974) 327–357.
- [44] J. Perez-Gil, Structure of pulmonary surfactant membranes and films: the role of proteins and lipid-protein interactions, *Biochim. Biophys. Acta* 1778 (2008) 1676–1695.
- [45] J. Perez-Gil, K.M. Keough, Interfacial properties of surfactant proteins, *Biochim. Biophys. Acta* 1408 (1998) 203–217.
- [46] J. Perez-Gil, T.E. Weaver, Pulmonary surfactant pathophysiology: current models and open questions, *Physiology* 25 (2010) 132–141.
- [47] P.M. Quinton, Physiological basis of cystic fibrosis: a historical perspective, *Physiol. Rev.* 79 (1999) S3–S22.
- [48] A. Ravasio, A. Cruz, J. Perez-Gil, T. Haller, High-throughput evaluation of pulmonary surfactant adsorption and surface film formation, *J. Lipid Res.* 49 (2008) 2479–2488.
- [49] A. Ravasio, N. Hobi, C. Bertocchi, A. Jesacher, P. Dietl, T. Haller, Interfacial sensing by alveolar type II cells: a new concept in lung physiology? *Am. J. Physiol. Cell Physiol.* 300 (2011) C1456–C1465.

- [50] A. Ravasio, B. Olmeda, C. Bertocchi, T. Haller, J. Perez-Gil, Lamellar bodies form solid three-dimensional films at the respiratory air–liquid interface, *J. Biol. Chem.* 285 (2010) 28174–28182.
- [51] S.I. Rennard, G. Basset, D. Lecossier, K.M. O'Donnell, P. Pinkston, P.G. Martin, R.G. Crystal, Estimation of volume of epithelial lining fluid recovered by lavage using urea as marker of dilution, *J. Appl. Physiol.* 60 (1986) 532–538.
- [52] B. Robertson, H.L. Halliday, Principles of surfactant replacement, *Biochim. Biophys. Acta* 1408 (1998) 346–361.
- [53] M.L. Ruano, K. Nag, L.A. Worthman, C. Casals, J. Perez-Gil, K.M. Keough, Differential partitioning of pulmonary surfactant protein SP-A into regions of monolayers of dipalmitoylphosphatidylcholine and dipalmitoylphosphatidylcholine/dipalmitoylphosphatidylglycerol, *Biophys. J.* 74 (1998) 1101–1109.
- [54] G. Schmitz, G. Muller, Structure and function of lamellar bodies, lipid–protein complexes involved in storage and secretion of cellular lipids, *J. Lipid Res.* 32 (1991) 1539–1570.
- [55] D. Schürch, O.L. Ospina, A. Cruz, J. Perez-Gil, Combined and independent action of proteins SP-B and SP-C in the surface behavior and mechanical stability of pulmonary surfactant films, *Biophys. J.* 99 (2010) 3290–3299.
- [56] S. Schürch, F.H. Green, H. Bachofen, Formation and structure of surface films: captive bubble surfactometry, *Biochim. Biophys. Acta* 1408 (1998) 180–202.
- [57] S. Schürch, R. Qanbar, H. Bachofen, F. Possmayer, The surface-associated surfactant reservoir in the alveolar lining, *Biol. Neonate* 67 (1995) 61–76.
- [58] A.G. Serrano, J. Perez-Gil, Protein–lipid interactions and surface activity in the pulmonary surfactant system, *Chem. Phys. Lipids* 141 (2006) 105–118.
- [59] L.N. Suri, L. McCaig, M.V. Picardi, O.L. Ospina, R.A. Veldhuizen, J.F. Staples, F. Possmayer, L.J. Yao, J. Perez-Gil, S. Orgeig, Adaptation to low body temperature influences pulmonary surfactant composition thereby increasing fluidity while maintaining appropriately ordered membrane structure and surface activity, *Biochim. Biophys. Acta* 1818 (2012) 1581–1589.
- [60] A. Suwabe, R.J. Mason, D. Smith, J.A. Firestone, M.D. Browning, D.R. Voelker, Pulmonary surfactant secretion is regulated by the physical state of extracellular phosphatidylcholine, *J. Biol. Chem.* 267 (1992) 19884–19890.
- [61] Y. Suzuki, Y. Fujita, K. Kogishi, Reconstitution of tubular myelin from synthetic lipids and proteins associated with pig pulmonary surfactant, *Am. Rev. Respir. Dis.* 140 (1989) 75–81.
- [62] H.W. Taeusch, J. Bernardino de la Serna, J. Perez-Gil, C. Alonso, J.A. Zasadzinski, Inactivation of pulmonary surfactant due to serum-inhibited adsorption and reversal by hydrophilic polymers: experimental, *Biophys. J.* 89 (2005) 1769–1779.
- [63] A.S. Verkman, Lung disease in cystic fibrosis: is airway surface liquid composition abnormal? *Am. J. Physiol. Lung Cell. Mol. Physiol.* 281 (2001) L306–L308.
- [64] T.E. Weaver, C.L. Na, M. Stahlman, Biogenesis of lamellar bodies, lysosome-related organelles involved in storage and secretion of pulmonary surfactant, *Semin. Cell Dev. Biol.* 13 (2002) 263–270.
- [65] J.A. Zasadzinski, T.F. Alig, C. Alonso, J.B. de la Serna, J. Perez-Gil, H.W. Taeusch, Inhibition of pulmonary surfactant adsorption by serum and the mechanisms of reversal by hydrophilic polymers: theory, *Biophys. J.* 89 (2005) 1621–1629.