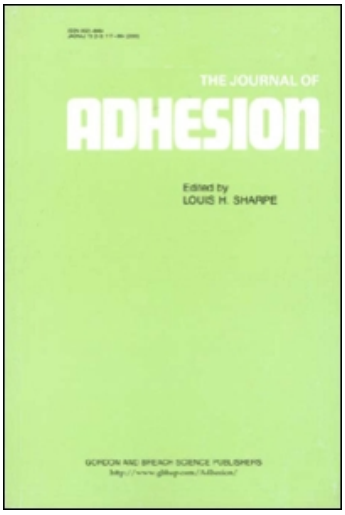

The Journal of Adhesion
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713453635

INFLUENCE OF SURFACTANT PROTEIN C IN β-SHEET CONFORMATION ON THE SURFACE DILATATIONAL BEHAVIOR OF PULMONARY SURFACTANT COMPONENTS SPREAD ON THE SURFACE OF A CAPTIVE BUBBLE

R. Wüstneck[a]; N. Wüstneck[a]; S. Pietschmann[a]; U. Pison[a]
[a] Charité Campus Virchow-Klinikum, Department of Anesthesiology and Intensive Care Medicine, Berlin, Germany

Online publication date: 10 August 2010

To cite this Article Wüstneck, R. , Wüstneck, N. , Pietschmann, S. and Pison, U.(2004) 'INFLUENCE OF SURFACTANT PROTEIN C IN β-SHEET CONFORMATION ON THE SURFACE DILATATIONAL BEHAVIOR OF PULMONARY SURFACTANT COMPONENTS SPREAD ON THE SURFACE OF A CAPTIVE BUBBLE', The Journal of Adhesion, 80: 9, 797 — 814
To link to this Article: DOI: 10.1080/00218460490480806
URL: http://dx.doi.org/10.1080/00218460490480806

The Journal of Adhesion, 80: 797–814, 2004

ISSN: 0021-8464 print/1545-5823 online
DOI: 10.1080/00218460490480806

Taylor & Francis
Taylor & Francis Group

INFLUENCE OF SURFACTANT PROTEIN C IN β-SHEET CONFORMATION ON THE SURFACE DILATATIONAL BEHAVIOR OF PULMONARY SURFACTANT COMPONENTS SPREAD ON THE SURFACE OF A CAPTIVE BUBBLE

R. Wüstneck
N. Wüstneck
S. Pietschmann
U. Pison
Charité Campus Virchow-Klinikum, Department of Anesthesiology and Intensive Care Medicine, Berlin, Germany

Surfactant protein C (SP-C) exists in α-helical and β-sheet conformation. The influence of these conformations on the surface behavior of spread layers containing dipalmytoyl phosphatodylcholine and the surfactant protein B was investigated. The β-sheet conformation of SP-C, which is formed during the preparation procedure at high lipid/protein concentrations, was confirmed by using circular dichroism (CD) and FTIR spectroscopy. Using a captive bubble device, the surface pressure/area isotherms, the surface dilatational elasticity and viscosity, and the main relaxation times of a transient stress relaxation were determined. Surface layers containing SP-C in β-sheet have no protein squeeze-out plateau. The surface dilatational elasticity is increased, which leads to a film of low compressibility.

Received 27 November 2003; in final form 22 April 2004.

The financial support by the Deutsche Forschungsgemeinschaft (grant Pi 165/10-1/2) is gratefully acknowledged. The EU partially supported these studies (EFRE 2000–2006 2ü/1, "Nano-Carrier"). The authors are grateful to U. Bentrup for kind assistance with the FTIR experiments.

Twenty years ago the pendant drop was a technique to measure surface tension with an accuracy of ± a few mN/m—too poor for many life science applications. Today the pendant drop and the captive bubble are used as a microfilm balance and an interfacial rheological device with accuracy better than ±0.1 mN/m. As such these methods have expanded beyond the borders of classical surface science and are now used for biological and medical applications. Thank you Wilhelm Neumann for helping us get there.

One of a collection of papers honoring A. W. Neumann, the recipient in February 2004 of *The Adhesion Society Award for Excellence in Adhesion Science, Sponsored by 3M*.

Address correspondence to Rainer Wüstneck, Charité Campus Virchow-Klinikum, Department of Anaesthesiology and Intensive Care Medicine, Augustenburger Platz 1, D-13344 Berlin, Germany. E-mail: rainer.wuestneck@charite.de

The relaxation times were increased by more than one order of magnitude, and the decays after surface compression and dilatation did not approach each other. Therefore, the surface behavior of layers containing SP-C in β-sheets remarkably differs from those containing SP-C in α-helical conformation. The presence of β–SP-C prevents the film from meeting the function of a pulmonary surfactant layer.

Keywords: SP-C; β-sheet; Axisymmetric bubble shape analysis; Surface rheology; Pulmonary surfactant; Hydrophobic proteins

INTRODUCTION

The function of proteins in biological membranes is strongly related to their amino acid sequence and their three-dimensional structure [1, 2]. Experimental determination of the conformation and secondary structure of a protein provides insight into its function. Recently, the pathogenesis of neurodegenerative diseases with different etiologies, such as Alzheimer's disease, scrapie in sheep and goats, bovine spongiform encephalopathy and other systemic amyloidosis, were related to the extracellular deposition of amyloid [3, 4]. Amyloid fibrils have a common structural motif of the β-pleated sheet conformation, and amyloid-forming proteins with α-helical structure undergo α-helix→β-sheet transition [5].

Amyloid-like fibrils are also formed by the hydrophobic pulmonary surfactant protein C (SP-C) and were found in lung washings from patients with alveolar proteinosis [6]. SP-C is a transmembrane lipopeptide containing 35 amino-acid residues in which residues 9–34 form an α-helix [6] in the natural bronchoalveolar lavage. Transformation of pure SP-C in organic solution from α-helix into β-sheet was reported after one-week incubation at a protein concentration of 4.6 mg/ml [6, 7] and during the preparation of the protein at high lipid/protein concentration [8].

The two positively charged residues of SP-C, arginine and lysine, at positions 11 and 12, are thought to interact with the negatively charged head groups of phospholipids, which should increase the rigidity of surface films. Note that the rigidity of the lipid–peptide interaction may be decreased towards the C-terminal end because it contains small or nonpolar residues only, which makes this part more mobile in a phospholipid layer. Furthermore, SP-C is assumed to influence the fluidity of the surrounding lipids v*ia* the extremely stable polyvaline helix [9]. However, the specific functional role of SP-C for breathing and the consequences of α-helix→β-sheet conversion of this protein are unknown.

Recently, we investigated the interfacial behavior of model surfactant layers that contained DL-dipalmitoyl phosphatidylcholine (DPPC), surfactant protein B (SP-B), and SP-C, the latter being α-helical ($\alpha-$SP-C) [10], and reported surface pressure/area isotherms of pure SP-C in β-sheet conformation (β-SP-C) [11]. The consequences of $\beta-$SP-C being present in mixed layers, especially in terms of surface dilatational rheological parameters, such as dilatational elasticity and viscosity, are unknown. The aim of the present study was to fill this knowledge gap by characterizing the surface behavior of mixed DPPC + SP-B + SP-C layers using a surface film preparation that contains SP-C in an antiparallel β-sheet conformation and does not differ in other respects from those investigated before [10]. The captive bubble device was used as a microfilm balance [12], with further improvements done to the device to obtain surface dilatational parameters by oscillating bubble experiments in the frequency range of human breathing [13], and by recording surface stress relaxation [14].

MATERIAL AND EXPERIMENTAL DETAILS

Materials

Chloroform and methanol were p.a. grade and purchased from Baker (J. T. Baker B. V., Deventer, Holland). DL-dipalmitoyl phosphatidylcholine (DPPC) was purchased from Sigma (Aldrich, Germany) and used without further purification (99% purity). Water (Millipore) was double distilled. The surface tension of pure water was 72.4 ± 0.2 mN/m at 23°C, as determined by the captive bubble technique. The films were spread on pure water. All glass vessels and the measuring cell of the captive bubble device were cleaned in KOH-saturated isopropanol.

Isolation of SP-B and SP-C

The pulmonary surfactant was obtained from cell-free sheep lung lavage fluid after 2 h centrifugation at 53,000 g. The pellet was homogenized in 1.64 N NaBr for density gradient centrifugation at 100,000 g overnight [15, 16]. The pellicle was removed, washed, and homogenized in 4 ml water, and the hydrophobic surfactant components were extracted into chloroform/methanol [17]. The hydrophilic components of ovine pulmonary surfactant were discharged, the remaining solvents containing the hydrophobic surfactant components were evaporated in vacuum at 40°C, and residues were weighed. The residue containing the hydrophobic components of pulmonary surfactant from chloroform/methanol

extraction (lipids, SP-B, and SP-C) was resolved in 1.5 ml acidified (5% 0.1 N trifluoroacetic acid) chloroform/methanol (1:1, v/v). Surfactant proteins were isolated using a semipreparative High Performance Liquid Chromatography (HPLC) column (250 × 10 mm ID) with Vydac C4, a butyl silica gel [18]. SP-B and SP-C fractions were collected, the solvent was evaporated, and the purified proteins weighted and then redissolved in chloroform/methanol (1:1, v/v) to give a final concentration of ~0.8–1.0 mg/ml. Aliquots were stored at −20°C. The protein storage concentration of SP-B and SP-C was 0.8–1.0 mg/ml.

On SDS polyacrylamide gel electrophoresis (16.5% Tris-tricine gels) under nonreducing conditions, SP-B showed a single wide band centered at ~29 kDa. SP-C showed only one band at ~5 kDa.

The circular dichroism (CD) and Fourier transform infrared (FTIR) spectra of the SP-C isolated in this way corresponded to antiparallel β-sheets [8].

Based on the assumption that the SP-B–to–SP-C ratio in pulmonary surfactant is 1:3 (by weight), experiments were performed with a DPPC + SP-B + SP-C mixture containing 17 wt% of SP-B and 5.7 wt% of SP-C, which corresponds to 0.25 mol% of SP-B dimer (relative to DPPC) and 3 mol% SP-C. The molecular weight of ovine SP-B is 17,380 (dimer, 158 amino acid residues), and that of SP-C is 4,200 (dipalmitoylated form, 35 amino acid residues).

Determination of Secondary Structure of the Proteins in Spread Layers

To determine the secondary structure of the isolated SP-C, we used CD and FTIR spectroscopy as described earlier [8]. Briefly, the special feature of the CD experiments is sample preparation by spreading the proteins onto a quartz surface. The spreading amount of proteins was 80 μg. Before spreading, the plates of 1.25 mm thickness were cleaned with KOH-saturated isopropanol and then soaked in pure water to hydrate the surface of the quartz glass, thus maintaining maximum similarity to the air–water interface in monolayer experiments. Far ultraviolet (UV) CD spectra were obtained at room temperature using a JASCO J715 spectropolarimeter (Jasco, Germany). After 5 min storage at room temperature to allow the solvent to evaporate, 1–4 quartz glass plates were placed into the measuring chamber with minimum space between individual plates. The area per plate was 100 mm^2; the cross section was 10 mm. The spectra were recorded at 20 nm/min scan rate, the response time was 2 s, and the band width was 1 nm. During each measurement the chamber was flushed with nitrogen. Four consecutive scans from 260–185 nm were accumulated

and averaged. The CD spectra were recorded in millidegrees of ellipticity as a function of wavelength.

FTIR spectra were recorded using a Bruker IFS 66 spectrometer (Bruker, Germany) equipped with a DTGS detector. The spectra were analyzed using the OPUS/IR2 program. Protein layers were obtained by spreading chloroform/methanol protein solution on self-pressed KBr plates. The solvent was allowed to evaporate for 5 min and then the plate was placed into the measuring chamber. During measurements the chamber was flushed with dry air, and one hundred consecutive scans were accumulated and averaged.

Surface Pressure Measurements

Surface pressure (π) *versus* area (A) measurements were performed using an airtight captive bubble cell, as was described in detail earlier [19, 20]. The surface pressure, $\pi = \gamma_0 - \gamma$, is the difference between the surface tension of the subphase, γ_0, and the film-covered surface, γ. The π-A isotherms were obtained by continuous variation of the internal cell pressure, which yields a compression of the bubble volume and a corresponding reduction of the surface area. The surface tension was determined by using axisymmetric captive bubble shape analysis (ADSA-CB) developed by Prokop *et al.* [21].

SP-B and SP-C were dissolved in chloroform/methanol (3:1, v/v). The protein solutions were spread at the air bubble surface using a 0.5 µl Hamilton syringe. The bubble size in all experiments was 70 µl. The amount of spreading solution was minimized to 0.1 µl to avoid influences of the spreading solvent on the surface behavior of the monolayer, as described earlier [19]. Evaporation of the extremely small samples before spreading was prevented by additional aspiration of an air cushion of at least 0.1 µl after filling the syringe needle with the spreading solution. The spreading solution was slowly injected onto the surface of the bubble under continuous video monitoring. Ten minutes after solvent evaporation and equilibration of the surface film, protein monolayers were continuously compressed. To evaluate the spreading quantity of protein, each spreading experiment was repeated at least 5 times. The compression rate chosen was $7.4 \times 10^{-4}\,\mathrm{nm^2 s^{-1}}$, which corresponds with quasi equilibrium conditions.

Harmonic Captive Bubble Oscillation Experiments

The harmonic oscillation of the internal pressure in the measuring cell causes a bubble volume oscillation. This, in turn, causes harmonic oscillation of both the bubble area and the interfacial tension. A piezo

transmitter was connected to the measuring cell and inserted into the liquid phase. The transmitter was controlled by an arbitrary frequency generator (Grundig AFG 100, Germany), which allows oscillation of the bubble volume in a frequency range of 0.001–1 kHz. For our measurements, we used frequencies in the range of 10^{-2} to 2.5×10^{-1} Hz and bubble volume amplitudes of 0.5 µl to avoid destruction of surface structures [22]. The frequency range covers both the human respiration frequency (4×10^{-2} to 2×10^{-1} Hz) [23] and the range of frequencies, characterized by maximum values of the rheological parameters for DPPC and DPPC + SP-C (1.7×10^{-2} Hz) [24, 25]. The interfacial tension and the bubble area were determined by recording 10 bubble profiles per second at 9 different levels of surface pressure and 9 different frequencies. The elasticity was determined by the ratio between the surface pressures *via* surface area amplitude; the viscosity by using the phase shift between the oscillation of surface area and pressure [13]. A spline-fit was used to create planes of elasticity and viscosity *via* frequency and surface pressure, based on a symmetric 9×9 frequency/surface pressure matrix. The confidence intervals (95% confidence level) of the elasticities were about 1–4%; those of the corresponding viscosities were in the order of 10–15%.

Transient Stress Relaxation Experiments

Transient stress relaxation experiments were carried out to discover rearrangement processes, which occur when the steady state of a surface layer is rapidly changed by a sudden reduction of the bubble area. To avoid surface destruction, changes of the bubble area were not more than 10–20%. The decay of π was recorded after the pressure jump. There is usually a spontaneous response, which can be used to calculate an elasticity parameter and a delayed one that can be described by an exponential function, which yields a relaxation time. This time constant is characteristic of the readjustment of a new steady state of the film. For further details, see Wüstneck *et al.* [10]. In practice, the delayed response includes different relaxation processes with different characteristic times. Therefore, a distribution for these processes was assumed that yields a main relaxation time and a homogeneity parameter and describes the stress relaxation more properly than a single exponential approach [14]. For discussing respiration processes, extremely slow relaxation processes are less important. Therefore, we restricted the time of our experiments to 120–400 s.

RESULTS

The Secondary Structure of SP-B and SP-C

To demonstrate the secondary structure of the surfactant proteins we recorded CD and FTIR spectra. The far UV CD spectra of the proteins SP-B and β–SP-C are shown in Figure 1. The spectrum of SP-B showed the shape and all typical features of α-helical structure: negative ellipticity bands near 222 nm and 208 nm, and a positive band at 192 nm. In contrast, the spectrum of SP-C showed a negative band near 216 nm and a positive band between 195 and 200 nm, which points to an antiparallel β-sheet secondary structure. There was no α-helical SP-C detectable in this preparation of SP-C.

The FTIR spectra of the proteins are displayed in Figure 2. The spectrum of the SP-B was characterized by the presence of an amide I band component centered at 1656 cm^{-1}, accompanied by some contributions at lower wave number. Amide I bands centered around 1650–1658 cm^{-1} are generally considered to be characteristic for α-helices [26]. The position of the amide I band at 1656 cm^{-1} thus confirmed the expected α-helical structure for SP-B. The spectrum of the SP-C showed the amide-I band components at 1628 cm^{-1}

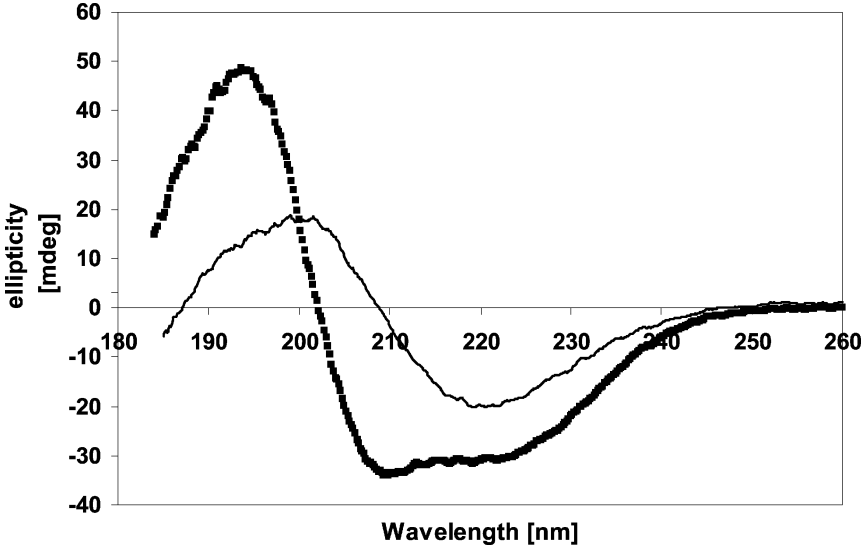

FIGURE 1 Far UV CD spectra of spread SP-B, and ß–SP-C layers on quartz sheets. Black squares typical for α helix conformation of SP-B; lines, SP-C in antiparallel β-sheet conformation.

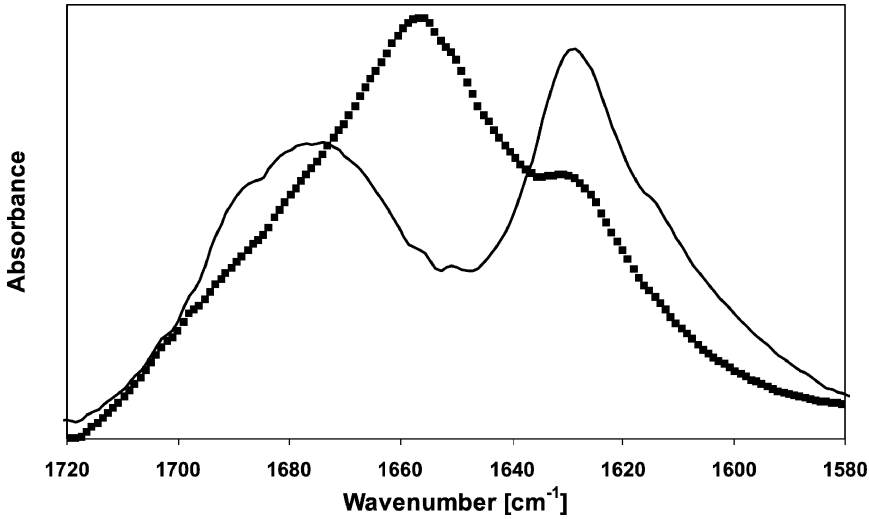

FIGURE 2 FTIR spectra of spread SP-B, and β–SP-C layers. Protein chloroform/methanol solutions (80 µg of protein) were spread onto a KBr plate. Black squares, SP-B; lines, β–SP-C.

and at 1676 cm^{-1}. These bands correspond to antiparallel β-sheet conformation of the protein. These data are in good agreement with our CD results.

π/A Isotherms

The π/A isotherm obtained with the system DPPC + SP-B + SP-C in β-sheet conformation was compared with the isotherm found with DPPC + SP-B + SP-C in α-helical conformation (Figure 3). The shape of the graph for the two systems was quite different. Although the mixing ratios did not differ, there was a well-pronounced squeeze-out plateau for the system containing α-SP-C at $\pi \approx 51$ mN/m. In contrast, the run for monolayer compression of the system with β–SP-C did not show such a plateau but a kink point instead at 0.45–0.48 nm^2/molecule. Further compression led to a rise up to a film pressure of about 70 mN/m. The run for layer expansion repeated the shape of that for compression with a hysteresis. Interestingly, there was no drastic breakdown of surface pressure immediately when the film expansion started. Also the layer expansion/layer compression (LE/LC) plateau at a film pressure of about 10 mN/m

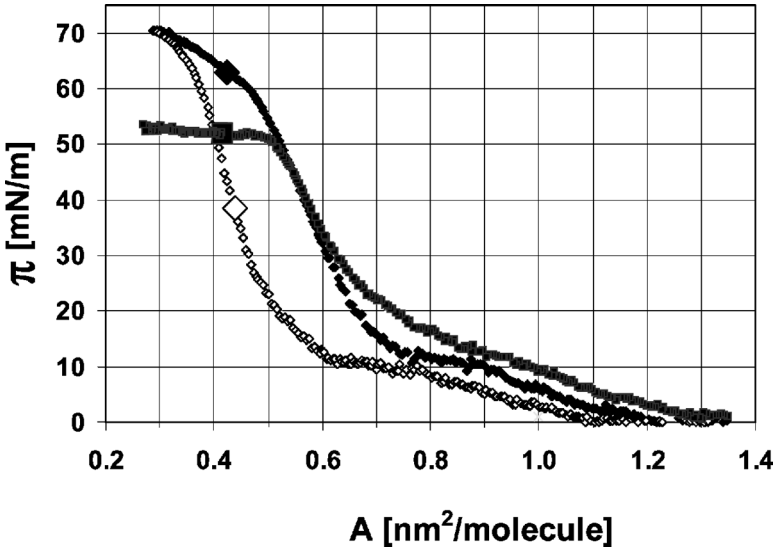

FIGURE 3 Quasi-equilibrium π/A isotherms of mixed spread monolayers of DPPC + 3 mol% SP-C + 0.25 mol% SP-B. Black squares, compression isotherm of α–SP-C; diamonds, isotherm of β–SP-C; black diamonds, compression; white diamonds, expansion.

was well pronounced and repeated that found for compression. In some experiments of layer compression for films containing β–SP-C the phenomenon of *bubble-clicking* was observed for pressures higher than 50 mN/m, which is characteristic for unstable surface structures.

Harmonic Oscillation Experiments

Comparisons between the surface dilatational elasticities and viscosities as a function of the surface pressure and the frequency of oscillation for DPPC + 3 mol% SP-C + 0.25 mol% SP-B are given in Figures 4 and 5. SP-C in α and β conformation behaves similar concerning both elasticity-frequency (no frequency dependence) and viscosity-frequency dependence (maximum values at low frequencies). In contrast, the maximum values of elasticity at the surface pressure of the starting squeeze-out process were definitely higher in the systems containing β–SP-C than those of α–SP-C (Figures 4a and 4b). Notice that there was only a less-pronounced stationary minimum in

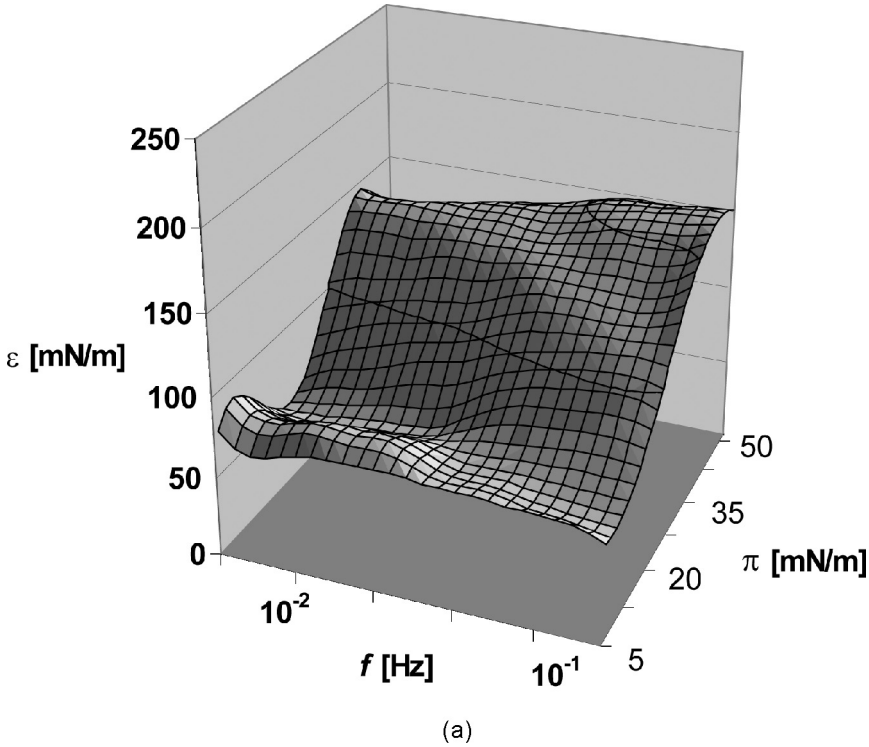

FIGURE 4 Surface dilatational elasticity, ε, of spread DPPC + 3 mol% SP-C + 0.25 mol% SP-B layers depending on surface pressure and frequency: (a) α–SP-C, (b) β–SP-C (See Color Plate I).

the range of the kink point in the π/A isotherms for systems containing β–SP-C (Figure 4a). The amplitudes of oscillation for the systems containing α–SP-C were too small to give measurable results for dilatational elasticity for π meeting the squeeze-out plateau exactly. That means the surface dilatational elasticity for systems containing α–SP-C became zero for this plateau range.

In contrast to the dilatational elasticities, the dilatational viscosities were lower for the systems containing β–SP-C (Figure 5a) compared with those of α–SP-C (Figure 5b). In the frequency range of human breathing ($\approx$0.05–0.2 Hz) the surface dilatational viscosities were lower for the system containing β–SP-C, whereas the elasticities were higher for systems containing α–SP-C. These results show that the films containing β–SP-C are more elastic, *i.e.*, less compressible, than those containing α–SP-C.

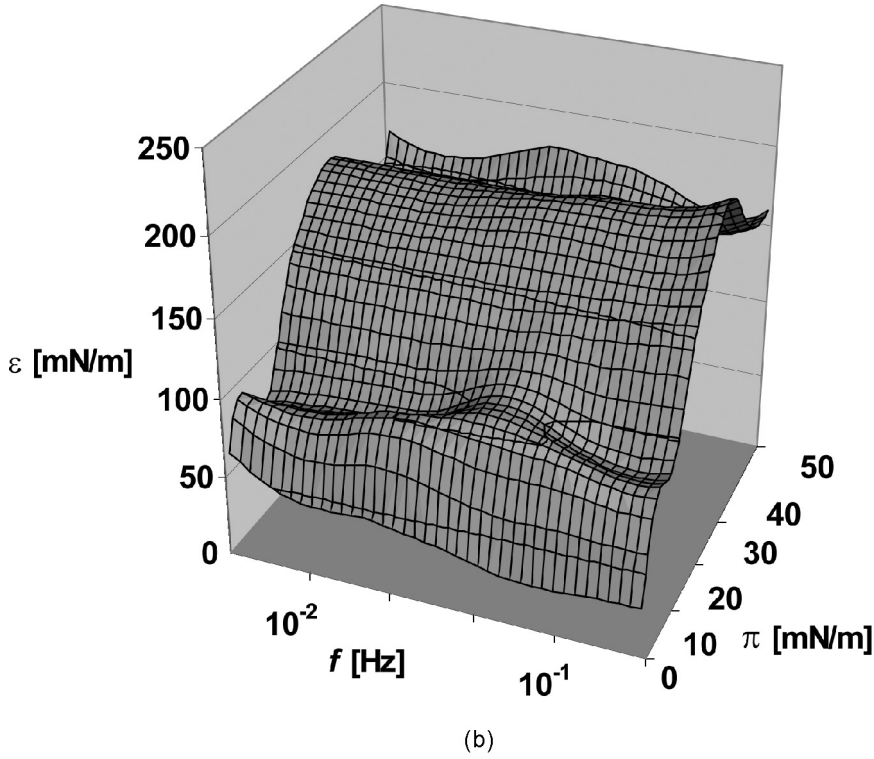

FIGURE 4 (Continued).

Surface Stress Relaxation

Starting from a film pressure in the range of the squeeze-out plateau, a transient jump of the pressure always led to a rapid recovery of the plateau film pressure to $\approx 51\,\mathrm{mN/m}$ for the system DPPC + SP-B + α-SP-C. For the system with β−SP-C and starting the relaxation experiments also at $\approx 51\,\mathrm{mN/m}$, *i.e.*, near the kink point in the π/A isotherm (see Figure 3), the recovery curves after compression or expansion never led to similar π. Figure 6 shows representative recovery curves for the two mixtures containing α-SP-C or β-SP-C.

Table 1 contains the calculated mean values for the relaxation time, τ. The values were calculated from 10 different relaxation experiments with different starting values of π after the pressure jump. The homogeneity parameters [14] were in the range 3.4−7.0, which proves the relaxation processes to be quite narrow by distributed, *i.e.*, the Poisson distribution used in the calculation procedure is a sufficient

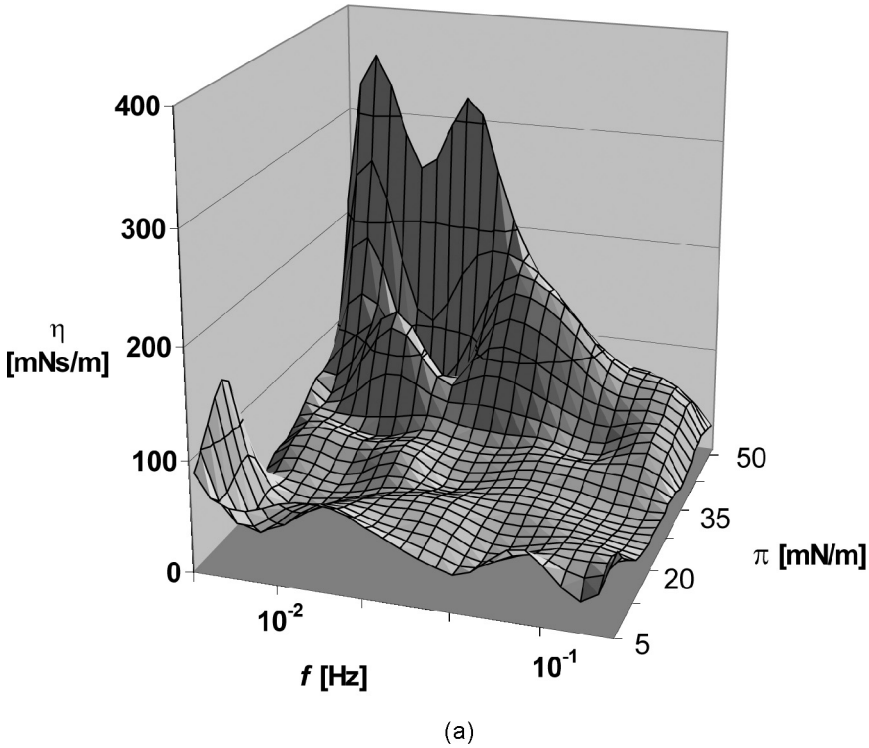

FIGURE 5 Surface dilatational viscosity, η, of spread DPPC + 3 mol% SP-C + 0.25 mol% SP-B layers depending on surface pressure and frequency: (a) α–SP-C, (b) β–SP-C (See Color Plate II).

approximation. The relaxation times determined from the recovery curves for the system DPPC + SP-B + β–SP-C were definitively higher than those reported for the system with α–SP-C [10] by more than one order of magnitude. This is the most drastic difference caused by the conformation of SP-C and may be important for the physiological function of pulmonary surfactant layers.

DISCUSSION

The interfacial behavior of the system DPPC + SP-B + α–SP-C is remarkably different from the system that contains β–SP-C. The presence of β–SP-C results in a continuous increase of the surface pressure during compression up to the collapse region, changes the squeeze-out mechanism, reduces the film compressibility, and changes the stress relaxation behavior.

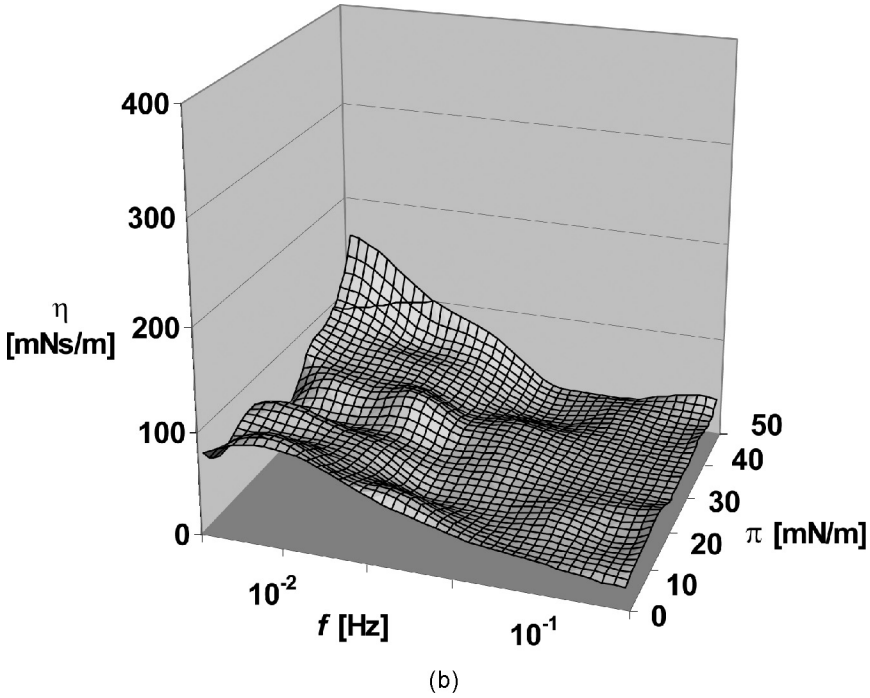

FIGURE 5 (Continued).

A continuous increase of the surface pressure during compression up to the collapse region is characteristic for mixtures containing β−SP-C but not for α−SP-C. The quasi-static π/A isotherm of the mixture DPPC + SP-B + β-SP-C increases continuously, passes a kink point at about 51 mN/m, and ends up at the collapse region. The π/A isotherm of the mixture DPPC + SP-B + α−SP-C shows a pronounced plateau at 51 mN/m, which is in good agreement with the equilibrium surface tension realized for adsorbed systems [27]. The collapse of the layer formed with β−SP-C occurs at a surface coverage higher than 0.32 nm^2/molecule, *i.e.*, slightly above that of a pure DPPC layer [20], but definitively below the cross section of a DPPC molecule. That means there must be a formation of three-dimensional structures for systems that contain SP-C in β conformation, as would be expected for α−SP-C. At the present time there is no experimental information about the nature of these structures. A possibility could be layer folding [20, 28−30]. This would explain the shape of the π/A isotherm during expansion, which shows a relatively weak hysteresis

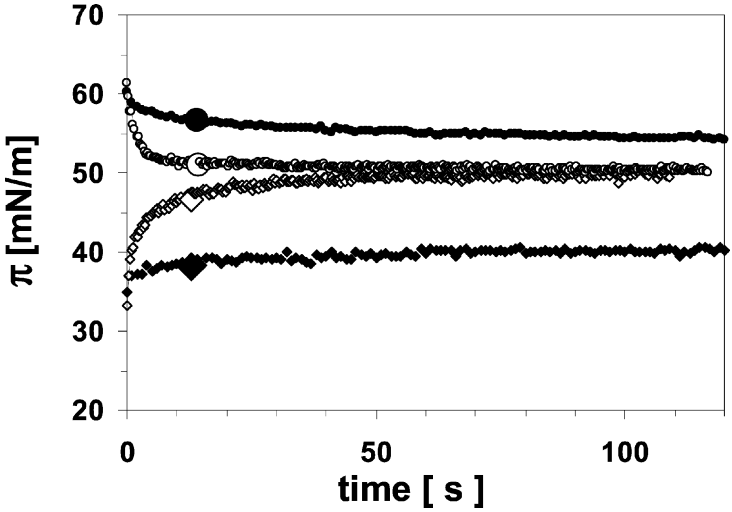

FIGURE 6 Surface pressure decay after a transient bubble volume change of spread layers, DPPC + 3 mol% SP-C + 0.25 mol% SP-B; circles, after transient increase of pressure in the measuring cell; diamonds, after transient decrease of pressure in the measuring cell; white symbols, α−SP-C; black symbols, β−SP-C.

and which does not show a sharp breakdown, as is expected for a collapsed layer.

The squeeze-out mechanism is strongly affected by the conformation of SP-C, which is the most conspicuous difference between the two conformers. Systems containing α−SP-C show a squeeze-out plateau in the π/A isotherm, systems containing β−SP-C do not. In the presence of β−SP-C the proteins in the film are hindered from being removed from the surface through compression. It might well be that the presence of β−SP-C prevents the formation of a surface-associated surfactant reservoir. These changes are reflected by the dynamic properties of the film in the whole range of surface pressure.

TABLE 1 Main Relaxation Times Determined after Transient Layer Compression or Expansion Using the Decay of the Surface Pressure [14]

	Main relaxation time τ_{max}[s]	
Mixture	Compression	Dilatation
DPPC + 3 mol% α−SP-C + 0.25 mol% SP-B	2.5 ± 0.25	4.3 ± 0.38
DPPC + 3 mol% β−SP-C + 0.25 mol% SP-B	66.8 ± 15.6	61.5 ± 8.6

The film compressibility is reduced by β−SP-C, but is infinite in the presence of α−SP-C in the surface pressure plateau range. Films containing β−SP-C are more rigid and more elastic, in general, compared with those that contain α−SP-C. In addition, the surface dilatational elasticity shows a weak pronounced stationary minimum at a film pressure of about 45 mN/m but further increases during compression. There is no range of infinite compressibility for films containing β−SP-C. It should be noticed that there are only a few data in the literature about the influence of secondary structure of proteins on surface rheological behavior. Only for gelatin/surfactant layers it was shown that both anionic and cationic surfactants cause a $\alpha \rightarrow \beta$ conversion of gelatin at interfaces, which influenced the interfacial shear rheology [31].

The stress relaxation is greatly influenced by SP-C conformation. In the presence of α−SP-C, surface pressures above 51 mN/m could be obtained only during rapid compression [10]. When the compression is stopped above the plateau value of 51 mN/m, the surface pressure of these films rapidly falls to the plateau pressure of about 51 mN/m due to the short relaxation time. The same holds for expansion. This fast relaxation observed for films containing α−SP-C is caused by SP-B, because films without SP-B relax only with diminished velocity [10]. While SP-B is important for the relaxation velocity, the conformation of SP-C modifies the speed of relaxation. In the presence of β-folded SP-C, the relaxation times of surface stress are increased and even exceeded those found for films, which contain only α−SP-C [10]. Therefore, the recovery process is hindered in the presence of β−SP-C, thus enabling higher surface pressures even when the layer is slowly compressed (quasi-equilibrium conditions). Such increase of relaxation times would not meet the demands of a film that should allow a fast reconstruction of the pulmonary layer *in vivo*. The dynamic process of compression and expansion of the interface that occurs during breathing must have a velocity higher than the breathing frequency. In all probability the formation of a surfactant reservoir is prevented by β−SP-C. This could be obtained only with α−SP-C.

How do our data transform into the *in vivo* situation? It is well known that temperature has an influence on surface rheology. Both dilatational elasticity and viscosity decrease with increasing temperature. Although normal body temperature in humans is 37°C, we used 23°C for our experiments to work out the rheological differences between layers containing α−SP-C *versus* β−SP-C. Therefore, further studies are necessary to show the relevance of the present data under more physiological conditions. However, some findings of our study can be generalized, because of the following aspects: First, the principal interfacial rheological features of pulmonary surfactant

components remain when the temperature is increased up to physiological values [25]. In particular, the stress relaxation is only negligibly influenced by temperature, which was first derived by Maxwell [32] and also experimentally shown for spread phospholipid layers [24]. Second, the π/A isotherms measured in our study using an artificial pulmonary surfactant system are well comparable with π/A isotherms obtained for other artificial pulmonary surfactant systems, including buffered subphases [33–37]. Third, the plateau value of the π/A isotherm of the system containing α–SP-C measured in our study is in good agreement with the equilibrium surface tension reported for adsorbed films and at sufficiently high concentrations of pulmonary surfactant in the bulk phase [27]. These values agree well with the surface tension measured in lungs under *in vivo* conditions, even when taking into account the particularities of the delicate technique used [38–42]. These arguments justify that the data reported in this study are at least partially valid under physiological conditions. Especially, velocity of relaxation is almost independent of temperature and hardly influenced by pH and ionic strength.

Breathing is supported by high film compressibility of the thin lipid/protein layer lining the alveoli of mammalian lungs and, in most circumstances, takes place at a film pressure alternating around the protein squeeze-out plateau, where the compressibility of the films is very high but decreases when the surface pressure becomes higher or lower than the equilibrium value. The surface behavior of the artificial pulmonary surfactant mixture investigated here shows that the presence of β–SP-C definitely contradicts these prerequisites of breathing.

At present, our data from *in vitro* experiments do not discriminate in detail between the physiological function of SP-C in α *versus* β conformation. Nevertheless, our data support the view that α–SP-C meets the dynamic necessities of the surface film that covers the alveoli lining layer in mammalian lungs, whereas β–SP-C does not. Further studies on physiological functions of pulmonary surfactant need to address the different rheological behavior of SP-C in its two conformation states.

REFERENCES

[1] Dobson, C. M., Sali, A., and Karplus, M., *Angew. Chem. Int. Ed.* **37**, 868–893 (1998).
[2] Eaton, W. A., Munoz, V., Hagen, S. J., Jas, G. S., Lapidus, L. J., Henry, E. R., and Hofrichter, J., *Annu. Rev. Biophys. Biomol. Struct.* **29**, 327–359 (2000).
[3] Soto, C., *Mol. Med. Today* **5**, 343–350 (1999).
[4] Kelly, J. W., *Curr. Opin. Struct. Biol.* **6**, 11–17 (1996).

[5] Guijarro, J. I., Sunde, M. T., Jones, J. A., Campbell, I. D., and Dobson, C. R. M., *Proc. Natl. Acad. Sci.* **95**, 4224–4228 (1998).
[6] Gustafsson, M., Thyberg, J., Naslund, J., Eliasson, E., and Johansson, J., *FEBS Lett.* **464**, 138–142 (1999).
[7] Szyperski, T., Vandenbussche, G., Curstedt, T., Ruysschaert, J. M., Wuthrich, K., and Johansson, J., *Protein Sci.* **7**, 2533–2540 (1998).
[8] Wüstneck, N., Wüstneck, R., Perez-Gil, J., and Pison, U., *Biophys. J.* **84**, 1940–1949 (2003).
[9] Johansson, J. and Curstedt, T., *Eur. J. Biochem.* **244**, 675–693 (1997).
[10] Wüstneck, N., Wüstneck, R., and Pison, U., *Langmuir* **19**, 7521–7527 (2003).
[11] Wüstneck, R., Fainermann, V. B., Wüstneck, N., and Pison, U., *J. Phys. Chem. B* **108**, 1766–1770 (2004).
[12] Li, J., Miller, R., Wüstneck, R., Möhwald, H., and Neumann, A. W., *Colloids Surf. A: Physicochem. Eng. Aspects* **96**, 295–299 (1995).
[13] Wüstneck, R., Moser, B., and Muschiolik, G., *Colloids Surf. B: Biointerfaces* **15**, 263–273 (1999).
[14] Wüstneck, R., Enders, P., Ebisch, T., and Miller, R., *Thin Solid Films* **298**, 39 (1997).
[15] Hawgood, S., Benson, B. J., and Hamilton, R. L., Jr., *Biochemistry* **24**, 184–190 (1985).
[16] Pison, U., Tam, E. K., Caughey, G. H., and Hawgood, S., *Biochim. Biophys. Acta* **992**, 251–257 (1989).
[17] Folch, J., Lees, M., and Sloane Stanley, G. H., *J. Biol. Chem.* **226**, 497–509 (1957).
[18] Bünger, H., Kaufner, L., and Pison, U., *J. Chromatogr. A* **870**, 363–369 (2000).
[19] Wüstneck, R., Wüstneck, N., Vollhardt, D., Miller, R., and Pison, U., *Mater. Sci. Eng. C-Biomimetic Supramol. Syst.* **8–9**, 57–64 (1999).
[20] Wüstneck, N., Wüstneck, R., Fainerman, V. B., Pison, U., and Miller, R., *Colloids Surf. A: Physicochem. Eng. Aspects* **164**, 267–278 (2000).
[21] Prokop, R. M., Jyoti, A., Eslamian, M., Garg, A., Mihaila, M., del Río, O. I., Susnar, S. S., Policova, Z., and Neumann, A. W., *Colloids Surf. A* **131**, 231–247 (1998).
[22] Wüstneck, R., Wüstneck, N., Moser, B., and Pison, U., *Langmuir* **18**, 1125–1130 (2002).
[23] Roussos, C. and Campbell, E. J. M., Respiratory muscle energetics. Section 3: The Respiratory System, in *Handbook of Physiology*, P. T. Macklem and J. Mead (Eds.) (American Physiological Society, Bethesda, MD, 1986), Vol. III, pp. 481–509.
[24] Wüstneck, R., Enders, P., Wüstneck, N., Pison, U., Miller, R., and Vollhardt, D., *Phys. Chem. Comm. (E J.)* **11** (1999).
[25] Wüstneck, N., Wüstneck, R., Fainerman, V. B., Miller, R., and Pison, U., *Colloids Surf. B: Biointerfaces* **21**, 191–205 (2001).
[26] Pelton, J. T. and McLean, L. R., *Anal. Biochem.* **277**, 167–176 (2000).
[27] Schürch, S., Schürch, D., Curstedt, T., and Robertson, B., *J. Appl. Physiol.* **77**, 974–986 (1994).
[28] Ding, J., Takamoto, D. Y., von Nahmen, A., Lipp, M. M., Lee, K. Y., Waring, A. J., and Zasadzinski, J. A., *Biophys. J.* **80**, 2262–2272 (2001).
[29] Takamoto, D. Y., Lipp, M. M., von Nahmen, A., Lee, K. Y., Waring, A. J., and Zasadzinski, J. A., *Biophys. J.* **81**, 153–169 (2001).
[30] Gopal, A. and Lee, K. Y. C., *J. Phys. Chem. B* **105**, 10348–10354 (2001).
[31] Wüstneck, R. and Krägel, J., Characterisation of gelatin/surfactant interaction and its relevance to liquid film coating, in *Studies of Interface Sciences*, D. Möbius and R. Miller (Eds.) (Elsevier, Amsterdam, 1998), Vol. 7, pp. 433–490.
[32] Maxwell, J., *Philos. Mag.* **4**, 129–145, 185–217 (1868).

[33] Grigoriev, D. O., Krägel, J., Akentiev, A. V., Noskov, B. A., Miller, R., and Pison, U., *Biophys. Chem.* **104**, 633–642 (2003).
[34] Krol, S., Ross, M., Sieber, M., Kunneke, S., Galla, H. J., and Janshoff, A., *Biophys. J.* **79**, 904–918 (2000).
[35] Kramer, A., Wintergalen, A., Sieber, M., Galla, H. J., Amrein, M., and Guckenberger, R., *Biophys. J.* **78**, 458–465 (2000).
[36] Knebel, D., Sieber, M., Reichelt, R., Galla, H.-J., and Amrein, M., *Biophys. J.* **83**, 547–555 (2002).
[37] Putz, G., Walch, M., Van Eijk, M., and Haagsman, H. P., *Biophys. J.* **75**, 2229–2239 (1998).
[38] Im Hof, V., Schürch, S., Straub, R., and Gehr, P., *Eur. Respir. J.* **3**, 257–262 (1990).
[39] Schürch, S., Goerke, J., and Clements, J. A., *Proc. Natl. Acad. Sci. USA* **73**, 4698–4702 (1976).
[40] Schürch, S., Bachofen, H., and Weibel, E. R., *Respir. Physiol.* **62**, 31–45 (1985).
[41] Im Hof, V., Gehr, P., Gerber, V., Lee, M. M., and Schürch, S., *Respir. Physiol.* **109**, 81–93 (1997).
[42] Schürch, S., Bachofen, H., and Possmayer, F., *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **129**, 195–207 (2001).