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Surface activity and film formation from the surface associated material of artificial surfactant preparations

Marie Palmblad [a,*], Magnus Gustafsson [b], Tore Curstedt [a], Jan Johansson [b], Samuel Schürch [c]

[a] *Department of Clinical Chemistry, Karolinska Hospital, S-171 76 Stockholm, Sweden*
[b] *Department of Medical Biochemistry and Biophysics, Karolinska Institutet, S-171 77 Stockholm, Sweden*
[c] *Respiratory Research Group, Faculty of Medicine, The University of Calgary, Calgary, Alta., Canada T2N 4N1*

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Abstract

Surfactant proteins B and C (SP-B and SP-C) are present in natural derived surfactant preparations used for treatment of respiratory distress syndrome. Herein the surface activity of an SP-C analogue (SP-C(LKS)), a hybrid peptide between SP-C and bacteriorhodopsin (SP-C/BR) and a model peptide (KL_4) was studied with a captive bubble surfactometer (CBS). The peptides were mixed with either 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC)/phosphatidylglycerol (PG) (7:3, by weight) or DPPC/PG/palmitic acid (68:22:9, by weight) at a concentration of 1 mg/ml in HEPES buffer, pH 6.9 and a polypeptide/lipid weight ratio of 0.02–0.03. In some lipid/peptide preparations also 2% of SP-B was included. Adsorption, monitored as surface tension vs. time for 10 min after bubble formation did not show discernible differences for the whole set of preparations. Equilibrium surface tensions of approximately 25 mN/m were reached after 5–10 min for all preparations, although those with SP-C/BR appeared not to reach end point of adsorption within 10 min. Area compression needed to reach minimum surface tension of 0.5–2.0 mN/m was least for the KL_4 preparation, about 13% in the first cycle. 3% SP-C(LKS) in DPPC:PG (7:3, by weight) reached minimum surface tension upon 27% compression in the first cycle. If DPPC:PG:PA (68:22:9, by weight) was used instead only 16% area compression was needed and 14% if also 2% SP-B was included. 3% SP-C(LKS) in DPPC:PG (7:3, by weight)+2% SP-B needed 34% compression to reach minimum surface tension. The replenishment of material from a surface associated surfactant reservoir was estimated with subphase depletion experiments. With the 2% KL_4 preparation incorporation of excess material took place at a surface tension of 25–35 mN/m during stepwise bubble expansion and excess material equivalent to 4.3 monolayers was found. When 2% SP-B was added to 3% SP-C(LKS) in DPPC:PG (7:3, by weight) the number of excess monolayers increased from 1.5 to 3.6 and the incorporation took place at 30–40 mN/m. When SP-B was added to 3% SP-C(LKS) in DPPC:PG:PA (68:22:9, by weight) the number of excess monolayers increased from 0.5 to 3.4 and incorporation took place at 40–50 mN/m. With 2% SP-C/BR incorporation took place at 40–45 mN/m, frequent instability clicks were observed and excess material of approximately 1.1 monolayer was estimated.

Keywords: Pulmonary surfactant; Hydrophobic protein; SP-B; SP-C; Analogue; Surface activity; Captive bubble surfactometer

Abbreviations: RDS, respiratory distress syndrome; SP, surfactant protein; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; PG, phosphatidylglycerol; PA, palmitic acid; CBS, captive bubble surfactometer; MALDI-TOF, matrix-assisted laser desorption ionisation-time of flight; γ_{min}, surface tension at minimum bubble size; γ_{max}, surface tension at maximum bubble size; PEEP, positive end expiratory pressure

* Corresponding author. Fax: +46-8-5177-6165; E-mail: marie.palmblad@lab.ks.se

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1. Introduction

Pulmonary surfactant is a complex material synthesised and excreted by alveolar type II cells. The main constituents are phosphatidylcholine, especially 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and phosphatidylglycerol (PG). A minor but essential part of surfactant are the proteins. The hydrophobic surfactant proteins B and C (SP-B and SP-C) are probably both involved in the alveolar air/liquid surface film formation. The hydrophilic surfactant proteins SP-A and SP-D are C-type (Ca^{2+}-dependent) collagenous lectins and are thought to act primarily in the host-defence system. SP-A and SP-D do however also interact with lipids in vitro. SP-A causes lipid aggregation in the presence of Ca^{2+} and is essential for the tubular myelin formation in vivo [1]. Lipids accumulate in the lungs of SP-D knock out mice, which suggests a role of SP-D in the metabolism of surfactant lipids [2,3].

Decreased levels of surfactant in premature babies result in respiratory distress syndrome (RDS) and is treated with exogenous surfactant isolated from animal tissue by extraction with organic solvents. These preparations contain, apart from the phospholipids, 1–2% (by weight) SP-B and SP-C [4,5]. The polypeptides improve surface activity dramatically compared to pure lipid preparations [6].

The primary and secondary structures of SP-B and SP-C and a tertiary structure of SP-C in solution have been determined [5,7,8]. SP-B is a dimeric molecule composed of two identical polypeptide chains of 79 amino acids with an interchain disulphide bridge and may interact with two lipid bilayers and bring them into close proximity [5,9]. This has been supported experimentally in several studies [10–13]. It has been shown in vivo that homozygous SP-B deficient mice can not expand their lungs and die within minutes of birth [14]. SP-C is a lipoprotein composed of 35 amino acid residues with an α-helical domain between residues 9–34 [8]. The helix is composed mostly of valyl residues and is embedded in a lipid bilayer and oriented in parallel with the lipid acyl chains [5,8]. SP-C is thought to influence the thickness and fluidity of the surrounding lipid layers via the extremely stable poly-valyl helix [5]. Two palmitoyl groups are covalently linked to cysteine residues in positions 5 and 6 in the N-terminal part of the peptide. Surface activity in vitro of chemically deacylated SP-C [15–17] indicates that palmitoylation is essential for good adsorption, mechanical stability and reformation of surfactant film at the interface as well as low film compressibility. With SP-C(Leu), an SP-C analogue where the poly-valyl helix has been replaced with poly-leucyl residues in order to avoid aggregation [18], improvements in surface activity are observed after palmitoylation. Most pronounced is the increased mechanical stability and improved incorporation of surfactant material from the surface associated reservoir [19]. Although the different components of pulmonary surfactant have been largely characterised the interaction mechanism at the molecular level is not fully understood. Synthetic polypeptide analogues of the surfactant proteins or their fragments can be used as tools to further approach this issue. In the present study we have investigated the surface activity of three synthetic polypeptides combined with lipid mixtures or lipid mixtures and native porcine SP-B. SP-C(LKS) is a polyleucine SP-C analogue (Table 1) where three leucines have been replaced with lysine residues in order to locate positive charges around the helical circumference. This makes it easy to handle and self-polymerisation is avoided [20]. SP-C/BR is a hybrid peptide (Table 1) where positions 13–35 of SP-C have been replaced with the amino acid sequence of positions 42–64 of bacteriorhodopsin, which is a known transmembrane α-helix [21].

Table 1
Amino acid sequence of SP-C and the polypeptides investigated

	1	10	20	30
Human SP-C	F G I P C C P V H	L K R L L I V V V V	V V L I V V V I V G	A L L M G L
SP-C(LKS)	- - - - S S - - -	- - - - - - L K L L	L L K - L L L K L -	- - - - - -
SP-C/BR	- - - - - - - - -	- - - F Y A I T T L	V A A I A F T L Y L	S L L L G Y
KL_4	K L L L L K L L L	L K L L L L K L L L	L K	

Bars indicate residues identical with those in human SP-C.

Finally, KL_4 (Table 1) which has been designed from the N-terminal part of SP-B [22] was included. In the present study, adsorption, film compression needed to achieve minimum surface tension, maximum surface tension and stability upon dynamic cycling as well as the existence of excess surface associated material have been investigated in the captive bubble surfactometer (CBS).

2. Materials and methods

2.1. Peptide synthesis, purification and characterisation

Two analogues of SP-C, SP-C(LKS) [20] and SP-C/BR [21] as well as the SP-B model peptide KL_4 [22] (Table 1) were synthesised by use of stepwise solid phase technology and the *tert*-butyloxycarbonyl (t-BOC) chemistry in an Applied Biosystems 430A instrument. Reagents and t-BOC amino acids for peptide synthesis were from Perkin-Elmer. Cleavage of the resin–peptide bond and deprotection of the side-chains were carried out in anhydrous hydrogen fluoride/metoxybenzene/dimethylsulfide, 10:1:1 (v/v/v) for 1.5 h at 0°C. Protecting groups and scavengers were removed by repeated extractions with diethyl ether. The peptides were extracted from the resin with dichloromethane/trifluoroacetic acid (TFA) 3:1 (v/v) or with 40% (v/v) acetic acid for SP-C/BR, followed by rotary evaporation. The crude peptide extracts of KL_4 and of SP-C/BR were dissolved in ethanol and purified with reversed phase HPLC using a C_{18} column (Vydak 2.2×250 mm) and a linear gradient of 60% aqueous methanol/0.1% TFA and isopropanol/0.1% TFA over 40 min with a flow rate of 0.7 ml/min [23]. SP-C(LKS) was purified with exclusion chromatography on Sephadex LH-60 in chloroform/methanol 1:1 (v/v) containing 5% H_2O. An aliquot of 10 mg at a concentration of approximately 100 mg/ml was applied on a Sephadex LH-60 column (40×1 cm) and eluted with a flow rate of 6–8 ml/min [20]. SP-B was obtained from porcine lungs as described previously [6]. Identification and quantification were made by amino acid analysis with an LKB Alpha Plus analyser.

Molecular masses were determined by matrix-assisted laser desorption ionisation-time of flight (MALDI-TOF) mass spectrometry (Lasermat 2000, Finnigan MAT) calibrated with vasoactive intestinal peptide ($[M+H]^+$ 3326.8) or with porcine insulin ($[M+H]^+$ 5778.6).

2.2. Preparation of peptide/lipid mixtures

DPPC, PG and palmitic acid (PA) were purchased from Sigma Chemical Co. (St Louis, MO, USA). The lipids, dissolved in chloroform/methanol 98:2 (v/v), were mixed in the proportions DPPC:PG:PA 68:22:9 (w/w/w) or DPPC:PG 7:3 (w/w). Surfactant preparations were prepared by adding SP-C(LKS) alone or SP-C(LKS) and SP-B in each of the lipid mixtures at a total polypeptide/lipid ratio of 0.02–0.05, by weight as previously evaluated [20]. KL_4 alone or SP-C/BR alone were added to the DPPC:PG:PA mixture at polypeptide/lipid weight ratios of 0.02 in accordance with earlier studies [18]. The mixtures were evaporated under nitrogen and suspended at room temperature in 10 mmol/l HEPES buffer pH 6.9 containing 140 mmol/l NaCl and 2.0 mmol/l $CaCl_2$, at a lipid concentration of 1 mg/ml.

2.3. Surface activity measurements

A captive bubble surfactometer [24] was used to determine the area, volume and surface tension of a surfactant film at the bubble air–water interface. The bubble shape was monitored using a video system, digitised and used to calculate surface tension and area of captive air bubbles [25]. Measurements were made during lipid adsorption and compression and expansion of a bubble under quasi-static or dynamic conditions. After substituting the surfactant in the subphase by normal saline (depletion experiments) quasi-static cycles were conducted.

2.3.1. Adsorption studies

An atmospheric bubble of 6–7 mm in diameter was injected into the well-stirred surfactant suspension of the sample chamber. Time zero for adsorption was taken, when the bubble showed a Laplacian shape, which occurred within 0.15 s. The bubble was then left undisturbed for 10 min. Adsorption was completed when a surface tension of 23–25 mN/m was reached.

2.3.2. Quasi-static cycles

After adsorption the bubble was stepwise compressed with a 2–3 s delay after each step until further compression did not affect bubble height, indicating that minimum surface tension was reached. Subsequently the bubble was expanded in the same manner until the original volume was reached (for details see [26]). The cycle was repeated four times, after 2 min inter-cycle delay. Each peptide/lipid preparation was examined in triplicate. The average surface tension calculated after each step of cycles 1 and 4 were plotted against the relative bubble area. The area measured after adsorption with the equilibrium surface tension of 23–25 mN/m was set to 1.0.

2.3.3. Dynamic cycles

After adsorption as above the bubble was compressed and expanded continuously for 20 cycles at a rate of 20–30 cycles/min. Compression was not terminated when minimum surface tension was reached, instead the bubble was overcompressed by approximately 20%. By overcompressing the bubble at minimum surface tension a collapse phase is formed, which might remain associated with the interface or might be lost to the surrounding subphase [27].

2.3.4. Subphase depletion

After bubble adsorption as above the surfactant preparation was washed out and substituted with 0.15 mol/l NaCl, 1.5 mmol/l $CaCl_2$. 3–5 quasi-static cycles were then performed. After compression to minimum surface tension in the first cycle the bubble was expanded to its original size and allowed to rest for 2 min, followed by stepwise expansions of the diameter of approximately 0.5 mm at each step. After each step there was a 2 min waiting period during which the bubble shape flattened, indicating a decrease in surface tension due to adsorption of material from a surface associated surfactant reservoir [28]. After 3–4 adsorption steps with a 2 min delay in between the second cycle was started by compressing the bubble to minimum surface tension. Stepwise expansion was repeated 3–4 times after each cycle until no adsorption took place during the 2 min delay and surface tension rose rapidly on bubble expansion. From these manipulations the adsorption from the surface associated reservoir was estimated. Since there was no surfactant in the surrounding subphase the decrease in surface tension after each expansion was due to the material in excess of the interfacial monolayer. The bubble area at minimum surface tension of the first cycle was compared to that of the final cycle and the amount of excess material is expressed as number of monolayers.

2.4. Statistical analysis

Where applicable, data points in the graphs represent means ± S.E.M., $n = 3$. Multiple mean comparisons were done using a one-way ANOVA with the Newman–Keuls method.

3. Results

3.1. Adsorption

After bubble formation had taken place the preparations containing 3% SP-C(LKS)+2% SP-B required 5 min to reach a surface tension of 25 mN/m. The surface tensions reached by adsorption at each time interval appeared to be slightly higher without SP-B, but the differences were not discernible at the 0.05% level (Fig. 1). The presence of PA did not influence the adsorption rate, neither in the presence nor in the absence of 2% SP-B in the preparations with 3% SP-C(LKS).

In the preparation with 2% KL_4 the surface tension was around 35 mN/m at time zero and adsorption was completed in 6 min, while 2% SP-C/BR did not reach end point of adsorption (25 mN/m) within 10 min (data not shown). However, no significant differences were found in the surface tensions reached on adsorption at each time interval when all samples were considered.

3.2. Quasi-static cycles

Film area compression needed to compress the surfactant films in a quasi-static manner from equilibrium surface tension of approximately 25 mN/m to a minimum surface tension of 0.5–2.0 mN/m was determined for the different surfactant preparations. The preparation with 3% SP-C(LKS) in DPPC:PG

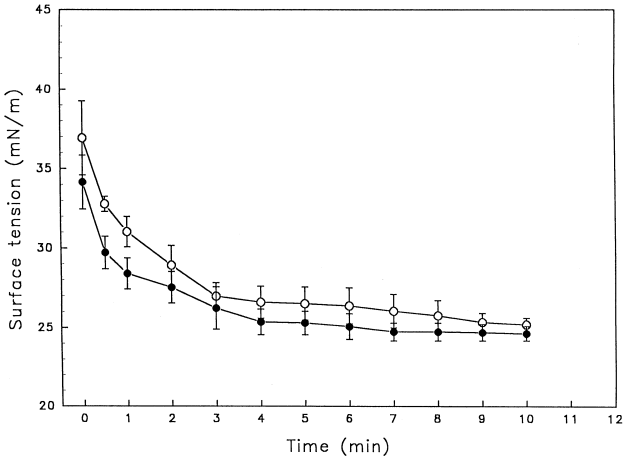

Fig. 1. Time course for adsorption of SP-C(LKS) in DPPC/PG/PA (68:22:9, by weight) with and without SP-B. Surface tension is plotted vs. time. Open circles, 3% SP-C(LKS); and filled circles, 3% SP-C(LKS)+2% SP-B.

7:3 (w/w) needed an area compression of approximately 27% to reach a minimum surface tension of 1 mN/m in the first cycle, with only a slight improvement in the fourth cycle. With PA in the lipid mixture, the area compression needed to reach minimum surface tension decreased to about 16% (Fig. 2A). When both 3% SP-C(LKS) and 2% SP-B were present in DPPC:PG 7:3 (w/w), but without PA, almost 30% area compression was needed in the first cycle and 20% in the fourth. For the preparation with 3% SP-C(LKS) and 2% SP-B in DPPC:PG:PA 68:22:9 (w/w/w) only 14% area compression was needed (Fig. 2B). The 2% KL_4 preparation needed an area compression of about 13% in the first cycle with some improvement by the fourth cycle, while the 2% SP-C/BR surfactant improved from 30 to 24% from the first to the fourth cycle (Fig. 3), but the change was not statistically discernible ($P > 0.05$).

In summary: (1) Films from all preparations could be compressed quasi-statically to obtain minimum surface tensions below 2 mN/m. (2) Films formed from the 2% KL_4 preparations required area compressions of about 13% to achieve minimum surface tensions of approximately 1 mN/m, similar to those needed for the films with 3% SP-C(LKS)+2% SP-B in the presence of PA. Films from the 2% SP-C/BR preparations required relatively large film area compressions ($\sim$30%) to reach minimum surface tension, similar to those needed for the preparations with 3% SP-C(LKS)+2% SP-B, without PA. These area compressions were significantly larger than those for the preparations with 3% SP-C(LKS)+2% SP-B with PA.

3.3. Dynamic cycles

All samples exhibited a minimum surface tension below 1 mN/m upon continuous cycling at a rate of 20–30 cycles/min. The preparation with 3% SP-

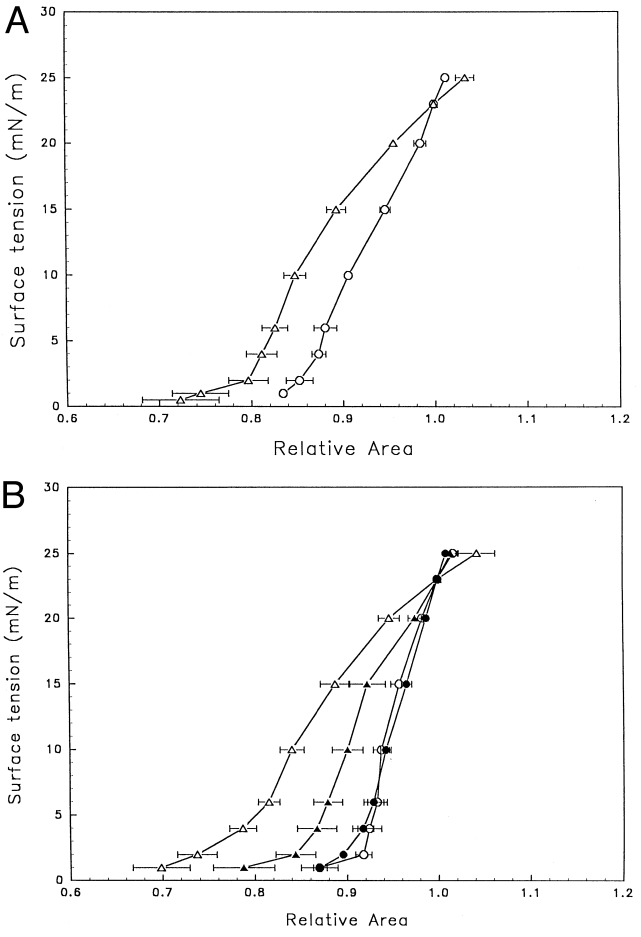

Fig. 2. (A) Quasi-static compression isotherms of SP-C(LKS) in lipid mixtures with and without PA. Surface tension is plotted vs. relative area for the first cycles. Open circles, 3% SP-C(LKS) in DPPC/PG/PA (68:22:9, by weight); open triangles, 3% SP-C(LKS) in DPPC:PG (7:3, by weight). (B) Quasi-static compression isotherms of SP-C(LKS)+SP-B in lipid mixtures with and without PA. Surface tension is plotted vs. relative area for the first (open symbols) and fourth (filled symbols) cycles. Circles, 3% SP-C(LKS)+2% SP-B in DPPC/PG/PA (68:22:9, by weight); triangles, 3% SP-C(LKS)+2% SP-B in DPPC:PG (7:3, by weight).

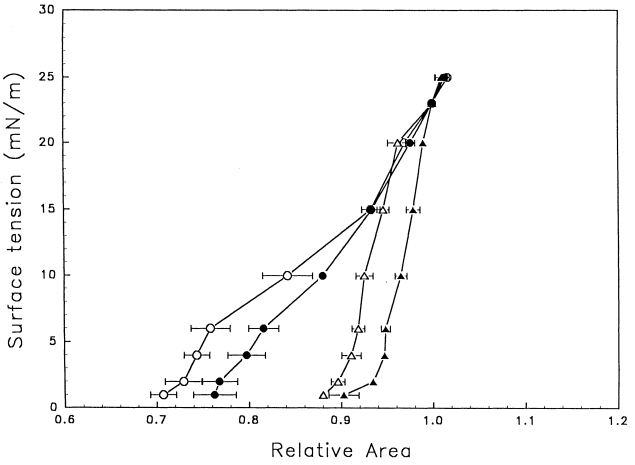

Fig. 3. Quasi-static compression isoterms of 2% SP-C/BR and 2% KL_4 in DPPC:PG:PA (68:22:9, by weight). Surface tension is plotted vs. relative area for the first (open symbols) and fourth (filled symbols) cycles. Circles, 2% SP-C/BR; triangles, 2% KL_4.

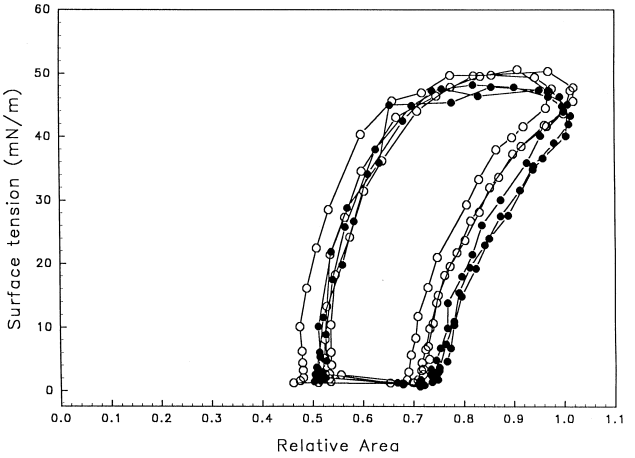

Fig. 4. Dynamic (20–30 cycles/min with approximately 20% overcompression) surface tension–area relationships of 3% SP-C(LKS) in DPPC/PG/PA (68:22:9, by weight) with and without 2% SP-B. Three consecutive dynamic cycles centred at cycle 10 are shown. Open circles, 3% SP-C(LKS); filled circles, 3% SP-C(LKS)+2% SP-B.

C(LKS) in DPPC:PG 7:3 (w/w) showed a maximum surface tension of 48–49 mN/m, similar to the maximum surface tension (50–51 mN/m) of the same preparation with PA. The addition of 2% SP-B to the preparations with 3% SP-C(LKS) ± PA reduced the maximum surface tension slightly to 46–47 mN/m, but these values were not discernible from those without SP-B ($P > 0.05$). In Fig. 4, cycles from preparations with 3% SP-C(LKS) and PA ± 2% SP-B are shown. The 2% KL_4 preparation showed a maximum surface tension of 44–45 mN/m and a region of high compressibility around 20 mN/m. 2% SP-C/BR showed a maximum surface tension of 50–51 mN/m, and at minimum surface tension sudden drops in bubble area combined with increments in surface tension, consistent with bubble clicks, were observed (Fig. 5).

The total film area compressions were 50–60%, including the reduction in film area at near zero minimum surface tension, as seen in the horizontal part of the dynamic curves (Figs. 4, 5, 6). The films from the 2% KL_4 preparations reduced the surface tension to below 1 mN/m upon an area reduction of only 18%, which is significantly lower than for all the other preparations ($P < 0.01$) (Fig. 5). The films from SP-C/BR and those from the 3% SP-C(LKS) ± SP-B but without PA required the highest film area reductions to reach minimum surface tension (34–38%). Adding PA to the above SP-C(LKS) preparations reduced the film area compressions to approximately 30% ($P < 0.01$) (Fig. 6).

3.4. Subphase depletion

In the preparations with 3% SP-C(LKS) in DPPC:PG 7:3 (w/w) without PA, incorporation of surface associated reservoir material occurred at

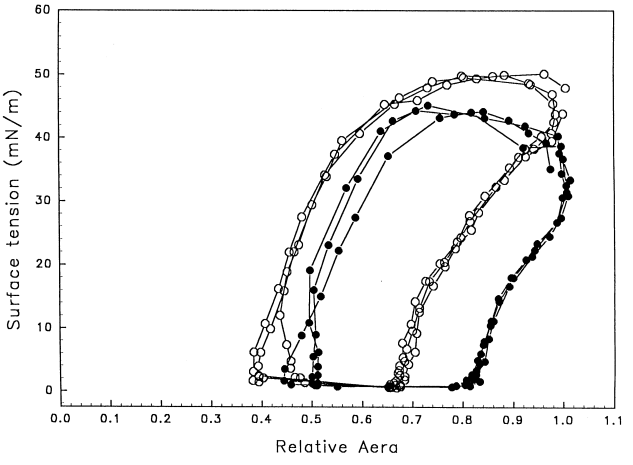

Fig. 5. Dynamic (20–30 cycles/min with 20% overcompression) surface tension–area relationships of 2% SP-C/BR and 2% KL_4 in DPPC:PG:PA (68:22:9, by weight). Three consecutive dynamic cycles centred at cycle 10 are shown. Filled circles, 2% KL_4; open circles, 2% SP-C/BR.

30–40 mN/m during stepwise bubble expansion. Minimum surface tension below 1 mN/m was reached in all cycles (Fig. 7A). Area compression needed to reach near zero minimum surface tension was around 30% in all cycles. No squeeze out plateaus were observed during compression, although sudden surface tension increases with associated drops in surface area (consistent with clicks) at minimum surface tension were observed. A surface associated reservoir equivalent to 1.5 monolayers in excess of one monolayer was calculated.

In contrast when 3% SP-C(LKS) was combined with the lipid mixture DPPC:PG:PA surface activity deteriorated (Fig. 7B). Incorporation of surface associated reservoir material occurred at 40–50 mN/m during stepwise bubble expansions. Minimum surface tension increased upon cycling from 2 mN/m in the first cycle to 5 mN/m in the third cycle, and area compression needed to reach 5 mN/m was 45%. There was a region of high film compressibility (squeeze out plateau) at about 20 mN/m on the third compression and clicks at minimum surface tension were observed. There was only material of 0.5 monolayers in excess of one monolayer associated with the surface film.

In the depletion studies with 3% SP-C(LKS)+2% SP-B in DPPC:PG 7:3 (w/w), without PA, incorporation of surface associated reservoir material occurred at 30–40 mN/m during stepwise bubble expansion (Fig. 8A). A minimum surface tension of zero was reached in all cycles and the area compression needed to reach zero minimum surface tension was 22% in the first cycle and 24% in the fifth. No squeeze out plateaus were observed during compression and clicks did not occur. 3.6 monolayers of excess material were calculated.

With 3% SP-C(LKS)+2% SP-B but using DPPC:PG:PA 68:22:9 (w/w/w) as the lipid mixture the adsorption upon stepwise increase of the bubble size occurred at the somewhat higher surface tension

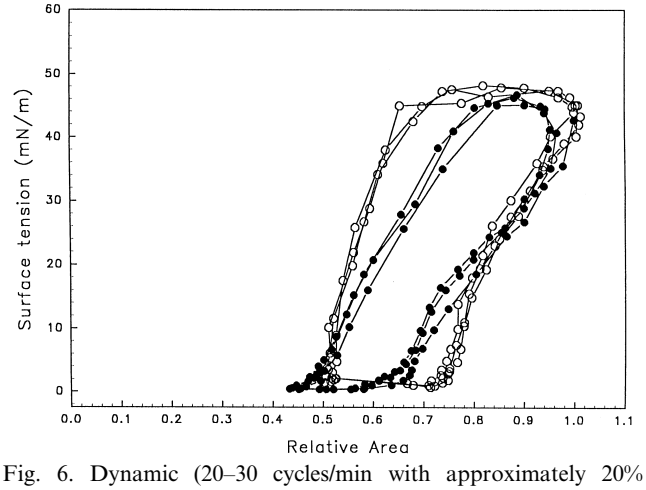

Fig. 6. Dynamic (20–30 cycles/min with approximately 20% overcompression) surface tension–area relationships of 3% SP-C(LKS)+2% SP-B in lipid mixtures with and without PA. Three consecutive dynamic cycles centred at cycle 10 are shown. Filled circles, 3% SP-C(LKS)+2% SP-B in DPPC:PG (7:3, by weight); open circles, 3% SP-C(LKS)+2% SP-B in DPPC/PG/PA (68:22:9, by weight).

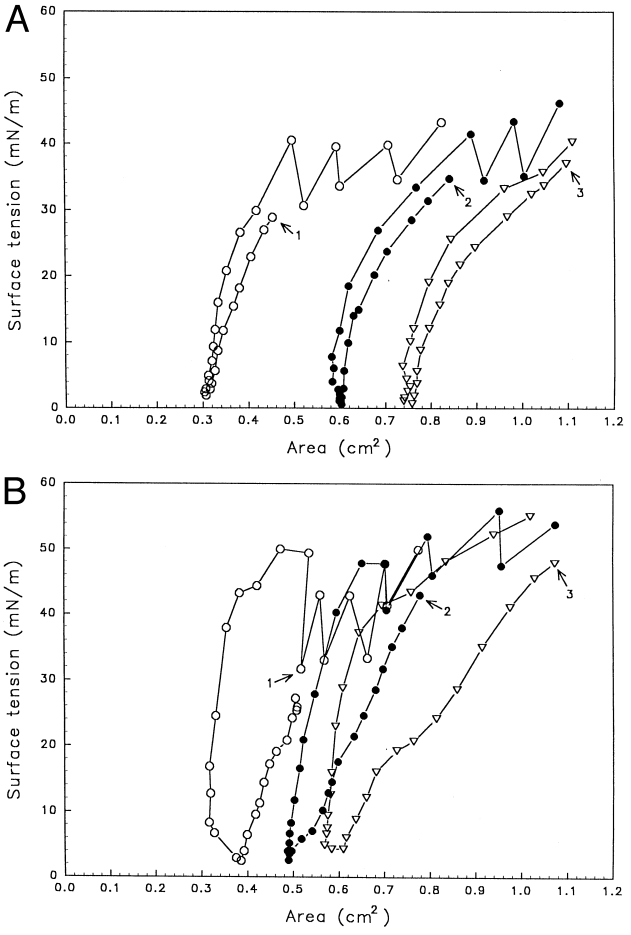

Fig. 7. Series of consecutive quasi-static compression expansion cycles conducted at increasing bubble areas, after surfactant depletion and replacement of subphase with buffer (initial bubble formation in 1 mg/ml surfactant). The plot represents consecutive quasi-static cycles, starting with the first cycle at the left. (A) 3% SP-C(LKS) in DPPC:PG (7:3, by weight). (B) 3% SP-C(LKS) in DPPC/PG/PA (68:22:9, by weight).

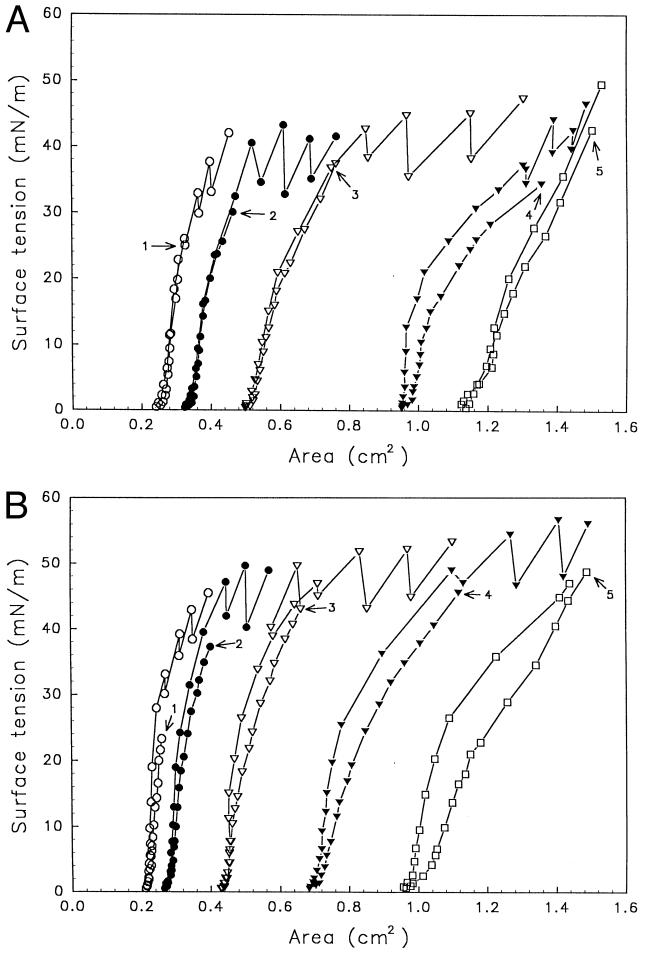

Fig. 8. Series of consecutive quasi-static compression expansion cycles after surfactant depletion and replacement of subphase with buffer as in Fig. 7. (A) 3% SP-C(LKS)+2% SP-B in DPPC:PG (7:3, by weight). (B) 3% SP-C(LKS)+2% SP-B in DPPC/PG/PA (68:22:9, by weight).

around 40–50 mN/m (Fig. 8B) than when PA was absent. Minimum surface tensions around zero were reached after surface area compressions of 15% for the first cycle and 35% for the fifth. No squeeze out plateaus were observed during compression and sudden increase in surface tension at minimum surface tension did not occur. Excess material equivalent to 3.4 monolayers were calculated.

The 2% KL_4 preparation in DPPC:PG:PA 68:22:9 (w/w/w) incorporated material from surface associated reservoir at 25–35 mN/m during stepwise bubble expansion. Near zero surface tension was reached in all cycles with a surface area compression around 30% throughout the experiment. Squeeze out plateaus were observed during compression in the fourth and fifth cycle and small instability clicks were observed at end compression for each cycle. 4.3 monolayers in excess of one monolayer were calculated (Fig. 9A).

With 2% SP-C/BR in the same lipids the surface tension for adsorption upon stepwise bubble expansion was 40–45 mN/m and the area compression needed was 43% in the first cycle and 32% in the third. Instability clicks were observed as well as high compressibility regions on compression at approximately 20 mN/m. Approximately one monolayer excess material in the surface associated reservoir was calculated (Fig. 9B).

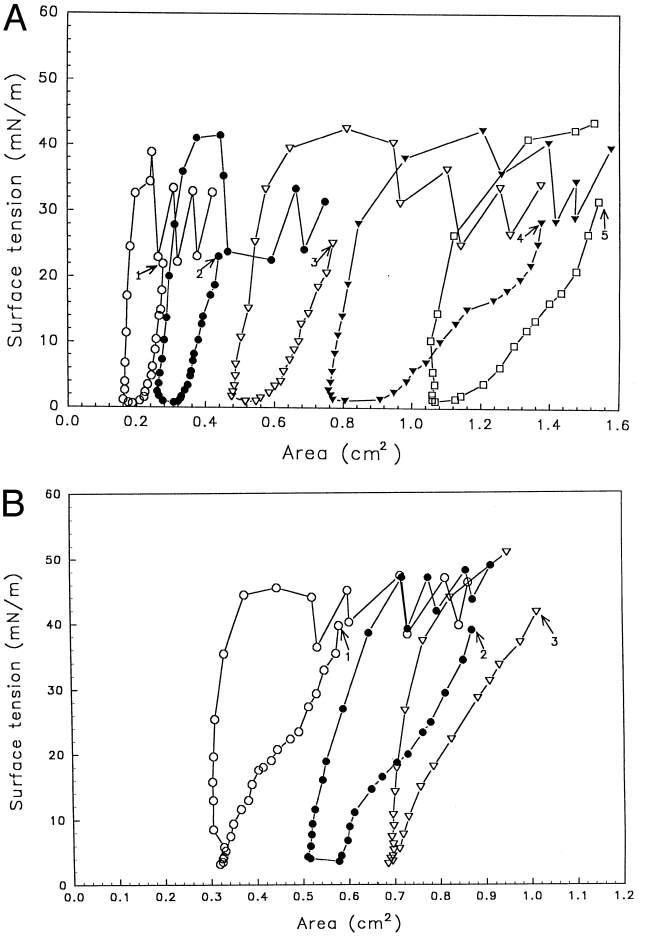

Fig. 9. Series of consecutive quasi-static compression expansion cycles after surfactant depletion and replacement of subphase with buffer as in Fig. 7. (A) 2% KL_4 in DPPC:PG:PA (68:22:9, by weight). (B) 2% SP-C/BR in DPPC:PG:PA (68:22:9, by weight).

4. Discussion

There is no consensus theory established for the mechanisms of interaction between the surfactant proteins and lipids. SP-B has been shown to induce lipid vesicle mixing, especially in the presence of PG [29], probably by cross-linking lipid membranes by means of ionic interactions. In that way SP-B could maintain a surfactant reservoir close to the air–water interface. It has been suggested that the biological activity of SP-C is to enhance surfactant spreading, and that this is an effect of the α-helical conformation of the peptide rather than of the amino acid sequence [21] (for definitions of adsorption and spreading see [30]). Although present in a monolayer both SP-B and SP-C are able to promote insertion of phospholipid molecules to the interface [31,32]. SP-C/BR was designed as a representative α-helical transmembrane peptide, and showed in mixtures with lipids rapid spreading on a Wilhelmy balance [21]. It has been stated that preparations with quick adsorption, that is, rapid accumulation also exhibit rapid spreading [30]. In the CBS spreading activity is not studied per se but adsorption is monitored by measuring the reduction in surface tension with time. The surface activity of the SP-C/BR preparation was inferior to other peptide/lipid mixtures studied. The preparation did not completely adsorb during 10 min, exhibited clicks indicating instability upon dynamic cycling, and showed a limited reservoir of excess surfactant material, or limited incorporation into the surface active film of such material. One explanation could be the less hydrophobic character of the bacteriorhodopsin α-helix compared to that of native SP-C or its analogues (Table 1).

We have studied the effect of the addition of PA to the SP-C(LKS) lipid mixture. PA is often added to artificial surfactant preparations and it often increases the adsorption rate in the pulsating bubble surfactometer (PBS) (unpublished observations) and makes the suspensions more homogeneous. In the CBS, no effect of PA on adsorption was noted. However, addition of PA to the lipid mixture decreased the film area compression needed to reach minimum surface tension during quasi-static and dynamic cycling (Figs. 2, 6). This is in agreement with earlier findings [33]. On the other hand, PA seems to reduce the amount of surface associated material or its incorporation into the surface active film (Fig. 7).

The effect of adding SP-B to SP-C(LKS) in lipid mixtures with and without PA was studied. Addition of SP-B increased the adsorption rate slightly, but the increase was not discernible from that observed on the preparations without SP-B, and the adsorption was not comparable to that of natural surfactant preparations. Earlier studies using the PBS [20] have demonstrated a decrease of surface tension at maximum bubble radius (γ_{max}) upon the addition of SP-B, which was interpreted as an indication of SP-B maintaining a surfactant reservoir. In the CBS, the decrease of maximum surface tension upon dynamic cycling after addition of SP-B (Fig. 4) was not as pronounced as in the PBS [20] were it decreased from 42 to 33–35 mN/m. The different results might be related to the higher surfactant concentrations used in the PBS, ∼10 mg/ml, vs. 1.0 mg/ml in the CBS. It has been shown in earlier studies [27] that maximum surface tension upon dynamic cycling is dependent on the degree of compression. In the present study, the films were compressed dynamically to a total area compression of 50–60%, which might be higher than the real film area compression in the PBS, which nominally should be 50% but might be lower due to film spreading in the capillary tube in the device [34]. Relatively high film area compressions upon dynamic cycling reduce film reformation upon bubble expansion, causing higher maximum surface tension and larger film area compressions needed to achieve near zero surface tension compared to films cycled with less area compressions [35].

Both SP-B and SP-C promote lipid adsorption and spreading at an air–water interface, but SP-C appears to be more efficient than SP-B [5]. SP-B seems to promote lipid insertion more efficiently from surface associated vesicles or multilamellar structures than SP-C [36]. SP-B seems to be involved both in the formation of the surface associated reservoir by promoting bilayer–bilayer or bilayer–monolayer contact [10–13] and to replenish the interface upon expansion [37]. The most pure experiment for studying replenishment from the surface associated surfactant reservoir is subphase depletion experiments. When 2% SP-B was added to the mixture of 3% SP-C(LKS) in DPPC:PG 7:3 (w/w), the number of

monolayers in excess of one monolayer increased from 1.5 to 3.6. When 2% SP-B was present in the mixture of 3% SP-C(LKS) in DPPC:PG:PA 68:22:9 (w/w/w) the number of calculated monolayers in excess of one monolayer increased from 0.5 to 3.4, but incorporation of surface active material occurred at somewhat higher surface tension. The KL_4 preparation exhibited excess material of 4.3 monolayers and thus mimics the action of SP-B under these conditions. Interestingly this was not observed in the PBS, where no decrease of γ_{max} was seen when KL_4 was added to the preparation of 3% SP-C(LKS) in DPPC:PG 7:3 (w/w) [20]. However, in the PBS, effects due to phenomena related to the bulk suspension are not clearly separated from those related to the surface associated material. In this investigation the KL_4 peptide was superior or equal to the best combination of SP-C(LKS) and SP-B in the different experimental protocols. Under dynamic conditions KL_4 surfactant seems to produce a monolayer enriched in DPPC which is continuously purified by squeeze out at a surface tension of approximately 20 mN/m.

There are no generally accepted parameters for in vitro evaluation of artificial surfactant preparations. Different preparations have been optimised individually, using different in vitro methods. Furthermore, many in vitro methods only measure a specific aspect of surfactant activity. The CBS offers a broad approach in optimising the preparations and a possibility to find a combination for which results from in vitro studies better correlate with those from in vivo animal models.

In summary, the adsorption rates were slow for all samples compared to animal derived natural surfactant preparations and were not significantly influenced by the inclusion of either PA or SP-B. In the quasi-static experiments addition of PA, especially in combination with SP-B, to SP-C(LKS)/DPPC/PG decreased the compression needed to reach minimum surface tension. Dynamic compression of KL_4 containing films were less effected by overcompression than those containing SP-C(LKS) and SP-B. After subphase depletion SP-B containing preparations increased the ability of the film to incorporate surfactant material from the surface associated reservoir. Also surface films of KL_4 preparations had similar properties and seem to act in an SP-B manner under these conditions.

Recently different artificial, synthetic peptide containing, surfactant preparations have been found useful for replacement therapy in experimental models and clinically, i.e. KL_4 surfactant [38,39] and rSP-C surfactant [40]. The performance of an artificial surfactant is however dependent on a PEEP (positive end expiratory pressure) value [41]. In treatments with natural surfactant preparations there is no need for ventilation with PEEP to prevent the lungs from collapse at end expiration. This indicates that further improvements can be made. Improvement of in vitro surface activity upon addition of SP-B has been shown [20]. There is not yet reported a recombinant expression of SP-B, nor does it appear possible to synthesise large quantities of SP-B due to its size and complexity. Absence of SP-B is fatal in the neonatal period [42] which has been confirmed in knock out mice [14]. Since SP-B is needed for survival, its significance is high. Addition of SP-B or a functionally related analogue that has the same ability of keeping lipid bilayers in close proximity can improve in vitro surface activity [9]. This might be a way to find more efficient artificial surfactant preparations for replacement therapy.

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