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Biochimica et Biophysica Acta 1778 (2008) 291–302
Biochimica et Biophysica Acta
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Interaction between chitosan and bovine lung extract surfactants

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Received 7 April 2007; received in revised form 27 September 2007; accepted 2 October 2007
Available online 14 October 2007

Abstract

The interaction between a cationic polyelectrolyte, chitosan, and an exogenous bovine lung extract surfactant (BLES) was studied using dynamic compression/expansion cycles of dilute BLES preparations in a Constrained Sessile Drop (CSD) device equipped with an environmental chamber conditioned at 37 °C and 100% R.H. air. Under these conditions, dilute BLES preparations tend to produce variable and relatively high minimum surface tensions. Upon addition of "low" chitosan to BLES ratios, the minimum surface tension of BLES–chitosan preparations were consistently low (i.e. <5 mJ/m^2), and the resulting surfactant monolayers (adsorbed at the air–water interface) were highly elastic and stable. However, the use of "high" chitosan to BLES ratios induced the collapse of the surfactant monolayer at high minimum surface tensions (i.e. >15 mJ/m^2). The zeta potential of the lung surfactant aggregates in the subphase suggests that chitosan binds to the anionic lipids (phosphatidyl glycerols) in BLES, and that this binding is ultimately responsible for the changes in the surface activity (elasticity and stability) of these surfactant–polyelectrolyte mixtures. Furthermore the transition from "low" to "high" chitosan to BLES ratios correlates with the flocculation and de-flocculation of surfactant aggregates in the subphase. It is proposed that the aggregation/segregation of "patches" of anionic lipids in the surfactant monolayer produced at different chitosan to BLES ratios explains the enhancing/inhibitory effects of chitosan. These observations highlight the importance of electrostatic interactions in lung surfactant systems.

Keywords: Polyelectrolyte; Surface tension; Zeta potential; Respiratory distress syndrome

1. Introduction

Respiratory distress syndrome (RDS) is a condition that affects preterm neonates (nRDS) with premature lungs that are not yet capable of producing lung surfactants, and adults (ARDS) whose lungs are afflicted by a serious injury that typically produces inactive lung surfactant films [1,2]. The introduction of surfactant replacement therapy in the 1980s has reduced the mortality of nRDS patients to less than half [1–3]. Unfortunately, lung surfactant therapy has been ineffective in reducing the mortality of ARDS patients or nRDS patients where the alveolar fluid is severely compromised by inhibitory factors such as serum proteins [1,2,4–6].

Lung surfactants were first defined by Pattle as a complex mixture of phospholipids and proteins [7,8]. After calculating the Laplace pressure in alveoli of different sizes, Pattle concluded that the surface tension (γ) of the alveoli should be close to zero in order to avoid alveoli collapse. In general, it is necessary to ensure that surface tensions of 5 mJ/m^2 or less are attained at the end of the expiration to prevent lung collapse [1,4,5,9].

Lung surfactants are produced and recycled by Type II pneumocytes. On average, lung surfactants are composed of 70–80% phosphatidylcholines (PC), 10–15% phosphatidylglycerols (PG), 7–10% proteins, 4–7% neutral lipids (mostly cholesterol), and minor fractions of other phospholipids [10–12]. Dipalmitoylphosphatidylcholine (DPPC) is the main PC species (50% of total surfactant) in lung surfactants, and has a net zero charge.

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0005-2736/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamem.2007.10.003

Dipalmitoylphosphatidylglycerol (DPPG) is the main PG species and is negatively charged at physiological conditions. DPPC is considered to be the molecule responsible for producing near zero surface tension upon compression [1]. However, DPPC requires the presence of surfactant proteins and ionic lipids to facilitate the exchange of material with the subphase [13,14]. Neutral lipids (in particular cholesterol) have been found to influence the lateral phase separation of lung surfactant films, and the spreading properties of lung surfactant aggregates [15]. There are four lung surfactant proteins: SP-A, SP-B, SP-C, and SP-D [16]. SP-A is the most abundant of the proteins and is hydrophilic and negatively charged. SP-B is a hydrophobic and positively charged protein which is considered essential to lung surfactants since SP-B deficiency is a lethal condition [17].

Various exogenous surfactant formulations have been produced for surfactant replacement therapy [1]. Bovine Lipid Extract Surfactant (BLES) is one of these. BLES is obtained using a solvent extraction process that removes water soluble components such as SP-A and SP-D. BLES has shown to be an effective surfactant in the treatment of neonatal respiratory distress syndrome (nRDS), and is one of the exogenous lung surfactant replacement preparation approved in Canada [18,19]. BLES contains nearly 40–45% DPPC, ~35% unsaturated PCs, 10–12% PGs, ~2% phosphatidylinositol (PI), ~3% phosphatidylethanolamine (PE), ~1.5% lyso-bis-phosphatidic acid (lyso-bis-PA), and ~2.5% sphingomyelin (SM), 1–2% proteins SP-B and SP-C. The neutral lipids (including cholesterol) are removed after washing the extracted material with acetone [18,20–22]. The range in composition indicated above for key components like SP-B and SP-C proteins, DPPC and PGs is to be expected from natural sources like bovine lungs, and can be expected to lead to batch to batch variations.

Numerous additives are currently under consideration to improve the effectiveness of exogenous lung surfactant formulations. A number of these additives are surface active protein analogs (in particular the cationic protein SP-B) [23,24], and cationic antibiotics such as polymyxin B [25,26]. Nonionic polymers such as dextran or polyethylene glycol (PEG) have also been considered [20,27–34]. However, these nonionic polymers need to be formulated at relatively high concentrations, and this leads to viscous solutions that are difficult to instill and have sometimes been found detrimental to the formulation [35]. The mechanism of action of these nonionic polymeric additives is based on a depletion–attraction effect [28,36] as ionic moieties are not present in the polymers. The depletion attraction mechanism proposes that, at high polymer concentrations, the difference in osmotic pressure between the polymer solution and the liquid in between the lipid vesicles is high enough to induce the "fusion" of the vesicles into larger aggregates. It is believed that these larger aggregates produce a rapid exchange of material with the surfactant film adsorbed at the air/water interface, leading to films enriched in DPPC that yield lower surface tensions.

Hyaluronan, an anionic polymer, has also been used to produce the same effects using lower polymer concentration [37,38]. While hyaluronan has produced a substantial improvement in terms of achieving inhibition reversal with low polymer concentrations (thus reducing the viscosity of the formulation), it is less effective than chitosan at improving the surface activity (fast adsorption, high dilatational elasticity) and reversing BLES inhibition [39].

A previous study has found that the chitosan concentration needed to reverse BLES inhibition is 500 times lower than that required by PEG to achieve the same effect [39]. Given the low concentrations at which chitosan is effective, and the fact that chitosan itself is not surface active (equilibrium surface tension of chitosan alone is 67.4 ± 0.3 mJ/m^2 — see Ref. [39]), it was proposed that the electrostatic interactions between anionic lipids and chitosan might be responsible for the observed phenomena [39]. However, a corroboration and understanding of this electrostatic interaction hypothesis between chitosan and BLES was yet to be pursued. In previous work [39] only a limited range of chitosan concentrations (no higher than 0.1 mg/ml) was considered and the optimal chitosan dosage was not systematically studied. The main objective of this work is to investigate the interaction between BLES and chitosan and to determine the optimal chitosan dosage for different surfactant concentrations. To this end, the surface tension of several chitosan–BLES formulations have been evaluated using dynamic compression/expansion cycles that mimic physiological conditions. The relation between BLES concentration and optimal chitosan dosage (defined as the concentration of chitosan that produces lowest minimum surface tension upon compression) is explored via optical micrographs and zeta potential measurements of the subphase surfactant aggregates.

2. Materials and methods

2.1. Materials

Bovine Lipid Extract Surfactant (BLES) was provided by BLES Biochemicals Inc. (London, ON, Canada) and used without further purification [20,27,28,40]. BLES was stored at −20 °C with an initial concentration of 27 mg lipids/ml and distributed into 1 ml glass vials in a N_2 atmosphere. On the day of experiment, one vial was first incubated in a 37.5 °C water bath for 1 h, before dissolving in a salt solution of 0.6% NaCl and 1.5 mM $CaCl_2$ [39]. BLES preparations containing 0.5, and 2.0 mg lipids/ml solution were prepared. These BLES preparations had a pH value of 5–6.

Chitosan (Cat. No. 448869, M_r 612 kDa, degree of deacetylation 75–85%) was purchased from Sigma-Aldrich. Further characterization for this chitosan can be found elsewhere [41]. 100 mg Chitosan was first dissolved in 9 ml 0.05 M hydrochloric acid overnight to ensure complete dissolution. The prescribed amount of chitosan (according to the specific formulation) was withdrawn and added to the NaCl/$CaCl_2$ salt solution on the day of the experiment [39]. For each experiment, the chitosan solution was added to an equal volume of the BLES suspension. The final pH of these chitosan–BLES suspensions ranged from 5 to 5.5. The chitosan concentration in lung surfactant preparations ranged from 0.01 to 0.5 mg/ml.

2.2. Methods

2.2.1. Surface tension measurements

After gentle vortexing, the BLES–chitosan mixture was loaded into a motor-driven syringe (2.5 ml, #1002, Gastight), ready for dynamic surface tension measurements using the Constrained Sessile Drop (CSD) surfactometer. The CSD apparatus allows measurements of low surface tension in lung surfactant systems regardless of the surfactant concentration [40,42]. The details of the design and operation of the CSD device has been described elsewhere [40,42,43]. The operation of the CSD device begins when a sessile drop of the

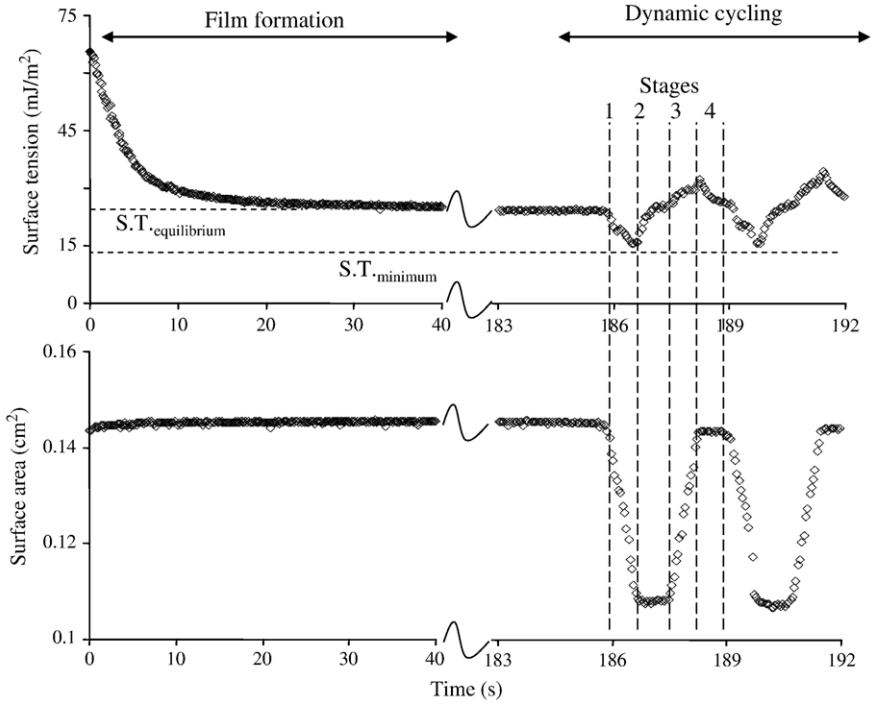

Fig. 1. Surface tension and surface area output for film formation and dynamic cycling experiments. BLES 0.5 mg/ml compressed at 3 s/cycle periodicity in humid (100% R.H.) air. Stages 1 through 4 correspond to film compression, film relaxation, film expansion and film re-absorption respectively.

test liquid is formed on a circular horizontal surface of a stainless steel pedestal (3 mm diameter). The pedestal has a sharp-knife edge (60° angle of approach) to prevent the spreading of the test liquid when the lipid film reaches a near zero surface tension at the end of the compression stage. During the experiments, the droplet and the pedestal are enclosed in a chamber that allows the control of gas composition and temperature. The humidity in the chamber was kept constant at 100% relative humidity at 37 °C. A CCD camera (Model 4815-5000, Cohu Corp., Poway, CA) was used to acquire the images of the drop throughout the experiment at a rate of 15 images/s. The acquired images were processed by a digital video processor (Snapper-8, Acsire Silicon Ltd., Uxbridge, UK) and stored in a workstation (Sun Blade 1500, Sun Microsystems Inc., Santa Clara, CA) for further analysis by Axisymmetric Drop Shape Analysis (ADSA) [43].

Table 1
Surface tension at equilibrium ($S.T._{eq}$), minimum surface tension ($S.T._{min}$), and dilatational elasticity (ε) of dilute (0.5 mg/ml) BLES preparations (at 10% area reduction) compressed with a periodicity of 3 s/cycle and 20% area reduction at the end of compression in pre-humidified air (100% R.H.)

BLES Batch	$S.T._{eq}$ (mJ/m^2)	$S.T._{min}$ (mJ/m^2)	ε (mJ/m^2)	Experiment Month-year
A	23.1	5.5±0.8	101.7±2.9	Feb-05
B	22.6	11.9±0.8	77.6±8.6	May-05
C	26.0	6.5±0.9	103.0±12.7	Jul-05
D	24.1	17.6±0.3	–[a]	Aug-06
D	23.7	15.3±0.2	–[a]	Sep-06
E	23.5	20.4±1.1	–[a]	Jun-06
E	24.3	19.3±0.1	–[a]	Nov-06
E	24.6	14.7±0.4	–[a]	Dec-06
F	22.6	5.5±0.5	109.8±3.5	May-07

Notes:
[a] These batches showed signs of film collapse before or at 10%, area reduction, thus no elasticity was calculated.

ADSA determines surface tensions by numerically fitting the shape of the drops to a theoretical profile generated using the Laplace equation of capillarity. Typical output of ADSA includes surface tension, surface area and volume of the drop.

The dynamic compression/expansion experiments are carried out by periodically injecting/withdrawing liquid from the drop through a motor-controlled syringe (controller 18705/6, Oriel Instruments, Stratford, CT). The cycle periodicity and the volume injected/extracted are programmed into the motor controller.

The surface tension procedure encompasses two stages. The first stage involves forming a drop on the pedestal (within 0.5 s) and tracking the surface tension over time until the film reaches the equilibrium surface tension value. This is the film formation stage illustrated in Fig. 1. In this stage, the surface tension of the suspension decreases from an initial surface tension of nearly 70 mJ/m^2 to surface tension values ranging from 20 to 25 mJ/m^2 at equilibrium [1].

Dynamic cycling started once the equilibrium surface tension has been reached. As shown in Fig. 1, the dynamic cycling consists of four sub-stages: compression, relaxation, expansion and re-adsorption. One of the main performance parameters that is obtained from these measurements is the minimum surface tension (ST_{min}) reached at the end of each compression (See Fig. 1). It is important to note that this

Table 2
Surface active properties of BLES 0.5 mg/ml preparations containing chitosan

Chitosan concentration (mg/ml)	ST_{min} (mJ/m^2)	Dilatational elasticity (ε) (mJ/m^2)	Relaxation rate (γ') (mJ/m^2-s)
0	11.9±0.9	86±10	9.6±2.7
0.01	7.2±0.5	101±8	15±3.0
0.05	2.8±0.2	169±5	2.6±1.5
0.0625	3.2±1.2	199±3	0.43±0.08
0.10	3.5±0.5	174±2	0.87±0.23
0.15	8.1±0.6	119±5	11.0±0.91
0.25	17.9±0.4	–[a]	–[a]

[a] The film collapsed before reaching $A=0.9Ao$.

Table 3
Surface active properties of BLES 2.0 mg/ml preparations containing chitosan

Chitosan concentration	ST_{min}	Dilatational elasticity (ε)	Relaxation rate
(mg/ml)	(mJ/m^2)	(mJ/m^2)	(mJ/m^2-s)
0	9.7±0.4	84±4	11.5±0.9
0.02	7.9±0.3	94±3	7.2±0.5
0.05	8.7±0.7	121±2	5.1±0.7
0.10	2.7±0.1	168±6	1.2±0.9
0.15	3.4±0.2	167±7	1.9±0.7
0.25	17.4±0.7	– [a]	– [a]
0.50	18.5±0.5	– [a]	– [a]

[a] The film collapsed before reaching $A=0.9$Ao.

minimum surface tension depends on the compression conditions, specifically the rate and extent of compression. Throughout this work we use a compression periodicity of 3 s/cycle and a compression ratio (fraction of surface area reduction) of 20% to simulate normal breathing conditions [1,43].

For each of the formulations presented in Tables 1–3, four or more droplets were tested in CSD experiments, and the results are expressed as the mean±95% confidence interval ($n>4$ unless otherwise indicated).

2.2.2. Dilatational elasticity calculation

Dilatational elasticity is a property (ε) of the surfactant film that defines the magnitude of surface tension reduction for a given film compression (surface area reduction). To calculate this value, the following equation was used [43]:

$$\varepsilon = \left(\frac{A}{\mathrm{Ao}}\right)\frac{d\gamma}{d\left(\frac{A}{\mathrm{Ao}}\right)}. \quad (1)$$

A is the surface area of the drop at any time, Ao is the surface area of the drop at the beginning of the compression stage (stage 1 in Fig. 1), and γ is the surface tension at any time during the compression. To calculate $d\gamma/d(A/\mathrm{Ao})$ the 3rd compression cycle of every $\gamma - A/\mathrm{Ao}$ isotherm was isolated (this is when steady state cycling was reached) and fitted to a 4th order polynomial equation. The slope ($d\gamma/d(A/\mathrm{Ao})$) of the fitted curve evaluated at half compression ($A/\mathrm{Ao}=0.9$) was calculated to obtain the dilatational elasticity value according to Eq. (1). These values are listed in Tables 1–3. These calculations were conducted using a digital differentiator, Savitzky–Golay (SG) filter [44], which takes the first derivative of the 4th order equation by moving a convolution mask, based on a piecewise least-squares polynomial fitting, over the experimental data. For some of the formulations presented in Tables 1–3, the surfactant film (adsorbed at the air/water interface) collapsed even before reaching a compression of $A/\mathrm{Ao}=0.9$, in which case no elasticity values are reported. The onset of film collapse occurs when further reduction of surface area of the drop does not yield a reduction in surface tension. The onset of film collapse and the collapse surface tension are reported in Tables 1–3.

2.2.3. Surface tension relaxation

To assess the relaxation effects, the rate of surface tension increase ($\gamma' = d\gamma/dt$) at the beginning of the relaxation stage (Fig. 1, stage 2) was calculated. To calculate γ', the $\gamma - t$ data obtained during the relaxation stage of the 3rd cycle was also fitted to a 4th order polynomial, and the slope ($\gamma' = d\gamma/dt$) in the middle of this relaxation stage was obtained using the SG filter introduced above. The calculated values of γ' are listed in Tables 2 and 3.

2.2.4. Optical microscopy

The aggregates present in the subphase of BLES–chitosan preparations were observed using an inverted optical microscope (Olympus IMT-2, Lake Success, NY). Images were taken by a high-resolution digital camera (Sony XCD-SX900, Toronto, ON, Canada).

2.2.5. Zeta potential measurements

The zeta potential of the surfactant aggregates was determined using a Delsa 440SX Zeta Potential Analyzer (Coulter–Beckman, Miami, FL). The measurements were made at room temperature (25 °C). To prevent the saturation of the light scattering detectors, each sample was diluted by a factor of 10 in a 0.9% NaCl solution. Zeta potential measurements of diluted and undiluted 0.5 mg/ml BLES had the same zeta potential, thus confirming that the dilution procedure does not change the zeta potential of the aggregates.

3. Results and discussion

3.1. Dynamic surface tension of dilute (0.5 mg/ml) BLES suspensions compressed in pre-humidified air

The surface activity of BLES formulations was tested by compressing these preparations in an environmental chamber filled with air pre-saturated with water (100% R.H.). It has been reported that air pre-saturated with water has a detrimental effect on the ability of lung surfactant preparations to achieve a low surface tension upon compression [43,45–47]. Furthermore, when the same lung surfactant preparations are compressed in dry air (less than 20% R.H.) the minimum surface tension is consistently low (i.e. less than 5 mJ/m^2) [43,46]. It has been proposed that the hydration of the lung surfactant film adsorbed at the air/water interface causes a fluidization effect that promotes film relaxation, leading to high minimum surface tension upon compression [43].Various factors, such as the reduced content of neutral lipids (cholesterol in particular) and surfactant protein SP-A in BLES preparations have been proposed as the causes of these film hydration–fluidization phenomena [43,46].

Over the last three years, i.e. the period in which dilute BLES preparations (0.5 mg/ml) have been evaluated using an environmental chamber with pre-humidified air (using the CSD device), it has been observed that, in the absence of additives, the minimum surface tension (obtained during dynamic compression cycles) of these preparations varies widely from batch to batch. Table 1 summarizes the surface tension at equilibrium, minimum surface tension, and dilatational elasticity of various batches of BLES preparations.

In Table 1, the minimum surface tension of dilute (0.5 mg/ml) BLES preparations ranges from as low as 5.5 mJ/m^2 to as high as 20.4 mJ/m^2. While there are significant variations in the minimum surface tension, the equilibrium surface tension values are relatively constant for all batches. Furthermore, low elasticity values, and the onset of film collapse, are observed for those batches with minimum surface tensions larger than 10 mJ/m^2. These observations highlight that, in the presence of pre-humidified air, the surface activity (expressed in terms of minimum surface tensions) of dilute BLES preparations tends to be highly variable.

The reasons behind the high values of minimum surface tensions of dilute BLES preparations in the presence of pre-humidified air are not completely clear. However, it should be reiterated that BLES is a complex mixture of lipids and proteins. In dilute BLES preparations, relatively small changes in compositions in key components such as SP-B (from 1% to 2%) could be responsible for drastic changes in the surface activity. However, in clinical applications, high surfactant dosages (up to 100 mg/kg) are instilled using concentrated solutions of BLES (~27 mg/ml) that buffer these changes in surfactant composition, and yield the desired performance observed in clinical practice [19].

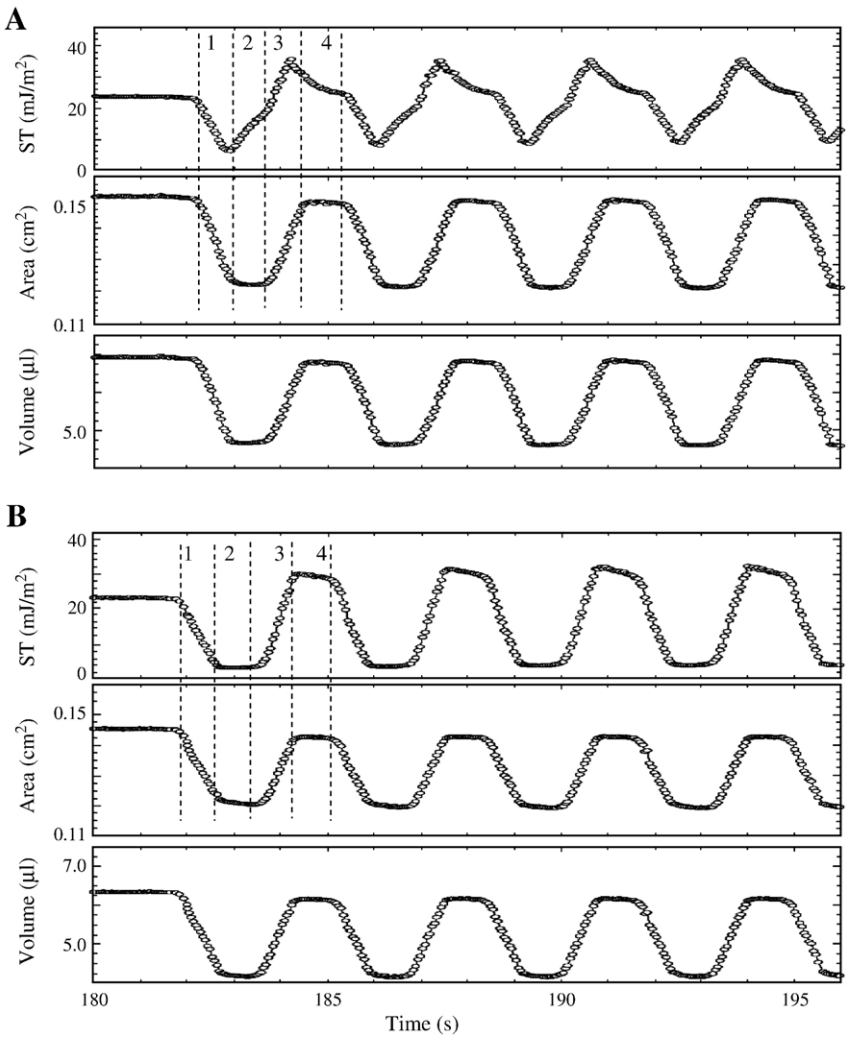

Fig. 2. Surface tension–area–volume (*S–A–V*) example output for 0.5 mg/ml BLES (part A) and 0.5 mg/ml BLES +0.1 mg/ml chitosan (part B) compressed at 37 °C, 100% R.H., 3 s/cycle periodicity, approximately 20% surface area compression.

3.2. Effect of chitosan on BLES suspensions compressed in pre-humidified and dry air

Fig. 2 presents a sample output (surface tension–area–volume) for the dynamic cycling of BLES 0.5 mg/ml and a lung surfactant formulation containing BLES 0.5 mg/ml and chitosan 0.1 mg/ml compressed in pre-humidified air (100% R.H. air). Fig. 2 shows the typical cycle to cycle reproducibility in terms of surface area, and drop volume is observed from the third cycle onwards. All the results presented in this work consider values obtained at the third cycle or shortly afterwards. Comparing the surface tension and surface area output for BLES alone (Fig. 2A) and BLES+chitosan (Fig. 2B), it is evident that the addition of chitosan enabled BLES preparations to achieve low and stable surface tensions during the compression stage 2.

In order to determine the effect of chitosan on different batches of BLES compressed in 100% R.H. air, the minimum surface tension obtained with different batches of BLES (containing 0.5 mg/ml and 2.0 mg/ml of BLES) is plotted in Fig. 3, as a function of the chitosan/BLES mass ratio. The addition of chitosan consistently reduces the minimum surface tension of BLES batches, independently of the minimum surface tension of the original batch in the absence of chitosan.

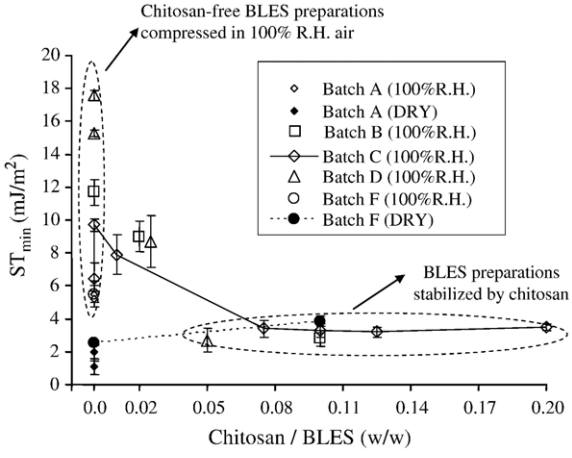

Fig. 3. Minimum surface tension (end of compression) as a function of chitosan/BLES weight ratio for 0.5 and 2.0 mg/ml BLES preparations compressed to a 20% surface area reduction using a cycle periodicity of 3 s/cycle.

Another important feature of Fig. 3 is that the minimum surface tensions of BLES+chitosan systems compressed in pre-humidified air are similar to those obtained with a batch of BLES compressed in dry air (batches A and F) with and without added chitosan. The data in Fig. 3 suggests that chitosan helps prevent the fluidization of the surfactant film at the air/water interface induced by the hydration effects of pre-humidified air (100% R.H.) [43]. Furthermore, the data presented in Fig. 3 shows that the fluidizing effect of pre-humidified air on dilute surfactant preparations represents a suitable and simple framework to investigate the lung surfactant–chitosan interactions. Thus, BLES–chitosan mixtures compressed in 100% R. H. air are convenient to investigate the interaction between these two species since no additional species (such as protein inhibitors) are involved. The studies presented in the following sections were conducted using batches B and C (Table 1) unless otherwise specified.

3.3. Effect of chitosan dosage on 0.5 mg/ml BLES suspensions

A close inspection of Fig. 2A reveals that, at the end of the compression stage (stage 3) the surface tension of the BLES-alone preparation increases due to relaxation whereas the surface tension of BLES–chitosan preparations (Fig. 2B) remains constant and low up to the beginning of the expansion stage (stage 4). The fact that the addition of chitosan suppressed the surface tension relaxation (i.e. surfactant film relaxation) is consistent with the stabilizing effect of chitosan against the film fluidization effect of humidity (preceding section). This example indicates that additives like chitosan affect various film properties and that the analysis of the results should not concentrate on minimum surface tensions only (Fig. 3), but on the changes of the mechanical properties of the surfactant film adsorbed at the air/water interface. For this reason, the changes in film elasticity and relaxation are analyzed as a function of chitosan dosage in this section. Table 2 summarizes the surface active properties of mixtures of BLES (0.5 mg/ml) and chitosan, as a function of chitosan concentration.

According to Table 2, the minimum surface tension decreased when the chitosan concentration increased from 0.01 mg/ml to 0.05 mg/ml and remained low (less than 5 mJ/m^2) within the range of 0.05 mg/ml to 0.1 mg/ml. The same trend was previously observed within the range of 0.01 mg/ml to 0.1 mg/ml chitosan [39]. However, the minimum surface tension increased for systems containing more than 0.1 mg/ml of chitosan. When the chitosan concentration was increased to 0.25 mg/ml, the surfactant film collapsed at a high surface tension of approximately 18 mJ/m^2.

The quality of the film can be assessed using the values of dilatational elasticity (ε) and the rate of surface tension relaxation (γ') [43]. The dilatational elasticity (or its reciprocal value — compressibility) reflects the "stiffness" of the surfactant film [48], and the rate of surface tension relaxation reflects the stability of the film [43]. The values of dilatational elasticity (ε) and the rate of surface tension relaxation (γ') in Table 2 suggest that the optimal chitosan dosage range (for 0.5 mg/ml BLES systems) lies between 0.05 mg/ml and 0.1 mg/ml chitosan. These chitosan–BLES preparations with high ε and low γ' values are also the systems that yield the lowest minimum surface tensions during dynamic compression. Further increase in the chitosan concentration to 0.15 mg/ml produces a reduction of the elasticity, an increase in relaxation rate and minimum surface tension.

When the concentration of chitosan in the BLES preparation is 0.25 mg/ml, the lung surfactant film collapses when it reaches a surface tension of 17.9 mJ/m^2. Such film collapse at high surface tension is typically observed in inhibited lung surfactant films [1]. This chitosan-induced inhibition resembles the inhibition produced by cationic peptides reported by Brummer et al. [49], and is also consistent with the fact that high intravenous dosage of chitosan induces lung injury [50]. It is clear, therefore, that although the addition of chitosan improves the surface tension of BLES preparation, there is an overdose threshold where chitosan becomes an inhibitor in itself. This will be discussed in the following sections.

3.4. Stoichiometric chitosan–BLES (N^+/N^-) ratio and its influence on film properties

The N^+/N^- "stoichiometric ratio parameter" relates the number of moles of ionic groups in the polymer to the number of ionic groups in the phospholipids. This N^+/N^- parameter was introduced by Sennato et al. to study the colloidal stability of liposome–polyelectrolyte systems [51,52]. It has been proposed that the electrostatic interactions (binding) between the cationic groups of chitosan (N^+) and negatively charged lipids (N^-, i.e. phosphatidylglycerols) in BLES could be responsible for the observed improvement in minimum surface tension [39]. Similarly, it has been proposed that the interactions between N^+ and N^- groups are also responsible for the inhibitory effects of chitosan (chitosan overdosage) [49]. To evaluate this electrostatic (binding) hypothesis, the properties of the surfactant film formed at the air/water interface (ε, γ') for 0.5 mg/ml BLES (Table 2) and 2.0 mg/ml BLES (Table 3) are interpreted in terms of the stoichiometric N^+/N^- dose ratio (Figs. 4 and 5). This "dose ratio"

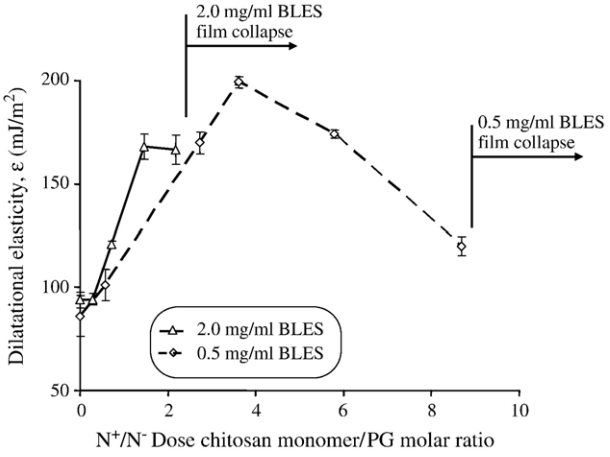

Fig. 4. Dilatational elasticity (ε) as a function of chitosan monomer/PG dose ratio (N^+/N^-) for 0.5 mg/ml and 2.0 mg/ml BLES formulations. All systems were compressed in 100% R.H. air.

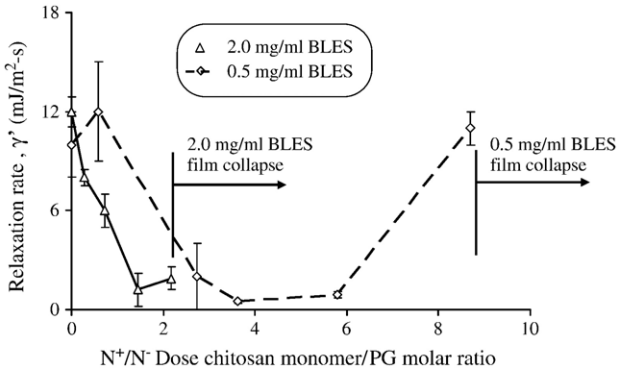

Fig. 5. Relaxation rate (γ') as a function of chitosan monomer/PG (N^+/N^-) dose ratio for 0.5 mg/ml and 2.0 mg/ml BLES formulations. All systems were compressed in 100% R.H. air.

is calculated based on the total concentration of chitosan and BLES in the preparations.

The minimum surface tension values of the 2.0 mg/ml BLES preparations diminish with increasing chitosan concentration until an optimal chitosan concentration (0.15 mg/ml) is reached. Based on the minimum surface tension values in Table 3, the optimal chitosan dosage for 2.0 mg/ml BLES preparations lies between 0.1 and 0.15 mg/ml chitosan. The maximum values of dilatational elasticity (ε) and minimum relaxation rate (γ') also occur at 0.15 mg/ml chitosan. However, using the same chitosan dosage in 0.5 mg/ml BLES does not produce low minimum surface tensions. The "optimal" chitosan dosage increases as the surfactant concentration increases, an observation consistent with the chitosan–BLES binding hypothesis, suggesting the existence of an optimal stoichiometric ratio.

The electrostatic binding between polyelectrolytes and ionic phospholipids has been studied in polyelectrolyte-coated liposomes [51,52]. For systems containing liposomes produced with ionic phospholipids, abrupt changes in zeta potential and liposome size occur near $N^+/N^-=1$. To explore this concept, Fig. 4 presents the values of dilatational elasticity (ε) plotted against the stoichiometric dose ratio (chitosan monomer (N^+)/phosphatidyl glycerol (N^-)) for the formulations presented in Tables 1 and 2. This N^+/N^- dose ratio was calculated as [52]:

$$\frac{N^+}{N^-}=\frac{\alpha_{dac}\times\alpha_{ionC+}\times C^+}{\alpha_{ionC-}\times C^-}\times\frac{MW_{C^-}}{MWm_{C^+}} \quad (2)$$

where C^+ is the concentration of chitosan in the suspension (in mg/ml), MWm_{C^+} is the molecular weight of the monomeric unit in chitosan (glucosamine, MW=179 mg/mmol), C^- is the concentration of the anionic phosphatidylglycerol (assumed to represent 10% w/w of the BLES concentration — see Ref. [28]), and $MW_{\overline{C}}$ is the molecular weight of palmitoyl–oleyl phosphatidyl glycerol–POPG (MW=767 mg/mmol) which is assumed to encompass the different PG species [21,53]. The parameter α_{dac} is the degree of deacetylation of chitosan (80%). The parameters, α_{ionC+} and α_{ionC-} are the degree of ionization of chitosan and the anionic lipids (PGs), respectively. The degree of dissociation of both chitosan and PGs is dependent on the pH of the solution. Maltseva et al., have shown that in salt solutions similar to those used in lung surfactants, PG molecules are fully dissociated (negatively charged) for pH values higher than 5 [54]. In the case of chitosan, this molecule is charged at pH values below 6.5 [55]. Within the pH of 5.5 to 6.0 (the pH of BLES preparations) the chitosan and PG molecules are dissociated and, therefore, $\alpha_{ionC+}=\alpha_{ionC-}\sim 1$. If BLES and chitosan were mixed at the physiological pH of 6.5–7.0 the PG species would remain dissociated, but the degree of dissociation of chitosan (α_{ionC+}) could be reduced to 0.7 or less [55]. In those cases the values of N^+/N^- should be adjusted using Eq. (2). Additionally, in the calculation of N^+/N^- in Eq. (2), the fraction of cationic proteins SP-B and SP-C that may be bound to the ionic lipids have not been considered, which may result in an overestimation (of up to 10%) of the N^- groups. Using this normalization procedure, the dilatational elasticity data is presented in Fig. 4 for 0.5 mg/ml BLES and 2.0 mg/ml BLES as a function of the N^+/N^- dose ratio.

For 0.5 mg/ml BLES formulations, the maximum elasticity ($\sim$200 mJ/m^2) is reached when the N^+/N^- dose ratio is approximately 4. Further addition of chitosan produces lower elasticities and film collapse when $N^+/N^->9$. It is noted that time-dependent effects were observed for formulations that produced extremely large aggregates (flakes).

The elasticities of films of 2.0 mg/ml BLES formulations increase with increasing N^+/N^- dose ratio up to $N^+/N^-\sim 2$ (0.15 mg/ml chitosan) where an elasticity of 175 mJ/m^2 is reached (nearly twice that of the BLES-only system). Systems formulated with N^+/N^- dose ratios higher than 2.2 experience film collapse and high minimum surface tensions.

Fig. 5 presents the rate of surface tension relaxation (γ') as a function of chitosan/PG dose ratio (N^+/N^-). As the N^+/N^- ratio increases, the relaxation rate decreases (more stable films) until the film reaches a minimum relaxation rate (optimal formulation). This optimal formulation is achieved when the N^+/N^- ratio is close to 2 for 2.0 mg/ml BLES, and close to 4 for the 0.5 mg/ml BLES formulation.

Both the elasticity and the relaxation rate of BLES formulations are a function of the N^+/N^- ratio and this supports the hypothesis that the binding between the positively charged chitosan and negatively charged lipids (phosphatidyl glycerol) is responsible for the observed changes in surface activity.

To understand these binding ratios, it is necessary to consider the potential configurations that the polymer and the ionic lipid may acquire. If the area per molecule occupied by a glucosamine group in chitosan is approximately 25 Å^2 (see Ref. [56]) and the area occupied by a fully compressed phosphatidyl glycerol group is approximately 40 Å^2 (see Ref. [57]), it is possible to calculate the N^+/N^- ratio that produces a flat "patch" of chitosan adsorbed on PG molecules. In such a case, $N^+\times 25\ Å^2 = N^-\times 40\ Å^2 \geq N^+/N^- = (40\ Å^2)/(25\ Å^2)\sim 1.6$. This theoretical binding ratio is of the same order of magnitude as the optimal range of N^+/N^- ratios obtained for 0.5 mg/ml and 2.0 mg/ml BLES. However, it should be noted that the ratios calculated using Eq. (2) do not take into account that there is a fraction of the chitosan that remains in the solution and is not bound to the

lipid [58]. Such unbound chitosan may also help explain the difference in optimal range observed for 0.5 mg/ml and 2.0 mg/ml BLES systems.

If this binding hypothesis is correct, then the changes in surface activity should also correlate with changes of the surface charge of the surfactant film adsorbed at the air/water interface. Ideally, the surface charge of the surfactant film adsorbed at the air/water interface should be measured to track the changes in film composition/binding. However the use of surface potential instruments (such as those used in Langmuir troughs) or AFM imaging using Kelvin probes requires spreading procedures or film sampling protocols that may affect the chemical environment of the suspension. Acknowledging that the zeta potential of the surfactant aggregates in the subphase may not be the same as the surface potential of the film, the measured zeta potential of the surfactant aggregates is an indication of the binding between chitosan and the anionic lipids in BLES [59], and can be used as an indirect estimate of surface binding.

3.5. Zeta potential BLES–chitosan subphase aggregates

Fig. 6 presents the zeta potential of BLES aggregates as a function of the chitosan/PG (N^+/N^-) ratio for 0.5 mg/ml and 2.0 mg/ml BLES preparations. The zeta potential of both formulations in the absence of chitosan is slightly negative (−10 to −15 mV), and as the N^+/N^- increased, this zeta potential increases due to the adsorption of chitosan. The zeta potential of both preparations (0.5 mg/ml and 2.0 mg/ml BLES) becomes zero when the N^+/N^- ratio is close to 1. This is consistent with the idea that at the point where the positive and negative charges are balanced, the zeta potential of the aggregates should be zero. A similar observation was made by Brody et al. [51] in their study of the interaction between liposomes produced with cationic lipids and DNA (negatively charged) using zeta potential measurements.

The interaction between chitosan and PC and PC/PG liposomes has been investigated (using zeta potential measurements) for the purposes of producing chitosan-encapsulated liposomes

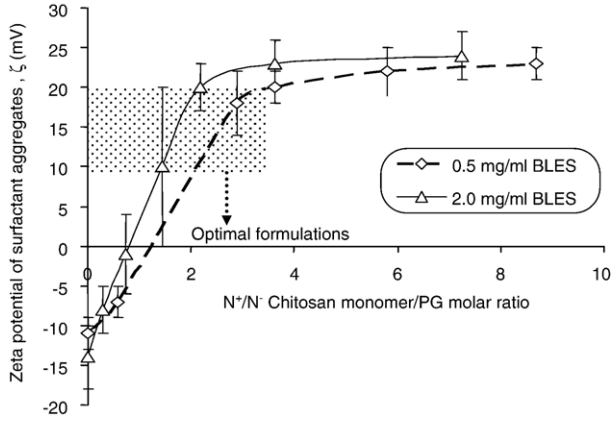

Fig. 6. Zeta potential of BLES–chitosan suspensions (pH=5.5) containing 0.5 mg/ml BLES and 2.0 mg/ml BLES as a function of chitosan monomer/PG (N^+/N^-) molar ratio.

(also known as chitosomes) for drug delivery purposes [60–62]. In these studies, the presence of PG was shown to be essential to the production of negative zeta potential of chitosan-free liposomes (typically −20 to −25 mV) and its subsequent binding to chitosan to form a "flat" adsorbed layer that coats the liposome [61]. Furthermore, it has been proposed that this flat layer of adsorbed chitosan was irreversibly bound to the liposome since, after various rinses, it remained on the surface of the liposome and produced a zeta potential of +25 to +30 mV [61].

In Fig. 6 the transition into a zeta potential plateau region (saturation) begins at around +20 mV. The data in Fig. 6 also shows that this plateau transition begins at around a N^+/N^- ratio of 2 for 2.0 mg/ml BLES and a N^+/N^- ratio of about 4 for 0.5 mg/ml BLES. The maximum elasticity and minimum relaxation values for both of these formulations occurs when the zeta potential of the surfactant aggregates range from +10 mV to +20 mV (area highlighted in Fig. 6). This optimal formulation "range" is adjacent to the plateau transition region.

The relation between zeta potential and surface active properties is not specific to chitosan-lung surfactant preparations. Davies et al. [59] also found that using different mixtures of lung surfactant and electrolyte solutions, different zeta potential values could be achieved, and that zeta potential values ranging from −7 to 0 mV produced minimum surface tensions of 1 mJ/m^2 or less. Davies et al. also observed that inhibited lung surfactant preparations had zeta potential values of either −30 mV or less, or +30 mV or more.

Despite the similarities between the work of Davies et al. (who used multivalent cations) and the work presented in this article (chitosan–cationic polyelectrolyte), there are important differences that need to be clarified. First, the multivalent cations only bind to a single lipid or, at best, to neighboring lipids, but a single cationic polymer chain is capable of binding to numerous anionic lipids present in the surfactant film. The second difference is that the optimum performance of chitosan–BLES systems occurs in the range of +10 mV to +20 mV and not at +0 mV. This could be explained by the fact that in multiple binding (to form a flat polymer monolayer), there are a number of unbound positive sites in the polymer. This multiple binding has been reported by various researchers who use lipid–polyelectrolyte mixtures to produce self-assembled monolayers (SAMs) [63–66].

3.6. Aggregation–segregation effects induced by chitosan

The addition of chitosan to BLES preparations also produces changes in the aggregation state of the lung surfactant. Fig. 7 presents typical micrographs for 0.5 mg/ml and 2.0 mg/ml BLES without chitosan, with optimal chitosan concentration, and with an excess chitosan concentration that yields inhibited surfactant films. According to Fig. 7, the addition of an optimal dosage of chitosan produces large flocks composed of small vesicles. Guo et al. [60] observed the same aggregation on PC–PE liposomes in the presence of chitosan. However, the addition of excess chitosan (0.25 mg/ml chitosan, see last column of pictures in Fig. 7) produces a de-aggregation of the flocks.

It is well known in the polymer/colloid literature that the addition of polyelectrolytes to suspensions of oppositely

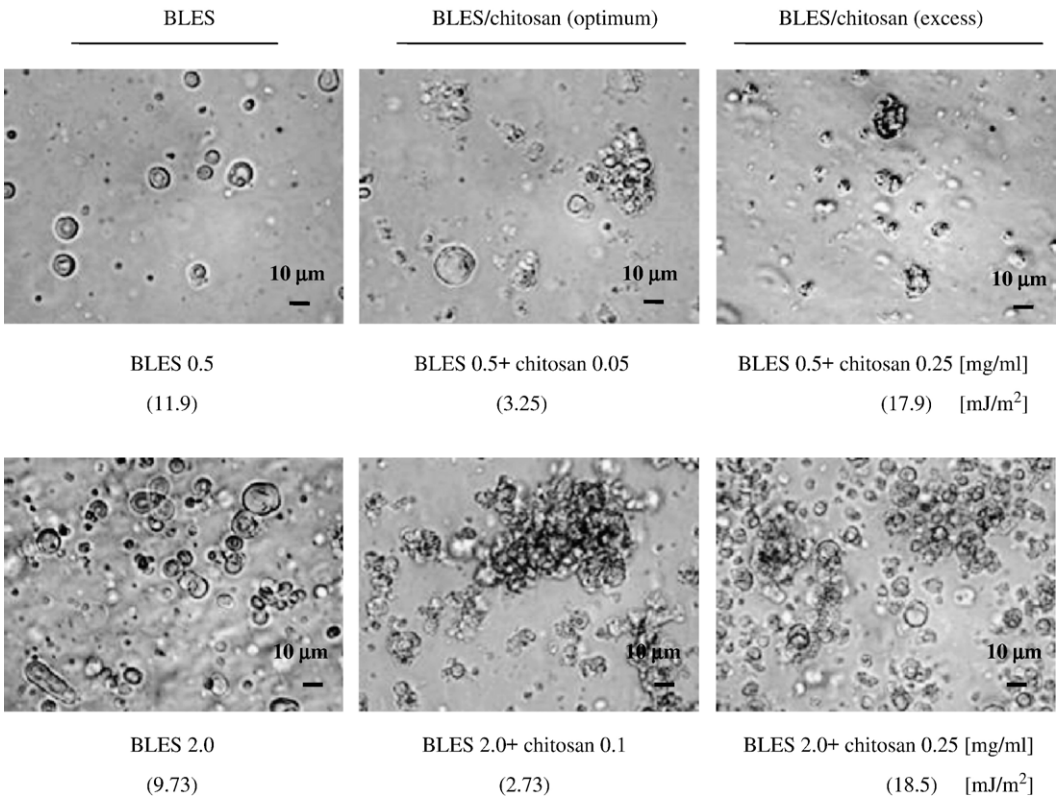

Fig. 7. Optical micrographs of surfactant aggregates in suspension. The number in parenthesis corresponds to the minimum surface tension achieved during dynamic cycling.

charged particles produces aggregation of these particles when the zeta potential approaches zero, and that addition of excess polyelectrolyte induces charge reversal and particle de-aggregation [67–70]. The same flocculation/de-aggregation phenomenon has also been observed in mixtures of polyelectrolytes with oppositely charged liposomes [51,52].

In order to correlate the morphology of the surfactant aggregates with their surface activity, Fig. 8 presents the surface tension-area compression loops for the systems of Fig. 7. For both BLES concentrations (0.5 mg/ml and 2.0 mg/ml) presented in Fig. 7, the addition of an optimal chitosan dosage allows the compression to be more efficient (minimal film relaxation, maximal surface tension reduction, and minimal hysteresis). The addition of a high chitosan dosage (within the plateau region of Fig. 6) yields BLES preparations that undergo film collapse at a surface tension of 20–22 mJ/m^2.

As deduced from the comparison between Figs. 7 and 8, the formation of large surfactant aggregates is typically associated with surfactant films that yield low minimum surface tension upon dynamic compression [1,4,5,28].

In the case of nonionic polymers, the depletion–attraction mechanism is the one responsible for the formation of large surfactant aggregates which is associated with the increase in the rate of film formation and reversal of surfactant inhibition by serum proteins [28,36]. The depletion–attraction mechanism requires that a polymer should not be attracted/adsorbed by the surface of the particle, and it is even more efficient when the polymer has the same charge as the surface of the particle (the reason why hyaluronan is more effective than dextran or polyethylene glycol) [71]. The data in Fig. 6 clearly shows that chitosan adsorbs on the surface of the aggregates and opposes the interaction which would be prescribed by the depletion–attraction mechanism.

The micrographs of Fig. 7 suggest that in optimal BLES–chitosan formulations chitosan caused the adhesion of lipid membranes (flocculation of vesicles), but that a chitosan overdose induced a de-flocculation of such aggregates. In the region leading to the plateau dosage (noted as optimal formulations in Fig. 6), chitosan seems to serve as a "bridge" between the negative groups of different vesicles. The same bridging effect has been used to explain the interaction between negatively charged DNA and cationic lipid vesicles [52]. However, with increasing chitosan dosage, the inter-vesicle bridging appears to be replaced by the formation of intra-vesicle patches which leads to individual vesicles decorated with a layer of chitosan. Such individual vesicles repel each other by virtue of their net positive charge and the lack of inter-vesicle bridge.

These flocculation and de-flocculation effects may also be at play between patches of ionic lipids adsorbed at the air/water interface. The literature on self-assembled monolayers (SAMS) between polyelectrolytes and monolayers of mixtures of ionic and zwitterionic lipids supports the idea of a "flat" adsorption configuration of the polyelectrolyte bridging ionic lipids of opposite charge [56,63–66,72,73]. Such bridging among ionic lipid patches at the interface would explain the superior elasticity and stability of optimal BLES–chitosan preparations.

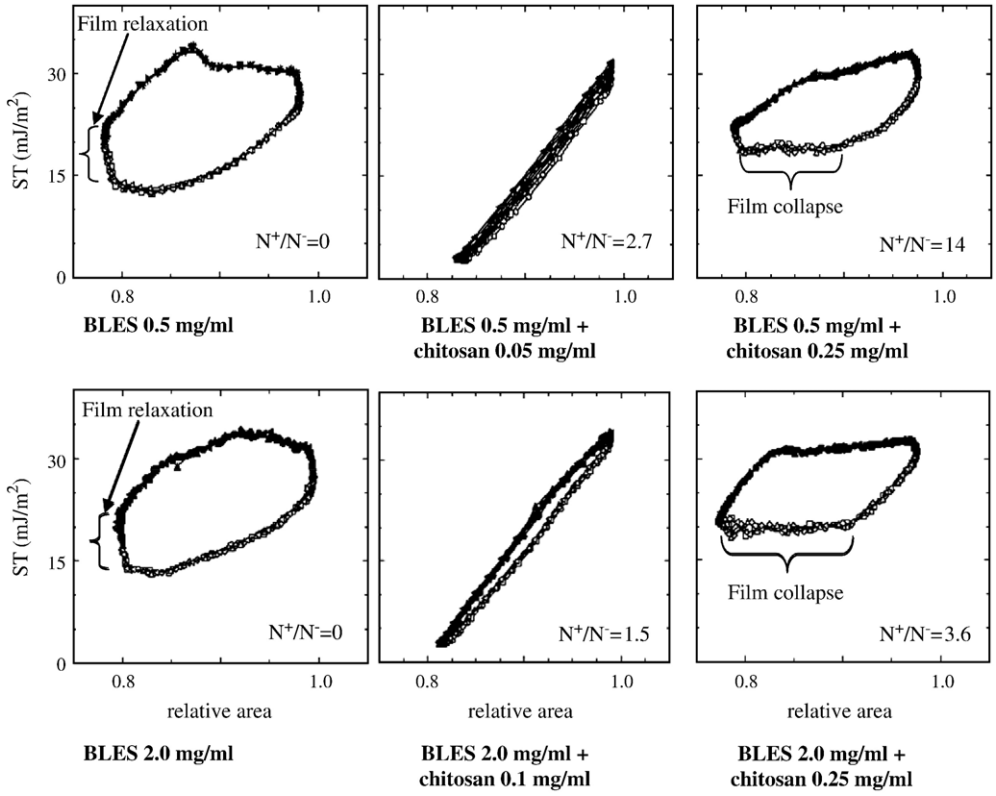

Fig. 8. Surface tension (ST)–relative area compression cycles for BLES–chitosan preparations (composition shown in the figure). All systems were compressed in 100% R.H. air.

The de-flocculation phenomena observed in Fig. 7 parallels the segregation phenomena observed in polyelectrolyte-ionic lipid monolayers (adsorbed at the air/water interface), where at high polyelectrolyte concentrations, patches of polyelectrolyte-bound ionic lipids form separate phases [74]. Furthermore this lipid demixing (segregation of ionic lipid patches) effect has been observed in various systems containing mixtures of anionic lipids, zwitterionic lipids and cationic proteins [75], and has been associated with an increase of fluidity of the monolayer [75,76]. Such fluidization of the film would explain the poor mechanical properties (film collapse in particular) of BLES–chitosan systems formulated within the zeta potential plateau region.

4. Conclusions

When dilute BLES-only preparations are compressed in the presence of pre-humidified air, the minimum dynamic surface tension of these systems is relatively high and variable, likely due to hydration–fluidization effects discussed in previous studies. This variability can be suppressed with the addition of an optimal chitosan concentration that depends on the concentration of BLES. This optimal range is correlated with the binding of positive charges in chitosan and the anionic lipids in BLES. This binding to BLES was confirmed by zeta potential measurements that show that the addition of chitosan to BLES produces an increase in zeta potential of the subphase aggregates from about −12 mV to +25 mV. Furthermore, it was shown that the "optimal dosage range" occurs when the zeta potential of the aggregates is between +10 mV and +20 mV. This zeta potential range is the same for both 0.5 mg/ml and 2.0 mg/ml BLES preparations.

The changes in dynamic surface tension and zeta potential also correlate with changes in the colloidal stability (flocculation and de-flocculation) of surfactant subphase aggregates. Within the optimal chitosan dosage range, the subphase aggregates form "clusters" of surfactant vesicles that adhered to each other.

These observations highlight the importance of electrostatic interactions on the properties of lung surfactant systems. Furthermore, the electrostatic binding principles discussed here are likely relevant to understanding the essential role that cationic proteins such as SP-B and SP-C play in lung surfactant systems.

Acknowledgements

This work was supported by a grant from the Canadian Institutes of Health Research (MOP-38037) and an Open Fellowship from the University of Toronto to Ningxi Kang. We also thank Dr. David Bjarneson of BLES Biochemicals Inc. for his generous donation of the BLES samples.

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