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Restoring the charge and surface activity of bovine lung extract surfactants with cationic and anionic polysaccharides

Edgar J. Acosta [a,b,*], Zdenka Policova [b], Simon Lee [b], Andrew Dang [b], Michael L. Hair [b], A. Wilhelm Neumann [b]

[a] Department of Chemical Engineering and Applied Chemistry, University of Toronto, 200 College Street, Toronto, ON, Canada M5S 3E5
[b] Department of Mechanical and Industrial Engineering, University of Toronto, 5 King's College Road, Toronto, ON, Canada M5S 3G8

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ABSTRACT

Chitosan, a cationic polysaccharide, has been found to improve the surface activity of lung surfactant extracts in the presence of various inhibitors. It has been proposed that chitosan binds to anionic lipids (e.g. phosphatidyl glycerols) in lung surfactants, producing stable lipid films at the air–water interface. This binding also reverses the net charge of the surfactant aggregates, from negative to positive. Unfortunately, positively charged aggregates may adsorb or interact with the negatively charged epithelial tissue, leading to poor surfactant performance. To address this issue an anionic polysaccharide, dextran sulfate (dexS), was used as a secondary coating to reverse the charge of chitosan–lung surfactant extracts without affecting the surface activity of the preparation. The dynamic surface tension and zeta potential of bovine lipid extract surfactant (BLES) containing chitosan chloride (chiCl) and dexS were evaluated as a function of dexS concentration. These studies were conducted in the absence and presence of sodium bicarbonate buffer, and in the absence and presence of bovine serum used as model inhibitor. It was determined that using an appropriate concentration of dexS, especially at physiological pH, it is possible to restore the negative charge of the surfactant aggregates, and retain their surface activity, even in the presence of bovine serum. High concentrations of dexS affect the binding of chiCl to BLES, and the surface activity of the preparation.

1. Introduction

Lung surfactants are mixtures of phospholipids (~85% of the mixture), surfactant proteins (~10%), and neutral lipids like cholesterol (~5%). These surfactants are secreted by Type II pnuemocytes into the alveolar fluid [1,2]. One of the main roles of lung surfactant is to form stable films of lipids adsorbed at the air–water interface which, upon compression, form a semi-solid film with near zero surface tension. This near zero surface tension reduces the difference in Laplace pressure among large and small alveoli, preventing lung collapse. It has been proposed that a minimum surface tension (upon compression) of 5 mJ/m^2 or lower is necessary to prevent lung collapse [3]. Unfortunately, the lack of lung surfactant (common in pre-term neonates) or its malfunction in the presence of inhibitors such as blood proteins, unsaturated fatty acids or lysolecithins increase the risk for lung collapse and poor blood oxygenation, a condition known as respiratory distress syndrome (RDS).

Surfactant replacement therapy, which involves instilling surfactants extracted from the lungs of swine and cattle into the lungs of the patient, has been successful in neonatal-RDS (or nRDS) when combined with appropriate ventilation techniques [4,5]. However, this therapy is still ineffective to reduce the mortality of patients with dysfunctional surfactant, a condition also known as acute-RDS or ARDS [6].

In the development of new, more effective surfactant therapies one needs to consider the differences in composition between the fully functional (complete) lung surfactants, and the composition of lung surfactant extracts. The current methods of surfactant extraction use organic solvents that completely remove the hydrophilic surfactant proteins SP-A and SP-D, as well as a fraction of the hydrophobic, and cationic surfactant proteins SP-B and SP-C [2,5]. The surfactant protein SP-A is negatively charged at the physiological pH of 6.9, and it is involved in the stabilization of lung surfactant aggregates in tubular myelin [7,8]. On the other hand, surfactant proteins SP-B and SP-C are positively charged at pH 6.9. Complete lack of surfactant protein SP-B is lethal [9,10]. Furthermore, SP-B and SP-C are said to interact with the anionic lipids (phosphatidyl glycerols) of lung surfactant to stabilize the tubular myelin and the surfactant film at the air–water interface [11,12].

Nonionic polymers such as dextran and polyethylene glycol have been used, in part inspired by SP-A, to induce the formation of large surface active surfactant aggregates [13–15]. These nonionic polymers are effective in accelerating the adsorption of surfactant aggregates *in vitro*, but there are conflicting results *in vivo* [16]. When evaluated in fully humid environment (100% RH) polyethylene glycol is ineffective

* Corresponding author. Department of Chemical Engineering and Applied Chemistry, University of Toronto, 200 College Street, Toronto, ON, Canada M5S 3E5. Tel.: +1 416 946 0742; fax: +1 416 978 8605.
E-mail address: edgar.acosta@utoronto.ca (E.J. Acosta).

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against bovine serum inhibition [17]. Anionic polymeric additives (e.g. hyaluronan) have also been evaluated and shown to be more effective than nonionic polymers [18,19].

Additives that could emulate the hydrophobic and cationic nature of SP-B and SP-C have also been considered. A synthetic lysine–leucine cationic peptide, KL_4, has been used as a SP-B analog; recombinant forms of SP-C, and the cationic lipopeptide polymyxin B have all improved the properties of lung surfactant extracts in the presence of various inhibitors [20–23].

More recently, chitosan base (or simply chitosan), a cationic polysaccharide has been used as additive in lung surfactant formulations, finding that it is up to 1000 times more effective than the nonionic polyethylene glycol in reversing surfactant inhibition associated with albumin [24,25]. Furthermore, mixtures of BLES and chitosan in its hydrochloride form (protasan or chitosan chloride, chiCl) have been found to retain their surface activity in the presence of albumin, cholesterol, fibrinogen, and cholesterol, and reverse the inhibitory action of serum [26]. The difference between ChiCl and chitosan base is that the base only ionizes and dissolves at low enough pHs, whereas the hydrochloride form was turned into a soluble chloride salt form by dissolving the chitosan base in hydrochloric acid, followed by lyophilization of the resulting salt.

It has been proposed that the enhancement of the surface activity of the extracts with the addition of cationic additives is associated with the binding of the cationic groups of the additive to patches of anionic lipids such as phosphatidyl glycerols [2,25]. This hypothesis is supported by thermodynamic models that explain the binding of cationic peptides to anionic lipids [27].

There have been several studies that show that most cationic additives, in particular chitosan base and chiCl, do not produce any toxic effect on bronchoalveolar tissue cultures [24,28,29]. However, there is one aspect that needs further consideration, i.e. the fact that when using cationic additives, and in particular chitosans, the charge of the surfactant aggregates is affected. The most significant change in this charge has been reported for BLES preparations formulated with chitosan base (i.e. not the hydrochloride form). In that case, the zeta potential of the surfactant aggregates changed from values near −15 mV to values close to +20 mV for optimal chitosan base-BLES formulations [25]. This modification of the charge of the aggregates may create significant changes in the way these aggregates interact with the rest of the components of the alveolar fluid and the alveolar epithelial tissue itself. In fact, in drug delivery applications, originally negatively charged nanoparticles are purposely turned positive with the aid of chitosans, to improve uptake by epithelial tissue [30]. Similarly, we have observed, in preliminary studies that upon contact with epithelial tissue, a partial (and sometimes total) disappearance of positively charged aggregates [31]. Furthermore, other cationic proteins or polyelectrolytes used in various medical treatments sometimes create problems including exacerbated response of the immune systems and lung edema [32–35].

In order to take advantage of the wide range of cationic additives for lung surfactant extracts, it is necessary to minimize the risks associated with these additives by modifying the charge of these surfactant–additive complexes. To meet this challenge, it is useful to turn the attention to the layer-by-layer polyelectrolyte assembly technique initially introduced by Decher et al. [36,37]. According to this technique, by sequentially adsorbing layers of anionic and cationic polymers over a surface it is possible to create “fuzzy” thin films with either negative or positive charges, depending on the number of layers applied. This approach has been used in numerous applications, including drug delivery and tissue engineering [38].

Before selecting a suitable anionic polyelectrolyte it is necessary to consider the interaction between chitosan and epithelial tissue. Several researchers interested in drug delivery applications have studied the binding of chitosan to mucin (anionic polyssacharide secreted by some epithelial cells) to understand the binding of chitosan-coated particles to tissue [39–41]. The work of Qaqish and Amiji is of particular interest because they found that the binding of mucin to chitosan is relatively weak compared to the binding of dexS (dexS) to chitosan [39]. This implies that if chitosan is exposed to dexS it will preferentially adsorb to this polyelectrolyte rather than adsorbing onto the mucin-coated epithelial tissue. Therefore, in this work, dexS is used to reverse the charge of BLES–chiCl preparations. Fig. 1 presents a schematic illustrating this idea. Furthermore, it is hypothesized that this change in the charge of the surfactant aggregates should not affect their surface activity. In the first part of this work, the zeta potential and surface activity of BLES–chiCl preparations are evaluated as a function of dexS concentration. The surface activity was evaluated using a constrained sessile drop (CSD) cycled to simulate the compression–expansion cycles during normal breathing conditions [42,43]. To evaluate the ability of these preparations to remain active in the presence of inhibitors, their activity was evaluated in the presence of bovine serum [44,45]. These studies were repeated in the presence of sodium bicarbonate used to simulate the physiological pH.

2. Materials and methods

2.1. Materials

BLES (Bovine Lipid Extract Surfactant) was provided by BLES Biochemicals Inc., London, Ontario, Canada. BLES contains mainly

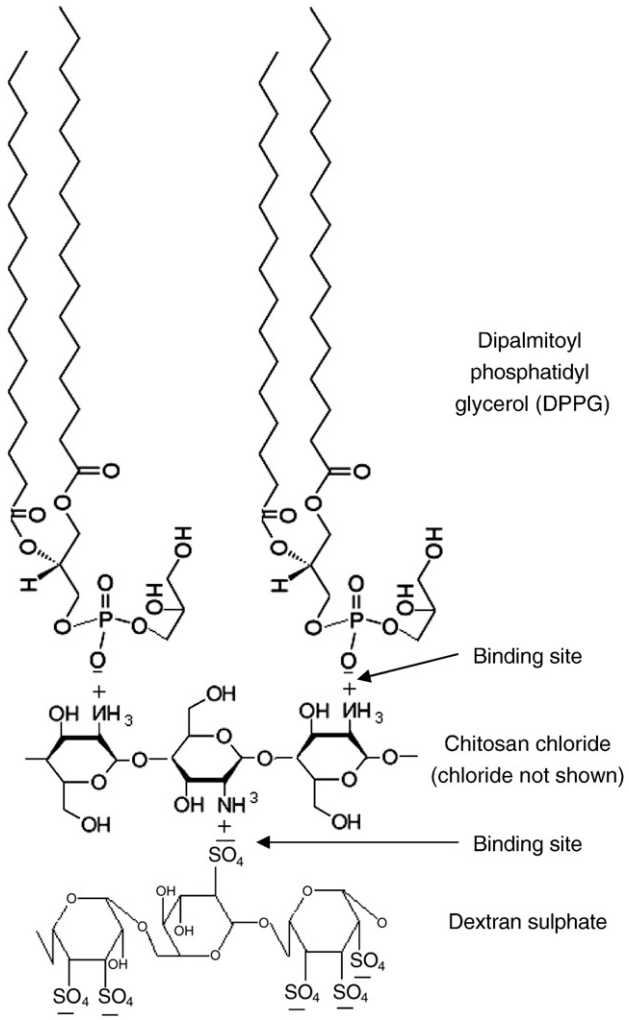

Fig. 1. Schematic of the hypothetical binding between the anionic lipids in BLES, represented by dipalmitoyl phosphatidyl glycerol (DDPG) with chitosan chloride (chiCl, protasan) and dextran sulfate (dexS).

phospholipids and two surfactant-associated proteins, SP-B and SP-C. Concentrated (27 mg/ml) BLES samples were stored as an aqueous suspension in glass vials under N_2 atmosphere at −20 °C until the day of the experiment. For all experiments, except those presented in Table 1, a BLES batch received in Winter of 2008 was used. The experiments of Table 1 were conducted using a BLES batch received in Fall of 2009.

Chitosan hydrochloride (Protasan Cl) 213 kDa was purchased from Novamatrix (Norway) (Protasan UP Test Kit, #4219001). These water-soluble chitosans have a degree of deacetylation of 75–90%. Protasan 213 contains molecules ranging from 150,000 to 400,000 g/mol. Dextran sulfate 500 kDa (product # D8906), sodium bicarbonate, and bovine serum (B8655, 60 mg/ml protein) were also purchased from Sigma-Aldrich (Oakville, Canada).

2.2. Methods

2.2.1. Sample preparation

Frozen BLES (27 mg lipids/ml as received) samples were thawed in a 37.5 °C water bath for 1 h, before being diluted using a salt solution containing 0.6% NaCl and 1.5 mM $CaCl_2$. The concentration of calcium chloride is important because, similarly to chitosan, it binds to anionic lipids; the concentration used in this work is representative of the levels found to in the alveolar fluid in plasma [46,47]. The prescribed amount of a stock solution of chitosan chloride (1 mg/ml) was added in the NaCl/$CaCl_2$ salt solution. The pH of the resulting BLES–chitosan chloride was 5.4 ± 0.1. The concentration of BLES was 2 mg/ml in all preparations. The concentration of chitosan chloride was 0.15 mg/ml. Finally, the prescribed volume of bovine serum was added to the surfactant mixture. The final pH of these BLES–chitosan chloride-serum mixtures was 6.9 ± 0.1. For systems involving sodium bicarbonate the order of addition was: BLES + electrolyte solution + chitosan chloride + bicarbonate + bovine serum.

2.2.2. Surface tension measurements

The surface activity of BLES–chitosan chloride-serum preparations was evaluated using a Constrained Sessile Drop (CSD) surfactometer in conjunction with Axisymmetric Drop Shape Analysis (ADSA). The design and operation of the CSD device has been described in detail elsewhere [24,25,43]. Briefly, to start any CSD operation, a sessile drop of the 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 surface tension reaches a near zero value at the end of the compression stage. During the experiments, the droplet is enclosed in a chamber with controlled temperature (37 °C) and humidity (100% relative humidity). After the drop is formed, it is undisturbed until the equilibrium surface tension of ~25 mJ/m^2 is reached (typically completed in 3 min). After that time, the surfactant suspension is injected into and out of the droplet, via a motor-controlled syringe, to produce dynamic expansion and compression cycles of the droplet with a periodicity of 3 s/cycle. The ratio between the drop area at the end of compression to the initial drop area was adjusted to approximately 80% (equivalent to 20% area compression), in order to mimic normal breathing in adults [1,43]. Images of the drop (20 images/s) during dynamic cycling were collected by a CCD camera (Model 4815-5000, Cohu Corp., Poway, CA). The acquired images were processed by a digital video processor (Snapper-8, Active Silicon LTD) and stored in a workstation to be later analyzed by ADSA to calculate the surface tension, surface area and volume of the drop during the cycle [25,42,43]. The results are expressed as the mean ± 95% confidence interval ($n \geq 4$ unless otherwise indicated). Typical surface tension – volume – area output of ADSA for 2.0 mg/ml BLES + 0.15 mg/ml chiCl and 50 µl/ml serum and for complete lung surfactant-serum during dynamic cycling are presented in Fig. 2.

Table 1
Dynamic surface tension properties (minimum interfacial tension and elasticity) of formulations prepared with 2 mg/ml BLES, 0.15 mg/ml Protasan 213 kDa, and the prescribed concentrations of dextran sulfate (dexS) and sodium bicarbonate ($NaHCO_3$). Dynamic compression conditions: 3 s/cycle, 20% compression in 100% R.H. air.

DexS	$NaHCO_3$	pH	1 h − γ_{min}	3 h − γ_{min}	1 h − ε	3 h − ε	ζ
mg/ml	mM		mJ/m^2	mJ/m^2	mJ/m^2	mJ/m^2	mV
0	0	5.29	2.6 ± 0.7	2.1 ± 0.1	143 ± 6	164 ± 7	+28 ± 9
0	0.4	6.4	8.1 ± 1.9	15 ± 1	80 ± 36	45 ± 2	−7 ± 9
0	1.5	6.98	17 ± 1	16.8 ± 0.1	8 ± 7	35 ± 3	−12 ± 9
0.04	0	5.38	3.3 ± 0.3	2.5 ± 0.2	127 ± 13	138 ± 2	+13 ± 5
0.04	0.4	6.4	5.4 ± 0.6	17.6 ± 0.1	99 ± 12	20 ± 3	−5 ± 6
0.04	1.5	7.09	5.6 ± 2.2	3.6 ± 0.3	74 ± 14	124 ± 7	−14 ± 3

2.2.3. Zeta potential measurements

The zeta potential of the surfactant aggregates was determined using a Delsa 440SX Zeta Potential Analyzer (Coulter-Beckman, Miami, FL). To prevent the saturation of the light scattering detectors, each sample was diluted by a factor of 10 in a 0.9% NaCl solution. Additional information on this method is available elsewhere [25].

2.2.4. Dilatational elasticity (ε)

The dilatational elasticity (ε) quantifies the ability of a surfactant film to reduce the surface tension (γ) for a given compression (reduction in surface area), such that $\varepsilon = d\gamma/(dA/A) = d\gamma/d(\ln A)$. In terms of fractional area(A/Ao), then $\varepsilon = d\gamma/d\ln(A/Ao)$ where Ao is the maximum surface area of the bubble . To calculate $d\gamma/d\ln(A/Ao)$ the compression stage of the 3rd cycle was considered, which corresponds to the steady state performance of the film. The data of γ versus ln(A/Ao) for that compression were fitted to a 4th order

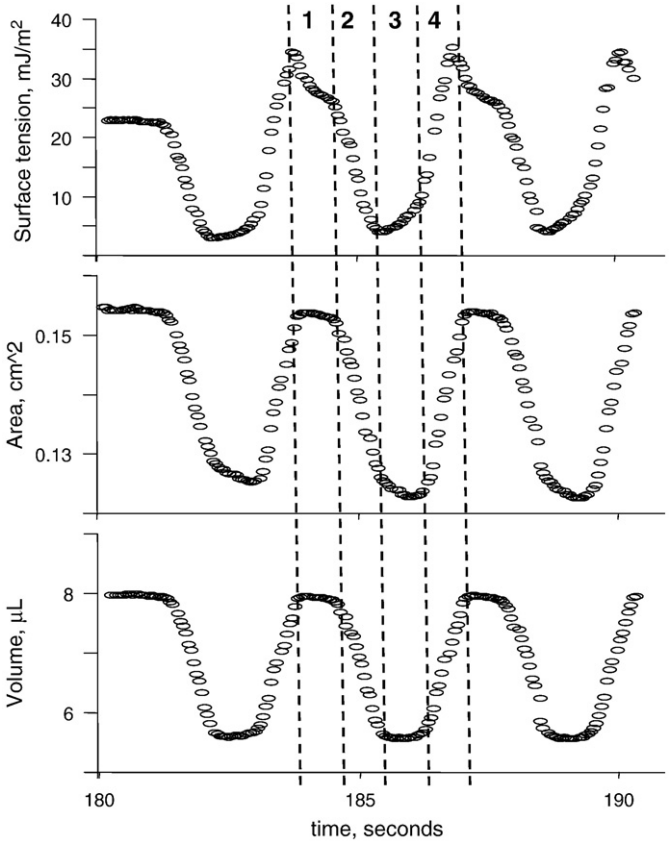

Fig. 2. Surface tension – Area – Volume output of ADSA for 2.0 mg/ml BLES + 0.15 mg/ml chiCl (chitosan chloride, protasan), in the presence of 50 µl/ml of bovine serum. Cycling conditions: 3 s/cycle periodicity, ~20% area reduction, 37 °C, 100% relative humidity. Stages 1–4 of the compression cycle are shown.

polynomial equation. The slope, $d\gamma/d\ln(A/Ao)$ of the fitted curve was evaluated at half compression. Further details of this calculation are provided elsewhere [25,43]. Further to reporting the elasticity of the surfactant films, in some cases the onset of film collapse is also reported. This film collapse occurs when further reduction of surface area of the drop does not yield a reduction in surface tension (this is equivalent to a film elasticity of zero during that part of the compression) [1].

3. Results

3.1. BLES–chitosan chloride (chiCl)–dextran sulfate (dexS)

Fig. 2 presents a typical ADSA output of a dynamic cycling experiment for a system containing 2 mg/ml BLES, 0.15 mg/ml chiCl (213 kDa) and 50 μl/ml of bovine serum used as a model inhibitor. This ratio of BLES and chiCl was found to be optimal in a previous study [26]. Fig. 2 shows the four stages of the dynamic cycling experiment. In stage 1 (adsorption) the surfactant adsorbs/spreads at the air–water interface and the surface tension approaches the equilibrium surface tension while the volume of the drop is held constant. In stage 2 (compression), liquid is drawn from the droplet, reducing the volume and surface area of the drop, and the surface tension of the suspension. At the end of this compression stage the surface tension of this formulation should be equal to or lower than 5 mJ/m^2 to minimize the risk of lung collapse [1,2]. During the compression stage it is also desirable to obtain the largest possible reduction in surface tension with a minimum reduction in surface area in order to prevent over-distending the lungs. The ratio between the surface tension reduction ($d\gamma$) and the reduction of surface area (dA) is expressed in terms of dilatational elasticity of the film (ε) [25]. In stage 3 (relaxation) the volume of the drop and surface area remain approximately constant, and the surface tension of the film may increase (relax) if the film is not completely stable, as in the case of Fig. 2. In stage 4 (expansion) liquid is injected into the drop which increases the volume, surface area and surface tension of the drop. In this work, the performance of the BLES–chitosan formulation of Fig. 2 is used as a benchmark for surface activity. It is necessary to verify that the modification of the BLES–chiCl formulation with the anionic polysaccharide, dexS, does not affect the surface activity of the formulation alone or in the presence of the inhibitor (serum).

Fig. 3 presents the zeta potential of surfactant aggregates prepared with 2 mg/ml BLES, 0.15 mg/ml Chitosan (same base preparation of Fig. 2) as a function of concentration of dexS. The data in Fig. 3 confirm that dextran-free BLES–chiCl formulations produce positively charged aggregates, as in the case of BLES and chitosan base [25]. In those cases, the positive charge of the aggregates has been associated with the unbound positively charged amino groups in chitosan [25]. The hypothesis was that the negatively charged groups in dexS would bind to the positive charges in chitosan and eventually reverse the charge of the aggregate, according to the schematic of Fig. 1. The data in Fig. 3 support this hypothesis since increasing the concentration of dexS the zeta potential of the surfactant aggregates shifts towards negative values, and eventually reverses when 0.06 mg/ml of dexS is added. After that charge reversal point, further increase in the concentration of dexS only results in marginal decreases in zeta potential.

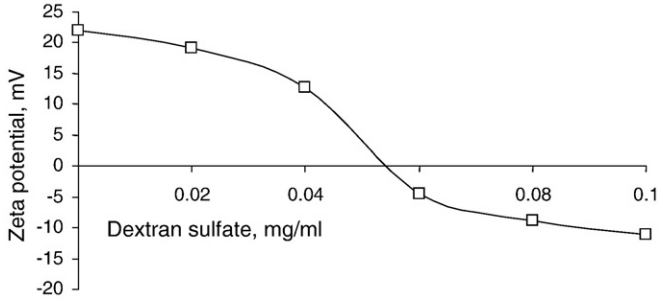

Fig. 3. Zeta potential of preparations containing 2.0 mg/ml BLES + 0.15 mg/ml chiCl (protasan) in saline solution, as a function of dexS concentration.

To evaluate the effect of the addition of dexS on the surface activity, dynamic cycling experiments were conducted with formulations prepared with 2.0 mg/ml BLES + 0.15 mg/ml chiCl and different concentrations of dexS. Fig. 4 presents the minimum surface tension of these preparations as a function of dexS concentration. At intermediate dexS concentrations (0.02 mg/ml and 0.04 mg/ml) there is a slight increase in the minimum surface tension, however, at the concentration of 0.06 mg/ml of dexS the minimum surface tension reached a value close to that of BLES–chiCl alone (~3 mJ/m^2). At concentrations of 0.08 and 0.1 mg/ml of dexS the minimum surface tension increases substantially.

In order to understand the observed changes in minimum surface tension with the addition of dexS, the surface tension-area cycling isotherms for systems containing 0, 0.06 mg/ml and 0.1 mg/ml dexS are presented in Fig. 5. According to that figure, the system of BLES and chiCl alone responds quite well to the compression as it achieves near 2 to 3 mJ/m^2 at about 15% compression (reduction of the initial area of the drop, $A/Ao = 0.85$). On the other hand, the system formulated with 0.06 mg/ml dexS requires a 20% compression ($A/Ao = 0.80$) in order to achieve a similar surface tension. However, the preparation containing 0.1 mg/ml dexS does not adsorb well (surface tension before compression near 40 mJ/m^2) and the surfactant film seems to collapse at a surface tension of 20 mJ/m^2. Further to these observations, the calculated elasticity (ε) of the BLES–chiCl system is 130 mJ/m^2, the elasticity of the system containing 0.06 mg/ml dexS is 120 mJ/m^2, and the system containing 0.1 mg/ml dexS is 90 mJ/m^2. These values are consistent with previous findings for chitosan base and BLES, where the most active systems can reach elasticity values ranging from 120 to 200 mJ/m^2, and BLES-only systems have an elasticity near 80 mJ/m^2 [25,43].

3.2. BLES–chiCl–dexS in serum

To study the activity of the BLES–chiCl system with dexS in 50 μl/ml serum (model inhibitor), zeta potential and dynamic cycling experiments were conducted for the formulations of Fig. 3. The zeta potential of these BLES–ChiCl–dexS-serum systems is presented in Fig. 6 as a

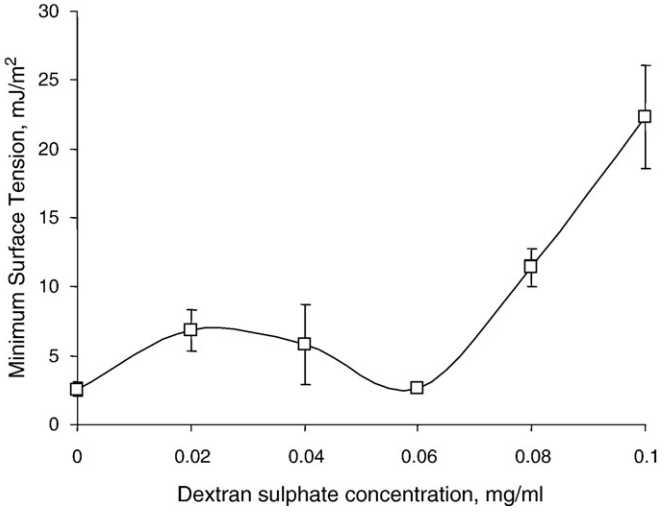

Fig. 4. Minimum surface tension of formulations containing 2.0 mg/ml BLES + 0.15 mg/ml chiCl (protasan), as a function of dexS concentration. Compression conditions described in Fig. 2.

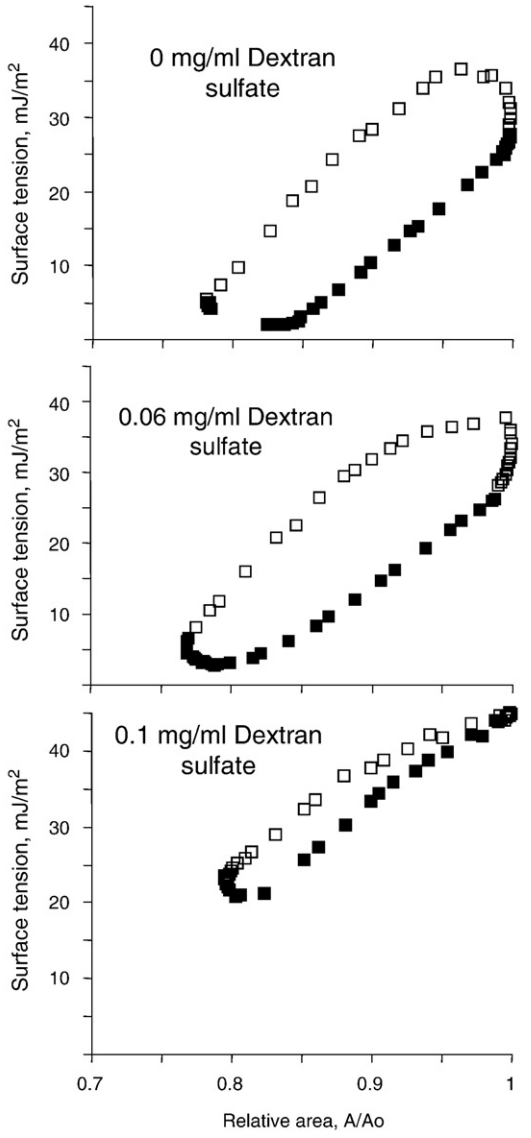

Fig. 5. Surface tension – relative interfacial area (cycling) isotherm for formulations containing 2.0 mg/ml BLES + 0.15 mg/ml chiCl (protasan) and 0, 0.06, and 0.1 mg/ml dexS, compressed at 3 s/cycle, 100% R.H., 37 °C.

function of dexS concentration. It is important to note that in the presence of serum, the zeta potential of BLES–chiCl is reduced from +20 mV to about +5 mV. This change in zeta potential is due to the fact that serum contains bovine albumin and other biomolecules that serve as buffers (pH of the suspension to a pH of 6.9 ± 0.1).

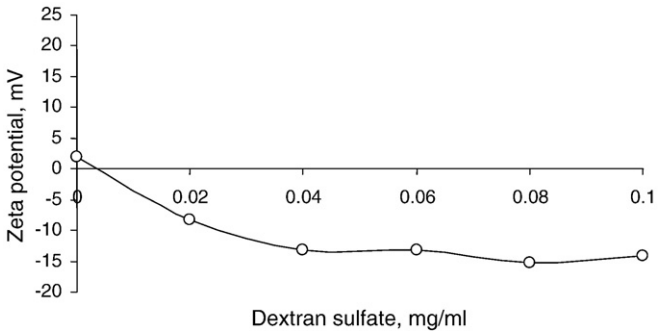

Fig. 6. Zeta potential of preparations containing 2.0 mg/ml BLES + 0.15 mg/ml chiCl (protasan) in saline solution, as a function of dexS concentration, in the presence of 50 μl/ml of bovine serum.

Furthermore some of the proteins in serum, mainly albumin, are negatively charged and they could adsorb on the positive groups in chitosan. However, the binding of albumin to chitosan is relatively weak [39]. Serum proteins could also insert themselves in the surfactant membrane. The reduction in zeta potential values with increasing dexS concentration reaches a plateau at a concentration of 0.04 mg/ml dexS.

The dynamic cycling isotherms of selected BLES–chiCl–dexS formulations in serum are presented in Fig. 7. The top part of the figure (no dexS) corresponds to the same system as Fig. 2. That BLES–chiCl system reaches a minimum surface tension of 5 mJ/m^2, and maintains a dilatational elasticity of 120 mJ/m^2. In the presence of 0.02 mg/ml dexS the system does not appear to undergo film collapse but the elasticity reduces to 70 mJ/m^2 and the film is highly unstable, as shown by the large surface tension relaxation at the end of the compression cycle. This instability is responsible for the hysteresis in the compression cycle. For the preparation containing 0.06 mg/ml dexS (an optimal formulation in the absence of serum), the surfactant film collapses at a surface tension of 18 mJ/m^2.

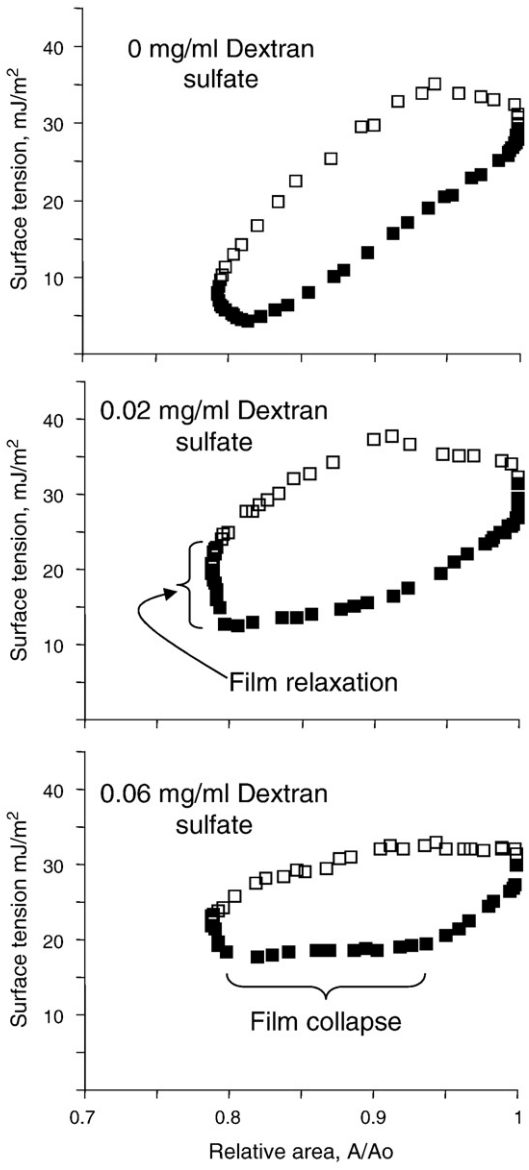

Fig. 7. Surface tension – relative interfacial area (cycling) isotherm for formulations containing 2.0 mg/ml BLES + 0.15 mg/ml chiCl (protasan) and 0, 0.02 and 0.06 mg/ml dexS in the presence of 50 μl/ml of bovine serum. These systems were compressed at 3 s/cycle, 100% R.H., 37 °C.

3.3. BLES–chiCl–dexS–bicarbonate systems

As will be explained in more detail in the Discussion section, the poor surface activity observed with systems containing high dexS concentration might be linked to the high binding affinity between chitosan and dexS. This ChiCl–dexS binding may remove chiCl from the BLES–chiCl. Based on the observations of Fig. 6, it is possible that increasing the pH of the system (one of the effects of adding serum to the system) may lead to more negative zeta potentials and lower necessary dexS concentrations to reverse the charge of BLES–chitosan aggregates. To test this hypothesis and observe the effect of pH on zeta potential, the zeta potential and surface activity of BLES–chiCl–dexS preparations was evaluated in the presence of 1.2 mM of sodium bicarbonate. This concentration of sodium bicarbonate is compatible with bicarbonate concentrations measured in the lungs and with previous studies with lung surfactants [1]. Fig. 8 presents the zeta potential of these systems as a function of dexS concentration. The addition of 1.2 mM of bicarbonate decreased the zeta potential of the BLES–chiCl aggregates from +20 mV (Fig. 3) to +7 mV (Fig. 8). Furthermore, the dexS concentration required to reverse the charge of BLES–chiCl was reduced from 0.06 mg/ml (Fig. 3) to about 0.03 mg/ml (Fig. 8). According to Fig. 8, the zeta potential of the system containing 0.06 mg/ml DexS and 1.2 mM bicarbonate is −15 mV, close to the zeta potential of the original surfactant aggregates [25].

The dynamic cycling isotherms of selected BLES–chiCl–dexS systems in the presence of 1.2 mM of bicarbonate are presented in Fig. 9. In this case, all the formulations can reach surface tensions near or even below 5 mJ/m^2 and that there is no evidence of film collapse. For the system BLES–chiCl–bicarbonate, the elasticity of that film is 130 mJ/m^2. That system also has the largest film relaxation, which suggests that the BLES–chiCl–bicarbonate systems are somewhat unstable. The formulation prepared with 0.04 mg/ml dexS has an elasticity of 125 mJ/m^2. The elasticity of the preparation with 0.06 mg/ml dextrate sulfate is 93 mJ/m^2.

A second series of BLES–chiCl–dexS–bicarbonate formulations was evaluated using a different batch of BLES (fall of 2009) to evaluate the effect of pH, and time in more detail. A summary of the minimum surface tension and elasticity after 1 and 3 h of preparation is included in Table 1 for formulations containing 0, 0.4 and 1.5 mM of bicarbonate, and 0 and 0.04 mg/ml dextran sulfate. Table 1 also includes the pH and zeta potential of these formulations. For the system of BLES–chiCl the minimum surface tension in Table 1 is similar to the BLES–chiCl systems obtained with the previous batch of BLES (see Fig. 4). The performance of this formulation tends to slightly improve between 1 and 3 h after preparation. However, for BLES–chiCl–bicarbonate systems the increase in pH from 5.3 to 6.4 and 7.0 with the addition of 0.4 and 1.5 mM of bicarbonate brings about an increase in the minimum surface tension, and a reduction in the zeta potential. Comparing with the results of the previous batch (top graph of Fig. 9), the batch of fall 2009 is more susceptible to the addition of bicarbonate.

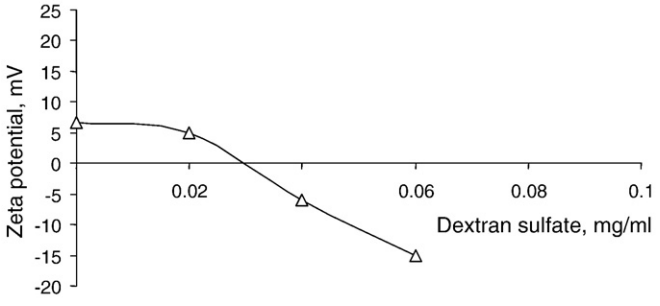

Fig. 8. Zeta potential of preparations containing 2.0 mg/ml BLES + 0.15 mg/ml chiCl (protasan) in saline solution containing 1.2 mM of sodium bicarbonate, as a function of dexS concentration.

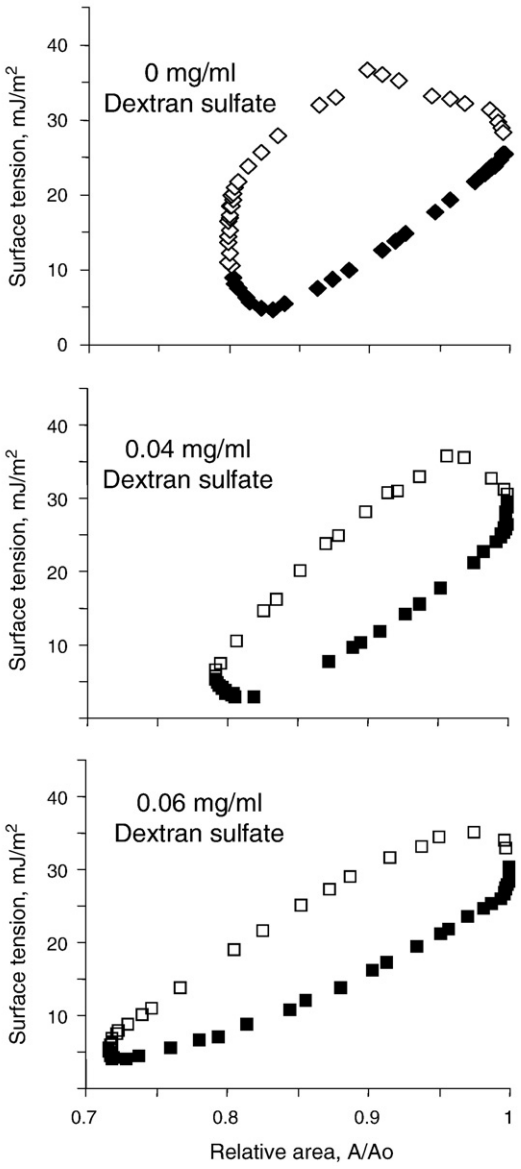

Fig. 9. Surface tension – relative interfacial area (cycling) isotherm for formulations containing 2.0 mg/ml BLES + 0.15 mg/ml chiCl (protasan) and 0, 0.04 and 0.06 mg/ml dexS in the presence of 1.2 mM sodium bicarbonate. These systems were compressed at 3 s/cycle, 100% R.H., 37 °C.

Another observation from Table 1 is that the addition of dextran sulfate produced negligible changes in pH, but it produced a modest reduction in the zeta potential of the surfactant aggregates. The system of BLES–chiCl–dexS–0.4 mM bicarbonate produced a relative low minimum surface tension of 5.4 mJ/m^2 after 1 h of preparation, but this tension eventually rose to 17.6 mJ/m^2 after 3 h. This increase in minimum surface tension was accompanied by the formation of large flocks that settled at the bottom of the syringe used to inject the surfactant solution into the CSD device. The formation of these large flocks is consistent with the near zero zeta potential of this formulation. The system of BLES–chiCl–dexS–1.5 mM bicarbonate produced a minimum surface tension of 5.6 mJ/m^2 after 1 h of preparation that reduced to 3.6 mJ/m^2 after 3 h. This formulation retained a “milky” appearance throughout the course of the experiment, which is consistent with the formation of stable aggregates with negative zeta potential of −14 mV. The formulation containing 0.04 mg/ml dexS and 1.5 mM bicarbonate prepared with

the BLES of Fall 2009 had similar performance to the formulation prepared with 0.04 mg/ml dexS and 1.2 mM bicarbonate and the previous BLES batch, suggesting robustness of this particular combination. Systems with 0.06 mg/ml dexS and the Sept. 2009 BLES batch (results not shown) produced formulations with high minimum surface tensions (>15 mJ/m^2). Such behavior was observed in the previous BLES batch at a concentration of 0.08 mg/ml dexS (Fig. 4).

3.4. BLES–chiCl–dexS–bicarbonate-serum systems

To evaluate if these formulations containing sodium bicarbonate can remain surface active in the presence of serum, dynamic cycling studies were carried out using the same systems of Fig. 9, but including 50 µl/ml serum in the preparation. The resulting dynamic cycling isotherms are presented in Fig. 10. All the formulations shown in Fig. 10 can reach minimum surface tensions lower than 5 mJ/m^2 without showing signs of film collapse. The system of BLES–chiCl (no dexS) shows signs of significant surface tension relaxation. The elasticity of that system is 122 mJ/m^2, and that level of elasticity is maintained in the presence of dexS (ε = 127 mJ/m^2 and 130 mJ/m^2 for 0.04 mg/ml and 0.06 mg/ml dexS, respectively). The zeta potential of the BLES–chiCl (no dexS) system was close to 0 mV whereas the zeta potential of the system containing 0.04 mg/ml dexS was −10 mV and that the system containing 0.06 mg/ml of dexS was close to −18 mV.

The combination of BLES, chiCl, dexS in bicarbonate buffered solution is capable of remaining surface active and negatively charged at physiologically relevant conditions and in the presence of bovine serum used as model inhibitor. BLEX–chiCl–dexS at pH 6.9 form stable "milky" suspensions that resemble the colloidal stability of the original lung surfactants.

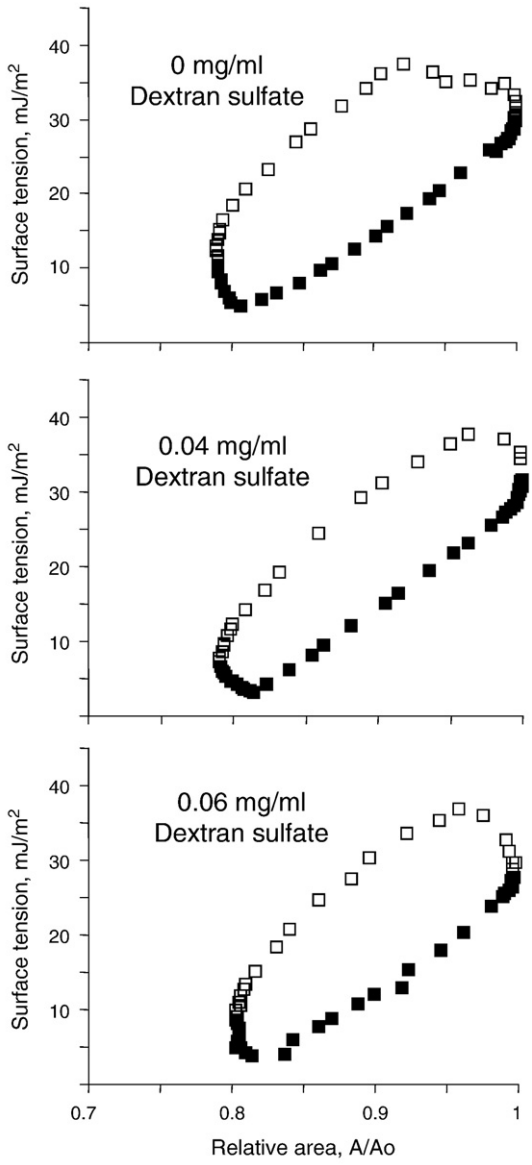

Fig. 10. Surface tension – relative interfacial area (cycling) isotherm for formulations containing 2.0 mg/ml BLES + 0.15 mg/ml chiCl (protasan) and 0, 0.04 and 0.06 mg/ml dexS in the presence of 1.2 mM sodium bicarbonate, and 50 µl/ml of bovine serum. These systems were compressed at 3 s/cycle, 100% R.H., 37 °C.

4. Discussion

The binding between the anionic lipid (DPPG) and chitosan proposed in Fig. 1 is compatible with a patch adsorption concept proposed to explain the binding of anionic lipids to cationic proteins and later extended to BLES–chitosan systems [25,27]. According to that concept, the chitosan binds preferentially to patches of anionic lipids in the lung surfactant aggregates, and in lung surfactant films adsorbed at the air–water interface [25]. It has been proposed that the optimal ratio between chitosan and BLES is obtained when the area occupied by the molecules of the anionic lipids is equal to the area occupied by the chitosan. From that ratio, it was proposed that approximately 3 positively charged glucosamine groups of chitosan (assuming complete dissociation and deacetylation of chitosan) bind to two molecules of the anionic lipid (DPPG or a DPPG-like molecule), each carrying one negative charge.

Dextran sulfate (dexS) used in this work is extracted from bacterial cultures, and therefore there is not a fixed molecular structure that can be used to represent the molecule. The structure presented in Fig. 1 shows the basic backbone of dexS, and the fact that each glucose unit can contain one or more sulfate groups. The supplier of dexS indicates that there are approximately 2 sulfate groups for each glucose unit [48].

The literature on polyelectrolyte complexes (PEC) of dexS and chitosan suggests that when the number of positive charges of chitosan (n^+) is equal to the number of negative charges in dexS (n^-), i.e. at $n^-/n^+ = 1$, large net-zero charge flocks of PEC form and flocculate, and that at other ratios smaller PEC particles form with either net positive or negative charge [39,49–51]. In this work, chiCl is already pre-adsorbed to the surfactant aggregates, and according to the optimal binding ratio discussed above and illustrated in Fig. 1, only one third of the positive charges are available for binding with dexS, and therefore one would expect a net-zero charge when $n^-/n^+ = 1/3$. To test these ideas, the zeta potential values of Fig. 3 were plotted as a function of the n^-/n^+ ratio. The value of n^+ was calculated based on the concentration of chiCl in the preparation (0.15 mg/ml), the weight of the repeating unit (glucosamine hydrochloride, 215 g/mol) assuming 100% deacetylation and 100% dissociation. The value of n^- was calculated based on the concentration of dexS, the weight of the repeating unit (a disulfate glucose unit, 365 g/mol), and assuming that there are 2 negative charges per repeating unit.

Fig. 11 presents the zeta potential and elasticity values for BLES–chiCl–dexS preparations versus the calculated n^-/n^+ ratio. According to this figure, the charge of the aggregates reverses for an n^-/n^+ ratio of 0.5, which is certainly lower than the value of 1 expected for chiCl–dexS complexes but is still higher than the expected value of 0.33 for BLES–chiCl–dexS. This suggests that a fraction of chitosan forms BLES–chiCl–dexS complexes, and another fraction forms chiCl–dexS polyelectrolyte complexes. The elasticity values shown in the bottom of Fig. 11 indicate that the addition of dexS to BLES–chiCl does not reduce the elasticity of the surfactant preparation substantially for n^-/n^+ ratios lower than the

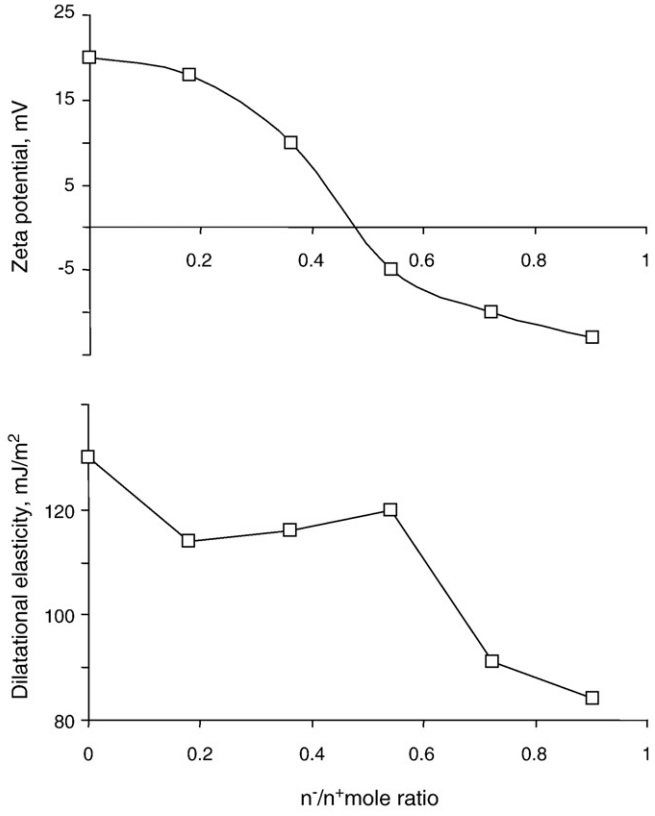

Fig. 11. Zeta potential (top) and dilatational elasticity (bottom) of preparations containing 2.0 mg/ml BLES + 0.15 mg/ml chiCl (protasan) in saline solution, as a function of the ratio between the anionic groups from dexS and the cationic groups from chiCl.

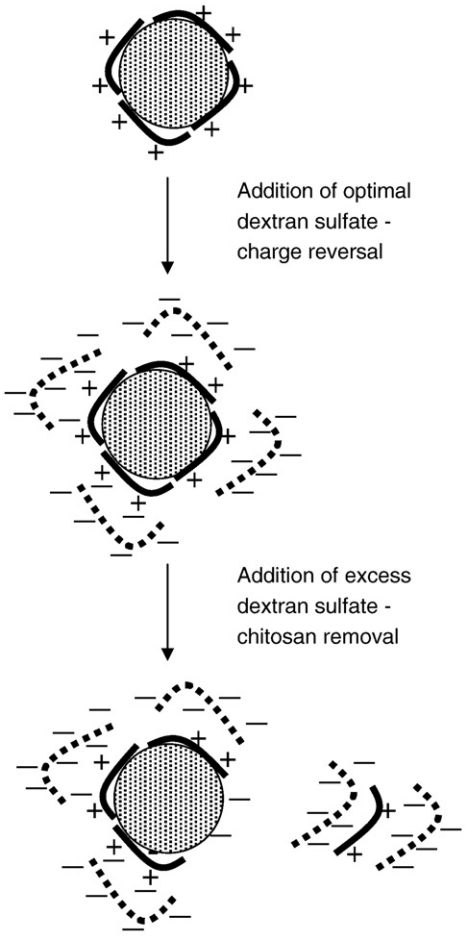

Fig. 12. Schematic of the binding of dexS to BLES–chiCl aggregates, and the chitosan losses from the surfactant aggregates in the presence of excess dexS, leading to the formation of chiCl–dexS complexes.

ratio needed for charge reversal. For n^-/n^+ ratios larger than the charge reversal ratio, the elasticity of the films decays substantially to values close to the elasticity of BLES-alone films [25,43]. This sharp decay in elasticity suggests that the BLES–chiCl complex is being affected by the presence of dexS. Certainly, as the n^-/n^+ ratios approach a value of 1, it is more likely that the complex chiCl–dexS is favored over the complex BLES–chiCl–dexS. This tendency of chiCl to bind exclusively to dexS was referred to as "chitosan removal" in the results section. This idea of chitosan removal is illustrated in the schematic of Fig. 12.

The introduction of bicarbonate into the formulation increased the pH of all the preparations. This increase in pH produced a decrease in zeta potential for all formulations, an effect that is likely associated with a reduction in the degree of ionization of chitosan chloride. A review of the literature did not yield any reports on degree of dissociation of chiCl with pH, but there is evidence that at pH values of 7 and higher chitosan hydrochlorides return to their insoluble chitosan base form [52]. This increase in pH seems to affect the binding of chiCl to BLES, as seen in the results of Table 1. The addition of dextran sulfate, being a neutral salt, does not affect the pH of the formulations, but it further reduces the zeta potential of the surfactant aggregates. When dextran sulfate is added at concentrations low enough to avoid chitosan removal (e.g. 0.04 mg/ml dexS), and added before sodium bicarbonate it helps protect the BLES–chiCl binding. This protective effect against the potential dissociative effect of higher pHs suggests irreversible BLES–chiCl associations in the presence of polyelectrolyte multilayers. Such irreversible effects have been observed in other polyelectrolyte assemblies [53,54].

The variability of the performance of BLES batches evaluated in ADSA–CSD at 100% R.H. and at the relatively low surfactant concentration of 2 mg/ml (BLES clinical concentration ~ 27 mg/ml) has been reported before, as well as the use of chitosan to suppress this variability [25]. Consistent with those observations, preparations of BLES–chiCl formulated with different batches of BLES had similar performance. However, the performance of BLES–chiCl–bicarbonate was batch dependent. The reason for this variability is not understood but it might be linked to the variability in the fraction of anionic lipids and cationic peptides in BLES [2]. Furthermore, the importance of this variability in clinical applications has not been established. On the other hand, BLES–chiCl preparations containing an appropriate concentration of dexS and enough bicarbonate to produce negatively charged stable suspensions, maintain their surface active even when using different BLES batches.

5. Conclusions

In this work it was shown that it is possible to use an anionic polyelectrolyte, dextran sulfate (dexS), to restore the negative charge (physiologically compatible) of surfactant aggregates prepared with mixtures of BLES and chitosan hydrochloride (chiCl), while retaining their surface activity. It was also determined that at high dexS/chiCl ratios the preparations were less surface active and more susceptible to serum inhibition. Introducing sodium bicarbonate to facilitate the charge inversion of BLES–chiCl–dexS aggregates led to formulations that retained their surface activity in the presence of serum used as model inhibitor. These BLES–chiCl–dexS–bicarbonate preparations produced stable milky suspensions that have the potential to be effective in surfactant therapy.

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