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Restoring the activity of serum-inhibited bovine lung extract surfactant (BLES) using cationic additives

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ABSTRACT

In this work four cationic additives were used to improve the surface activity of lung surfactants, particularly in the presence of bovine serum that was used as a model surfactant inhibitor. Two of those additives were chitosan in its soluble hydrochloride form with average molecular weights of 113 kDa and 213 kDa. The other two additives were cationic peptides, polylysine 50 kDa and polymyxin B. These additives were added to bovine lipid extract surfactant (BLES) and the optimal additive–surfactant ratio was determined based on the minimum surface tension upon dynamic compression, carried out in a constrained sessile drop (CSD) device in the presence of 50 μl/ml serum. At the optimal ratio all the BLES-additive mixtures were able to achieve desirable minimum surface tensions. The optimal additive–surfactant ratios for the chitosan chlorides are consistent with a previously proposed patch model for the binding of the anionic lipids in BLES to the positive charges in chitosan. For the peptides, the optimal binding ratios were consistent with ratios established previously for the binding of these peptides to monolayers of anionic lipids. The optimal formulation containing these peptides were able to reach low minimum surface tension in systems containing 500 μl/ml of serum, matching the effectiveness of a lung surfactant extract that had not undergone post-separation processes and therefore contained all its proteins and lipids (complete lung surfactant).

1. Introduction

There are several conditions that may lead to poor blood oxygenation associated with respiratory distress syndrome (RDS), however the lack or malfunction of lung surfactants is one of the most common conditions linked to this syndrome [1,2]. In pre-term neonatal patients diagnosed with RDS (neonatal RDS or nRDS) the syndrome is often associated with the lack of surfactants in the alveolar fluid. In patients diagnosed with Acute RDS (ARDS) the lungs may collapse due to surfactant malfunction caused by various surfactant inhibitors [1,3]. Surfactant therapy (instillation of surfactant extracts from animals) has reduced the mortality of nRDS patients from nearly 70% to less than 20%, but it has been ineffective in the treatment of ARDS [3–6].

Various inhibitors are associated with surfactant malfunction, including proteins like albumin and fibrinogen, and lipids like cholesterol, lysolecithins and unsaturated fatty acids. Bovine and human serum has been used as a broad spectrum surrogate to simulate surfactant inhibition [1,3]. Various formulations can overcome the action of low serum content (<30 μl/ml), but cannot handle higher serum content [7–9]. To assess the appropriate serum content to evaluate surfactant inhibition one should consider that bronchoalveolar lavages of ARDS patients contain up to 25 mg/ml of albumin [10]. Since bovine serum contains approximately 40 mg/ml of albumin suggests that approximately 600 μl/ml of serum is needed to evaluate surfactant inhibition.

One method used to evaluate surfactant inhibition *in vitro* involves measuring the surface tension of exogenous surfactants compressed and expanded under physiologically relevant conditions. In this work a constrained sessile droplet device (CSD) is used to measure the dynamic surface tension using the Axisymmetric Drop Shape Analysis (ADSA) [2,11–13]. Surfactant inhibition is typically characterized by either high minimum surfactant tension ($\gamma_{min} > 5$ mJ/m^2) and/or catastrophic film collapse (film collapse at high surface tensions) [1]. A typical surface tension–area–volume ADSA-CSD output for these studies is presented in Fig. 1 for a 2 mg/ml bovine lung extract surfactant (BLES) with 10 μl/ml of bovine serum, and a complete bovine lung surfactant with 750 μl/ml serum. This complete surfactant of Fig. 1 was obtained by saline lavage from calf lungs and simply lyophilized without further solvent separation steps (used to produce BLES) that remove surfactant proteins SP-A and SP-D and part of the proteins SP-B and SP-C. As indicated by Fig. 1, a common exogenous surfactant (BLES) cannot reach low surface tensions ($\gamma_{min} \sim 20$ mJ/m^2)

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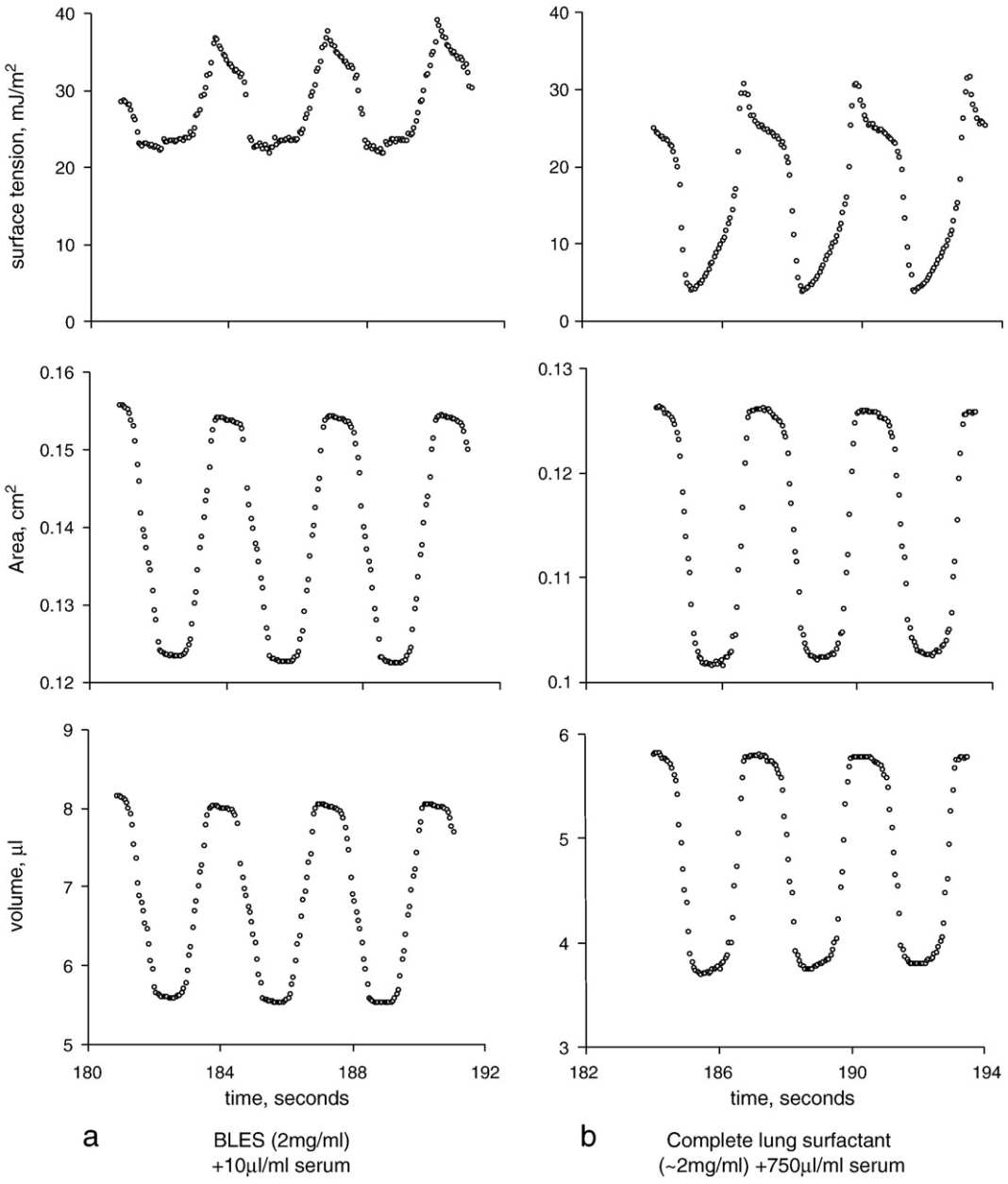

Fig. 1. Dynamic surface tension, area and volume output from ADSA for (a) 2 mg/ml BLES in the presence of 10 µl/ml serum, and (b) complete lung surfactant (~2 mg/ml) in the presence of 750 µl/ml serum. Dynamic cycling conditions: 20% compression (reduction in area), 3 s/cycle, 100% R.H., 37 °C.

when evaluated in the presence of serum and in humid air. However, a complete lung surfactant can produce minimum surface tensions of 5 mJ/m^2 or less even in the presence of 750 µl/ml of serum.

The difference between BLES and the complete lung surfactant used in Fig. 1 is that in order to produce BLES the surfactant lavage has undergone further purification with organic solvents that remove the hydrophilic surfactant proteins SP-A and SP-D, part of the cholesterol, and part of the surfactant proteins SP-B and SP-C [2,6]. Furthermore, the process of solvent extraction may also disrupt the original structure of lipid–protein complexes. Several additives have been proposed in order to make up for these changes in surfactant compositions. For example, neutral and hydrophilic polymers like dextran and polyethylene glycol have been used as surfactant additives [2,7,14–18]. Promising results have been observed *in vitro* with these additives, in particular against albumin-induced inhibition, but there are conflicting results in vivo [19]. Furthermore, mixtures of BLES and polyethylene glycol evaluated with ADSA-CSD were effective against 2.5 mg/ml albumin, but ineffective against 10 µl/ml of serum [20]. Anionic polymers such as hyaluronan and mucins have been found effective at reversing serum inhibition [20–22]. Nonionic and anionic polymers are said to simulate the role of SP-A in the preparations [2,7,18]. On the other hand, a cationic polysaccharide, chitosan, has been found to be more effective than nonionic and anionic polymers, and produce formulations that are consistently active regardless of the batch to batch variability of the extract [23,24]. A recent study has shown that chitosan chloride is effective at resisting the action of various inhibitors including albumin, fibrinogen, serum (50 µl/ml), and cholesterol [25]. Other cationic additives like polylysine, recombinant surfactant protein-C, synthetic peptide KL_4 and the biosurfactant polymyxin B have been proposed as additives that simulate the role of proteins SP-B and SP-C, which are essential for lung surfactant activity [26–30]. However, their effectiveness against high serum content has not been evaluated. These findings led to the hypothesis that using the proper concentration of cationic additives in BLES it is possible to overcome serum levels that simulate the inhibitory conditions of ARDS patients. In this work, four

cationic additives—chitosan chloride with molecular weights of 113 kDa and 213 kDa; polylysine (50 kDa); and polymyxin B—were evaluated as additives in BLES preparations containing various levels of bovine serum. The molecular structure of these cationic additives is illustrated in Fig. 2. Dynamic surface tensions obtained using ADSA-CSD were used to assess the activity of these formulations. Zeta potential measurements were used to assess the binding of these cationic additives to the anionic lipids in the surfactant aggregates.

2. Materials and methods

2.1. Materials

BLES (bovine lipid extract surfactant) was provided by BLES Biochemicals Inc., London, Ontario, Canada. BLES is an organic solvent extract from a bovine source, and contains mainly phospholipids and two surfactant-associated proteins, SP-B and SP-C. Concentrated (27 mg/ml) BLES samples were stored in glass vials under N2 atmosphere at −20 °C until the day of the experiment. The suspension of the complete lung surfactant was also provided by BLES Biochemicals.

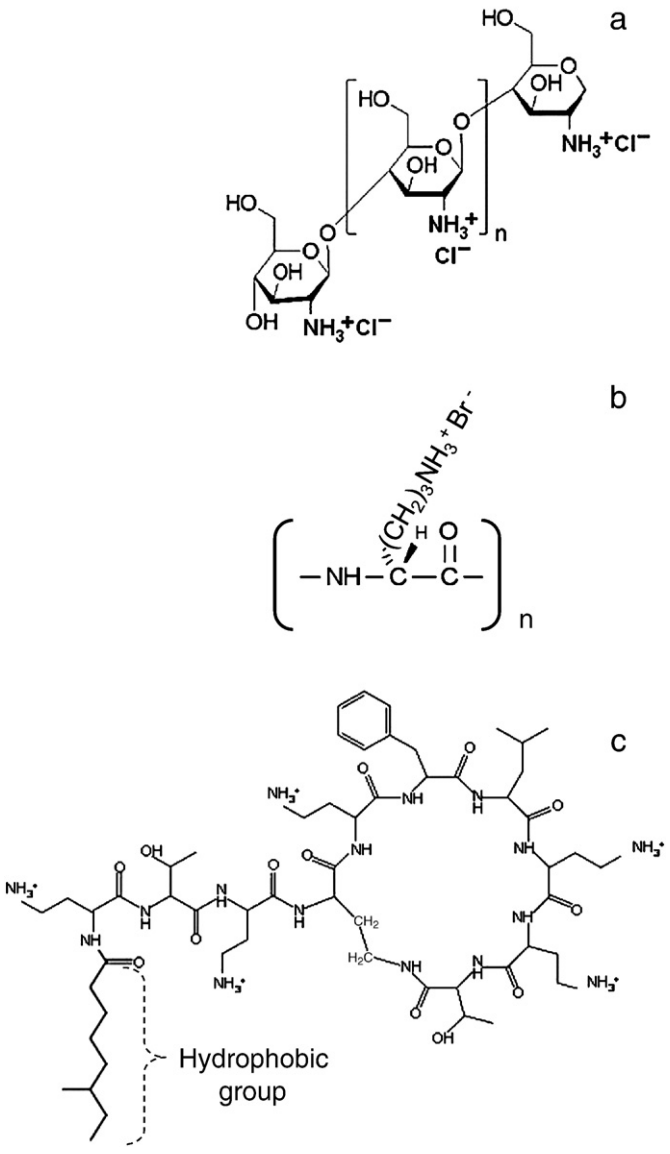

Fig. 2. Molecular structures of (a) chitosan chlorides (protasans), (b) polylysine, and (c) polymyxin B.

Chitosan hydrochloride (Protasan Cl) 113 kDa and 213 kDa were purchased from Novamatrix, FMC BioPolymer AS, Drammen (Norway) (Protasan UP Test Kit, #4219001). These water-soluble chitosans have a degree of deacetylation of 75–90%. Protasan 113 contains a distribution of molecules with molecular weights ranging from 50,000 to150,000 g/mol and protasan 213 contains molecules ranging from 150,00 to 400,000 g/mol. Poly-D-lysine hydrobromide was purchased from Sigma-Aldrich and contain molecules ranging from 30,000 to 70,000 g/mol (product code P-2636). Polymyxin B (molecular weight 1301 g/mol) was purchased from Sigma-Aldrich (product code P1004). Bovine serum was also purchased from Sigma-Aldrich (product code B8655) (Lot No. 127H9001, protein content: 60 mg/ml determined by the Biuret method).

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 in a salt solution containing 0.6% NaCl and 1.5 mM $CaCl_2$ [41,42]. The concentration of BLES was 2 mg/ml in all preparations. The prescribed amount of a stock solution of the cationic additive (chitosan chloride 2 mg/ml, polylysine 1.0 mg/ml, and polymyxin B 1.0 mg/ml) was added to the NaCl/$CaCl_2$ salt solution. Various concentrations of each cationic additive were also evaluated, ranging from 0 to 0.3 mg/ml. The pH of these BLES–cationic additive preparations ranged from 5.3 to 5.7. Bovine serum (stored in aliquots at −20 °C) was added to the surfactant mixture. The final pH of these BLES–cationic additive–serum mixtures ranged from 6.5 to 7.0.

2.2.2. Surface tension measurements

The BLES–cationic additive–serum preparations were gently mixed using a Vortex-Genie, Model K-550-G mixer, and their surface activities were examined using a Constrained Sessile Drop (CSD) configuration in conjunction with Axisymmetric Drop Shape Analysis (ADSA). The design and operation of the CSD configuration has been described in detail elsewhere [13,23,24]. 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 spread 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 and the pedestal are enclosed in a chamber that allows control of humidity and temperature of the air in the chamber to 100% relative humidity at 37 °C. The liquid drop was first left undisturbed for 3 min or until the equilibrium surface tension of ~25 mJ/m^2 was reached. Dynamic expansion and compression cycles of the test droplet were carried out at a periodicity of 3 s/cycle. The ratio between the interfacial area reached at the end of compression to the initial area was adjusted to approximately 20%, in order to mimic normal breathing in adults [1,13,31]. Sequential images (20 images/s) of the drop during dynamic cycling were collected by a CCD camera (Model 4815-5000, Cohu Corp., Poway, CA), and later analyzed by ADSA to get a typical output including surface tension, surface area and volume of the test drop [12,13,24].

For each of the formulations presented in this paper, four or more droplets were tested and the results are expressed as the mean ± 95% confidence interval ($n \geq 4$ unless otherwise indicated). Typical surface tension–volume–area outputs of ADSA for BLES–serum and for complete lung surfactant–serum during dynamic cycling are presented in Fig. 1. Using these data, dynamic cycling isotherms can be obtained by plotting the surface tension versus the relative area of the droplet (area at any time divided by the maximum surface area during the cycle). The dynamic cycling isotherms for the systems of Fig. 1 are presented in Fig. 3.

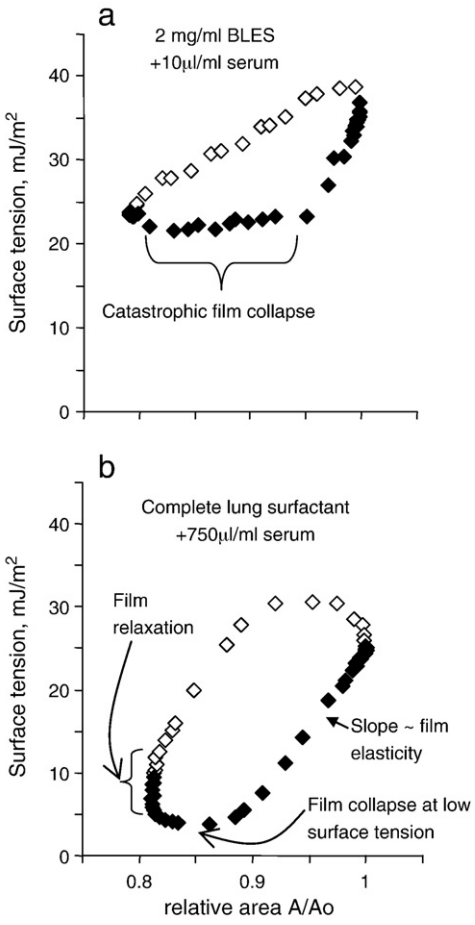

Fig. 3. Cycling isotherms (dynamic surface tension versus relative surface area) (a) 2 mg/ml BLES in the presence of 10 μl/ml serum, and (b) complete lung surfactant (~2 mg/ml) in the presence of 750 μl/ml serum. Dynamic cycling conditions: 20% compression (reduction in area), 3 s/cycle, 100% R.H., 37 °C.

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 [24].

3. Results

3.1. Surface activity of BLES and complete lung surfactant

Fig. 1 shows that the minimum surface tension of BLES + 10 μl/ml of serum is close to 22 mJ/m^2, and for complete lung surfactant + 750 μl/ml serum it is close to 5 mJ/m^2. This indicates that this BLES preparation is inactivated by 10 μl/ml serum since it cannot achieve low minimum surface tensions (~5 mJ/m^2 or less) when compressed at 20% compression (fractional reduction in surface area) with a 3 second/cycle periodicity, in 100% R.H. air at 37 °C (physiological conditions). It is important to note that the surface activity of surfactants in 100% R.H. air is markedly less than in dryer air at R.H. < 100% R.H [13,32,33].

The data in Fig. 1 can also be used to produce a cycling isotherm by plotting the surface tension versus the relative area during cycling. Fig. 3 presents the cycling isotherms for the BLES–serum and complete lung surfactant–serum systems. The solid symbols in Fig. 3 represent the data obtained during the compression stage, and the open symbols the data obtained during the expansion stage. In Fig. 3a (BLES–serum) one observes that by reducing the area by more than 10% of its maximum value there is no further reduction in surface tension (i.e. film collapse). In Fig. 3b (complete lung surfactant–serum) there is also film collapse but in this case it takes place at low surface tensions (~3 mJ/m^2), and therefore is not a cause for concern. The reduction in surface tension with reduction in relative area (slope in Fig. 3b) is steep, corresponding to a high film elasticity ($\varepsilon = [A/A_o]\, d\gamma/d[A/A_o]$) [13]. Finally, Fig. 3b also illustrates the relaxation of the surfactant film, characterized by an increase in the surface tension after the compression of the film has ended and before the expansion commenced. Although this feature of the compression cycle of ADSA-CSD might not be physiologically correct in all cases, it gives the opportunity to study the stability of the film. Film relaxation is generally undesirable as it reflects instability of the film. Furthermore it is important to clarify that film relaxation is typically observed in systems exposed to 100% R.H. at 37 °C [13].

3.2. Surface activity of BLES + cationic additives

In order to evaluate the surface activity of BLES with cationic additives in the presence of 50 μl/ml serum, their minimum surface tension during dynamic cycling was obtained, as a function of the concentration of the cationic additive. Fig. 4 presents the minimum surface (γ_{min}) tensions obtained with mixtures of BLES and chitosan chlorides (protasans), polylysine, and polymyxin B. For the case of protasans (Fig. 4a), between 0.15 and 0.2 mg/ml of the polymer the minimum surface tension is below 5 mJ/m^2. This range of concentration is consistent with that obtained by Saad et al. in the absence of inhibitors, and is slightly higher than the one reported by Kang et al. for chitosan (not chitosan hydrochloride) mixed with 2 mg/ml of BLES [24,25]. The data in Fig. 4a also suggest that optimal formulations (lower γ_{min}) with 2.0 mg/ml BLES require less protasan 113 kDa (~0.15 mg/ml) than protasan 213 kDa (~0.2 mg/ml). Furthermore, the "V" shape of the minimum surface tension curves in Fig. 4 has been observed before in the case of chitosan–BLES mixtures. In those cases, it has been proposed that an overdose of chitosan leads to a hydration and fluidization of the film, and film collapse [24].

For the case of peptides, polylysine and polymyxin B an optimal additive concentration was also found in each case. For polymyxin B this occurs at a relatively low concentration (near 0.1 mg/ml, or 5% on a weight basis) but for polylysine this occurs at the relatively high concentration of 0.2 to 0.25 mg/ml of the peptide (~10% w/w). These findings are consistent with those of Calkovska et al. who found that curosurf (a porcine-extracted surfactant) with 2% of polymyxin B outperformed curosurf alone when challenged with albumin as inhibitor, reducing the frequency of lung collapse in rabbits [29]. A previous experience with polylysine as surfactant additive was not successful [27]. As will be discussed later, recent studies suggest that the problem with those earlier studies is that the molecular weight of polylysine (14 kDa) was too low to produce any significant binding with the anionic lipids. One interesting observation with regards to peptides is that their overdose effect is not as pronounced as that experienced by the polysaccharides. This is a desirable feature for cationic peptide formulations, as it suggests that these formulations are more robust than the ones based on cationic polysaccharides.

Fig. 5 presents the dynamic cycling isotherms for these preparations. In the case of the low molecular weight protasan (113 kDa), there is no film collapse observed, and no relaxation, leading to systems with minimal hysteresis. For the case of the higher molecular weight protasan (213 kDa) no film collapse was observed, but substantial relaxation was noticeable at the end of the compression step, suggesting that protasan 213 kDa formulations are less stable. The preparations containing polylysine and polymyxin experienced some film collapse at surface tensions near 3 mJ/m^2 and no significant relaxation.

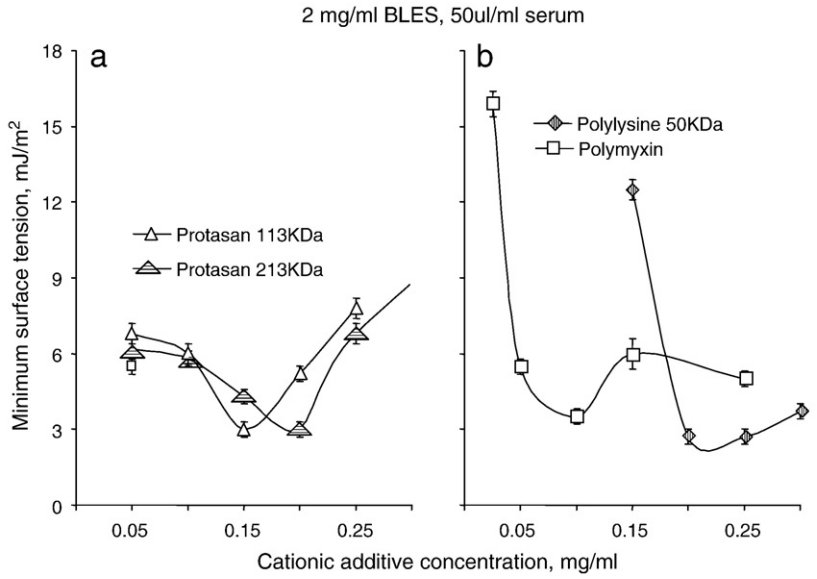

Fig. 4. Minimum surface tension of mixtures of 2 mg/ml BLES in the presence of 50 µl/ml serum as a function of cationic polymer concentration. Dynamic cycling conditions: 20% compression (reduction in area), 3 s/cycle, 100% R.H., 37 °C.

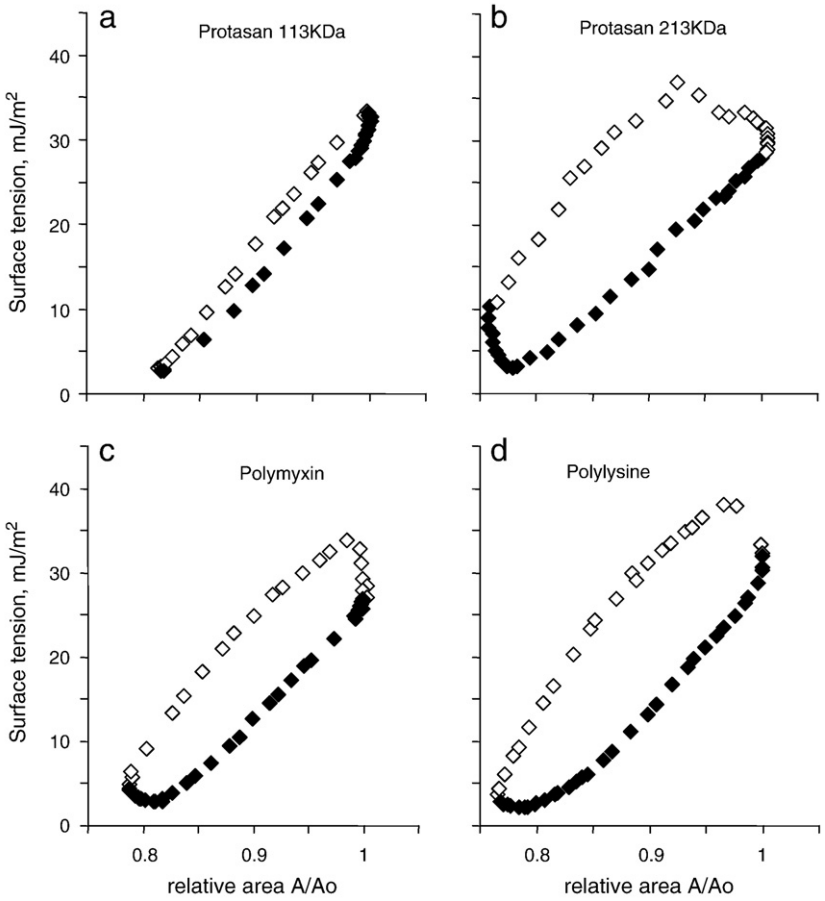

Fig. 5. Cycling isotherms for the optimal formulations of Fig. 4. All formulations contain 2 mg/ml BLES and 50 µl/ml bovine serum. The concentration of the optimal cationic additive is (a) 0.15 mg/ml protasan 113 kDa, (b) 0.20 mg/ml protasan 213 kDa, (c) 0.1 mg/ml polymyxin B, and (d) 0.2 mg/ml polylysine 50 kDa. Dynamic cycling conditions: 20% compression (reduction in area), 3 s/cycle, 100% R.H., 37 °C.

3.3. Binding of cationic polymers to lung surfactants

Previous work has shown that the zeta potential of the surfactant aggregates reflects the binding of chitosan to BLES [24]. In that case, the binding of this cationic polymer to the negatively charged surfactant aggregates reverses the charge of these aggregates with increasing chitosan concentration. The same transition is observed for BLES–protasan mixtures in Fig. 6a. For these polysaccharides there is a substantial increase in the charge of the aggregates within the range of 0 to 0.1 mg/ml for protasan 113 kDa and from 0 to 0.15 mg/ml for

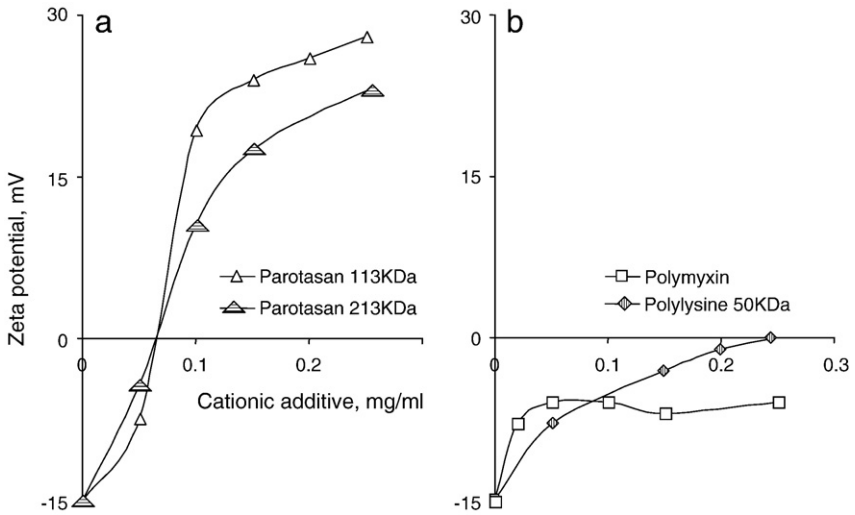

Fig. 6. Zeta potential of the aggregates of mixtures of 2 mg/ml BLES in mixture with (a) cationic polysaccharides (protasan 113 kDa and 213 kDa), and (b) cationic peptides (polymyxin and polylysine); as a function of the concentration of the cationic additive. pH = 5.5, electrolyte solutions containing 0.9% NaCl.

protasan 213 kDa. Further increase in protasan concentration only produces a marginal increase in the charge of the aggregates. The transition into this region of marginal zeta potential increase has been associated with the optimal formulation in the case of BLES + chitosan preparations [24]. In this case, the transition points observed for BLES + protasan are also consistent with the optimal formulations reported in Fig. 4. Furthermore, the fact that the transition into the marginal region takes place at a lower protasan 113 kDa concentration than that of protasan 213 kDa suggests that protasan 113 kDa binds more strongly to the anionic lipids in BLES.

For the cationic peptides polylysine and polymyxin (Fig. 6b), the zeta potential does not turn positive, but it does shift towards higher zeta potential values. For the case of polymyxin there is a clear plateau region after 0.05 mg/ml of polymyxin (2.5% mass basis), which is consistent with the data in Fig. 4b that shows that desirable minimum surface tensions are obtained using 0.05 mg/ml to 0.1 mg/ml of polymyxin. For polylysine, the change in zeta potential is more gradual and it only appears to approach a plateau value at concentrations close to 0.20 to 0.25 mg/ml of the peptide. The fact that the cationic peptides do not produce the highly positively charged aggregates obtained with cationic polysaccharides suggests that the binding between these types of additives and BLES is different from that of chitosans and BLES.

To illustrate the effect of cationic additive binding on surfactant aggregation, Fig. 7 presents micrographs of BLES–cationic additive preparations formulated at the optimal conditions identified in Fig. 4. To interpret these results it is important to keep in mind that BLES without additives produces a suspension of 10–20 μm vesicles, as seen in BLES-only micrographs presented in previous articles [23,24]. The images in Fig. 7 show that using all these cationic additives at optimal formulation conditions induces the formation of larger surfactant aggregates. The same observation has been made previously when using chitosan as cationic additive [23,24].

3.4. Resistance of cationic additives–BLES against serum inhibition

The maximum serum content that can be handled by BLES + cationic additives was evaluated by determining the minimum surface tension of these optimal formulations (from Fig. 4) as a function of serum content. These minimum tensions are presented in Fig. 8.

For BLES alone, even as little as 10 μl/ml of serum is enough to inhibit this surfactant preparation. With protasans, the formulation retains its surface activity in the presence of 50 μl/ml of serum, but they become inactive when the serum content increases to 100 μl/ml. On the other hand, the polylysine formulation retains its surface activity in the presence of 500 μl/ml of serum, but they are inactive in 750 μl/ml of serum. Finally, polymyxin B produces a similar minimum surface tension to that of complete lung surfactant throughout the range of serum content of 0 to 750 μl/ml. Thus, by combining 2 mg/ml BLES with 0.1 mg/ml polymyxin B it is possible to simulate the inhibition resistance of complete lung surfactant.

Fig. 9 presents the dynamic cycling isotherm for 2 mg/ml BLES + 0.1 mg/ml polymyxin B in the presence of 750 μl/ml of serum, and a superimposed isotherm for the complete lung surfactant (Fig. 3b). While both formulations can reach low surface tensions, the complete surfactant can reach this low tension with only 10% compression, which is indicative of the higher elasticity of the surfactant film.

4. Discussion

The differences in surface activity of BLES and complete lung surfactant in the presence of serum, as illustrated by Figs. 1 and 3, should be seen in light of the methods used to extract lung surfactants from intact bovine lungs. In the case of the complete lung surfactant, a saline solution is used to wash the lungs, and is later lyophilized to a concentrated solution. Thus none of the proteins or lipids of the lung surfactants is removed. On the other hand, BLES requires two additional purification steps. The first step involves an extraction with chloroform and methanol to recover the lipids and remove the hydrophilic proteins SP-A and SP-D. The second step involves the removal of cholesterol and neutral lipids from the surfactant extract through an “acetone” wash.

The surfactant protein SP-A is the most abundant of the four surfactant proteins (~7% in the complete surfactant), it has a large molecular weight (~30 kDa) and it assembles in a tertiary structure of up to 750 kDa. SP-A has been associated with tubular myelin formation and the spreading of the surfactant film at the air/water interface [34,35]. In the case of cholesterol, its role on lung surfactant physiology is more controversial since at low concentration (~5% of total lipids) it improves the mechanical properties of the film, but at high concentrations it fluidizes the surfactant film, inhibiting formulation [36,37].

Besides the loss of SP-A and cholesterol, the solvent extraction method used in the preparation of BLES and other commercial surfactant extracts also removes between 50% and 80% of the cationic and hydrophobic surfactant proteins SP-B and SP-C [2,6]. The positively charged surfactant protein SP-B (molecular weight ~9 kDa) is an essential protein since its absence from lung surfactants

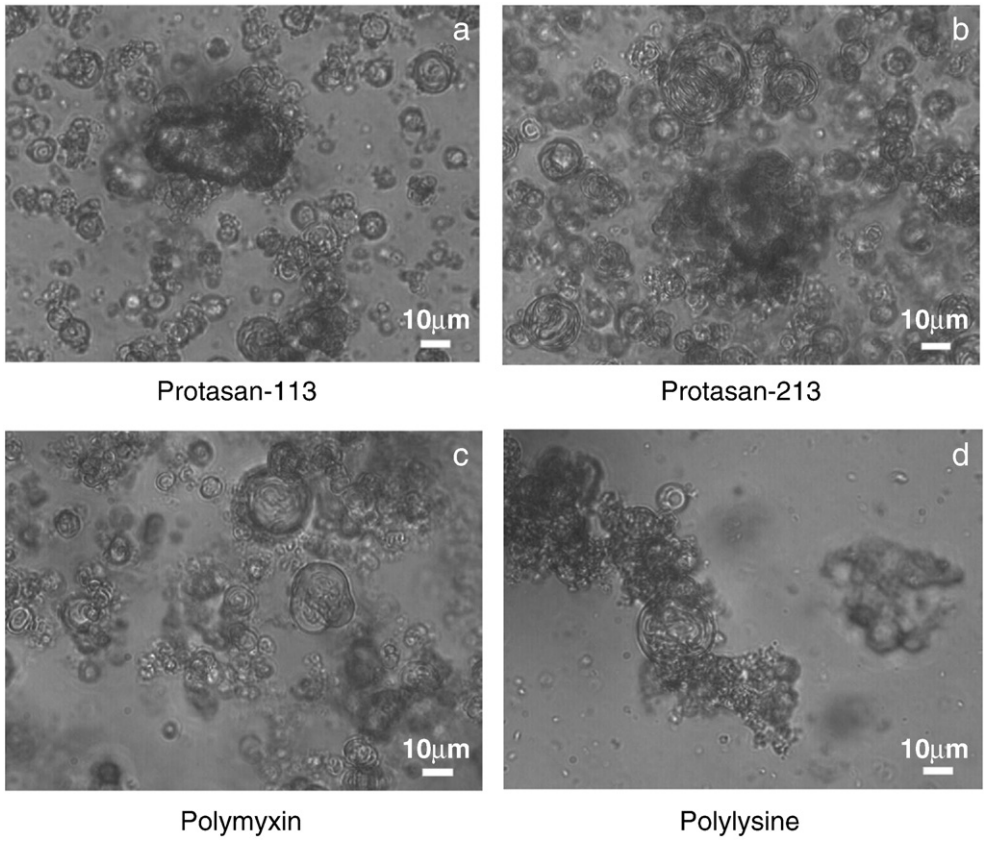

Fig. 7. Micrographs of optimal formulations of BLES and cationic additives. All formulations contain 2 mg/ml BLES, and: (a) 0.15 mg/ml protasan 113 kDa, (b) 0.20 mg/ml protasan 213 kDa, (c) 0.1 mg/ml polymyxin B, and (d) 0.2 mg/ml polylysine 50 kDa.

leads to respiratory failure [38,39]. SP-B, along with the more hydrophobic cationic protein SP-C (~4 kDa) is said to be inserted in the lipid membrane, facilitating the formation of large surfactant aggregates, tubular myelin formation and film spreading [35,40]. The content of SP-B in complete lung surfactant is typically 1–2% (of the total lipids), and SP-C is close to 2–3% [2].

Chitosan chlorides and polylysine, similar to SP-B and SP-C, have positive charges that can bind to anionic lipids, but they do not have the lipophilic moieties of these proteins. Polymyxin, on the other hand, has the positive charge characteristic of SP-B and SP-C, and lipophilic moieties. The lipophilic moieties of polymyxin may explain the fact that polymyxin outperformed chitosan chlorides and polylysine in preventing serum inhibition.

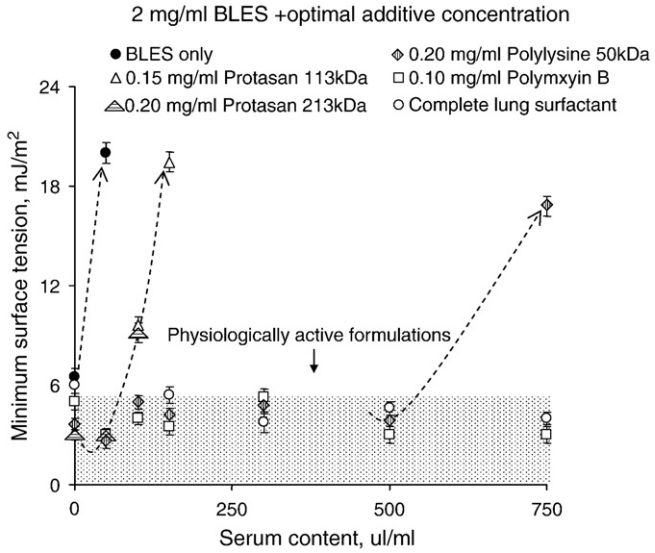

Fig. 8. Minimum surface tension of optimal formulations of 2 mg/ml BLES and different cationic cationic additives (formulations of Fig. 5) as a function of bovine serum content. Dynamic cycling conditions: 20% compression (reduction in area), 3 s/cycle, 100% R.H., 37 °C.

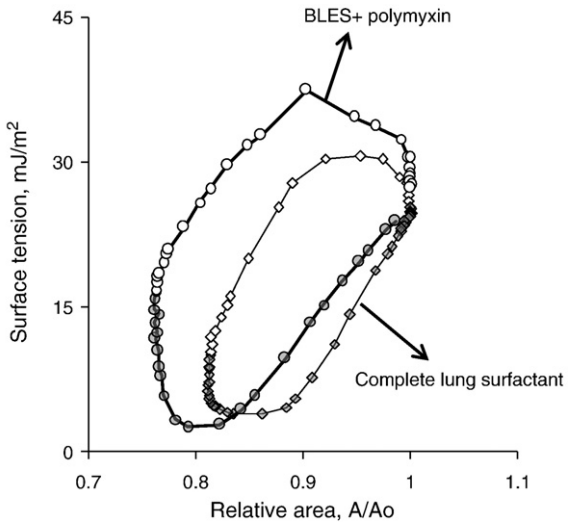

Fig. 9. Cycling isotherms for 2 mg/ml BLES + 0.1 mg/ml polymyxin (circles), and complete lung surfactant (diamonds) in the presence of 750 μl/ml bovine serum. Dynamic cycling conditions: 20% compression (reduction in area), 3 s/cycle, 100% R.H., 37 °C.

The optimal ratio between a cationic additive and BLES has been previously discussed for the case of chitosan–BLES preparations [24]. In that work, it was proposed that the cationic groups ($-NH_3^+$) of chitosan bind to patches of anionic lipids (phosphatidylglycerols) in BLES. That patch hypothesis is consistent with an optimum chitosan concentration of 0.1–0.15 mg/ml for 2.0 mg/ml of BLES [24], and an optimal chitosan chloride (protasans) concentration of 0.15–0.2 mg/ml for 2 mg/ml BLES (Fig. 4a). The hypothesis of patch adsorption is consistent with thermodynamic models on the adsorption of cationic proteins on mixed films of anionic and neutral lipids [41]. These patches have been observed in vesicles produced with fluorescence labeled lipids [42]. Despite the fact that the chitosan chlorides are not as potent additives to counteract serum inhibition as the cationic peptides, their possible use in surfactant formulations cannot be discarded. These polymers displayed excellent properties in resisting the action of potent inhibitors such as cholesterol and fibrinogen [25]. One interesting property of chitosan is that it can compact molecules of unsaturated lipids that inhibit surfactant films, and this may explain their performance as surfactant additives [43]. With regards to safety issues associated with chitosan chloride in surfactant therapy, it is noted that micro-particle systems containing protasan 213 kDa are being used in pulmonary drug delivery [44]. There is also evidence that chitosan chlorides are compatible with the alveolar epithelial tissue, and that they do not trigger a cytotoxic response or an increase in tissue permeability that could lead to lung edema [45].

One of the differences between chitosans and peptides is that chitosans are rigid molecules that cannot penetrate lipid layers [46], but peptides are more flexible and could, in principle, infiltrate lipid membranes [47]. It has been proposed that the ability of cationic proteins SP-B and SP-C to insert themselves in surfactant bilayers is important in improving the properties of lung surfactants [2,48]. The lack of membrane insertion ability of chitosans could explain their inferior performance against serum inhibition. Furthermore, chitosans are known to adsorb either flat or in loop conformations on surfaces [49] which suggests that the unbound NH_3^+ groups of chitosan are exposed to the aqueous environment, which would explain the highly positive zeta potential of BLES–chitosan formulations.

The binding between polylysine and anionic lipids has been explored by various researchers [50–52]. It has been found that polylysine may adopt alpha-helix, random coils or even beta-sheet configurations when bound to anionic or mixed lipid monolayers. Unfortunately, there is no further confirmation at this time of the polylysine configuration in BLES–polylysine systems. Thus, further studies are necessary to investigate the secondary structures of polylysines bound to lung surfactants and the role of these structures in improving the properties of lung surfactants. Part of the role of polylysine in improving surfactant properties may be related to their ability to dehydrate lipid bilayers [50,52].

With regards to the effect of molecular weight on the binding of these additives, the data in Figs. 4 and 6 suggest that the lower molecular weight chitosan chloride has a stronger binding to the anionic lipids in BLES since it requires less protasan of 113 kDa than 213 kDa to reach the optimal concentration. Considering that their binding ratio and performance in mixtures with BLES is similar to that of chitosan 6000 kDa [23,24] one is inclined to conclude that molecular weight is not a highly significant variable in these formulations. However, that statement should be considered with some caution as the minimum molecular weight of polymers evaluated in this study is 50 kDa (i.e. polylysine). It has been found that polylysine with molecular weights below 15 kDa have a weak binding or no binding at all to phosphatidylglycerol lipids [50]. This finding is consistent with the fact that in a previous study polylysines with molecular weights of 2.3 to 14 kDa were found to be ineffective as lung surfactant additives [27].

The use of polylysine in surfactant therapy should be carefully considered. Polylysine and other cationic proteins have been found to trigger pulmonary edema and to increase the permeability of epithelial tissue [53,54]. However, in those studies polylysine was directly administered to the subject, getting polylysine in direct contact with the epithelial tissue. In the case of polylysine pre-bound to surfactant lipids it is less likely that polylysine could produce any significant effect on the tissue. However, the safety of polylysine–surfactant formulations is an issue that should be considered in the future.

One disadvantage of polylysine compared to SP-B is the lack of hydrophobic amino acids in its backbone (e.g. proline, isoleucine, and valine) that are present in the backbone of SP-B. These hydrophobic amino acids are said to provide surface active properties to SP-B and further contribute to its insertion into lipid layers [48]. In the case of SP-C, this peptide has palmitoyl (a C16–16 alkyl carbon fatty acid) groups connected to an alpha-helix secondary structure rich in valine [55]. Some of these hydrophobic properties are simulated by polymyxin B (Fig. 2c). Polymyxin has one hydrophobic group containing 9 alkyl carbons, and two hydrophobic amino acids: leucine and phenylalanine. Furthermore, polymyxin B has 5 lysine amino acids that provide five cationic binding points with anionic species like phosphatidylglycerols. Studies on dipalmitoyl phosphatidyl glycerol (DDPG)–polymyxin B monolayers confirm that this molecule binds to five neighboring DPPG molecules, and that at that ratio there is a shift in the compression isotherm of this mixture, and an increase in the collapse surface pressure of DPPG [56,57]. Vibrational sum frequency generation spectroscopy studies suggest that polymyxin B dehydrates the anionic groups of phosphatidyl glycerol, and that the lipophilic group of polymyxin B interacts with the lipophilic portion of the lipid layer [58]. Based on the fact that about 10% of lipids in BLES are anionic, and using the optimal stoichiometric ratio of 5 anionic lipids to one polymyxin B, one would predict that the optimal polymyxin concentration (for 2 mg/ml BLES) is close to 0.07 mg/ml. This estimated optimal concentration is close to the value of 0.1 mg/ml polymyxin B obtained from the data of Fig. 4. Based on the data of Fig. 8, polymyxin B is an excellent additive for BLES, suggesting that all three properties of this molecule—being cationic, a peptide, and hydrophobic are relevant to improving the performance of extract surfactants in the presence of bovine serum.

The use of polymyxin B in surfactant therapy is less of a concern since this antibiotic has already been used in the treatment of septic conditions in the lungs [30,58,59]. However, it is important to keep in mind that polymyxin B is a histamine-releasing agent that may cause over-responsive (allergic) reactions in some patients [60,61]. These undesirable responses have been observed when polymyxin is instilled or inhaled directly into the lungs, but it is expected that polymyxin pre-bound to lipids would be less active (at histamine release) than polymyxin alone.

One important point that needs to be addressed is that the optimal cationic additive/lipid ratio obtained from the data in Fig. 4 cannot be extrapolated to other surfactant preparations. The lipid composition in different surfactant extracts can vary, and therefore an optimization procedure similar to that illustrated in Fig. 4 is necessary in order to formulate an optimal surfactant preparation. Furthermore, bovine serum is useful to evaluate the activity of the formulations against some blood proteins (particularly albumin), but there are other powerful inhibitors like fibrinogen, cholesterol, and lipases that need to be considered [62].

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