Effect of humidity on lung surfactant films subjected to dynamic compression/expansion cycles

Edgar J. Acosta¹,*, Roya Gitiafroz², Yi Y. Zuo², Zdenka Policova², Peter N. Cox³, Michael L. Hair², A. Wilhelm Neumann²

¹ Department of Chemical Engineering and Applied Chemistry, University of Toronto, 200 College Street, Toronto, Ont., M5S 3E5 Canada
² Department of Mechanical and Industrial Engineering, University of Toronto, 5 King’s College Road, Toronto, Ont., M5S 3E5 Canada
³ Department of Critical Care Medicine, The Hospital for Sick Children, 555 University Avenue, Toronto, Ont., M5S 3E5 Canada

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Abstract

The surface activity of bovine lipid extracted surfactant (BLES) preparations used in surfactant replacement therapy is studied in dynamic film compression/expansion cycles as a function of relative humidity, surfactant concentration, compression rate, and compression periodicity. BLES droplets were formed in a constrained sessile droplet configuration (CSD). Images obtained during cycling were analyzed using axisymmetric drop shape analysis (ADSA) to yield surface tension, surface area, and drop volume data. The experiments were conducted in a chamber that allowed both humid (100% RH), and “dry” air (i.e. less than 20% RH) environments. It was observed that in humid environments BLES films are not stable and tend to have poor surface activity compared to BLES films exposed to dry air. Further analysis of the data reveal that if BLES films are compressed fast enough (i.e. at physiological conditions) to avoid film hydration, lower minimum surface tensions are achieved. A film hydration–relaxation mechanism is proposed to explain these observations.

Keywords: Dynamic cycling; Lung surfactant; Hysteresis; Compressibility; Humidity; Hydration

1. Introduction

1.1. Lung surfactants and the respiratory distress syndrome (RDS)

The concept of “lung surfactants” was introduced by Pattle, who recognized that these surfactants are a complex mixture of phospholipids and proteins (Pattle and Thomas, 1961). According to Pattle and earlier studies by Von Neergaard (1929) the surface tension (γ) of the alveoli should be close to zero to avoid alveolar collapse due to the difference in Laplace pressure (∆Pl) among alveoli of different size. In general, it is considered that surface tensions near 1 mJ/m² or less are reached at the end of the expiration in healthy individuals (Hall et al., 1993; Schürch et al., 1989). Respiratory distress syndrome (RDS) is a clinical condition defined by the onset of poor blood oxygenation due to lung injury, and lack or malfunction of lung surfactant (Petty, 2003). RDS is typically classified into neonatal RDS (nRDS) and acute RDS (ARDS) (Petty, 2003; Notter, 2000; Zuo and Neumann, 2005a).

With the introduction of lung surfactant replacement therapy, the rate of mortality of nRDS patients decreased from 50% to approximately 20% (Notter, 2000). Unfortunately, lung surfactant therapy has not been effective in ARDS patients (~40%), where surfactant inhibition is associated with the pathology (Petty, 2003; Notter, 2000; Taeusch, 2000; Zasadzinski et al., 2001). Thus, the importance of pursuing a deeper understanding of how the surface activity is affected by the composition of the lung surfactant preparation, the presence of various inhibitors, and the role of the gas-phase (i.e. air humidity, carbon dioxide in air, air pollutants, etc.).

Recent studies from our group have shown that the rate of formation of BLES (a lung surfactant preparation) films and the stability of these films under compression are affected by humid air (Zuo et al., 2005b, 2006). Here the effect of humidity on BLES preparations is further explored in dynamic cycling experiments that mimic physiological respiration parameters such as surface area reduction and respiration periodicity.

* Corresponding author at. Tel.: +1 416 946 0742; fax: +1 416 978 8605. E-mail address: acosta@chem-eng.utoronto.ca (E.J. Acosta).
Lung surfactants are produced and recycled by Type II pneumocytes, and they are composed of 85–90% phospholipids, 7–10% proteins, and 4–7% neutral lipids, mostly cholesterol. The phospholipids are composed of phosphatidyl cholines (PC ~80%), phosphatidyl glycerols (PG ~10%), and minor proportions of phosphatidyl inositol (PI), phosphatidyl serine (PS), phosphatidyl ethanolamine (PE), and sphingomyelin (SPH). Dipalmitoyl phosphatidyl choline (DPPC) is the most abundant phospholipid in lung surfactants (Notter, 2000). When DPPC films are compressed, they form a closely packed monolayer at the air–water interface, reducing the surface tension to values of 5 mJ/m² or less (Notter et al., 1980).

Although lung surfactants are complex mixtures, it has been shown that after a series of compression cycles, surfactant molecules that are “less surface active” are squeezed out of the film, thus producing DPPC-rich films that yield lower minimum surface tensions (Notter, 2000; Zuo and Neumann, 2005a; Goerke, 1998). Surfactant proteins also play an important role on the physico-chemical properties of the surfactant film. There are four surfactant-associated proteins: SP-A, SP-B, SP-C, and SP-D (Possmayer, 1988). While SP-A and SP-D are hydrophilic proteins that are negatively charged (at physiological pH), SP-B and SP-C are hydrophobic proteins, positively charged and of smaller molecular weights (Pérez-Gil and Keough, 1998; Possmayer et al., 2001). It has been shown that SP-B and SP-C proteins are essential to maintain the surface activity of lung surfactant preparations, in particular, SP-B deficiency has resulted lethal in mice experiments (Pérez-Gil and Keough, 1998; Possmayer et al., 2001). It has also been shown that SP-A deficiency is not lethal, suggesting that this protein is not essential for adequate lung surfactant activity. However, there is evidence that SP-A improves the effect of SP-B on rapid film formation, and the formation of tubular myelin (Notter, 2000; Possmayer et al., 2001). While the role of SP-A on the surface activity of lung surfactants has not been completely clarified, it is believed that both SP-A and SP-D may play a more important role as immunological “sensors” (Haagsman and Diemel, 2001).

There are four types of exogenous surfactant preparations used in surfactant replacement therapy: synthetic phospholipid mixtures (e.g. Exosurf), extracts from lung tissue (e.g. Survanta, Cuporin), extracts from lung lavages (e.g. BLES, CLSE), and whole surfactant from amniotic fluid (Notter, 2000). In this work, we concentrate on bovine lipid extracted surfactant, and whole surfactant from amniotic fluid (Notter, 2000). In a separate study, air bubbles formed in a 0.5 mg/ml BLES preparation inside a CB device, were compressed once and while the bubble was pre-humidified, the surface tension raised shortly after the bubble was compressed (Zuo et al., 2006). In this study, we investigate the effect of humidity on surfactant activity.

Axisymmetric drop shape analysis (ADSA) is a methodology which has been developed for the in vitro assessment of the surface activity of lung surfactants (Zuo and Neumann, 2005a). The drops or bubbles can be generated using three basic measuring configurations: pendant drop (PD), constrained sessile drop (CSD), and captive bubble (CB).

Conceptually, ADSA determines surface tensions by numerically fitting the shape of experimental drops/bubbles to theoretical Laplacian profiles given by the classical Laplace equation of capillarity. Typical output of ADSA is surface tension, contact angle, drop/bubble superficial area and volume, and curvature of the drop/bubble at the apex. ADSA also features a high processing speed (i.e. 1–2 s/image) (Zuo et al., 2004).

In ADSA—CSD, a sessile drop of the surfactant suspension (4–8 μl drop) is formed on a circular horizontal surface of a pedestal made of stainless-steel (SS316) with a diameter of approximately 3 mm. The pedestal features a sharp-knife edge to prevent the test liquid from spreading beyond the horizontal surface of the pedestal, i.e. preventing film leakage at low surface tensions (Yu et al., 2004). The CSD configuration eliminates film leakage (Goerke and Clements, 1986), and the concentration restriction of the CB configurations (Zuo et al., 2004). Other advantages are: (1) ease of operation; (2) smaller sample volume; (3) good control of environmental conditions (e.g. humidity) (Yu et al., 2004; Zuo and Neumann, 2005a).

During the experiments, the droplet (surfactant suspension) as well as the pedestal is enclosed in an environmental control chamber (see Fig. 1), which allows the control of humidity and gas composition. The volume of the droplet is controlled by a motor-driven syringe. A motor-controller is used to carry out the volume cycling program. At the beginning of each run, a number (typically 4) of drops containing the surfactant suspension are formed on the surface of the pedestal and subsequently suctioned off the pedestal to rinse the surface of the pedestal and purge air out of the system. This rinsing procedure minimizes changes of surfactant concentration during the course of the experiment. After the final drop is formed, the surface tension is tracked for at least 5 min to ensure that the drop reached equilibrium surface tension before commencing the dynamic cycling.

1.3. Effect of humidity on the surface activity of lung surfactants

Recent studies conducted in humid and dry environments of BLES preparations have shown that humidity does play a role in the surface activity of BLES films adsorbed at the air–water interface. In particular it was found that when drops containing 0.5 mg/ml of BLES were produced in humid environments, the adsorption time was close to 3 min, which contrasts with the almost instantaneous adsorption obtained for droplets formed in dry environments (less than 20% RH) (Zuo et al., 2005b). These results have been confirmed by all three ADSA techniques (CSD, CB, PD). Short adsorption times (the time required to achieve equilibrium surface tension) are correlated with low surface tension under compression and overall good performance of the surfactant preparation (Zuo and Neumann, 2005a).

In a separate study, air bubbles formed in a 0.5 mg/ml BLES preparation inside a CB device, were compressed once and while compressed, the surface tension was monitored over the course of 5 min (Zuo et al., 2006). When a bubble of dry air was introduced, the surface tension remained low throughout the experiment, but when the air was pre-humidified, the surface tension raised shortly after the bubble was compressed (Zuo et al., 2006). In this study, we investigate the effect of humidity on adsorption at the air–water interface.
BLES films subject to dynamic compression/expansion cycles that simulate physiological breathing. It should be highlighted that studies of this kind have been scarce because of the difficulty to control the composition of the gas in contact with the surfactant preparation. The development of ADSA–CSD with environmental chamber (Fig. 1) has made possible the study of the surface activity of lung surfactants in environments with controlled gas composition.

Although scarce, there are some previous studies to the ones described above where the effect of environmental factors such as temperature (Goerke and Gonzales, 1981), pH, electrolyte concentration (Scarpelli et al., 1965; Davies et al., 1986; Amirkhanian and Merritt, 1995), humidity (Colacicco et al., 1976; Wildeboer-Venema, 1980), and gas composition (Wildeboer-Venema, 1984) have been studied. Colacicco et al. (1976) first addressed the effect of humidity on the surface activity of DPPC films, concluding that the surface activity of DPPC films was compromised by 100% relative humidity (RH) at 37°C, perhaps due to the hydration of DPPC films. Wildeboer-Venema (1980), after observing similar behavior in lung surfactant extracted from dogs, speculated that the decrease in film stability might be due to the penetration of water molecules through the fatty acid chains of the phospholipid film (i.e. hydration of the tails).

Apparently these findings were ignored because they seemed inconsistent with the fact that lung surfactant films are always in contact with 100% RH air in the lungs, and because it is assumed that in captive bubble surfactometers (CBS) and bubble pressure methodologies the bubble is saturated with water vapor (100% RH). One advantage of CSD methodology over CBS and bubble pressure methods is that it allows accurate control of gas composition such that the effect of humidity, in this case, can be studied. As will be shown latter, the dynamic nature of the compression/expansion cycle is an important factor to consider when interpreting the surface tension results obtained at 100% RH. This work highlights the importance of considering the mechanics of ventilation along with the lung surfactant preparation properties to produce effective lung surfactant replacement therapies.

2. Materials and methods

2.1. Materials

The lung surfactant used in this study is called bovine lipid extract surfactant (BLES; BLES Biochemicals Inc., London, Ont., Canada). BLES is stored frozen in sterilized vials with an initial concentration of 27 mg/ml. This preparation is diluted to 5 and 0.5 mg/ml with a preparation containing 0.6% NaCl and 1.5 mM CaCl₂ on the day of the experiment. The water used in the experiments is demineralized and glass distilled.

2.2. Methods

The CSD experiments used the following protocol: first, to ensure a clean air–water interface prior to adsorption, formation of a sessile drop is completed rapidly within 0.5 s, precisely controlled by a programmable motor-controller (18705/6, Oriel Instru., Stratford, CT). The droplet is then left undisturbed to allow adsorption of the lung surfactant film. Complete film
formation is achieved when surface tension reaches an equilibrium value of approximately 25 mJ/m² (Zuo and Neumann, 2005a). Dynamic cycling is commenced immediately after film formation. This cycling is conducted by periodically adding and withdrawing test liquid into and out of the droplet by means of a motor-driven syringe (2.5 ml, #1002, Gastight, Hamilton Corp., Reno, NY) with periodicity of 3 s/cycle (physiological conditions) and 10 s/cycle (Zuo and Neumann, 2005a). Different compression ratios (CRs), defined as the ratio of the total area reduction upon compression to the initial surface area prior to compression, were tested. About 5%, 10%, and 20% CRs are reported in this study. The system temperature is thermostatically maintained at 37 ± 0.2 °C by a water bath (Model RTE-111, Neslab Instruments Inc., Portsmouth, NH).

A CCD camera (Model 4815-5000, Cohu Corp., Poway, CA) is used to acquire images throughout the experiment at a rate of 30 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 ADSA. The entire experimental set-up, except the computer and water bath, is mounted on a vibration-free table (Technical Manufacturing Corp., Peabody, MA, USA).

The humid air is generated by filling the humidity control reservoir (see Fig. 1) with distilled water 1 h before the experiment to ensure that the chamber becomes saturated with water vapor, i.e. the relative humidity (RH) is ∼100% at 37 °C. To provide a low relative humidity environment (dry air), the humidity control reservoir is filled with anhydrous CaSO₄ to adsorb the moisture from the air contained in the chamber. The relative humidity at this condition is less than 20% RH. A humidity probe Omega RH411 (Omega, Stamford, CT, USA) was used to measure the humidity under these conditions.

Typical output of ADSA includes surface tension, drop/bubble surface area, and drop volume. Using these data, the minimum surface tension during cycling, film surface tension relaxation, and film dilatational elasticity are calculated. Desirable surface properties are low minimum surface tension, low surface tension relaxation, and high dilatational elasticity. All experiments were reproduced at least in triplicate. Fig. 2 shows an example of an ADSA–CSD surface tension–surface area–drop volume output as a function of cycling time.

![Fig. 2. Typical surface tension–area–volume ADSA output for 0.5 mg/ml BLES formulations compressed to a 20% area reduction at 3 and 10 s/cycle periodicity, in humid and dry air.](image-url)
3. Results and discussions

3.1. Dynamic cycling studies

The dynamic surface tension studies were carried out to evaluate the combined effect of four different variables on the surface activity of lung surfactants: air humidity (100% RH air and dry air), lung surfactant concentration (0.5 and 5 mg/ml), dynamic cycling periodicity (3 and 10 s/cycle), and surface area compression ratio (5%, 10%, and 20%). Each of the 24 experiments was repeated at least in triplicate. Average values as well as the standard deviations of the measurements are reported throughout this article.

Typical surface tension–area–volume output from ADSA are presented in Fig. 2 for three different experiments: (A) humid air, 0.5 mg/ml BLES, 3 s/cycle, 20% compression; (B) humid air, 0.5 mg/ml BLES, 10 s/cycle, 20% compression; (C) dry air, 0.5 mg/ml BLES, 10 s/cycle, 20% compression. As will be seen later, these curves summarize some of the most important findings of this work. Fig. 3 shows the surface tension versus cycling time curves of three experiments for the systems introduced in Fig. 2. The reproducibility of the experiments of Fig. 3 is typical of the experiments reported throughout this work.

It is important to recall that during the dynamic cycling studies, it is the volume and not the surface area of the droplet that is controlled with the motor-driven syringe during the compression and expansion cycles. Thus, the maximum and minimum volume achieved during the expansion and compression cycles is the same for all three experiments presented in Fig. 2. In “dry air” experiments, there is some level of drop evaporation, which is reflected in a decrease in the minimum plateau volume in Fig. 2C. The surface area obtained in these CSD experiments is a result of the balance between the change in volume of the droplet and the change of droplet shape due to changes in surface tension.

Although the maximum and minimum volumes of the droplets in Fig. 2 are approximately the same for all three systems, the minimum surface area of Fig. 2B is lower than in A and C, likely as a result of the higher minimum surface tension experienced by this system.

Fig. 2 shows that when the lung surfactant preparation is exposed to dry air, a lower minimum surface tension is attained. By comparing Fig. 2A and B it is also apparent that when BLES films are exposed to humid air, reducing the cycling periodicity (faster cycling) yield lower minimum surface tensions.

The surface tension–area–volume curves in Fig. 2 confirm our previous observations (Zuo et al., 2005b, 2006) that humidity...
affects the surface activity of lung surfactants. In other words, the presence of water vapor in air not only retards the adsorption of the surfactant, but it also prevents the lung surfactant film from attaining low minimum surface tensions. Nonetheless, the mechanism of how humidity affects the properties of the film seems to be a more complex story, since the minimum surface tension also depends on the rate of film compression.

3.2. Effect of humidity and compression periodicity

To show the effect of humidity in more detail, the relative surface area and surface tension versus a normalized time for the systems presented in Fig. 2B and C (humid and dry air systems, respectively) is given in Fig. 4. This normalized cycle time represents the actual time divided by the periodicity of the cycle.

In Fig. 4, the relative area curves for both the system exposed to dry air and the system exposed to humid air coincide for a large part of the compression cycle, but they reach different minimum surface areas. At the end of compression, the lower surface tension in the case of the drop exposed to dry air allows for this drop to be more deformed, i.e. less spherical, and hence since the volumes are the same, the area of this drop is larger that of the drop exposed to humid air. This means that the drop exposed to humid air is slightly more compressed.

The surface tension curves show that in both, humid and dry air, the surface tension during compression are similar up to the point prior when both systems reach a surface tension of 15 mJ/m². After this surface tension, the rate of surface tension reduction is less for the surfactant film exposed to humid air. At the end of the compression stage the surface tension reached for the case of humid air is close to 7–8 mJ/m², while in the case of the dry air the minimum surface tension is close to 2–3 mJ/m². This difference may be significant, since it has been estimated that surface tensions below 5 mJ/m² are required to ensure patient survival (Notter et al., 1980; Notter, 2000). Another important feature of Fig. 4 is that after the compression stage has been completed (normalized time ~0.5), and constant surface area is reached, the surface tension of the systems exposed to humid air relaxes to 15 mJ/m² before the expansion stage begins. The system exposed to dry air showed no relaxation.

Fig. 5 shows the surface area and surface tension versus normalized cycle time for systems exposed to humid air, containing 0.5 mg/ml of BLES, compressed 20% but at different cycle periodicity (3 and 10 s/cycle).

According to Fig. 5, the surface tensions of the lung surfactant films compressed at 3 s/cycle and the film compressed at 10 s/cycle are the same (during the compression cycle) until they reach a surface tension near 15 mJ/m². Beyond that surface tension, the lung surfactant film compressed at 10 s/cycle changes its mechanical properties, becoming more compressible (i.e. more fluid-like). Remarkably the film compressed at 3 s/cycle does not undergo this transition. The lung surfactant film exposed to humid air and compressed at 3 s/cycle seems to emulate the properties of films exposed to dry air.

The transition point at 15 mJ/m² has also been observed in systems containing 5 mg/ml of BLES and compressed in 100% RH and 10 s/cycle. The reason for this transition is still unclear, but maybe related to a transition in the film composition or structure for surface tensions of 15 mJ/m² or less. Alonso et al. (2004) have noted that for surface pressures larger than
50 mJ/m² (or surface tensions lower than 22 mJ/m²) pure liquid condensed phases enriched in saturated lipids (especially DPPC) are formed. Wuestneck et al. (2002) have also found that while pure DPPC spread films can produce surface pressures of 70 mJ/m² when slowly compressed, mixed surfactant films containing DPPC, SP-B, and SP-C cannot reach surface pressures beyond 51 mJ/m² unless they are compressed at high compression speed (equivalent to low cycle periodicity). This is consistent with the observations of Hall and collaborators (Piknova et al., 2002). In particular SP-B was found to accelerate the rate of surface tension relaxation and adsorption (Wuestneck et al., 2002). Based on these observations it would seem that the entrapment of surfactant proteins in the liquid condensed phase may lead to a relaxation phenomena induced by SP-B and SP-C that occur at surface tensions less than 20 mJ/m². Due to the limited number of scenarios evaluated in this study we cannot produce a broad statement that a phase transition occurring at 15 mJ/m² for films exposed to humid air. Later in this work we will use an average rate of surface tension reduction to interpret the correlation between rate of compression and rate of surface tension relaxation.

Under physiological conditions, the periodicity of the respiration is close to 3 s/cycle (Notter, 2000). Thus, the findings of Fig. 5 can explain the apparently contrasting observations that humidity affects the surface activity of lung surfactant films (Zuo et al., 2005b, 2006), and yet at the same time these films are, in fact, exposed to 100% RH air in the lungs (Notter, 2000).

Fig. 6 shows the surface tension–relative area (A/A₀) curves for the systems shown in Fig. 2. Three important parameters are identified in these curves: the minimum surface tension during the cycle, the dilatational elasticity (ε = dy/[d(A/A₀)] = (A/A₀)[dy/d(A/A₀)]), and the surface tension relaxation (rise in surface tension before expansion begins). Higher values of surface elasticity are desirable because, for a given compression ratio, lower surface tensions are attained. Surface tension relaxation gives rise to surface tension hysteresis; this increases the amount of work required to compress and expand the film and reflects the instability of the film (Lu et al., 2002, 2005).

According to Fig. 6, the surfactant film formed in dry air shows the lowest minimum surface tension, lowest film relaxation and the largest compression slope (higher film elasticity). On the other hand, the film exposed to humid air and compressed with a periodicity of 10 s/cycle had the lowest surface activity and the largest film relaxation and hysteresis.

Another important feature of Fig. 6 is that the surface tension–relative area slope (elasticity) changes during the compression/expansion process.

Fig. 7 presents the values of dilatational elasticity during compression as a function of surface tension for 0.5 mg/ml BLES formulations, 20% compression ratio, 3 and 10 s/cycle periodicity, in the presence of humid and dry air.
or compressibility in lung surfactant systems (Zuo et al., 2006; Wuestneck et al., 2002, 2003).

According to Fig. 7, the dilatational elasticity of the lung surfactant films during compression varies with the degree of compression (and thus with surface tension). For surface tensions larger than 10 mJ/m², the elasticity of the film exposed to dry air increases as the surface tension reduces. In contrast to the dry air case, if the lung surfactant film is compressed in the presence of humid air (and at 10 s/cycle periodicity), the dilatational elasticity decreases with decreasing surface tension. The values of dilatational elasticity obtained for humid air are more typical of those values found in the literature for mixed monolayers containing DPPC (between 50 and 100 mJ/m²) (Nanaumi et al., 2000; Miller et al., 1997).

Another significant feature of Fig. 7 is that when the lung surfactant film is compressed in humid air at a shorter periodicity (3 s/cycle), the dilatational elasticity remains almost constant throughout the compression stage. For the system exposed to humid air and compressed at 10 s/cycle the dilatational elasticity is significantly lower, especially at lower surface tensions. Thus, the presence of water molecules in air affects the properties of the compressed film, and this effect seems to be related to the rate of surface tension reduction.

This finding is consistent with the effect of humidity on the rate of surface tension reduction during adsorption reported by Zuo et al. (2005b). Specifically, the time of lung surfactant film formation (complete adsorption) in the presence of humid air was close to 5 min, whereas in the presence of dry air, it was instantaneous. Again, the presence of humid air hinders the rate of surface tension reduction.

### 3.3. The hydration-film fluidization mechanism

The experimental evidence that humidity plays a role in the surface activity of lung surfactants is not completely novel, but has been largely overlooked. The findings of Colacicco et al. (1976) and Wildeboer-Venema (1980) show that the minimum surface tension achievable in the presence of humid air and at 37°C is higher than that of films exposed to dry air. As an example, using the surface tension—percentage compression area data for dipalmitoyl lecithin (DPL) of Colacicco et al. (1976), the dilatational elasticity of films formed in dry air is 57 mJ/m² at 10% compression whereas the dilatational elasticity of films formed in humid air is 34 mJ/m² at the same compression level. Colacicco et al. (1976) also show that if the experiments are run at room temperature, the difference between dry and humid air is not significant. To understand this it is necessary to recall that these surfactant films consist of dipalmitoyl phosphatidyl choline (DPPC), and other anionic phospholipids. Consequently the mixture has a lower gel to liquid crystal transition temperature than the pure DPPC (41.5°C). At 37°C the surfactant monolayer is closer to its transition temperature and thus shows a more fluid-like behavior (Notter, 2000). Under this condition, the packing and orientation of the phospholipid tails exposed to air could be easily disturbed by water vapor present in humid air that could condense in various locations on the surface of the film.

The self-assembled monolayer community has paid closer attention to the effect of ambient conditions on the surface activity of their systems. Chen and Israelachvili (1992) evaluated the effect of humidity and vapors of organic molecules on the swelling of polymer-surfactant self-assembled monolayers, and they proposed that humidity induce a complicated dynamic phenomenon that involves surfactant overturning or “flip-flop” and lateral (two-dimensional) diffusion that evolves into a more fluid-like monolayer. According to their findings, if the surfactant monolayer is composed of ionic surfactants (e.g. phosphatidyl glycerols) or surfactants with “loose” tail packing (e.g. unsaturated phospholipids) film hydration is likely. Dipalmitoyl phosphatidyl glycerol (DPPG) is the second most abundant saturated phospholipid in BLES and this could help explain the apparent “hydration” observed when BLES films are compressed in humid air.

The results of Colacicco and Wildeboer-Venema could be explained by the fact that at 25°C, the film is well below its transition temperature to liquid crystal, and thus the surfactant film is in a “semisolid” state impervious to hydration, as explained by Chen and Israelachvili (1992).

Another important piece of the puzzle is the relationship between surface tension and film hydration. Damodaran (1998) has developed a thermodynamic relationship between water activity in the monolayer (film hydration) and surface tension. Although that equation does not account for the contribution of surfactant adsorption, it does reflect that, in order to achieve larger surface pressures, the activity of water at the interface should be near zero; in other words, the surfactant monolayer should be nearly dehydrated. More importantly, Damodaran validated the trend proposed in his model by showing that water-activated enzymes that adsorb at the air–water interface in the presence of dipalmitoylphosphatidyl ethanolamine (DPPE) become inactive when the surface pressure is larger than 30 mJ/m².

Hall and collaborators have introduced the notion that in lung surfactant preparations that contain mixtures of phospholipids and proteins, low and stable surface tensions are found only when compressing the surfactant film fast enough that an ordered liquid structure with high viscosity is produced (Crane and Hall, 2001; Piknova et al., 2002; Smith et al., 2003). According to Hall’s kinetic model (Yan et al., 2005) it is easy to imagine that any change in the composition of the surfactant mixture that would make the film more fluid may affect the metastability of such films. The observations made about compression periodicity are certainly in line with this concept, and suggest that humidity may be one of those factors that tend to increase the fluidity of the film, probably through a mechanism of hydration. Hydration would certainly introduce disorder into a structured liquid (via flip-flop or lateral diffusion) leading to the relaxation of the film and increase in surface tension.

By combining all these individual pieces of evidence it is possible to propose a mechanism in which the film becomes dehydrated when the phospholipid molecules become more closely packed during compression. If, at the same time, the air surrounding the film is saturated with water, the water molecules in air would tend to re-hydrate the surfactant film. The balance
between these two opposing forces depends on the dynamic characteristics of the film and the compression process. If the film is quickly compressed to a semisolid state, the film will be impervious to hydration effects; otherwise hydration may induce undesirable phospholipid “flip-flop” and lateral diffusion, eventually leading to film fluidization.

3.4. Analysis of combined effects

Thus far the discussion has been centred in three particular cases out of the 24 scenarios evaluated, since these three cases exemplify the most important conclusions of this work. In an effort to summarize the results of all 24 scenarios, Fig. 8 presents the minimum surface tension of all the experiments considered in this work. Fig. 8A shows the minimum surface tension for the systems exposed to humid air, and these are notably higher than the minimum surface tension of the systems exposed to dry air (Fig. 8B). This is consistent with the hydration–fluidization mechanism proposed above. In all cases, by increasing the compression ratio, the minimum surface tension is reduced as predicted by the dilatational elasticity equation. The periodicity of compression (striped bars, 10 s/cycle versus solid bars, 3 s/cycle) does not make a substantial difference with the exception of the systems exposed to humid air and compressed 20%. In this case the compression with a periodicity of 3 s/cycle is the fastest of all compression scenarios, and seems to be fast enough to prevent, in part, the hydration of the surfactant film.

The influence of BLES concentration (white bars, 0.5 mg/ml versus gray bars, 5 mg/ml) on minimum surface tension is significant when the film is slightly compressed (5% compression) in both humid and dry air conditions. This observation is consistent with the fact that, on increasing the concentration of lung surfactant, the rate of film formation (surfactant adsorption) increases and the film is enriched in saturated phospholipids such as DPPC and DPPG (Lu et al., 2003; Schirch et al., 1994). The greater phospholipid content of these films makes them more surface active, resulting in a lower minimum surface tension. At larger compressions this effect is not noticeable because the “squeezing” effect during cyclic compression helps to increase the concentration of saturated phospholipids in the film (Notter, 2000; Zuo and Neumann, 2005a). Another important surface activity parameter is the dilatational elasticity of the film during compression, summarized in Fig. 9. The values reported here correspond to the elasticity calculated using the procedure described for Fig. 7. The values obtained at half compression are reported. This half compression value is equivalent to $A/A_0 = 0.975$ for 5% compression experiments and $A/A_0 = 0.90$ for 20% compression. In systems that experienced film collapse before the end of compression (i.e. 5 mg/ml BLES preparations formed in dry air and compressed 20%), the half compression was taken as the half point (in terms of relative area $A/A_0$) between the beginning of the compression and the $A/A_0$ value when the film collapses.

![Fig. 8. Minimum surface tension of BLES films compressed in 100% RH (A) and in dry air (B). The films were compressed using 5%, 10% and 20% compression ratios; periodicity of 3 and 10 s/cycle; and BLES concentrations of 0.5 and 5 mg/ml.](image)

![Fig. 9. Dilatational elasticity of BLES films at half compression for systems exposed to humid (A) and dry air (B). The films were compressed using 5%, and 20% compression ratios; periodicity is 3 and 10 s/cycle; and BLES concentrations of 0.5 and 5 mg/ml, respectively.](image)
To interpret Fig. 9 it is necessary to keep in mind that larger values of dilatational elasticity are desirable because lower surface tensions can be achieved using less compression (thus less work).

At 5% compression, increasing the surfactant concentration improves the film elasticity, likely due to the larger content of saturated phospholipids in the film. This is consistent with the lower minimum surface tension observed in Fig. 8. The same trend applies to all the systems exposed to dry air.

For all the systems exposed to humid air their dilatational elasticity is lower than the elasticity of the systems exposed to dry air. This observation is consistent with the proposed mechanism of film hydration–fluidization, since a more fluid-like interface can be compressed with a small increase in surface pressure.

Although increasing the surfactant concentration usually improves the elasticity of the film, this trend does not prevail for the case of films compressed 20% in humid air. This observation may be explained by the data presented in Fig. 10 in which 5 mg/ml BLES films compressed 20% in humid air show the largest surface tension relaxation; suggesting that larger surfactant concentrations may not only facilitate film formation but also film relaxation, which is undesirable.

Fig. 10 shows the rate of surface tension relaxation ($r_{relax}$) after the compression stage for selected systems. The $r_{relax}$ value was calculated as the slope ($dγ_{relax}/dt_{relax}$) of the surface tension–time data (fitted to a first order polynomial) during the period of time that the drop remains compressed ($t_{relax}$) at a constant volume. It should be noted that under physiological conditions, alveoli recoil occurs immediately, and thus the film does not remain compressed one-third of the cycle time as it was programmed in these experiments. Although this lag time is essentially an experimental artifact, it is a convenient way to address the film relaxation phenomenon.

The data in Fig. 10 indicate that little or no significant relaxation is experienced by BLES films compressed in dry air. For the systems compressed in humid air, this relaxation rate is significantly larger, especially for those systems compressed by 20%.

The data for the rate of surface tension relaxation at 20% compression in humid air can be interpreted in terms of the film hydration–fluidization hypothesis. Briefly, films exposed to humid air are more susceptible to hydration; the slower the rate of compression (i.e. periodicity of 10 s/cycle) the greater the chance for the relatively slow hydration to take place. The hydration brings about film fluidization, which may then result in film collapse and thus an increase in surface tension (film relaxation).

Another important feature of Fig. 10 is that by increasing the surfactant concentration for films compressed in humid air at 20% compression, the rate of surface tension relaxation also increases. As mentioned earlier, increasing in surfactant concentration improves the rate of film formation (Lu et al., 2003). This observation suggests that increasing the surfactant concentration also accelerates the relaxation process back to the equilibrium surface tension after the compressed film collapses.

Finally, the film hydration–fluidization mechanism suggests that the difference in the surface activity of films compressed under dry and humid air is related to the rate of hydration, and that this rate of surface hydration correlates with the rate of surface tension relaxation. In order to test this postulate, the rate of surface tension reduction was calculated for BLES formulations exposed to humid air, i.e. $r_{wet}$, and dry air, $r_{dry}$. To obtain these rates the surface tension–time data obtained during compression was fitted to a first order polynomial, and the rate was obtained as the first derivative of this linear equation. Although this linear approximation is questionable, it is adequate for the present purpose.

Fig. 11 shows the correlation between $r_{dry} - r_{wet}$ and $r_{relax-wet}$, where $r_{dry}$ and $r_{wet}$ correspond to formulations containing the same BLES concentration, and compressed at the same ratio and periodicity; $r_{relax-wet}$ corresponds to the surface tension relaxation experienced by the film exposed to humid air. It should be noted that in Fig. 11 only the compressions at 5% and 20% were considered (they represent the extreme cases) thus 16 scenarios (corresponding to eight data points) are included.

According to the regression of Fig. 11, $r_{dry} - r_{wet} = 2r_{relax-wet}$, or in other words $r_{dry} - r_{relax-wet} = r_{wet} + r_{relax-wet} = r_{intrinsic}$, where $r_{intrinsic}$ can be interpreted as the rate of surface tension relaxation.
reduction if the surfactant film is in equilibrium with the water vapor present in the gas phase.

Rearranging we have that: $r_{\text{dry}} = r_{\text{intrinsic}} + r_{\text{relax-wet}}$ and $r_{\text{wet}} = r_{\text{intrinsic}} - r_{\text{relax-wet}}$. This mathematical rearrangement suggests that the rate of surface tension reduction during the compression in dry conditions is increased by the dehydration of the film ($+r_{\text{relax}}$), whereas in the case of films exposed to humid air, the rate of surface tension reduction is decreased by the hydration of the film ($-r_{\text{relax}}$).

From the discussion above it can be inferred that, although the uncompressed surfactant film might be in equilibrium with the water vapor at the given temperature (e.g. 37°C), the compressed film is not in equilibrium, and thus susceptible to re-hydration. On the other hand, films exposed to dry air dehydrate as evidenced by the reduction in drop volume observed in Fig. 2.

The dehydration of the films exposed to the dry air and the hydration of the films exposed to humid air explain the contrasting behavior of dilatational elasticity observed in Fig. 7. Following the discussion above, when the film is hydrated, it becomes less elastic, and the opposite happens when is dehydrated. When the compression periodicity is short enough for the hydration effects not to take place ($\sim 3$ s/cycle) the dilatational elasticity remains almost constant.

As a physiological implication, these findings suggest that when using conventional mechanical ventilation (CMV) combined with lung surfactant replacement therapy, the frequency of ventilation is an important variable to consider. Additionally, in adults, the periodicity of respiration could vary from 5 to 25 s/cycle (Roussos and Campbell, 1986), thus in certain cases of ARDS this large periodicity and 100% RH in the lungs may be a culprit in the poor effectiveness of surfactant replacement therapy.

It should also be kept in mind that the observations discussed here for the exogenous lung surfactant preparation (BLES) do not necessarily apply to natural lung surfactants where the presence of SP-A may help compensating the effect of humidity in air. At the same time, this disclaimer does not apply to other surfactant replacement formulations that do not contain SP-A and thus are likely to produce the same response.

In addition to being devoid of SP-A, BLES preparations are almost devoid of cholesterol as well. While some researchers propose that cholesterol only increases fluidity of the film and leads to film instability, recent findings suggest that different species have developed an appropriate level of cholesterol to phospholipid ratio that balances rapid film adsorption, and film stability (Bernardino de la Serda et al., 2004; Orgeig and Daniels, 2001). The 5–10% of cholesterol in natural lung surfactant seems to facilitate the formation of an ordered liquid-phase upon fast compression, more or less cholesterol seem to affect this phase transition (Bernardino de la Serda et al., 2004). In addition to changes in cholesterol content, changes in the ratio of SP-B and SP-C to lecithin may play a role in the sensitivity to moisture. As recalled, Wuestneck et al. (2002) found that while SP-B increased the rate of surfactant re-adsorption after expansion, it also increased the rate of surface tension relaxation. In any case one could expect that changes of protein and cholesterol composition would modify the response of lung surfactants under dynamic cycling.

The low cholesterol content in BLES also brings up the question of bilayer–monolayer transition (e.g. vesicle “unzipping”) to form the film (facilitated by cholesterol) and monolayer-collapse transition (also facilitated by high cholesterol concentration). We have noticed that, indeed rapid bilayer to monolayer transition is a necessary condition to attain low surface tension upon dynamic cycling (Zuo et al., 2005b), and that humid air tends to inhibit this transition. On the other hand, the experiments with 5 mg/ml of BLES reveal that this fast bilayer to monolayer transition is not a sufficient condition, and that in some cases leads to an increase in the fluidity of the monolayer and facilitates the monolayer-collapse transition. Thus, we should expect that the optimum phospholipid, cholesterol, and protein concentration in natural lung surfactant are not set by chance, but as a result of a delicate balance of these transitions, and environmental conditions.

4. Conclusions

The effect of humidity, surfactant concentration, compression ratio, and compression periodicity were evaluated for an exogenous lung surfactant preparation (BLES). The minimum surface tension achieved at the end of compression, film dilatational elasticity and film relaxation were evaluated for these systems. It is concluded that humidity does play a role on the surface activity of exogenous lung surfactant systems, but that the extent of this effect depends on the composition of the surfactant film and the way the compression is carried out. Under physiologically relevant conditions (i.e. 20% compression, 3 s/cycle periodicity, humid air) the effect of humidity is reduced since, according to a film hydration–fluidization mechanism, the rate of film hydration (which leads to poor surface activity) is lower than the rate of film dehydration by compression. Moreover it was found that
although in most cases the increase in surfactant concentration leads to more surface active films, this trend is reversed in humid air since the larger surfactant concentration facilitates the relaxation back to the equilibrium surface tension values.

Complete validation of the proposed mechanism requires further research into the degree of hydration of the film in these various scenarios. From the biomedical point of view, this work suggests that increasing the rate of film compression, it is possible to avoid the problem of film hydration. Finally, it is necessary to recall that natural lung surfactants contain SP-A and up to 10% cholesterol that may help stabilizing the film against hydration. Such effects remain to be studied.

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