Impairing effect of fibrinogen on the mono-/bi-layer form of bovine lung surfactant

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Abstract Lung surfactant (LS), a lipid–protein mixture responsible for alveolar stability, is inhibited by serum proteins leaked into the lungs in disease. Interaction of bovine lipid extract surfactant (BLES), a clinical replacement lung surfactant, with serum protein fibrinogen (Fbg) was studied employing various structural and biophysical techniques in adsorbed films and bulk bilayer dispersions. Surface tension area isotherms of the adsorbed films revealed the suppression of interfacial activity of BLES by Fbg (adsorption and surface tension reduction). Fbg, predominantly associated with the fluid phase of BLES films, resulted in the aggregation of the gel lipid domains as evidenced by atomic force microscopy. BLES bilayer dispersion showed phase transition from a diffused gel to liquid–crystalline phase in the temperature range 10–35 °C as studied by differential scanning calorimetry (DSC). Fbg resulted in the shift of peak to a higher transition temperature for the maximal heat flow ($T_{\text{max}}$) of BLES dispersions. Combined Raman and FTIR spectral studies of the BLES/Fbg dispersions revealed that Fbg altered the $-\text{CH}_2-$, $-\text{CH}_3$, and $-\text{PO}_4^-$ vibrational modes of the phospholipids present in BLES, suggesting the condensing and dehydrating effect of the protein on surfactant. Studies suggest that Fbg, by directly interacting with the gel lipids in LS in bulk dispersions, alter the packing of the films formed at the interface, and can be used as a specific model for lung disease.

Keywords BLES · Fbg · AFM · DSC · FTIR · Raman

Introduction

Lung surfactant (LS), responsible for nor airway patency, is inhibited by serum proteins leaked into the lungs in diseases such as acute respiratory distress syndrome (ARDS) [1]. Previous studies performed using different serum proteins on synthetic or semi-synthetic LS suggest most serum proteins were non-specific in their interactions with these surfactants. Bovine lipid extract surfactant (BLES$^\text{TM}$) is a clinically used natural LS preparation obtained from bovine lung lavage (washings) [2]. BLES, developed in Canada, is currently being used for the treatment of neonatal and acute respiratory distress syndrome (ARDS) [3]. BLES contains all the phospholipids and proteins commonly present in most mammalian LS, however, the hydrophilic SP-A, SP-D, and the neutral lipids (cholesterol) are removed during extraction to minimize immune reactions and better surface activity [3].

There are a number of previous reports involving in vitro studies on the surfactant inhibition by serum proteins and the possible mechanism involved in such inhibitions can be used as models for ARDS [4–9]. Some of these studies explored the effects of change of ions, pH, and temperature fluxes, as well as serum protein levels in the dysfunction and inhibition of
various surfactant preparations [5]. Many serum proteins such as albumin [6], fibrinogen (Fbg) [7–12], C-reactive protein (CRP), and globulin [2, 5], etc., have been found to inhibit LS function, by preventing the reduction of surface activity of the lipid–protein films [10–15]. However, in most of the previous studies, protein concentration was varied in the non-physiological or non-pathological range and various synthetic surfactant preparations were used [5]. A generalized correlation could not be achieved from the combined results. In a recent study by Paviniato et al. [12], it has been reported that the hydrophobic macromolecular entities can affect the packing of both the hydrocarbon chain as well as the head group of the phospholipids in LS, at very high amounts. Another previous study by Panda et al. [4] on injured surfactant from rat lungs revealed that serum proteins inhibit LS functions even at very low concentration.

Fibrinogen (also called serum Fbg, plasma Fbg, and factor I) is the main protein of the blood coagulation system, produced by the parenchymal cells of the liver. Previously, the influence of Fbg or other serum proteins on LS have been performed using extremely high physiological concentrations [2, 7–11, 15]. Some reports are also available where lower path-physiological amounts of serum proteins were used; however, the obtained results failed to show any major effects on LS dysfunction [5, 16]. Those studies revealed that Fbg and CRP have greater inhibitory effects than albumin, when used at high amounts; however, at low amounts, the results were inconclusive [15, 17]. A recent study also suggests that Fbg in low amount may actually have a protective role in surfactant recycling [18]. A number of these previous studies are difficult to compare and contrast, since different surface tension measuring devices and synthetic surfactants were used, films were made by solvent spreading, the bulk-phase dispersions were not studied, as well as no structural information were available on such systems.

The present study attempts to determine the interaction of LS with Fbg at path-physiological concentration as well as at higher concentration, in monolayer films and bilayer dispersion. The dispersions were used to adsorb such films of a well-defined extracted bovine lung surfactant (BLES), and the films were studied structurally. Different complementary techniques, viz., interfacial adsorption, dynamic compression–expansion isotherms, Langmuir–Blodgett film imaging by atomic force microscopy (AFM) were undertaken to understand the interaction of Fbg with BLES monolayers, as previously done for another serum protein albumin [6]. Raman, FTIR spectroscopy, and differential scanning calorimetry (DSC) studies yielded correlated information on the interaction of Fbg with BLES bilayers. These correlated studies suggest specific alteration of LS lipid packing in bulk bilayers and films, very different than those obtained previously with albumin by us [6], and some of the studies on Fbg by others.

Materials and methods

Materials

Bovine lipid extract surfactant (BLES™) was obtained as a 5-mL vial of 27 mg mL⁻¹ suspension in saline from BLES Biochemicals Inc., London, ON, Canada. The phospholipid DPPC (1, 2-dipalmitoyl-sn-glycero-3-phosphatidylcholine), fibrinogen (fraction-1; type 1 from human serum containing 90 % clottable protein) in dry form, de-lipidated bovine serum albumin (BSA) (protease free, fraction V, 99 %) in powdered form, and Trizma. HCl were obtained from Sigma Chemical Co. (St. Louis, MO, USA). HPLC-grade chloroform, methanol, and AR-grade sodium chloride were obtained from Fisher Scientific (Ottawa, Ontario, Canada). All the experiments were carried out in NaCl-Trizma-HCl buffer, pH 7.0, unless otherwise stated. Glassware used in the monolayer experiments was all chromo-sulfuric acid washed and rinsed thoroughly in distilled water, and dried at 180 °C for 2 h prior to use. This was done to remove all organic and surface-active impurities from the glassware as previously discussed elsewhere [19].

The buffer used in the study was prepared by adding 150-mM NaCl and 5-mM Trizma hydrochloride to 1 L of double-distilled water, mixed well, and the pH was adjusted to 7.0 using 0.1-M NaOH. In some studies on Langmuir-Blodgett film deposits for AFM imaging, doubly distilled water was used as subphase, since salt crystallization affected the film structure [17].

Methods

For all the studies described in this paper, viz., monolayer adsorption, FTIR, Raman, DSC, and AFM. Fbg used was dissolved in the saline buffer at a concentration of 10 mg mL⁻¹ (the maximum solubility of fibrinogen in saline). The dissolved fibrinogen was then mixed with BLES after appropriate dilution at (0.1:1 to 5:1, Fbg/BLES, w/v). These smaller amounts of Fbg, added to BLES, were physiologically/pathologically relevant to what was observed in injured lungs for soluble proteins [4].

Preparation of DPPC vesicles

Quantitative amount of DPPC was taken in a small round bottomed flask, and 500 μL of chloroform–methanol mixture (3:1, v/v) was added to it. The contents in the flask were vacuum evaporated using a rotary evaporator for 15 min at 37 °C to remove the solvents. Traces of the solvent were removed by drying under nitrogen followed by desiccating overnight. The required volume of buffer was added to the flask and vigorously vortexed for 5 min at 46 °C. This temperature was chosen since it is above the chain melting
temperature of DPPC (41 °C). Under such conditions multilamellar vesicles are formed as previously reported [20, 21].

Adsorption isotherms

Change in the surface tension values with time (adsorption isotherm) were carried out in a 6.28-mL small cylindrical teflon cup. Measurements were made at controlled room temperature (23±1 °C) with a 0.6-mL subphase of NaCl-Trizma-HCl buffer at pH 7.0, which was stirred continuously to minimize diffusion resistance. Adsorption experiments were initiated by injecting 400 μL of BLES or BLES-Fbg dispersions to the clean surface. stirred, surfactant-free subphase to give a final volume of 6 mL. Adsorption to the surface (determined by surface tension drop) was then measured as a function of time in seconds, using a Wilhelmy plate as discussed in detail in previous studies [6, 21].

Surface balance

A modified Langmuir-Wilhelmy surface balance (Langmuir Mini Trough. Applied Imaging. UK) with a teflon ribbon barrier was employed, the design and construction has been described previously [22]. The dimension of the teflon trough gave a surface area of approximately 500 cm², which was considered as 100 % of the monolayer area for the isotherms. Surface tension as function of the monolayer surface or pool area was used in all studies, because accurate area per molecule information could not be calculated for adsorbed films. A roughened Wilhelmy platinum dipping plate was used to measure the surface tension [22, 23] and a motorized teflon barrier operated by a forward-reverse switch located on the instrument was used to compress and expand the monolayer.

Initially, NaCl-Trizma-HCl buffer, and pH 7.0 was used as the subphase representing an air-buffer interface and having a surface tension of ~72 mN m⁻¹. BLES dispersed in buffer containing either no Fbg (control) or varying amount of Fbg was injected using a Hamilton syringe just under the air-water interface of the subphase. One hour was allowed for adsorption and equilibration of the film. After equilibration, compression and expansion of the film by and the isotherm was obtained at a controlled room temperature (23±1 °C). By compressing and expanding the monolayer, transition of the surfactant from fluid to condensed (gel-like) phase could be initiated and monitored through the inflection in isotherms [6, 20, 23, 24].

AFM

Adsorbed monolayer of BLES, with or without Fbg, was deposited onto freshly cleaved mica substrate by way of Langmuir-Blodgett deposition technique. After the attainment of desired surface tension, the film was allowed to equilibrate for 10 min. Pre-immersed mica substrate was then lifted with an upstroke of 0.22 mm/s [6, 25]. The Langmuir-Blodgett deposition on mica was mounted on the magnetic steel disk of the AFM scanner (Scanner J) where the samples were imaged within 1 h of deposition by a Nanoscope® scanning probe microscope (Veeco Instruments. Nanoscope IIIa) in contact mode [23]. Contact mode imaging was preferred since the small height differences between gel-fluid boundaries were not prominent in the tapping mode as discussed elsewhere [6, 20, 23, 25]. Images of the samples were flattened using the Nanoscope IIIa software and analyzed to determine the height differences (by section analyses) between the observed domains [23, 25].

DSC

DSC studies on the samples were carried out in the temperature range 10–45 °C using a Mac-2 differential scanning calorimeter (Serial no. 025, MicroCal. LLC Inc., Northampton, MA, USA) [26]. This range was chosen so as to include the main transition in DPPC, which occurs at 41 °C and to avoid thermal denaturation of Fbg, which occurs at 50 °C [27, 28]. Scanning rate was 30 °C h⁻¹ throughout all the DSC experiments. A total of three scans were recorded for each sample, with a 60-min break in between for the phospholipid to cool back to the starting temperature of 10 °C [28]. The scans obtained were later baseline normalized (to kcal mole⁻¹ of phospholipids) using the MicroCal™ Origin. The second or third scan out of the three cycles have been shown in the data, as they are considered to be more refined and thus, more representative, as well these processes were reversible [28]. There were no appreciable differences between the third, fourth, and fifth cycles out of the five heating and cooling curves of each sample with Tₘₐₓ or enthalpy change, and thus, the data is displayed without averaging or error bars.

FTIR spectroscopy

FTIR measurements were carried out with a Bruker Tensor 27 Infrared Spectrometer (Bruker, Billerica, MA, USA). This instrument is equipped with a MIRacle attenuated total reflection (ATR) accessory allowing rapid and easy Fourier transform analysis of liquid and solid samples [23]. A demountable FTIR liquid cell (Pike Technologies. Madison, WI, USA) was used. Initially, the two disk-shaped window materials (made up of CaF₂) were sealed in place using the aluminum needle plate and Teflon alignment posts as guides. The sealed assembly was placed in a demountable liquid cell holder, and sample dispersion was then injected in-between the liquid cell window. Absorbance value of the background was subtracted from the corresponding numbers of the sample containing buffer alone. The spectrometer had 0.001 cm⁻¹ resolution.
Raman spectroscopy

Raman spectral measurements were obtained using a LabRAM confocal microscope (Horiba Jobin Yvon Inc., Edison, NJ, USA) with a grating (1800 grooves/mm), a Leica microscope equipped with a long-working distance objective with a magnification factor of ×50, and a Peltier CCD detector. The spectra were recorded by using the 532-nm green laser (Ar ion laser) line for excitation. The D0 filter (no attenuation) and acquisition times of 30 s were used in acquiring the spectra. Spectra were also obtained from 15-min to 2-h acquisition time without any major changes observed from the 30-s spectra, with a spectral resolution as used for FTIR [6, 23]. However, the short time period used in collecting the spectra was chosen to avoid drying effect, especially during heating scans, as the samples were placed in an open microscope cuvette with a heating-cooling chamber [6, 20].

Results

Adsorption and monolayer isotherm studies

Adsorption over time for BLES dispersion in combination with Fbg are shown in Fig. 1 (I) and the compression-expansion isotherms of such adsorbed films are shown in Fig. 1 (II). Pure BLES dispersion attained a surface tension (γ) value of 30 mN m⁻¹ (near the equilibrium γ, 25 mN m⁻¹) within 600 s. In case of pure Fbg, the surface tension reached the value of 50 mN m⁻¹ after 600 s. In case of BLES + Fbg mixtures, the decrease in γ was suppressed by Fbg in the same time period and this suppression effect was strongest in case of 1:10 (w/w) BLES: Fbg. At equilibrium, γ around 50–55 mN m⁻¹ for BLES + Fbg mixtures were significantly higher than the equilibrium γ of 30 mN m⁻¹ for pure BLES. Results suggest that Fbg minimally inhibited BLES adsorption onto an air-water interface.

Figure 1 (II) compares multiple compression-expansion cycles of the adsorbed films of (A) BLES and (B) BLES + Fbg mixtures (1:1, w/w). Compression-expansion cycles were carried out at a speed of 7.3 cm² s⁻¹. The amount of added BLES to the subphase (1.62 mg) was kept constant. Pure BLES films showed a reduction of γ to as low as ~1 mN m⁻¹, upon complete compression and in combination with Fbg, as in (B), the surface tension was only reduced to 27 mN m⁻¹. This suggests that the protein interfered with the packing of the lipid films which subsequently prevented the attainment of low surface tension value.

Change in the compressibility of BLES films with increasing amount of Fbg (in w% with respect to BLES) is shown in the bar graph [Fig. 1 (III)]. Film compressibility is defined as the amount of films pool or surface area reduction at a fixed surface tension (15 mN m⁻¹) drop, by the intercepts shown in (B) [6]. The films are considered to be less compressible with larger area compression required for fixed surface tension reduction [6]. The percent change in pool area increased with increasing Fbg in BLES: Fbg mixtures (1:0, 1:0.5, 1:1, 1:10 and 0:1, w/w). This trend was observed for cycle 2 (shown in blue) and cycle 5 (green) as shown in Fig. 1 (III).

Atomic force microscopy (AFM)

Film morphology of the samples used in monolayer study was also examined by AFM imaging. Representative AFM images of BLES in combination with different amount of Fbg are shown in Figs. 2 and 3. It was observed that the films experienced transitions from liquid expanded state (fluid) to liquid condensed state (gel-like) upon compression [23, 25, 29]. The liquid condensed (LC) or gel-like domains (formed when films were compressed to γ=52 mN m⁻¹) of BLES in the image are observed as bright (greater height) nearly circular domains. This was due to a more perpendicular tilt of the lipid molecules in the LC phase compared to the plane of the film and the surrounding (more isotropic) liquid expanded phase (LE) or fluid-like phase of lower height [23, 24]. These LC domains in BLES exhibited approximately a height difference of 1.5 nm (15 Å) as determined by the section analysis of representative images (Fig. 3a). Pure Fbg film images unlike BLES, showed no LC domains or appreciable features but rather a more homogenous phase (Fig. 2c). All images of pure protein films showed small bright regions of aggregated proteins against a smooth background. All pure-protein images also showed horizontal trails of tip scanning, possibly caused by contact of the tip with the protein aggregates or possibly by the tip picking up such aggregates during scanning.

In Fig. 2a, the BLES condensed domains were observed as clear and distinct structures, more pronounced in the three dimensional images and section analysis shown in Fig. 3. Section analyses of these condensed domains showed that they were ~1.5 nm above the surrounding fluid phase. In BLES: Fbg (1:1, w/w) mixtures (Fig. 2b), the BLES domains (formed at a γ=52 mN m⁻¹) were still intact and distinct with a slight reduction in height from 1.5 nm (as seen in BLES alone, Fig. 3a) to 1.1 nm (Fig. 3b). Other structures seen in the image as a continuous stream of peaks (height of the peaks ~1.2 nm) surrounding the BLES gel domains may be fibronogen or its aggregates, suggesting the protein has propensity to act on the boundaries of the gel-fluid lipids. These small height differences are significant, since a number of previous AFM studies have shown that the proteins act on the gel–fluid phase boundaries either dissolving such boundaries reducing the
height \([4, 6, 20, 23-25]\) or increasing the tilt of gel lipids perpendicularly by further condensing them as seen in this study. In the BLES/Fbg films (Fig. 2c, d), ribbon-like structures of the protein aggregating the gel domains were observed, suggesting the fact that although the protein was mainly present in the fluid phase, some aggregate adhered to the gel-fluid boundary, and thus further caused the gel domains to form a lipid-protein network.

Differential scanning calorimetry (DSC)

DSC heating endotherms of multilamellar dispersions of DPPC alone (A) and with Fbg (1:10, w/w) (B) are shown in Fig. 4. Figure 4a illustrates the typical sharp phase transition of DPPC at 41 °C for all the three cycles. With DPPC/Fbg at 1:10 (w/w) (Fig. 4b) the transition was slightly broadened. While considering the heating cycle effect, it was observed that the main transition peaks observed in cycles 2 and 3 were split, the second peak observed to a slightly higher temperature (42 °C). Results suggest that Fbg could further condense some gel lipids in the bilayers or can induce two separate gel phases in such bilayers.

Figure S1 shows the first scan of the heating-cooling endotherms of BLES and Fbg separately over a temperature range of 10 to 70 °C. BLES showed a broad transition peak at 28 °C and Fbg denaturation peak was observed at 50 °C [27]. The three consecutive heating scans of BLES were found to be reproducible with all the scans showing a broad transition at 28 °C. This suggested that the gel to liquid-crystalline transition in BLES was reversible. However, the melting or denaturation peak of pure Fbg was not reversible. This was revealed through the appearance of flat line in scans 2 and 3 in the DSC.

Figure 5 shows the melting behavior for BLES and with various amounts of Fbg. Although the samples were scanned for five cycles, however, the third scan is shown for clarity. The thermograms were found to be reversible, no significant hysteresis was noted. BLES alone displayed a broad transition and a \(T_{max}\) of 28 °C, which shifted to higher values (34 °C) with increasing amount of Fbg in BLES. With 1:0.1 [Fig. 5, curve b] and 1:0.5 (w/w) [curve c] BLES + Fbg mixtures, the phase transition was delayed only by 1 °C. On the other hand the \(T_{max}\) increased to 34 °C with added Fbg (1:1) [curve d] and 35 °C with 1:1.4 (Fig. 5e). This shift in \(T_{max}\) of BLES with increasing amount of Fbg clearly revealed that Fbg interacted with BLES gel lipids by condensing them further or only allowing them to melt at significantly higher temperature.

Vibrational spectroscopy: FTIR and raman studies

Both the complete FTIR and Raman spectra of BLES dispersions are shown in Fig. 6. Such studies were carried out in
order to understand the impact of Fbg on the packing of the hydrocarbon chain as well as the head group of the phospholipids (phosphate) present in BLES (supplementary section). Spectra of BLES dispersions were also recorded in presence of bovine serum albumin in order to make comparison. The different vibrational modes include symmetric and asymmetric (a) methyl group (terminal hydrocarbon chain), (b) methylene group, (c) C–C stretching of hydrocarbon backbone, and (d) the phosphate head group [as shown in the Supplementary section Fig. S2–S7]. Raman spectra were recorded in the temperature range 10–45 °C in order to understand the chain melting in the bilayer [29–33]. Symmetric and asymmetric stretching of the methylene/methyl groups (appearing in the region 2,800–3,000 cm$^{-1}$) are shown in Figure S2 (Supplementary section). Peaks at 2,850 and 2,920 cm$^{-1}$ were exhibited by BLES which correspond to the symmetric and asymmetric stretching for the methylene group, respectively. With increasing amount of added Fbg, the peaks became broadened with a mildly shift in both cases.
the effect of BSA, it was noticed that although BSA resulted in broadening of the peaks, however, no significant shift in peak positions occurred (Figure S2, panel b). Besides, the effect was insignificant in the low concentration range of BSA [6].

According to the report of Arrondo et al. [30], the peak appearing at 1,224 cm\textsuperscript{-1} corresponds to the characteristic asymmetric stretching of the phosphate head group of phospholipid molecules. Effects of Fbg and BSA on such vibrational modes have been graphically presented in Figure S3 (Supplementary section). With added Fbg to BLES dispersion, the peak was shifted to lower frequency. Unlike Fbg, BSA resulted in an upshift in the peak position.

While considering the effect of temperature (as shown in Figure S4, Supplementary section) it was observed that apart from broadening, the peak appearing at 2,847 cm\textsuperscript{-1} (assigned as the symmetric stretching frequency) was upshifted with increasing temperature. While the peak intensity at 1.062 (representative vibration of the extended all-trans configuration) receded continuously with increasing temperature, intensity of the peak at 1.091 cm\textsuperscript{-1} (assigned to the random lipid-like configuration) showed a reverse trend (as shown in Figure S5, Supplementary section). Other two characteristic features of the BLES dispersions include: (a) the lipidic CH\textsubscript{2} band at 2,886 cm\textsuperscript{-1} and the band at 2,937 cm\textsuperscript{-1} for the terminal methyl group. It was noted that Fbg resulted in the broadening of the 2,886 cm\textsuperscript{-1} band and at the same time, the 2,937 cm\textsuperscript{-1} peak intensity increased. The effects were almost similar at different temperatures (the results are shown in Figure S6). It may be noted that due to the limited solubility of Fbg, effects of higher amount of Fbg could not be studied. A clear variation was noted from the \(I_{2937}/I_{2886}\) vs. temperature profile, as shown in Figure S7.
Discussion

The small inhibition of the surface tension lowering capacity of BLES by Fbg can be explained possibly by the high interfacial activity of serum protein. Fbg, being partially hydrophobic, can occupy the surface through competitive adsorption with BLES lipids. Thus, Fbg can prevent adsorbed BLES molecules from forming a compact monolayer, which does not reduce surface tension to low values upon compression. With increasing Fbg content, BLES adsorption slightly decreased and longer time was necessary to reach equilibrium surface tension. The high surface activity of Fbg could be seen in the rapid adsorption of the pure protein to low surface tension (Fig. 1a), and with BLES lipids, slightly increase the time required to reach equilibrium surface tension. Similar trends of adsorption inhibition were observed in previous studies with major serum proteins [2, 5, 6, 34].

Previous studies with different soluble serum proteins also suggest that adsorption surface activity of various LS preparations are somewhat inhibited in a concentration-dependent mechanism [2, 5, 6]. Although different techniques were used from ours, the change of concentration of Fbg from 1.0.5 to 1:1 (BLES/Fbg) did not show any significant difference (Fig. 1a). This could be either due to the lack of sensitivity of the adsorption apparatus, or some other mechanisms at play, which did not discriminate between low and high amounts of proteins. However, all such adsorbed films containing Fbg showed significant changes in compressibility and well as lowering of surface tension in a concentration dependent manner (Fig. 1c).

Compression–expansion isotherms of such films of BLES/Fbg (1:1, w/v) suggest that Fbg did not allow the lipids in LS films to reach very low surface tension. Normally, when LS monolayer is compressed, the fluid lipids from the films are presumed to get squeezed out or transformed into the gel lipids resulting in the attainment of low surface tension [24, 33]. Fbg either directly or indirectly interacts with these gel lipids and/or alters their packing somehow, so that they cannot reach low γ. This suggests that the main effect of the protein is on the BLES lipids and on the interface rather than on adsorption.

However, the AFM images (Fig. 2) did not show major disruption of gel domains at least in the low concentration range of Fbg. Higher amount Fbg probably prevents the BLES gel lipid adsorption to the monolayer (at least the layer of lipids in the bilayers in contact with Fbg), and compression of such (more fluid lipid) monolayer does not allow the attainment of lower surface tension. Also, the difficulty in compressing the BLES films with an increase in the amount of Fbg in such films suggests that Fbg may be competing for space with BLES lipids at the surface proportional to their concentration in the subphase. As in the case of pure Fbg films [Fig. 1, panel III], the area compressibility of the protein was seen to be minimal, and in such films, γ minimizes at 30 mN m⁻¹ even at maximal compression. This suggests that if fibrinogen occupies a large area in the BLES films, these films would behave more like the pure protein systems rather than those of the surfactant lipids. A related possibility is that if Fbg binds to the solid (gel) lipids of BLES in the bulk phase, the monolayer films formed from such aggregated material may not be able to reduce γ as efficiently. However, in either case, the initial distribution of pure fibrinogen into pure protein films and its high adsorption rate may be a critical factor in inhibiting LS activity.

AFM is emerging as a promising tool for investigating the topography or structures of soft biological materials [35], as well as interaction of soluble proteins with LS [6, 25, 33]. To the best of our knowledge, only a single study has employed AFM to image structures in natural dysfunctional LS film formed from extraction of the such material from injured rat lungs [4]. In that study, it was observed that the dysfunctional LS films had dramatic re-distribution of the gel–fluid domains, with significant changes between the gel–fluid domain heights. In the present study, Fbg was seen to be inserted in the lipids in fluid phase while also somewhat aggregating the gel domains (Figs. 2 and 3). Pure BLES monolayer could attain
surface tension to as low as ~1 mN m⁻¹, whereas in the presence of Fbg, at all the amounts studied, BLES could not attain such low values [Fig. 1, panel III]. The AFM images suggest that Fbg not only penetrates into the fluid phase of the films but also, at higher concentration, abolishes any remnants of gel phase. It is possible that such mixed BLES films are mostly made of pure Fbg (homogenous AFM features as seen in Fig. 2d) since the gel phase was significantly abolished to a more fluid-like system. However, due to the hydrophobicity of the protein, some aggregation of the protein into ribbon-like structures may, suggest a different redistribution of the proteins in contact with one or more subset of BLES lipids.

Fbg also induced two sets of domains, one with the gel lipids and the other with fluid lipids as observed by AFM at low concentration. This could probably explain the broadening and the splitting of the DPPC peak appearing at 41 °C (Fig. 4a) in the presence of Fbg as seen in the DSC studies. The protein probably interacts with a certain number of DPPC molecules and these Fbg-DPPC complexes require melting at a slightly higher temperature. However, while the DSC of BLES with Fbg did not show any major peaks, the main transition peak \( T_{\text{m}} \) was shifted to slightly higher temperatures (Fig. 5). In case of BLES (unlike DPPC), Fbg probably has a cumulative effect on its overall transition or interaction with the gel and fluid lipids additively in BLES. This interaction can be seen in the AFM images where Fbg occupied the fluid phase but aggregated the condensed gel domains (Fig. 2c, d).

The split in the transition peaks of DPPC with Fbg could be due to segregation of certain fraction of DPPC molecules by Fbg in the outer layers of multilamellar vesicles, which melt at high temperature. The other population of DPPC in the inner layers were not affected and thus showed the transition peak of the pure lipid. Such an assumption was based on the fact that when Fbg was added to the MLV dispersions after the preparation of the vesicles were complete, as appropriate to those events in the lung, where lamellar bodies come in contact with serum materials only in the outside bilayer [1, 2, 20]. From this observation, one can speculate that Fbg has a slight chain ordering effect by aggregating a certain fraction of DPPC molecules, in LS. However, in case of BLES due to the presence of large fluid–gel phase separation, Fbg affected the melting temperature significantly, probably by acting on the domain boundaries [24]. A recent study suggests that small amounts of fibrinogen may, in fact, have a protective effect on surfactant lipids during film cycling [18]. Other studies have suggested that at very high surfactant lipid concentrations, Fbg is not as effective in disrupting preformed LS films [11].

In the FTIR and Raman studies, the peak intensities and the wavenumber shifts of various bands in BLES alone and some studies on BLES + Fbg or with bovine serum albumin were performed [Supplementary data S2–S7]. The regions of interest were mainly the asymmetric and symmetric bands in the C–H stretching region (2,800–3,000 cm⁻¹) and the asymmetric and symmetric phosphate bands appearing at 1,224 cm⁻¹ and 1,087 cm⁻¹, respectively, of the head group region (1,000–1,500 cm⁻¹) [30–32, 36–39]. In previous spectroscopic studies on lipid mixtures, it was noted that these peaks, especially the 2,800–3,000 cm⁻¹ range are most susceptible to changes in temperature and additives [39]. In the present study, the C–H stretching modes were all shifted to a higher wave number in the presence of Fbg, suggesting a condensing effect of the protein on BLES lipids, consistent with the DSC data.

A previous Raman study had also shown Fbg can affect the head group region of the phospholipid dimyristoylphosphatidylcholine (DMPC) and makes them more polar [30]. The phosphate stretching bands in BLES shifted to a lower frequency with Fbg indicating that the environment surrounding the head group region of BLES phospholipids was more hydrated [Supplementary data Fig. S7]. This was in consensus with others [30] who observed the asymmetric phosphate band appeared at 1,220 cm⁻¹ with hydrated DPPC, and 1,240 cm⁻¹ with DPPC in “dry” state. They concluded that the phosphate in the head of the DPPC forms more hydrogen bonds with the surrounding water molecules in its fully hydrated state (wave number of 1,220 cm⁻¹ being monitored.) Fbg is also negatively charged at pH 7 [36]. Whether Fbg further hydrates the head group region by forming more hydrogen bonds with water, or binds to some specific groups (positively charged choline) of BLES phospholipids, eventually enhancing the H-bonding network, both possibilities can explain our Raman data.

Further studies are required probably to explore this somewhat unique condensing effect of Fbg on surfactant lipids, compared to other serum proteins as well as native serum. However there should be systematic studies on the changes of molecular conformational order of the surfactant in the bilayer as well as adsorbed films made from such bilayer, as well as take into account the presence or absence of surfactant proteins.

**Summary and conclusions**

As a model for ARDS, this study suggests that Fbg, a serum protein uniquely affects the structure–function properties of lung surfactant as studied in vitro. Bilayers of surfactant were affected by Fbg specifically interacting with the gel phospholipids, as seen by a condensing effect in DSC and Raman/FTIR studies. Adsorbed films formed from such surfactant bilayers with Fbg showed re-distribution of the gel–fluid domains, as well as the protein probably aggregating such domains at high concentrations. Future studies are underway.
to further understand these effects including whole serum, specific surfactant proteins, and means to reverse such inhibitions by polymers and other agents.

References