Chapter 1

Effect of Serum, Cholesterol and Low Density Lipoprotein on the Functionality and Structure of Lung Surfactant Films

Abstract: Lung surfactant is a complex mixture of lipid and protein, responsible for alveolar stability, becomes dysfunctional due to alteration of its structure and function by leaked serum materials in disease. Serum proteins, cholesterol and low density lipoprotein (LDL) were studied with bovine lipid extract surfactant (BLES) using Langmuir films, and bilayer dispersions using Raman spectroscopy. While small amount of cholesterol (10 wt %) and LDL did not significantly affect the adsorption and surface tension lowering properties of BLES. However serum lipids, whole serum as well as higher amounts of cholesterol, and LDL dramatically altered the surface properties of BLES films, as well as gel-fluid structures formed in such films observed using atomic force microscopy (AFM). Raman-spectroscopic studies revealed that serum proteins, LDL and excess cholesterol had fluidizing effects on BLES bilayers dispersion, monitored from the changes in hydrocarbon vibrational modes during gel-fluid thermal phase transitions. This study clearly suggests that pathophysiological amounts of serum lipids (and not proteins) significantly alter the molecular arrangement of surfactant in films and bilayers, and can be used to model lung disease.

J. Oleo Sci. 63, *(12) 1333-1349 (2014)*

1. Introduction

Pulmonary or lung surfactant（LS）is secreted by the alveolar type II epithelial cells[1]. From its secreted bilayer form（lamellar bodies）LS transforms into tubular myelin and gets adsorbed at the air – liquid interface to form a tightly packed monomolecular film. The film reduces the surface tension to low value as

the area of the alveolar interface decreases upon expiration. The monomolecular film becomes compressed to near 0 mN/m value and this prevents alveolar collapse and maintains air - way patency. Lipids are the main components of pulmonary surfactant. Among them, dipalmitoylphosphatidylcholine（DPPC）and phosphatidylglycerol（DPPG）are the most characterized and abundant phospholipids. While DPPC（40 - 50 wt％ of the lipid pool）is responsible for maintaining the surface tension near zero during compression, presence of other fluid lipids are also essential in order to re-spread the com-pressed film easily[2,3]. Beside the phospholipids, there are four surfactant associated proteins, SP - A, SP - B, SP - C and SP - D. While SP - A and SP - D are hydrophilic, the other two surfactant proteins（SP - B and SP - C）are hydrophobic and attenuates the surface tension of LS[4,5]. The most abundant neutral lipid in pulmonary surfactant is cholesterol, amounting to 5 - 10 wt％(or 10 - 20 mol％)of the phospholipids[6]. Even though the presence of cholesterol in surfactant has long been recognized, however, little is known about its function[2,6,7]. Some studies suggests that in the presence of the hydrophobic surfactant proteins cholesterol is essential for fluidity control[6], surface tension reduction and adsorption of other phospholipids onto the monolayer[8-10]. However previous studies also suggest that excess cholesterol also has deleterious effects, *e.g.*, it retards the achievement of minimum surface tension to near zero value during complete film compression and decrease of post collapse re-spreading[11].

In adult respiratory distress syndrome（ARDS）and acute lung injury（ALI）elevated levels of serum materials such as soluble proteins and some lipids are found in the extracted LS. These LS are also found to have reduced surface activity, although the cause for this is not clear[11]. An early study by Haas and Longmore showed that although the lung can produce cholesterol much larger in amount than its own requirement, the main mechanism used in the production of surfactant cholesterol is the use of lipoprotein cholesterol supplied form the blood, since about 2 wt％ of the cholesterol is from endogenous synthesis[12]. Other studies have established that lipoproteins such as low density lipoproteins（LDL）not only supply cholesterol to surfactant but also affect the secretion of surfactant from the type - II cells[1,6,13].

Previous studies have shown that cholesterol present in LS in physiological amounts has no deleterious effect on LS function[8]. However in ARDS and ALI some serum materials, which leak from the capillaries into the fluid lining of the lung, inactivate LS. Most previous studies have suggested that it may be serum soluble proteins which can inactivate surfactant, however *in vitro* studies require very high amounts of proteins to provide such inactivation. It is known that some serum proteins which leak from the capillaries into the fluid lining the lungs may render LS inactive. However, some recent studies have suggested that excess amounts of cholesterol or other serum lipid components are far more potent inhibitor of LS[8-10]. Previous studies on serum albumin, C - reactive protein, fibrinogen and others have shown that these proteins affect the structure-function properties of LS in very high nonpathophysiological amounts when studied *in vitro*[14-17]. However there are evidences that serum protein levels in pathophysiological lungs only increase by three folds, which cannot inactivate surfactant. In an earlier study we have shown that cholesterol levels in such lungs only increases by two fold, and completely inactivates surfactant[18]. In another study we have also observed that whole serum is a far more potent inactivator of LS than its soluble protein fraction[19].

Bovine lipid extract surfactant（BLES）a clinically used surfactant developed in Canada is used for treating patients with ARDS[11]. The material contains all surfactant lipids and proteins except the hydrophilic SP - A and SP - D, and the neutral lipid cholesterol, which are synthetically removed. This allows *in vitro* studies with BLES with various increments of cholesterol or serum proteins[8-11]. In a previous study we have used fetal calf serum（FCS）with BLES and have found that the serum（with its lipids and proteins）is 200 times more potent inactivator of LS than its protein component albumin[19]. FCS is very similar in composition to human serum. Human serum mainly consists of albumin, fibrinogen, CRP, globulin among other proteins, and also ions, amino acids, sugars（fructose and glucose）as well as serum lipids and lipoproteins such as LDL[20-22].

In this study we have investigated the normal and pathophysioligcal amount of FCS, and serum lipids（cholesterol and LDL）with BLES using a set of correlated biophysical and structural methods previously used to study

surfactant dysfunction mainly with proteins[14,17,19]. Detailed iatrascan and matrix assisted laser desorption / ionization – time of flight（MALDI-TOF）suggested the exact composition of BLES and FCS lipid components. Langmuir surface balance and adsorption studies suggested the alterations of surface activity of the BLES by FCS lipids, as well as atomic force microscopy（AFM）suggested the structural alteration of the gel-fluid domain distribution in such functionally altered films. Raman-spectroscopy was applied to such BLES / additive bilayer dispersions used to form such surface films, to suggest alteration of molecular packing of the surfactant phospholipid chains in such bilayers from monitoring hydrocarbon vibrational modes during thermal transitions.

2. Materials and methods

2.1. Materials

Samples were prepared with supplied exogenous lung surfactant, bovine lipid extract surfactant（BLES®）dispersion and 10 and 20 wt% of fetal calf serum（FCS）, cholesterol, and low density lipoprotein（LDL）. The clinically used BLES suspension（27 mg/mL）was a generous gift from Dr. Dave Bjarnson of BLES® Biochemicals Inc.（London, Ontario, Canada）and was used without further modification. FCS （7 mg/mL）, LDL（5 mg/mL）, and cholesterol （crystalline form）were all purchased from Sigma-Aldrich Inc（.USA）, and were used as received. HPLC grade solvents, chloroform and methanol, were purchased from Fischer Scientific（Ottawa, Ontario, Canada）. Most samples were studied in the buffer using 0.15 M NaCl-Trizma®. HCl buffer at pH 7 to maintain optimum pH and ionic conditions for LS. The NaCl-Trizma®. HCl buffer used in this study was prepared by the addition of 150 mM NaCl and 5 mM（～0.08 g）Trizma hydrochloride in 1 L of double distilled water and the pH was adjusted to 7 by titrating with 0.1M NaOH[14]. Each sample was prepared by incubating desired amount（10 and 20 wt%）of FCS, cholesterol and LDL with appropriate amounts of BLES followed by thorough mixing. Small aliquots were diluted appropriately with buffer to be used for all monolayer and bilayer model studies. This was done to ensure that the similar stock of samples was used in all experiments and to avoid heterogeneity of sample concentration and composition.

All solution preparation and experiments were conducted using double distilled water（ddH_2O）. Glassware used were washed by perchloric acid followed by rinsing with ddH_2O and were dried for 2 hr. at 180 °C to remove any organic and surface active impurities[23].

2.2. Lipid extraction of samples

While conducting the monolayer, AFM, Raman and some mass spectrometric studies on BLES and BLES + cholesterol, BLES was extracted in hydrophobic solvents from aqueous dispersions according to the Bligh and Dyer method [24]. Briefly, in this method, 0.8 mL of BLES dispersion（27 mg/mL）was mixed with 3 mL of chloroform: methanol（3:1, v/v）, in a test tube and homogenized by mechanical shaking. One part of chloroform along with one part of double distilled water was added then and again vortexed to ensure uniform mixing. The mixture was then centrifuged for 2 - 3 minutes at 1000 rpm in order to separate the organic and aqueous layers. The organic phase at the bottom layer was carefully taken out into a glass vial with a glass pipette without disturbing the aqueous layer. This extraction process was repeated with remaining aqueous layers using 2:1 chloroform - methanol. All organic extracts were kept in a single vial. It was then placed under nitrogen gas flow to dry and kept overnight in a vacuum desiccator to make it free from trace amounts of organic solvent. Cholesterol was added to BLES and after the addition the sample was suspended in the chloroform - methanol mixed solvent, which was then dried under nitrogen gas and re-suspended in a saline buffer[19]. The BLES - cholesterol mixture was re-suspended in a saline buffer, by vortexing above 30 °C, to obtain multi-lamellar vesicles as observed using electron microscopy by methods discussed elsewhere[25]. To identify the lipid components of both FCS as well as LDL, the lipid portions of each sample were extracted by using modified Bligh and Dyer method[19,24]. The organic layer of each was dried under nitrogen and the residue was then used for analysis by matrix - assisted laser desorption ionization - time of flight mass spectrometry（MALDI - TOF MS）using 0.5 M 2, 5-dihydroxybenzoic acid（DHB）matrix.

2.3. Methods

Pure BLES as well as BLES in the presence of 10 and 20% serum or cholesterol or LDL were chosen for the present set of studies. Selections of these amounts of additives were not arbitrary since these concentrations are pathophysiologically relevant as noticed in diseased as well as in the normal lung surfactant lavages[11,18]. Adsorption, surface tension area isotherm and AFM measurements were performed to investigate the function and structure of monolayer films, whereas the use of Raman spectroscopy was to examine the bilayer phases. Experiments were performed at a controlled ambient temperature of 23 ℃, except Raman spectroscopy, which were conducted in the temperature range 10 - 40 ℃. The studies were conducted with this ambient temperature since AFM studies could not be performed at higher temperature, as well as the Langmuir surface balance had a subphase volume of 5 L, which cannot reach higher temperature equilibrium without surface evaporation. A previous study on higher temperatures suggested that there are no major or significant differences in adsorption and surface tension changes between ambient and physiological temperatures for BLES[19].

2.3.1. Adsorption kinetics studies

Adsorption experiments were carried out by surface tension study（plate detachment method）. Sample dispersions were injected thorough a rubber valve into a cylindrical teflon cup of 5 mL capacity and a surface area of 6.28 sq. cm[14]. Samples were homogenized by constant stirring and adsorption of sample to the interface was determined by the drop in surface tension, by using a Wilhelmy platinum dipping plate.

2.3.2. Langmuir surface balance studies

A Langmuir Wilhelmy balance（Applied Imaging, London, England）was used to record the surface tension（γ）– area isotherm for the adsorbed films. This balance has an initial open area of 500 cm^2, which was large enough to allow compression of films to lower γ values very close to 1 mN/m[14,17,19]. A motorized leak-proof rectangular teflon tape barrier was used to compress and expand the monolayer films while a platinum Wilhelmy plate hanging from a

force transducer detected the change of γ[19]. Prior to beginning each experiment, the trough was thoroughly cleaned with chloroform: methanol（3:1, v/v） mixture and then with ddH_2O. Each time the interface was suctioned off and the trough was dried. The trough was filled with ddH_2O to simulate an air - water interface. BLES and BLES + 10 and 20 wt% samples of serum or LDL or cholesterol （systems） dispersions were allowed to form adsorbed films with an initial γ drop close to 60 mN/m. Compression and expansion of monolayer films were conducted at a rate of 2 mm^2/sec. Compression allowed the lipid monolayer to undergo a fluid to gel phase transition. Details of the Langmuir - blodgett method using this trough have been previously published[14,17,19]. This balance was also used to deposit films using Langmuir - blodgett transfer technique at various γ on pre-immersed freshly cleaved mica substrate for AFM studies[16,26]. Adsorbed films were compressed to the desired γ values. Pre-immersed mica disc was then vertically lifted out off the subphase with an upstroke of 1 mm / sec in order to get the Langmuir - blodgett films[27]. A standard surface tension vs. percent film area protocol was adopted for showing the surface tension - area isotherms data instead of the standard surface pressure - area per molecule isotherms. This was done due to technical difficulties in calculating the exact area per molecule of "adsorbed" films as well as comparing the γ data with some previous studies using captive bubble methods, where surface area data are redundant and cannot be calculated with accuracy[8]. The details of the techniques and physics of the Langmuir balance and trough have been previously published[16].

2.3.3. Atomic force microscopy（AFM）

Langmuir - bodgett deposits of BLES, with or without additives, were used for structural studies using AFM. A teflon dipping head with three freshly cleaved mica disks was lowered into the water subphase before the sample film was made. Compression（2 mm^2/sec） from ～60mN/m to stepwise decrease of three desired surface tensions（52, 42 and 32 mN/m, respectively） was conducted and the compression was kept stopped during each deposition. When the desired surface tension was attained, the dipping head was slowly retracted vertically at a rate of 1 mm^2/ sec[19]. Monolayers were deposited on flat surfaces of mica, and were imaged with a Nanoscope III an atomic force microscope（AFM, Digital

Instruments, CA, USA). A silicon nitride tip, attached to a cantilever was laterally moved across the surface of the deposited sample by the Nanoscope software in contact mode[16,26,28]. Samples were imaged within two hours from the films being deposited to prevent dehydration[19]. The images were processed using IGOR Pro software (WaveMetrics, Portland, Oregon, USA) to produce 2D, 3D and sectional images, similar to those obtained using Nanoscope III software. To easily compare domain sizes and heights, each field size, Z-scale and deposited surface tensions of all samples were kept reasonably close to the best of our ability. At least 3 - 4 random spots were scanned for each sample and the best representative images are displayed.

2.3.4. Raman spectroscopy

In the present study a Raman spectral - microscope (RMS) (LABRAM confocal microscope, Horiba Jobin Yvon, Edison, NJ, USA) with a grating of 1800 grooves/mm was used. A Leica microscope with a long working distance objective (50X) attached to the spectroscope with a Peltier CCD detector allowed for direct imaging of the samples while collecting spectra at different temperatures of heating - cooling cycles. A 532 nm green laser (diode pumped solid state laser) line was excited to yield the spectra. Each spectrum had an accumulation time of 6 - 20 seconds and was passed through the D_0 filter to find the best representative average spectrum. The shortest time (1 minute per degree) for collection was chosen to prevent sample evaporation and drying[14]. Samples were taken in a small glass cuvette and were placed in the sample chamber of the microscope. The temperature was controlled by a thermostated water bath (5 - 50 °C), and spectrum collected for each 2 °C increments of heating or cooling to induce phase transitions. Details of similar methods have been published previously[14,27,29-31].

2.3.5. MALDI-TOF MS

For matrix assisted laser desorption - ionization time of flight mass spectrometry（MALDI - TOF MS）studies on lipid analysis an applied Biosystems voyager system 1027（Voy-ager-DE^{TM}）mass spectrometer was used to analyze the lipid components of BLES, LDL and serum lipid extracts to specifically measure the difference of cholesterol and cholesterol esters in these samples, as well as some studies were followed up using Iatroscan. 100 μL of samples dissolved in chloroform : methanol（3:1, v/v）were injected in the MALDI by the methods discussed in detail elsewhere and the profiles obtained as intensity as a function of mass / charge（m/z）[32].

3. Results and discussion

3.1. Composition of serum and surfactant

To identify lipid components of whole FCS, both MALDI - TOF and IATRO-SCAN studies were carried out. BLES extract was also used in MALDI - TOF mass spectrometry. Figure 1 shows the spectra for（a）BLES,（b）serum lipids and（c）LDL. BLES exhibits an intense peak at 735 m/z（molecular wt. of DPPC＋H^+）. This is the parent ion peak for DPPC, confirming that BLES contains mostly DPPC. The other peaks in 700 - 800（m/z）range are for other phosphocholine classes especially the fluid 16:0/18:1 PC. In order to investigate the lipid classes comprising whole serum［Figure 1（a）］, the spectra suggests a variety of lipids but showed the high intensity peak of cholesterol at approximately 369 m/z. Cholesterol is not detectable as a whole molecule, but detectable only upon the elimination of water as shown in Figure 1（b）[33]. The highest peak for LDL samples is possibly cholesterol ester fragments as shown in Figure 1（c）. These lipids and there fragments were also previously detected by others[32,34 32, 33] and our Iatrascan results support these findings. The data from the Iatroscan of serum are also shown in Table 1 where the serum lipids were extracted from the FCS. The highest amount of lipids was stearoyl esters（37% cholesterol ester）and phospholipids（21%）. The Iatroscan method combined thin layer chromatography and flame ionization detection[33,35]. With this method,

a lipid mixture（serum）, was separated using silicic acid coated quartz rods and then quantified using flame ionization detection to measure the lipid classes present[35].

With the present level of knowledge it is also uncertain about the source of extra cholesterol from LDL and serum. The mass spectral data（Figure 1）do not provide any clear idea about excess cholesterol. The bulk of cholesterol in LDL and probably in serum is supposedly to be found as cholesterol esters, the MALDI - TOF data shows that some of the cholesterol is mainly detected in its free form in the serum lipid extract as well as LDL. The main peak in the mass spectra（Figure 1）at 369 m/z is possible either the fragmented form of cholesterol ester or free cholesterol from some other source.

Table 1. Iatroscan data of serum: the components present in serum and their percent composition.

Lipid Classes	*Lipid Composition* (%)
Hydrocarbons	5.86
Steryl esters/wax	37.85
Ketones	2.90
Triacylglycerols	6.51
Free fatty acids	11.51
Alcohols	4.90
Sterols	6.14
Acetone mobile polar lipids	3.30
Phospholipids	21.02

Previous mass spectral study of LDL also suggests that the 369 m/z peak is a deoxygenated cholesterol, ［molecular wt, M.W., of cholesterol – OH ＝386 – O（16）＝370 or cholesterol + H^+］, and is the major peak. The other major peak at 496 could possibly be fragments of cholesterol ester[33,34]. The calculated number for complete cholesterol ester peak at 671 m/z is very small as well as contained a Na^+ ion and was difficult to detect in MALDI - TOF of LDL[34]. A more recent study has shown that cholesterol esters are more potent inhibitors of BLES than free cholesterol[10].

3.2. Adsorption isotherms

Adsorption isotherm of BLES in the absence and presence of serum, LDL and cholesterol is presented as a function of time（Figure 2）. Adsorption of lipids onto the air - water interface over time（seconds）was recorded by monitoring γ continuously for 300 seconds. Measurements were carried out in the presence of 10 and 20 wt% serum, cholesterol and LDL with BLES keeping the total volume constant by dilution with buffer. Each adsorption curve represents the average of three replicate experiments, with the standard deviation represented by error bars. Results are also summarized in Table 2. Pure BLES adsorbed（$\gamma \approx 20$ mN/m）within 100 seconds on to the air - water interface. With the addition of serum and LDL to BLES γ shifted to higher values and after 300 seconds observed γ value of these two systems were almost double that of pure BLES. The adsorption of BLES did not change significantly with the addition of cholesterol. These results suggest that serum and LDL did not allow BLES to be adsorbed rapidly（to equilibrium γ）to the air - water interface. It should be noted that physiological amounts of cholesterol（10 or 20%）had no significant effects on adsorption. Adsorption occurs when there is a propensity for the more hydrophobic regions on the outer surface of a molecule to repel from interaction with the aqueous environment towards the air, while the hydrophilic portion would be drawn towards the aqueous environment[4,5,18,36,37]. Adsorption studies were conducted to determine the exact mechanism in which BLES surface activity is compromised by serum and its components.

Adsorption of BLES and BLES + additive mixtures（Figure 2）showed that BLES + serum and BLES + LDL samples had prevented adsorption of the BLES film to an equilibrium value（～25 mN/m）. For these samples γ values could not go below 50 mN/m. However BLES + cholesterol samples, on the other hand, were able to reach equilibrium γ in a short time of adsorption, with very little effect compared to BLES alone.

The inhibition of LDL and serum could perhaps be explained by competitive adsorption of specific components of serum or by prevention of the specific BLES lipids to be able to absorb [37]. Since the Raman studies show that the

bilayers of BLES were directly affected by serum and LDL, we would rather assume the latter idea.

Table 2. Summary of the equilibrium surface tension data for the adsorbed BLES films in the presence of different additives at 25 °C.

Adsorbed sample films	*Surface tension (γ /mNm^{-1}) at 1, 150 and 300 sec of adsorption to air - water interface*		
	γ_1	γ150	γ300
BLES	71	35	25
BLES+10wt% Serum	73	58	53
BLES+20wt% Serum	74	61	59
BLES+10wt% Cholesterol	70	21	31
BLES+20wt% Cholesterol	65	26	27
BLES+10wt% LDL	73	59	56
BLES+20wt% LDL	72	59	50

However, since the AFM studies show that serum and LDL caused dramatic changes in BLES film structure, it is reasonable to assume that some of the additive components could have competitively adsorbed on the surface from such bilayers. Serum derived proteins as well as cellular lipids are normally surface active and can also adsorb onto the air - water interface, forming films, just like LS. These materials are known to impair LS through specific biophysical interactions as suggested above.

An earlier study by Holm *et al.*[15], showed that albumin and fibrinogen, by way of competitive adsorption, interfered with the adsorption of specific surfactant components to the air - water interface. A recent study of ours showed that fibrinogen adsorbs competitively to the air - water interface, as well aggregate the gel domains in BLES films[14]. Competitive adsorption described serum proteins that formed a film or parts of the film at the air - water interface and prevents or delays surfactant adsorption by occupying space in films or the air - water interface and preventing the surfactant aggregates from reaching the interface due to lack of space[15]. The results from an *in vitro* study suggest such a

mechanism[14]. Using Wilhelmy balance and pulsating bubble surfactometer studies to measure adsorption, Holm *et al*.[15] measured the attenuation of the overall γ with surfactant alone or with the addition of inhibitors. It was found that albumin competed with LS for the air - water interface during adsorption when simultaneously added with the surfactant.

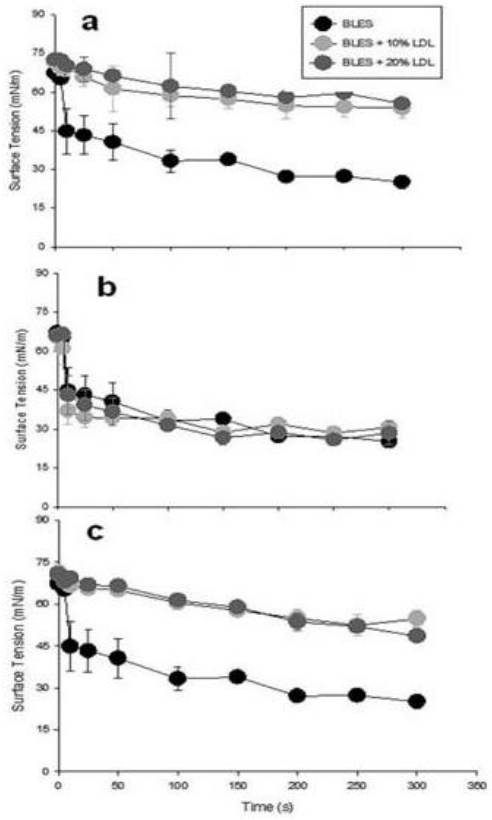


Figure 2. Adsorption isotherms (γ vs. time) of BLES dispersions in the presence of 10 and 20 wt% (a) LDL, (b) cholesterol, and (c) serum at 25±1 °C. Each plot is an average of 3 independent experimental sets. Standard deviation is shown by the error bars of n=3 experiments.

Some conflicting results have been experienced in a study by Gunasekara *et al*. in 2008[38]. They examined two different mechanisms of surfactant inhibition: i）competition of the air - water interface and ii）impairment of the surfactant film by itself. They studied serum proteins, albumin and fibrinogen, in concentrations similar to those in diseased lungs and when additives were added before or after formation of BLES films. In their study, minimal delay or rapid adsorption was noticed in BLES film formation with the presence of a preformed

protein film. They concluded that surfactant inhibition was likely caused by a dysfunctional film instead of inhibition by way of competitive adsorption of the serum proteins[38]. This contradicted the previously reported result[11,19]. This may be due to different surfactant concentrations used in the studies as well as insertion of the proteins in the surfactant dispersions by different methods. However unlike these proteins, cell membrane lipids and free fatty acids, such as oleic acid, are shown to also readily adsorb and penetrate the films and form mixtures with lung surfactant lipids[21,30,34,39]. Previous study on the effects of lysophosphotidylcholine（LPC）on lung surfactant shows that, LPC induced inhibition of LS due to interactions within the surfactant film during compression. LPC was shown to readily adsorb and penetrate the film and efficiently mix with the film itself[39]. In this study, it was mentioned that due to its conical or 'wedged' shape, LPC possibly perturbed the packing of the disaturated phospholipid（DPPC）and thus prevented the low surface tension values being achieved as in normal surfactant films. This may be a possible mechanism by which cholesterol or its ester may interact with gel lipids in BLES.

As mentioned above, the effect of cholesterol on BLES adsorption was minimal. It has been previously shown that enhancement of adsorption rate of surfactant phospholipids occurs to the equilibrium γ, by physiological levels of cholesterol. This is credited to fluidizing effects of cholesterol on phospholipid mixtures high in DPPC content[11,40]. It was suggested however, that this fluidity of the phospholipid may disrupt such films from reaching low γ upon compression[11]. However, Vockerath *et al.*[36], showed that normal cholesterol present in the surfactant can lower surface tension more efficiently than those seen in its absence.

In the present study, it is possible that excess cholesterol could not affect the adsorption rate but the ability of such surfactant films to reach low γ is significantly affected. This may have been with an increase in the fluidity of bilayer dispersions that would help in the rapid spreading of surfactant but would also make the LS film too fluid to lower γ upon compression as evident from the experimental results. In another previous study no difference in the behaviour of surfactant containing various amounts of cholesterol with respect to adsorption（film formation）was noted[8]. This study pointed out that both the normal and

dysfunctional surfactants are able to reduce the γ to equilibrium, but only a fully functional surfactant can achieve very low surface tension values upon compression. However most studies have shown that excess cholesterol can slightly impede surfactant adsorption rate[7-10].

3.3. Langmuir surface balance studies

Multiple compression and expansion cycles of the adsorbed films of BLES and BLES - serum, BLES - LDL and BLES - cholesterol was performed and compared. The same concentration of sample lipid（100 μg/mL） per group was added in each of the three trials, and the films were formed by initial adsorption of the dispersions. Compression - expansion cycles were carried out at a speed of 2 mm^2/Sec after initial equilibrium adsorption of the films to ～62 mN/m. Figure 3 compares compression - expansion isotherms of（a） BLES in buffer with（b） 10 wt% serum,（c） 20 wt% serum,(d)10 wt% cholesterol,（e） 20 wt% cholesterol,（f） 10 wt% LDL, and（g） 20% LDL. Pure BLES film, when fully compressed, could attain the γ to a minimum of near ～1 mN/m value from an initial value of 60 mN/m. Upon addition of serum to BLES, there occurred a drastic shift of the minimum surface tension to higher values（30 mN/m）. Addition of LDL resulted in similar behavior as in the case of serum. No significant change was observed in presence 10 wt%（physiological amount） cholesterol compared to pure BLES. But excess(20 wt%)cholesterol lifted the minimum to a γ～15 mN/m. Such an observation implies that excess cholesterol significantly obstructs BLES ability to reach a low γ, but not as much as serum or LDL. Upon addition of serum to BLES（Figure 3(b) and （c)), there occurred a significant increase in the minimum γ （γ_{min}） to 30 mN/m compared to the low values for pure BLES. Changes in γ_{min} were also observed upon the addition of similar amounts of LDL （Figure 3 （f） (g)). Addition of cholesterol in physiological amounts （10%） resulted in γ min values comparable to BLES(γ_{min}～1 mN/m） films, [Figure. 3(d) (e)]. When cholesterol was added in excess（20 wt%） the γ_{min} could not attain low surface tension（15 mN/m）. Results suggest that excess cholesterol affects BLES ability to reach a low γ, however the effect was not as dramatic as that of serum or LDL. It is evident

from the results that the serum, LDL and cholesterol impair the ability of BLES films to lower γ. Isotherms of BLES + 10wt% serum and BLES + 20wt% serum are shown in Figure 3(b)(c). The inhibitory effects are clearly evident in both the cases. There occurred no change in the isotherm plateau area, suggesting that the material did not undergo 'squeeze out' phenomena and therefore could not be removed easily even after multiple cycling.

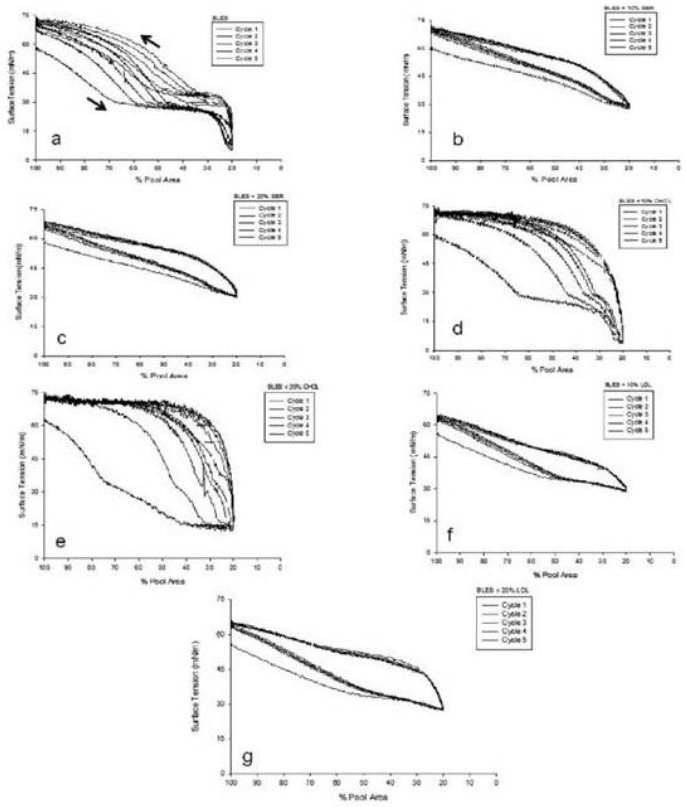


Figure 3. Surface tension - area isotherms of 5 cycles of dynamic compression - expansion at a rate of 2 mm^2 /sec for (a) pure BLES, (b) 10 wt% serum, (c) 20 wt% serum, (d) 10 wt% cholesterol, (e) 20 wt% cholesterol, (f) 10 wt% LDL and (g) 20 wt% LDL adsorbed films. Each experiment was conducted in triplicate and the best representative graph from a single experiment is shown for clarity. All isotherms were plotted as percentage of film area change versus surface tension (γ). The downward arrow shows the direction of compression and the upward of expansion.

Histograms of the minimum and maximum γ that were attained during the fifth cycle of compression and expansion of monolayer films are shown in Figure 4. Each experiment value shown in the bottom panel of Figure 4 the average of

triplicate experiments, where initial values, close to ～ 60 mN/m increased to ～70 mN/m after the fifth cycle. The data shown in the bottom panel of Figure 4 suggest that upon addition of 10 and 20 wt% serum, the minimum γ increased to ～28 and ～30 mN/m respectively, clearly demonstrating that serum prevented the surface activity of BLES films to reach low γ.

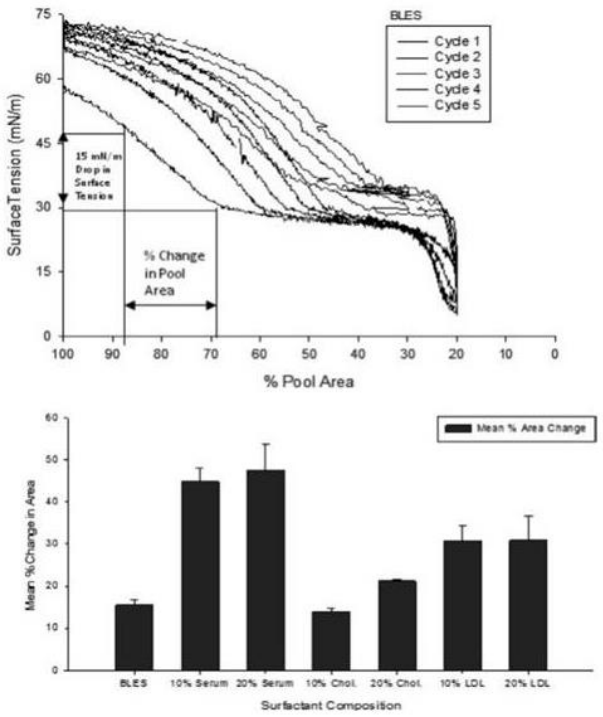


Figure 4. Effects of serum, cholesterol and LDL on compressibility of BLES films based on mean % pool area compression required for a surface tension drop of 15 mN/m (C_{15} values). Serum and LDL had to be compressed two fold more in area than the ones of pure BLES.

Compressibility of BLES and BLES + serum or cholesterol or LDL films were calculated by obtaining C_{15} values as discussed elsewhere[14,16,18]. Briefly, the C_{15} values suggest the total film area（compressibility）required to drop the surface tension of the films by 15 mN/m during film compression from an initial value of 45 mN/m［Figure 4（top）］. The greater the percent（%）area change, for the equivalent drop of γ, the more incompressible the films are. This calculation was previously applied to dysfunctional as well as normal LS films[18]. These studies had shown that for dysfunctional films of LS, large area compressions

were required to drop γ compared to significantly smaller areas required for native surfactant or BLES alone[16,18]. Requirement of large areas of compression indicates that probably materials, which inhibit LS surface activity, cannot be easily removed from the surface film either by 'squeeze-out' or by subphase vesicle formation. It was difficult to remove the serum as well as other component（cholesterol）from dysfunctional surfactant by repeated cycling and thus such films could not achieve low γ[41]. Figure 4（top panel）shows the C_{15} values calculated from a γ change from 45 to 30 mN/m（the change of total area）since serum and LDL films could not be compressed below 30 mN/m even at maximal compression. As shown in the bar graph（Figure 4, bottom panel）, in order to lower BLES films γ by 15 mN/m, the film had to be compressed only by 15% area（highly compressible）. The lowest compressibility（or highest incompressibility）was exhibited by BLES in the presence of serum and LDL where these films required 45% and 30% compression respectively. When cholesterol was added to the films, such films were found to be further difficult to compress than pure BLES.

Figure 3 showed the compression-expansion isotherms for BLES and BLES ＋additive films. As evident from the plots, all materials except 10% cholesterol prevent the BLES films reaching close to 1 mN/m surface tension. When 10% cholesterol films are further compressed, there occurs indeed the formation of gel type domains along with some other 'spiky' centre. In a previous study we suggested that these spikes may also be possibly cholesterol crystals squeezing-out of the films in air and this may be a mechanism of small amounts of cholesterol remaining in the film upon expansion[16,23]. Somehow serum, cholesterol and LDL interact with the BLES lipids to prevent complete compression of the monolayer and also interact with the gel lipids to prevent LS films to reach a low γ. Cholesterol may somehow bind to some of the lipid components in the monolayer which in turn prevents the LS monolayer from fully functioning and the LS from reaching a low surface tension. Multiple compression - expansion cycles, on BLES（Figure 3(a)）shows that the plateau region changes with increasing number of cycles. This suggests that materials from films probably were 'squeezed out' and caused film refining with specific surfactant components as suggested by others[1,2,18,42]. The term 'squeeze out'

refers to the removal of material above or below the plane of the air - water interface. It was proposed that this 'squeeze out' process allows the removal of the nonlipid or other fluid phospholipid constituents off the lipid monolayer, thus allowing for the enrichment of DPPC in such films. As more compression - expansion cycles are preformed, the more enriched with DPPC the monolayer becomes which allows the surfactant monolayer to achieve low γ values [23,42].

In comparison, Figure 3(d), BLES + cholesterol (10%) shows different isotherms than those with 20%(Figure 3(e)). With BLES + 10% cholesterol (as shown in Figure 3(d)), a low γ value could be achieved, which agrees with the fact that the 10% physiological amount of cholesterol naturally found in the LS, does not affect the film's ability to reach very low γ. The plateau region for BLES + 20% cholesterol (Figure 3(e)) is, however different, since there appears no significant plateau (squeeze-out) region. This is due to the fact that when excess cholesterol is added, this excess cholesterol could not have been 'squeezed out' as easily as those of other components of LS. The attainment γ_{min} for BLES + 10% cholesterol (Figure 3(d)) is ~5 mN/m, which is almost the same as for BLES alone, and lower than that for BLES + 20% cholesterol (Figure 3(e)) which had a minimum γ of ~15 mN/m. This trend is also evident in Figures 3(f) - (g) with the addition of both 10% and 20% LDL to BLES. These results suggest that serum and LDL materials can uniformly mix well with the BLES lipids in films and cannot be easily removed. These results are in agreement with those found in a previously conducted study[6], where a captive bubble surfactometer (CBS) was used to investigate the effects of addition of 10% (healthy lung) and 20% (injured lung) by wt of cholesterol to BLES[11]. It was found that with 10% cholesterol added to BLES films, a low (close to 1 mN/m) value of surface tension, similar to normal LS, could have been sustained for a period of time. Samples that contained BLES + 20% cholesterol however could not attain a low γ[11,18].

Cholesterol and LDL are difficult to remove since they are lipids. Cholesterol also has a high affinity for DPPC[23]. The phospholipid DPPC having two saturated palmioyl chains can pack tightly in films (and thus produces gel

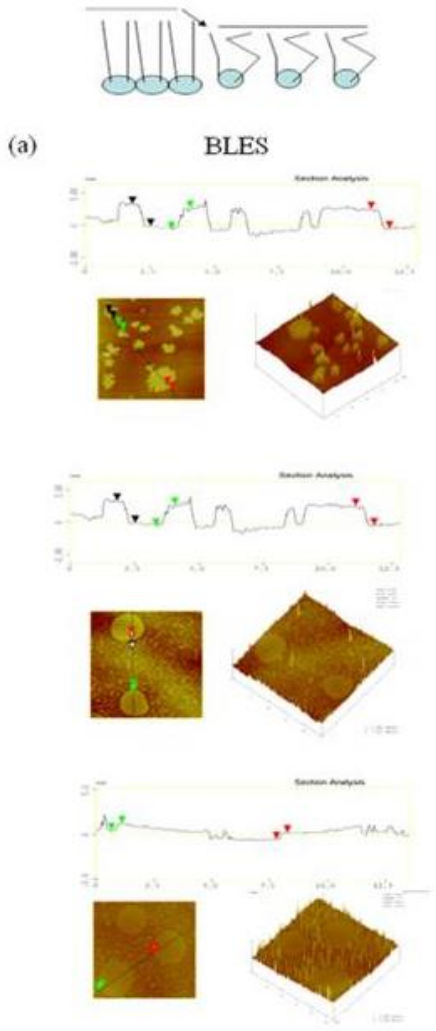
domains）due to their chains being in the all - trans conformation[37,43]. Cholesterol will normally fluidize these chains or cause packing perturbation and such films would not be expected to reach low γ[7,8,23]. However, when present in physiological amounts, they may also rigidify the fluid chains of fluid phospholipids such as 16:0/18:1（palmitoyl-oleyl-PC）which are also present in significant amounts in BLES（Figure 1(b)）. This balance of rigidity of chains in LS may be overturned by excess cholesterol, mainly since cholesterol is difficult to remove from the lipid environment either by increasing compression or multiple cycling as evident from Figure 3(a). These effects are far more evident and dramatic for serum and LDL, suggesting possibly cholesterol may not be the only factor in surfactant inhibition. Their inability to be removed would interfere with the tight packing of DPPC lipids as well as the DPPC gel domain formation in the surfactant monolayer. Previously hole-like domains and the absence of gel domain structure has been observed on dysfunctional surfactants which had about twice the amount of normal cholesterol and prevented films from reaching low γ[18].

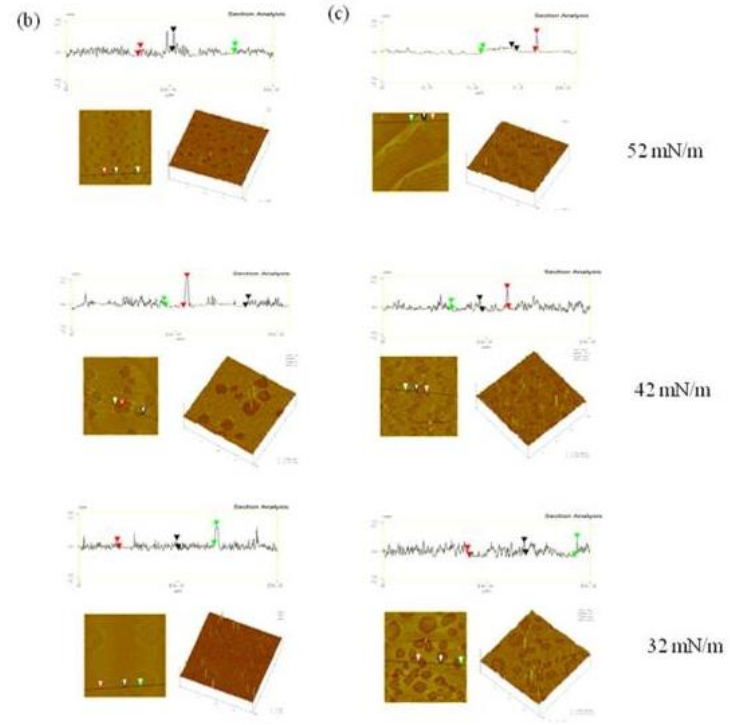
By examination of the C_{15} values as seen in Figure 4, it was evident that the compressibility of BLES films got dramatically altered when serum or LDL were added. This might have been due to the competition of these materials with BLES for the formation of the monolayer or some portions of these materials may have somehow bound to BLES molecules and thus prevents the formation of a surface active film. Since serum and LDL contains proteins, it is possible that there may be a twofold mechanism of surfactant inhibition.

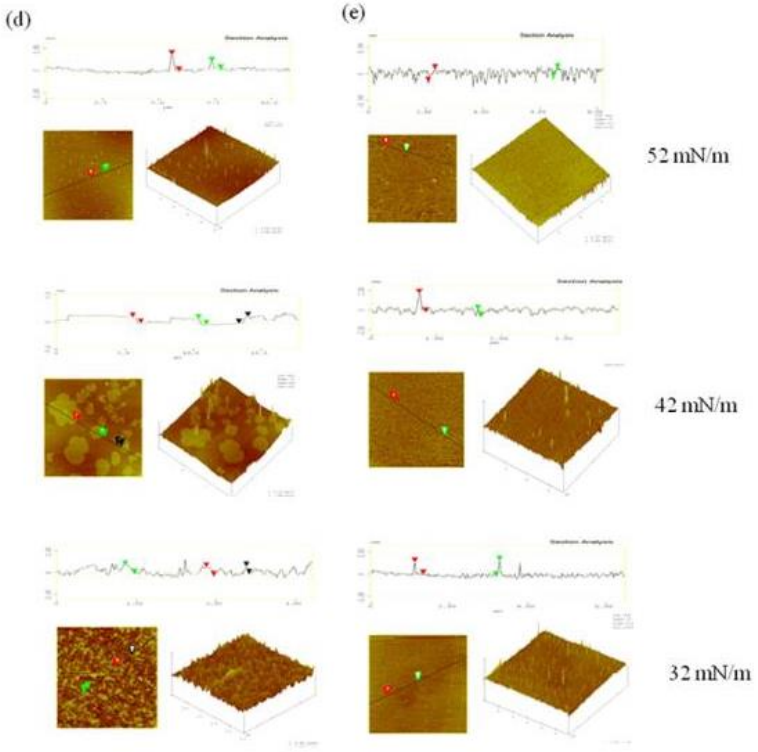
3.4. Atomic force microscopy（AFM）studies

The samples previously used in film functionality studies were deposited on freshly cleaved mica substrates by Langmuir - blodgett transfer technique and were examined using contact mode AFM. The images shown in Figure 6 are representative images of BLES and BLES + 10 and 20 wt% serum or cholesterol or LDL films compressed to a surface tension of 52 mN/m prior to being deposited on mica. It is evident from the images that upon compression, the monolayer films had undergone the transition from fluid（liquid expanded）to gel（liquid condensed）phase[42]. The gel domains were formed upon compression

of films at 52 mN/m. In Figure 6(a), the BLES domains appeared brighter (or higher than surrounding phase), compact and more circular in shape. This was caused by the formation of a more compact organization of lipid molecules in the gel phase. Based on representative section analysis of corresponding Figures (as shown in Figure 5), the height of the BLES gel domain was found to be ~1.2 nm higher than the surrounding fluid phase. Comparing Figure 6(a)-(g), it was evident that even though all the images were taken with the deposits at 52 mN/m, there appeared large differences in surface heterogeneity due to the composition of the films.




(b)
(c)
52 mN/m
42 mN/m
32 mN/m


(d)
(e)
52 mN/m
42 mN/m
32 mN/m

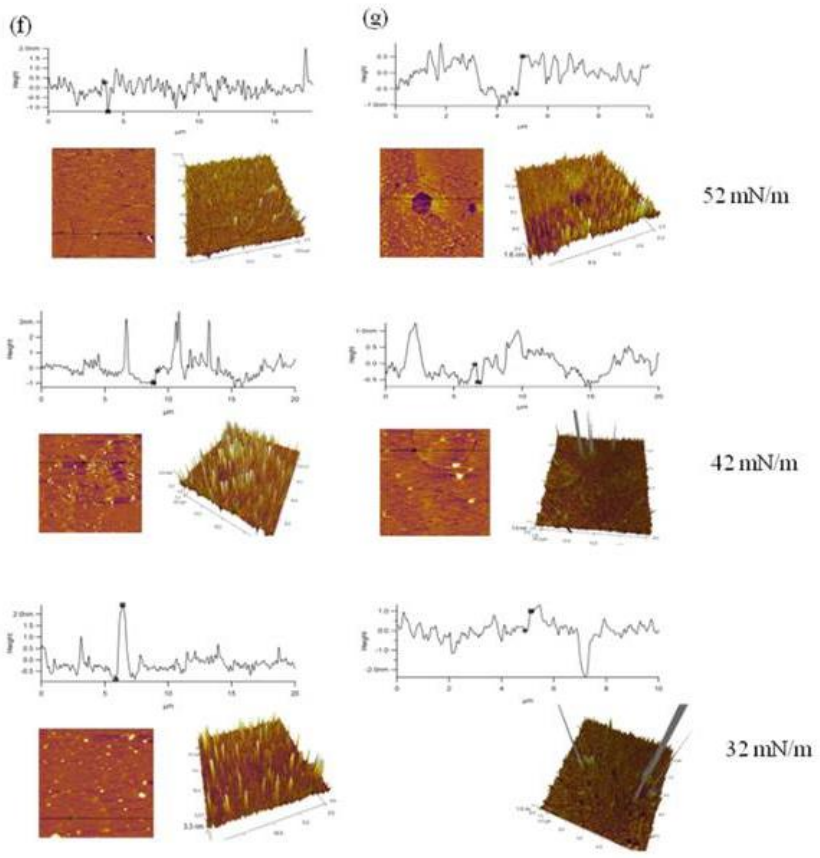


Figure 5. Height differences (section analysis) between the gel and fluid phases of the AFM image of BLES + additive films at γ 52, 42, and 32 mN/m for (a) pure BLES and BLES with (b) 10wt% serum, (c) 20wt% serum, (d) 10wt% cholesterol, (e) 20wt% cholesterol, (f)10wt% LDL and (g) 20wt% LDL. Both three dimensional (3D) and sectional analysis.

BLES gel domains were clearly disrupted in films with the additives. As evident from Figure 8（b）and（c）addition of serum resulted in the areas with holes as well as some areas with spiked（sharp height differences）domains in the center. Figure 8（d）shows that with the addition of cholesterol（10 wt%），small micro domains（with heights of gel phase）were also formed, which disappeared upon the addition of higher amounts（20 wt%）of cholesterol as shown in Figure 9（e）. Also as shown in Figure 8, 10 wt% cholesterol films at further compression（γ＝42 mN/m），larger fluid like domains with spiked centers were observed. Figure 9（f）shows no gel or other domains（hole-like）structures, and the film showed a more solid like homogenous appearance. From

Figure 9（f）and（g）, it becomes clear that higher amount of LDL（20 wt%）prevents BLES gel domain formation as well as appearance of the hole-like domains. The data collectively suggest that LDL, excess cholesterol and serum significantly perturbed the packing of gel lipids in BLES.

As shown in Figure 5, the sections analysis of the 3D images of the films of at γ＝52, 42, and 32 mN/m respectively, the height differences in these films between domains and holes can be measured. For BLES (Figure 5 (a)), gel domains as high as 1.6 nm were formed in a continuum of surrounding fluid phase.

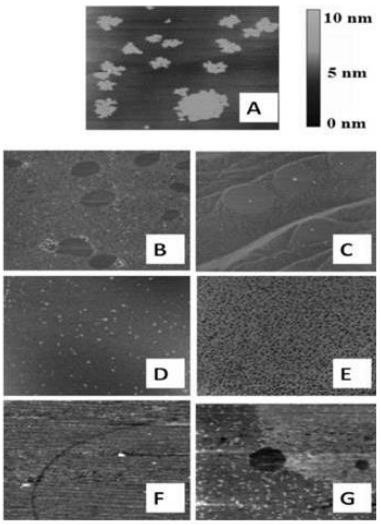


Figure 6. Representative contact mode AFM images of Langmuir - Blodgett film deposited (imaged in air in contact mode) at equivalent γ of 52 mN/m for (a) Pure BLES, and BLES in presence of (b) 10 wt% serum, (c) 20 wt% serum, (d) 10 wt% cholesterol, (e) 20 wt% cholesterol, (f) 10 wt% LDL, and (g) 20 wt% LDL. Image field sizes shown were taken at 10 μm×10 μm (X-Y plane) and height differences (Z plane) are shown by the 10.0 nm bar, indicated in (a). The condensed domains are about 1.2 nm above the surrounding fluid regions. The bright regions represent condensed gel domains and other structures which are 0.5 - 1 nm higher than the surrounding phase. Each frame represents a scan area of 10 μm×10 μm.

When serum was added to BLES, hole like structures appeared along with some spiky structures inside the domains（Figure 5（b）and（c））. The holes are ～2 nm in depth and the spiky domains are seen to be at a height range of 4 - 7 nm.

Upon the addition of cholesterol in smaller amounts as in（d）, further micro domains appeared and became larger upon compression.

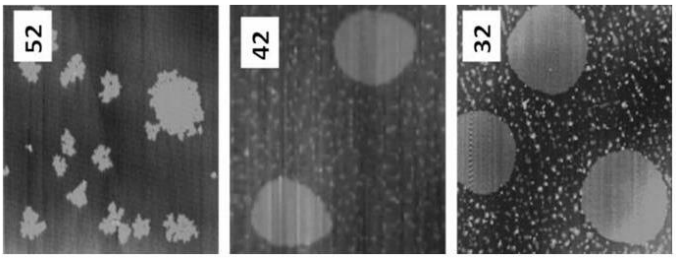


Figure 7a. Representative AFM images of films of BLES deposits taken at γ = 52, 42, 32 mN/m respectively in each vertical panel. Each frame represents a scan area of 10 μm X 10 μm.

Spiky centers were formed which then disappeared upon further compression, and the film turned more homogenous upon further compression. These domains were close to about 1 nm high compared to the intermediate phase between them. Cholesterol, added in excess（20 wt%）, prevented domain formation to occur, and the homogenous film appearance was again observed.

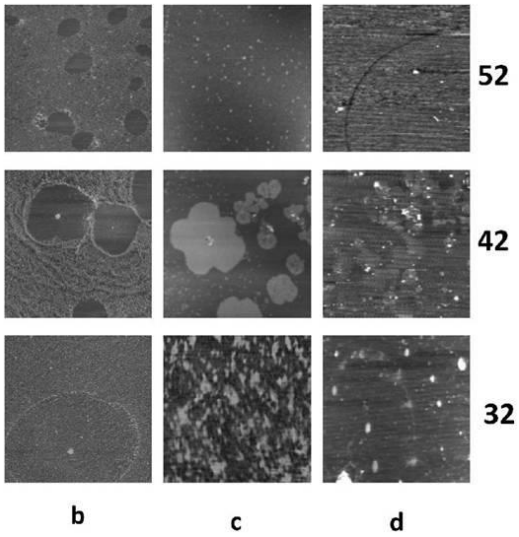


Figure 8b-d. Representative AFM images of films of BLES + 10% serum (b), 10% cholesterol (c) and 10 wt% LDL (d). Deposits were taken at γ = 52, 42, 32 mN/m respectively in each vertical panel. Upon addition of materials to BLES films, the gel domain (bright circular areas) formation is altered. Each frame represents a scan area of 10 μm×10 μm.

Various previous studies have used the method to study lipid films as well as those with serum proteins[7,11,14,17,28]. Our study examined the effects of serum or serum components in the formation of BLES domain. It was concluded that serum materials separately or in conjunction with one another can prevent BLES domain formation by interacting with the DPPC lipids or forming their own monolayer film. Deposits of the same samples used in the monolayer studies were taken at γ 52, 42, and 32 mN/m, respectively.

Representative AFM images have been presented in Figure 6. Prevention of BLES domain formation was seen in all but the 10% cholesterol deposit γ ＝42 mN/m. This was likely to do with the physiological amount of cholesterol that was found in the lung somehow helping to form these domains, such as lipid rafts in cell membranes. Evident from Figure 6, serum and LDL were able to form areas of holes and also their own domains. The domains in the holes were due to components of serum and LDL preventing surface active components of LS to adsorb so that they could not form a surface active film. The hole-like areas were perturbed DPPC gel domain remnants or separate domains of serum components（possibly cholesterol esters）or a combination of both of both[19].

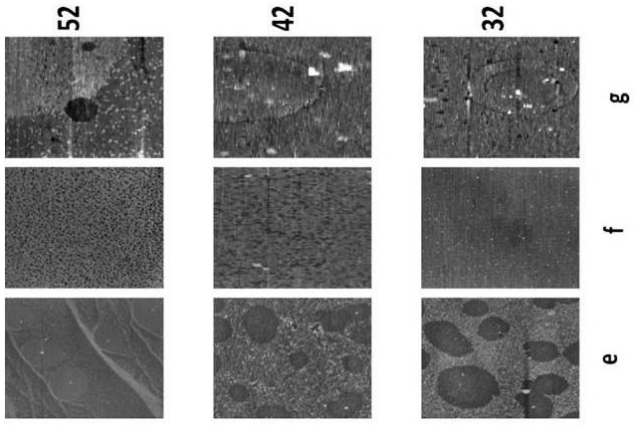


Figure 9e-g. Representative AFM images of films of BLES with 20 wt% serum (e), 20 wt% cholesterol (f) and 20 wt% LDL (g). Deposits were taken at γ 52, 42, 32 mN/m respectively in each vertical panels. Upon addition of materials to BLES films, the gel domain (bright circular areas) formation is altered. In (e) and (g) the domains formed exhibit lower height profile compared to the surrounding phase, suggesting that these circular domains may be fluid in nature. Each frame represents a scan area of 10 μm×10 μm.

The ‘spiky’ area seen in the various samples, especially BLES + 10% cholesterol, could possibly be the cholesterol domains, or in other cases, some combination of perturbant and BLES lipids. Previous studies with low amounts of cholesterol（2 - 8 wt %）in DPPC and BLES films, suggested that the lipid remains in the films at high compression by a unique mechanism rather than being directly removed from the film by squeeze-out to the subphase[19,23]. By AFM and fluorescence, it was observed that cholesterol in highly compressed films form multilayer structures, as well a possibly crystallizes into solid forms, above the plane of the monolayer. Upon expansion these structures are readsorbed or dispersed back in the films[19,23].

Domain height is due to the tilting of the DPPC fatty acid chains in gel phase, more perpendicular with respect to the plane of the monolayer, compared to those in the fluid phase as shown in Figure 5(a). Cholesterol when added in excess completely abolished any domain formation. This was due to the fact that when cholesterol exceeds the DPPC concentration, the sterol may start to interact with the unsaturated lipids and thus completely keep the monolayer in a fluid state[11]. Previous AFM and fluorescence studies suggest that normally soluble proteins or other bulky perturbants were preferably adsorbed in the fluid phase of lipid films because of the loose packing of the lipids in that phase[14,19]. This most often resulted in decreased size and amount of gel or condensed domains as the addition of the perturbant increased. An earlier study using fluorescence microscopy showed that high concentrations of serum had caused the formation of smaller BLES domains possibly due to its presence in the fluid phase which inhibited DPPC lipids to segregate into gel domains upon compression[19]. Also in the present case the domains that were not probed, may have been formed by serum component. In agreement with these findings, an even earlier study showed how injuriously ventilated lungs, similar to diseased lungs, showed increases in serum protein and cholesterol[18]. Detail mechanisms for the formation of different types of domains in these “diseased” lungs were proposed in that study. In the present case, very few, if any, DPPC - rich domains were present at low surface tension（more compressed films）and these domains are noticeably smaller and covered less of the total surface area, despite similar DPPC content. These intermediate domains were found to contain areas of some fluidity seen

from non-exclusion of fluorescent probes[18]. This is viewed as different types of domain height differences between the two phases in AFM[16]. The larger heights of the gel domains（circular areas）are due to tilting of the fatty acyl chains of the PLs in gel phase when there occurs a tighter packing induced by lateral compression of the films as well as due to the molecules orientation being more perpendicular to the air - water interface[11,16].

3.5. Raman spectroscopy studies

Raman spectroscopy can suggest specific bond vibrations in a single phospholipid system or an average of signals from specific bonds（C-H, C-C, PO_4）in a complex system such as BLES. Higher shift in wave number indicates more fluidity or increasing number of *gauche* bonds, whereas lower shift indicates more *trans* bonds or more rigidity of the hydrocarbon chains[29,31]. Both serum and LDL increased fluidity of the BLES films, while cholesterol had slight fluidizing（10 wt%）and rigidifying（20 wt%）effects as shown in Figure 11（a - c）. The CH_2, CH_3, and C - C skeletal bands could clearly be defined, as well as temperature dependent shifts in some of the bands which allow for a relative measurement of the phase transition of BLES and BLES with additives could be monitored[17,19]. This phase change monitoring allow correlating the structural features observed in films（due to the 2D lateral phase transitions induced by compression）with those that occurred in the bulk（bilayer）material with the change of temperature, since the films were formed from such bilayer dispersions. The correlations suggested significant as serum or LDL.

In the spectra of the lipid class（Figure 10), the strongest peaks that were mostly evident in BLES were the bands from 2800-3000 cm^{-1}. These corresponded to the CH_2 symmetric stretching and asymmetric stretching modes at 2850 and 2890 cm^{-1} respectively. These bands were sensitive to the *trans* - *gauche* conformation changes in the acyl chains of lipids. Another Raman band of similar interest was that of the CH_3 symmetric stretch（2930 cm^{-1}）and asymmetric stretch（2960 cm^{-1}）, as it provided information from the interior core of the bilayers. The Raman spectra of BLES and BLES + serum or cholesterol or LDL in the 2800 -3000 cm^{-1} regions at different temperatures

have been shown in Figure 11. These Raman peaks, for specific phospholipids and their bond vibration designations, have been determined previously by others[38,44]. The symmetric CH_2 stretch (ν_s CH_2), at 2850 cm^{-1}, the asymmetric stretch (ν_{as} CH_2), at 2890 cm^{-1}, and the symmetric CH_3 stretch (ν_s CH_3), at 2930 cm^{-1} were prominent for BLES and BLES + 10 wt% serum, 10 wt% cholesterol and 10 wt% LDL systems. For BLES, in (a), the ν_{as}, showed a trend for a broadening of the ν_{as} CH_2 band with the increase in temperature from 25 – 40 °C. For temperatures lower than 25 °C, narrowing of the band was observed. Another trend was the shift in wave number of the ν_{as} CH_2 peak with the increase in temperature. It was also evident from the intensity of the ν_{as} CH_2 peak compared to the ν_s CH_2 peak. At higher temperature intensity of ν_{as} CH_2 was less than ν_s CH_2 and it increased with decreasing temperature. The ν_s CH_3 peak at 25 °C was less intense than that of the ν_{as} CH_2 peak, and as the temperature increased, so did the ν_s CH_3 intensity. These trends were consistent with a change in conformation of the lipids as a function of temperature, where an increased fluidity of the hydrocarbon chain or *gauche* conformation had been observed at higher temperatures. At lower temperatures (10 - 25 °C) however, the hydrocarbon chains were more "gel-like" or rigid with *trans* conformation[8,14]. The effects of serum, cholesterol, and LDL on BLES however were not seen quite clearly, due to high noise or low signal/noise ratio ascribed to protein auto-fluorescence expected from the serum soluble proteins or from apoproteins of LDL. Also any clear cut peaks from pure serum and LDL were not observed due to their high protein content. By plotting the shift of the wave number of the ν_s CH_2 peak versus temperature, small changes in conformation were seen, as shown in Figure 11. The wave number of the ν_s CH_2 (2850 cm^{-1}) was plotted against temperature for the average of three independent experiments and the error bars represent standard deviation. These plots can suggest the order parameters of the phospholipid chains as discussed in details elsewhere[8,14]. A shift in wave number towards higher frequency side indicated more fluidity or higher number of *gauche* bonds, whereas lower shift indicated more *trans* bonds or more rigidity.

The temperature induced changes in the wave number of the ν_s CH_2 band of BLES + serum, BLES + cholesterol and BLES + LDL are shown with those of

BLES in Figure 11. For the BLES curve, a more sigmoid shaped plot was obtained which might be attributed to a broad phase transition observed between 10 – 40 °C, the midpoint of the transition being about 27 °C, which was previously measured by differential scanning calorimetry（DSC）[14].

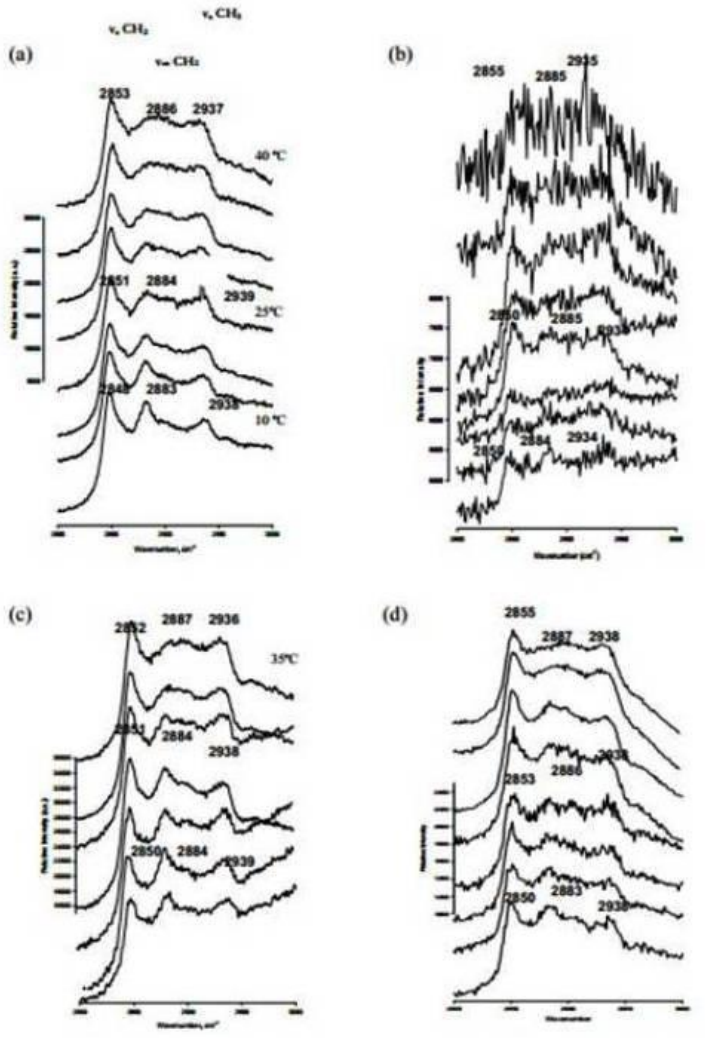


Figure 10. Raman spectra of the 2800-3000 cm^{-1} range for (a) BLES, (b) BLES + 10wt% serum, (c) BLES +10wt% cholesterol, and (d) BLES + 10wt% LDL. Each graph is a representative of three different experimental trials. Similar trends were shown for BLES and BLES + 20wt% serum, 20wt% cholesterol, and 20wt% LDL samples. Temperature range indicated from 10 ^{0}C - 40^0 C.

Figure 11 (a) and (c) describe the fluidizing effects of serum and LDL, respectively, on BLES. The fluidizing effects were reflected from the increase in shift to higher wave numbers for all points on the curve throughout the temperature range. Upon addition of cholesterol however, the shifts of wave numbers were not so significant compared to BLES alone. A slight shift to higher wave numbers were resulted in the 20 wt% cholesterol system, however the effects were not significant, suggesting cholesterol might have rigidified as well as fluidized the system depending on the temperature.

The systems containing LDL (Figure 11(c)), as well as the ones with serum (Figure 11(a)), clearly show a shift of all wave numbers of higher values suggesting a fluidizing effect of the BLES system with these additives. Therefore it could be concluded that both serum and LDL fluidized the bilayer, which in turn prevented the formation of gel domains in films formed from such bilayers, and this might have affected LS function. Cholesterol seemed to increase fluidity of the gel phase at lower temperatures and to rigidify the fluid phase at the higher temperatures. The Raman studies directly showed that serum and LDL affected surfactant molecular packing in bilayers.

Raman spectroscopy involves the study of vibrational modes of a system. It can be used to study solids and even liquids and solutions because water is Raman inactive unlike Fourier transform infrared spectroscopy (FTIR) where water vibrations at 3000 cm^{-1} and H-bonding suppress the hydrocarbon vibrations peaks at the 2000 - 3000 cm^{-1} range[27,29-31]. Figure 11 describes the variation in the Raman shift for adsorbed BLES film in the absence and presence of different additives. It is noted that there occurs an up shift in the ν_s CH_2 peak position with an increase in temperature. The shift in wave number in higher numbers implies an increase in the fluidity of the system. Since the fluidity of BLES bilayers gets directly affected by additives, it is assumed that the films formed from such bilayers would also lead to an increase in fluidity[37,43]. Serum and LDL addition seem to cause a more fluid bilayer at all temperatures and it is probably due to this fluidity that prevention of BLES domains formation occurs in the films, thereby inactivating a functioning surfactant or by the lipids ability to be tightly packed.

The bilayer phase changes observed from the order parameter profiles（Figure 11）from the Raman spectra suggest that LDL and serum alters the fluidity of such membranes, however excess cholesterol does not show any significant effects.

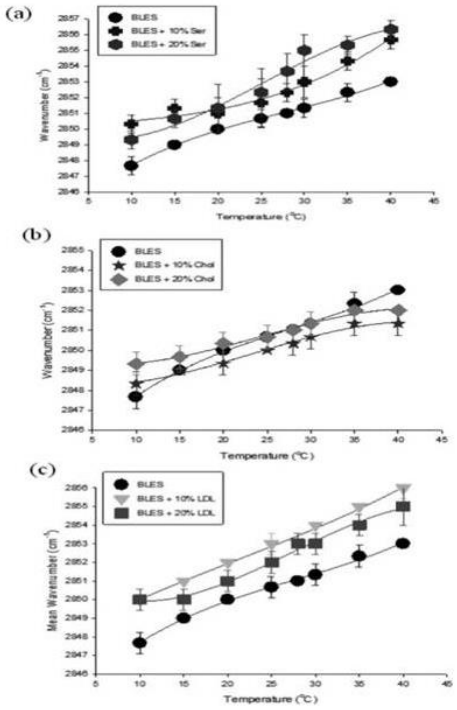


Figure 11. Vibrational wave number shift for BLES compared to (a) BLES + serum, (b) BLES + cholesterol and (c) BLES + LDL. The temperature range was from 10 – 40 °C for all graphs. Each graph is the mean of wave number shifts from three independent experiments and the error bars represent the standard deviation.

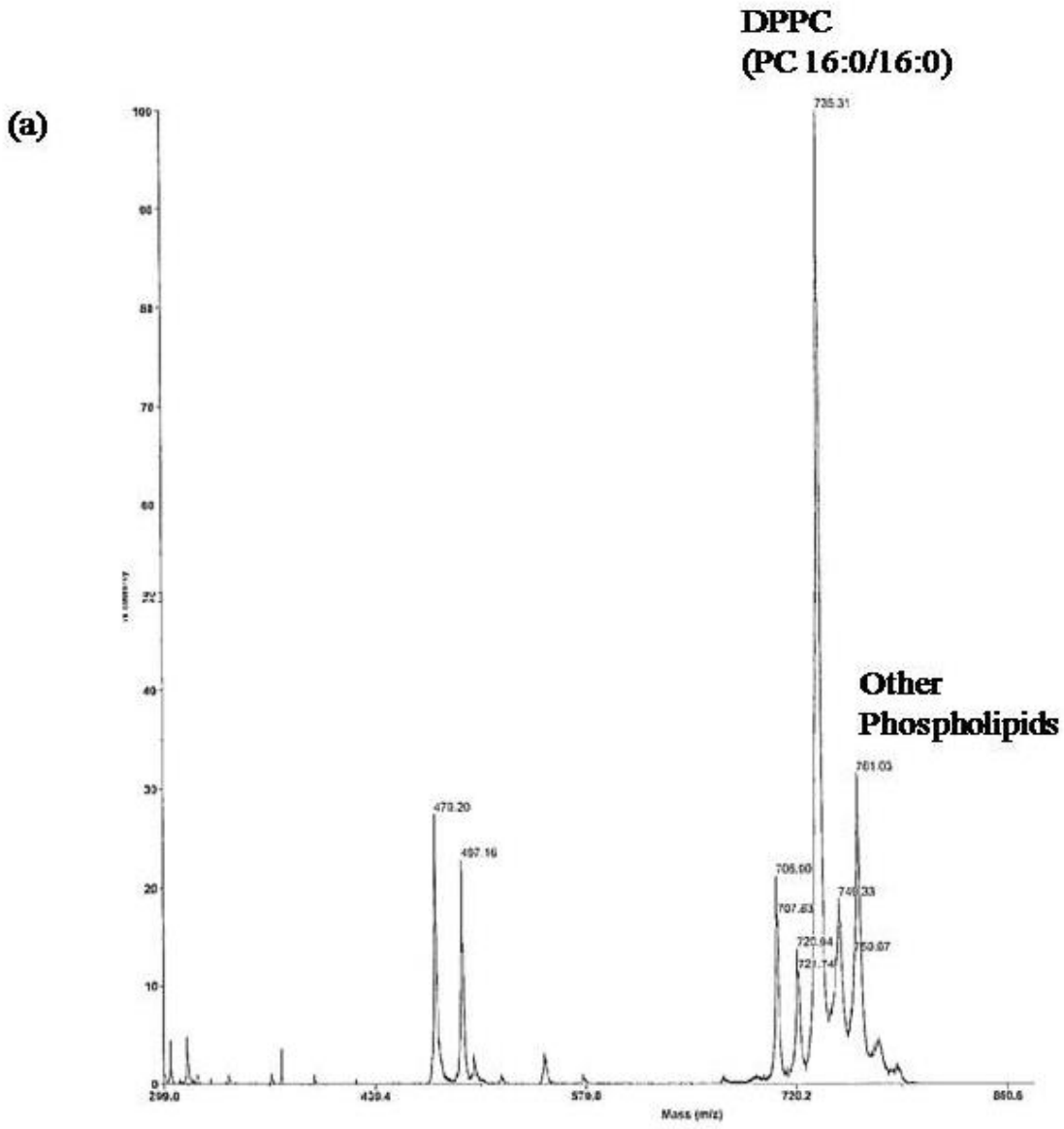
It is also puzzling to notice how the cholesterol or its ester in LDL directly affects the BLES bilayers, since the esterified form of cholesterol in LDL（or in serum LDL）cannot directly enter BLES bilayers simply by lipid exchange, since in normal situations these are entered into type II cells by the receptor mediated pathway. Whatever is the case, certain materials from LDL and serum seem to cause fluidity changes of bilayers as detected in the Raman spectroscopy, and this causes possible structure function changes in the films being adsorbed from such bilayers. Previous studies had shown that cholesterol can interact with SP - B and SP - C in LS bilayers which were present in BLES[45]. Other studies using NMR and X-ray diffraction show cholesterol to slightly increase the order

parameter of BLES bilayer in the gel phase albumin to induce specific fluid-like domains in such bilayers[37,43]. Although these studies do not suggest any clear cut mechanism of bilayer interactions of LDL and serum with BLES, our Raman data clearly suggest possibilities of different modes of interaction of these serum materials with BLES compared to cholesterol in normal or excess amounts. It is possible that the apoproteins present in LDL as well as serum soluble proteins such as albumin and others could have probably interacted with SP – B / SP - C present in BLES and thus causes the changes in BLES bilayers, other than the effects already induced by cholesterol or its esters[10]. There may possibly be a dual mechanism involved here requiring further investigation with serum or LDL protein components. This could be considered as the future perspective.

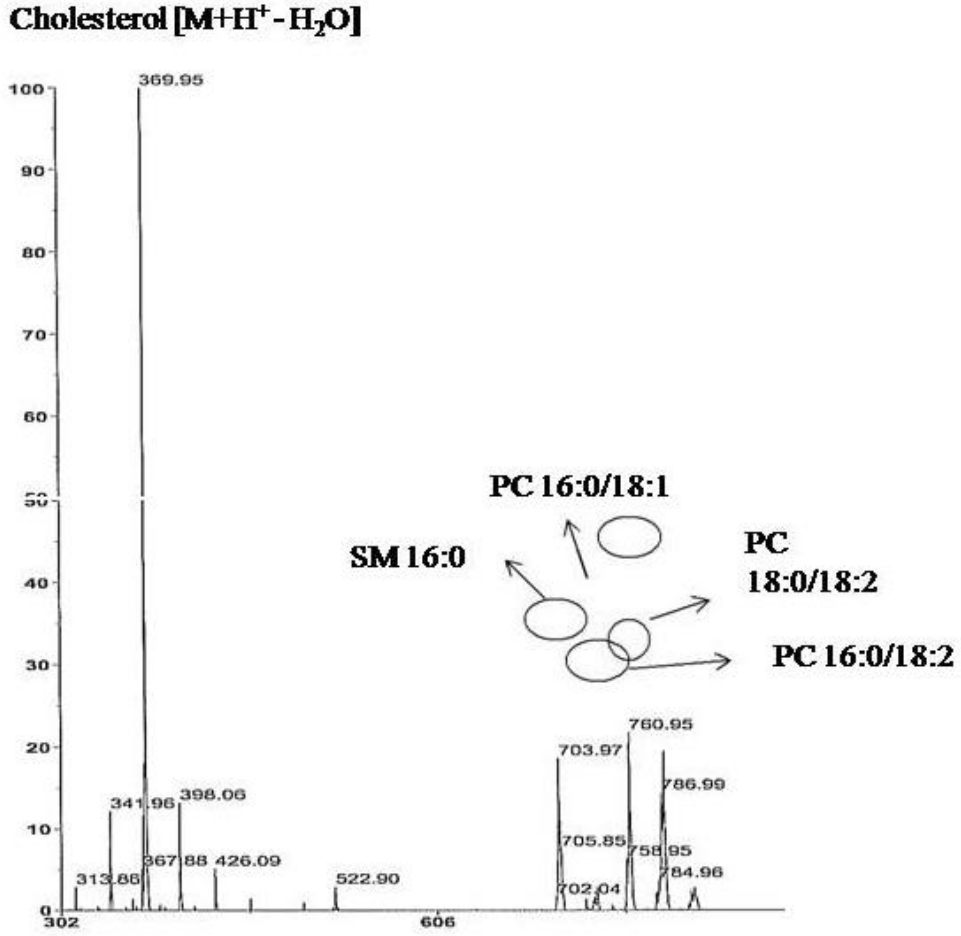
4. Summary and conclusions

Complete physicochemical studies involving the composition, functionality and structure of the adsorbed BLES films in the absence and presence of three additives, *viz*., cholesterol, serum and LDL were performed. The study includes the pathophysiological amounts of these materials found in lungs in disease. The additives altered the bilayer and film packing, surface activity and structures of surfactant, suggesting possible molecular rearrangement and disordering of surfactant in disease. Since excess cholesterol or its esters may actually arrive inside the lung through LDL transport, it is cholesterol that is the most potent inactivator of LS, than leaked soluble serum proteins, since lipids are far more hydrophobic and more difficult to remove from films during dynamic cycling. Whole serum, serum proteins and its lipid components may specifically interact with the surfactant films and bilayers by either separate mechanisms, or synergistically. In future studies, specific lipid components of serum such as HDL, one of the major studies requires the specific separation of serum lipid and serum protein fractions and observing their structure function correlations in inactivating surfactant. The specific molecular rearrangements observed in our study of domains in films need to be further explored in detecting the exact composition of the variety of domain structures using possibly fluorescently labeled LDL, cholesterol and serum proteins. However these studies need to be conducted in a manner using similar sets of biophysical methods which yield structure function correlates of bilayers and films, as well as uses

pathophysiological amounts of materials, so that an over simplification of the models in previous studies can be rectified.



(b)


Cholesterol [M+H+ - H2O]
369.95
100
90
80
70
60
50
40
30
20
10
0
302
606
PC 16:0/18:1
SM 16:0
PC
18:0/18:2
PC 16:0/18:2
760.95
703.97
786.99
705.85
758.95
784.96
702.04
341.96
398.06
313.86
367.88
426.09
522.90

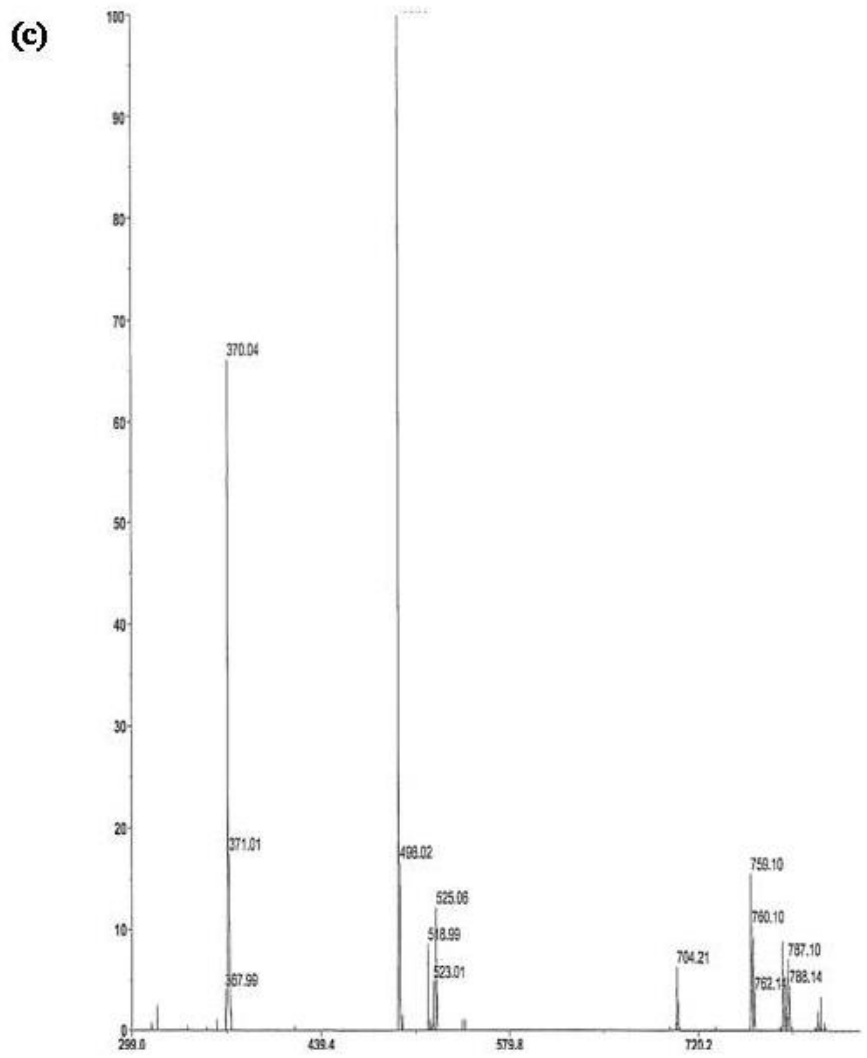


Figure 1. MALDI-TOF mass spectrometry spectra of (a) BLES and (b) serum lipids (c) LDL and (d) table of serum lipids by Iatroscan. For the BLES sample, DPPC was the most abundant. As for the serum lipid extract, cholesterol was the most abundant. Several other phospholipid classes are detected in the 700+ m/z range.

References

References are given in BIBLIOGRAPHY under Chapter I (pp. 173 - 176).