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Review Article

Composition, Structure and Biophysical Functions of Pulmonary Surfactants: Their Deficiency and Strategy for Remedy

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Pulmonary surfactant (PS) is a complex mixture of lipids and proteins that forms a monomolecular film at the lung-air interface. It serves dual purposes: (i) lowers the surface tension; and (ii) participates in innate immune defense of the lung. Surface tension lowering property of PS is achieved by forming a surface film (monolayer) which is highly enriched in dipalmitoylphosphatidylcholine. Among the four surfactant proteins (SP), SP-A and SP-D are hydrophilic, SP-B and SP-C are hydrophobic and are also biocompatible in all the mammalians. This article gives a brief account of the history of research on PS, anatomy of lung, alveolar metabolism, composition and methodologies adopted for *in vitro* evaluation of the PS.The possible molecular mechanism of film formation (adsorption), and of film adaptation to surface changes (phase transitions) during the process of respiration have been described in detail. Major disorders of the surfactant system related with its clinical consequence, and the potentials of surfactant therapy in the treatment of some of these disorders have been discussed.A brief account of the evolutionary development of pulmonary surfactants has also been presented.

Keywords: Pulmonary Surfactant; Surfactant Proteins; Adsorption; Phospholipid; Langmuir Isotherm; Microscopy; Exogenous Surfactant

Background

In the 1950s and 1960s, a respiratory disease, misleadingly named hyaline membrane disease (HMD), was the world's most common cause of infant morbidity, especially among the preterm babies. The infant morbidity led to the need of search for the understanding of lung function.

Scientists and clinicians, working together, discovered the existence and necessity of pulmonary surfactant (PS) and then attempted to figure out how to overcome its dysfunction. The year 1929 is considered as the starting date in the history of PS when Kurt von Neergaard's experiments revealed that the required pressure for filling the lungs with air was higher than filling it with liquid [Von Neergard, 1929]. The alveoli can achieves near zero surface tension at the lung-air interface following the principle of the Young and Laplace. In 1946, it was reported that lung tissue has a remarkably high content of the lipid, dipalmitoyllecithin (currently known as dipalmitoylphosphatidylcholine). Studies on the effects of nerve gases on lungs, significantly contributed to the understanding of the physiology of PS [Clements, 1957]. It was proposed that alveoli, made of lung fluid material, can achieve substantial stability through the quantity and quality of the surface-active materials available at the lung-air interface. Subsequently, with the help of modified surface balance, it could be demonstrated that surface tension can drop to low values upon compression of surface films from lung extracts [Klaus *et al.*, 1961]. There exists similarity in surface activity of dipalmitoylphosphatidylcholine (DPPC) and the phospholipids isolated from freshly lavaged bovine lung. HMD, now known as respiratory distress syndrome (RDS), is due to the deficiency or

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dysfunction of PS [Avery and Mead, 1959]. Since 1959, scientists from various disciplines flocked to the field, which led to dramatic decline in infant morbidity due to RDS.

DPPC is produced during the development of the lung and secreted into the alveolar space by type II pneumocytes [Gluck *et al.*, 1971]. A diagnostic test, using the lecithin/sphingomyelin ratio of amniotic fluid, was subsequently developed to determine the maturity of the fetal lung. Hallman *et al.* [Hallman *et al.*, 1977] discovered the importance of phosphatidylglycerol (PG) in contributing to surfactant spreading and the decreased levels of this phospholipid in children caused suffering from RDS. A landmark could be achieved by the first successful treatment of neonatal RDS with exogenous surfactants. [Fujiwara *et al.*, 1980]. It was found that lipid extracts were not sufficient alone for effective surfactant function, and there came the role of surfactant proteins.This resulted in a rapid expansion of research in the molecular biology, and the structure and properties of PS proteins. A new nomenclature for surfactant proteins A, B, C and D was proposed by Possmayer *et al.* [Possmayer *et al.*, 1988].

Anatomy of Lung and Alveolar Metabolism

The lung is an organ (6% of the body volume) with a large inner surface, continuously in contact with the environment. To facilitate optimized exchange of gases during respiration, the lung consists of highly branched airways (bronchi) ending in air sacs (or alveoli) to maximize the area of gaseous exchange (Fig. 1).

The bubble-shaped alveoli have high curvature: 1 cm^3 of lung tissue has a total gas exchange surface area of 300 cm^2. Warm blooded animals require high rate of oxygen uptake, thus the large surface area of lung is essential [Schmidt-Nielsen, 1997]. Oxygen diffuses from the alveoli to the capillaries, and carbon dioxide leaves the capillaries and diffuses into the alveoli. PS prevents lung collapse (Fig. 1B) by lowering the surface tension on the inner surface of the alveoli to very low values. Clements first proposed that the surface tension of a film varies with the surface area [Clements, 1957]. Decreased surface tension due to the decrease in surface area prevents the alveoli from collapse during end expiration.

Weibel *et al.* [Weibel, 1968], with the help of electron microscopy, proposed that a substantial area of the alveolar surface is lined with a thin aqueous layer which acts as a source of PS, its morphological transformation, its adsorption, desorption and recycling. These events are commonly referred to as alveolar metabolism of PS [Goerke and Gonzales, 1981], and are schematically shown in Fig. 2. PS is synthesized by pulmonary type II epithelial cells, processed and packed into lamellar bodies, structures consisting of closely packed multiple bilayers. Subsequently, PS is secreted into the aqueous subphase (hypo-phase) of the alveoli, where the lamellar bodies undergo transformation into a new

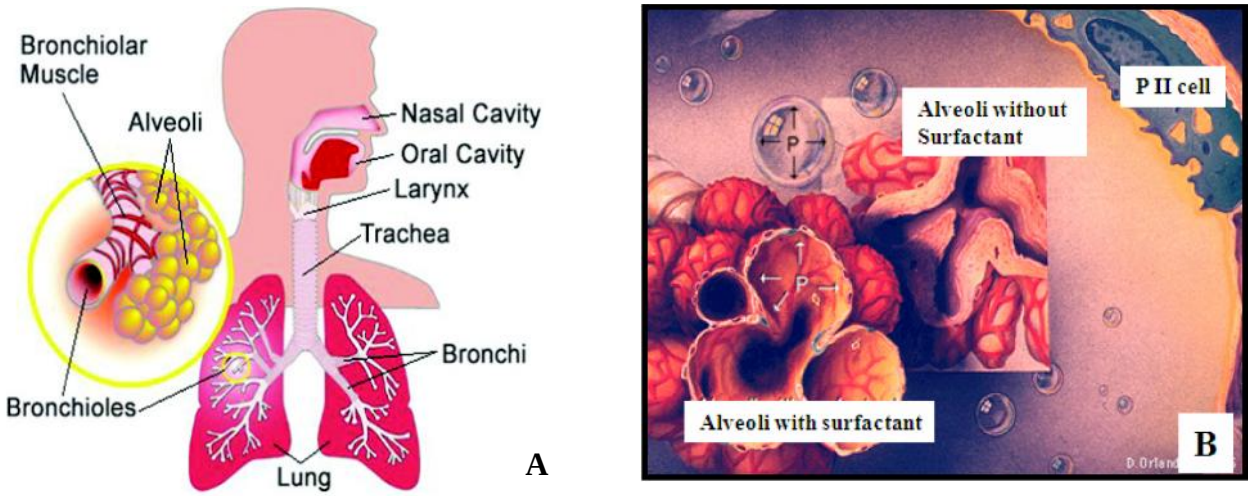

Fig. 1: (A) Structure of the human lung; (B) Difference in alveolar structure in presence and absence of PS. Adapted from Nag [Nag *et al.*, 1999]

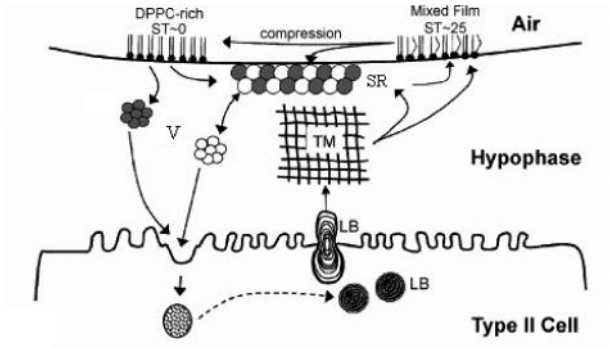

Fig. 2: Alveolar metabolism of Pulmonary Surfactant (reproduced from [Goerke, 1998] with permission from Elsevier)

morphological form called tubular myelin (TM, Fig. 3). TMs are large square elongated tubes, ranging in size from nanometers to microns.

The surfactant components are subsequently released from TM to form a surface active film at the air-water interface of alveoli through rapid adsorption. It consists of a surface monolayer, one or more lipid bilayers/multilayers closely and functionally associated with the interfacial monolayer. These multilayers form the surface-associated "surfactant reservoir" [S Schurch *et al.*, 1995]. The surfactant film is periodically compressed and expanded during breathing, by modulating the surface tension in the lungs. After performing its physiological functions, surfactant is released as small vesicles. Some of this spent surfactant is taken up by the alveolar macrophages while most of the remainder is cleared from the alveolar space by endocytosis back into type II cells. These cells recycle part of the surfactant components into lamellar bodies. Some surfactant apparently flows into the tracheae and is eventually swallowed. The estimated turnover period of PS is surprisingly short, ranging from 4 to 11h [Baritussio *et al.*, 1981].

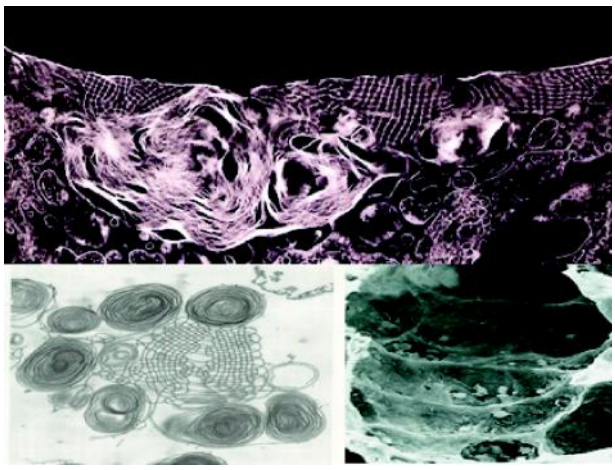

Fig. 3: Electron microscopic image of lung surfactant reservoir (Tubular myelin) (Reproduced from [S Schurch *et al.*, 1995], with permission from the Karger Publishers)

Composition and Related Functions of PS

Comparative biological studies suggest that PS exists in all air breathing vertebrates, with somewhat differing compositions [Postle *et al.*, 2001]. However, the composition of mammalian PS is remarkably similar among diverse species, *i.e.*, approximately 90% lipids and 10% proteins by weight (Fig. 4).

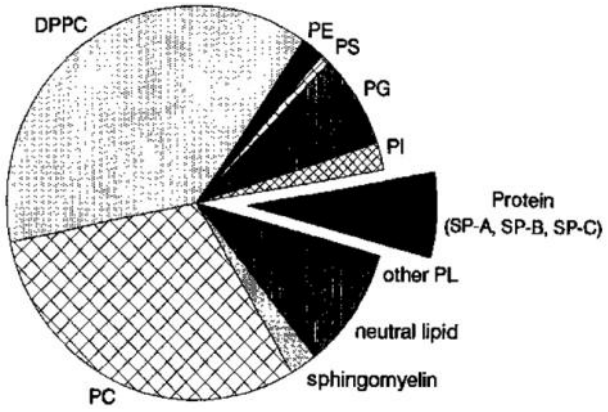

Fig. 4: A general composition of pulmonary surfactant (reproduced from [Nag and Keough, 1993] with permission from the Elsevier)

Four proteins are associated with PS (SP)-A, -B, -C and -D, based on the nomenclature proposed by Possmayer [Possmayer *et al.*, 1984]. These proteins are divided into two groups, SP-B and SP-C are two small hydrophobic proteins, while SP-A and SP-D are large hydrophilic proteins.

Lipids

The main constituents of PS are lipids and the composition of the lipid pool is quite different from that of other membrane systems (Table 1). The lipids mainly consist of phospholipids, (PL, 90-95 wt%) with a small amount of neutral lipids (5-10 wt%). Among the PL components, in all mammalian species, surfactant contains high amounts (approximately

Table 1: General composition of the mammalian pulmonary surfactant (reproduced from [Panda *et al.*, 2004] with permission from the Elsevier)

Molecular species	Mass	%Total ± S.D.
16:0, 14:0 PC	706.5	7.3±0.28
16:0, 16:0 PC*	720.6	5.8±0.66
16:0, 16:1 PC	732.6	13.4±1.04
16:0, 14:0 PC (DPPC)	734.6	37.1±0.66
16:0, 18:2 PC	758.6	5.8±0..63
16:0, 18:1 PC	760.6	25.4±0.98
18:0, 18:2 PC + 18:1,18:1 PC	786.8	2.8±0.63
18:0, 18:1 PC	810.6	2.4±0.42
16:0, 16:1 PG	719.4	11.4±0.73
16:0, 16:0 PG (DPPG)	721.5	16.1±0.71
16:0, 18:2 PG	745.5	12.9±0.62
16:0, 18:1 PG (POPG)	747.5	34.4±0.89
18:0, 18:1 PG	769.5	9.3±0.96
18:0, 18:2 PG+ 18:1,18:1 PG	771.5	8.9±0.73
16:0, 18:1 PI	8.5.5	6.9±0.48

*16:0a, 16:0 PC, alkyl ether PC

80%) of phosphatidylcholine with DPPC, the long-chained disaturated zwitterionic PL as the most abundant constituent with a few exceptions. Representative mass spectra of mammalian lung surfactant are shown in Fig. 5. Many species also contain significant levels of palmitoylmyristoylphosphatidylcholine (PMPC)or unsaturated, *e.g.*,

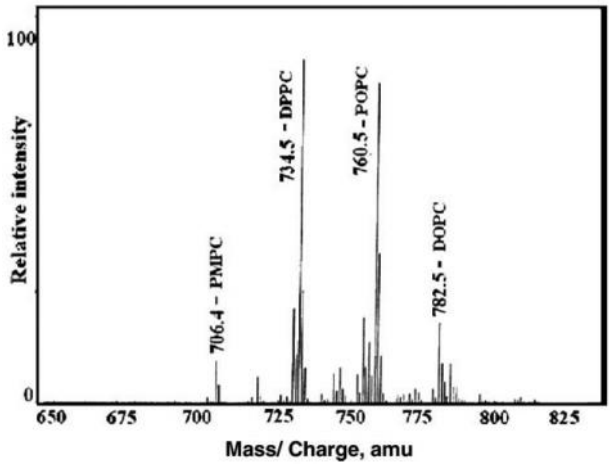

Fig. 5: ESIMS-spectra of the major phosphocholine (PC) species present in goat lung surfactant. Expanded diagram between 650 and 850 Da is illustrated (reproduced from [Mukherjee *et al.*, 2008])

palmitoyloleoylphosphatidylcholine (POPC) [Veldhuizen and Haagsman, 2000]. Apart from PC, other classes of PL are mainly anionic (*e.g.*, phosphatidylglycerol (PG), phosphatidylinositol (PI), and lyso-bis-phosphatidic acid), and non-PC zwitterionic PL (*e.g.*, phosphatidylethanolamine (PE) and sphingomyelin) [Veldhuizen and Haagsman, 2000].

Cholesterol is the most abundant neutral lipid present in PS [Veldhuizen and Haagsman, 2000]. DPPC is responsible for generating a near-zero surface tension at the interface during compression. The two saturated acyl chains of the lipid form a compact monolayer capable of achieving low surface tension values without collapsing. The unsaturated PC lipids are important in the formation of a lipid reservoir, in the initial adsorption of lipids to the interface and in the regulation of surface tension during the respiratory cycle. They also have other intracellular functions, *viz.*, lamellar body assembly, transport or secretion, although specific role of the unsaturated lipids still remain unclear.

The high amount of anionic phospholipid (PL), phosphatidylglycerol (PG) in PS is important. PC-PG mixtures have better adsorptivity than the individual component, even in the presence of SP-B and SP-C. SP-B and SP-C being positively charged, enhance the surface activity of the oppositely charged lipid mixtures. The level of PI, (also negatively charged), is usually low, with some exceptions. Cholesterol increases the fluidity of the DPPC-rich surfactant membrane systems; other minor lipids like lysophosphatidyl choline (lyso-PC), phosphatidyl ethanolamine (PE) induce the structure of curvature in TM [Veldhuizen and Haagsman, 2000].

Surfactant Proteins

Surfactant protein A, the first surfactant-specific protein to be detected, has a molar mass of 26 kDa. [Timothy E. Weaver *et al.*, 1985]. The primary amino acid sequence of SP-A shows that has four structural domains: (1) a short N-terminal segment; (2) a proline-rich collagen-like domain; (3) a neck region; and (4) a carbohydrate recognition domain. The active form of SP-A is an octadecamer built up from six trimers which forms an open flower bouquet [Voss *et al.*, 1988]. SP-A is involved in surfactant function and homeostasis, including: (a) TM formation [Clark *et al.*, 1995], (b) protection of the surface film against

protein inhibition [Strayer *et al.*, 1996], (c) enhancement of SP-B's surface activity [Venkitaraman *et al.*, 1991], and (d) regulation of uptake and secretion of surfactant by type II cells [Horowitz *et al.*, 1993], *etc*. SP-A also has a role in host defense [Henk P. Haagsman, 1998]. Surfactant dysfunctions due to the lack of SP-A are subtle and show up only in stressed situations [Veldhuizen and Haagsman, 2000].

Surfactant protein B is a hydrophobic protein that consists 79 amino acids and forms a homodimer of 17 kDa [Hawgood *et al.*, 1987]. Being hydrophobic, it interacts with lipids, as evidenced by electron spin resonance [Perez-Gil *et al.*, 1995], and fluorescence anisotropy [Baatz *et al.*, 1990]. Due to the presence of three intramolecular disulfide bridges and one intermolecular disulfide bridge, it exists in the dimer form [Johansson *et al.*, 1991]. Fourier transform infrared experiments have shown that 27-45% of the secondary structure of SP-B is helical, independent of the presence of lipids or calcium [Pastrana *et al.*, 1991]. SP-B assists the formation of lamellar body inside the type II cell. Lethal respiratory distress is caused by SP-B deficiency in humans [Klein *et al.*, 1998]. Promotion of lipid adsorption to the air-liquid interface, formation of TM, re-spreading of films from collapse phase [Taneva and Keough, 1994], reuptake of surfactant by type II cells [Horowitz *et al.*, 1996], stabilization of monolayer lipid films, membrane binding, membrane fusion, *etc*., are some of the functions of SP-B. SP-B can bring two membranes in close proximity. SP-B is also found in host defense; it can inhibit bacterial growth [Kaser and Skouteris, 1997]. Detection of SP-B in the gastrointestinal tract (as well as SP-A and SP-D) [Eliakim *et al.*, 1989], further supports more extended function of SP-B than just surface activity related ones.

The extremely hydrophobic surfactant protein C is the PS protein in the true sense as it is exclusively present in PS [Timothy E Weaver and Whitsett, 1991]. Its only site of synthesis discovered so far is the alveolar type II cell. SP-C is a 35 amino acid peptide which is formed from a 21kDa pro-protein after cleavage of the N- and C-terminal precursor parts. Other conserved characteristics are two positive charges and mostly two palmitoylated cysteines in the N-terminal part, flanked by 2 proline residues [Curstedt *et al.*, 1990]. The structure of the protein in an apolar solvent has recently been resol ved by NMR, which features a valyl-rich K-helix formed by amino acids 9-34. This correlates well with secondary structure analysis by circular dichroism and FT-IR [Pastrana *et al.*, 1991]. In contrast to SP-B, there are only a few activities of SP-C, which mostly overlap with SP-B's activities, *e.g.*, promotion of lipid adsorption onto the air-liquid interface, re-spreading of films from their collapse phase, re-uptake of surfactant by type II cells, and stabilization of the monolayer lipid film [Qanbar *et al.*, 1996]. All these functions are extracellular, and except for surfactant re-uptake, it also contributes to film homeostasis.

Surfactant Protein D

It is argued that SP-D is not a true surfactant protein. Only a small part of SP-D (less than 10%) is associated with surfactant phospholipids [Possmayer *et al.*, 1984], and the production of SP-D is not exclusive in the lung; SP-D mRNA is also found in gastric tissue [Fisher *et al.*, 1989]. The mature human SP-D polypeptide chain contains 355 amino acid residues, and the molar mass of this protein is 43 kDa under reducing conditions. The monomeric subunit of SP-D consists of four regions similar to that of SP-A. Electron microscopy reveals a highly homogenous quaternary structure of SP-D in the form of a cross [J Lu *et al.*, 1993]. SP-D does not behave like a classical surfactant protein. It has been demonstrated that SP-D binds to several bacteria (*Escherichia coli, Klebsiella pneumoniae, Salmonellapara typhi, and Pseudomonas aeruginosa*), and alveolar macrophages (Kuan *et al*., 1992). It also helps in bacterial agglutination, protection against nonbacterial microorganisms and viruses, *etc.* [Escamilla *et al.*, 1992].

PS at the Air-Water Interface

The five most important surface properties of an effective PS are (1) rapid film formation by adsorption of surface active materials from the hypophase; (2) achievement of near-zero surface tension on film compression, to prevent alveolar collapse and reduce the work of breathing; (3) low film compressibility which allows achievement of a near-zero surface tension on minimal compression; (4) a stable film which maintains low surface tension over hours; and (5) effective replenishment of surfactant molecules at the air–liquid interfaces during surface expansion.

In vitro Evaluation of PS

The progress in understanding the proper functions of PS and its constituents has paralleled the invention of new experimental techniques. Various *in vitro* techniques are commonly used for studying the surface properties of PS under dynamic conditions which simulate those present *in vivo* in the pulmonary system.

Langmuir-Wilhelmy Balance (LWB)

One of the first model systems used to examine surface activity of several surfactant components is the Langmuir-Wilhelmy balance, introduced by Clements [Clements, 1957] (Fig. 6). In this method, the surface tension at an air-water interface is directly measured by a small plate (or a filter paper) attached to a balance. One of the major advantages of this system is that a well-defined monolayer of lipids and proteins can be spread at the air-liquid interface. Animated version of the Langmuir-Blodgett surface balance is freely available in the website (https:// wn.com/langmuir_ blodgett_animation). Radiography and different microscopic measurements of PS films can be done as an attachment with the LWB [Nag *et al.*, 1998]. The use of fluorescence labeled proteins or lipids enables visualization of the formation of lipid-protein domains at varying conditions. With these experiments, considerable insights into the structure and dynamics of the surface film have been obtained. They are used in conjunction with Brewster angle microscopy [Piknova and Hall, 2001], fluorescence microscopy [Harbottle *et al.*, 2003], confocal microscopy [Bagatolli, 2006], scanning near-field optical microscopy [Flanders *et al.*, 2001], atomic force microscopy (AFM) [Harbottle *et al.*, 2003], grazing incidence X-ray diffraction [Alonso *et al.*, 2005], infrared spectroscopy [Phang *et al.*, 2005], and time of flight secondary ionization mass spectrometry (ToF-SIMS) [Harbottle *et al.*, 2003] for film imaging/ analysis of PS, and they can provide valuable information that complements the traditional tensiometry techniques. Amongst them, AFM has been used most extensively for PS studies. The PS monolayer in the LWB is transferred to mica sheet (or other substrates) by the Langmuir-Blodgett (LB) technique, and the fluid and gel phase regions within the lipid are determined [Harbottle *et al.*, 2003]. One of the disadvantages of the Wilhelmy balance is that it is a relatively static method for surface tension measurements. The surface area can be varied by moving a barrier at the surface, but this is a slow process. Despite this limitation, important information can be obtained for which it continues to be a potential instrument to determine surface film structure and activity of surfactant components.

The biophysical property of pulmonary surfactant can conveniently be studied by an *in vitro* method using a Langmuir surface balance. Its use is a well-developed technique in studying the amphiphilic monolayer [Harbottle *et al.*, 2003]. Amphiphiles, dissolved in suitable organic solvent (which are highly vaporizable), are spread over the air-water interface in a Langmuir surface balance. After sufficient time is allowed for the evaporation of solvent and equilibration of the solvent spread film, it is subjected to compression (Fig. 6).

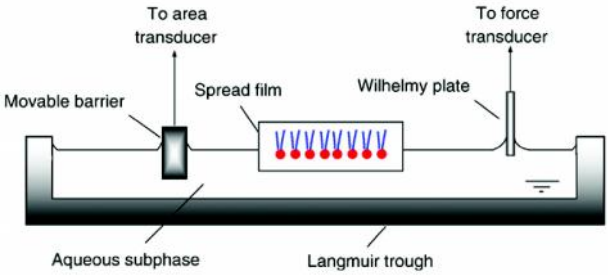

Fig. 6: Schematic of a Langmuir-Wilhelmy balance (LWB). The surfactant film is usually formed by spreading at the air-water interface of the aqueous subphase filling the Langmuir trough. Moving the barrier to the right decreases the area of the spread film, thereby increasing surface pressure (www.wn.com)

The technique can simply provide a number of parameters, *viz.*, lift-off area (the area from which the surface pressure starts rising), limiting molecular area, film compressibility and the maximum attainable surface pressure (also known as collapse pressure) of the monomolecular film. Besides, the mutual miscibility among the components can conveniently be studied by such a device. The two dimensional phase transition, occurring in the monomolecular films, can be processed in obtaining the film compressibility. The reciprocal of compressibility called compression modulus, *i.e.*, C_s^{-1} is defined as:

$$C_s^{-1} = -A\left(\frac{\delta\pi}{\delta A}\right)_T \quad (1)$$

where, A and π represent the film area and surface pressure, respectively. In the case of pure surface, C_s is infinite as surface pressure does not change with area.

The lower the compressibility, the more beneficial is the surfactant as less of an area change is required for reduction of surface tension, leaving large surface area for gas exchange.

Pulsating Bubble Surfactometer (PBS)

PBS, first describedby Enhorning in 1977 [Enhorning, 1977], consists of a small sample chamber, connected to the atmosphere by a small capillary (Fig. 7). An air bubble attachedto the capillary is pulsated by varying the pressure inside the cuvette which generates a dynamic '*in vitro* alveolus' system. Surface tension is calculated using the law of Young and Laplace from the pressure gradient across the bubble. The maximum and minimum surface tensions during cycling indicate the adsorption capability of the sample and the enrichment in DPPC of the monolayer, respectively. The advantage of the system is that it is a fast and easy method to investigate surface activity of samples. The main disadvantage is the accuracy of the measurement which decreases due to leakage of material through the capillary. At low surface tension values, the bubble flattens and sometimes detaches from the capillary which also reduces its accuracy. But, since its invention, experiments utilizing the PBS have significantly contributed to the understanding of surface tension reduction by PS and the role of the surfactant proteins in this process [Possmayer, 1990].

The Young-Laplace law states that the pressure ($\Delta \boldsymbol{P}$) required to distend a sphere is directly proportional to the surface tension (g) at the spherical interface and inversely proportional to the radius (r) of the sphere as shown in the following equation [Possmayer, 1990]:

$$\Delta P = \frac{2\gamma}{r} \qquad (2)$$

The unique ability of PS to decrease the surface tension of the interface during expiration and increase the surface tension during inspiration, helps in stabilizing the alveoli. However, in the preterm newborns, due to the deficiency of PS, the lung collapses as the alveoli can not achieve lower surface tension (Fig. 1B). [Avery and Mead, 1959].

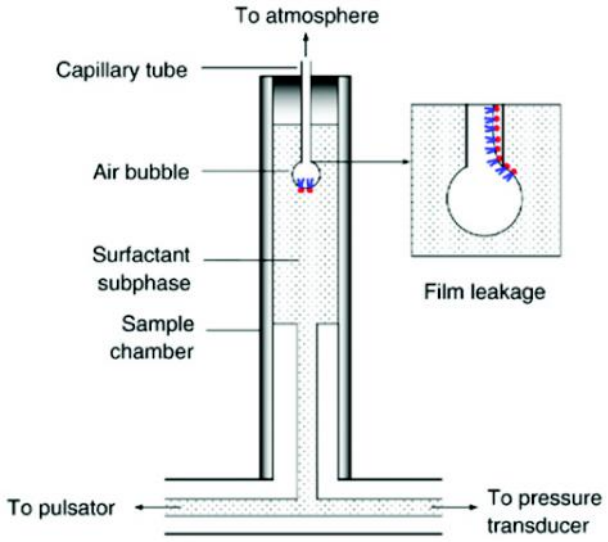

Fig. 7: Schematic diagram of a PBS. The polyacrylamide chamber filled with the sample to be tested is positioned on the pulsating unit. Sufficient fluid is withdrawn by the pulsator such that the sample water level moves down through the capillary and a bubble with minimum radius is created. The pressure difference across the bubble is recorded to monitor surfactant adsorption, and is reported as surface tension. The bubble is then pulsated between minimum and maximum radius and surface tension is recorded. The inset illustrates film leakage (reproduced from [Zuo *et al.*, 2008] with permission from the Elsevier)

Captive Bubble Surfactometer (CBS)

CBS was introduced by Schürch [S Schurch *et al.*, 1989] and it is a modified PBS (Fig. 8). In this an air bubble is introduced into a buffer solution in an air-tight cuvette. The bubble floats against a hydrophilic agar gel that fills the upper part of the cuvette.

A thin layer of buffer solution separates the bubble from the agar gel, thereby preventing interaction between the agar and the hydrophobic phospholipids/proteins at the air-water interface of the bubble. It is more leak-proof than the PBS. Surface tension is calculated from the shape of the bubble which is monitored by a video camera. The shape changes from sphere at high surface tensions to oval at low tensions can be achieved by changing the pressure inside the cuvette, done either manually by changing the volume of the subphase or by using a pressure device. The CBS is a dynamic fast technique which resembles natural breathing frequencies. In addition, the CBS is more accurate and reproducible than the PBS [Putz *et al.*, 1994]. A major disadvantage

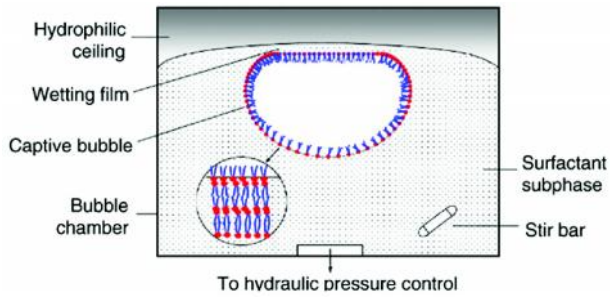

Fig. 8: Schematic diagram of a CBS. The captive bubble floats against the hydrophilic ceiling but is separated from it by a thin wetting film. The inset shows multilayer structures of the adsorbed PS at the air–water interface of the bubble. The surface tension is determined by bubble shape analysis. Surface area manipulations are conducted by altering the hydraulic pressure in the chamber (reproduced from [Zuo *et al.*, 2008] with permission from the Elsevier)

of CBS over PBS is more time consumption of the former.

Other In vitro Methods

Other *in vitro* methods for assessing the surface activity of surfactants, include constrained sessile drop [Wulf *et al.*, 1999], micro-bubble stability studies [Berggren *et al.*, 1992], capillary surfactometer [Enhorning and Holm, 1993], oscillating drop surfactometer [Meier *et al.*, 2000], pendant drop (PD) tensiometry [Kwok *et al.*, 1994], axisymmetric drop shape analysis [Cabrerizo-Výlchez *et al.*, 1999], etc.

Adsorption of PS

Rapid adsorption of PS onto the air-alveolar interface is important for its proper function. Phosphatidyl-choline reduces surface tension quite effectively, but functions poorly in the lung when used alone, as it adsorbs slowly at the air-liquid interface [Hall *et al.*, 1992]. PS lacks the ability to restore the mechanical characteristics of an excised lung depleted of surfactant by repeated lavage [Hall *et al.*, 1992]. During the breathing cycle, for a constant replenishment of surface materials PS needs the intrinsic capacity to transit rapidly from its three dimensional arrangements in the hypophase into the two-dimensional film at the surface. Samples of surfactant purified from alveolar lavages adsorb very quickly [Perez-Gil *et al.*, 1992a], and they usually have a mixture of the three dimensional physical forms of surfactant including secreted lamellar bodies, tubular myelin (TM) and other loosely packed bilayer forms. Presence of surfactant proteins and other unsaturated PL species improve the adsorptivity of PS, but it is still inferior to the natural materials [Perez-Gil and Keough, 1998]. Dispersed bilayers, reconstituted from natural surfactants, show better surface activity [S H Yu and Possmayer, 1986]. SP-B and SP-C proteins are critical in promoting rapid transit of bilayer-based surfactant structures from the subphase to the interface; SP-B is usually more active than SP-C, and their effects are additive [Z. Wang *et al.*, 1996]. The adsorption process can be better understood by the combinations of Fig. 2 and Fig. 9 (the final step is exemplified in Fig. 9).

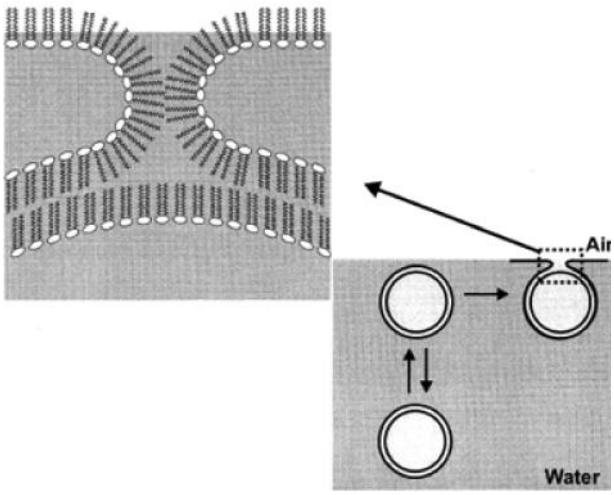

Fig. 9: Enlarged view of final step of adsorption; transfer of surfactant in the air-liquid interface (reproduced from [Schram and Hall, 2001] with permission from the Elsevier)

Transport of Surfactant to the Surface

Surfactant adsorption onto the interface is a concerted kinetic process of molecules and assemblies. Changes in the environmental conditions, like increase in hydration, change in the concentration of Ca^{2+}ion, increase in pH, induce unpacking of lamellar bodies and interfacial adsorption. SP-B and SP-C influence packing of bilayers and monolayers. TM is associated with highly surface active material as a precursor and a reservoir for the surface film [H. P. Haagsman *et al.*, 1989]. For an *in vitro* reconstitution of TM the minimal components required are DPPC, PG, SP-A, SP-B and Ca^{2+} [Suzuki *et al.*, 1986]. Bilayers at the

corners of TM are under very high curvature strain. These regions are the initiation sites for concerted transfer into the interface, a process potentiated by SP-A. SP-B and SP-C can independently promote interfacial adsorption of phospholipids [Perez-Gil *et al.*, 1992a]. SP-B induces lipid vesicle aggregation and fusion [Cruz *et al.*, 1997] which, in turn, enhances the cooperative movement of materials to the surface. Studies support that SP-B, and not SP-C is the essential protein for surfactant film dynamics and stabilization [Veldhuizen and Haagsman, 2000]. Inactivation of SP-B leads to a complete inhibition of lamellar body adsorption but has no effect on the adsorption of native surfactant purified from bronchoalveolar lavage.

Transfer of Surfactant into the Air-liquid Interface

Phospholipid bilayers need to be disrupted to allow the phospholipid molecules to move to the interface. SP-B and SP-C perturb packing of phospholipid molecules in model monolayers and bilayers [Perez-Gil *et al.*, 1995]. These increase the apparent polarity of the surface of DPPC bilayers, which relaxes the membrane packing to certain extent, and thus facilitates phospholipid adsorption. Perturbation of bilayers by SP-B usually occurs through the interaction of cationic amphipathic K-helical segments of the protein with the surface of surfactant bilayers. SP-C perturbs the acyl chain packing of phospholipid bilayers [Perez-Gil *et al.*, 1995] and monolayers and thus facilitates the exchange of molecules between both the structures. With increasing surface pressure (defined as the extent to which a film reduces the surface tension of a clean interface, denotes the two-dimensional equivalent pressure in three dimensions, or the force exerted by an interfacial film on its linear confining boundaries, with units of force length^{-1}) the hydrophobic K- helix of SP-C reorients in monolayers, and acts as a 'lever' to raise the phospholipid acyl chains in the interface and, therefore, facilitates insertion of more lipid molecules. SP-B fluidizes the monolayer at low to intermediate surface pressure, while SP-C efficiently increases compressibility at higher surface pressure. Stacked bilayers, adjacent to the monolayer, are formed solely in the presence of surfactant proteins. Compounds with mixed acyl groups also promote the fusion of vesicles with the interface. Adsorption of PS at the air-water interface lowers the surface tension (γ) at rates that initially decrease progressively, but accelerate near the equilibrium [Loney *et al.*, 2011]. SP-B and SP-C are the key constituents, both for achieving the surface tension (γ) at which the acceleration for the adsorption process occurs. The surface tension at which the rate of adsorption increase is unaffected by the concentration of protein in the films. Insertion into a more densely occupied surface occurs more slowly as g approaches its equilibrium value (γ_e). PS initially demonstrates this kinetics, lowering g at rates that progressively low down. In the lung, where PS exerts its biological function,when the alveolar air-water interface expands during inhalation, the rise of γ above γ_e is limited [Inoue *et al.*, 1982]. These discrete processes of adsorption have different dependence on phospholipid composition and its concentration both in the subphase and at the interface. Initially, when the surface tension is high relatively little material is there at the interface. At this stage the adsorption rate depends on the concentration of the surfactant in the subphase. The transport process that begins in the subphase, to the interface might be the crucial step in achieving the equilibrium surface tension. Subsequently, when a significant amount of surfactants are adsorbed at the interface, the dependence shifts. The rates vary only with surface tension and are unaffected by subphase concentration, suggesting a process that depends only on material at the surface. Schram *et al.* [Schram *et al.*, 2001] have carried out a detailed thermodynamic study of the adsorption process of PS and have concluded that although the thermodynamic barrier to adsorption of the surfactant lipids is primarily the entropy of transition, the hydrophobic proteins accelerate adsorption by reducing the enthalpy of activation.

Phase Behavior of PS

The function of PS is considered in terms of two phase transitions. The first rapid one is the conversion of bilayer vesicles into the interfacial film (adsorption), and the second transition (must be avoided) is the collapse from the interface. The mechanistic steps of adsorption have been explained in detail in the previous section. When the monomolecular films of PS are compressed at the air-water interface, they exist under equilibrium condition up to a maximum surface pressure (π_e), above which they collapse from the two-dimensional interface to form three-dimensional

structures. PS films are sufficiently rigid and can exist at higher surface pressures in metastable states for few tens of minutes [S F Schurch and Roach, 1976]. This characteristic of PS is important for the stabilization of small air spaces of the lungs. Phase behavior is an essential determinant for both the precursor structures; bilayers for adsorption, during which insertion of surfactant constituents into the interface raises the surface pressure to π_e, and for the metastability of the monolayers that allows compressed films to avoid collapse and reach surface pressures above π_e [Piknova *et al.*, 2002]. When dispersed in aqueous phase, at optimum temperature and pressure, most PL molecules spontaneously form bilayers, or vesicles. With increasing temperature, PL bilayers melt from an ordered gel (L_β) phase (solid phase) to a disordered liquid-crystalline (L_α) phase at the main transition temperature (T_m), which permits diffusion of individual PL molecules within each leaflet of the bilayer, referred to as "fluidity". Addition of cholesterol transforms PL bilayers in L_β and L_α phases into a liquid-ordered (L_o) phase and a liquid-disordered (L_d) phase, respectively. The overall fluidity of the bilayers in the four phases is $L_\beta < L_o < L_d < L_\alpha$. Thus,cholesterol has a fluidizing effect on lipids in the gel state (because it disrupts packing) and a condensing effect on fluid membranes (because it interacts with disordered chains and stabilizes them).

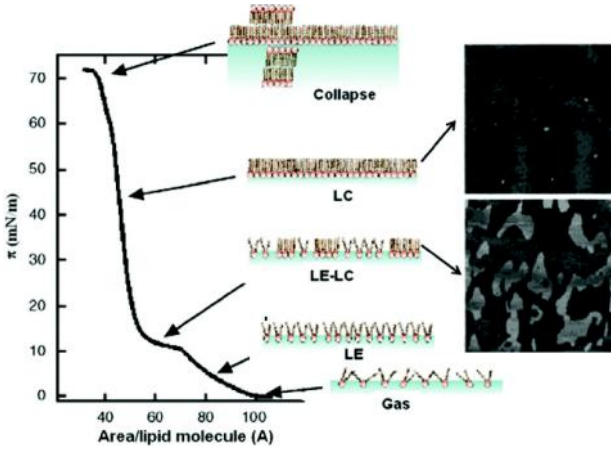

Fig. 10: π-A isotherm and characteristic film structures of a DPPC monolayer studiedby Langmuir balance and AFM at 298K. (LE: liquid-expanded phase, LC: liquid-condensedphase, LE–LC: liquid condensed-liquidexpanded coexistence)(reproduced from [Feng *et al.*, 2002] with permission from the American Chemical Society)

Monolayers

Experimentally, the phase behavior of PS monolayers is studied in a Langmuir film balance [Feng *et al.*, 2002], by monitoring surface pressure (π) *vs.* area (*A*) at constant temperature. Fig. 10 shows the π-A isotherm and phase transition of DPPC in a Langmuir film balance. At large surface areas, monolayers are 'gaseous'; as the monolayer is compressed it is transformed into the liquid-expanded (LE) phase. Further compression leads to a first-order transition to the 'liquid-condensed' (LC) phase. This generates a surface with LE-LC coexistence where the decreased surface area is taken up by forming a greater proportion of LC phase. In single component monolayer, the LE-LC coexistenceis marked by a plateau in the isotherm; for multicomponent mixtures, the plateau may be absent. Nucleation and growth of the LC phase in the monolayer can be directly visualized as "domain" formation which is a pressure induced crystallization process by fluorescence microscopy (fluorescent dyes preferentially locate in the more disordered LE phase and are expelled from the better-ordered LC phase) [Nag *et al.*, 1998] or by atomic force microscopy (AFM) [Panda *et al.*, 2004].

Above the phase transition temperature (T_m) of lipid, only the LE phase is present. Most of the disaturated phospholipid molecules undergo two dimensional phase transition. The unsaturated lipids present in native and replacement lung surfactants, such as POPC or POPG, have T_m below 20°C [Takamoto *et al.*, 2001], and hence always form LE phases at physiological conditions. The disaturated PCs (DPPC) and PGs present in PS have high T_m (41°C) and form LC phases [Crane and Hall, 1999]. The LC domains in mixtures containing saturated and unsaturated PCs and PGs consist of the saturated lipids, with the fluid phases made up of the unsaturated lipids and surfactant proteins in the monolayer [Discher *et al.*, 1999]. The fraction of LC phase depends on the surface pressure, average molecular area, temperature, and on the ionic species present in the subphase. Over a range of surface pressures and temperatures between 20 and 37°C, the hydrocarbon chains in the LC domains of DPPC exhibit a lattice, with molecules tilted in a non-symmetry direction [Bringezu *et al.*, 2001]. On compression, the tilt angle

decreases, but tilts greater than 25 degrees are observed at π above 40 mNm^{-1}. This is due to the large area required for the well-hydrated polar head group of DPPC relative to the smaller area required for the hydrocarbon chain. Palmitic acid and hexadecanol have relatively small headgroup areas relative to the chains. Hence, mixtures of palmitic acid or hexadecanol with DPPC can specifically interact to produce new crystalline close packings, and thus increase the LC-LE ratio of DPPC rich monolayers [Bringezu *et al.*, 2001]. On the other hand, cholesterol and other neutral lipids appear to have the opposite effect on PS monolayers [Discher *et al.*, 1999]. So the DPPC films in the absence and presence of palmitic acid (or hexadecanol) differs in the compressibility and are referred to as LC and ''solid'' states, respectively. The DPPC monolayer possesses the same degree of translational order in both the cases. The X-ray diffraction studies show that the hydrocarbon chains of the molecules are aligned parallel to each other; they are either tilted (LC) with respect to the water surface or perpendicular to it (solid) [Discher *et al.*, 1999]. On further compression, the monolayer becomes unstable and it can (1) fracture and break, (2) buckle, or (3) lose material depending on the elastic and solubility properties of the monolayer [Crane *et al.*, 1999]. Surface pressure at this collapsed stated is called collapse pressure (π_c). Beyond π_c, molecules cannot maintain their ordered 2D arrangement and begin to form multilayers (3D) (Fig. 11).

The most impressive characteristic of PS is the prolonged metastability of the interfacial films *in situ*

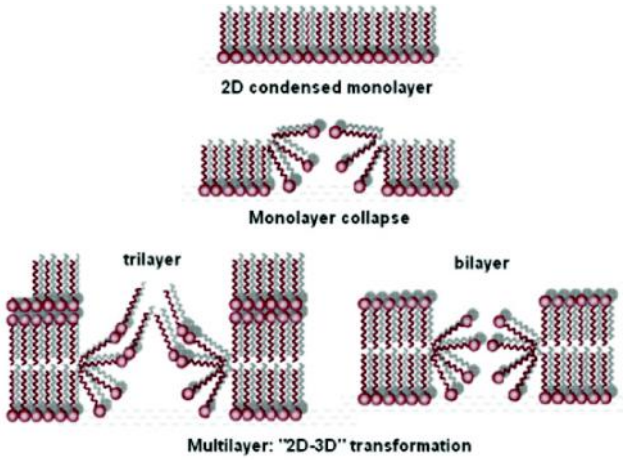

Fig. 11: Schematic diagram of film collapse

at surface pressures well above π_c [S Schurch, 1993]. Resistance to collapse indicates the rigid structures of the solid film. In order to maintain the operation of the smaller alveoli, compressibility should remain relatively low, and the surface tension should be stable over few minutes. As DPPC is the only constituent that can form a condensed phase at physiological temperatures, therefore the film must be greatly enriched in DPPC. Separation of DPPC from other constituents produces solid/fluid coexistence at the interface. Incase of pure phospholipids, natural surfactant (having all the components) or microscopic studies on simple model system have revealed the coexistence of solid/fluid phases. Both fluorescence microscopy and Brewster angle microscopy (BAM) can distinguish isolated domains surrounded by continuous phase which have irregular shapes, invariant optical thickness during compression and anisotropy expected for a condensed phase [Discher *et al.*, 1999]. The discontinuous domains grow in proportion with the added DPPC and extend up to pure DPPCconcentration. This provides indirect evidence that the condensed phase contains mainly DPPC. Two mechanisms have been proposed to produce such a film [Perez-Gil and Keough, 1998]. Firstly, DPPC selectively adsorbs at the interface, and secondly, non-DPPC constituents get selectively excluded.

Formation of Surfactant Reservoir

Surfactant reservoir is a complex membrane system comprised of several tightly packed surfactant bilayer (membranes) structures closely associated with the interfacial monolayer [Possmayer *et al.*, 2001]. Wilhelmy balance studies clearly evidence that lipids that are squeezed out during compression of the monolayer, stay in close proximity to the interface [Taneva and Keough, 1994]. During the next expansion, these lipids can re-insert. This dynamic cycling can be performed without significant loss of material in the presence of SP-C and SP-B. SP-B and SP-C promote insertion of lipids from vesicles associated with a preformed spread phospholipid monolayer. These associated vesicles are difficult to wash away with extensive flushing of the subphase, even in the absence of calcium ions, indicating that these are firmly attached to the monolayer. There are evidences that SP-B and mostly the palmitoyl chain of SP-C facilitate formation of surface associated

surfactant-reservoir by bridging the monolayer to the lipid bilayers underneath [Qanbar *et al.*, 1996]. Model studies suggest that there exists a continuous and dynamic flow of surface active lipid species between the monolayer directly located at the interface and the associated bilayer structures [Perez-Gil, 2002]. During compression, unsaturated phospholipids, surfactant proteins are selectively "squeezed-out" of the interface which refines the composition of the interfacial film to end in a DPPC-enriched state.

Re-spreading or Renewal of Expanded Surfactant Film

It is anticipated that re-expansion of the interface containing a film and a collapsed phase that is rich in DPPC would lead to incomplete re-spreading and insufficient refilling of the available surface. The result is that during film expansion there is transient production of 'empty' surface spaces. Replenishment of the surface can happen via relaxation of compressed components of the film, adsorption from subphase structures as discussed earlier and re-spreading of both collapse phases and selectively excluded phases (Fig. 12).

Monolayers prepared from organic extracts of surfactant behave like the whole natural preparations in this respect, indicating the important role of surfactant proteins in re-spreading the lipid on to the monolayer [Holm *et al.*, 1996]. The portion of SP-B or SP-C which appears to remain in the collapsed phase, and also in the selectively excluded phase, can enhance the rate of redistribution of material in the interface upon expansion. Epifluorescence microscopy of monolayers and atomic force microscopy of Langmuir-Blodgett films confirm that the appearance of films after compression and re-expansion to low surface pressure (π) closely resembles that of films during their initial compression stages. The exclusion of the protein occurring during compression can be reversed on expansion since the material excluded from the interface seems to be closely associated with the monolayer. The hydrophobic proteins associated with the excluded phases help to form associated multilayers at the surface which have significance not only in surface replenishment, but also in surface stability. The presence of the protein containing folds extending into the subphase at high compression also provides a mechanism for incorporation of new material into the interface upon expansion. Negatively charged lipids in the monolayer as well as in the vesicles are also relevant for rapid re-spreading [Possmayer *et al.*, 2001].

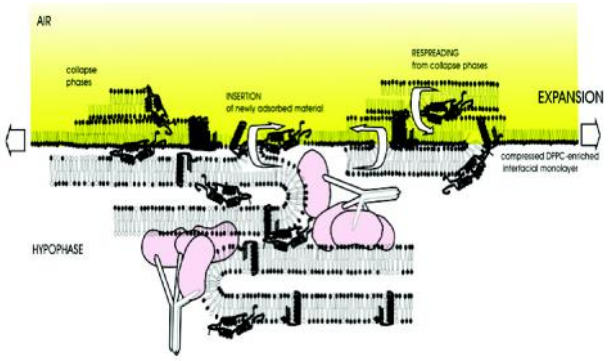

Fig. 12: Re-spreading of surfactant film on expansion (reproduced from [Perez-Gil and Keogh, 1994] with permission from the Elsevier)

Hysteresis

Results of cycling experiments (repeated compression and expansion of the PS film) depend both on the speed of cycling and the range of pressure or surface coverage. A monolayer compressed to the maximum, followed by an expansion, simulates the breathing processes although slowly. The reversible squeeze-out procedure is changed when the film is repeatedly over-compressed into the collapse region. Such procedure yields irreversible collapsed structures, which causes an effective loss of surface active material. *In vitro* studies have shown that some of the materials excluded from the monolayer permanently leave the surface to form small subphase structures that have reduced the tensioactive properties [Gross and Narine, 1989]. This continuous removal of surface active material is responsible for the existence of fractions of surfactant differing in structure (density, composition, lipid/protein ratio, etc.) and function (surface activity) in the alveolar lavages. The processes of conversion of surfactant into the different sub-fractions have been studied *in vitro* by 'cycling' experiments [*Gross and Narine*, 1989]. Isotherms of monolayers formed from the whole surfactant show less hysteresis on compression-expansion cycles consistent with more rapid replenishment than those prepared from DPPC or other pure phospholipid systems [Perez-Gil *et al.*, 1992b].

Collapsed Phase

Microscopic studies have documented the detailed structures formed during the process of collapse that must be avoided in the lungs. Simple model systems indicate two forms in particular: bilayer folds from the monolayer that can extend hundreds of micrometers into the subphase and buds [Gopal and Lee, 2001] that can grow into stacked bilayer disks immediately adjacent to the monolayer [Amrein *et al.*, 1997]. Surfactant extracts or preparations derived directly from PS form only the budded structure. For both the forms, the collapsed material reversibly reinserts into the expanding surface. One of the first theories of collapse [Diamant *et al.*, 2001] hypothesizes that in a heterogeneous monolayer, the interface may not be as flat as is generally assumed. Lipid monolayers, as opposed to bilayers, usually have a finite spontaneous curvature, arising either from the shape of the lipid molecule itself, or from electrostatic interactions. Variations in composition and local molecular order can lead to differences in the spontaneous curvature and the bending modulus between domains. Hence, at and near the boundaries between the domains, the monolayer may be locally curved. The finite surface tension causes the monolayer to relax to a flat profile away from the domain boundaries, and limits the curvature at the domain boundaries. As the monolayer is compressed, the surface tension of the monolayer decreases. The curvature at the domain boundaries then grow, eventually causing the monolayer to overlap itself leading to the formation of a multilayer patch, *i.e.*, monolayer collapse. Monolayer collapse initiates at domain boundaries in model PS monolayers. Thus phospholipid films collapse by flow of the complete monolayer into the third dimension, rather than by a standard phase transition in which constituents diffuse across the interfacial boundary.

Dysfunctional Surfactant: Inhibition and Disorders of PS

If the quantity and/or quality of PS are inadequate or surfactant is inactivated, the work of breathing increases and causes neonatal RDS. The lungs of the preterm babies dying of RDS have a mean surface tension of 30 mNm^{-1} as compared to 8 mNm^{-1} in normal babies. The baby takes the first good deep breath, as any baby would. But if the newborn's immature lungs lack PS (or is inactivated), the alveoli tend to collapse when the baby breaths out which means breathing-in requires extra effort, as if every breath is like the first breath after birth. This extra effort tires out the newborn's diaphragm and tears the lung tissues with leakage of fluid into the airspaces, causing additional impairment of lung mechanics and gas exchange and leads to inflammation [Wrobel, 2004]. Many adult diseases like ARDS and lung injury also have dysfunctional PS system due to inactivation of surfactant. In the presence of PS, 2% of the total energy is utilized for breathing, whereas in its absence this increases to 33% of the total energy.

Surfactant inhibition or inactivation is a process that decreases or abolishes the normal surface activity of PS. The major inhibitory factors include plasma proteins, unsaturated membrane PL, lysophospholipids, free fatty acids, supra physiological levels of cholesterol [Notter, 2000], reactive oxygen species [Rodriguez-Capote *et al.*, 2006], pollutants [Bakshi *et al.*, 2008], etc. Surfactant inhibition can also arise from degradation of PS lipids by phospholipases or of surfactant proteins by proteases. Relative amounts of these degradation agents increase during microbial infection and through secretion by leukocytes and type II cells with pulmonary inflammation [Griese *et al.*, 1997]. Human meconium also adversely affects the surface tension-lowering activity of PS [K W Lu *et al.*, 2013]. Warriner *et al.* [Warriner *et al.*, 2002] have reported concentration dependent surfactant inhibition impact of fibrinogen, albumin, and IgG. Seeger *et al.* [Seeger *et al.*, 1993] have reported the inhibitory effect of hemoglobin along with fibrinogen and albumin on calf lung surfactant extracts, Curosurf, Survanta (commercial surfactants), *etc*. Lysophosphatidyl-choline is a superior inhibitor than albumin [Holm *et al.*, 1985]. Both the hydrophilic and hydrophobic nanoparticles induce surfactant inhibition [Hu *et al.*, 2013], the interaction mechanism between the nanoparticles and surfactant films were studied by AFM. Enhanced inhibition by hydrophobic nanoparticles as compared to hydrophilic nanoparticles is proposed to be due to their retention at surfactant film [Beck-Broichsitter *et al.*, 2011]. Anesthetics such as halothane [Molliex *et al.*, 1994], toxic agents such as polyurethane smoke [Oulton *et al.*, 1994], or certain drugs [Henk P. Haagsman and van Golde, 1985] can negatively influence the biosynthesis and function of PS *in vivo* and *in vitro*. Several other factors which

impair PS synthesis and function are nitrogen dioxide exposition [Mengel *et al.*, 1993], ozone exposure [Henk P. Haagsman and van Golde, 1985], shortage of copper during the gestation period (associated with a lower birth weight and neonatal lung abnormality [Abdel-Mageed *et al.*, 1994], iron-transferrin accumulation in epithelial lining fluid (promoting the formation of free radicals) [Hallman *et al.*, 1994], *etc*. There are mainly two distinct inhibition mechanisms: one due to competitive adsorption of plasma proteins, and other due to mixing and fluidizing an otherwise stable PS film by lipids. The former mechanism mainly influences surfactant adsorption while the latter prevents the films from reaching low surface tension (γ) [Zuo *et al.*, 2008].

RDS and ARDS are also caused due to shortage of PS and leakage of serum proteins to the alveolar space. Lungs of infants dying from RDS contain all normal components except tubular myelin. As SP-A and SP-B are essential for the formation of tubular myelin, these proteins are nonfunctional or missing [Moya *et al.*, 1994] in RDS patients. Pulmonary SP-B deficiency (named *congenital alveolar proteinosis*), originates from a deficiency of SP-B mRNA [Nogee *et al.*, 1993]. The SP-B deficiency is also associated with SP-A and SP-C abnormalities. Alveolar proteinosis is a disease in which the quantity of the alveolar material is increased but its composition is changed. Most notably, the content of SP-A is elevated and is often present as a complex with immune globulins [Kuroki and Akino, 1991]. There is an accumulation of SP-D in the lungs of alveolar proteinosis patients [Crouch *et al.*, 1993]. Silicosis in humans and animals causes somewhat similar increase in alveolar material as in alveolar proteinosis [Lesur *et al.*, 1995]. In cystic fibrosis, bronchiolar lavage of infants with associated pulmonary inflammation or infection showed the saturated phosphatidylcholine/total phosphatidylcholine ratio to be lower and the SP-A concentration to be higher than in either normals or non-inflamed cystic fibrosis patients [Hull *et al.*, 1997].

Overcoming inhibition plays a key role in developing new surfactant formulations for RDS and ARDS treatment. This can be done by two ways:

Optimizing Lipid and Protein Contents

Increase in the PL concentration of surfactant preparations can effectively reverse plasma protein-induced and meconium induced inactivation. Novel surfactant preparations were developed consisting synthetic C16:0 diether phosphonolipid (DEPN-8) in combination with 1.5 wt.% purified bovine SP-B/C [Z. Wang *et al.*, 2003] or mini-SP-B [Walther *et al.*, 2007]. The synthetic surfactant is highly resistant to inhibition due to serum proteins, phospholipase A2, and lyso-PC, both *in vitro* [Z. Wang *et al.*, 2003] and in excised rat lungs [Z. Wang *et al.*, 2007]. Optimizing the content of surfactant proteins is another effective means to reverse surfactant inactivation. Superior performance of modified natural surfactants over protein-free synthetic surfactants clearly demonstrates the importance of SP-B and SP-C analogs in overcoming surfactant inactivation. Addition of peptide analogs of SP-B and/or SP-C improves the resistance to surfactant inactivation [Walther *et al.*, 2000]. Adding SP-A to lipid extract surfactants increase their resistance to inactivation due to blood proteins [Cockshutt *et al.*, 1990] and meconium [Sun *et al.*, 1997]. *In vitro* experiments showed that small amounts of SP-A can significantly enhance adsorption and dynamic surface activity of lipid extract surfactants, thereby increasing their effectiveness at reduced PL concentrations.

Using Water-Soluble Polymers as Surfactant Additives

Water-soluble polymers as additives to clinical PS can drastically enhance its activity. First, they improve the surface activity of dilute surfactant preparations (mainly by enhancing adsorption). Second, they can effectively counteract surfactant inactivation due to a variety of inhibitory substances (such as plasma proteins and meconium), thus having a potential to enhance the clinical efficacy of surfactant therapy [Dargaville and Morley, 2000; Taeusch and Keough, 2001]. So far nonionic (*e.g.*, PEG [Taeusch *et al.*, 1999] and dextran [Kobayashi *et al.*, 1999], anionic (*e.g.*, hyaluronan ([K W Lu *et al.*, 2005]) and cationic (*e.g.*, chitosan [Zuo *et al.*, 2006]) polymers have been used. Many *in vitro* [K W Lu *et al.*, 2005; H. William Taeusch *et al.*, 2008; L M Y Yu *et al.*, 2004] and *in vivo* animal studies [Kobayashi *et al.*, 1999] have shown that these polymers significantly improve the surface activity of different clinical surfactants and reverse surfactant inactivation.

Exogenous Surfactants

RDS, ARDS and other lung diseases can be prevented by administering exogenous surfactant via the airways. The efficacy of this therapeutic approach, first demonstrated in animal models [Fujiwara *et al.*, 1980] could be confirmed in a large number of randomized controlled clinical trials. In the beginning, exogenous surfactants were made only from lipids as the structure and functional significance of the SPs were not known. In two early trials, aerosolized DPPC was used as a surfactant substitute without impressive effects. Later on, effective artificial surfactants for clinical use were prepared by mixing DPPC with spreading agents such as serum high density lipoprotein, unsaturated phosphatidylglycerol (PG), hexadecanol, *etc*. The formulations without surfactant proteins showed limited short-term therapeutic effects. Treatment of infants with RDS with isolated surfactant or synthetic surfactant has a beneficial effect on the alveolar ventilation.Surfactants can be used prophylactically or given to infants who have developed the disease, though the former is more effective than the rescue therapy, especially in infants under 28 weeks gestation, and in infants weighing less than 1,000 g [Kendig *et al.*, 1991]. But the rescue therapy is normally used as they are less oxygen dependent and less quantity of surfactant is required [Jobe, 1993]. In different surfactant preparations, there are only small differences in the rate of mortality or bronchopulmonary dysplasia [Been *et al.*, 2009]. With time, the effects of surfactant treatment of premature infants have considerably increased. Mortality and morbidity of the preterm neonates have significantly been reduced as an effect of surfactant therapy, although it has also shown limited positive effects with ARDS patients [Frerking *et al.*, 2001]. Administration of the surfactant can be done both by instillation and by nebulization though the latter gives a better distribution [Ueda *et al.*, 1994]. Exogenous surfactant probably associates with components of the endogenous surfactant.

Exogenous surfactants can be categorized, based on surfactant apoprotein content [Lewis and Veldhuizen, 2003] as (1) surfactant from human amniotic fluid, containing both the hydrophobic and hydrophilic proteins, (2) modified natural surfactants derived from either bovine or porcine sources, which contain only SP-B and SP-C (*e.g.*, BLES, Curosurf, Infasurf and Survanta) (3) synthetic surfactants that contain simplified peptides or recombinant surfactant protein analogs (*e.g.*, Surfaxin and Venticute), and (4) protein-free synthetic surfactants that consist of only PL components (mainly DPPC) and additives (*e.g.*, ALEC and Exosurf). Human amniotic fluid lacks commercial capacity due to its limited source. Animal-derived surfactants are superior to the synthetic preparations [Been *et al.*, 2009], although they too have limitations, *viz.*, (i) batch-to-batch variation in composition and risk of transmission of microbes; (ii) absence of the hydrophilic proteins (SP-A and SP-D), which are removed during the purification processes that can impose potential immunological hazards; (iii) economical synthesis or recovery of human SP-A is difficult as its host protective benefits are lost; (iv) variation of content of hydrophobic proteins (SP-B and SP-C), PL (especially DPPC, PG and PI), neutral lipids (mainly cholesterol) and additives (such as the palmitic acid and triacylglycerol in different surfactant preparations; (v) high cost, approximately US$500 per dose for premature infants. A new generation of clinical surfactants is under development [Curstedt and Johansson, 2006], which are fully synthetic and contain synthesized surfactant protein analogs. Both preclinical and clinical trials are conducted using synthetic surfactants with the addition of simplified SP-B/C-like peptides. Surfaxin is reported to be efficacious with preterm infants and in preliminary studies with ARDS [Moya *et al.*, 2005]. Venticute, a preparation based on recombinant human SP-C, is being investigated in a trial involving direct lung insult-induced ARDS. While the advantages of synthetic surfactant peptides are obvious, synthesizing fully functional analogs of hydrophobic surfactant proteins is not a trivial task. The SP-B molecule is too large and structurally complex to be easily synthesized. SP-C molecules are structurally unstable in pure form and tend to aggregate. So far, none of the synthetic preparations are known to be more efficacious than the natural preparations. Waring *et al*. [Waring *et al.*, 2005] have reported the synthesis of a number of simplified SP-B analogs which mimic the biophysical and physiological properties of the whole hydrophobic protein. Reports on the synthesis of a number of peptides with SP-B and SP-C like properties are available in the literature [Seurynck-Servoss *et al.*, 2006]. A major goal of PS research is to generate"designer" surfactants that fully or closely

mimic the biophysical properties of the endogenous surfactant and highly resist inactivation.

Evolution of Surface Activity Related Functions of Vertebrate PS

The evolutionary biological studies of lungs of different vertebrates has been undertaken by Orgeig *et al.* [Orgeig *et al.*, 2007]. In the animal kingdom, evolution of the respiratory system over the past 300 million years has produced an amazing variety of structures to satisfy a wide range of oxygen demands in vertebrates. Lungs differ considerably in structure, embryological origin, and function among the vertebrate groups. The bronchoalveolar lung of mammals is a branching "tree" of tubes leading to millions of alveoli. In humans there are ~25 branches and 300 million alveoli. This structure allows for the generation of an enormous respiratory surface area (up to 70 m^2 in adult humans). In the non-mammals, lungs are bag like with either smooth walls or large bellow-shaped respiratory units (termed faveoli) extending from the outer wall of the lung into a central air space. Animated versions on the lungs and pulmonary respiration and ventilation in humans can be found in the website: https://www.youtube.com/watch?v=Lo74zm3ULyk.

Birds have the most strikingly different lung structure, with a pair of small parabronchial lungs connected to a series of air sacs. Air is propelled via the air sacs, which act like bellows, in a unidirectional manner through the lung. The lungs consist of a series of tubes (parabronchi) from which emanate the very small diameter, rigid air capillaries, that represent the site of gas exchange. But all lungs have one common characteristic. They are internal, fluid-lined, gas-holding structures that inflate and deflate cyclically. Free animations on the avian pulmonary systems are available freely in the YouTube: Lungs and Pulmonary respiration and ventilation in humans – Biology: https://www.youtube.com/watch?v=Lo74zm3ULyk.

PS is present in the lungs of all air-breathing vertebrate groups; even in gold fish swim bladders [Orgeig *et al.*, 2007]. Lamellar bodies and TM are observed in lungs of reptiles, birds and amphibians [*Orgeig et al.*, 2007]. The alveolar metabolism of lower vertebrates is similar to the mammalian vertebrates. In all vertebrates, the major PL is PC and DPPC that forms the monolayer at the interface. Temperature has a profound effect on the lipid fluidity. DPPC has T_m of 41ºC but addition of unsaturated phospholipids (USPs) or cholesterol decreases the T_m of the mixture, improves the spreadability, and facilitates adsorption of PS. In mammals, SP-B and SP-C help in the adsorption process whereas cholesterol and USP are 'squeezed out' so that the monolayer is enriched in DPPC as explained in previous sections. The vertebrates with lower body temperature (amphibians, fish and 'colder' reptiles) have higher cholesterol/DPPC content than warmer reptiles, birds and mammals [Orgeig *et al.*, 2007]. The fish surfactant, dominated by cholesterol, occasionally used for respiration is termed as the "protosurfactant" [Daniels and Orgeig, 2001] (Respiration in reptiles: https://www.youtube.com/watch?v=kWMmyVu1ueY). Temperature also controls the lipid composition of PS. At low body temperature, animals respond by selectively increasing the cholesterol content to maintain the fluidity.

Surface activity of PS of fish, amphibians, birds and reptiles is low. Only few reptiles and mammals have surface active PS. Lower vertebrates, particularly those with low body temperature and surfactant with low DSP content have purely detergent like surfactants (they reduce surface tension but are not able to vary surface tension greatly with surface area). As the composition of the surfactants is influenced by body temperatures and function is related to the structure of their lungs, a highly surface active material may not be required for optimal lung function in lung fish [Johansen *et al.*, 1966]. The faveoli of birds, air breathing fishes are up to 1000 fold larger than alveoli of mammals of similar body size, which confers faveolar stability by substantially reducing the collapse pressure. In addition, it has enhanced backbone of elastin and collagen and an inner trabecular network that supports and stabilizes the interconnecting units [McGregor *et al.*, 1993]. Reptile, fish and amphibian lungs are at least an order of magnitude more compliant than mammalian lungs. While the alveoli of the mammalian lung comprise by far the majority of the lung volume, faveolar volume in unicameral lungs comprises only a very small percentage of total lung volume.

PS exists not only in alveoli but also in bronchioles and small airways. The main function of airway surfactant is to maintain potency of the conducting

airways, *i.e.*, to prevent cohesion of bronchiolar walls by keeping the water lining spread out and by decreasing surface tension of the airway mucus lining [Enhorning *et al.*, 1995]. The latter function is particularly important in diving mammals (*e.g.*, seals) and in reptiles because these animals often collapse part, or all of their lungs as part of their diving and expiratory cycles [Orgeig *et al.*, 2007]. PS also helps the narrowest airways to remain open, thereby reducing the resistance to air flow and controlling fluid balance in the lung.

PS also acts as an anti-adhesive that prevents adhesion of adjacent epithelial surfaces at low lung volumes or when the alveoli fold in on each other during expiration. This anti-adhesive function is critical for species that lack conducting airways, a diaphragm where the lungs also receive structural support. The anti-adhesive property of surfactant is, therefore, a biological manifestation of the ability of the lipids to lower surface tension. The pattern and mode of breathing of nonmammalian vertebrates indicate that an anti-adhesive function might be essential for the animals, during the period of low body temperature [Orgeig *et al.*, 2003].

Therefore, there is marked differences in the functionalities of surfactants in the lungs of non-mammals from that in mammals.Acting as an anti-adhesive is the primitive function of PS in lower vertebrates, whereas alveolar stability and increasing lung compliance are restricted to the mammals.

Summary and Conclusion

The knowledge regarding the formation and dynamics of the PS film at the air-liquid interface has increased significantly over the years. Surface film consisting of a DPPC monolayer in close contact with multilayers is the active structure at the interface. Several dynamic processes occur in the film during breathing including, selective adsorption of DDPC enriched domains, specific squeeze-out of non-DPPC lipids, lipid reservoir formation, *etc*. Each process requires the combined action of both lipid and protein components of the PS. DPPC is the main component of PS responsible for lowering of surface tension in the lung, the surfactant proteins, particularly SP-B and SP-C, are crucial in providing it with full physiological and physical activities. SP-B and SP-C can greatly accelerate lipid adsorption; they can also influence the DPPC containing surface films to reduce the surface tension to near zero. SP-B is more effective in adsorption while SP-C plays the significant role in the lipid reservoir formation. SP-A further augments the ability of SP-B. SP-C also accelerates reinsertion of the collapse-phase lipid into the monolayer during film expansion, and provides mechanical stability to the surface film. Role of the minor lipids is still far from clear. Direct imaging with fluorescence, Brewster angle and atomic force microscopy clearly show that the interactions between SP-B, SP-C, and other lipid components of synthetic and animal surfactant extracts are non-ideal and lead to novel morphologies important for PS function. With SP-B, collapse occurs in a reversible process in which the monolayer is flexible enough to fold, while retaining sufficient cohesion to prevent loss of material to the subphase. The folds have the same composition as the monolayer, and reversibly re-incorporate into the monolayer on expansion. These two- to three-dimensional transitions allow collapse to occur at elevated surface pressures while making it possible for the protein and unsaturated lipid components to remain associated with the monolayer, facilitating rapid re-spreading.

Since the first direct surface tension measurements of PS using home-made LWB half a century ago, many more *in vitro* tensiometric techniques have been developed with its specific advantages and disadvantages. They enable direct film imaging and domain formation, topography, molecular orientation, electrical surface potential, film thickness, etc.

A thorough understanding of the functions of the lipids and proteins in PS provides a mechanism-based rationale for the design of the replacement surfactants for the treatment of RDS. The most driving motivation for studying PS is its clinical application. Recent studies have provided new evidence for the actual inhibition mechanisms of PS due to both proteins and lipids (*viz.*, cholesterol). Overcoming surfactant inhibition plays a central role in resolving the clinical constraints on surfactant therapy in ARDS. Added polymers can enhance the PS performance by resisting inhibition. However, there are still plenty of scopes to improve the efficiency of synthesized designer surfactants, although the existing marketed formulations are quite successful in combating the RDS.

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