Current perspectives in pulmonary surfactant — Inhibition, enhancement and evaluation

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ABSTRACT

Pulmonary surfactant (PS) is a complicated mixture of approximately 90% lipids and 10% proteins. It plays an important role in maintaining normal respiratory mechanics by reducing alveolar surface tension to near-zero values. Supplementing exogenous surfactant to newborns suffering from respiratory distress syndrome (RDS), a leading cause of perinatal mortality, has completely altered neonatal care in industrialized countries. Surfactant therapy has also been applied to the acute respiratory distress syndrome (ARDS) but with only limited success. Biophysical studies suggest that surfactant inhibition is partially responsible for this unsatisfactory performance. This paper reviews the biophysical properties of functional and dysfunctional PS. The biophysical properties of PS are further limited to surface activity, i.e., properties related to highly dynamic and very low surface tensions. Three main perspectives are reviewed. (1) How does PS permit both rapid adsorption and the ability to reach very low surface tensions? (2) How is PS inactivated by different inhibitory substances and how can this inhibition be counteracted? A recent research focus of using water-soluble polymers as additives to enhance the surface activity of clinical PS and to overcome inhibition is extensively discussed. (3) Which in vivo, in situ, and in vitro methods are available for evaluating the surface activity of PS and what are their relative merits? A better understanding of the biophysical properties of functional and dysfunctional PS is important for the further development of surfactant therapy, especially for its potential application in ARDS.

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Abbreviations: ADSA, axisymmetric drop shape analysis; AFM, atomic force microscopy; ALI, acute lung injury; ARDS, acute respiratory distress syndrome; BLES, bovine lipid extract surfactant; CBS, captive bubble surfactometer; CSD, constrained sessile drop; DPPC, dipalmitoyl phosphatidylcholine; DPPG, dipalmitoyl phosphatidylglycerol; LE, liquid-expanded; LWR, Langmuir–Wilhelmy balance; PBS, pulsating bubble surfactometer; PC, phosphatidylcholine; PD, pendant drop; PEG, polyethylene glycol; PC, phosphatidylglycerol; PL, phospholipid(s); PMPC, palmitoyl–myristoyl phosphatidylcholine; POPC, palmitoyl–oleoyl phosphatidylcholine; POPG, palmitoyl–oleoyl phosphatidylglycerol; PS, pulmonary surfactant; RDS, respiratory distress syndrome; SP, surfactant protein; TC, tilted-condensed; TEM, transmission electron microscopy; Tm, main transition temperature; T, surface pressure(s); Tm, equilibrium spreading pressure; γ, surface tension(s); γm, equilibrium surface tension; γm and γm, minimum and maximum surface tensions (upon film oscillation), respectively

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1. Introduction

The entire alveolar surface of mammalian lungs is lined with a thin fluid continuum, called the alveolar lining layer [1]. This lining consists of an aqueous hypophase covered by a film of pulmonary surfactant (PS) [2]. The major function of this PS film is to reduce the surface tension of the alveolar surface, thereby contributing significantly towards maintaining the normal mechanics of respiration [3].

First by lowering alveolar surface tension, PS reduces the energy required to inflate the lungs thereby increasing pulmonary compliance (i.e., the ratio of lung volume change to an applied distending pressure). Second by decreasing elastic recoil, PS reduces the likelihood of alveolar collapse during expiration. As a result, the lungs can easily maintain patency with a small transpulmonary pressure of less than 10 cm H2O [4].

PS exists not only in alveoli, but also in bronchioles and small airways. The main function of airway surfactant is to maintain patency 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 [5,6]. This 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 [7,8]. When it comes to humans, PS can play an important role in protecting the epithelial cells from damage by air thrusts during reopening of collapsed airways, a common condition in mechanical ventilation [9]. In addition to the classical functions relevant to surface tension reduction, PS possesses a number of other functions which make it physiologically indispensable. These functions are summarized in Table 1 [10–13]. Nevertheless, this review will be restricted to the surface tension-relevant properties of alveolar PS.

Deficiency or dysfunction of PS causes severe respiratory disease. Neonatal respiratory distress syndrome (RDS), the major disease of PS deficiency worldwide, is due to prematurity [14]. Premature infants suffering from RDS exhibit increased work of breathing, decreased lung compliance, prominent atelectasis (alveolar collapse) with reduced functional residual capacity (FRC, i.e., the lung volume remaining at the end of expiration), impaired gas exchange, and diffuse interstitial edema [14]. The actual incidence of RDS in premature infants declines greatly with increasing gestational age. It is estimated that RDS generally affects 10% of all premature infants in developed countries [15]. In 2002 RDS affected an estimated 24,000 newborns in the USA alone [15].

Displaying symptoms similar to RDS, acute lung injury (ALI) and its more severe form, the acute respiratory distress syndrome (ARDS), occur as a rapid onset of respiratory failure that can affect patients of any age [16]. ARDS affects approximately 150,000 people per year in the USA and has a case fatality rate of approximately 30–40% [17]. The pathogenesis of ARDS is still not fully understood but surfactant inhibition, due to leakage of a variety of inhibitory substances (e.g., serum proteins, hemoglobin, and certain lipids) into the alveolar space, is believed to be an operative cause induced by primary pathogenesis such as extensive lung inflammation, trauma, severe pulmonary infection, near-drowning, oxygen toxicity, or radiation damage [16,17].

Exogenous surfactant replacement therapy, in which either synthetic or modified natural PS (extracted from bovine or porcine sources) is delivered into the patients' lungs, has been established as a standard therapeutic intervention for patients with RDS [18]. Surfactant therapy has also shown limited positive effects with ARDS patients [11,19–21].

Therefore, the study of PS has not only physiological but also clinical significance. This paper reviews the history and recent progress in PS research. Due to the interdisciplinary nature of these studies, a large body of literature in a variety of areas including biochemistry, biophysics, physiology, colloid and interface science, biomedical and clinical applications, is available. This review will focus on the biophysical properties, especially surface activity, of PS. Other

Table 1

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areas closely related to the biophysical studies are also covered, albeit to a lesser extent. The remainder of this paper is organized into the following three parts: (1) Fundamentals of PS, including a brief history of the discovery of PS and its clinical applications, alveolar surfactant metabolism, component functionality, and interfacial properties. The focus of this part is on the biophysical properties of functional PS. (2) Clinically used PS preparations and the challenge of ARDS treatment. We will emphasize biophysical inhibition of PS and the reversal of this inhibition by water-soluble polymeric additives, a recent research focus in developing inactivation-resistant surfactant formulations. (3) Methods for evaluating the surface activity of PS. Although in vivo, in situ and in vitro methods will be discussed, focus will be on the in vitro methodologies.

2. Fundamentals of pulmonary surfactant

2.1. A brief history of the discovery of surfactant and its clinical applications

The initial indication for PS can be traced to 1929 when von Neergaard first demonstrated the importance of interfacial forces for lung mechanics [22]. By comparing the recoil pressure (i.e., the tendency to collapse) of lungs filled with air and lungs filled with aqueous solution, von Neergaard demonstrated that alveolar surface tension contributed significantly to lung recoil. He also hypothesized that the alveoli might contain a special surface active material capable of lowering alveolar surface tension. However, he was not able to demonstrate that surface active materials were present in the lungs, using the methods available to him [23].

von Neergaard’s findings drew little attention. In 1955, Pattle reported in his classic paper, published in Nature [24], that small bubbles generated in lung extracts initially decreased in size but then stabilized at 40–50 μm diameter. Pattle concluded that the surface tension of these microbubbles likely fell to near 0 mN/m. Otherwise, the pressure difference across the bubble interface, predicted by the Laplace equation of capillarity (see Section 4), would cause the bubbles to deflate rapidly. Initially, Pattle erroneously concluded that the film responsible for the zero surface tension was composed of an insoluble protein. Pattle later corrected this error by identifying the film as a lipid–protein complex [25].

In 1957, Mead et al. reported the dependence of lung compliance on surface tension forces [26]. They observed that, when an excised lung was filled with air, the quasi-static (step-by-step) transpulmonary pressure observed during inflation was higher than that observed during deflation, a phenomenon they called “pulmonary volume–pressure hysteresis”. The hysteresis largely disappeared with lungs filled with saline. They attributed this difference to the effect of surface tension forces operating at the air–water interface of the lung. Interestingly, they noted a large discrepancy (∼10-fold) between the surface area predicted by their measurements (assuming alveolar surface tension to be 60 mN/m as with serum) and the surface area calculated by histology.

Also in 1957, Clements published the first direct surface tension measurements on lung extracts using his modified Langmuir balance (see Section 4) [27]. He found that the surface tension of saline extracts from animals’ lungs decreased from 46 to 10 mN/m when the surface area was reduced. He also calculated the compressibility of these films to be as low as 0.01 (mN/m)^2, consistent with a strong ability to stabilize the lungs against collapse at the end of expiration. This in vitro surface activity of PS, i.e., its ability to decrease surface tension to remarkably low values upon film compression, was later confirmed by Schuch et al. in situ through direct surface tension measurements in excised lungs [28].

The clinical correlation between PS deficiency and RDS was first established by Avery and Mead in 1959 using a Clements-type Langmuir balance [29]. They reported that very little surface active material could be recovered from the lungs of infants who died of hyaline membrane disease, now known as RDS. However, surfactant was found in lung extracts of infants who died from nonpulmonary diseases, provided that their birth weight was more than 1000 g. Avery and Mead hypothesized that the lack of surfactant due to prematurity was responsible for RDS. The pathological relationship between prematurity and surfactant deficiency was later confirmed by Brumley et al. who found that the PS system does not mature until late in gestation [30].

In 1980, Fujiwara et al. reported the first successful trial of surfactant replacement in RDS infants using a surfactant extracted from bovine lungs [31]. Since that discovery, surfactant replacement therapy has become a standard therapeutic intervention for RDS patients. Partially owing to surfactant therapy, the mortality rate of premature infants in the USA due to RDS fell by 24% in 1990 and has continued to decrease thereafter [32,33].

In 1967, Ashbaugh et al. first described a syndrome of acute respiratory distress in adults which displayed some symptoms similar to RDS in premature infants [34]. This syndrome is now referred to as ARDS. Inspired by the symptomatic similarity between ARDS and RDS, surfactant replacement therapy has been tested in treating ARDS [35]. It was found that surfactant therapy generally showed a positive effect on the respiratory aspects of ARDS [19,20,36]. Gaseous exchange is normally improved in the patients, although the effects on the disease as a whole can be variable and are largely dependent on dosing, administration, timing and the actual surfactant preparation used [37,38]. However, ARDS is a complicated disease which can affect non-respiratory systems, such as liver and kidney, leading to multiorgan failure [37,38]. With the exception of two trials conducted with pediatric patients suffering from a selected etiology consistent with direct lung involvement and using a highly surface active, modified natural surfactant (Infasurf) [39,40], surfactant treatment has not demonstrated a significant impact on mortality from ARDS. As a result, clear indications of a distinct surfactant-mediated decrease in mortality or improvement in ventilatory care of ARDS patients are still lacking and further definitive clinical trials, possibly using “designer” surfactant preparations (see Section 3), are required.

Excellent reviews on the history of the discovery of PS and its clinical applications can be found elsewhere [23,41–46].

2.2. Alveolar surfactant metabolism

Using electron microscopy, Weibel and coworkers [24,47,48] have convincingly demonstrated that a substantial area of the alveolar surface is lined with a thin aqueous layer. The overall continuity of this lining layer was definitively established by studies on rat lungs using low-temperature electron microscopy [49]. It was found that the aqueous layer has an average thickness of 0.14 μm over the alveolar surface, 0.89 μm in alveolar corners, with an overall area-weighted average thickness of 0.2 μm [49]. Although very thin, this aqueous layer is considerably larger than that required to accommodate a bulk phase comparable to the surfactant film on top, which is in molecular dimensions. This aqueous subphase is essential for PS metabolism because it provides a medium for surfactant secretion, morphological transformation, adsorption, desorption and recycling [14]. These events are commonly referred to as the life cycle or alveolar metabolism of PS [3].

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 of the alveoli, where the lamellar bodies undergo transformation into a morphological form called tubular myelin. The formation of tubular myelin is dependent on the presence of surfactant apoproteins and calcium [50–52]. Tubular myelin is composed of large square elongated tubes, constituted primarily of phospholipids and proteins, ranging in size from nanometers to microns [53,54]. It appears that the surfactant components are subsequently released from tubular myelin to form a surface active film at the air–water interface.
interface of alveoli by rapid adsorption. Traditionally, the PS film has been considered to be a monomolecular film, i.e., a monolayer [55,56]. However, accumulated evidence from studies using electron microscopy [57–59], captive bubble tensiometry [59–61], autoradiography [62], atomic force microscopy [63,64], and neutron reflection [65] all suggest that at least part of the PS film is thicker than a single monolayer. It consists of a surface monolayer plus, in places, one or more lipid bilayers, closely and apparently functionally associated with the interfacial monolayer. These multilayers form the so-called surface-associated “surfactant reservoir” (detailed later) [60].

After de novo adsorption, the surfactant film is periodically compressed and expanded during breathing. This action modulates surface tension in the lungs. After performing its physiological functions, surfactant is released as small, unilamellar 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 trachea and is eventually swallowed. The estimated turnover period of PS is surprisingly short, ranging from 4 to 11 h [66]. Reviews on the metabolism of PS can be found in [67,68].

2.3. Surfactant composition

Comparative biological studies suggest that PS exists in all air-breathing vertebrates, although with somewhat differing compositions [69–71]. However, the composition of mammalian PS is remarkably similar among diverse species, i.e., approximately 90% lipids and 10% proteins by weight [72]. The composition of bovine pulmonary surfactant is remarkably similar among diverse species, i.e., approximately 90% lipids and 10% proteins by weight [72]. The composition of bovine surfactant, as shown in Fig. 1, is representative of most species [73]. The lipids consist mainly of phospholipids (PL, ~90–95 wt.%), primarily cholesterol [68,74]. Among the PL components, phosphatidylcholine (PC) is the most prevalent class, accounting for ~80% of the total PL. Although notable exceptions exist [70], in most cases about 30–60% of the PC is dipalmityl phosphatidylcholine (DPPC) (16:0/16:0 PC), a long-chained, most prevalent class, accounting for 10% proteins by weight [72]. The composition of bovine surfactant as a group accounts for ~50% of the total PL. 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 trachea and is eventually swallowed. The estimated turnover period of PS is surprisingly short, ranging from 4 to 11 h [66]. Reviews on the metabolism of PS can be found in [67,68].

Four proteins have been found associated with PS [75]. They are named surfactant protein (SP)-A, -B, -C and -D, based on the nomenclature proposed by Possmayer [76,77]. SP-A (monomeric MW 26–38 kDa) and SP-D (43 kDa) are large, multimeric, hydrophilic glycoproteins. They are members of the Ca2+-dependent carbohydrate-binding collectin (collagen-like lectin) family. In contrast, SP-B (MW 8.7 kDa) and SP-C (4.2 kDa) are smaller and extremely hydrophobic. Among these surfactant associated proteins, SP-A is the most abundant by mass, accounting for about 5 wt.% of PS, with SP-D, accounting for about 0.5%. SP-B and SP-C together constitute approximately 2% (~1:3 in bovine surfactant).

SP-A is the most abundant surfactant protein by mass, but not in terms of molar ratio. Taking bovine surfactant as an example, for every SP-A octadecamer (composed of six trimers) there are approximately 3 SP-B dimers, 45 palmitoylated SP-C and 12,500 PL molecules. There are about half as many SP-D dodecamers (composed of four trimers) as SP-A octadecamers in the alveolar space, but SP-D is soluble and only a small proportion of this collectin will be associated with surfactant.

Reviews on the PL and protein compositions of PS and its component functionality can be found elsewhere [10,14,74,75,78–84].

2.4. Phospholipid phase behavior

Discussion of the biophysical properties of PS requires some basic knowledge of PL phase behavior. The phase behavior of PL is commonly studied with monolayers, bilayers, or vesicles [83]. The phase behavior of PL can be profoundly varied by the addition of cholesterol. Here, we briefly review the PL and PL-cholesterol phase behavior and lipid polymorphism relevant to PS study. Recent reviews on the PL phase behavior of bilayers [84], monolayers [86], vesicles [87], and lipid polymorphism [88,89] can be found elsewhere.

2.4.1. Bilayers

When dispersed in aqueous phase, at room temperature and atmospheric pressure, most PL molecules spontaneously form bilayers (Fig. 2(a)), where the hydrophobic PL fatty acid groups shield each other from being exposed to water. The formation of closed vesicles (i.e., liposomes, Fig. 2(b)–(d)) completes this water-avoidance process, resulting in energetically optimized structures. With increasing temperature, PL bilayers without cholesterol “melt” from an ordered gel (Lβ) phase to a disordered liquid–crystalline (Lα) phase at the main transition temperature (Tα). This fatty acid disorder permits diffusion of individual PL molecules within each leaflet of the bilayer, a property referred to as “fluidity”.

Addition of a sterol such as cholesterol transforms PL bilayers in Lα and Lβ phases into a liquid-ordered (Lα) phase (sometimes referred to as β phase) and a liquid-disordered (Lβ) phase, respectively. The overall fluidity of the bilayers in the above mentioned four phases would be Lβ < Lαc < Lαβ < Lα. This is because when intercalated between the closely packed Lα PL molecules, cholesterol disrupts (fluidizes) the ordered structures. On the other hand, when associated with mobile Lβ PL molecules, cholesterol condenses and increases the packing density of the disordered structure [90]. Thus the overall effect of cholesterol is to have 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). However, the actual phase behavior of PL-cholesterol mixtures is complex and depends greatly on both the temperature and cholesterol mol fraction [91].

The readers are cautioned that some authors have not yet adopted the above nomenclature and may, for example, refer to plasma membrane lipids, which contain cholesterol, as being in the Lαβ phase. It should also be noted that the actual phase behavior of PL bilayers can be more complicated and intermediate phases, such as the ripple phase, can exist between the main Lβ and Lαβ phases [85].

![Fig. 1. The composition of bovine pulmonary surfactant. DPPC: dipalmitoyl phosphatidylcholine; PC: phosphatidylcholine; PG: phosphatidylglycerol; PE: phosphatidylethanolamine; Lyso-bis-PA: lyso-bis-phosphatic acid; SM: sphingomyelin; DG: diacylglycerol; Chol: Cholesterol. Adapted from Yu et al. [73].](image-url)
2.4.2. Monolayers

With PL monolayers, water avoidance is achieved through extension of the fatty acid groups into the air (which is much more hydrophobic than water). The behavior of PL in interfacial monolayers can be correlated at least in part with the phase behavior of hydrated bilayers. Deposition of a small amount of a saturated PL, such as DPPC, on a surface balance will result in a monolayer in the dilute gaseous phase. Because van der Waals attractive forces are weak and the fatty acids can “wave” in air, the DPPC molecules can collide with one another, although they tend to remain separated. Reducing the surface area available to each lipid molecule by moving a barrier across the surface or by depositing additional DPPC will increase the surface concentration. At a certain point, all of the molecules will touch each other. A further compression will then lead to a detectable increase in surface pressure, which can be measured either as back pressure on the barriers or, as explained later, as a decrease in surface tension. Here PL molecules will no longer be in the dilute gaseous state but are in the more concentrated lipid-expanded (LE) phase where they interact, but the fatty acids remain mobile due to the presence of kinked gauche–gauche (also known as trans–gauche) configurations.

As the film is further compressed, some of the PL molecules will become more closely packed to generate the more ordered tilted-condensed (TC) phase (traditionally called the liquid-condensed (LC) phase) where all of the acyl groups are in the extended all-trans (straight) configuration [86]. At this point, further decreases in surface area will generate LE-TC coexistence where the decreased surface area is taken up by forming a greater proportion of TC phase [86,92–94]. Nucleation and growth of the TC phase in PL monolayers can be directly visualized as “domain” formation using fluorescence microscopy or atomic force microscopy (AFM) (detailed later). Within the TC domains, PL molecules interact side by side and the fatty acids tilt in a parallel fashion with a tilting angle smaller than the PL molecules in the LE phase. (See Fig. 3(b) for a schematic of the different chain tilting configurations. There is an inherent positive curvature with bilayers which is exaggerated here; (b) Unilamellar vesicle, which is similar in form to a tennis ball; (c) Multilamellar vesicle, which is similar to an onion; (d) Multivesicular body, where small vesicles are trapped within a larger vesicle; (e)–(g) Inverted hexagonal (HII) phase. (e) Cross section of a HII phase cylinder. Due to the small size of the PE headgroup, with a planar bilayer the mobile acyl groups would be exposed to water. Moving the headgroups together results in negative curvature of the resulting cluster. As a consequence the PE headgroups enclose a water pocket and the acyl groups extend outwards. (f) A HII phase cylinder, where the PE headgroups interact with the long column of water extending down the middle of the cylinder. Note that only two methyl groups per PE molecule are exposed to the exterior. (g) HII phase, where a number of the hydrophobic cylinders depicted in (f) interact together in a hexagonal pattern, thus minimizing water–fatty acid interactions.

The domain formation occurring during LE-TC phase transition due to monolayer compression can be considered as a process of pressure-induced crystallization. This raises a distinction between phase transition in monolayers and bilayers. While (bulk) pressure has only a small effect on bilayers, phase transition in monolayers is dependent not only on temperature but also on the surface pressure of the monolayers. With a monolayer in the LE-TC coexistence state, increasing the temperature will lead to a reduction in the size and possibly the number of the TC domains. However, simply increasing the surface pressure will restore the domains. This is true for temperatures up to and, in practice, somewhat above the corresponding $T_m$ for bilayers of that particular PL [92–98]. For example, it has been observed that highly compressed DPPC monolayers only melted at temperatures of 48–55 °C, well above the DPPC bilayer $T_m$ of 41.5 °C [97]. In essence, this means that compared to bilayers, monolayers do not possess a characteristic $T_m$. Rather, the “$T_m$” for monolayers is highly dependent on the surface pressure.
Analogous to bilayers, incorporating cholesterol into a DPPC monolayer changes the TC phase to a liquid-ordered (LO) phase, whereas adding this sterol to an unsaturated POPC monolayer converts it to a liquid-disordered (LD) phase. The LO phase is more fluid than the TC phase and the LD phase is less fluid than the LE phase, leading to an order of fluidity: TC < LO < LD < LE.

2.4.3. Nonbilayer phases

In addition to bilayer structures, certain PL molecules upon hydration can self-assemble into some nonbilayer structures, such as the inverted hexagonal (HII) phase and cubic phases [88,89]. These nonbilayer phases have a curved morphology which contrasts with the planar morphology of lamellar phases. As shown in Fig. 2(g), in HII phase, PL molecules are arranged cylindrically with the polar headgroups facing toward the interior of the structure. The formation of nonbilayer phases arises due to the molecular shape of the PL [88,89]. Typical bilayer-forming molecules, such as PC, both solid and fluid, have a cylindrical shape with nearly equal headgroup and hydrocarbon areas. Such molecules pack into sheets which can fit readily into bilayers. In contrast, nonbilayer-forming lipids, such as fluid PE, exhibit a cone-shape with a small headgroup area and large hydrocarbon area. This molecular shape prevents them from packing.

Fig. 3. Schematic representation of pulmonary surfactant adsorption. (a) Diffusion of a surfactant vesicle/bilayer towards the air–water interface. In this schematic, the surfactant bilayers consist mainly of DPPC, fluid PC (such as POPC), PG (DPPG and POPG) and PE, in similar proportion to BLES. (b) Fusion of the vesicle/bilayer with the interface stabilized by SP-B. Note that PE molecules could help stabilize the negative curvature generated at the fusion pore/neck monolayer and fusion pore bilayer interfaces. The monolayer contains tilted-condensed (TC) and liquid-expanded (LE) domains composed of saturated and unsaturated phospholipids, respectively. (c) Fusion of the vesicle/bilayer with the interface stabilized by SP-C. Note that the bilayer can be also connected to the interfacial monolayer by a SP-C molecule. Such SP-B and SP-C stabilized fusion necks/pores can occur simultaneously and independently during the adsorption of surfactant. Note that SP-B and SP-C are not drawn to scale and the proportions of these hydrophobic proteins to PL are not representative.
into a planar bilayer arrangement without allowing water to slip in between the headgroups and wetting the acyl groups. Consequently, monolayers comprised of such lipids have a spontaneous tendency to form structures with negative curvature [100].

Formation of H$_2$ phase is promoted by increasing the temperature, introducing acyl chain unsaturation, and decreasing pH [101,102]. Unsaturated PE is found to be particularly effective in forming H$_2$ phase [89]. In addition, certain proteins or peptides are found to modulate the tendency of lipids to form H$_2$ phase [88]. The nonbilayer phases, such as the H$_2$ phase, have found particular interest in the study of membrane fusion [89,103].

2.5. Biophysical properties

At least three biophysical properties of PS are considered essential for normal respiratory physiology, especially in the neonatal period. These are: (1) rapid adsorption, (2) very low surface tension upon film compression, and (3) effective film replenishment upon film expansion [3,84,104,105].

2.5.1. Rapid adsorption

A functional PS should adsorb rapidly to form a film at the air–water interface of the lungs, probably within a few seconds or faster. Analogous to bilayer fusion, the adsorption of surfactant aggregates to an air–water interface likely consists of two sequential steps [106–109]. As shown in Fig. 3(a), the surfactant aggregates must first diffuse to the subphase closely adjacent to the interface. This diffusion step is higher dependent on the surfactant concentration in the subphase [110,111]. In the second step, as shown in Fig. 3(b) and (c), the surfactant aggregates need to fuse with the interface. This fusion step involves “unzipping” and spreading of the surfactant PL vesicles at the interface. Although PL vesicles are held together by weak van der Waals forces, the major cohesive force arises from the so-called hydrophobic effect, because considerable energy is conserved through the formation of vesicles. Therefore, unzipping of PL vesicles needs to overcome an energy barrier, which arises during unravelling of the PL vesicles in water, before the hydrophobic fatty acid chains become exposed to the atmosphere [106,107]. Not surprisingly, adding fluid PL to DPPC decreases the energy barrier as they increase overall disorder. Anionic PL such as fluid PG has been shown to be particularly effective. However, recent studies indicate that with either native or modified PS, or the lipids derived from surfactant, the factors mentioned above play only a small role. Rather, it appears that the formation of a fusion neck or pore, perhaps similar to those observed with viral membrane cell fusion or bilayer fusion, contributes even more to the adsorption process [101,102]. For example, addition of lyso-PC, which should increase disorder but would hinder the formation of negatively curved structures with concave hydrophilic faces as present in fusion necks, markedly prolongs adsorption times [112].

Addition of SP-B and SP-C counteracts the energy barrier limiting adsorption, especially in the presence of anionic PL (such as PG), possibly by introducing electrostatic interactions [107,109,113]. SP-B and SP-C significantly promote adsorption [107,109,113] and reinsertion of PL vesicles from the surfactant multilayers into the interfacial monolayer during film expansion [114]. Perhaps the most effective way for SP-B or SP-C to promote adsorption would be to stabilize neck structures (as shown in Fig. 3(b) and (c)) [101,115]. However, to date, direct evidence, for example by microscopy or by demonstrating the presence of negatively curved structures such as the H$_2$ phase, is still lacking [112].

During adsorption, films of a physiologically competent surfactant can decrease the surface tension of the air–water interface from ~70 mN/m to ~20–25 mN/m at 37 °C, but no further [84]. At this final surface tension range, the PL molecules at the air–water interface are in thermodynamic equilibrium with the molecules in the bulk phase [84]. This equilibrium surface tension ($\gamma_{eq}$) corresponds to the equilibrium spreading pressure ($\pi_e$) of PL, which is the highest surface pressure obtained by spreading excess saturated or unsaturated PL at the air–water interface with organic solvent or by placing dry crystals of the PL at the interface [84]. Increases in surface pressure beyond $\pi_e$ can only be achieved at a fixed temperature by lateral film compression.

It should be noted that surface pressure ($\pi$) and surface tension ($\gamma$) are linearly correlated by

$$\pi = \gamma_0 - \gamma$$

where, $\gamma_0$ is the surface tension of a clean air–water interface at the experimental temperature, approximately equal to 70 mN/m at 37 °C. Thus, $\pi$ corresponds to the extent that a film decreases $\gamma$. Surface tension, $\gamma$, with quantitatively equivalent units of mN/m or mJ/m$^2$, is commonly used in lung physiology and biology as a measure of the energy used to create a unit area of a new interface in air. The $\gamma$ of a liquid can be directly measured by monitoring the shape of a sessile or pendant drop of the liquid (detailed in Section 4). Alternatively, $\gamma$ can be measured from the additional force exerted on a suspended plate or other object due to capillary rise, upon contact with the liquid surface as, for example, with the Wilhelmy plate or the Du Noüy ring method [116].

Surface pressure, $\pi$, is the concept usually used in the physical chemistry of monolayers where surface energy is interpreted in terms of intermolecular forces [117]. The original Langmuir balance measured $\pi$ directly using a barrier connected to a pressure transducer [118]. $\pi$ is a more fundamental concept under this circumstance as it offers the direct 2D monolayer analog of the bulk phase (3D) pressure. As a result, many equations of state derived for the bulk phase can be directly applied to monolayers without significant reformatting. In the remaining part of this review, both $\gamma$ and $\pi$ will be used dependent on convenience and clarity, with the corresponding value included, where deemed helpful to the reader.

2.5.2. Film stability at low surface tensions

Although limited, the available experimental evidence indicates that $\gamma$ of the alveolar surface falls to very low values during expiration [28,119–121]. Accordingly, upon compression, competent PS films should lower $\gamma$ to near-zero values [119], in other words, increase $\pi$ to a value close to 70 mN/m at 37 °C. Equally important, this low $\gamma$ should be achieved by only a slight film compression, i.e., no more than 20–30% area reduction, corresponding to the maximum variation of the alveolar surface area during normal tidal breathing [121,122]. Hence, the film should have a low compressibility (dln $\pi$/d$\gamma$) of less than 0.01 (mN/m)$^{-1}$. Upon expansion, the PS film should only moderately increase $\gamma$ and remain close to $\gamma_{eq}$ [120]. The complete compression–expansion loop should have limited hysteresis, reflecting minimized film collapse and efficient film replenishment.

These premier biophysical properties of PS, however, seemingly create a paradox. Rapid adsorption requires fluid PL molecules with highly mobile amphiphilic structures [116]. However, physicochemical studies have established that monolayers formed by fluid amphiphilic molecules generally collapse rapidly when $\pi$ is increased beyond $\pi_e$, for example, in a Langmuir balance [123]. To sustain stable (or metastable) $\pi$ higher than $\pi_e$, a monolayer must consist of highly rigid, insoluble surfactant molecules [117,124]. Experiments with Langmuir monolayers demonstrate that only such stiff molecules allow the formation of highly ordered, tightly packed, solid-like films in a condensed phase, thus being capable of increasing $\pi$ higher than $\pi_e$ without monolayer collapse. Fully hydrated DPPC bilayers have a $T_m$ near 41 °C. Hence, DPPC is traditionally taken to be the only significant component of PS capable of reaching high $\pi$ at physiological temperatures. This led to the classical model of surfactant function which suggested that the alveoli were stabilized by a monolayer highly enriched in DPPC [125–127]. Note that such a monolayer is in a metastable state, confined by the $\pi_e$ (~45 mN/m) and the collapse pressure (~70 mN/m) of DPPC.
However, adsorption of vesicles of stiff molecules, such as hydrated DPPC bilayers below 41 °C, is very slow. (Actually, dry DPPC will not adsorb below \(T_m\). This is because PL molecules do not hydrate well unless the fatty acids of the PL are mobile and this occurs at \(T_m\).) No single component in endogenous PS is able to both adsorb rapidly and withstand \(\pi\) higher than \(\pi_c\) at 37 °C for a time period long enough to avoid alveolar collapse. This dilemma has been rationalized by the so-called “squeeze-out” hypothesis [125–127]. The squeeze-out model predicts a rapid cooperative adsorption of DPPC and other unsaturated PL components to the air–water interface in which the unsatisfactory adsorption of DPPC is compensated for by the presence of unsaturated PL, neutral lipids, and, most importantly, the surfactant associated proteins. SP-B and SP-C independently promote rapid adsorption of PL [75,81]. SP-A further enhances adsorption in the presence of SP-B and calcium ions [128–131]. These proteins enhance adsorption much more than the effects of unsaturated PL. The squeeze-out hypothesis proposed that after adsorption, the fluid non-DPPC components, which are less effective in lowering \(\gamma\), are selectively squeezed out of the film during film compression. This likely occurs with the help of SP-B and SP-C [63,64,132–140]. This selective squeeze-out process would result in a condensed, highly DPPC-enriched film that would be responsible for the further reduction of \(\gamma\) to near-zero values upon further film compression [125–127].

Although theoretically plausible, both the classical model and the squeeze-out hypothesis have been challenged by recent studies on monolayer phase transition/selection using direct film imaging [141–149]. As shown in Fig. 4, when examined in a Langmuir balance at room temperature, pure DPPC monolayers show a plateau of LE–TC phase coexistence at \(\pi\) of 8–13 mN/m [86]. The TC phase consists of domains which exclude fluorescent dyes (detected by epifluorescence microscopy) [86,150] and extend ~1 nm higher than the LE phase (detected by AFM) [141,151]. At \(\pi\) beyond the plateau region, pure DPPC monolayers consist of a nearly homogeneous TC phase [86,141]. In contrast, as shown in Fig. 4, LE–TC coexistence in PS monolayers clearly persists to a \(\pi\) of at least 40 mN/m [141–143,149]. The results from recent fluorescence microscopy studies have demonstrated that LE–TC phase coexistence in the PL fraction of PS persists even at \(\pi\) approaching 70 mN/m (i.e., at near-zero \(\gamma\)) [146]. The TC domains contain mostly DPPC and small amounts of other disaturated PL such as DPPG (16:0/16:0 PG) [152] and perhaps PMPC [153], while the LE domains contain mostly unsaturated components. As revealed by fluorescence microscopy, the TC domains account for a total area fraction of 30–40% of the PS films, roughly corresponding to the percentage of disaturated PL [146,147]. These findings clearly demonstrate that a TC film composed of almost pure DPPC is not required for reaching high \(\pi\). This obviously contradicts the classical model, which contends that a monolayer highly enriched in DPPC stabilizes the alveoli at end-expiration [125–127].

The question as to how surfactant films with mixed LE and TC domains attain \(\pi\) near 70 mN/m remains. The TC domains are enriched in DPPC and hence are intrinsically stable at high \(\pi\). However, how the LE domains, which account for most of the surface area, persist with high \(\pi\) remains unknown. The most obvious explanation is that with appropriate Langmuir balances and experimental conditions, the PL fraction of PS can be compressed into a metastable film that acts like a “solid”, despite being predominantly in a LE phase [146].

A potential explanation for this seemingly paradoxical situation is that these LE domains may form a sort of matrix in which the TC domains are uniformly distributed [149,154,155]. This can be envisioned as a kind of composite material or be analogous to an alloy [75]. This suggestion is supported by recent studies using AFM. With a higher resolution than fluorescence microscopy, AFM has

![Fig. 4. Compression isotherms and characteristic film structures for monolayers of bovine lipid extract surfactant (BLES) and DPPC, studied by the Langmuir–Wilhelmy balance and AFM at room temperature. The AFM scan areas for BLES films are 20×20 \(\mu\)m and for DPPC films are 50×50 \(\mu\)m. The enlargements of the AFM images for BLES at 30 mN/m (1×1 \(\mu\)m) and for DPPC (10×10 \(\mu\)m) show the nanometer-sized TC domains. The AFM images of BLES clearly show LE–TC phase separations and transitions as the surface pressure increases from 20 to 40 mN/m. Nanodomains are observed at 30 mN/m and increase in number up to 40 mN/m; in contrast, microdomains increase in area from 20 to 30 mN/m but decrease from 30 to 40 mN/m. At 50 mN/m, multilayers that contain stacks of PL bilayers appear (see the surface plot of the AFM image). The transition from a monolayer to multilayers is apparently reflected by the plateau at 40–50 mN/m in the compression isotherm of BLES. In contrast to BLES, DPPC monolayers only show LE–TC phase coexistence and transition at 8–13 mN/m. (The kink shown in DPPC isotherm at ~50 mN/m is likely an artefact due to film leakage to the Teflon ribbon.) The TC phase in DPPC monolayers also consists of microdomains and nanodomains. After this transition region, indicated by the rising plateau in the DPPC isotherm, DPPC monolayers consist of nearly homogenous TC phase. AFM images of BLES are adapted from Zuo et al. [149]. AFM images of DPPC are courtesy of Dr. Eleonora Keating, University of Western Ontario.)](image-url)
revealed numerous nanometer-sized TC domains embedded within the LE phase, in DPPC and DPPC/DPPG monolayers [155]. The number and size of these nanodomains depend on the presence and concentration of surfactant proteins [155]. Recently, such nanodomains have been demonstrated in a modified natural surfactant (bovine lipid extract surfactant, BLES) with and without SP-A [149]. It was found that when π of BLES films was increased, the original micrometer-sized TC domains (i.e., microdomains) dissociated into many nanodomains (Fig. 4). As a result, at 40 mN/m the BLES monolayers contained only a few micromdomains but many nanodomains uniformly embedded within the LE phase. The total area of these microscale and nanoscale TC domains accounted for ~40% of the surface area [149], approximately equal to the total fraction of disaturated PL in BLES [153]. Topographic analysis also suggested that these nanodomains, similar to their microscale counterparts, are composed of disaturated PL [149,155]. Due to the composite structure, such surfactant monolayers could be both flexible and stable.

A second mechanism for attaining high π (low γ) arises from the 3D multilayer structures formed when the composite monolayers are further compressed beyond a π close to the πc of PL (i.e., 40–45 mN/m) [149]. Such a 3D architecture consists of the interfacial monolayer plus interconnected bilayers closely associated with the interface (see the surface plot at 50 mN/m in Fig. 4) [63,149,156,157]. The monolayer-to-multilayer transition is reflected in the compression isotherm of PL films by a plateau at 40–50 mN/m, as shown in Fig. 4. This 3D architecture could provide additional stability by acting as a scaffold or scaffold to resist further compression and hence allow the films to attain π above πc [149,154]. This would be particularly true if, as has been proposed, the excluded multilayers are bridged to the interfacial monolayer through SP-B and SP-C (see Fig. 3(b) and (c) for schematics) [136,157–159].

It should be noted that the compression-driven monolayer-to-multilayer transition generally occurs at a π close to the πc of PL at which multilayers are formed during adsorption, i.e., the surfactant reservoir (detailed later). This coincidence may indicate that these two multilayer structures are formed due to the same molecular basis, i.e., accumulation of excess material at the air–water interface beyond saturation. In other words, if there is insufficient free surface area to accommodate the PL in the interfacial monolayer, the excess material becomes surface-associated multilayers [62,84]. Actually, theoretical studies have suggested that there may be a slight deviation between the πc at which 3D nuclei coexist with a 2D monolayer in an adsorbed film and the π at which 3D nucleation initiates in a Langmuir monolayer during compression [160]. However, such a deviation may not be distinguishable for a complicated biological system like PS. Recent molecular dynamics simulations suggested that the compression-driven multilayer structures could protrude into the aqueous subphase, as folding bilayers, instead of extending into the air [161]. This further indicated that the multilayer structures formed during compression appear to be similar, if not identical, to the surface-associated surfactant reservoir formed during adsorption, both functionally and structurally.

Here, it is important to distinguish the monolayer-to-multilayer transition occurring at πc from the two other phenomena/hypotheses. First, it is different from the “true” collapse of PS films near π of 70 mN/m. During the film collapse at 70 mN/m, the PL molecules are primarily irreversibly lost into the bulk phase in the form of small aggregates/vesicles [162]. Hence further film compression causes no increase in π above the collapse pressure. In contrast, the monolayer-to-multilayer transition appears to represent a reversible, partial collapse of the interfacial monolayer occurring near πc. The resultant multilayer structures apparently remain closely attached to the interfacial monolayer as they can readily re-spread to the interfacial monolayer during film expansion [63,149]. Second, the monolayer-to-multilayer transition is intrinsically different from the “squeeze-out” predicted by the classical model [125–127]. The 40–45 mN/m squeeze-out model mentioned above predicts that PS films maintain stability by selectively excluding fluid non-DPPC components from the interfacial monolayers. Consequently, the interfacial monolayer after formation of the multilayered protrusions would be expected to be purified with DPPC. However, recent AFM and Kelvin probe force microscopy studies have found that the topography and electrical surface potential of the interfacial monolayer appear to be heterogeneous even after the multilayer formation [157,163]. Hence, the multilayers would appear to show no absolute differences from the interfacial monolayer in terms of composition. A similar conclusion has been derived from time-of-flight secondary ion mass spectrometry (ToF-SIMS) studies of compressed DPPC/DPPG/SP-C films [156]. Autoradiographic studies also indicated that the lipid composition of multilayers formed during adsorption is similar to the interfacial monolayer [62].

In addition to the above indicated PL phase transition and multilayer model based on microscopic studies, another hypothesis has been proposed by Hall and coworkers to explain how PS monolayers could reach low surface tensions [147,148,164]. This group found that monolayers of the PL of PS or a single-component fluid PL can be transformed to a metastable structure when the monolayers are compressed to π higher than πc (actually higher than ~55 mN/m) using a sufficiently rapid compression, the so-called “supercompression”, in a captive bubble surfactometer (CBS) [164,165]. Once transformed by a supercompression, the physicochemical properties of the initial fluid films are markedly altered. These films (for example a POPC film that usually collapses at πc when compressed slowly in a Langmuir balance) can withstand high π for prolonged periods, even if the films are expanded back to π below πc [164,165]. These workers proposed that these films are compressed so quickly that they do not have enough time to collapse near πc, but rather form an amorphous, non-crystalline structure (termed a “jam” phase). This can be considered similar to 3D liquids supercooled towards a glass transition [147,148]. A recent study of the melting behavior of supercompressed PS, DPPC and POPC monolayers clearly suggested that a supercompressed PS film is different from either a pure DPPC or a pure POPC monolayer [97,98]. So far, no detailed microscopic examination of the film structure after supercompression is available, probably due to technical difficulties.

It should be noted that the PL phase transition model and the supercompression model are not necessarily conflicting. A recent AFM study of BLES monolayers, conducted in a Langmuir balance at room temperature, showed that after a rapid compression (close to the rate of supercompression for extracted surfactants, defined by Hall et al., i.e., ~4% area per second [147]) the monolayers consisted of mainly nanometer-sized TC domains [149]. Although only a low π of 30 mN/m was examined [149], this observation suggested an alternative interpretation of the supercompression model: the rapid compressions likely facilitate formation of TC nanodomains rather than micromdomains because the latter structures require longer times for assembling. Monolayers containing evenly distributed TC nanodomains could be stable despite containing a large proportion of LE phase since they could act as an alloy or a composite material, as explained above. Upon compression, such monolayers would be expected to have little chance of fracture. Further, should fractures occur at extreme compression, they would not be readily propagated because they would have to proceed around the intervening TC nanodomains.

2.5.3. Surface-associated surfactant reservoir

In order to function appropriately in the lung, a competent PS must be able to maintain a near equilibrium γ during inspiration [28,120]. Dynamic compression–expansion cycling studies conducted with the pulsating bubble surfactometer (PBS) or the CBS indicate that PL uptake into the interface occurs too quickly to be explained by de novo adsorption of PL vesicles from the subphase. Hence this can be best explained by re-spreading from a surface-associated surfactant reservoir, formed during adsorption and/or film compression, and
functionally associated with the interfacial monolayer. The initial evidence for the surfactant reservoir came from CBS studies where it was shown that although bulk phase PS was removed by washing, the remaining surface film contained highly surface active material in excess of the interfacial monolayer [60]. This concept has been reinforced by numerous studies using electron microscopy, autoradiography, fluorescence microscopy, and AFM, all of which revealed excessive PL material associated with the surface monolayer [61–65,136,158,166,167].

Although notable exceptions exist, as a general rule, PS preparations that adsorb well also tend to function well to reduce γ to low values during film compression and to re-spread effectively during film expansion. This correlation likely exists because the surfactant reservoir can be created during adsorption and situations promoting rapid adsorption (e.g., high surfactant concentration, presence of SP-A, lack of inhibitors) favor formation of the multilayered reservoir. Once established, the interfacial surfactant monolayer can exchange surface active material with the surfactant reservoir during film compression and expansion. Although not fully understood, it is evident that SP-B and SP-C contribute to reservoir formation (as shown in Fig. 3(b) and (c)), most likely at independent sites, as does SP-A in the presence of SP-B and calcium [60,62,131,168,169].

In closing Section 2, we would like to emphasize that functional PS films may reach low γ (high π) by forming kinetically-controlled nanoscale TC domains. These nanodomains are uniformly distributed in the LE phase, thus forming a 2D alloy-type structure which imparts both flexibility and stability to the monolayer. Upon further compression, such an alloy structure also facilitates partial collapse of surfactant films on the domain boundaries [170,171]. The resultant multilayer structures could provide additional stability to PS monolayers, thereby allowing the attainment of very low γ. It should be evident that the biophysical properties of PS, although dependent on surfactant PL, are directed by the surfactant apoproteins SP-B, SP-C, and to a lesser extent by SP-A. These surfactant proteins influence the extent of nanodomain formation. They also facilitate formation of multilayers which act as the surfactant reservoir. The biophysical performance of PS monolayers is significantly enhanced by the surfactant reservoir. A complete understanding of the biophysical properties of PS on a mechanistic level, however, is still unavailable. Both the classical and the modern models have limitations [147]. Recent reviews on the molecular interactions of PS components and their contributions to the biophysical properties of PS films can be found elsewhere [105,147,154,172].

3. Inhibition of clinical surfactants and reversal thereof

3.1. Different types of clinical surfactants

A number of exogenous surfactants have been tested in clinical or preclinical trials for treating RDS and ARDS. As summarized in Table 2, these therapeutic preparations generally fall into four categories based on surfactant apoprotein content [11,14,36,173]. They are: (1) whole 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 the hydrophobic surfactant proteins 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). Among these surfactant preparations, human amniotic fluid lacks commercial capacity due to its source limitation. The synthetic surfactants devoid of proteins have become unpopular due to their relatively poor clinical performance [174]. ALEC, which was licensed in the UK, has been withdrawn from the market. Exosurf is no longer available in the USA.

Both preclinical animal experiments and clinical practice suggest that the animal-derived surfactant preparations are superior to the synthetic preparations [174–176]. However, these modified natural surfactants also have some limitations. The general concerns of animal-derived products are batch-to-batch variation in composition and potential risk of transmission of microbes. In addition, the surfactant protein contents in the modified natural surfactants currently available for clinical use can be very low compared with the endogenous surfactant. Because of immunological considerations, none of the modified natural surfactants currently available contains the hydrophilic proteins (SP-A and SP-D), which are removed during the purification processes used in manufacturing the products. This is

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Surfactant preparations used clinically and preclinically</th>
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<tbody>
<tr>
<td><strong>Trade name</strong></td>
<td><strong>Source</strong></td>
</tr>
<tr>
<td>Human surfactant containing all surfactant proteins</td>
<td>Human amniotic fluid</td>
</tr>
<tr>
<td>Modified natural surfactant containing hydrophobic proteins SP-B and SP-C</td>
<td>Bovine lung lavage</td>
</tr>
<tr>
<td>Alvedact</td>
<td>–</td>
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<tr>
<td>BLES</td>
<td>Calf lung lavage</td>
</tr>
<tr>
<td>Infasurf</td>
<td>Bovine lung lavage + DPPC + palmitic acid + tripalmitin</td>
</tr>
<tr>
<td>Survanta</td>
<td>Porcine lung lavage</td>
</tr>
<tr>
<td>Curosurf, Infasurf and Survanta</td>
<td>Porcine lung lavage</td>
</tr>
<tr>
<td>Synthetic surfactant containing simplified peptides or protein analogs</td>
<td>SP-B-like peptide (KL4)+ lipids</td>
</tr>
<tr>
<td>Surfaxin</td>
<td>Recombinant SP-C (rSP-C)+ lipids</td>
</tr>
<tr>
<td>Venticute</td>
<td>Recombinant SP-C (rSP-C)+ lipids</td>
</tr>
<tr>
<td>Synthetic surfactant free of proteins</td>
<td>ALEC</td>
</tr>
<tr>
<td>Exosurf</td>
<td>DPPC + PG</td>
</tr>
</tbody>
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ALEC: artificial lung-expanding compound; BLES: bovine lipid extract surfactant.
because, unlike the low molecular weight hydrophobic proteins, SP-A and SP-D are multimeric glycoproteins and consequently impose potential immunologic hazards. Economically synthesizing or recovering human SP-A from natural sources is difficult. Thus, the host protective benefits of these proteins are lost. The content of the hydrophobic proteins (SP-B and SP-C) in different surfactant preparations varies but can be significantly lower than the endogenous surfactant. For instance, Survaanta contains only ~1/8 SP-B and ~1/2 SP-C found in the endogenous bovine surfactant [177]. Curosurf contains only ~1/3 SP-B and ~1/2 SP-C found in the endogenous porcine surfactant [177]. The in vitro surface activity of different animal-derived surfactants and their sensitivity to inhibition vary significantly [177,178], presumably related to the different protein contents of these products.

As a result of different animal sources (bovine or porcine) and different production procedures (bronchoalveolar lavage or lung mincing), the modified natural surfactants differ not only in their protein contents but also in the compositions of PL (especially DPPC, PG and PI), neutral lipids (mainly cholesterol), and additives (such as the palmitic acid and triacylglycerol supplemented in Survanta). A detailed comparison of lipid and protein compositions of the modified natural preparations can be found in a recent review by Blanco and Perez-Gil [179]. The different PL contents can result in a significant difference in the fluidity of different preparations and hence different behavior of adsorption. The neutral lipids and additives likely play an important role in altering the surface rheological properties of different clinical preparations [180,181]. It should be noted that despite the evident biophysical differential and clinical superiority of some animal-derived surfactants over others, statistical differences in mortality or days in neonatal intensive care units related to different surfactant preparations have not been demonstrated [174–176].

Another limitation of animal-derived surfactants is the relatively high cost, at approximately US$500 per dose for premature infants [182]. These costs are mainly associated with the high expense of conducting quality control with relatively small batches of biologically-derived products and recovery costs for the very expensive clinical trials that must be conducted. When treating ARDS patients, large surfactant amounts, multiple doses and a continuous supply will be required. This will further increase the cost of surfactant therapy. Socioeconomic analysis shows that surfactant replacement therapy is only cost-effective in developed countries [182]. In the developing countries, surfactant therapy is used only sparingly due to the lack of an affordable clinical surfactant and the costs associated with intensive care facilities. For instance, RDS affects ~7–12% of newborns in India and is associated with a high mortality rate due to the lack of tertiary care, including surfactant treatment [183]. In China, surfactant therapy has been introduced into clinical practice but only limited to the highly developed regions of the country [184]. The high cost of surfactant has prevented its worldwide distribution for neonates and considerably more so for adult therapy. Thus, there is an urgent need for developing an inexpensive surfactant preparation.

A new generation of clinical surfactants is under development [185–189]. These preparations are fully synthetic and contain synthesized surfactant protein analogs. The long-term goal of this research is to develop synthetic surfactant preparations that fully or closely mimic the biophysical properties of the endogenous surfactant and highly resist inactivation. Mass production could result in a more reasonable price. Both preclinical and clinical trials have been conducted using synthetic surfactants with the addition of simplified SP-B/C-like peptides.

The initial surfactant protein mimic studied was KL4, a short amphipathic peptide consisting of leucine amino acids with a lysine moiety introduced at every fifth residue. This structure is based on a positively charged helical segment of SP-B [190]. KL4 enhances the adsorption of DPPC/PG/palmitic acid mixtures and interestingly, low levels of SP-A further augment this adsorption [191]. Recent studies have demonstrated that KL4 can promote formation of lipid protrusions with DPPC/DPPG mixtures [192]. Although KL4 exhibits a number of interesting SP-B-like properties, it is still considered a first generation protein mimic. The manner in which this peptide replicates SP-B properties is still being investigated [192–194]. Nevertheless, KL4 is being used as the basis of a wholly synthetic surfactant, Surfaxin, which has been reported to be efficacious with premature infants and in preliminary studies with ARDS [195–197].

Another wholly synthetic surfactant is Venticute, a preparation based on recombinant human SP-C (rSP-C), which has been utilized in a number of clinical trials. Venticute is presently being investigated in a trial involving direct lung insult-induced ARDS [198–201].

While the advantages of synthetic surfactant peptides are obvious, synthesizing fully functional analogs of hydrophobic surfactant proteins is unfortunately not a trivial task. The SP-B molecule is too large and structurally complex to be easily synthesized. Folding this hydrophobic protein to generate the three intra- and one inter-disulphide bonds has proven particularly arduous. The palmitoylated cysteine residues in SP-C have been replaced by phenylalanines. However, SP-C molecules are structurally unstable in pure form and tend to aggregate. A major goal of such research is to generate “designer” surfactants with properties particularly well suited for ARDS and other specific lung diseases. So far, none of the synthetic preparations available have proven more efficacious than the natural preparations [176,189]. In addition, the presence of potentially dangerous reaction side-products and the possibility of adverse metabolic effects related to intracellular processing of synthetic compounds must be considered.

Nevertheless, it is important to note that significant progress is being made in this direction. Waring and associates have generated a number of simplified SP-B analogs such as mini-SP-B (contains N-terminal and C-terminal sections of SP-B) which mimic the biophysical and physiological properties of the whole hydrophobic protein [202,203]. Barron and coworkers have produced a number of peptides with SP-B and SP-C-like properties [204,205]. Such compounds would have the advantage of being protease resistant. A number of recent reviews offer in-depth discussion of these novel approaches [185–189,206,207].

3.2. Surfactant inhibition

Surfactant inhibition, or inactivation, refers to those processes that decrease or abolish the normal surface activity of PS. Such processes may interfere with the PL adsorption to form a functional surfactant film, prevent the film from reaching low γ upon compression, or affect re-spreading of PL during expansion. A number of substances have been reported to inhibit PS. The major inhibitory factors include plasma proteins, unsaturated membrane PL, lysosphospholipids, free fatty acids, meconium (fetal feces expelled during stress), and supraphysiological levels of cholesterol [11,14]. Surfactant inhibition can also arise from degradation of surfactant lipids by phospholipases or of surfactant proteins by proteases. These degradative agents, normally present in the alveolus at very low levels, can be increased during microbial infection and more importantly through secretion by leukocytes and type II cells with pulmonary inflammation [208–211]. In addition to the above, surfactant can be compromised by reactive oxygen species [212,213] and by pollutants [214,215] but these latter effects will not be covered here.

3.2.1. Surfactant inhibition by plasma proteins

 Leakage of plasma proteins into the alveolar space due to an impaired alveolar-capillary barrier is an early event in the pathogenesis of ARDS [208]. The mechanism by which PS is inactivated by plasma proteins is not yet fully understood. Evidence is available demonstrating that albumin, fibrinogen, and hemoglobin can inhibit PS by competitive adsorption [216–221]. These protein inhibitors are
surface active. When mixed with PS they compete with PL for the air–water interface by spontaneous adsorption. Although larger than individual PL molecules, these protein molecules are water soluble and hence can quickly reach the interface by molecular diffusion. In contrast, the insoluble PL of PS adsorb to the air–water interface by cooperative diffusion of large molecular aggregates (especially vesicles), followed by vesicle “unzipping” and monolayer spreading (see Section 2 for detail). Consequently, the protein molecules can adsorb to the air–water interface quicker than the surfactant PL. Once adsorbed, the protein film excludes the PL from entering the interface by creating a steric and/or electrostatic energy barrier [222,223]. In keeping with this view, surfactant injected under preformed protein films adsorbs slowly and takes longer to reach low ζ during compression–expansion cycling [218]. Surfactant inhibition due to competitive adsorption can be largely overcome in vitro by increasing the surfactant concentration [220] or adding SP-A [130,224] because these approaches enhance adsorption kinetics of PL.

Not surprisingly, surfactant inhibition depends on the plasma protein/surfactant ratio [220]. The alveolar hypophase normally contains only small amounts of soluble proteins [225–227]. For instance, Rennard et al. [228] and Ishizaka et al. [229] have estimated the albumin concentration in the epithelial lining layer to be 3.7 to 4.9 mg/mL, corresponding to ~10% of the plasma values of albumin (i.e., ~40 mg/mL). The concentration of PS in the alveolar hypophase has been estimated to be 30 mg/mL in rats and 100 mg/mL in rabbits [230], calculated on the basis of surfactant PL recovery by lavage [231] and an average alveolar lining layer thickness of 0.2 μm [49,232]. Since inhibition normally requires a much larger amount of protein than surfactant [220], it appears that normal lungs maintain a considerable safety factor.

However, in ALI/ARDS, the alveolar space experiences markedly increased concentrations of plasma proteins, due to capillary leakage, with average levels of 25 mg/mL and individual values of over 100 mg/mL being reported [229]. (The high values, above plasma levels, probably arise during epithelial recovery when ions and water are eliminated more rapidly than proteins [225].) In contrast, the concentrations of surfactant PL and/or surfactant associated proteins decrease due to inflammation, oxidation and simple dilution [208,233]. Consequently, competitive adsorption may play a role in inactivating surfactant under these circumstances.

Although considerable effort has been directed toward studying the effects of plasma proteins on surfactant adsorption, it should be stressed that these proteins can inhibit surfactant function in other ways. Warriner et al. demonstrated that albumin interfered with re-spreading of a model surfactant PL mixture, DPPC/POPG/palmitic acid [221]. This inhibition occurs when ζ of 50 mN/m or higher are attained, where 50 mN/m corresponds to the γc of albumin. This implies that only when ζ rises to the equilibrium for albumin can this protein adsorb and interfere with PL re-adsorption/re-spreading. This is consistent with the inability of albumin to inhibit high levels of surfactant [220] and with the ability of DPPC, spread onto albumin films to a low ζ, to displace this protein from the interface [234,235]. Nevertheless, it should be noted that the model PL system described above [221] did not contain SP-B or SP-C, either of which tends to maintain the surfactant films near a ζ of 25 mN/m, i.e., the γc of PL. Unless inactivated, the hydrophobic surfactant proteins would protect the film against attaining the high ζ required for this inhibition mechanism [236,237].

In addition to the above example, recent work has provided further evidence that albumin may inhibit surfactant by mechanisms other than only interfering with surfactant PL adsorption. Both NMR [238] and X-ray diffraction [239] studies suggested that albumin directly interacted with PL bilayers of BLES. Such interactions thinned the PL bilayers and altered the distribution and motion of PL in bilayers/monolayers. AFM studies demonstrated that serum altered the microstructures of BLES monolayers [240,241]. As recently revealed by AFM and confocal fluorescence microscopy, albumin can remain within the LE phase of the PL of BLES up to a π of 40 mN/m, significantly higher than the πc of albumin [i.e., ~20 mN/m] [241]. By remaining at the surface and mixing with the surfactant films, albumin increases the film compressibility and may also disturb the normal PL phase transition and separation [241]. Such inhibitory effects cannot be effectively mitigated by repeated compression–expansion cycles in a Langmuir balance [241]. These studies suggest new biophysical mechanisms for surfactant inhibition due to plasma proteins, and possibly also indicate that surfactant–protein interaction/binding may play a role in the inhibition process.

Investigations of surfactant inhibition have emphasized serum albumin because at 35–50 mg/mL, it is the predominant plasma protein, accounting for about half of the plasma proteins [242]. The availability of cheap, highly purified albumin is a likely consideration. However, it must be mentioned that albumin is a very weak inhibitor compared to a number of other plasma proteins. For example, early studies by Seeger’s group demonstrated that fibrin generated from fibrinogen by a variety of methods was considerably more potent than albumin in inhibiting a variety of surfactant preparations [230]. This mechanism appears to involve coagulation of surfactant PL and protein molecules, resulting in the hyaline membranes for which RDS was originally named [23].

Surfactant function is also markedly hampered through interaction with C-reactive protein (CRP) [243,244]. CRP was originally discovered through its ability to bind the polar phosphorylcholine groups of PC. This binding and the corresponding inhibition can be blocked by water-soluble phosphorylcholine. Simultaneous increases in CRP and decreases in SP-A in the alveolar space are conditions associated with ALI [245]. In contrast to albumin, CRP markedly inhibits surfactant function at 50 wt/wt. % of the surfactant and this inhibitory effect cannot be relieved by repeated compression–expansion cycling. Recent studies suggested that CRP inhibits PS by fluidizing the surfactant PL films, i.e., a completely different mechanism from the competitive adsorption of albumin [246,247]. As will be discussed shortly, this mechanism is commonly associated with surfactant inhibition by unsaturated lipids. As in the case of albumin and fibrinogen, adding SP-A at low levels relative to surfactant blocks the inhibitory effects at a fraction of the inhibitory plasma proteins’ concentration [246,247]. The mechanism by which SP-A reverses CRP-inhibition appears to be also different from the case of albumin. It was suggested that direct interaction between SP-A and CRP was responsible for preventing inhibition due to this serum protein [246,247]. As discussed above, however, SP-A overcomes albumin-induced inhibition mainly by enhancing surfactant adsorption.

3.2.2. Surfactant inhibition by lipids

Although not nearly as well studied as protein inhibition, a second inhibition mechanism involving unsaturated membrane PL, lysosphospholipids, free unsaturated fatty acids (such as oleic acid), bile acids, diacylglycerol and cholesterol has been identified [248–251]. Some of these lipids have detergent properties and can be referred to as soluble amphipathic lipids because they form micelles [252]. None of these lipids are bilayer formers, although they can be incorporated into bilayers to some degree. Lysosphospholipids are PL containing a single fatty acid chain per molecule and are generated by phospholipase A2 secreted by white blood cells and likely type II cells, particularly during ALI and ARDS [253]. Bile acids, which are strong detergents, are present in meconium, along with a number of other lipids, mucous glycoproteins [254], and phospholipases [255]. Such lipid substances can be considered diluents of the specific surfactant lipid assembly. Thus, insertion and mixing of these unsaturated amphipathic lipid and fatty acid molecules with the surfactant PL molecules would significantly fluidize the PL monolayers and could promote early collapse, thus preventing low ζ from being reached. The inactivation due to lipid penetration cannot be effectively overcome by raising surfactant concentration [220,248,249].

Cholesterol is a special case of lipid inhibition since it is not very amphipathic. Cholesterol appears to be an inherent component of
natural surfactant [74, 256, 257]. Most isolated mammalian surfactants which have been examined contain 5–10 wt.% (10–20 mol%) cholesterol [74, 256–258]. Cholesterol is also present in premammalian vertebrates where it can reach very high levels [256]. Although cholesterol has been associated with surfactants for a very long time, phylogenetically, and its presence in mammalian surfactants has long been recognized, the precise physiological role of this steroid in surfactant remains somewhat ambiguous.

Early studies on model membranes and PS extracts revealed that the presence of cholesterol tended to decrease film stability at low γ [73, 128], presumably due to the increased fluidity of the LO phase (see Section 2 for the description of PL-cholesterol phase behavior). For this reason, methods were developed for removing cholesterol from BLES and Curosurf [73, 84]. These observations were apparently related to the use of the Langmuir balance and the PBS, which will be described in Section 4. More recently, studies with the CBS have demonstrated that the levels of cholesterol in natural surfactant and its lipid extracts do not inhibit surfactant biophysical activity when assayed with this device [251, 259, 260]. This difference is attributed to the lack of edge effects (i.e., barriers, walls, and capillary) which make the CBS less susceptible to the loss of PL from the air–water interface (i.e., so-called “film leakage”, detailed later in Section 4).

While cholesterol at 5–10 wt.% had no negative effects on surfactant function with the CBS, a large number of studies have demonstrated supraphysiological levels are detrimental [163, 251, 258, 260, 261]. Elevations in surfactant cholesterol have been reported in a number of animal models of ARDS such as high-stretch ventilation-induced ALI [262], oleic acid-induced ALI [263] and oxidant-induced ARDS [264]. Elevated cholesterol has also been detected with ARDS patients due to fat embolism syndrome [265]. A recent randomized, controlled clinical trial found a remarkably elevated level of neutral lipids, including diacylglycerol, triacylglycerol, and cholesterol, in the bronchoalveolar lavage fluids of patients with ARDS arising from various predisposing factors [201].

Although the mechanism of cholesterol-induced inhibition has not been fully elucidated, several groups, including our own, have demonstrated alterations of surface-derived films using AFM [163, 260, 261]. In particular, the ability to generate DPPC-rich condensed domains within the LO/LD phase and to generate PL multilayers (surfactant reservoirs) appears impaired with the addition of high levels of cholesterol. Whether these two surface aspects of surfactant function are related has not been clearly established.

The detrimental effects of high cholesterol on surfactant function raise the question as to whether it should be included in the synthetic and semi-synthetic clinical preparations currently being formulated, or removed from those modified natural surfactants which contain this steroid. Bernardino de la Serna et al. [266] suggested that small amounts of cholesterol may play a crucial role in phase separation in porcine surfactant bilayers by inducing the formation of Lα and Lβ phases. Malcharek et al. [261] found physiological levels of cholesterol (10 mol%) stabilize DPPC/DPPG/SP-C films by strengthening the surface-associated multilayer structures. Nevertheless, to date, the authors know of no negative effects of omitting cholesterol from clinical surfactants, although admittedly the verdict is clearly still out. Since high levels of cholesterol are clearly deleterious, it would appear judicious to avoid including cholesterol in clinical preparations. This would build in a potentially important safety factor by providing a sink for the elevated endogenous sterol.

3.3. Overcoming surfactant inhibition

Overcoming inhibition will play a key role in developing new surfactant formulations for ARDS treatment. Two general approaches to be discussed here are (1) optimizing lipid and protein contents, and (2) using water-soluble polymers as surfactant additives.

3.3.1. Optimizing lipid and protein contents

Simply increasing the PL concentration of surfactant preparations can effectively reverse plasma protein-induced inactivation [220]. It was reported that increasing surfactant concentration also mitigated meconium-induced inactivation [267]. As a new direction in optimizing the lipid components of surfactant, Notter and coworkers have developed a novel surfactant preparation which consists of a synthetic C16:0 diether phospholipid (DEPN-8) in combination with 1.5 wt.% purified bovine SP-B/C [268] or mini-SP-B [269]. Compared to the glycerophospholipids in other surfactant preparations, DEPN-8 is structurally resistant to phospholipase degradation [268]. This synthetic surfactant was found to be highly resistant to inhibition due to serum proteins, phospholipase A2, and lyso-PC, both in vitro [268] and in excised rat lungs [270]. A potential advantage of this preparation is that, being resistant to phospholipases (A1, A2, and D), DEPN-8 could be cycled into lamellar bodies and secreted back into the alveolar without degradation. This would greatly prolong the effectiveness of this synthetic preparation. A potential problem might be that this synthetic lipid may not be routed in the same manner as DPPC. However, the presence of significant quantities of 1-alkyl ether PCs in marsupial surfactants, particularly the Tasmanian devil [70], indicates that ether lipids are compatible with normal processing in type II cells.

Optimizing the content of surfactant proteins is another effective means to reverse inactivation. The superior performance of modified natural surfactants over protein-free synthetic surfactants clearly demonstrates the importance of SP-B and SP-C in overcoming surfactant inactivation. Addition of peptide analogs of SP-B and/or SP-C also improves the resistance to surfactant inactivation [202, 203, 271, 272]. Adding SP-A to lipid extract surfactants increased their resistance to inactivation due to blood proteins [130, 224] and meconium [273]. 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 [129, 131, 274]. In animal experiments, surfactants containing SP-A [275, 276] showed higher resistance to inactivation than the surfactants without SP-A.

3.3.2. Using polymeric additives

Taeusch et al. [277] and Kobayashi et al. [278] first introduced the concept of using low-cost, water-soluble, nonionic polymers, such as dextran and polyethylene glycol (PEG), as additives to clinical PS. The benefits from these polymeric additives were found to be twofold [279–281]. First, they can improve the surface activity of dilute surfactant preparations (mainly by enhancing adsorption) and hence are capable of decreasing the cost of surfactant therapy. 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 in treating ARDS.

The polymers tested so far cover a surprisingly broad range, including nonionic (e.g., PEG [277] and dextran [278]), anionic (e.g., hyaluronan [282]) and cationic (e.g., chitosan [283]) polymers. Many in vitro [222, 277, 278, 282–290] and in vivo animal studies [278, 291–297] have shown that these polymers can significantly improve the surface activity of different clinical surfactants and reverse surfactant inactivation. The encouraging results from these preclinical studies make these polymers very promising for the development of low-cost and inactivation-resistant surfactant formulations.

The increasing experimental success led to exploration of the underlying mechanism responsible for the enhancement. These polymers are hydrophilic. Alone they have no or only poor surface activity [283, 288]. When mixed with the inhibitory substances, such as albumin, they do not alter the interfacial properties of the inhibitors significantly [283]. Therefore, it is unlikely that these polymers enhance surface activity by directly accumulating at the interface or reverse inactivation by directly interacting with the inhibitors. Yu et al. [288] found that...
nonionic polymers, such as PEG, improved the adsorption of dilute surfactant preparations as a function of both polymer concentration and molecular weight. These experimental results fit very well with a polymer-induced depletion–attraction model.

Depletion–attraction is a non-specific, polymer-induced entropic force dependent on both the concentration and molecular weight of the polymer [298,299]. Depletion–attraction forces are routinely used to promote fusion of cells in culture likely by the same mechanism that improves surfactant function. The simplified model described here will be followed by a more rigorous mathematical formulation. Consider two surfactant vesicles approaching each other. At a certain point the smaller, non-deformable polymer molecules will no longer fit in the space available between the vesicles and so will be excluded (i.e., depleted) from this space. The resulting enrichment in areas other than the region between the two vesicles produces an increase in osmotic pressure in those regions. This increased osmotic pressure draws water from the polymer-depleted region, thus forcing the two vesicles together (i.e., attraction). The initial effect will be to promote membrane–membrane (bilayer) fusion or aggregation. The resulting larger vesicles/aggregates will be even further subject to depletion–attraction forces because they are more effective in eliminating the polymer between them. A similar process will occur at the air–water interface and the resultant depletion forces will drive the vesicle towards the interface, thus decreasing the energy barrier for surfactant adsorption. It should be noted that some nonionic polymers such as PEG (10 kDa) may adsorb to the air–water interface due to a weak surface activity (γ ≈ 60 mN/m [288]). This may weaken the depletion forces near the surface region (personal communication with Dr. Tonya Kuhl, UC Davis).

In physicochemical terms, the depletion forces described above arise when two surfaces approach each other closely in a solution of non-adsorbing polymers. As shown in Fig. 5, when the distance between the two surfaces-vesicles decreases to approximately two times the radius of gyration of the polymer chains (2Rg), the polymer molecules in the region between these two surfaces are depleted since otherwise the polymer coils lose configuration entropy, i.e., they will be deformed or involved in other random changes in morphology. Consequently, an osmotic pressure appears between the bulk solution that contains the polymer and the polymer depletion zone of radius Rg between the two surfaces. The osmotic effect generates a net force of attraction between these two surfaces and hence causes flocculation. Kuhl et al. [300,301] have provided solid evidence that depletion–attraction is responsible for the fusion of pure PC vesicles in PEG solutions by direct force measurements using a surface force apparatus. They also found a change from attraction to repulsion with increasing molecular weight of PEG [300,302]. This turnabout was due to adsorption of PEG molecules onto the surfaces of the PC vesicles, which eliminates depletion–attraction but raises steric repulsion between the vesicles [302]. A similar turnabout has been reported when BLES was mixed with a high molecular weight PEG (300 kDa), where surfactant adsorption was completely abolished [288].

The depletion force can be quantitatively estimated from the classical model first developed by Asakura and Oosawa [303]. When considering the depletion force between two flat surfaces, this model can be simplified as follows [304].

The depletion pressure (P) between two flat surfaces is:

\[ P = -\frac{\rho kT}{2h} \]

where \( \rho \) is the molecular density of the polymer, i.e., the number of molecules per unit volume (m\(^{-3}\)); \( \rho = 6.022 \times 10^{23} \text{ C}_{\text{w}} / \text{M}_{\text{w}} \) where \( C_{\text{w}} \) and \( M_{\text{w}} \) are the polymer concentration (wt.%) and molecular weight, respectively; \( k \) is the Boltzmann constant, 1.38 \times 10^{-23} \text{ (J/K)}; \( T \) is the absolute temperature (K).

Fig. 5. Schematic representation of the polymer-induced depletion–attraction mechanism in promoting surfactant aggregation and adsorption. Note that this mechanism only applies to non-adsorbing polymers such as low and medium molecular weight polyethylene glycol (PEG). The depletion forces arise when two vesicles approach each other, or when a vesicle approaches the air–water interface, at a surface-to-surface distance no more than two times the radius of gyration of the polymer chains (2Rg). In these regions, the polymer molecules are depleted since otherwise the polymer coils lose configuration entropy. Consequently, an osmotic pressure appears between the bulk solution and the polymer depletion zone. The osmotic effect will draw water away from the depletion zone, thereby generating a net force of attraction between these two vesicles. This causes flocculation and “pushes” the lipid aggregates to the air–water interface. As indicated by the interaction potential – vesicle-to-vesicle distance (E–D) curve, the attraction force due to PEG-induced depletion can be as large as the van der Waals force in the absence of the polymer. The profiles of interaction potentials with/without PEG are adapted from Kuhl et al. [300].
The net attractive interaction potential \( E \) over the depletion region \( D \) at \( D<2R_g \) is:

\[
E(D) = -\int_D^{2R_g} \rho kT dD = -\rho kT (2R_g - D) = E_0 [1 - D/(2R_g)].
\]

(3)

At contact of the two surfaces (i.e., at \( D=0 \)),

\[
E_0 = E(0) = -2R_g \rho kT.
\]

(4)

To relate the interaction potential between two flat surfaces to the adhesion force \( F \) between two identical spheres of radius \( R \), one can use the Derjaguin approximation, which gives:

\[
F(D) = \pi R E(D).
\]

(5)

Substituting Eq. (4) into Eq. (5), gives

\[
F = \pi R E_0 = -2\pi R_g \rho kT = -\frac{1.2 \times 10^{-20} \pi R_g C_w kT}{M_w}.
\]

(6)

Eq. (6) predicts the polymer-induced depletion–attraction force \( F \) between two vesicles with a similar size proportional to the vesicle radius \( R \), polymer size \( R_g \), polymer concentration \( C_w \), and temperature \( T \), and inversely proportional to the molecular weight of the polymer \( M_w \). Strictly speaking, \( R_g \) in Eq. (6) should be replaced with the thickness of the depletion layer, which can be experimentally estimated from the distance between two approaching vesicles when they start experiencing the attractive force [300]. In addition, \( C_w \) in Eq. (6) should be replaced by the activity at high polymer concentrations.

Using Eq. (6), it is estimated that PEG \( (M_w=8\text{ kDa}, R_g=5\text{ nm}) \) at a concentration of 50 mg/mL \( (C_w=5\text{ wt.\%}) \) can induce a depletion force of \( ~50\text{ pN} \) in a colloidal system of vesicles with an average radius of \( 100\text{ nm} \) at \( 37 \degree C \). This force is in the same order of magnitude as the contribution of van der Waals attraction that is always present even in an aqueous suspension free of polymers [300]. Although being an extremely simplified model, the depletion forces predicted from Eq. (6) were found to correspond very closely to those directly measured using a surface force apparatus [300]. More complicated calculations result in no significant deviation from this simplified model [300].

Zasadzinski et al. [223] have developed a more detailed thermodynamic model to study the mechanisms of surfactant inactivation due to serum proteins and to explain how polymers such as PEG overcome this inactivation by depletion–attraction. Using the classical Smolukowski analysis of colloid stability, this theoretical model confirmed that the polymer-induced depletion force promotes adsorption of PL vesicles/aggregates of PS, thereby overcoming competitive adsorption of serum proteins. This model predicts that the adsorption rate of PL vesicles/aggregates increases only linearly with the bulk surfactant concentration but exponentially with decreasing the energy barrier for adsorption [223]. Hence, the use of water-soluble polymers appears to provide a very effective and inexpensive way to overcome surfactant inactivation.

The depletion–attraction model predicts that adding nonionic polymers would alter the surfactant morphology in the bulk phase, i.e., introducing larger surfactant vesicles/aggregates with the depletion forces. Morphological alterations of surfactant due to the presence of inhibitors and/or polymers were evident under optical microscopy [223,283] and transmission electron microscope (TEM) [267,305,306]. Optical microscopy detected that PEG added to different clinical surfactant preparations induced large aggregated structures [223]. Using Cryo-TEM, Gross et al. [305] found that meconium altered the morphology of Curosurf from spherical unilamellar and multilamellar vesicles to smaller and elongated structures with nonuniform curvatures. At high magnifications, these workers observed disruption of PL vesicles when Curosurf was mixed with meconium or taurocholic acid, a bile acid in meconium. This may indicate that meconium inhibits surfactant in part by penetrating the bilayer structures of the PL vesicles. Ochs et al. [306] found that adding dextran into meconium-inhibited Curosurf reversed the surfactant morphology from small, irregular structures to large lamellar body-like, unilamellar and multilamellar structures. Together with the reversal of surfactant morphology, the surface activity of Curosurf was also restored [306]. All of these observations suggest a correlation between morphology of surfactants and their surface activity, which is consistent with the biophysical analysis of surfactant subfractions (detailed in Section 4) [307–309].

It should be stressed that the surfactant morphology – surface activity correlation discussed above appears to be only valid for surfactants mixed with nonionic polymers, where depletion–attraction is the major driving force for flocculation of PL vesicles. Surfactants with the addition of ionic polymers such as hyaluronan do not necessarily follow this rule. Both freeze-fracture TEM [310] and turbidity [290] studies found that low-concentration hyaluronan did not induce as much aggregation as high-concentration PEG but enhanced surface activity more. This indicates that ionic polymers enhance surface activity of surfactants by a different mechanism than the nonionic polymers (detailed later).

### 3.3.3. Beyond surface activity enhancement

The use of water-soluble polymers as additives to clinical PS could potentially break new ground in the development of cost-effective, inactivation-resistant formulations that are highly favorable for ARDS treatment. The early in vitro tests mainly focused on the improvement in surface activity, i.e., rapid adsorption, low \( \gamma_{min} \) upon compression, and high resistance to surfactant inactivation. However, recent studies suggest that a number of further considerations beyond surface activity enhancement will be necessary for further development.

First, the efficacy of different polymers seems to be dependent on the clinical surfactant to which the polymer is added. Lu et al. [292] found that both in vitro and in vivo dextran was more effective when mixed with Curosurf while PEG was more effective when mixed with Survant. The specificity of a polymer for a certain surfactant preparation may imply a specific molecular interaction between the polymeric additive and certain PL/protein components which are varied in different surfactant preparations. If such a specific interaction does exist, the depletion–attraction mechanism needs to be amended as, theoretically, depletion–attraction ought to be non-specific. In addition, this means that the experimental results obtained from one polymer-surfactant combination may not necessarily be generalizable to the others.

Second, the performance of a polymer may depend on the specific experimental approach used for evaluation. It has been shown that different lung injury models yield different alveolar environments that can play a critical role in determining the overall efficacy of the surfactant therapy [311]. Lu et al. found that PEG (10 kDa) significantly improved the efficacy of surfactant therapy in meconium-induced [295], acid-induced [294], and endotoxin-induced [294] animal ARDS models. However, Campbell et al. [312] reported an adverse effect of PEG in a saline lung lavage model. With these latter studies, rabbits treated with mixtures of BLES and PEG showed greater hypoxemia, lower lung compliance, and higher hypercapnia compared to those administered only BLES. The deleterious effects of PEG in the saline lung lavage model were confirmed by a different group using a different animal (rats) and a different surfactant (Survanta) [313]. These deleterious effects were attributed to increased pulmonary edema due to the addition of PEG which has a high capacity to bind water. In the saline lung lavage model, this side-effect of PEG can overwhelm its beneficial effects on enhancing surface activity. These negative reports constitute a serious caveat to the use of polymers as a surfactant additive.

In addition, it should be noted that most previous in vitro studies involving polymers used serum proteins (usually albumin), whole
serum, or meconium as model inhibitors. A recent in vitro study has shown that PEG cannot effectively overcome surfactant inhibition due to supraphysiological levels of cholesterol [314]. These experimental results, on the one hand, are consistent with the argument that PEG overcomes surfactant inhibition mainly by enhancing surfactant adsorption, which has been compromised in the presence of plasma proteins. On the other hand, these results also show the inability of PEG to deal with surfactant inhibition by lipids, such as cholesterol, which inhibit surfactant by a completely different mechanism. Given the fact that cholesterol may play a significant role in ARDS [201,315], the clinical potential of the polymeric additives in treating ARDS is still to be confirmed.

Third, polymer dosing has not always been optimized. A PEG (10 kDa) concentration of 50 mg/mL was recommended in a number of early studies [277,294,295]. However, it has been reported that a much lower concentration near 20 mg/mL was sufficient to render a satisfactory surface activity in vitro [285,288]. Further increasing PEG concentration yielded no apparent improvement in surfactant adsorption [288]. Similarly, a much lower concentration of hyaluronan than previously used [282,293] was found to be sufficient to produce satisfactory performance both in vitro and in vivo [291]. High polymer concentrations can introduce difficulty in drug delivery due to increased viscosity [283,297], increased osmotic stress in the lungs [313], and possible impaired pulmonary gas exchange due to reduced gas diffusivity in the alveolar lining layer [316]. Hence, it is necessary to optimize the polymer dosing relative to the particular in vivo situation.

The difficulty in endotracheal instillation due to viscous surfactant preparations may be mitigated to some degree by using a new approach of drug delivery, i.e., bronchoalveolar lavage with a large volume of dilute surfactant. A recent study has shown that a mixture of 10 mg/mL Curosurf and 30 mg/mL dextran (69 kDa) improved meconium clearance and lung function in rabbits with meconium aspiration [296]. Compared to bolus delivery, bronchoalveolar lavage with dilute surfactant–polymer mixtures may help to directly remove inhibitors from the alveolar space and facilitate drug delivery. Another study showed that dextran improved the efficacy of Curosurf given at a low dose but not at a high dose in a lung injury model induced by tracheal instillation of albumin [297]. This further demonstrates the negative effect of increased viscosity on the efficacy of surfactant formulations.

Finally, the use of ionic polymers with PS clearly requires considerably more investigation. Ionic polymers are superior to nonionic polymers in requiring lower effective concentrations. Hyaluronan (1240 kDa), an anionic biopolymer inherently existing in the alveolar fluid, was able to prevent serum-induced surfactant inactivation at a concentration 40 times lower than that of PEG (10 kDa) (1.25 mg/mL vs. 50 mg/mL) [222]. Chitosan (612 kDa), a cationic biopolymer derived from fully or partially deacetylated chitin, was shown to enhance the surface activity of dilute BLES and to reverse albumin–induced inactivation at a concentration 1000 times lower than PEG (10 kDa) (0.05 mg/mL vs. 50 mg/mL) [283,289]. The reduced polymer concentration facilitates drug delivery and minimizes osmotic stress in vivo. In spite of these advantages the mechanisms by which the ionic polymers interact with PL of PS are still vague. When the added polymer moieties are polyelectrolytes, a combination of electrostatic interactions and polymeric effects (such as depletion–attraction) is expected [283,289,290]. At extremely low concentrations, however, the polymer-induced depletion force (as predicted by Eq. (6)) would be weak and only secondary to the electrostatic interactions. Moreover, if the ionic polymers adsorb to the surface of PL vesicles by non-specific electrostatic binding, the depletion force would be more or less eliminated. Although direct experimental evidence for PS is still lacking, studies of pure PL vesicles [317,318] and monolayers [319,320] both suggested that chitosan interacted with PL mainly through electrostatic interactions.

When using ionic polymers as surfactant additives, it is also important to keep in mind that the introduced electrostatic interactions may disturb the charge balance in surfactant. It was found that the effects of chitosan and hyaluronan on the surface activity of BLES were critically dependent on the polymer-to-PL ratio [283,289]. Above the optimal range of this ratio, i.e., at higher polymer concentration, an inhibitory effect on surface activity was observed [283,289]. These findings are consistent with previous reports that addition of polycations could inactivate surfactant as a function of the cationic additives-to-surfactant PL ratio [321,322]. Determination of the optimal ratio in vitro appears to require characterization of the pH and ionic strength of the electrolytes, especially the calcium concentration [289]. The ratio dependence of the ionic polymers complicates the surfactant formulation and would be best, if not only, tested using in vivo animal models (see Section 4).

To conclude Section 3, it has become apparent that, compared to ALI and ARDS, surfactant treatment of premature infants with RDS is relatively straightforward. One must simply recognize the signs that significant RDS is imminent and administer surfactant before serious lung injury occurs. This explains why the relatively poor protein-free synthetic surfactants employed in early trials were beneficial. They merely had to provide sufficient surfactant PL substrate to mix with endogenous alveolar pools of surfactant and/or cycle through the developing type II cells to maintain infants until they could generate sufficient endogenous surfactant of their own. Nevertheless, establishing surfactant therapy for neonates with basically healthy lungs took many years.

ARDS has clearly proven much more difficult. Part of the reason that ALI and ARDS are so difficult clinically is the lack of appropriate early markers for identifying those patients whose disease will not resolve spontaneously. Further difficulty arises from the multiple insults involved in lung injury and surfactant inhibition. Early pilot trials with infants clearly demonstrated the futility of introducing surfactant into lungs filled with surfactant inhibitors and this led to early treatment protocols with RDS. For a number of reasons, including the lack of dependable markers and expense, pulmonologists do not have the option of treating ARDS early and in practice are almost always faced with multiple inhibitory mechanisms arising from the original insult and including membrane and lysospholipids as well as various plasma proteins. In addition there is the release of proteases, phospholipases and other disruptive agents from lung tissue. To give one example, it has been shown that lyso-PC, at levels which do not affect the biophysical activity of surfactant, sensitizes surfactant to protein inhibition [249]. Another example is meconium aspiration syndrome, which involves many inhibitory agents including bile acids, fatty acids, proteins and other substances [14,173,250,323]. Under these circumstances, surfactant function will be inhibited by both competitive adsorption and film penetration and likely some other mechanisms. Consequently, the actual mechanism of inhibition in the case of ARDS can be expected to be quite complicated due to additive and synergistic effects of different inhibitors [324]. Different biophysical mechanisms of surfactant inhibition have been summarized in Fig. 6.

As it stands, overcoming inhibition will play a crucial role in the development of clinical surfactants for ARDS treatment. The use of low-cost, water-soluble polymers as surfactant additives has proven to be a promising approach in this direction. The early studies focused on the nonionic polymers, such as dextran and PEG. These polymers likely contribute to improving surface activity and overcoming inhibition by plasma proteins through a depletion–attraction mechanism. However, generating a large enough depletion force to enhance surfactant adsorption requires high concentrations of these polymers, thus introducing difficulties in tracheal drug delivery and causing increased osmotic stress in the lungs. More recent studies exploit the ionic polymers, such as hyaluronan and chitosan, which require a much lower effective concentration than the nonionic polymers.
Nevertheless the mechanism by which these ionic polymers benefit surfactant function and resistance to inhibition is still unclear. Considerable attention should be given to the negative reports of using polymers in animal trials. A number of in vitro studies have also demonstrated adverse effects of adding polyelectrolytes to surfactants, depending on the polymer-to-PL ratio. Taking into account the difficulty in controlling a fixed polymer-to-PL ratio, before and after drug delivery, we suggest that in vivo animal trials are crucial for testing the polymeric additives, especially the ionic polymers.

4. Methods for evaluating pulmonary surfactant

Methods for assessing the surface activity of PS fall into three categories: in vivo, in situ and in vitro techniques.

4.1. In vivo methods

As discussed in Section 2, a functional surfactant must be able to rapidly form a surface film at an air–liquid interface and to reduce \( \gamma \) to very low values under dynamic compression. When exogenous surfactant is administered to a surfactant-deficient lung, these biophysical characteristics should translate into lung compliance and, as a consequence, improved blood oxygenation. However, the in vivo situation is more complicated since the physiological response is not only determined by the biophysical properties of the surfactant, but also by how this material is delivered to the terminal airspaces and how it is metabolized once deposited [331]. For this reason evaluation of newly developed exogenous surfactants utilizing in vivo experimental models is critical prior to clinical usage of such preparations. These in vivo methods contribute first-hand physiologically relevant information on the factors influencing surfactant therapy, such as the efficiency of different surfactant preparations, the effects of different means of administration, dosing and timing, etc.

Various in vivo models have been developed to evaluate the efficacy of surfactant replacement therapy on preterm and term animals. Accordingly, the commonly used in vivo models can be generally divided into those primarily involving surfactant deficiency, such as the premature animal model and the saline lung lavage model in adult animals, and those primarily involving surfactant dysfunction or inactivation, such as the meconium aspiration, high-stretch ventilation, and acid aspiration models [332,333]. The overall objective of the latter models is to investigate the therapeutic potential for the specific lung injury rather than an examination of the biophysical properties in vivo. As such, a detailed discussion of those studies is beyond the scope of this review and we will focus solely on the surfactant-deficient models.

Evaluation of surfactant efficacy in premature animals has been performed mainly with premature rabbits [273,334–336]. In these experiments the rabbit fetuses are delivered by caesarean section at a gestational age of 27 or 28 days, at which time the lungs are still surfactant-deficient. The premature rabbits are connected to a pressure controlled ventilator in a 37 °C plethysmography setup in which the peak pressure at which each rabbit is ventilated can be controlled individually. Surfactant can be administered directly into the lungs of the animals through a bolus injection via an endotracheal tube. The main outcome of surfactant efficacy in this model is dynamic lung compliance during ventilation.

The two main advantages of the preterm rabbit model are the size of the preterm rabbit lung which ensures optimal surfactant distribution throughout the lung with a relatively simple instillation procedure, and the ability to rapidly perform measurements on a large number of animals simultaneously. Disadvantages include the relatively short period of observation which does not allow for detailed metabolic assessments of the exogenous surfactant, and limited flexibility in terms of ventilation strategy during the analysis. This model has been utilized extensively to test exogenous surfactant, including studies comparing the efficacy of different surfactants, testing protein inhibition and examining the physiological activity of different surfactant subtypes [273,334–336].

A second preterm animal model that has been utilized is the premature lamb [337,338]. Although conceptually similar to the preterm rabbit model, this model has the advantage of a larger animal size which more closely mimics the premature infant observed in RDS. This model allows for more extensive measurements on blood gases, surfactant distribution, and metabolism. On the other hand, the premature lamb is considerably more labour-intensive and expensive. In general, this model is suitable for more extensive preclinical studies for exogenous surfactant therapies.

The saline lavage model in adult animals has also been utilized extensively for testing surfactant preparations [309,339–343]. In general, this model involves connecting the animal to a mechanical ventilator and subsequently lavaging the lung with saline to remove the endogenous surfactant. One response to the lavage procedure will be the secretion of intracellular surfactant; thus the lavage procedure is repeated several times at regular intervals. Depletion of surfactant is generally reflected by low blood oxygenation, and can be confirmed by analysis of the amount of surfactant removed by the final lavage procedure. Subsequently, exogenous surfactant can be administered to the surfactant-deficient animal. The most relevant physiological
outcome of surfactant function in these studies will be blood oxygenation. The animals can be monitored for several hours which may reflect how the surfactant is handled in the airspace.

The adult saline lavage model has been employed in a large variety of species including rats, rabbits, pigs and sheep. In general, smaller species are utilized for simple comparisons between different surfactant preparations. Larger animal models may also test efficacy of surfactant preparations, but more often are utilized for other aspects that influence the surfactant therapy such as the delivery method or the effects of ventilation strategy after treatment.

In general, most in vivo studies examining the activity of surfactant have confirmed the previous in vitro data. For example, in vitro analysis of the biophysical activity of the different surfactant subfractions, i.e., the large aggregates (lamellar bodies, multilamellar vesicles, tubular myelin) and small aggregates (small unilamellar vesicles), indicated that the large aggregates represented the functional component of surfactant [307]. This was subsequently confirmed in vivo utilizing both the premature rabbit and the lavage rat models [309,335]. In another study Bailey et al. utilized lavaged rats to compare the physiological responses to an exogenous surfactant (BLES) with and without exposure to oxidation [339]. This study confirmed previous in vitro data that had demonstrated that oxidation impaired the surfactant function [153,212]. Importantly however, not all in vivo studies correlate with the in vitro biophysical activity. For example, as part of the study mentioned above, Bailey et al. [344] found that SP-A addition improved the biophysical activity of oxidized BLES in vitro, but this combination was not as effective as control BLES in vivo. Also, based on investigations in which the addition of PEG to surfactant was able to improve the exogenous surfactant properties in vitro, Campbell et al. [312] investigated this in the lavaged rabbit model. In contrast to the in vitro data, animals receiving surfactant with PEG had an inferior physiological response compared to animals receiving just surfactant. A subsequent study suggested that the administration of PEG in this model led to increased extracellular lung water thereby limiting its functionality (see also Section 3) [313].

4.2. In situ methods

Two in situ methods have been developed to estimate alveolar γ in excised lungs. One is the pressure–volume (P–V) method initially used by von Neergaard [22] in his early discovery of the importance of alveolar γ forces. This procedure was also used by Mead et al. [26], followed by standardized methods by Fisher et al. [345], Bachofen et al. [121,346] and Wilson [347,348]. This method indirectly determines alveolar γ by analyzing the P–V isotherms of excised lungs ventilated in a quasi-static manner. The principle of this method is as follows: inflating or maintaining the lungs at a fixed volume with air requires work against both tissue forces and γ forces. Filling the lungs with saline annihilates the air–liquid interface, thus leaving only the lung tissue forces. Hence, the difference between the P–V isotherms of air-filled and saline-filled lungs reflects the contribution of γ forces. In practice, this procedure is complicated by the need to remove all air from the lungs. This can be achieved by allowing the lungs to collapse completely. For instance, this can be aided by brief ventilation with pure O2 which, in contrast to N2-containing air, can be completely absorbed by the vasculature.

Another in situ method is the microdroplet technique first developed by Schuch and coworkers [28,119,120,349] in the 1970s. This is so far the only method truly capable of direct γ measurement in the lung. With this method, a droplet of a water-immiscible liquid (e.g., fluorocarbon) with known γ is deposited onto the alveolar surface of excised lungs using a micropipette. The droplet will spread on the surfactant lining layer of the alveoli to form a liquid lens. Theoretically, the equilibrium shape of this lens (its contact angle) is determined by the balance of γ of the three adjacent interfaces, i.e., air–lens liquid, air–lining layer, and lens liquid–lining layer interfaces, as predicted by the classical Neumann triangle relation [116]. Hence, the shape of the liquid lens can be used to estimate γ of the surfactant lining layer. In practice, the alveolar γ is estimated from the diameter of the liquid lens, monitored by a microscope. A calibration curve (γ vs. diameter of the lens) for the immiscible fluid is determined in a separate in vitro experiment using a Langmuir balance. It should be noted that this calibration procedure may introduce errors into the in situ γ measurements [350]. This is because the shape and diameter of the liquid lens is determined by the surface tension balance only for a sufficiently deep subphase. If the thickness of the subphase is similar to the penetration depth of the liquid lens, the lens can be significantly distorted by a disjoining pressure [351]. Consequently, the γ-diameter curve calibrated in a Langmuir balance, with a macroscopic subphase, could deviate from the in vivo situation, where the aqueous hypophase of alveoli is extremely thin (i.e., ∼0.2 μm, see Section 2).

The microdroplet method has also been successful applied to measure γ in vivo, for example, in the trachea of non-anesthetised horses [352].

4.3. In vitro methods

Because of their convenience, in vitro methods are most commonly used for examining the surface activity of PS. A variety of techniques have been developed for this purpose. Three widely used methods are the Langmuir–Wilhelmy balance, pulsating bubble surfactometer, and captive bubble surfactometer. In addition to these three, a method called the constrained sessile drop was recently developed for the in vitro assessment of PS. These four methods will be extensively discussed and compared. Other in vitro methods will also be introduced, albeit in much lesser detail. Other reviews on the three traditionally used in vitro methods are also available [14,84,104,105,353,354].

4.3.1. Langmuir–Wilhelmy balance

The classical Langmuir balance was introduced to surface science in the early years of the 20th century [118]. An insoluble surfactant monolayer, usually in organic solvent, is spread on top of a liquid subphase, usually aqueous, filling a trough. The film is confined by two barriers that move symmetrically, or by a fixed one on one side and a movable one on the other side. The film can be slowly compressed and expanded in a quasi-static fashion by the relative movement of the two barriers. The force acting on the floating barrier is measured by a horizontal force transducer, which directly indicates π. Clements [27,355–357] modified Langmuir’s original design to introduce the Langmuir–Wilhelmy balance (LWB) to the study of PS. As shown in Fig. 7, the LWB consists of a Langmuir trough constructed of Teflon and a Wilhelmy plate for measuring γ of the film-covered subphase. Recall that π and γ are coupled by Eq. (1) (see Section 2).

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**Fig. 7.** 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 (i.e., decreasing surface tension, according to Eq. (1)). The surface tension is determined by measuring the change in the vertical pull on the Wilhelmy plate.
This early tensiometric apparatus not only initiated a virtual explosion in surfactant research (Section 2), but also remains popular even now. The LWB is well suited for recording π-area isotherms of spread monolayers as the surface area per molecule can be precisely determined by controlling the amount of surfactant spread and the surface area available for spreading. This feature is particularly useful to characterize the surface rheological properties of individual PS components and their simple mixtures. Another primary advantage of the LWB is the capability for easy combination with a variety of microscopic and spectroscopic techniques [358], which are not readily amenable to the other in vitro methods. Such assemblies allow direct examination of molecular structure, orientation, domain formation, topography, electrical surface potential, and localized chemical composition of either surfactant films at the air–water interface or films transferred to a solid substrate using the Langmuir–Blodgett technique. The LWB has been used in conjunction with Brewster angle microscopy [143,146,359], fluorescence microscopy [63,142,143,146,167,359], confocal microscopy [87,360], scanning near-field optical microscopy [361,362], AFM [63,149,157,166,241,363–365], grazing incidence X-ray diffraction [364,366], infrared spectroscopy [367,368], sum-frequency generation spectroscopy [369,370], and ToF-SIMS [152,156,260]. Application of these film imaging/analysis techniques to PS studies has provided valuable information that complements the traditional tensiometry techniques.

In spite of the above merits, the LWB has a number of drawbacks for studying PS. First, it is not ideal for studying surfactant adsorption from the subphase as it requires a relatively large amount of liquid sample, i.e., usually no less than several tens of millilitres. Second, the LWB only allows relatively slow compression–expansion cycles as fast cycling creates waves at the air–water interface, which interfere with the γ measurement. Together with the difficulties in precisely controlling the physiological temperature and humidity, the LWB does not directly simulate respiration. Third, there can be a problem with the measurement accuracy of the Wilhelmy plate as it requires a 0° contact angle (according to Eq. (7)). The leakage can be minimized by keeping the capillary dry. However, the effect of leakage can be augmented when studying surfactant inhibition due to plasma proteins as these proteins can adsorb onto the capillary during bubble formation and these proteins, being hydrophilic, retain water, thus impeding drainage.

4.3.2. Pulsating bubble surfactometer

The pulsating bubble surfactometer (PBS) was first introduced by Enhorning in 1977 [376]. As shown in Fig. 8, the currently commercially available PBS consists of an air bubble formed in a disposable polyacrylamide chamber. The chamber contains only 20 μL of the test liquid and is immersed in a temperature-controlled bath. The bubble is formed by drawing air from the atmosphere through a capillary (bubble-on-a-tube model). Adsorption is monitored for 10 s. Subsequently, the bubble is oscillated by a pulsator between two fixed positions: a minimum radius of 0.4 mm and a maximum radius of 0.55 mm, which produces a maximum 50% reduction in surface area. The cycling frequency usually used is 20 cycles/min to mimic breathing. However, it can be changed from 0.02 to 80 cycles/min.

During oscillation, the maximum and minimum radii (R) of the bubble are monitored by a microscope. The pressure gradient across the bubble (∆P) is measured by a pressure transducer. Since the bubble communicates with the ambient atmosphere, the pressure gradient measured by the transducer corresponds to the negative pressure across the liquid phase. γ is calculated using the Laplace equation for a spherical interface

$$\Delta P = \frac{2\gamma}{R}$$

(7)

The PBS is highly efficient and time-effective. One measurement can be completed within 5 min and takes only ≈20 μL of sample. Therefore the PBS has been widely used for the quality control of clinical surfactants. In addition, it is superbly useful for monitoring and comparing the surface activity of different surfactant samples when many animal or clinical samples are generated. The rapid adsorption during expansion is indicated by the γ of the film at the maximum bubble radius and the ability to reach low γ is examined when the film is compressed to the minimum bubble radius. Another advantage of the PBS, which makes it superior to the LWB, is the fast pulsing rate that permits direct simulation of breathing.

Nevertheless, the PBS has some drawbacks. First, for a number of reasons, leakage is a serious problem. At low γ leakage can occur at both the inner surface (air–solid) and outer surface (liquid–solid) of
the capillary. The original PBS reported by Enhorning used home-made chambers with a Teflon capillary which reduced leakage onto the air–solid interface [376]. With the present polyacrylamide chamber, a thin film of water can be retained inside the capillary when the bubble is formed, thus leading to a much greater film area than anticipated (see the inset in Fig. 8). During pulsation, the water film can drain from the capillary but this can be variable [377]. For example, plasma proteins can adsorb onto the capillary during bubble formation and the proteins, being hydrophilic, retain water, thus impeding drainage. Hence, it is particularly problematic with studies of surfactant inhibition by plasma proteins where the apparent deleterious effects of these proteins can be augmented in the PBS due to leakage. Also because of leakage, the PBS is not suitable for studying film stability at $\gamma_{\text{min}}$ in a non-pulsating mode.

A second problem with the commercial PBS is the lack of flexibility in assessing the surface activity. For example, the time for adsorption to equilibrium is set at 10 s. While this can be changed, it cannot be done easily. As a result, animal and clinical samples are subject to expansion and contraction, often before $\gamma_{\text{eq}}$ is attained. This obviously has an impact on $\gamma_{\text{min}}$ and $\gamma_{\text{max}}$ as well as $\gamma_{\text{eq}}$ recorded for that particular sample. Lengthening the adsorption time would also help to minimize the amount of water remaining inside the capillary and therefore film leakage. In addition, the way that the current commercial apparatus functions is to initiate pulsation by expanding the bubble from the minimum radius at the end of the 10 s adsorption period. Thus, even if the sample has attained $\gamma_{\text{eq}}$, an additional 50% of area is imposed in 1.5 s (for pulsating at 20 cycles/min), making it impossible to relate initiation of compression to $\gamma_{\text{eq}}$. Moreover, although additional information can be accessed, the PBS usually records only the $\gamma$ of the bubble at the maximum and minimum radii, to be the $\gamma_{\text{max}}$ and $\gamma_{\text{min}}$ in a pulsating mode. The maximum reduction of bubble area, i.e., the compression ratio, is fixed at 50%, which prevents detailed study of surface rheology.

Finally, $\gamma$ measurements at low values using Eq. (7) are not reliable. Even for a small bubble less than 1 µL (≈1 mm in diameter), the assumption of spherical shape does not hold true when the $\gamma$ falls to values near 1 mN/m [378]. The effect of gravity on bubble deformation at low $\gamma$ and other effects of altering the bubble shape such as the hydrodynamic effects due to rapid pulsating have been extensively studied by Franes and coworkers [379–381]. Nevertheless, it should be stressed that in most situations where this apparatus is used to compare functional vs. non-functional surfactants, the estimations of $\gamma_{\text{min}}$ are more than adequate [378].

A recent development by Seurynck et al. [382] contributes to improving the current PBS. A real-time image acquisition system has been integrated into the traditional PBS, which allows the determination of $\gamma$ and surface area at any point of pulsating. In addition, instead of simply assuming a spherical shape, these workers fit the bubble profile to an ellipse. This yields enhanced accuracy compared to spherical fitting. Although still incapable of preventing film leakage, the real-time imaging facilitates visual detection of film leakage into the capillary. Those images with leakage can be discarded in data processing.

4.3.3. Captive bubble surfactometer

The captive bubble surfactometer (CBS) was first introduced by Schurch et al. in 1989 [383]. As shown in Fig. 9, an air bubble (2–7 mm in diameter) is introduced into a chamber where it floats against a ceiling coated with 1% agar gel. The agar coating renders the ceiling completely hydrophilic. Consequently, the bubble is separated from the ceiling by a thin wetting film of the surrounding aqueous liquid, thus avoiding adhesion to any solid support and apparently eliminating all potential pathways for film leakage [384]. It should be noted that the use of the agar coating is not always necessary provided that the ceiling is smooth and hydrophilic. It has been shown that a chamber made of stainless steel without the agar coating is also capable of maintaining a leakage-proof environment [385].

The original CBS chamber was constructed from a gastight syringe making it very difficult to spread films so that only adsorbed films were studied [383,384]. More recently, different film spreading techniques have been developed for the CBS [386,387]. With one spreading technique developed by Putz et al. [386], films are spread from organic-extracted surfactants onto the bubble surface with a microsyringe, followed by exhaustive subphase replacement to remove the organic solvent vapor. Another type of spreading technique was developed by Schurch et al. [387]. Films are spread/adsorbed at the bubble surface from an aqueous suspension of high-concentration surfactant using a microsyringe. To ensure the surfactant floats around the bubble surface to enhance spreading, the saline subphase can contain 10 wt.% sucrose, which increases the density of the subphase.

After film formation, the bubble can be compressed and expanded in either quasi-static or dynamic fashion by varying the hydraulic pressure in the chamber. The pressure can be changed by varying the chamber volume [383] or by regulating liquid flow between the chamber and an external reservoir (with the manufactured plastic chamber) [388]. The frequency of cycling can be varied from extremely slow (i.e., in the case of quasi-static cycling) to faster than 60 cycles/min.

In contrast to the PBS, the shape of the bubble in the CBS cannot be assumed to be spherical due to its relatively large size. (See Fig. 10(a) and (b) for two typical captive bubble images with different $\gamma$.) Instead, the bubble shape is controlled by the mechanical balance between the $\gamma$ forces and local gravity, according to the Laplace equation of capillarity:

$$\gamma \left( \frac{1}{R_1} + \frac{1}{R_2} \right) = \frac{2\gamma + \Delta g z}{R_0}$$

where $R_1$ and $R_2$ are the two principal radii of curvature at the studied point on the surface, which reflect the shape of the bubble; $R_0$ is the radius of curvature at the apex of the bubble; $\Delta g$ is the density difference across the interface; $g$ is the local gravitational acceleration; $z$ is the vertical distance from the apex to the studied point. Normalizing Eq. (8) yields a single nondimensional characteristic parameter, called the Bond number ($B$)

$$B = \frac{A\rho g R_0^2}{\gamma}$$

The Bond number reflects the relative effects of gravity and capillary forces on the shape of a drop/bubble. Hence, it is possible to determine $\gamma$ from the bubble shape since gravity is known. One way of characterizing $\gamma$ from the bubble shape is to use the height-to-diameter ratio of the bubble, as formulated by Malcolm and Elliott [389]. Bubble area and volume can be calculated from polynomial functions.
regressed from measurements of a series of sample bubbles with a wide range of height-to-diameter ratios [390].

A more accurate and automatic way to determine $\gamma$, surface area and volume from a bubble image is to use axisymmetric drop shape analysis (ADSA). ADSA was first developed by Neumann and associates in the 1980s [391]. ADSA has been continuously improved during the last two and a half decades [392–394]. Conceptually, ADSA determines $\gamma$ by numerically fitting the shape of experimental drops or bubbles to the theoretical profiles given by the Laplace equation of capillarity (Fig. 11). The input parameters of ADSA are $g$, $\Delta\rho$, and a number of coordinates of the experimental drop/bubble profile automatically detected by digital image analysis. ADSA was first applied to analyze captive bubble images by Prokop et al. [385]. A new generation of ADSA has been recently developed [394–396]. By using advanced image analysis, this new ADSA is capable of automatic $\gamma$ measurements of turbid fluids, such as PS in which optical noise is introduced by insoluble surfactant aggregates and sometimes satellite bubbles (see Fig. 10(a) for an example). This new ADSA was found to be particularly useful for studying surfactants with polymeric additives where system turbidity increases (see Section 3 for detail) [394–396].

Strictly speaking, determination of $\gamma$ from the shape of a drop/bubble is only applicable to an equilibrium condition since the Laplace equation (Eq. (8)) considers only the balance between the gravity and capillary forces. Under a highly dynamic condition, such as dynamic cycling in the CBS, the bubble profile may deviate from the theoretical profile predicted by the Laplace equation due to hydrodynamic effects (such as inertial and/or viscous effects caused by fluid flow). This may introduce errors in $\gamma$ measurement using ADSA. Liao et al. have systematically studied the hydrodynamic effects on the measurement of dynamic $\gamma$ using an oscillating bubble [380,381]. Results from the numerical simulation suggested that the hydrodynamic effects were negligible except at some extreme conditions, such as a highly viscous liquid oscillated at an extremely high frequency. For a bubble in aqueous media, only if the oscillatory frequency is slower than 10 Hz (i.e., 600 cycles/min) will the bubble profiles predicted from ADSA and from the model considering the hydrodynamic effects be in good agreement [381]. The effects of viscous forces on ADSA measurement were also studied by Freer et al. [397]. Similarly it was found that the viscous forces were only significant enough to alter the ADSA measurement for highly viscous liquid upon highly rapid dynamic oscillation.

TheCBS has been used to measure adsorption rate (time to equilibrium), $\gamma_{\text{min}}$, and $\gamma_{\text{max}}$, during quasi-static or dynamic compression–expansion cycling, and the percent of area reduction required to attain $\gamma_{\text{min}}$ from $\gamma_{\text{max}}$. The percentage of area reduction has been shown to be a more sensitive parameter than $\gamma_{\text{min}}$ in evaluating surface activity of PS, especially in the study of surfactant inhibition. In addition, owing to its leakage-proof capacity, the CBS is well suited for studying mechanisms of surfactant films, such as film compressibility, stability, and collapse at extreme $\pi$. For films adsorbed at low surfactant concentrations (0.2–0.5 mg/mL), near-zero $\gamma$ can be readily obtained by a moderate compression ratio (i.e.,<20% area reduction). This is in good agreement with the in situ measurements. Studies of

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**Fig. 10.** Typical images of captive bubble (CB), constrained sessile drop (CSD) and pendant drop (PD) at different surface tensions. (a) CB at 21 mN/m; (b) CB at 1.3 mN/m; (c) CSD at 47 mN/m; (d) CSD at 0.68 mN/m; (e) PD at 42 mN/m; (f) PD at 16 mN/m. The black particles in the CB images are insoluble surfactant aggregates suspended in the subphase, which introduce optical noise to the images. The arrow in (a) points at a satellite bubble formed during the experimental manipulations, which can have a very deleterious effect on the surface tension measurement. Arrows in the CSD and PD images point at the three-phase contact line. The three-phase contact line in (f) is not discernible due to film leakage. The PD and CSD images are courtesy of Ms. Zdenka Policova, University of Toronto.
Fig. 11. Principle of axisymmetric drop shape analysis (ADSA). ADSA measures surface tension from the shape of pendant drops, sessile drops or captive bubbles by comparing the experimental drop/bubble profiles (dots in the X-Z coordinate) with the theoretical Laplacian curves (curve in the X-Z coordinate). The experimental profiles are automatically extracted from the drop/bubble images using advanced image analysis. The theoretical Laplacian curves are generated by numerical integration of the Laplace equation of capillarity (Eq. (9)). ADSA determines surface tension by iteratively fitting the experimental profile to a family of theoretical curves until the best match is found. The best matched theoretical curve represents the experimental drop/bubble. ADSA solves this optimization problem by minimizing an objective function that consists of the sum of least squares of the minimum distance (i.e., normal distance, d_0) between the experimental points and the theoretical profiles. With the input of the local gravitational acceleration (g) and the density difference across the interface (Δρ) (see Eq. (9)), five parameters can be simultaneously optimized: the surface tension (γ), the curvature at the apex (1/R_0), the coordinates of the apex of the experimental profile (X₀, Z₀), and the vertical misalignment (α). The surface area and volume of the drop/bubble are calculated from the free surface of revolution of the best matched theoretical profile. Adapted from del Rio and Neumann [393].

film stability in the CBS are commonly performed in two different ways. Stability can be examined by compressing the bubble and holding the volume at a minimum value. A stable film is able to maintain the γ_{min} (in an isovolumetric fashion) without returning towards equilibrium for a prolonged period [307,374]. Similarly, stability can be examined by studying the rate of bubble area reduction required to maintain a constant γ, i.e., in an isobaric fashion [97,145]. Alternatively, stability can be examined by studying “bubble clicks”, i.e., a sudden decrease in bubble area associated with an increase in γ, which indicates partial collapse of the surfactant film coating on the surface [374,383]. Bubbles coated with a stable film have less tendency to click. The CBS is also flexible in terms of being able to vary independently the amount of film compression (i.e., compression ratio) and the speed of the compression (i.e., compression rate) [377]. Moreover, in conjunction with other techniques, the CBS is able to scrutinize a variety of surfactant characteristics, such as the surface-associated surfactant reservoir, using a subphase depletion technique [60] and a subphase solidification technique (solidification of the hypophase–surfactant lining complex in a sodium alginate solution by adding calcium ions) [59]. In addition to γ measurements, the CBS has been broadly expanded to other investigations, e.g., gas transfer properties of PS films [398,399], dissolution characteristics of anesthetic vapors and gases [400].

A major advantage of the CBS is the ability to conduct investigations at physiological and higher temperatures. For example, with the LWB, it is difficult to demonstrate that DPPC monolayers can attain n of 70 mM/m (i.e., near-zero γ) at 37 °C [95,96] and relatively few studies using this apparatus show π higher than 50 mM/m even at room temperature, when the films are compressed in a quasi-static fashion. In fact, results from a number of LWB studies have concluded that DPPC monolayers become unstable somewhat above 41 °C [92–94,401], which of course corresponds to the main transition temperature T_m for DPPC bilayers (detailed in Section 2). These experimental studies suggested that PL monolayers might possess a critical point. A critical point can be defined as the temperature and pressure above which the properties of two phases no longer remain distinct. For example, above 31 °C and 73 atm, CO_2 becomes supercritical, a fluid which has the characteristics of both a liquid and a gas. However, more recent investigations using the CBS have shown that spread DPPC monolayers melt between 48 and 53 °C and found no evidence for critical behavior [97,145]. While the case for critical behavior of PL monolayers depends on several arguments, these melting data make this phenomenon considerably less certain. In any event, such CBS studies emphasize the advantage of employing this apparatus for experimentation under extreme conditions.

Nevertheless, the CBS also has weaknesses. First, in contrast to the LWB, the γ and surface area (i.e., the bubble area) in the CBS is coupled. This is due to the fact that the area of a bubble is a function of not only its volume but also its shape as determined by the γ forces. When a surfactant-coated bubble is compressed, the bubble volume decreases, which tends to decrease the bubble area, and meanwhile, the γ decreases, which tends to increase the bubble area [374]. Hence, it is more difficult to precisely control the bubble area than its volume in the CBS. Nevertheless, in most cases this does not pose a serious concern on the recorded γ-area isotherms because the apparent area at each time is still calculated based on the same bubble image from which the γ is estimated. Second, compared to the PBS, operation and data processing of the CBS is relatively time-consuming. The problem of tedious data processing can be largely solved using ADSA [394–396]. Third, for adsorption studies, the maximum surfactant concentration is usually restricted to no more than 30 mg/mL [58]. This restriction arises from optical limitations since surfactant suspensions become murky and eventually opaque at increased concentrations. High-concentration surfactants can be studied using spreading techniques. Finally, the CBS may not ensure full humidification automatically. This concern arises when the CBS is used to study surfactant adsorption in which the rapidly adsorbed surfactant films may present a barrier to water evaporation, thus slowing down the process of humidification [374]. Although direct humidity measurements in the bubble are not yet available, there was clearly a difference in the adsorption kinetics and film stability of BLES films formed on bubbles of ambient air and prehumidified air [374,402].

4.3.4. Constrained sessile drop

The prototype of the constrained sessile drop (CSD) was first developed by Wulf et al. [403] for simultaneously measuring γ and the density of polymer melts. It was later modified by Yu et al. [404] for determining the γ of PS. As shown in Fig. 12, a sessile drop (4–8 μL) of PS rests on a pedestal, which employs a sharp knife-edge to prevent the drop from spreading, thus eliminating film leakage. The pedestal is machined from stainless steel with a diameter of 2.5 to 4 mm. The angle between the horizontal and the lateral surfaces of the pedestal is 45° to 60°. The pedestal has a central hole of 0.5 mm, through which the drop is connected to a surfactant reservoir of 0.5–0.75 mL. The surfactant reservoir is continuously stirred by a magnetic stir bar. Formation and oscillation of the sessile drop is performed by a programmable motor-driven syringe connected to the surfactant reservoir.

Film formation in the CSD can be conducted by adsorption or spreading. For adsorption studies, a drop can be formed within 0.5 s, which ensures adsorption at a fresh, clean air–water interface. Spreading on the CSD from either aqueous solution or organic solvent is also straightforward. After film formation, the drop can be oscillated at a predetermined compression ratio and compression rate, from very slow to very fast.

The CSD facilitates rigorous atmospheric control by means of an environmental control chamber. Full humidification, monitored by a hygrometer, is ensured by a water reservoir enclosed in the chamber. A gas manifold system (to produce different gas compositions) and a gas chromatography system (to analyze the gas composition) are under development. This development aims at studying the effect of different gas compositions on the surface activity of PS films. It has been reported that different gases, such as CO_2 [405] and fluorocarbon gases [406,407], affected the surface activity of PS and PL films.

So far, no apparent serious limitations have been found with the CSD. This device eliminates both the problems of film leakage, as in...
4.3.5. Other in vitro methods

In addition to the above four techniques, other in vitro methods are available for assessing the surface activity of surfactants. For instance, the surface activity can be examined by studying microbubble stability [408–410]. Enhorning and Holm developed a capillary surfactometer, which is especially suitable for examining the role of surfactant in maintaining airway patency [411]. This method has proven especially useful for research involving asthma. Meier et al. [412] developed an oscillating drop surfactometer in which a 1 μL pendant drop is oscillated in resonance with an exciter. This method simultaneously measures γ and energy dissipation at the surface, thus providing an approach to evaluate surface viscosity. The γ is calculated from the experimentally determined oscillation period by considering the Rayleigh instability. However, this method is restricted to γ_min of ~ 15 mN/m, at which point the drop is released. Bertocchi et al. [413] developed an inverted interface technique in which γ is estimated from the curvature of a submicron-sized pendant drop. Combined with fluorescence microscopy, this method permits the study of adsorption of single surfactant aggregates. However, this method does not allow compression and expansion of the surfactant films.

Neumann et al. [414] have developed a pendant drop (PD) tensiometry system, which has been used in conjunction with ADSA (axisymmetric drop shape analysis).

Table 3
Comparison of four in vitro methods for assessing the surface activity of pulmonary surfactant

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<td>LWB</td>
<td>Precisely-controlled molecular areas for mixtures of phospholipids</td>
<td>• Not readily adaptable for studying adsorbed films</td>
<td>• Study of surface rheological properties of surfactant monolayers</td>
</tr>
<tr>
<td></td>
<td>Easy assembly with different surface analysis techniques</td>
<td>• Difficult to control the environment</td>
<td>• Study of phospholipid phase behaviors in conjunction with microscopic and spectrometric methods</td>
</tr>
<tr>
<td></td>
<td>Commercially available (e.g., Kibron Inc., Espoo, Finland; KSV Instruments Inc., Helsinki Finland; Nima Technology Ltd, Coventry, UK)</td>
<td>• Not capable of direct simulation of breathing</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Commercial product lacks flexibility in assessing surface activity</td>
<td>• μ measured from a Wilhelmy plate may not be very accurate</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Commercially available (General Transco, Inc., Largo, FL)</td>
<td>• Film leakage at low γ</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Close simulation of alveolar environment</td>
<td>• Commercial product lacks flexibility in assessing surface activity</td>
<td>• Quality control of clinical samples</td>
</tr>
<tr>
<td></td>
<td>Leakage-proof capacity</td>
<td>• Film leakage at low γ</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Capable of studying both adsorbed and spread films</td>
<td>• μ measured at low values are inaccurate</td>
<td>• Rapid comparison of the surface activity of many different samples</td>
</tr>
<tr>
<td></td>
<td>Accurate and automatic γ measurement in conjunction with ADSA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Commercially available (Department of Physiology and Biophysics, University of Calgary)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Precisely-controlled experimental environment</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Little sample consumption (in μL range)</td>
<td>• γ and surface area are correlated</td>
<td>• Study of surfactant mechanisms</td>
</tr>
<tr>
<td></td>
<td>Leakage-proof capacity</td>
<td>• Difficult to operate and clean</td>
<td>• Comprehensive and accurate evaluation of surfactant properties</td>
</tr>
<tr>
<td></td>
<td>Capable of studying both adsorbed and spread films</td>
<td>• γ and surface area are correlated</td>
<td>• Miniaturized Langmuir balance for studying spread monolayers</td>
</tr>
<tr>
<td></td>
<td>Accurate and automatic γ measurement in conjunction with ADSA</td>
<td>• Limitation on the max. surfactant concentration when studying adsorbed films</td>
<td>• Study of gas transfer properties of surfactant films</td>
</tr>
<tr>
<td></td>
<td>Easy to operate and clean</td>
<td>• Uncontrolled humidity when studying adsorbed films</td>
<td></td>
</tr>
<tr>
<td></td>
<td>No limitation in surfactant concentration</td>
<td>• Not yet commercially available</td>
<td></td>
</tr>
<tr>
<td>CSD</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

for measuring surface activity of PS [288,402,415–417]. The PD method features advantages of simplicity and high accuracy. However, it suffers from film leakage when \( \gamma \) of the surfactant films is decreased to \( \sim 18 \text{ mN/m} \). [385]. (See Fig. 10(e) and (f) for PD images at different \( \gamma \). Film leakage is shown in Fig. 10(f).) The PD is well suited for the study of surfactant adsorption as the limitation of film leakage is relatively minor in such a case. As mentioned in Section 2, the \( \gamma_{	ext{cm}} \) of PS films is approximately 22–25 mN/m, which is well above the threshold value at which leakage may occur. Inspired by the CSD, a new PD method is under development in which the traditional capillary is replaced by a pedestal with a sharp knife-edge to prevent film leakage. The pedestal is similar to that used in the CSD but upside-down. Cabrerizo-Vilchez et al. have further developed the ADSA-based PD technique by introducing a novel coaxial capillary design for rapid subphase exchange in a pendant drop [418]. With this development, the PD can be used as a fully functional miniaturized alternative to the traditional Langmuir balance for the study of insoluble PL films.

In summary of Section 4, different in vivo, in situ, and in vitro methods have been developed for the evaluation of biophysical properties of PS. The in vitro methods outperform the others in terms of convenience of operation and efficiency in collecting data. Among the in vitro methods available, four tensiometry techniques, LWB, PBS, CBS and CSD have exhibited full capacity to simulate and measure all three important biophysical properties of PS, i.e., rapid adsorption, low \( \gamma_{	ext{min}} \) upon film compression, and limited \( \gamma_{	ext{max}} \) upon film expansion. The relative merits and disadvantages of these four methods are summarized in Table 3. Selection of a specific method depends on the measurements of interest and the desired accuracy.

5. Concluding remarks

After more than a half century of research, PS proves itself to remain a “super extraordinary juice” [42] full of surprises. The most attractive motivation for studying PS is perhaps its clinical applications. Though there is still room for improvement, such as the ongoing development of fully synthesized designer surfactants, surfactant therapy has been very successful in treating premature infants with RDS. However, the applicability to ARDS, and other pulmonary diseases such as asthma, has not clearly been established. Inactivation of PS undoubtedly contributes to its unsatisfactory performance in ARDS. In vitro biophysical studies led to two distinct inhibition mechanisms: inhibition due to competitive adsorption of plasma proteins, and inhibition due to mixing and fluidizing an otherwise stable PS film by lipids. This simple classification implies that the former mechanism mainly influences surfactant adsorption while the latter prevents the films from reaching low \( \gamma \). Recent studies using direct film imaging, however, have provided new evidence that the actual inhibition mechanisms due to both proteins and lipids, such as cholesterol, could be much more complicated. In addition, the exact relevance of these inhibition mechanisms to ARDS is still undefined. In ARDS, the endogenous and exogenous surfactants are likely to be inactivated by multiple mechanisms. The predominant mechanisms by which surfactant is inhibited in vivo are still unestablished.

We would suggest that overcoming surfactant inhibition plays a central role in resolving the clinical constraints on surfactant therapy in ARDS. The use of polymers as surfactant additives is an intriguing approach due to its potent preclinical performance in resisting inhibition and the simplicity in formulation, i.e., by simply mixing water-soluble polymers with currently available surfactant preparations. However, it should be noted that the selection of a polymeric additive and the determination of its optimal concentration require consideration of physiological factors and not just surface activity. Selection of an appropriate evaluation model representing clinical practice, examination of the pulmonary fluid balance and the interfacial gas transfer properties, plus the ease of tracheal instillation all need to be kept in mind for the preclinical tests. Some of these concerns can be minimized by using ionic polymers, which seem to be effective at much lower concentrations than nonionic polymers. However, introducing ionic polymers may disturb the electrostatic balance in the PS system, which must be carefully considered.

Since Clements conducted the first direct \( \gamma \) measurements of PS using his home-made LWB half a century ago, many more in vitro tensiometric techniques have been developed. These methods have their own relative merits and disadvantages. Selection of a particular method depends on the application and the desired accuracy. During the last decade, in combination with the LWB, more and more surface analysis techniques have been applied to studies of PS. Application of these techniques opens new horizons to the study of PS. These tools enable direct film imaging on domain formation, topography, molecular orientation, electrical surface potential, film thickness, and even localized chemical compositions. Combining \( \gamma \) measurements with information obtained from direct film imaging has provided unprecedented insight into the behavior of PS, such as the finding that pressure-driven phase transition/separator occurs in surfactant monolayers, and that the formation of multilayer structures from monolayers occurs at high \( \pi \).

Progress has also been made in combining film imaging techniques with \( \gamma \) measurements using drop shape methods. It has proven possible to directly apply fluorescence microscopy [419] and even scanning force microscopy [420] at the air–water interface of a bubble with an adjustable size. Although limitations still prevent these methods from acting as a fully functional miniaturized Langmuir balance with in situ film imaging capacity, it is not unreasonable to foresee that such a mini-balance-imaging assembly may be developed soon. The CSD could provide a promising drop configuration in this direction as the air–water interface in the CSD is readily accessible to microscopic facilities. With the aid of new techniques more insight would be gained towards the biophysical understanding of PS and eventually for the development of new inhibition-resistant surfactant formulations for ARDS treatment.

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References


[1972]


