

Review

Role of pulmonary surfactant components in surface film formation and dynamics

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Abstract

Pulmonary surfactant is a mixture of lipids and proteins which is secreted by the epithelial type II cells into the alveolar space. Its main function is to reduce the surface tension at the air/liquid interface in the lung. This is achieved by forming a surface film that consists of a monolayer which is highly enriched in dipalmitoylphosphatidylcholine and bilayer lipid/protein structures closely attached to it. The molecular mechanisms of film formation and of film adaptation to surface changes during breathing in order to remain a low surface tension at the interface, are unknown. The results of several model systems give indications for the role of the surfactant proteins and lipids in these processes. In this review, we describe and compare the model systems that are used for this purpose and the progress that has been made. Despite some conflicting results using different techniques, we conclude that surfactant protein B (SP-B) plays the major role in adsorption of new material into the interface during inspiration. SP-C's main functions are to exclude non-DPPC lipids from the interface during expiration and to attach the bilayer structures to the lipid monolayer. Surfactant protein A (SP-A) appears to promote most of SP-B's functions. We describe a model proposing that SP-A and SP-B create DPPC enriched domains which can readily be adsorbed to create a DPPC-rich monolayer at the interface. Further enrichment in DPPC is achieved by selective desorption of non-DPPC lipids during repetitive breathing cycles. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Pulmonary surfactant is a mixture of lipids and proteins which is secreted into the alveolar space by epithelial type II cells. The main function of surfactant is to lower the surface tension at the air/liquid interface within the alveoli of the lung. This is needed to lower the work of breathing and to pre-

vent alveolar collapse at end-expiration. The surface tension of the air/liquid interface within the lung reaches values close to 1 mN/m at low lung volumes [1]. A lipid monolayer enriched in dipalmitoylphosphatidylcholine (DPPC) then covers the interface and is responsible for this low surface tension. Bilayer lipid structures are attached to the lipid monolayer and together they make up the surface film, as was visualized by electron microscopy [2,3]. These bilayer structures are thought to be in direct contact with the monolayer and to form the lipid reservoir from which insertion of lipids and possibly proteins is

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achieved upon inhalation. One study showed tubular myelin, a surfactant specific lattice-like lipid structure, being in continuum with the monolayer, indicating that the lipids of tubular myelin very likely feed the monolayer and can be part of the surface film [4].

The role of the different components of surfactant in the surface film structure and formation is becoming more evident. This is largely due to new model systems with reconstituted purified surfactant components. However, much remains to be learned regarding the molecular mechanisms and actions of especially the surfactant proteins. We review the current knowledge on the formation and dynamics of the surfactant film, focusing on the role that model systems played in assigning separate functions to the individual surfactant components.

2. Composition of pulmonary surfactant

Surfactant is composed of approximately 90% lipids and 10% proteins. The four surfactant specific proteins are designated surfactant protein A (SP-A), SP-B, SP-C and SP-D. These proteins can be divided into two groups, SP-B and SP-C are two small hydrophobic proteins, while SP-A and SP-D are large hydrophilic proteins. In the subsequent sections, the structure, composition and some general functions of the surfactant components are described. Since SP-D does not appear to possess any activity related to reducing the surface tension, this protein will not be further discussed in the current review. For more information on this protein the reader is referred to other review articles [5–7].

2.1. Lipids

The main constituents of surfactant are lipids. The composition of the surfactant lipid pool is quite different from that of other membrane systems. In all mammalian species, surfactant contains high amounts (approximately 80%) of phosphatidylcholine (PC) [8,9]. Generally, it is assumed that approximately 60% of the PC is in the dipalmitoylated form, but lower amounts of DPPC have been found as well [10,11]. A recent report by Brouwers et al. [12], using a new sensitive HPLC method to determine PC spe-

cies, describes a lower value of DPPC of approximately 40% in porcine surfactant. Other constituents are 16:0, 18:2 PC (18%) 16:0, 18:1 PC (20%) and 16:0, 16:1 PC (13%). Phosphatidylglycerol (PG) comprises approximately 10% of the lipid pool and small amounts of (lyso) phosphatidic acid (PA), phosphatidylinositol (PI) and phosphatidylethanolamine (PE) are found. Cholesterol is the most abundant neutral lipid present in pulmonary surfactant.

DPPC is generally accepted as being the lipid responsible for generating a near-zero surface tension at the interface during compression [13]. The two saturated acyl chains enable the lipid to form a tightly packed monolayer which can generate these low surface tension values without collapsing. The unsaturated PC lipids might be important in the formation of a lipid reservoir, in the initial adsorption of lipids to the interface or in the regulation of surface tension during the respiratory cycle. Other functions in intracellular events, such as lamellar body assembly, transport or secretion, are also possible. However, no specific role for any of the unsaturated PC species has so far been proven.

The unusually high amount of PG in surfactant indicates a particular role for this acidic phospholipid. DPPC:PG mixtures have increased adsorption activity compared to PC mixtures, also when the hydrophobic proteins SP-B and SP-C are present. A direct interaction of the negatively charged PG with the positive charges of SP-B and SP-C seems likely to be involved and indications for interaction with SP-B have been observed [14–16]. The levels of PI, which is also negatively charged, are usually low, although some exceptions exist. Surfactants from adult rhesus monkey [17] and guinea-pig [18] have relatively high amounts of PI and lower amounts of PG compared to most other species. PG can be replaced by PI in SP-B mediated lipid mixing or adsorption experiments without any loss of activity [19,20]. Rabbits that were fed a high inositol diet creating a high PI/PG ratio in their surfactant showed no signs of surfactant or lung dysfunction [21] although another group observed differences in the lamellar body turnover rate in similarly fed rats [22]. It will be interesting to see whether specifically PG is required for a role in surfactant and/or surface film homeostasis or if any acidic lipid can substitute for PG.

The role of cholesterol and minor lipid components like lysoPC, PE or PA in surfactant is far from clear. Cholesterol likely increases the fluidity of the DPPC-rich surfactant membrane systems, and the minor lipid components might induce structures of curvature in, for example, tubular myelin. The influence of both the minor lipid components and cholesterol on surfactant function has not been extensively studied and it is obvious that more work has to be performed to achieve a better understanding of their roles.

2.2. Surfactant protein A

Surfactant protein A was the first surfactant-specific protein to be detected. Its monomeric form has a molecular mass of 26 kDa on SDS-PAGE with several bands observed at higher molecular masses [23]. These represent glycosylated forms due to sialic acid containing complex carbohydrates attached to asparagine [24]. The primary amino acid sequence of SP-A shows that the protein can be divided into four structural domains: (1) a short N-terminal segment; (2) a proline-rich collagen-like domain; (3) a neck region; and (4) a carbohydrate recognition domain. The active form of SP-A is an octadecamer built up from six trimers which forms an open flower bouquet [25] (Fig. 1). The formation of collagen-like triple helices results in a trimer structure followed by disulfide linkage of six of these trimers by the N-terminal segments.

The concepts concerning the function of SP-A have changed dramatically over the past few years. Initial *in vitro* experiments indicated that this protein is involved in surfactant function and homeostasis, including: (1) tubular myelin formation [26–28]; (2) protection of the surface film against protein inhibition [29–31]; (3) enhancement of SP-B's surface activity [32,33]; and (4) regulation of uptake and secretion of surfactant by type II cells [34–36]. In recent years, the emphasis of SP-A research has shifted towards its role in host defense [37–39]. SP-A-deficient mice, produced by targeting the SP-A locus by homologous recombination in embryonic stem cells [28,40], gave more insight into the *in vivo* function of the protein. These animals lack tubular myelin and are more susceptible to several pathogens, confirming the host defense role. On the other hand, the

animals unexpectedly have normal oxygenation, apparently contradicting the role of the protein in surface film formation. However, the surfactant dysfunctions due to the lack of SP-A might be more subtle and only show up in stress situations. Further characterization of these mice will definitely provide a better understanding of the complexity of the SP-A functions and their relative importance.

Because of the distinct structural segments of SP-A, several functions of the protein can be assigned to different segments. Much information regarding this structure–function relationship has been produced by synthesizing mutant forms of the protein in the baculovirus expression system with the mutations varying from point mutations up to deletions of whole segments. To describe a detailed overview of these studies is beyond the scope of this review and the reader is referred to other articles (for a review see [41]).

2.3. Surfactant protein B

Surfactant protein B is a hydrophobic protein that consists of 79 amino acids and forms a homodimer of ~17 kDa [32]. Due to its hydrophobicity, SP-B will interact with lipids. This has been observed using several techniques including electron spin resonance [14] and fluorescence anisotropy [16]. SP-B has a net positive charge and the positive charges of SP-B are thought to interact with anionic lipids [16,42]. Another characteristic feature of SP-B is that it contains three intramolecular disulfide bridges and one intermolecular disulfide bridge which results in the dimer form of the protein [43]. The mature protein is formed from a preprotein of 42 kDa after cleavage of both N-terminal as well as C-terminal sequences [44,45]. The preprotein can be arranged in three tandem repeats, based on the primary sequence and especially the conserved cysteine periodicity. This motif is also observed in the family of 'saposin-like proteins' [46,47]. The structure of one family member, NK-lysin, has been determined recently by NMR and shows that the protein contains five helices that interact with the membrane surface [48]. It is tempting to predict a similar structure for SP-B, but other models with varying lengths and number of α -helices within the protein have been proposed. One of them is shown in Fig. 1. One major difference

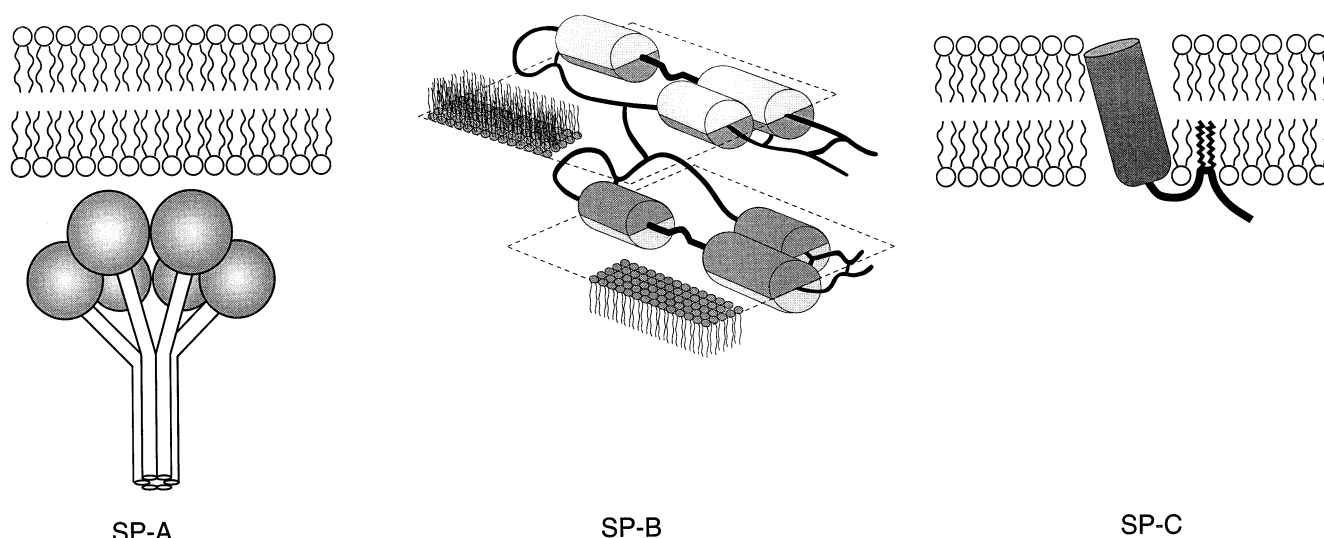


Fig. 1. Structure of the surfactant proteins A, B and C.

between SP-B and the other family members of the saposin-like family is the homodimer formation of SP-B.

Fourier transform infrared experiments have shown that 27–45% of the secondary structure of SP-B is helical, independent of the presence of lipids or calcium [49,50]. The axes of the helices lie nearly parallel to the interface of a lipid bilayer [51], which is in agreement with the proposed amphipathic character of the helices. It was observed that addition of SP-B to preformed lipid vesicles led to the formation of small bilayer disks [52,53]. The authors proposed that SP-B lined the edges of these disks protecting the phospholipid acyl chains from interaction with water. The disks fused rapidly into membranous bilayer sheets that assembled into multilayer stacks, which could be important for lamellar body formation inside the type II cell. Recent reports show that the exact interaction of SP-B with the membrane depends on the method of reconstitution [54,55]. SP-B reconstituted from lipid/protein mixtures penetrates deeper into the lipid bilayer than SP-B added to preformed liposomes and this influences its activity in Wilhelmy balance studies. This problem will make it more difficult to determine the exact structure of the protein in a lipid environment.

The importance of SP-B in surfactant is apparent from the lethal respiratory distress that is caused by SP-B deficiency in humans [56] and in SP-B knockout mice [57]. SP-B has been shown to have many

activities in *in vivo* and *in vitro* assays. These include promotion of lipid adsorption to the air/liquid interface [58], formation of tubular myelin [26,27], re-spreading of films from collapse phase [59], reuptake of surfactant by type II cells [60,61], stabilization of monolayer lipid films, membrane binding, membrane fusion and lysis [53]. Some of these functions will be discussed in more detail later in this review. The homodimer formation of SP-B is thought to be important for several of these functions. Each monomer can interact with a membrane system and in that way SP-B could bring two membranes in close proximity (Fig. 1). The connection to the fusogenic and aggregating properties of SP-B is easily pictured. However, no proof for this localization of the protein has been found and further studies using monomeric SP-B must still be performed [62].

A more surprising role of SP-B might be found in host defense. Recently, it was described that a synthetic SP-B peptide inhibits bacterial growth [63]. The fusogenic properties measured *in vitro* might be important for this function. The sequence homology of SP-B with other antibacterial peptides like the saposins and dermaseptins is consistent with this function. Finally, one study describes the detection of SP-B in the gastrointestinal tract (as well as SP-A and SP-D) [64] which also argues for a more extended function of SP-B than just surface activity related ones.

2.4. Surfactant protein C

Surfactant protein C is the only true surfactant protein in the sense that it is the one protein that seems to be present only in pulmonary surfactant [65]. The only site of synthesis discovered so far is the alveolar type II cell. SP-C is a 35 amino acid peptide which is formed from a 21-kDa proprotein after cleavage of the N- and C-terminal precursor parts. Its main characteristic is its extreme hydrophobicity. Other conserved characteristics are two positive charges at positions 11 and 12 (lysine and arginine) and two palmitoylated cysteines in the N-terminal part, flanked by 2 proline residues [66]. A reported exception is canine SP-C which has only one palmitoylated cysteine [58]. The structure of the protein in an apolar solvent has recently been resolved by NMR and features a valyl-rich α -helix formed by amino acids 9–34 [67] (Fig. 1). This structure correlates well with secondary structure analysis by circular dichroism and Fourier transform infrared. Using the latter technique, it was observed that the α -helical content increases even more in a lipid environment [68,69] and might extend to the two palmitoyl chains at position 5 and 6 in the peptide chain. The N-terminus has a less defined structure, but might form a β -sheet in a lipid environment.

The membrane spanning α -helix of SP-C contains 16 consecutive branched residues (Val, Ile and Leu) and is known to be a very stable and rigid structure. Recent studies describe that the α -helix is in a metastable phase when the protein is in solution and SP-C slowly transforms through several intermediates into thermodynamically favorable aggregates of SP-C with a mainly β -sheet conformation [70]. However, when SP-C was embedded in micelles this transformation was not observed. In addition, algorithms designed for the identification of transmembrane regions predict an α -helix for residues 13–33 of SP-C in a lipid environment, while a β -sheet conformation is predicted in aqueous solutions. Due to the metastability of the protein in solution, production of correctly folded synthetic SP-C has been problematic [71], although one study describes a very mild production method resulting in SP-C with a high α -helix

content [72]. We have recently shown that production of recombinant mature SP-C in the baculovirus expression system yields correctly folded protein [73], probably due to the lipid environment present while SP-C is produced. This also indicates that the precursor parts of SP-C are not required for this process.

The two acyl chains of SP-C constitute an intriguing part of this protein. In general, acylation of proteins might serve several purposes. For example, palmitoylation of cytosolic proteins might allow their attachment to membranes which is observed with many G-proteins [74,75]. Palmitoylation of integral membrane proteins is also observed and is often linked to receptor activation or translocation [76]. It is noteworthy that palmitoylation of these proteins often occurs at cysteines close to the membrane spanning helix, in the proximity of basic residues. This resembles the situation found in SP-C and may indicate a general palmitoylation motif. This is supported by our recent work which shows that mature SP-C produced in insect cells is palmitoylated as well [73]. Although only 15% of the total amount was in the dipalmitoylated form, it does imply that the mature sequence is sufficient for palmitoylation. In the case of SP-C, dynamic palmitoylation/depalmitoylation has not been observed and the palmitoyl groups seem to be a constant part of the protein. This would rule out that, at least in the extracellular lining fluid, palmitoylation of SP-C has a regulatory function. However, there have been several plausible roles described for the palmitoylation of SP-C, such as an effect on lamellar body assembly [77], SP-C's surface activity [71,78,79] and the orientation of the protein in bi- and monolayers [69,79].

In contrast to SP-B, there have only been a few activities described for SP-C, which largely overlap SP-B's activities. These are promotion of lipid adsorption into an air/liquid interface [80], respreading of films from the collapse phase [59], reuptake of surfactant by type II cells [60,61], and stabilization of the monolayer lipid film [78]. All these functions are extracellular and, except for surfactant re-uptake, contribute to film homeostasis. Whether SP-C also has intracellular functions in, for example, lamellar bodies is not clear.

3. Model systems

3.1. Methodology

The progress in understanding the function of surfactant proteins has paralleled the invention of new experimental techniques. Especially with regard to the hydrophobic proteins, for which a simple functionality assay is still lacking, these new techniques are of great value for assigning the various interactions involved in the complex life cycle of the surface film to distinct domains of the proteins. The general picture one obtains from the earlier experiments is that “SP-B is capable of doing everything SP-C does, but better”. However, this picture is fading with increasing sensitivity of the techniques and the cooperation of the surfactant proteins and lipids becomes increasingly clear. In this section the main model systems involved in determining the role of surfactant proteins and to a lower extent the surfactant lipids in surface film homeostasis will be described. A general overview of the model systems is given in Table 1.

3.1.1. Langmuir–Wilhelmy balance

One of the first model systems used to examine surface activity of several surfactant components was the Langmuir–Wilhelmy balance, introduced by Clements [81]. In this method, the surface tension at an air/water interface is directly measured by a small plate attached to an electro balance. One of the major advantages of this system is that a well-defined monolayer of lipids and proteins can be spread at the air/liquid surface. Other advantages are that radiog-

raphy and epifluorescent microscopy of the surface film can be easily performed. Especially the microscopy has been used quite extensively. The use of fluorescently labeled proteins or lipids enables visualization of the formation of lipid/protein domains at varying conditions. With these experiments, a lot of insight into the structure and the dynamics of the surface film can be obtained. One of the major disadvantages of the Wilhelmy balance is that it is a relatively static method for surface tension measurements. The surface area can be varied by moving a barrier at the surface, but this is a slow process which does not reflect a dynamic process like breathing. Despite these limitations, important information has been obtained and the Wilhelmy balance will continue to be a valuable instrument to determine surface film structure and activity of surfactant components.

3.1.2. Pulsating bubble surfactometer

The pulsating bubble surfactometer (PBS), first described by Enhorning in 1977 [82], consists of a small sample chamber which is connected to the atmosphere by a small capillary. An air bubble is attached to the capillary and this bubble is pulsated by varying the pressure inside the cuvette which generates a dynamic ‘in vitro alveolus’ system. The surface tensions are calculated using the law of Young and Laplace from the pressure gradient across the bubble. The maximum and minimum surface tensions during cycling are considered to be indicative of the adsorption capability of the sample and the enrichment in DPPC of the monolayer, respectively. The advantage of the system is that it offers a fast and easy method

Table 1

General overview of the mostly used model systems to test the surface activity of surfactant components

	Advantages	Disadvantages	Amount of material (mostly PL) used
CBS	dynamic system spread films possible	time-demanding procedure	0.2–0.5 µg (spread films) 0.2–1 mg (adsorbed films)
PBS	fast procedure dynamic system	leakage of material	0.05–0.5 mg
Wilhelmy balance	spread films possible lifting of surface possible	leakage of material no fast surface area changes possible	5–10 µg (spread films) 0.5–5 mg (adsorbed films)

to investigate surface activity of samples. The main disadvantage is that the accuracy of the measurement is decreased by leakage of material through the capillary. This makes the actual surface area covered by the film larger and precautions have to be taken when small differences between samples are assigned. A small modification in the normally used experimental procedure can prevent the capillary from being wetted (and thereby make the area covered by the film equal to that of the air bubble) [83]. Another disadvantage of the apparatus is that at low surface tensions, the bubble flattens and sometimes detaches from the capillary which reduces the accuracy of the PBS in the low surface tensions region.

Since its invention, experiments utilizing the PBS have significantly contributed to our understanding of surface tension reduction by pulmonary surfactant and the role of the surfactant proteins in this process. However, at our current stage of knowledge, the disadvantages of the technique make utilization of the PBS for more detailed mechanistic studies impractical. The technique remains useful for the detection of large differences in surface tension reducing activity among samples.

3.1.3. *Captive bubble surfactometer*

The captive bubble surfactometer was introduced by Schürch [84] and can be seen as an improved pulsating bubble surfactometer. An air bubble is introduced into a buffer solution in an airtight cuvette. The bubble floats against an hydrophilic agar gel that fills the upper part of the cuvette. A thin layer of buffer solution separates the bubble from the agar gel, thereby preventing interaction between the agar and the hydrophobic phospholipids and proteins at the air/water interface of the bubble. The absence of a tube penetrating the bubble surface prevents the possible leakage of interface material as observed by the PBS. The surface tension at the bubble surface is calculated from the shape of the bubble which is monitored by a video camera [85]. The shape changes from a more spherical shape at high surface tensions to more oval at low tensions. The bubble area can be increased and decreased by changing the pressure inside the cuvette. This is done either manually by changing the volume of the subphase inside the cuvette or by using a pressure device in the pres-

sure driven captive bubble surfactometer. This latter technique has been described more recently by Putz et al. [86]. The disadvantage of the pressure driven CBS is that the pressure inside the cuvette varies between two preset values that can produce over-compression of the film. However, this problem can be overcome by the introduction of a feed-back loop that prevents further compression when minimal surface tension is attained.

The CBS offers several advantages over the other methodologies described above. One of them being that it is a dynamic fast technique which resembles natural breathing frequencies. In addition, the CBS is more accurate and reproducible than the pulsating bubble surfactometer as demonstrated by a direct comparison experiment [83]. Especially with slowly adsorbing surfactants the CBS was recommended to be used for accurate measurements. This is partly due to a relatively simple difference: the fact that the subphase in a captive bubble experiment can be stirred. Comparable to the Wilhelmy balance, spread films can be used in this technique which offers the advantage that the exact composition of the surface film is known [87]. Only nanograms of protein are needed in this technique when spread films are used, which also makes this a very attractive method when small amounts of proteins are available.

A major disadvantage of captive bubble surfactometry compared to PBS is that it is very time consuming. The calculation of the surface tensions from the video images was until recently a very time-demanding process. However, software is being developed which allows the calculation of both surface tension and area of the bubble in real time. Still, when spread films are used, each experiment can take several hours due to the extended experimental protocol used for film spreading and washing plus replenishing of the subphase. When adsorbed films are used a few samples can be tested and analyzed within an hour which is approaching the experimental times used in PBS experiments.

3.1.4. *Scanning force microscopy*

Scanning force microscopy is a relatively new technique that will undoubtedly provide more detailed information about monolayer structure in the near future. Even though to date only very few pulmonary surfactant related articles have been published, the

sensitivity and especially the new perspective in comparison with the older techniques is very refreshing. Fluid and gel phase regions within the lipid monolayer can be determined and the activity of surfactant samples to create a lipid bilayer reservoir can be visualized. The technique involves spreading of a monolayer of lipids, with or without surfactant proteins, at the air/water surface in a Wilhelmy balance trough. The surface film is then transferred onto a mica sheet and scanned with a small tip which will experience local forces. The interaction force and, hence, the tip to sample distance is kept constant via feed-back and the film topology is traced. In the case of the pulmonary surfactant, a strong adhesion between the tip and the sample apparently proved detrimental to a good imaging. The best results so far were therefore obtained in a dynamic mode of operation of an SFM to overcome the adhesion efficiently [88]. The obtained profile reflects the reservoir forming activity of surfactant samples, but gives no information on other critical aspects of film behavior.

3.2. Data obtained from the model systems

3.2.1. Adsorption

The first adsorption experiments performed were monolayer experiments in the Wilhelmy balance. Interfacial characteristics of lipids from protein containing lipid vesicles in the subphase showed that SP-B and SP-C enhanced adsorption [19,80,89] in an additive way. The adsorption enhancing effect of SP-B and SP-C is also observed when these proteins are only present in a spread monolayer. This effect of the surfactant proteins on adsorption was confirmed in a variety of PBS and CBS experiments [78,87,90–96]. From the above studies it is clear that both hydrophobic proteins enhance adsorption, but the information on the relative activity of the proteins in the process is sometimes contradicting. For example, SP-C has a higher activity than SP-B in a PBS study [97] while SP-B is more active using the Wilhelmy balance [80]. The different experimental setup, not only in terms of the apparatus used, but also differences related to lipid compositions, protein concentrations, protein purification methods etc., is the most likely cause of these differences. Considerable discrepancy may also arise from the preparation

of the vesicles: their size determines very strongly their stability and, hence, the ability to adsorb.

A tendency towards a higher activity, on a molar basis, for SP-B is seen. However, taking all these published results into account, it is unlikely that the major functional difference between SP-B and SP-C lies in the absolute adsorption rate. Especially with regards to the adsorption to a clear interface, which only reflects the first breath after birth in an *in vivo* situation, it is unlikely that distinct properties for each of the hydrophobic proteins exist. As will be discussed below, other surface film related phenomena like DPPC enrichment of the monolayer during adsorption and reservoir formation, are more likely dependent on the specific characteristics of either SP-B and/or SP-C.

Wilhelmy balance studies have also demonstrated that SP-A can enhance the adsorption of SP-B containing samples [32,33]. This cooperative effect between SP-A and SP-B in this process was confirmed in PBS studies and was shown to be calcium dependent [29,98]. The minor lipid components of surfactant in the vesicles have been shown to increase adsorption of lipids to the monolayer as well [99–101]. This is not surprising since palmitic acid and lysoPC are known fusogenic compounds. Also plasmalogens have been shown to increase adsorption in CBS studies [102]. However, these effects may not represent a specific role of these lipids. It can be assumed that any compound, either proteins or lipids, that disturbs the rigidity of the DPPC-rich bilayers used in the vesicles, has to some extent a positive effect on adsorption. An interesting phenomenon is observed for cholesterol. This neutral lipid increases the adsorption rate at lower temperature (23°C) in PBS, and Wilhelmy balance studies [103,104], but has an inhibiting effect on adsorption at 37°C. However, the inhibiting effect at higher temperatures could not be reproduced in captive bubble studies [105], indicating that more studies are required to determine the role of cholesterol.

3.2.2. Enrichment of the monolayer in DPPC

The near-zero surface tension values obtained during static (Wilhelmy balance) or dynamic (PBS, CBS) compression of the surface film imply that the monolayer has to be enriched in DPPC. Two general mechanisms might be involved: (1) selective adsorp-

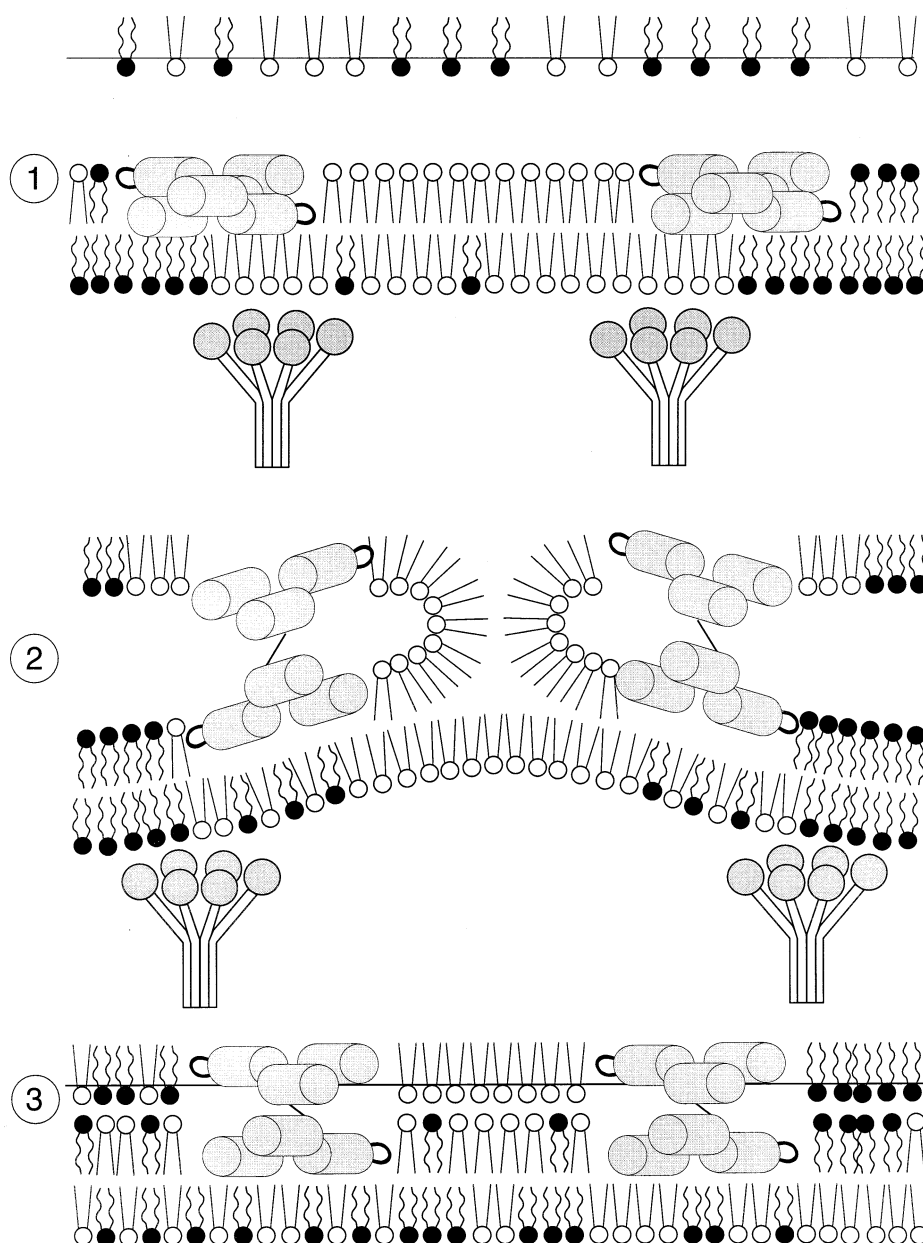


Fig. 2. Hypothetical in vitro model for specific DPPC adsorption to the interface. SP-B is embedded in the lipids of a unilamellar vesicle, while SP-A is present in the subphase. (1) SP-A and SP-B cooperate in the formation of DPPC enriched domains in the vesicle bilayer. (2) SP-B fuses the bilayer with the air/water interface and (3) a DPPC enriched domain is subsequently inserted into the interface.

tion of DPPC; or (2) selective removal of non-DPPC lipids. Experimental evidence suggests that both mechanisms occur.

(1) Evidence for selective adsorption stems mostly from CBS studies. In the CBS, adsorbed films of surfactant samples in suspension reached near-zero surface tensions at much lower area reduction of

the air bubble than expected based on the lipid composition of the surfactant [94]. A film consisting of pure DPPC requires 12–15% area reduction to reach near-zero surface tension from equilibrium, and the surfactant used in that study contained 45% DPPC. Theoretically, if the lipid composition of the film was the same as the bulk face, an area reduction of 55%

plus 12% would be required (plus selective non-DPPC desorption). Yet the authors observed that only a 30% reduction was required. Since the surfactant preparation only contained the surfactant proteins B and C, these are likely involved in this process. Reconstitution of SP-B and SP-C separately in lipid mixtures indicated that SP-B had a higher activity in this regard [3]. Interestingly, the rate of adsorption seems to be directly correlated to low compressibility of the surface films which reflects the enrichment in DPPC [86,95]. During a fast adsorption, sudden decreases in the surface tension, the so-called adsorption clicks, are observed. This reflects the movement of large amounts of lipids into the monolayer. The combination of adsorption clicks and low compressibility of the resulting film suggests that aggregates or domains highly enriched in DPPC are adsorbed during these clicks. It is tempting to speculate that one or more of the surfactant proteins plays a role in the formation of DPPC-rich lipid domains in tubular myelin or other lipid bilayer structures in close proximity of the interface. These DPPC rich domains are subsequently inserted or moved into the interface (Fig. 2). The domain formation or the existence of lipid microheterogeneities under the influence of surfactant proteins has been proposed before [15,106], but evidence is scarce and nothing is known about the mechanism by which this would occur.

Even though the adsorption clicks do not depend on the abundance of SP-A in the samples, a role for SP-A in the 'selective DPPC adsorbing' mechanism still seems likely. In a CBS study using surfactant extracts, smaller area reductions were needed to reach a low surface tension when SP-A was added to the subphase. It is known that SP-A preferentially binds DPPC [107] and interacts with the boundaries between condensed and fluid regions in DPPC monolayers (see also below) [108]. SP-A was shown to aggregate DPPC molecules in DPPC/cholesterol mixtures *in vitro* [109]. Unpublished studies in our laboratory also support the DPPC domain forming role of SP-A. In these studies, it was observed that SP-A can induce a so-called ripple phase in unilamellar liposomes composed of POPG and DPPC (Fig. 3). This ripple phase, or P_β phase, has first been described by Tardieu et al. [110]. Contrary to 'normal' fluid phases (L_α) or gel phase (L_β), the membrane

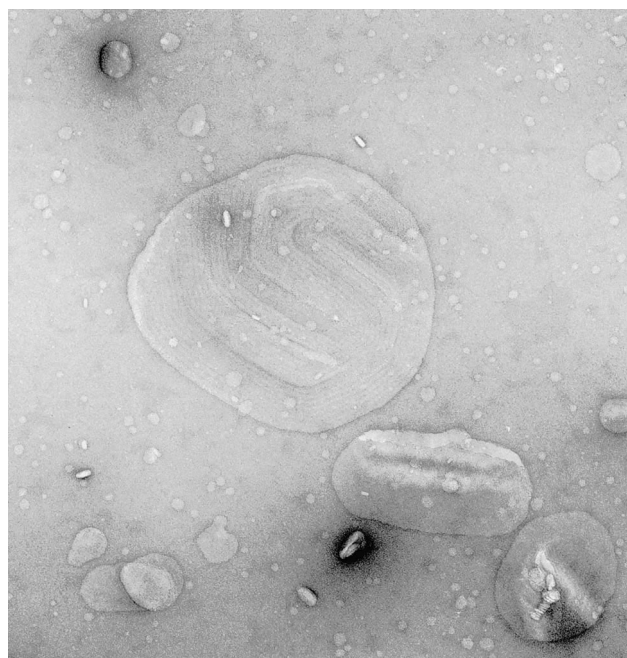


Fig. 3. Cryo-TM image of an SP-A induced ripple phase. Large unilamellar vesicles of DPPC:POPG in Tris/NaCl buffer were incubated with SP-A. After 5 min, a ripple phase is observed in the vesicles.

surface is wrinkled. A model to explain this phenomena has been proposed [111] in which the ripples are considered to be alternating liquid phase and gel phase regions, which in our experiments would probably represent POPG rich and DPPC rich domains, respectively. Comparable experiments with DPPC/eggPC mixtures were recently conducted as well by Palaniyar et al. [112]. Interestingly, they observed a corrugated membrane structure with this lipid mixture even without the presence of SP-A. They also suggest that this represents a phase separation between saturated and unsaturated phosphatidylcholines. SP-A was in these experiments preferentially localized in the 'valleys' of the corrugations which would represent the DPPC enriched phase.

Some results are not in line with a role for the hydrophobic proteins in the selective enrichment. Comparison of spread and adsorbed surface films on the Wilhelmy balance showed complete homology under the epifluorescent microscope [113,114]. The number and size of the liquid condensed phases in the monolayer as well as the localization of labeled SP-C in the fluid phase were indistinguishable for both methods of surface film formation. In CBS

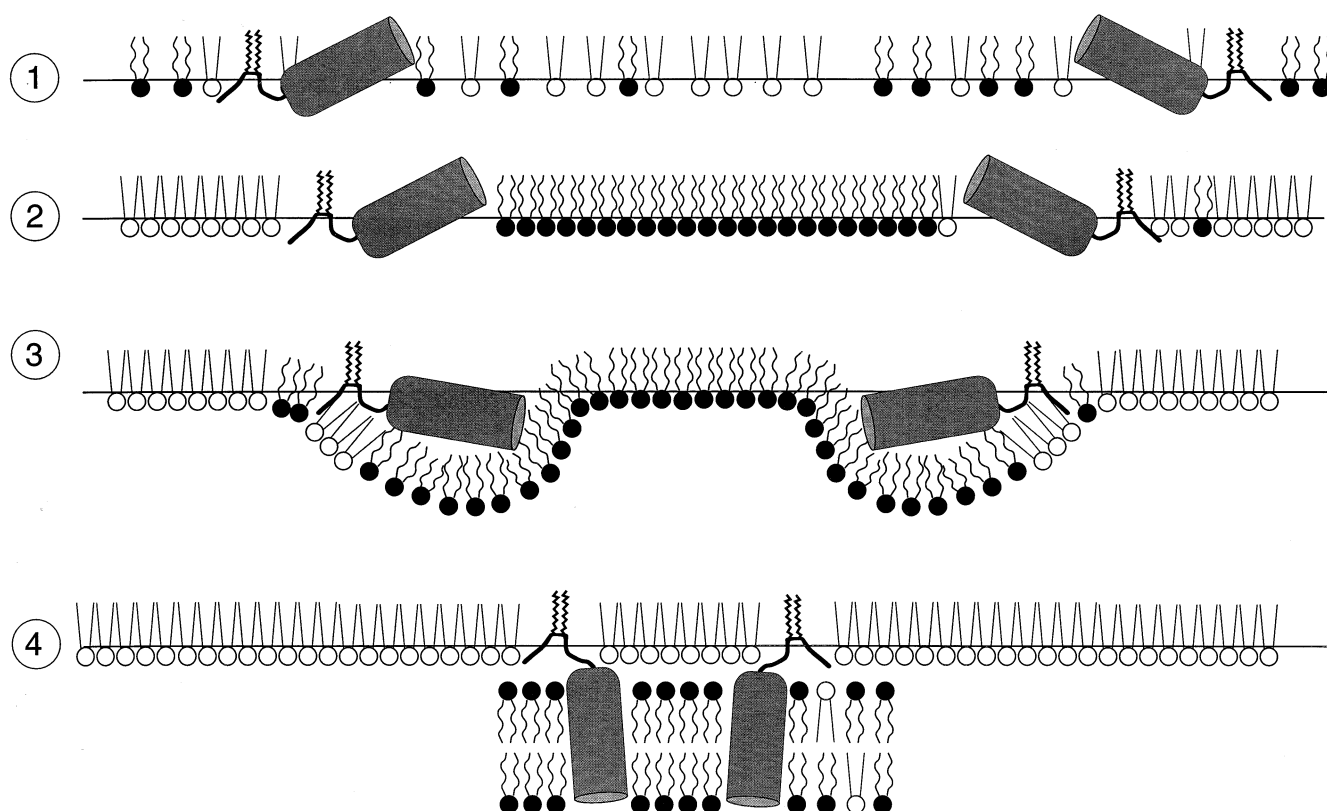


Fig. 4. Hypothetical in vitro model for selective squeeze out of non-DPPC lipids. (1) SP-C is present in the lipid monolayer at the air/liquid interface, as is the case in CBS or Wilhelmy balance studies. (2) The interface area is decreased (expiration) and fluid and gel phases are formed. SP-C localizes in the fluid phase containing non-DPPC lipids. (3) Overcompression of the interface, the monolayer deforms and lipids are squeezed out. (4) SP-C is also squeezed out, leaving the acyl chains in the monolayer, bridging the squeezed out lipids to the interface.

studies, subtle differences were observed between spread and adsorbed films. The compressibility was higher for adsorbed films, actually indicating a selective diminishing of DPPC adsorption [87]. Maximum and minimum surface tensions during cycling were at some stages higher for the spread films. However, the authors concluded that the differences did not reflect 'true' differences between the films, but differences caused by the experimental approach.

(2) Evidence for the selective removal of non-DPPC compounds during surface film compression has been obtained from studies utilizing the Wilhelmy balance, the PBS and the CBS. SP-B and SP-C both seem to be involved in the selective removal of non-DPPC compounds, despite some contrasting results. Both proteins localize in the fluid phases of lipid monolayers [88,115] (see below). Using surface pressure–area isotherms in Wilhelmy balance studies it was observed that SP-B is partially

squeezed out at a surface pressure of 40 mN/m (surface tension of 30 mN/m at 37°C) while SP-C is partially squeezed out at 55 mN/m (tension of 15 mN/m). SP-C is accompanied by 7–10 lipid molecules during the removal from the monolayer, contrary to SP-B which does not, or at least to a much lower extent, show this effect [116,117]. No specificity of SP-C for accompanying lipids has been proven [116,118], but combined with the above-mentioned localization in the fluid phase of the monolayer containing non-DPPC lipids [119] it seems reasonable to suggest that this is a non-DPPC lipid removing mechanism (Fig. 4). Supporting this hypothesis is the effect of SP-C incorporation on the minimum surface tension of spread films in CBS studies. Even though a lipid sample without SP-C also reached very low minimum surface tensions during cycling, the values obtained when SP-C was present were significantly lower [73]. The same effect is seen

when adsorbed films of SP-C/lipid mixtures are used [78]. However, in PBS studies, a smaller effect of SP-C in this respect was observed. Adsorbed DPPC/PG/SP-C films failed to reach low surface tensions even after extensive cycling. Contrary to this, a clear refinement of the monolayer during cycling was observed for SP-B mixtures indicative of selective non-DPPC squeeze out [97]. Interestingly, a recent report by Nag et al. describes that this effect of SP-B is specific for acidic lipids. A clear refinement of the surface film was observed for DPPC/PG/SP-B mixtures, but not with mixtures in which POPG was replaced by POPC [120].

3.2.3. Surface film reservoir formation

It is now generally accepted that the surface film at the interface does not consist solely of a monolayer of lipids, but of a complex membrane system composed of a monolayer with bilayer structures attached to it. Recently, the occurrence of the described structures *in vivo* was reported. Electron micrographs of the alveolar lining layer in rabbit lung showed sites with multilayers of lipids at the interface [2,3].

In model systems, indications for such structures have been observed. Wilhelmy balance studies have shown clearly that lipids that are squeezed out during overcompression of the monolayer, stay in close proximity to the interface [59]. During the next expansion, these lipids can re-insert. This dynamic cycling can be performed without significant loss of material when SP-C and especially SP-B were present.

Oosterlaken-Dijksterhuis et al. reported in 1991 that SP-B and SP-C promote insertion of lipids from vesicles associated with a preformed spread phospholipid monolayer [80]. These associated vesicles could not be washed away with extensive flushing of the subphase, even in the absence of calcium ions, indicating that they were firmly attached to the spread monolayer. These results also suggest that the reservoir does not solely consist of squeezed out lipids, but can at least partly be formed by attachment of subphase lipids. Similar results were obtained with spread surface films in the CBS where the protein/lipid reservoir stayed attached to the interface during dynamic cycling [121]. A tightly bound reservoir was also observed in CBS experiments

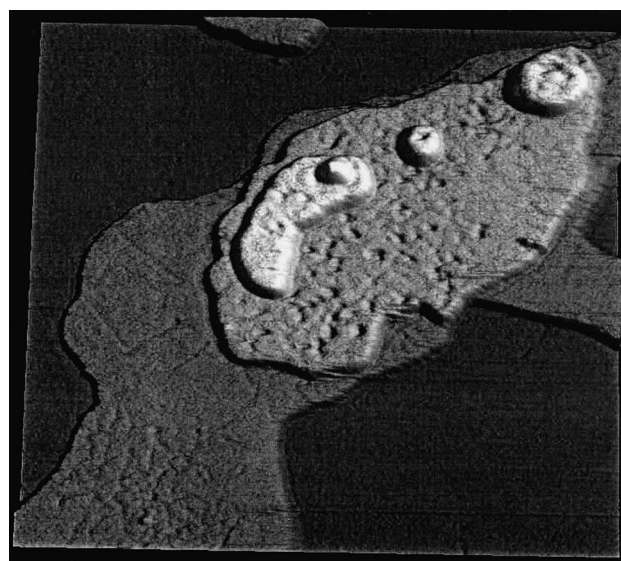


Fig. 5. Pseudo-three-dimensional view of squeezed out phospholipid bilayer stacks. Upon compression of a monolayer of lipids and SP-C, lipids are squeezed out but stay attached to the monolayer, forming the observed protrusions. Each single step is about 6 nm high. The picture is $1.2 \times 1 \mu\text{m}$. Permission for reprint of this figure was kindly granted by Dr. Matthias Amrein and the Biophysical Society.

when SP-C/lipid samples [78] or surfactant lipid extracts [2] were used. In these experiments, a film is absorbed after which the subphase is replaced by a salt solution without disturbing the bubble. Upon the next expansion the lipids in the reservoir can be re-inserted into the interface, lowering the surface tension. This effect was dependent on the palmitoyl chains of SP-C. This interesting finding may indicate a role of the acyl chains of SP-C in bridging the monolayer to the lipid bilayers underneath. SP-B had, contrary to the Wilhelmy balance studies, some reservoir-forming effect, but to a much lower extent than SP-C. Interestingly, SP-B_{1–25}, a peptide that represents the first 25 amino acids of SP-B, mimics many of the protein's activity, but not the surface reservoir formation. However, this activity could be introduced by synthetic dimerization of this peptide [127]. This suggests that the dimerized form of natural SP-B does play a role in the reservoir formation.

Recently new reports describing the formation of these reservoirs associated with the monolayer have been published [88]. Scanning force microscopy of collapsed SP-C containing surface films showed

that phospholipid bilayers were stacked in the SP-C rich regions and piles of up to four bilayers were found (Fig. 5). These stacks were not found in collapsed protein-free lipid films. Upon expansion of the surface area, these lipid stacks were inserted into the monolayer again. Similar results were obtained using fluorescently labeled SP-C in monolayers [122]. Upon reduction of the surface area, the fluorescence was quenched, indicative of increasing concentrations of SP-C in small domains. Further compression led to a constant surface pressure accompanied by an increase in fluorescence which is, according to the authors, representing the formation of multilayer stacks under the monolayer. It will be very interesting to see whether similar experiments with SP-B and especially non-acylated SP-C will also be in line with the results obtained by the CBS experiments.

3.2.4. Modulation of monolayer packing

The use of fluorescently labeled surfactant proteins and fluorescent lipid analogues has greatly enhance insight into the behavior of especially the hydrophobic surfactant proteins in the surface film. SP-B and SP-C seem to have the same effect on monolayers, observed by epifluorescent microscopy, which is that they create more, but smaller, gel-like condensed phases [115]. Both proteins localize in the fluid phase regions of the monolayers [115] and are squeezed out at increasing surface pressure (40 mN/m for SP-B and 55 mN/m for SP-C). Small amounts of the proteins stay within the monolayer, also at pressures of 65 mN/m. Re-insertion of the proteins is observed during re-expansion of the interface surface. If both proteins are present in a pure DPPG monolayer, but not in a DPPC:DPPG mixture, both are simultaneously squeezed out at 55 mN/m [123]. This phenomenon is calcium dependent and might indicate an association of both proteins. In pure palmitic acid monolayers SP-B and also SP-B_{1–25} induced a fine ‘fluid’ network around condensed domains at high surface pressures [124]. The authors conclude that the observed structural arrangement might actually be the main reason for the stability and flexibility of the surfactant film. However, the results of this study should be interpreted cautiously because of the monolayer composition and the high amounts of peptides used.

4. Summarizing conclusions and future directions

The knowledge regarding the formation and dynamics of the surface film at the air/liquid interface has increased significantly over the last decades. Starting with a simple DPPC monolayer model, it is now widely accepted that a surface film consisting of a monolayer in close contact with multilayers is the active structure at the interface. Several dynamic processes occur in the film during breathing including, amongst others, selective adsorption of DDPC enriched domains, specific squeeze-out of non-DPPC lipids, lipid reservoir formation and modulation of monolayer packing. Each process probably requires the combined action of both lipid and protein components of surfactant. In vivo experiments have revealed some structural information, but most detailed knowledge about this complex issue has to be gained by in vitro experiments using model systems.

By far the most research in this area concentrates on the role of SP-B and SP-C. Despite some apparently contradicting results, some clear differences regarding their surface active roles are now emerging between these two hydrophobic surfactant proteins. Especially the experiments utilizing the captive bubble surfactometer, in which several aspects of the surface film dynamics can be studied independently, have proven to be very useful in this respect. SP-B appears to be more effective in adsorption while SP-C plays a marked role in the lipid reservoir formation. However, it still remains remarkable, that two such structurally different proteins seem to possess so many overlapping activities. Since real proof of the interaction of SP-B and SP-C with the lipids in the surface film is lacking, one can only hypothesize about the molecular mechanism by which SP-B and SP-C fulfill their proposed roles. It will be a future challenge to come up with evidence of, for example, the lipid membrane bridging capabilities of both proteins. Also, the exact localization from which the hydrophobic proteins exert their function, monolayer or underlying bilayer or both, is an interesting issue that has to be clarified.

The role of the minor lipid components of surfactant is still far from clear. The high amounts of PG and DPPC and, to a lower extent, cholesterol seem

to distract the attention from the minor lipid components. However, abundance does not necessarily equal importance. Roles for the minor lipids in structural features of the surfactant film are bound to be discovered with the sensitive techniques that are available nowadays.

As with all in vitro experiments, one has to be very careful with the interpretation of the obtained results. Artefacts that arise due to the specific methodology are very common. The 'golden rule' to have only one variable per experiment combined with the complexity of the surfactant system will make it hard to determine the cooperation between the surfactant components. The determination of the activity of each component separately in a certain aspect does not lead to a complete picture by simply adding up the results. Knock-out mice can provide a useful tool to connect in vitro results with in vivo function. The SP-A knock-out mouse is in this respect a good example since many of the in vitro determined functions could be tested. SP-B and more recently SP-C knock-outs have been produced as well. As expected, the SP-B knock-out mice only survive a few hours after birth due to respiratory failure. Surprisingly, the lack of SP-C in the recently generated SP-C knock-out mice seems to have no influence on surfactant function and homeostasis [125]. These mice seem to possess no respiratory failure and no changes in surfactant pool sizes, phospholipid composition or oxygenation.

Despite some difficulties and the cautiousness that has to be taken in interpreting the results, the genetic techniques will definitely provide further insight into surface film behavior. Expression of SP-B mutant forms in a SP-B knock-out background has for example already been performed [126] and the behavior of the SP-C knock-out mice under stress situations will provide more detailed information on the function of the surfactant proteins. The variety of model systems that are now available and the in vivo studies will prove to be a very strong combination to clarify the molecular mechanism by which surface film homeostasis occurs.

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