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Review

Structure of pulmonary surfactant membranes and films: The role of proteins and lipid–protein interactions

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ABSTRACT

The pulmonary surfactant system constitutes an excellent example of how dynamic membrane polymorphism governs some biological functions through specific lipid–lipid, lipid–protein and protein–protein interactions assembled in highly differentiated cells. Lipid–protein surfactant complexes are assembled in alveolar pneumocytes in the form of tightly packed membranes, which are stored in specialized organelles called lamellar bodies (LB). Upon secretion of LBs, surfactant develops a membrane-based network that covers rapidly and efficiently the whole respiratory surface. This membrane-based surface layer is organized in a way that permits efficient gas exchange while optimizing the encounter of many different molecules and cells at the epithelial surface, in a cross-talk essential to keep the whole organism safe from potential pathogenic invaders. The present review summarizes what is known about the structure of the different forms of surfactant, with special emphasis on current models of the molecular organization of surfactant membrane components. The architecture and the behaviour shown by surfactant structures in vivo are interpreted, to some extent, from the interactions and the properties exhibited by different surfactant models as they have been studied in vitro, particularly addressing the possible role played by surfactant proteins. However, the limitations in structural complexity and biophysical performance of surfactant preparations reconstituted in vitro will be highlighted in particular, to allow for a proper evaluation of the significance of the experimental model systems used so far to study structure–function relationships in surfactant, and to define future challenges in the design and production of more efficient clinical surfactants.

Contents

1. Introduction

The lungs establish the largest surface contact that most air-breathing vertebrates have with their environment. Exposure of a sufficiently large surface to the air is required to facilitate appropriated

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levels of gas exchange to support metabolic functions [1,2]. A complex macromolecular system has evolved in the pulmonated organisms as part of their environmental interface, to provide optimal properties in terms of structural stability and accessibility to the air phase while raising an efficient barrier against environmental insults, including the entrance of pathogens. Pulmonary surfactant, a membrane-based lipid–protein complex, which is assembled and secreted onto the respiratory surface by specialized cells of the alveolar epithelium, contains molecular components simultaneously responsible for biophysical stabilizing activities [3] and innate defence mechanisms [4,5]. Which of these activities evolved first is a matter of debate, but it is becoming clear that the two functions, biophysics and defence, are now inseparably coordinated in the surfactant system.

Lack of an operative surfactant system is associated with severe respiratory dysfunctions [6–8]. The pulmonary surfactant system matures during the last few weeks of gestation, and babies delivered prematurely before a threshold amount of surfactant has been produced are at risk of developing Infant Respiratory Distress Syndrome (IRDS), a major cause of mortality and morbidity in neonates, particularly before supplementation with exogenous surfactant preparations was established as a routine therapeutical practice [9,10]. On the other hand, patients suffering from acute lung injury (ALI), arising from a number of different potential causes, often develop Acute Respiratory Distress Syndrome (ARDS) ending in severe respiratory failure due, at least in part, to inactivation of the surfactant system by inflammatory by-products and blood components leaked into the airways through a deteriorated alveolar-capillary barrier [11–13]. Much research in this field, carried out in the last decades, has been devoted to elucidating the role of the different lipid and protein components of surfactant, with the primary purpose of understanding the molecular mechanisms associated with surfactant function. An additional objective has been defining the minimal compositional requirements for an effective therapeutic surfactant [14–17]. Extensive knowledge is available today about structural and physicochemical properties of most surfactant components in simplified model systems, although we still do not fully understand how the whole surfactant complex is assembled and developed in vivo or the molecular mechanisms by which surfactant proteins and membranes modulate respiratory physiology. The clinical surfactant preparations available today are effective in preventing and treating IRDS in preterm babies, but have not proven effective in reverting or ameliorating ARDS, suggesting that clinical formulations are still suboptimal, but also that we need to get further insight into the molecular events defining surfactant action in the complex context of the alveolar spaces, both in normal and injured lungs.

Apart from the clinical importance of understanding structure–function correlations in surfactant, the pulmonary surfactant system constitutes an excellent example of how dynamic membrane polymorphism governs some biological functions through specific lipid–lipid and lipid–protein interactions assembled in highly differentiated cells [18–20]. Lipid–protein surfactant complexes are assembled in pneumocytes in the form of tightly packed membranes, which are stored in specialized organelles called lamellar bodies (LB). Upon secretion of LBs, surfactant develops a membrane-based network that covers rapidly and efficiently the whole respiratory surface. This membrane-based surface phase is organized in such a way that it permits an efficient exchange of molecules between the gas and the liquid regions in alveoli, enabling gases to reach the blood stream flowing on the other side of the thin alveolar-capillary barrier. At the same time, the respiratory surfactant layer optimizes the encounter between many different molecules and cells at the epithelial surface, in a cross-talk essential to keep the whole organism safe from potential pathogenic invaders [5].

The present review summarizes what is known about the structure of the different forms of surfactant, with special emphasis on current model systems on the molecular organization of surfactant membrane components. The architecture and behaviour shown by surfactant structures in vivo will be interpreted to some extent from the interactions and properties exhibited by different surfactant models as they have been studied in vitro, particularly addressing the possible roles played by surfactant proteins. However, the limitations in structural complexity and biophysical performance of surfactant preparations reconstituted in vitro will be particularly highlighted, to allow a proper evaluation of the significance of the experimental models used so far to study structure–function relationships in surfactant, and to define future challenges in the design and production of more efficient clinical surfactants.

2. Pulmonary surfactant: the molecules and the basic interactions

The composition of pulmonary surfactant has been discussed in detail in other reviews, including its analysis as a reference either to interpret differences in performance of current clinical surfactant preparations of natural origin [14] or to rationalize the selection of protein and lipid components used to produce new artificial surfactants [15,21]. The present review will then sketch only the main structural features, the potential interactions and some of the self-organization properties of what are considered the key compositional elements in surfactant [22,23] (see cartoon in Fig. 1). It is important to consider that composition of native pulmonary surfactant is usually studied using material obtained from bronchoalveolar lavage of animal lungs. This lavage may collect structures that could coexist in the airspaces but were assembled separately and/or play different functions, or from different locations of the respiratory tract. The traditional approach has been to fractionate the lipid/protein material obtained from lavage into what has been called *large aggregates* (LA), large membrane-based structures with relatively high density and very good surface activity, and *small aggregates* (SA), of lighter density and much less surface active [24–26]. These two fractions are considered as two different stages of surfactant in its morphological transformation in the respiratory cycle. However, the possibility that both LA and the SA fractions are intrinsically heterogeneous and could contain more than one type of structure, cannot be discarded.

2.1. The lipids and the membrane phases

In general terms, pulmonary surfactant is composed of around 80% phospholipids, 5–10% neutral lipids –mainly cholesterol–, and 8–10% proteins, with 5–6% of total surfactant mass being constituted by specific surfactant proteins [22]. The phospholipid fraction of surfactant is mainly responsible for forming surface active films at the respiratory air–liquid interface [19,23], but it also provides the scaffold or matrix on which the different surfactant structures are assembled. In the bulk phase of aqueous environments, phospholipids usually self-organize in the form of bilayers, which is also the structural form in which surfactant is assembled and stored by the pneumocytes. At polar/non-polar interfaces such as the air–liquid interface, phospholipids form oriented monolayers, with the headgroups oriented towards the aqueous phase and the hydrophobic acyl chains pointing toward the air. The higher the concentration of phospholipid molecules at the interface, the fewer the number of water molecules exposed to air and the lower the surface tension, which also defines a lower energy required to enlarge the surface exposed while opening the alveoli during inspiration [22]. In most mammals, half of surfactant phospholipids by mass is composed of disaturated species, mainly dipalmitoylphosphatidylcholine (DPPC), a scarce phospholipid species in other tissues. Evolution has probably selected DPPC as the main phospholipid species in surfactant because at physiological temperature, the saturated chains of DPPC can be packed to a very high density at the air–water interface, providing the large reductions of surface tensions required to stabilize the lung at the end of expiration [17,27]. The kinked chains of unsaturated phospholipid species –constituting

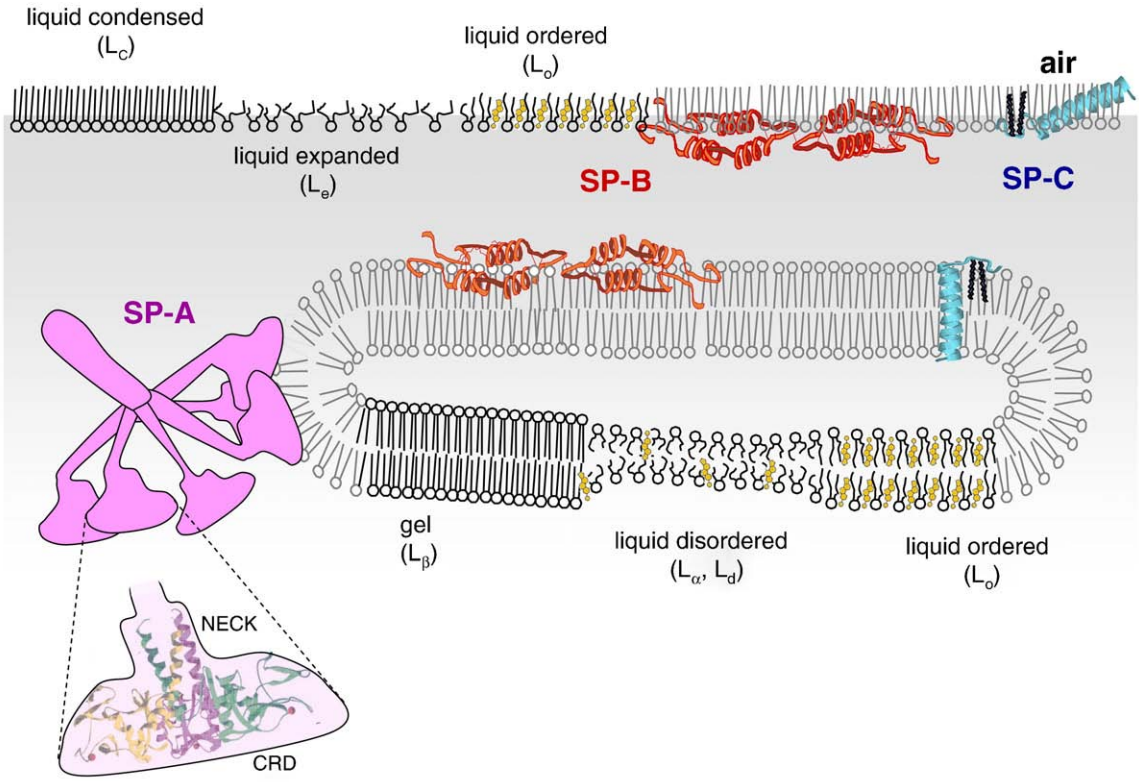

Fig. 1. Structure of lipid phases and membrane-associated proteins in pulmonary surfactant. The cartoon summarizes current models on the structure and orientation of the three proteins usually obtained associated with pulmonary surfactant membranes, SP-A, SP-B and SP-C. The representation of the oligomeric structure of SP-A has been inspired by electron microscopy pictures of the protein [54] and the three-dimensional structure determined for the CRD and neck domains of human SP-A [49] (code 1r13 at Protein Data Bank (PDB)), represented in the figure. The cartoon of the structure of SP-C has taken as a reference the conformation of the protein as determined by NMR in organic solvents [93] (PDB code 1spf). The model for the dimer of SP-B has been drawn from predictive studies of the potential position of helical segments in the protein [195]. Differences in organization and packing of phospholipids have been illustrated in gel, liquid disordered and liquid-ordered phases in bilayers, and in liquid-expanded and liquid-condensed two-dimensional phases in interfacial films. Potential distribution of cholesterol molecules (yellow) in ordered and disordered phases has been also represented.

roughly the other half mass of surfactant phospholipids– cannot be interfacially packed beyond a certain threshold and cannot therefore sustain low enough tensions [28]. This is also the rationale for DPPC constituting the main phospholipid component in all the clinical surfactant preparations available today, those surfactants being obtained from natural extracts or prepared with synthetic lipids [14,15]. Some challenging recent data from the group of Hall show that films made of unsaturated phospholipid species can also reach stably very low surface tensions, if they are compressed at high enough speed [29]. It has been proposed that very rapid compression takes the film into a highly dense vitreous-like state, also competent to sustain very low tensions. Interestingly, surfactant from heterothermic mammals adapted to live at relatively low temperatures contains unusually low levels of DPPC, which is substituted by certain amounts of palmitoleoylpalmitoyl phosphatidylcholine (PPPC). Packing and fluidity properties of membranes containing this unsaturated phospholipid species could be more favourable for surfactant function at low temperature than that of DPPC-enriched structures, which are optimal at around 37 °C [30]. Packing propensity is then a main feature to define surface activity and organization of surfactant structures, while the actual packing of surfactant phospholipids does not only depend on the acyl chain composition but also on temperature, the presence of other lipids such as cholesterol and, at the interface, the state of compression, as summarized in the cartoon of Fig. 1. At low temperatures, phospholipid bilayers are in *gel* phase (called L_{β}), in which the lipid molecules are highly ordered and show much reduced lateral mobility. If the phospholipid possesses saturated chains –such as DPPC– the gel phase is very highly packed. At temperatures above a threshold, known as the gel-to-fluid transition temperature or T_m, the bilayers melt to a *liquid–crystalline* fluid state (called L_{α}), in which the lipid molecules gain considerable mobility and are relatively disordered, meaning that the acyl chains undergo frequent *trans–gauche* isomerizations at their C–C bonds. For this reason, this fluid state is also usually known as a fluid-disordered phase. The melting temperature of DPPC bilayers is 41 °C, slightly above the physiological temperature in homeothermic mammals. Therefore, pure DPPC bilayers would not be entirely fluid at 37 °C. Bilayers made of unsaturated phospholipid species have much lower T_m values. Palmitoyloleoylphoshatidylcholine (POPC), for instance, the main unsaturated phospholipid in surfactant, has a melting temperature of −3 °C [31]. The presence in surfactant of a significant proportion of unsaturated phospholipid species reduces the melting temperature of surfactant membranes to values lower than that of pure DPPC [32,33], making surfactant membranes fluid at physiological temperatures, which is important to improve their dynamical properties as will later be discussed. The gel and fluid phases in bilayers have some correspondence with the order and packing defined by the organization of phospholipids in interfacial monolayers. Besides temperature, packing at the interface can be also be defined by changes in compression, i.e., changes in the surface available for the lipids to redistribute, as occurs during the breathing cycles or is modelled in surface balances in vitro. At low compression levels, phospholipid molecules have low packing density and a considerable configurational freedom at the interface, constituting what is called a *liquid-expanded* (L_e) phase [34,35]. In this interfacial L_e phase, the mobility and order of phospholipids would be qualitatively similar to that in the bilayer L_{α} phase. When compressed enough at the interface, phospholipids can reach a highly packed state, known as a *liquid-condensed* (L_C) phase. The high order and low mobility of phospholipid

molecules in this two-dimensional phase approach those shown by gel phase bilayers [36]. As a matter of fact, lipid monolayers can only be compressed to a liquid-condensed phase at temperatures below the corresponding T_m of bilayers of the same composition. Interfacial compression can proceed even further, to reach extreme packing states which are in principle not accessible to phospholipids in bilayers, and produce *solid-ordered* phases (S_O). These solid phases are not compressible any further, and behave as real two-dimensional solids: the individual molecules have essentially no translational mobility and the film breaks if compressed beyond a threshold, leading to *collapse* of the film [37].

Besides temperature and compression, packing of phospholipids in bilayers and interfacial films is also modulated by the presence of cholesterol. Cholesterol is an important component of native surfactant (5–10% by mass), and recent results indicate that cholesterol is an important element modulating the structure of surfactant membranes [32,38]. In spite of this, cholesterol is systematically removed from most clinical surfactant preparations available today [14]. Intercalation of enough cholesterol molecules into phospholipid bilayers and interfacial monolayers decreases packing and increases mobility of phospholipids in the ordered phases (gel in bilayers, liquid-condensed in monolayers), producing phases where the phospholipid molecules maintain a certain order –a reduced number of trans–gauche isomerizations– but that are much more fluid than tightly packed pure phospholipid [39–41]. On the contrary, disordered phospholipid phases gain order in the presence of cholesterol, because the intercalation of the planar steroid molecules reduces considerably the conformational entropy of the acyl chains. Presence of cholesterol, therefore, defines an organization of particular phases termed *Liquid-ordered* (L_o), ordered but fluid, in both bilayers and interfacial films. Phases or regions with low cholesterol in membranes or films remain intrinsically disordered at high temperatures or low compression levels, and known as the *Liquid-disordered* phase (L_d). The cartoon in Fig. 1 illustrates that packing, order and thickness (per layer) increase in general terms in the order $L_\alpha \approx L_d \approx L_e < L_o < L_C \approx L_{\beta'} < S_o$ in the different phases.

2.2. The membrane-associated surfactant proteins

The cartoon in Fig. 1 includes a representation of the three specific surfactant proteins, SP-A, SP-B and SP-C, which are obtained from alveolar lavage associated with pulmonary surfactant membranes and that are therefore considered as surfactant apolipoproteins [3,42]. A fourth surfactant protein, SP-D, is not usually associated with membranes [43,44] and will not be discussed in this review.

Proteins SP-A and SP-D are hydrophilic in nature and consist of large macromolecular assemblies belonging to the family of collectins, Ca^{2+}-dependent C-type lectins possessing both collagen-like and carbohydrate-recognition domains (CRD) [45]. The quaternary structure of SP-A consists of a hexamer of trimers. Each trimer contains a long triple-helical collagenous stem, interrupted at a given point by a flexible hinge, a helical bundle connector and a globular head, which contains the CRD domains [46–48]. Through the globular heads, whose three-dimensional structure has been determined [49], SP-A is able to bind multiple ligands, including sugars, Ca^{2+}, and phospholipids in a cooperative manner [48]. These activities allow SP-A to bind to the surface of multiple pathogens, including bacteria, viruses and fungi, contributing to their elimination from the airways as a part of the innate defence response [50]. Recognition of SP-A by specific receptors in alveolar macrophages stimulates phagocytosis and other pathogenicidal events [51–53]. Abundant details on the structure–function determinants and the specific activities of SP-A can be found elsewhere [4,43,45,48,50]. What is relevant to consider for the models discussed in this review is that SP-A associates with pulmonary surfactant membranes via the CRD domains [54,55], and that Ca^{2+}-dependent cooperative membrane binding and protein self-association enables SP-A to promote membrane–membrane aggregation as assayed in vitro [56–58] (Fig. 2). Although the globular CRD domains of SP-A interact rather superficially with phospholipid membranes [59,60], the protein–lipid interaction has both hydrophobic and polar contributions [48]. It has been proposed that SP-A recognizes ordered lipid patterns, and that this is the rationale behind a certain preference for the protein to interact with ordered membranes such as those in gel-like or in liquid-ordered phases [61,62], and also with ordered regions or the boundaries between ordered and disordered phases in interfacial films [57,63]. SP-A could have then evolved to bind to or contribute to clustering of laterally segregated DPPC-enriched domains thought to exist in pulmonary surfactant membranes and films.

SP-B is the most important protein in surfactant for sustaining respiratory physiology. Inactivation of the expression of the SP-B gene leads to a lethal respiratory failure at birth, as a result of the impossibility of maintaining the lungs open [64–66]. This protein is strictly required for the biogenesis of pulmonary surfactant and its packing into LBs [67,68]. On the other hand, extensive research in vitro has shown that SP-B is very efficient in transferring surface active phospholipid species from membranes into air–liquid interfaces [69–71]. Unfortunately, a model for the three-dimensional structure and the molecular mechanism of SP-B is still lacking. Analysis of the sequence of SP-B reveals that this protein belongs to the family of the saposin-like proteins, which possess small folds of around 80 amino acids containing amphipathic alpha-helices and three highly conserved disulphide bridges in invariant positions [72]. All the saposin-like proteins have activities related to interacting with and inserting to different extents into phospholipid membranes [72], but SP-B is the only member of the family which is permanently membrane-associated. This is in part due to the high hydrophobicity of the protein, which is soluble in organic solvents such as chloroform/methanol mixtures, making it to co-purify with lipids in the extraction methods used to obtain the lipid moiety of surfactant. Mature SP-B is produced in the pneumocytes as a result of the processing of a much larger precursor, proSP-B, of around 400 residues [73]. The protein is obtained from alveolar spaces in the form of a covalent dimer of two 79-amino-acid polypeptides. The cartoon in Fig. 1 illustrates that SP-B probably interacts more or less peripherally with phospholipid membranes and monolayers, with the main axis of its amphipathic helical segments orientated in parallel to the plane of the lipid layers [74–76]. The net positive charge of SP-B likely promotes a certain selective interaction of SP-B with the anionic phospholipid fraction of surfactant, and particularly with PG [77], although there is at present certain controversy as to whether SP-B prefers PG-enriched or PC-enriched membrane regions [78–80]. What has been clearly demonstrated is that SP-B distributes preferentially in disordered regions of membranes [32] and interfacial films [81]. Detailed physico-chemical studies have revealed that SP-B has a strong tendency to perturb phospholipid packing in membranes and films, and as a consequence, to promote leakage of aqueous content from lipid vesicles [82,83], exchange of phospholipid molecules between membranes [83–85] and even whole membrane fusion events –meaning complete merging of lipid membranes and aqueous contents– [82,83,86] (summarized in Fig. 2). It is thought that membrane perturbation by the amphipathic helical motifs of SP-B could be an important determinant in catalyzing the transit of phospholipid molecules from surfactant membranes into the interfacial surface active film. SP-B could in this sense promote formation and stabilization of certain non-bilayer intermediates required for efficient phospholipid dynamics [87]. A recent study has mapped significant structure–activity determinants in the sequence of SP-B, identifying segments particularly active in promoting membrane perturbation and protein-mediated membrane–membrane associations [83]. A cluster of aromatic residues near the N-terminal end of the molecule has been also proposed as an important determinant defining high affinity of SP-B to interact with the interfacial region of phospholipid bilayers and monolayers [70].

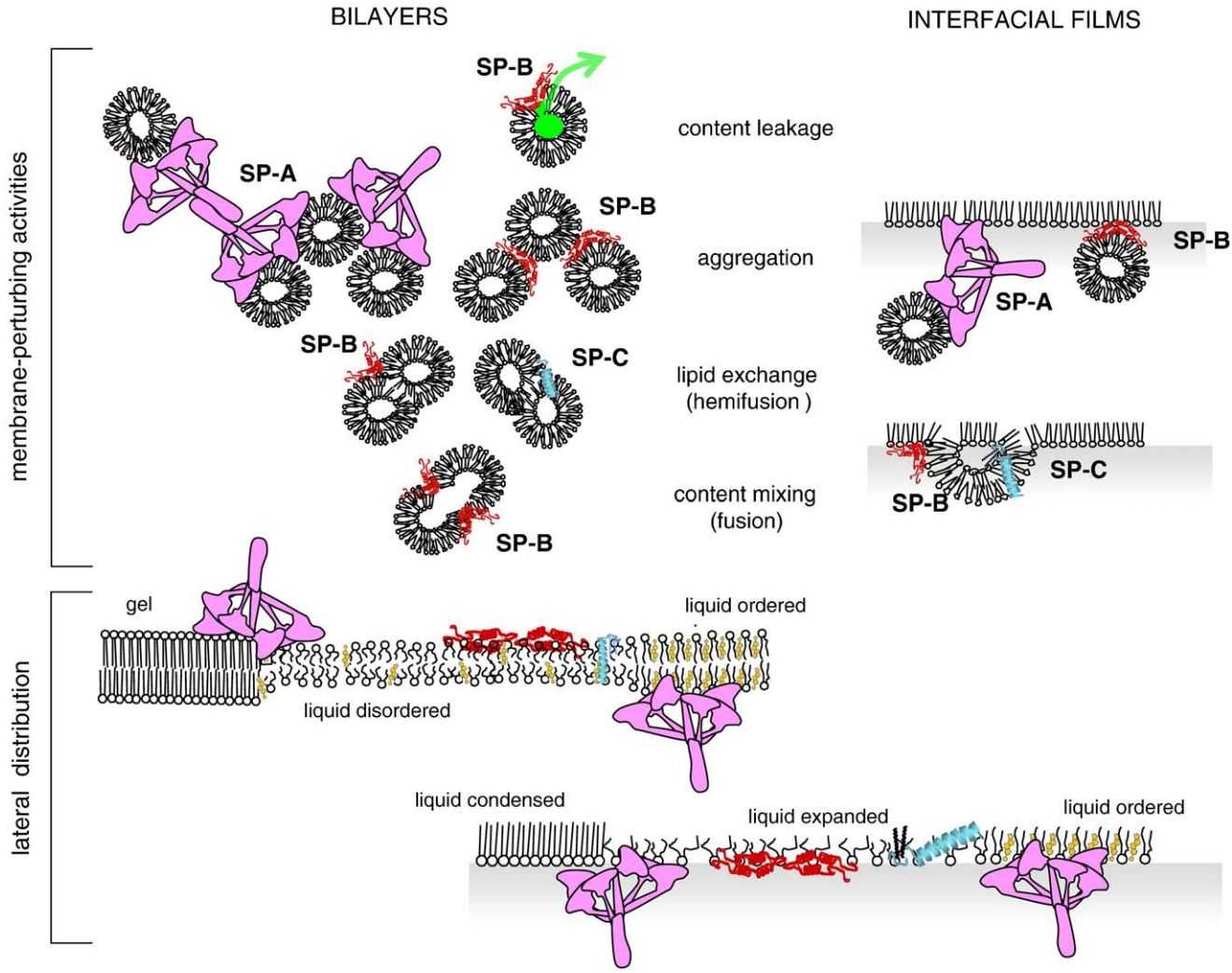

Fig. 2. Membrane-perturbing activities and lateral in-plane distribution of surfactant-associated proteins.

The biosynthesis of SP-C, the smallest of the surfactant proteins, is tightly coupled with the differentiation of mammalian lung tissue [88,89]. This fact and the lack of significant homology between SP-C and any other known protein lead to the general thinking that SP-C could be the most specific protein in terms of the biophysical activities of pulmonary surfactant. Surprisingly, animals lacking expression of an operative SP-C can breathe and survive [90], suggesting that the action of SP-C is not as critical as that of SP-B. However, since the first genetic experiments deactivating the SP-C gene were carried out, it has become clear that deficiencies in SP-C are also associated with severe respiratory pathologies [7,66,91], usually under a chronically adverse regime, suggesting that SP-C may also be essential to properly sustain long-term respiratory dynamics. Mature SP-C is a small very hydrophobic lipopeptide of 35 amino acids, containing palmitoylated cysteines, which is co-purified with SP-B and phospholipids in chloroformic extractions of pulmonary surfactant [92]. Its three-dimensional structure has been determined by NMR in chloroform/methanol solutions [93], consisting in a very regular and rigid α-helix covering approximately two thirds of the sequence and an unstructured N-terminal segment containing prolines and the palmitoylated cysteines. In membranes, the helical segment of SP-C adopts a transmembrane orientation [94], perfectly suited to traverse the thickness of a DPPC bilayer in a fluid state. During the biosynthesis of proSP-C, its transmembrane segment, which will be later the main part of the mature protein, orientates with its N-terminal end exposed to the cytosol and the C-terminal side to the lumen of the endoplasmic reticulum [95]. Assembly of SP-C into pulmonary surfactant membranes is coupled with proteolytic processing of proSP-C precursor, to liberate N-terminal and C-terminal propeptides. When situated at an air–liquid interface in vitro, mature SP-C orientates with the C-terminal end exposed to air [96] (see Fig. 1), but there is no clear idea as to whether SP-C is actually transferred into the interface at the alveolar spaces in vivo. Different experiments have shown that SP-C, as well as SP-B, partitions into disordered regions in both bilayers [32] and monolayers [81,97]. The summary in Fig. 2 also illustrates that SP-C has been shown to promote exchange of phospholipids between membranes [98] and between membranes and interfacial monolayers [99,100], activities associated with the well-known property of SP-C to promote rapid formation of interfacial phospholipid-based films [85,99,101,102]. Although the molecular mechanism by which SP-C promotes interfacial adsorption is not known, it has been proposed that the N-terminal segment of SP-C has a rather dynamic character [103,104], with the potential to interact and perturb packing in lipid structures –membranes [105] or films [106]– other than the one where the hydrophobic α-helix is inserted [3,107,108]. Direct evidences for this mechanism, however, are still lacking. A recent study suggests that the palmitoylated N-terminal segment of SP-C could be well suited to stabilize interdigitated-like phospholipid structures [109] such as those potentially taking part as intermediates in bilayer-to-monolayer conversions or in bilayer–bilayer fusion.

Palmitoylation of the N-terminal segment has been also proposed to play a role in the association of the protein and SP-C-containing membranes with very well ordered lipid structures such as those thought to exist at the highest compressed states of the interfacial film [110]. Palmitoylation maintained peptides designed to mimic the N-terminal segment of SP-C associated with lipid/peptide monolayers compressed to high pressures, while non-palmitoylated peptides were irreversibly lost. Taking into consideration that the main part of SP-C is squeezed-out from highly compressed interfacial films [111], it is conceivable that the N-terminal segment of the protein is the only motif that, thanks to acylation, maintains association of surfactant layers with the most compressed interfacial structures (as illustrated, for instance, in Fig. 2).

3. Membrane packing and unpacking in lamellar bodies

Pulmonary surfactant complexes are assembled in type II pneumocytes and stored in the form of tightly packed membranes in the LBs. These organelles belong to the endosomal–lysosomal pathway and their maturation requires proper trafficking of proteins and lipids along the regulated exocytic pathway [20]. Extensive studies have approached the way pulmonary surfactant proteins SP-B and SP-C are synthetised as large precursors in the endoplasmic reticulum and processed through different intermediates as they are targeted through the Golgi, the multivesicular bodies (MVBs) and LBs [73,112–114]. Although it was originally proposed that at least some amount of SP-A could be also associated with LBs [115] it is now widely assumed that SP-A is secreted into the extracellular media by a different pathway and it probably joins and interacts with surfactant membranes once in the alveolar spaces [116,117]. Maturation of the precursors of surfactant proteins SP-B and SP-C include several proteolytic steps, which are apparently coupled with the progressive acidification occurring in the exocytic compartments [20,73,112,118]. MVBs are organelles containing numerous internal unilamellar vesicles and are supposed to be the organelles preceding the final assembly and storage of surfactant [68,119]. Surfactant proteins and lipids also reach LBs once taken up again by endocytosis from the extracellular material and recycled into new surfactant [117,120]. However, no model has been proposed for the molecular mechanisms behind the biogenesis of the membranes as they are stored into the LBs, both from the point of view of how the particular lipid composition of surfactant is sorted and how the highly packed state of stored surfactant membranes is generated and stabilized. Understanding packing and organization of surfactant membranes in LBs would also provide clues about the mechanisms by which surfactant membranes are unpacked upon secretion and adsorb at the air–liquid interface.

3.1. Role of proteins in the biogenesis and organization of LBs

The study of genetic models in which the expression of surfactant proteins could be inactivated made clear very early that biogenesis of LBs is tightly connected with the expression and maturation of surfactant protein SP-B in type II cells. Lack of SP-B expression seems to be always accompanied, in both genetic animal models [64,68] and patients bearing inherited genetic deficiencies [6,8,66], of a deficit of LBs and an accumulation of MVBs in the pneumocytes. This observation confirmed that MVBs are probably the precursors of LBs, but also that the presence of the mature form of SP-B is somehow required for surfactant membranes to accumulate and pack in LBs. In the absence of SP-B, MVBs also accumulate a partially processed form of SP-C [121], indicating that full maturation of this protein requires either a direct interaction with SP-B or the type of membrane transformations that is promoted by SP-B. A hypothesis that has been proposed suggests that the proteases responsible for the last proteolytic steps in SP-C maturation might not have access to the lumen of the internal vesicles of MVBs unless SP-B exerts its lytic and fusogenic activities [73]. The membrane orientation of SP-C would be in principle preserved from the orientation of the protein at the endoplasmic reticulum and the Golgi membranes through the membrane budding process thought to be the source of the inner vesicles in MVBs. This orientation would then preclude proper processing of the domain exposed to the lumen of the vesicles –topologically equivalent to the cytosol– by proteases residing in the endosomal-like compartments of MVBs. Membrane reorganization promoted by properly matured SP-B would gain access to the lumen of the internal vesicles permitting the complete processing also of SP-C. The hypothesis is appealing, but it still has to be demonstrated. On the other hand, tight packing of membranes in LBs could include the type of membrane–membrane interactions that SP-B is able to promote, at least in vitro [69,82,86,122], but this situation has still to be confirmed. As stated above, complete SP-B and SP-C processing requires a proper acidification along the exocytic pathway. Acidic pH –thought to be around 5.5 in LBs– could be also a major driving force to trigger full membrane packing, once proteins and lipids achieve a proper conformation and state of charge [118,123].

In recent years, however, a protein that has gained a major leading role in the biogenesis of surfactant and LBs is a membrane protein called the ATP-binding cassette transporter A3 (ABCA3). This protein belongs to a family of membrane transporters, which use the energy from ATP hydrolysis to pump different molecules across cellular membranes [124,125]. Many of these transporters act in fact as *flippases*, proteins catalyzing the transmembrane movement of phospholipids, which would otherwise translocate at a very slow rate [126–128]. The ABCA3 protein is probably a lipid translocase selective for saturated phospholipid species. This would explain why genetic deficiencies in the ABCA3 gene lead to failures in the accumulation of saturated phosphatidylcholine (PC) species in surfactant, lack of lamellar bodies, and the development of fatal respiratory distress syndrome in newborns [129,130]. It has therefore been proposed that ABCA3 is responsible for pumping surfactant lipids into endosomal-like compartments to originate lamellar bodies [131,132], in conjunction with SP-B and SP-C, which would organize the lipids in the form of tightly packed membranes. Exogenously expressed ABCA3 leads in culture cells to the accumulation of PC and cholesterol into lysosomes [133], supporting this hypothesis. It is important to notice that several of these ABC-type flippases are responsible for maintaining asymmetry of most cellular membranes, through selectively pumping certain lipid species from one leaflet of membranes to the other [134–136]. In this sense, it is remarkable that no study has been published so far on the possible asymmetric distribution of lipid species at the two sides of surfactant membranes. Membrane asymmetry caused or maintained by flippases has been in fact proposed as a major driving force in the biogenesis and trafficking of other membrane systems [127,137].

Fig. 3 illustrates the possible mode by which pumping of saturated lipid species by ABCA3 could promote accumulation of surfactant membranes inside LBs. A simple but continuous energy-supported translocation of phospholipids from the outer into the inner leaflet of the compartment would create an imbalance in the number of phospholipid molecules towards the inner side of the membrane. Selectivity of the pumping activity in favour of saturated species would also originate an asymmetric character of the membrane. The progressive accumulation of phospholipids into the inner leaflet could only be relaxed upon creation of membrane folds, which would protrude membrane structures into the lumen of the compartment. The driving force of asymmetric lipid pumping to promote membrane budding has been already demonstrated [138]. Vesiculation initiated and promoted by a continuous pumping of lipids into the inner leaflet of the limiting membrane could be the main mechanism behind the biogenesis of MVBs. Alternatively, folds originating mainly from the inner leaflet of the limiting membrane could protrude membranes into the lumen, which would be enriched in the lipids selectively pumped by ABCA3. The activity of the ABCA3 transporter could then explain at the same time the accumulation of saturated species in surfactant

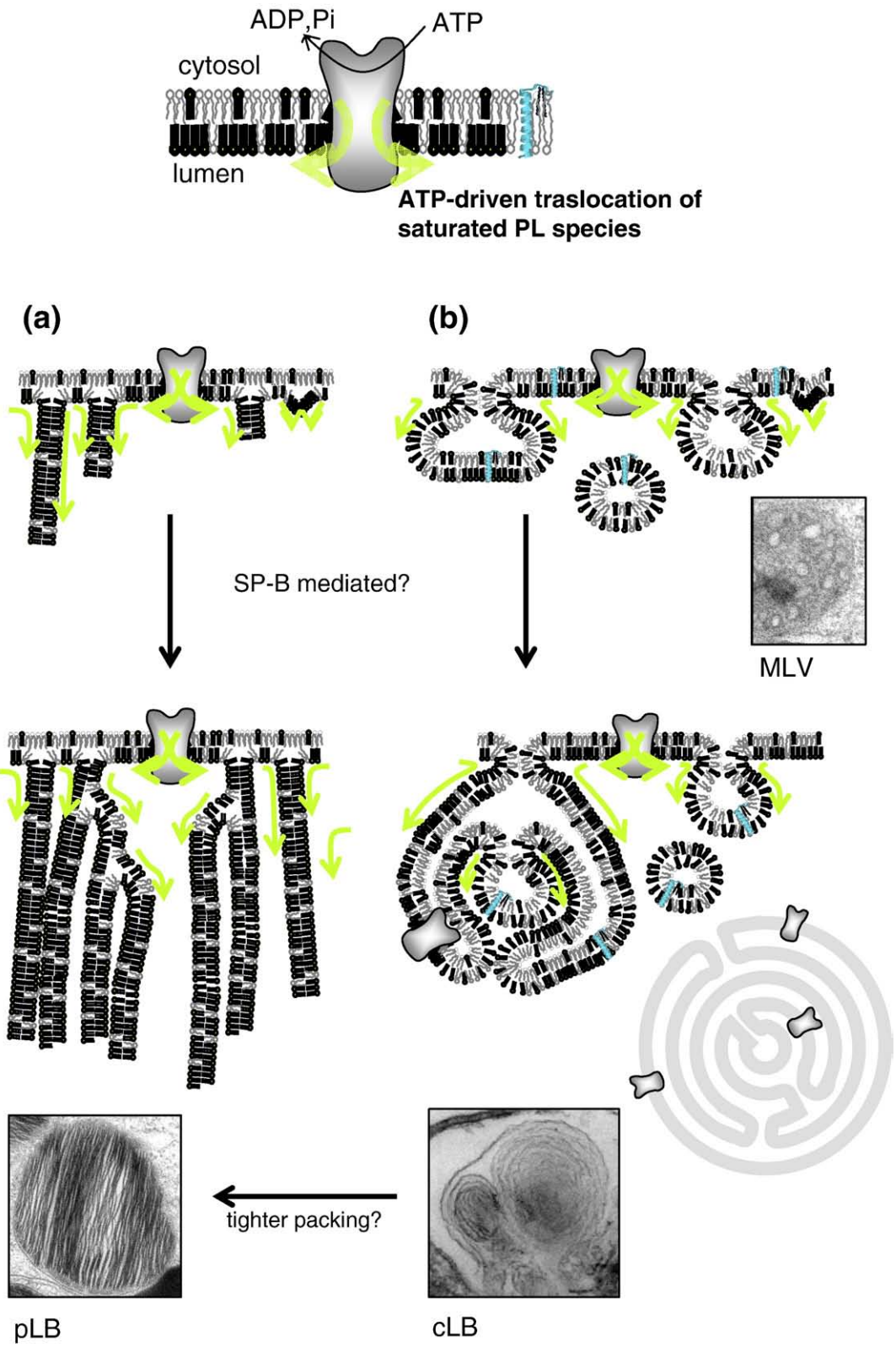

Fig. 3. Model for the activity and the role of the ATP-binding cassette protein ABCA3 in the biogenesis of lamellar bodies. The ATP-binding cassette ABCA3 uses the free energy of ATP hydrolysis to catalyze translocation of phospholipids across the limiting membrane of lamellar bodies, with selectivity towards saturated phospholipid species. The model in a) illustrates how ABCA3-promoted continuous pumping of phospholipids could initiate membrane folding from the inner leaflet of endosomal/lysosomal compartments. Alternatively, and as indicated in model b), accumulation of saturated phospholipids in the inner leaflet might promote budding and vesiculation to yield accumulation of inner vesicles as observed in multimesicular bodies (MVBs). SP-C has been included in the membranes just to show how the orientation of the protein could be maintained through LB biogenesis, following the insertion of proSP-C in the endoplasmic reticulum. Further accumulation and tight packing of parallel or concentric surfactant membranes might require the concerted action of SP-B, as discussed in the text. Concentric (cLB) and parallel (pLB) morphologies of surfactant membranes in LBs may reflect differences in the packing state of surfactant. Electron microscopy pictures of MVB and pLB were a generous gift of Dr. Mattias Ochs, from University of Bern. The electron microscopy picture of cLB was provided by Dr. Paul Dietl, from University of Ulm in Germany.

membranes –essential for the surface activity at the respiratory surface– and the driving force to produce and accumulate membranes in the lumen of MVBs, which in a later stage would be packed to produce the lamellar bodies. Properly matured SP-B might be required to drive membrane–membrane packing, which might be necessary for maximal accumulation of membranes. In the presence of ABCA3 but in the absence of SP-B, membranes could only maintain a loosely packed state such as the one in MVBs. The cartoon in Fig. 3 also illustrates that presence of ABCA3 in the inner membranes could promote further membrane vesiculation at different levels, potentially explaining the "onion-like" morphology of apparently concentric membranes frequently observed in LBs. However, the cartoon clearly illustrates that most of these membranes would be interconnected topologically –also with the limiting membrane–, a condition that could be very important for a rapid and cooperative unpacking of the whole structure upon secretion. A wide examination of previously published electron microscopy pictures of LBs suggests that considering their morphology, there are two main classes of these organelles. A picture of each of these two types has been included as an example in Fig. 3. Some of them appear to contain round –spherical 3D– concentric-like membranes, with frequent but clearly discrete contacts between them. Others accumulate very tightly packed stacks of membranes which are parallel to a main axis of the LB, and are apparently in a close contact at their whole surface. The two types of LBs could represent different stages of packing, which could depend on the establishment of possible protein-promoted membrane–membrane interactions. Participation of SP-B in promoting such membrane packing could at least partially explain its major role in LB biogenesis.

The ABCA3 machinery would then be the main factor responsible for converting the energy provided by ATP hydrolysis into a *sphere* of packed membranes, the LB, which could be energetically *poised* to rapidly expand and unpack once the limiting membrane is open, presumably upon secretion. An open question remains as to the potential role of creating and maintaining lipid asymmetry in surfactant membranes, which could define, together with probable asymmetric orientations of surfactant proteins in the membranes, the occurrence of specific lipid–protein and protein–protein interactions governing surfactant morphology at different membrane sides.

3.2. Unpacking LBs

The final packed state of LBs could suppose a *pre-loaded* structure, energetically primed to favour very rapid and efficient unpacking once secreted to the extracellular alveolar environment. Recent observation of type II pneumocytes under dark-field microscopy reveals that secretory vesicles, i.e. LBs, show a strong scattering contrast (Paul Dietl, unpublished observations), consistent with a gradient of dense packing from the center towards the most external layers. ABCA3-promoted packing of LBs might progressively increase membrane packing until internal pressure can no longer be overcome by the free energy liberated by ATP hydrolysis. Fusion of the limiting LB membrane with the plasma membrane of pneumocytes could almost instantaneously liberate the internal pressure, producing swelling of the membranes and the abrupt expulsion of membrane complexes usually observed upon secretion in electron microscopy pictures (i.e. in [139]) (see Fig. 4).

Fig. 4 summarizes some significant images and experiments illustrating relevant aspects of LB secretion and unpacking. The first interesting observation comes from an extensive work carried out by the group of Drs. Paul Dietl and Thomas Haller [18,140–142]. They have extensively used the fluorescent probe FM-143 as a marker for LB exocytosis. This fluorophore can be added externally to the extracellular medium of type II cell primary cultures, and only shows significant fluorescence when it inserts into phospholipid membranes [142]. FM-143 cannot traverse membranes, so it can only insert into and fluorescently label membranes exposed to the external environment. Pictures and cartoons in Fig. 4a illustrate how FM-143 induces a rapid increase in bright fluorescence, as soon as a given LB fuses with the plasma membrane and the fusion pore permits the entrance of the probe. Secreted LBs can be therefore easily distinguished from non-secreted organelles under a fluorescent microscope. The interesting feature is that apparently, all membranes inside the secreting LB become rapidly intensely labelled. This means that the internal membranes do not isolate different aqueous compartments inside each LB, but that the entire secreted membrane surface is simultaneously exposed to the external medium. A model of surfactant membrane packing such as the one suggested in Fig. 3 would be consistent with this observation. The work by Dietl et al. has also shown that once the fusion pore is formed, it still takes some time for the packed membranes to be completely expelled out of the cell [143]. Connection through the fusion pore of the internal medium of LBs with the extracellular environment probably triggers changes in membrane packing, due perhaps in part to re-hydration but also possibly to pH neutralization and other potential changes in the ionic environment, including a decrease in Ca^{2+} concentration, considering that calcium concentration inside LBs is fivefold higher than in the surrounding alveolar environment [144]. These changes could induce the partial unpacking of LB membranes often seen in just fused LBs (i.e. in the picture in Fig. 4b) (i.e. in [139]). Very often, unpacking of multilamellar arrays of LBs seems to proceed at defined peripheric points of the secreted body (as can be seen, for instance, in the picture of Fig. 4c), with inner layers being expelled out of the LB before the more external ones. A reason for that could be the increasing pressure gradient towards the center of the LB that could be implicit to the mechanism of progressive packing by ABCA3 pumping. One cannot discard the possibility that coupling of membranes through defined protein–protein interactions creates specific and localized structures that facilitate a cooperative unpacking. Microscopic observations also reveal that a main part of the secreted membranes retains a packed state, where a significant proportion of membrane–membrane contacts are retained [18,145]. The picture in Fig. 4d illustrates that the mechanism for membrane unpacking could include the slipping of some membranes over their neighbour ones, while maintaining their relative separation [146]. This feature suggests the possible existence of very dynamic, possibly protein-mediated, membrane–membrane interactions. Lipid–protein and protein–protein interactions could mediate membrane packing in the environmental context of the endosomal compartments, but lead to membrane unpacking under the different circumstances of the external medium. Optimization of the unravelling of LBs could require participation of other molecular elements such as additional proteins joining surfactant membranes once they are in the exterior hypophase milieu. SP-A secreted to the alveolar spaces from alternative pathways [146] could contribute to unpacking LBs mainly through conversion of multilamellar arrays into tubular myelin, as will be discussed later. It is important to consider, however, that parameters conditioning LB unpacking could be very different in the alveolar spaces in vivo, where both the respiratory dynamics and the secretion products of other cell types can also play a role, than in isolated cultured cells. Unpacking factors might even be subjected to modulation in real physiological situations.

An interesting observation is that in type II cell primary cultures, most secreted surfactant remains in the form of lamellar body-like packed particles for a relatively long time, from minutes to hours [143,146,147]. Though packed, these particles maintain a state which is highly competent for interfacial adsorption. Fig. 4e illustrates a typical experiment from those published by the group of Haller [146], showing how individual secreted LBs are very efficient in transferring surfactant material into the air–liquid interface [147]. As a single, packed, LB approaches and touches the interface it suddenly *explodes* and transfers almost instantaneously its material –lipids, protein and fluorescent markers– into the interface. Transferred material remains for a relatively long time of a few minutes in a defined region of the interface, as if there were limitations to free lateral diffusion of lipids –

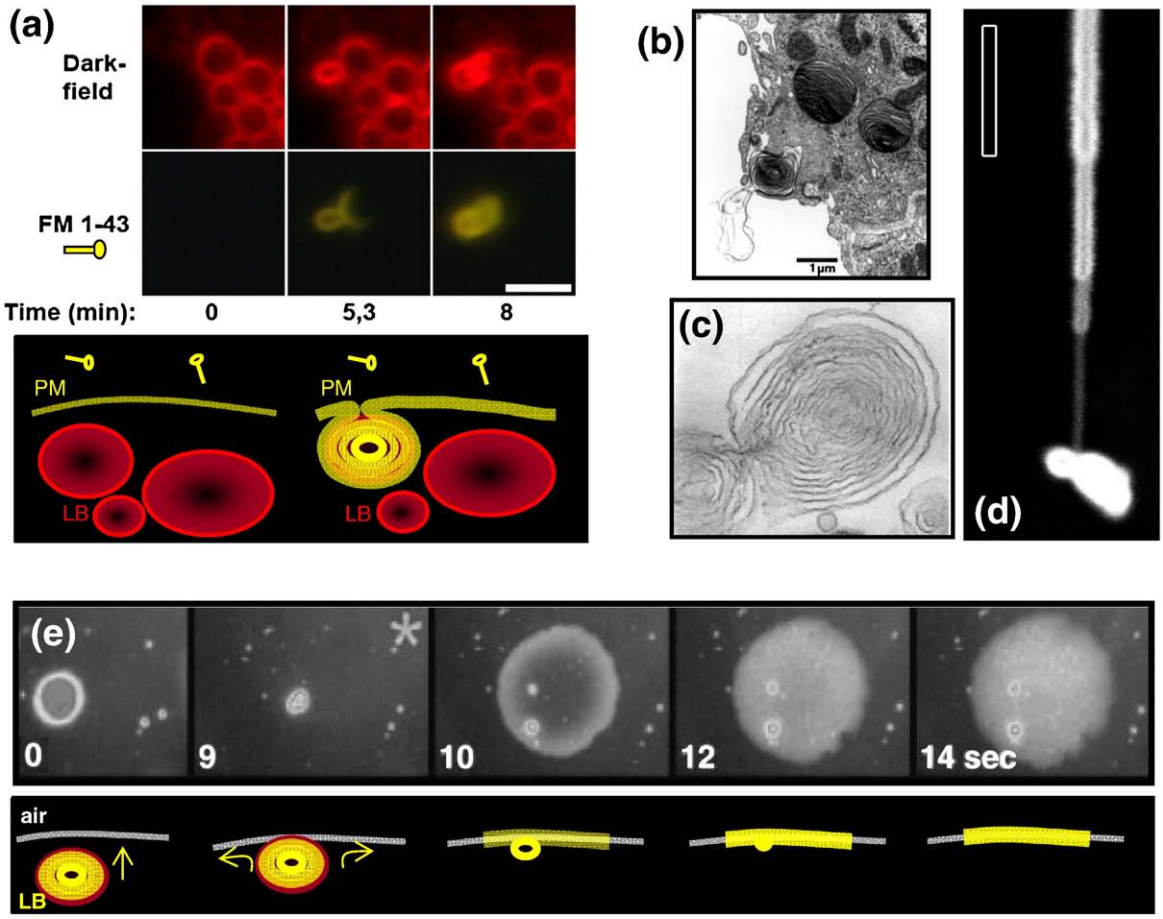

Fig. 4. Unpacking of secreted lamellar bodies. a) Combined dark-field and FM-143 fluorescence images of lamellar bodies as they are secreted by primary cultures of type II pneumocytes, at different time points after stimulation by secretagogues. The cartoon illustrates how the red dark-field image identifies intracellular lamellar bodies, which are not initially labelled by the yellow fluorescent probe FM-143 because it cannot cross the plasma membrane. Fusion of the limiting membrane of LBs with the plasma membrane, allows the entrance of the probe into the lumen of the secreting LB and heavy labelling of surfactant membranes. Microscopic images generously provided by Dr. Paul Dietl, from University of Ulm. b) Electron microscopy illustrating how surfactant membranes in LBs may swell and spontaneously unpack as soon as the organelle fuses with the plasma membrane (picture provided by Dr. Stephen Young from Pennsylvania University). c) Unpacking of internal surfactant membranes through specific peripheral locations of a lamellar body (electron microscopy by courtesy of Dr. Paul Dietl, Univ. of Ulm). d) Sliding of surfactant membranes during unpacking of a lamellar body (reproduced from [146] with permission). e) Sequence of microscopy pictures illustrating how a single surfactant lamellar body-like particle approaches the interface ($t=0$), touches it ($t=9$ s) and almost instantaneously "explodes" and transfer all the phospholipids into an inverted air–liquid interface. Fluorescently-labelled lipids transferred into the interface look confined in a defined interfacial region for long periods, indicating that lateral diffusion of lipid species is limited in the plane of the interface. Images reproduced from [146] with permission.

at least of the probes– along the interfacial plane. One could speculate that the network of lipid–protein interactions maintaining membrane packing in the secreted LBs could also be responsible for maintaining the material in a low-diffusible state upon transfer, either at the interface itself or as part of surface-associated multilayer complexes. The remarkable efficiency of the LB structure to be transferred at once into the interface could be essential for a rapid surfactant replenishment of the respiratory interface in vivo. The elaborated architecture of membrane arrangements in LBs could have been evolutionarily optimized for this purpose, and it is surely very distinct from the organization and the surface behaviour of the type of lipid/protein complexes composing the clinical surfactant preparations currently in use. Inverted phase experiments show that transfer of material from secreted LBs into the interface is modulated by surface tension [147], suggesting that most material could maintain a packed state until the precise moment when the interface needs to be replenished. Thus, packed lamellar bodies could be less prone to inactivation by binding serum components than the more exposed membranes of clinical surfactants [148]. In vivo, secreted but still packed LBs, ready to transfer material into the interface, could coexist with unpacked structures fulfilling complementary functions such as establishment of defence networks or organization of dense surface phases optimized for mechanical resistance under the demanding circumstances of respiratory physiology.

4. Ordered membrane organization in tubular myelin

Electron microscopy pictures of surfactant material purified from alveolar lavage or from whole lung tissue, reveal that a significant fraction of secreted surfactant membranes rearrange in a very unusual ordered network of membranes called tubular myelin (TM) [139,149]. This membrane-based structure has a remarkable architecture, which is not found in any other animal tissue. Material secreted from pneumocyte cultures only seldom shows TM figures (Dr. Paul Dietl, personal communication), suggesting that TM assembly requires both secreted surfactant and additional complementary elements, which are available at sufficient concentration in the alveolar spaces such as, perhaps, the collectin SP-A. The seminal reconstitution studies carried out by Suzuki's group [150] early established that TM organization requires the presence of phospholipids DPPC and PG, proteins SP-A and SP-B, and Ca^{2+}. Consistent with this observation, animals in which the expression of SP-A was inactivated lacked TM [151,152]. Interestingly,

these animals did not show any apparent respiratory dysfunction upon birth, suggesting that TM has no an essential role in the biophysical function of pulmonary surfactant. As SP-A is mainly implicated in innate defence [4], current thinking assumes that TM structure plays also some role in optimizing clearance of pathogens, although no rational hypothesis has been raised to explain how TM, and the particular organization that SP-A and SP-B adopt in such a structure, could enhance defence actions.

Still, the way surfactant proteins re-organize membranes to produce TM is remarkable, and deserves some discussion. As stated above, the SP-A macromolecule is a major determinant for TM organization. Membranes in TM arrange in an ordered pattern, whose dimensions are related with the molecular size of SP-A macromolecule. As a matter of fact, manipulation of the size of SP-A, through genetic modification of the segment of the gene codifying for the collagenic stem, gave rise to a correlation between the size of the protein and the dimensions of the membrane pattern in TM [54]. Architecture of TM must therefore rely on the properties of SP-A to interact with membranes and other surfactant proteins. Fig. 5 displays some significant pictures illustrating and interpreting how SP-A interacts with and modulates membrane structure at a macroscopic scale. The macromolecular character of SP-A assembly allows direct observation of the disposition of the protein when it associates with the surface of membranes [54,55], as illustrated in the electron micrographs of Fig. 5a. The typical disposition of SP-A at the surface of membranes consists of an apparent direct interaction of most of the globular heads –those containing the Ca^{2+}-dependent CRD domains– with the membrane, while the collagenous stem points more or less perpendicularly away from the membrane plane. This is consistent with experiments that determined that the phospholipid-binding motif of SP-A is located at the CRD globular domains [153,154]. The high resolution of the micrographs taken by Palaniyar et al. allows observation of an interesting feature. As the details magnified in Fig. 5a illustrate, the end of the collagenous stem of each SP-A molecule shows a clearly defined Y-shaped structure, which could be important to define specific interactions of this part of the protein with other SP-A molecules, receptors, etc. To our knowledge, no data has been provided so far on the possible three-dimensional structure of the N-terminal end of the SP-A assembly in spite of the fact

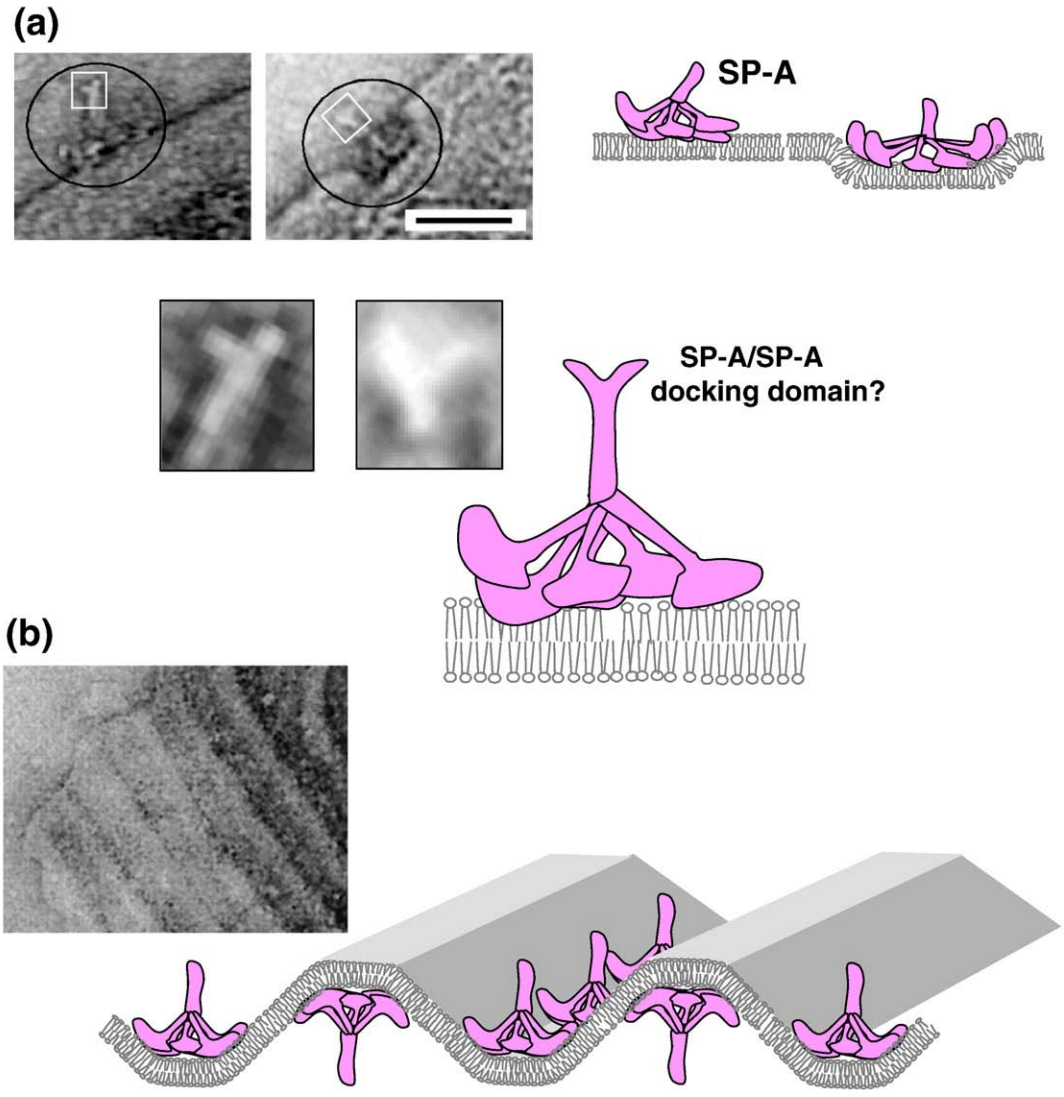

Fig. 5. Interaction of surfactant protein SP-A with phospholipid membranes. a) Electron microscopic pictures illustrating the interaction of hexadecameric SP-A with phospholipid membranes at a superficial (left micrography) or a curved membrane (right micrography) disposition. The cartoons on the right interpret how the interaction of only a few as opposed to practically all of the globular domains could be the difference between the two dispositions of the protein in the membranes. Details of SP-A structure enclosed in white boxes have been magnified below, to show how the N-terminal end of SP-A molecule seems to adopt a well-defined Y-shaped structure, which could be the basis for a potential protein/protein interacting motif. EM pictures have been reproduced from [54] with permission. b) Effect of SP-A to promote formation of "corrugated" phospholipid membranes, with defined grooves of regular dimensions. The EM picture (reproduced from [161] with permission) has been interpreted according to the cartoon.

that this segment of the molecule has been proposed as a critical element in lipid–protein and protein–protein interactions, including SP-A/SP-A network organization or interaction of SP-A with its cellular receptors [155,156]. Protein–protein interactions through the N-terminal end of their collagenous stems is a very important function for other collectins such as SP-D [60,157], but again no information is available about the structural context of such interactions. Most structural work with collectins has been mostly focused so far on the study of the folding and functional determinants of the globular domains [49,60,158,159].

In some pictures, such as the one on the right of Fig. 5a, binding of SP-A creates a deep depression in the membrane, with some localized markedly curved regions [54,55]. How SP-A could pass from the *planar* disposition at the membrane surface to the curved membrane situation is not known. A possibility is that the membrane depression would be created upon extensive interaction of the globular domains, and perhaps also the segments connecting the collagen with the globular heads, with the membrane, in a particular conformation. Lateral protein–protein interactions of the different globular heads from a given SP-A molecule could also be important to establish a rigid membrane-interacting protein platform. Another possibility is that interaction of SP-A with the membrane could sort the lipids laterally to segregate a region particularly enriched in saturated species. It has been demonstrated that SP-A has some preference to interact with ordered arrays of phospholipid headgroups such as the ones organized in ordered lipid phases [61,63,160]. SP-A-promoted curved membranes could be a result of creation by SP-A of boundaries between ordered rigid membrane phases and fluid, more deformable, membrane regions.

Whatever the molecular reason, the potential of SP-A to create membrane curvature and membrane deformations could be greatly enhanced in a cooperative manner at enough protein densities, as shown by several groups [161–163]. Fig. 5b shows a very suggestive picture in this respect, taken by the group of Dr. Nades Palaniyar. SP-A favours phospholipid membranes to adopt a *corrugated* structure, in which membrane shows distinct hills and valleys, with very regular dimensions [161]. A somehow corrugated structure was observed also in protein-free DPPC-containing lipid membranes, suggesting that the protein could interact and perhaps stabilize periodical lipid arrangements pre-existing in the membranes. Bilayers made of DPPC are known to form by itself a *ripple* phase [164] at temperatures between the pretransition –around 32 °C– and its main thermotropic transition, at 41 °C. A similar corrugated-like structure adopted by DPPC-enriched membrane regions could be the structure somehow stabilized by SP-A. It was demonstrated that the valleys in the membranes are associated with long-range SP-A alignments. Therefore, as illustrated in the cartoon in Fig. 5b, the corrugated membrane structure could also include deformations induced by alignment of SP-A molecules in the membrane. The alignment of SP-A could be the entropic consequence of a thermodynamically favourable segregation of SP-A-promoted deformed membrane regions. A similar mechanism has been recently proposed as a major force driving long-range protein-promoted membrane deformations [165,166]. Alternatively, specific lateral SP-A/SP-A interactions might mediate the extension of membrane deformations into the long grooves observed in corrugated membranes [167]. The ability of SP-A to self-associate in fibers and fibrous networks [55] could be the basis of the particular arrangements of the protein in the TM structure once the SP-A molecules get orientated at the surface of the membranes. The conclusion is that the combination of membrane–protein and protein–protein interactions of SP-A supplies the potential of SP-A to modulate membrane structure at a macroscopic scale.

Fig. 6 presents a model suggesting how SP-A interactions could mediate organization of TM architecture. Pictures in Fig. 6a illustrate the remarkable regular structure of membranes in TM [145]. Those transmission electron micrographs typically show that membranes in TM form a square lattice, with the membranes apparently crossing each other at regular distances. To our knowledge there is no data about whether intersecting membranes in TM are really fused, just apposed, or whether the crossing membranes are locations where lipids adopt non-bilayer structures. The correlation between the dimensions of the square pattern of TM and the molecular size of SP-A strongly supports the view that the lattice could be a consequence of regular deformations induced by SP-A arrays, such as those observed in corrugated membranes. Some pictures obtained by Palaniyar et al. revealed a very important feature [161], as shown in Fig. 6b. Fixation of TM membrane figures, followed by delipidization, allowed the observation under TEM of the presence in TM of an ordered network of protein, in the form of filamentous crosses occupying the lipid-free square spaces in TM tubes. Those protein-made crosses could be interpreted as an apposition of four SP-A molecules, contacting one to another through the N-terminal end of the collagenous stem. This disposition was suggested by Palaniyar et al. [54] and previously by others [168], but no clear model has been proposed for the way the membranes could be forced to adopt the TM network. However, in our opinion, the disposition of SP-A molecules in TM with contacts through their N-terminal ends immediately suggests a possible model for a transition from SP-A-promoted corrugated membranes into the square tubular structure of TM membranes, in a similar way to what it was already suggested by Palaniyar et al. [167], which is schematized in Fig. 6c. Two corrugated membranes could couple their grooves one to another by the direct interaction of the N-terminal collagenous ends of the SP-A molecules aligned in the valleys. Similar interactions could make SP-A promote lipid vesicle aggregation, with the important participation of the N-terminal segment of the protein [60,156]. Cooperative contacts between SP-A molecules regularly distributed in the two contiguous membranes would create a layer of square-shaped tubes. The square shape would be a consequence of the membrane deformation induced by SP-A, as observed already in the sharp edges of the grooves in corrugated membranes [161]. Apposition of two corrugated membranes through SP-A/SP-A interactions would also put in contact the membranes themselves at the “hills” between the “grooves”. Such membrane–membrane interactions could be supported or facilitated by the presence, in these abutting regions, of SP-B, a protein known to induce membrane–membrane interactions. This process could be followed by real membrane fusion [69,83,86,122], also required to assemble TM [150]. Apparently crossing membranes could then be really fused or perhaps only closely apposed via SP-B/SP-B or SP-B/membrane interactions. Similar association of not only two, but multiple SP-A-modelled corrugated membranes would extend TM-like structure in the third dimension. The model would predict that only SP-A dimers –formed through interaction of the N-terminal ends of two SP-A hexadecamers– would be enough to trigger membrane organization in the TM-like architecture. The protein crosses observed in TM structure [161] could be a consequence of either a two-dimensional projection of SP-A dimers orientated in two different perpendicular dispositions inside each tube or the interaction via their N-terminal ends of four different SP-A molecules –two dimers– at each node of the membrane network. Isolation and structural characterization of native-like SP-A suprao-ligomers would shed some light on this respect. It is interesting to notice that there are apparently some differences in the structure of some of the TM figures shown in the literature. In some pictures, it seems that the proteinaceous filaments –presumably SP-A– are more or less homogeneously distributed in the TM lattice (for instance, in Fig. 6b). However, in TM-like structures observed as directly forming from lamellar body unpacking, it is usually noticed that square tubules with two types of shapes coexist, which are alternately arranged (see, for instance, Fig. 6a and the magnified detail). SP-A-like structures seem to locate, in such “early” TM, only

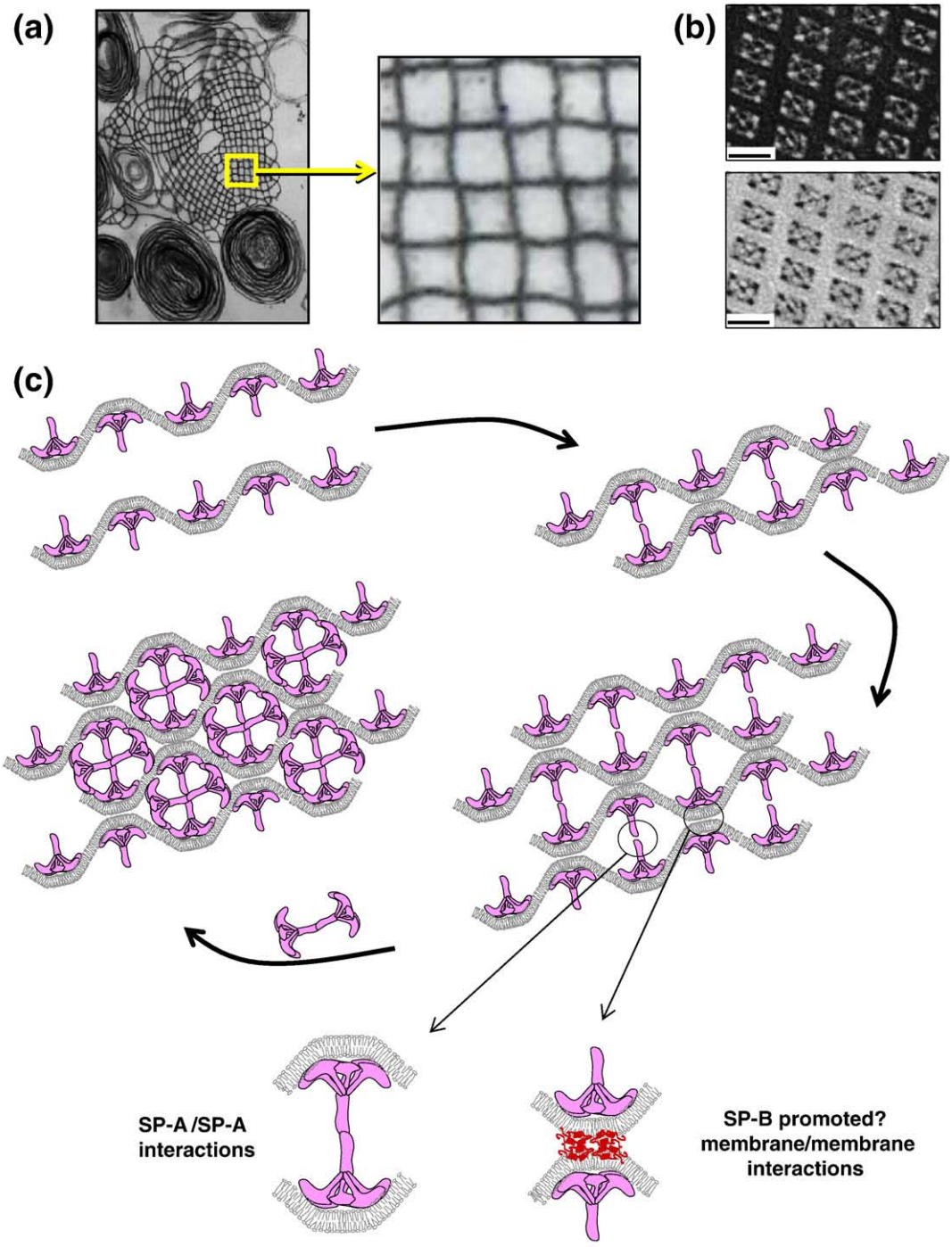

Fig. 6. Structure and molecular model for the assembly of tubular myelin. a) Electron microscopy picture illustrating how membranes from LBs can unpack to form the square-lattice network of membranes typical of tubular myelin. The magnified detail illustrates how the square tubes in TM that has been just assembled from unpacked LBs are of two types, with well differentiated sizes, with only the smaller ones containing SP-A-like fibrous structures (identified by arrows). The picture, by courtesy of Dr. Stephen Young, from Pennsylvania University. b) Protein lattices in delipidized TM samples, showing the regular disposition of proteinaceous cross-like figures in the square-lattice pattern defined by pre-existing membranes. Figures represent negative staining and its reverse contrast. Taken from [161] with permission. c) Model for the possible SP-A and SP-B-promoted assembly of TM network. Pairs of SP-A-corrugated membranes could couple via SP-A/SP-A interactions mediated by their N-terminal domains. Additional membrane layers could be assembled through protein–protein interactions. Membrane–membrane contacts could be favoured or progress towards real membrane fusion in the presence of SP-B, which has been shown to be required to reconstitute TM in vitro.

in alternate tubules. The model in Fig. 6c suggests that early TM could be an intermediate form upon the primary interaction of SP-A with just one side of the membranes, as they are progressively unpacked from LBs. Later interaction of further SP-A molecules with membrane tubes could complete the organization of the very regular, and probably rigid, structure of mature tubular myelin.

5. Interfacial membrane-based surfactant films under dynamic conditions

Membrane unpacking after surfactant secretion ultimately leads to the adsorption of lipid/protein complexes into the air–water pulmonary interface [146]. The stabilization by surfactant of the respiratory surface

through a dramatic reduction in surface tension strictly requires formation of an interfacial film competent to exclude most water molecules from their exposure to air [17]. The potential behaviour of interfacial surfactant layers has been extensively evaluated in simple surface balance models, where physiologically relevant conditions such as lipid and protein composition, compression–expansion rates, temperature, ionic environment of the subphase, etc, have been extensively studied [3,17,19,169]. However, it is becoming clear that surfactant films as they are formed in vivo are probably much more complex than the simple monolayers studied during the last 30 years as fundamental models of surfactant films. Therefore, care must be taken when extrapolating most of the features defined from the studies in basic surface balances to the real physiological situation.

Basic concepts widely assumed in pulmonary surfactant physical chemistry include that surfactant films must reduce surface tension to values near 0 mN/m at the end of expiration –in principle, the maximally compressed state of the respiratory surface– to avoid alveolar collapse [22]. Also, that just a phospholipid-based monolayer enriched in saturated species can sustain low enough tension upon compression, and only at temperatures below the T_m temperature of the corresponding gel-to-fluid phase transition of the phospholipids [27,170]. According to these assumptions, DPPC is the major component of surfactant in mammals because it is the phospholipid species optimally selected to form films capable of reaching extremely low tensions when compressed at 37 °C [17,22,23]. The recent data provided by the group of Hall showing that rapid compression of films made exclusively of unsaturated phospholipids can also produce very low tensions are challenging the classical model [29]. Unsaturated phospholipids, as well as other lipid and protein components of surfactant have been usually considered to play the role of modulating the properties of DPPC to facilitate its transit to the air–water interface [19,27,37,171,172]. Some of these other components were also proposed to promote a progressive enrichment of the interfacial film in DPPC [170,173,174], as this was supposedly the main component able to produce and sustain the lowest tensions. However, these concepts have arisen mainly from the study of the behaviour under different conditions of monomolecular films formed in surface balances by spreading lipid or lipid/protein mixtures from organic solvent solutions. The fact that the two hydrophobic proteins required to stabilize the lung, SP-B and SP-C, can be purified and manipulated in organic solvent has also facilitated their inclusion and study in such simplified models. This section will analyze the current basic ideas on the participation of surfactant proteins and lipid–protein interactions in interfacial dynamics as it has been mostly proposed from the models, within the scope of the potential relevance in the physiological context of the alveolar spaces.

Both, SP-B and SP-C, promote rapid transfer of phospholipids from membranes into interfacial films [71,100,102]. In vitro, adsorption to open interfaces at relatively diluted surfactant concentrations could end in an extensive conversion of bilayers into interfacial monolayers [87,175]. Such conversion may or may or not imply transfer of proteins themselves into the interface. At the high surfactant concentrations thought to exist in the alveolar surface, SP-B and SP-C probably promote the establishment of molecular connections between the secreted membrane-based structures and the surface film [69]. Those protein-promoted contacts could ensure a rapid flow of lipids between the different structures, which is probably important to facilitate replenishment of the interface with surface active molecules.

Compression of interfacial films formed in vitro produces very low surface tension, both in regular Langmuir-type surface balances [17,23,171,172] and in captive bubble surfactometers [176], as long as the film contains a sufficient proportion of saturated DPPC. Compression in Langmuir balances of films formed by whole native surfactant or its organic extract –containing all the lipids plus the hydrophobic surfactant proteins– show isotherms with very conspicuous plateaus [37,177], indicative of the existence of structural transitions occurring in the compressed films, which are required for the films to reach the lowest tensions. When the films are formed and compressed at much more physiologically relevant conditions in captive bubble surfactometers, i.e. at higher concentrations of surfactant in the hypophase and relative rapid compression rates, the films reach the lowest tensions with much less compression than in films in LB balances [176,178]. Interfacial films formed from native-like concentrated surfactant suspensions could therefore be particularly prepared to sustain very low tensions –high lateral pressures– with very little change in the surface area. Adsorption of good surfactant preparations at high enough concentrations in the hypophase has been shown to produce interfacial films comprised of several phospholipid layers, tightly associated with the interface [179] (see Fig. 7a). Similar interfacial multilayer arrays have been observed in electron microscopy pictures of lung tissue (an example is shown in Fig. 7b), using special procedures to fix and capture the lipid-enriched interfacial structures [180]. Other authors have been able to detect and analyze the repetitivity of membrane structure in surfactant multilayered films made of some partial surfactant preparations [181]. Compression of these multilayered films could yield a different behaviour in terms of the extent of compression required to reach the minimal tensions and in terms of stability, than the compression of simple monolayers. In the in vivo situation, the multilayered film would be probably interconnected with multiple surfactant membrane structures in the hypophase, including secreted lamellar body-like particles and tubular myelin arrays, increasing presumably the potential for the film to structurally stabilize the interface under dynamic conditions.

Monolayer experiments with films containing different proportions of surfactant proteins SP-B and SP-C have shown that the two proteins facilitate ordered and reversible compression-driven structural transitions. Compression of pure lipid films usually ends with an irreversible collapse, in which part of the lipid material is irreversibly lost towards the hypophase [28,37,161]. The presence of SP-B in films under compression results in the formation of associated bilayer patches (see AFM picture and cartoon in Fig. 7c), which can reinsert into the interface upon expansion [182]. Overcompression of lipid/protein films containing the lipopeptide SP-C also results in formation of multiple associated layers, presumably bilayers, which were not seen in the absence of the protein [183,184] (Fig. 7d). Maintenance of the association of the protein and the lipid/protein structures with the compressed films requires the palmitoylation of SP-C [110], suggesting that palmitoylation could permit this protein to associate simultaneously with and to sustain close contact between the two neighbour lipid layers. The model sketched in Fig. 7e suggests that SP-B could be particularly important in ensuring a rapid, and possibly bidirectional, flow of lipid molecules between the different surfactant structures and with the multilayer surface-associated film. Constitutive absence of SP-B compromises the synthesis and production of surfactant in its primary biogenesis [8,64], but the respiratory failure associated with an induced depletion of the protein in conditioned knock-out models [65,83] could rather be associated with the lack of a proper dynamic flow of lipids interconnecting the whole alveolar surface. SP-C might have, on the other hand, a major role in maintaining the integrity of the multilayered surface film, providing the particular properties of stability and mechanical resistance that only the well packed multilayered structure can sustain. The apparent reduced stability of the films formed by the surfactant of SP-C knock-out mice [90] would support this hypothesis, as well as the fact that the lack of an operative SP-C has been associated with the development over the long term of chronic respiratory failure [66,91].

In the alveolar spaces, formation of a thick enough surfactant multilayered film at the interface, interconnected with enough density of surfactant structures at the hypophase, could produce a relatively steady-state situation. The surfactant-loaded surface phase might then be optimized to sustain breathing dynamics needing very little change in area/volume of the terminal airways, where gas exchange is the primary objective. The observation that very significant

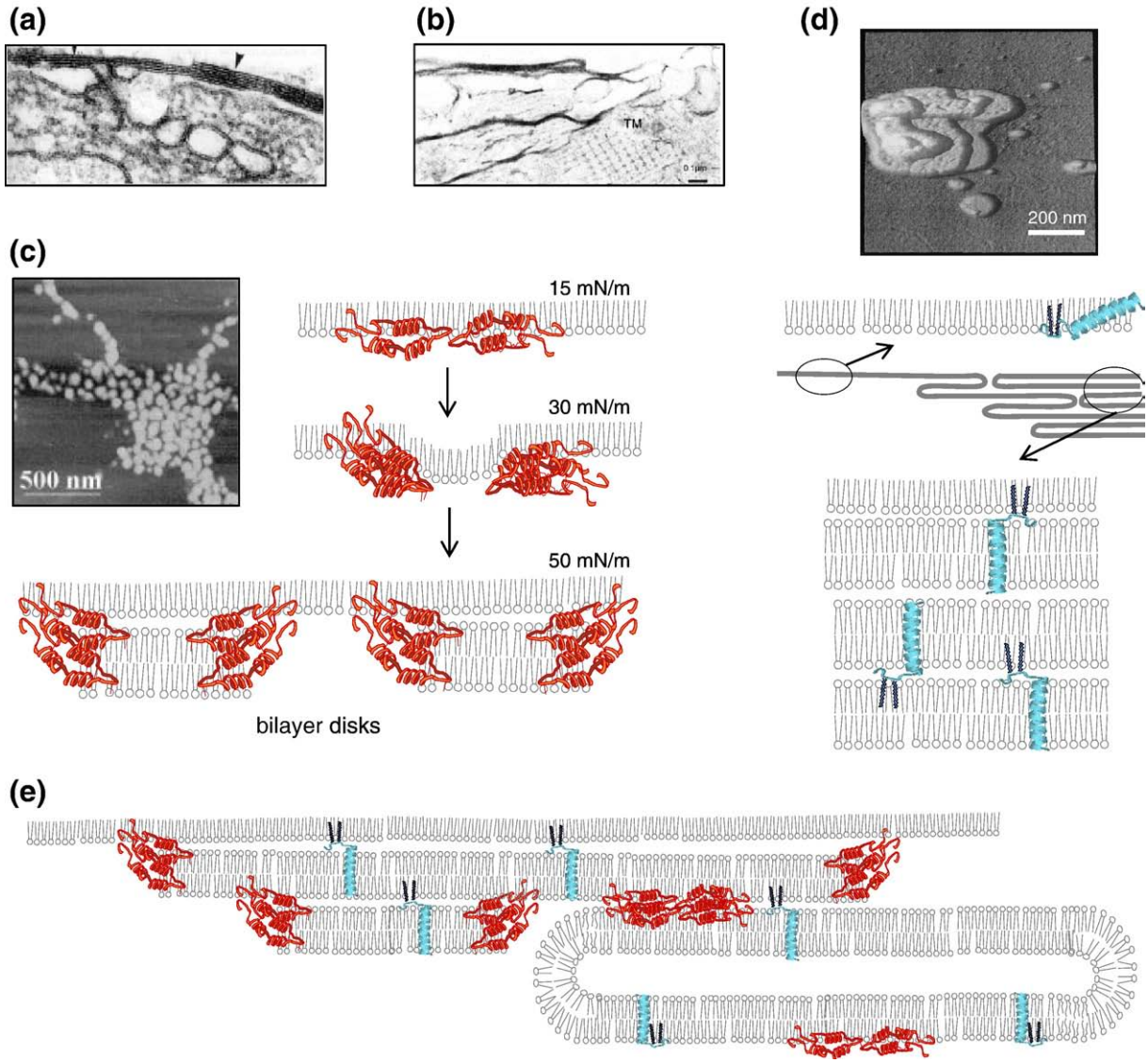

Fig. 7. Structure of multilayer interfacial surfactant films. a) Electron microscopy picture of a segment of the multilayer-like interfacial film formed by surfactant at the air–liquid interface by the lung epithelium (taken from [180] with permission). b) Multilayer-like film of natural surfactant adsorbed at the air–liquid interface of a captive bubble (from [179] with permission). c) AFM picture (from [182] with permission) of interfacial phospholipid films containing surfactant protein SP-B. The cartoon illustrates how the progressive compression of the films produce formation of lipid/protein discs of defined sizes, which are maintained in close contact with the interface presumably by lipid/protein interactions (adapted from [182]). d) Multilayer morphology of a compressed phospholipid film containing pulmonary surfactant protein SP-C, as observed by AFM (from [184] with permission). The cartoon below the AFM picture interprets the structure in terms of membrane layers sustained underneath the interface by simultaneous interaction of SP-C with neighbour surfactant layers. e) The model shows how in multilayered surfactant films, SP-B could be important to initiate protrusion of different membrane layers while palmitoylated SP-C could help to sustain close association of the different layers along the different compression–expansion respiratory cycles.

differences could exist in the change of the apparent alveolar volume during breathing when comparing a surfactant-containing with a surfactant-depleted lung [185], would support this view.

6. Lateral structure of pulmonary surfactant membranes and films

Much of the research during the last few years on the structure of pulmonary surfactant layers has been devoted to the analysis of the lateral in-plane organization of lipids and proteins in membranes and interfacial films. This organization probably explains, on one hand, the combination of saturated and unsaturated phospholipid species, charged lipids and cholesterol, which evolved to fulfil surfactant function in the defined context of temperature and mechanical constraints of mammalian lungs. On the other hand, an optimized lateral organization is likely important for pulmonary surfactant to simultaneously sustain its dynamic membrane polymorphism and a robust enough structural role in the airways.

The segregation of ordered and disordered regions in pulmonary surfactant membranes has been recently analyzed using giant unilamellar vesicle (GUV) technology [32,38] (see Fig. 8). Native surfactant membranes could be converted into micron-size fluorescently-labelled liposomes while maintaining its original lipid and protein organization. This approach has also been shown to preserve the original orientation of other membrane-associated proteins [186]. Microscopic observation of pulmonary surfactant GUVs under the microscope revealed the coexistence of two types of membrane regions. Part of the membrane surface was occupied by fluid-disordered domains, surrounded by a background of fluid-ordered phase, presumably enriched in DPPC and cholesterol. Proteins SP-B and SP-C partitioned selectively into the fluid-disordered regions. This lateral segregation of fluid phases was retained even after disorganization of the original structure by organic solvent extraction or the complete elimination of proteins, indicating that it is the particular lipid composition of surfactant that creates the coexistence pattern. The increase

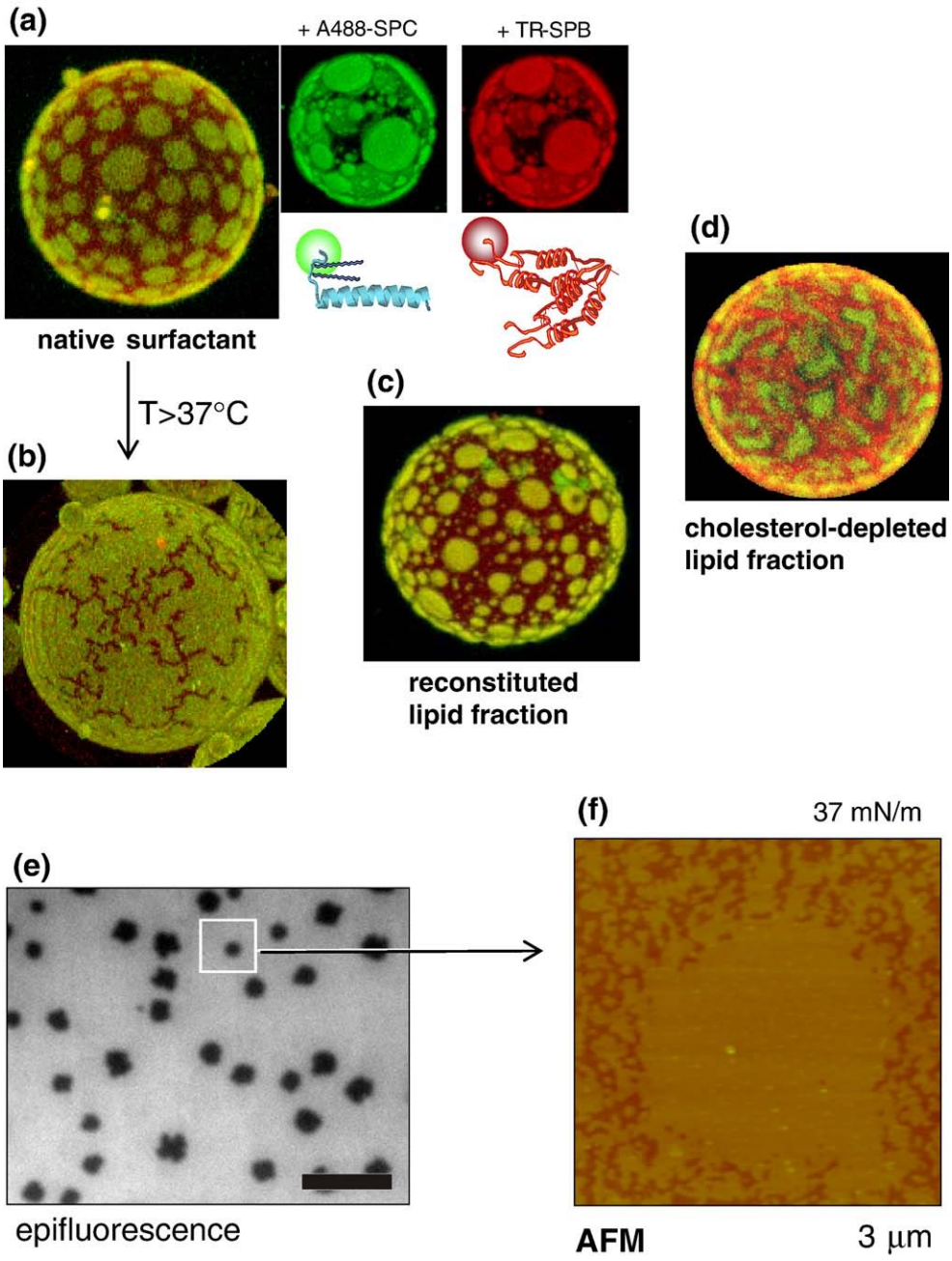

Fig. 8. Lateral structure of pulmonary surfactant membranes and films. a) Segregation of fluid-ordered (red, $DiIC_{18}$ labelled) and disordered (green, BODIPY-PC labelled) regions in native pulmonary surfactant membranes, observed in giant unilamellar liposomes (GUVs). Green and red liposomes on the right illustrate that both proteins, SP-B and SP-C, partition into the fluid-disordered membrane phase, as observed upon introduction into surfactant of traces of Alexa-488-labelled SP-C and Texas-Red-labelled SP-B. b) Heating of native surfactant membranes to temperatures above 37 °C produces melting of most liquid-ordered phase. c) GUVs prepared from a totally deproteinized lipid fraction of surfactant entirely mimic the coexistence of ordered/disordered fluid phases of native surfactant membranes, indicating that it is the lipid moiety of surfactant what drives lateral membrane organization. d) GUVs made of cholesterol-depleted surfactant always show a very polymorphic phase separation, consistent with gel/fluid phase coexistence instead of the fluid/fluid phase separation observed in native surfactant, illustrating the crucial role of cholesterol in modulating lateral membrane structure. Pictures *a–d* taken from [32]. e) Epifluorescence microscopy picture *d* from Bernardino de la Serna, Bagatolli and Perez-Gil. e) Epifluorescence microscopy image taken from a native pulmonary surfactant film compressed to 37 mN/m, showing segregation of condensed domains excluding a NBD-PC lipid probe. f) AFM image illustrating the nanoscopic structure of a condensed microdomain segregated from an apparently liquid-expanded background. Notice that bright expanded regions in epifluorescence really contain interconnected condensed nanodomains when observed under AFM (Antonio Cruz, David Schurch, Luis Vazquez, J. Perez-Gil, unpublished).

of temperature above a certain threshold caused melting of a substantial fraction of the liquid-ordered phase, supporting the idea that a sufficient proportion of saturated lipids with a melting transition above physiological temperature is strictly required as a basis for lateral segregation of phases in surfactant membranes. Removal of cholesterol also caused a major effect in the lateral membrane structure, promoting the change from an entirely fluid ordered/disordered coexistence into a gel/fluid-disordered type of segregation [32,187]. Cholesterol, therefore, seems to be an important determinant modulating phase behaviour, fluidity and dynamics in surfactant membranes. To what extent the presence of certain amounts of cholesterol contributes to optimize the functional performance of surfactant under the particular environmental conditions of the alveolar spaces, has still to be fully explored. In several animal models that have been studied, the level of cholesterol in surfactant seems to be a factor involved in physiologically-regulated responses to

environmental challenges [188–191]. On the other hand, abnormally high levels of neutral lipids have been found in poorly surface active surfactant obtained from acute distress syndrome patients [192], suggesting a deleterious effect of cholesterol on surfactant function. Mechanisms of adaptation of the surfactant system to challenging environmental conditions could include concerted changes of the proportion of cholesterol from or to alveolar reservoirs, as demonstrated in other air-breathing animals [190]. Determination of the real existence of such potential cholesterol alveolar reservoirs, as well as the nature of the molecular sensors supposedly connecting the alteration of breathing performance with adaptations of surfactant composition, is a challenging task. The increase in cholesterol could therefore be a consequence rather than a cause of a poorly-performing surfactant system. Proteins do not seem to play a major role in modulating the lateral structure of surfactant membranes, but show a non-homogeneous distribution based on their lipid phase partition properties. As said, SP-B and SP-C have been shown to segregate into the fluid-disordered regions of the membranes. The lateral distribution of SP-A has not been directly visualized, but some indirect evidence indicates that this protein would prefer to interact with ordered phases [57,61,63]. The lipid phase "scaffold" of surfactant membranes could then be a major determinant to initiate or coordinate the three-dimensional organization of membranes in surfactant structures via lipid–protein and protein–protein interactions. An important consequence extracted from these studies is that a minimum level of lipid complexity may be important in surfactant to provide the proper level of structural complexity. Surfactant preparations containing an excess of saturated phospholipids, too high electrostatic charge, or exacerbated proportions of cholesterol could be far from optimized even in the presence of substantial levels of proteins, especially under the environmental conditions of the alveolar spaces.

Segregation of different lipid- and protein-containing regions in interfacial surfactant films has previously been analyzed long ago, as compression was well known to be a major determinant of lateral organization. From the earliest experiments applying epifluorescence microscopy to the observation of interfacial lipid and lipid–protein films, it became clear that the ability of different films to reach the lowest compression-driven surface tensions was related with their propensity to achieve a highly packed condensed lateral structure upon compression [34,35,102]. Lateral condensation of simple films composed of DPPC or very simple binary or ternary combinations of DPPC and other lipid and protein species have been characterized in detail [34,35,69,71,81,97,102,106]. Lateral transitions in much more complex films, such as those formed by whole pulmonary surfactant or its organic extract have been studied in much less detail, and usually in a rather qualitative way. Compression of full complex surfactant films produce segregation of small apparently fluid-ordered domains [37,177], likely enriched in DPPC and cholesterol, and presumably excluding hydrophobic surfactant proteins SP-B and SP-C. This segregation occurs at pressures preceding those where the plateau originates in the isotherm, which is associated with three-dimensional folding transitions occurring in the films, suggesting that lateral sorting of lipid and protein components might be an important feature in defining a proper compression-driven behaviour. Segregation of ordered/disordered phases in full surfactant films is critically dependent on temperature [187], as occurs in surfactant membranes [32], but melting temperature of interfacial films could be a few degrees higher than that of bilayers [2]. Lipid composition in pulmonary surfactant could have therefore been optimized to provide phase transitions close enough to the physiological temperature, in such a way that membranes would be mostly fluid and disordered, and in consequence highly dynamic, while interfacial films could still support enough proportion of ordered phases to sustain the mechanical stability required to maintain proper breathing. In the recent years, the lateral structure of pulmonary surfactant membranes and films has been also extensively studied by atomic force microscopy (AFM), a technique providing resolution at a nanoscopic level, in the absence of spurious probes. Observations by AFM have given access to the analysis of a level of complexity at the nanometer scale in lateral organization that was not available before, confirming that lipids and proteins in surfactant are far from being homogeneously distributed also in membranes [32] and interfacial films [193]. The importance of this nanoscopic organization for the rheological properties and the functional activities of surfactant is only starting to be appreciated.

However, structural studies of the lateral organization of surfactant membranes and films could still provide only a very simplified picture of the real situation. As stated above, surfactant surface structure, as it is disposed in the thin water layer of the alveolar epithelium, could be much more complex than a single monolayer in a surface balance or a single bilayer in a giant liposome. The characterization of the actual lateral organization of lipids and proteins in the multilayer-like polymorphic three-dimensional structure of surfactant as it is interconnecting the whole subphase with the gas phase supposes a technical challenge that will have to be approached, possibly through new still-to-come methodological developments, before understanding the ultimate structure–function correlations of the system.

7. An integrated model of pulmonary surfactant structure at the alveolar spaces

Fig. 9 illustrates an integrated view of how pulmonary surfactant could develop a sort of *membrane surface phase* covering the respiratory surface. The term "surface phase" was first suggested by the group of Larsson [181,194], to point out that pulmonary surfactant should not be seen as a purely interfacial film at the air–liquid interface but as a complete structure developed in the aqueous lining layer as a whole. From our point of view, this is certainly an appropriate way to interpret surfactant membrane-based polymorphism at the alveolar airspaces, but in a much more complex view than discussed by Larsson et al. Part of pulmonary surfactant properties could come from a sophisticated pre-loaded structure, as assembled in the pneumocyte, with the potential to develop a complex polymorphism once secreted into the extracellular medium. But besides that, surfactant structures could be poised to integrate lipid–protein entities liberated into the airways from different origins and modulated by different environmental and physiological determinants. Just this complex biological "phase", developed as a *three-dimensional film* at the respiratory surface, could be competent to sustain simultaneously proper respiratory gas exchange, mechanical stabilization under the dynamic conditions imposed by breathing, and protection against pathogenic invaders. The disruption of such a complex structure that is likely associated with lung injury could have many more consequences than a mere impairment of the ability of surfactant to produce very low surface tensions. A global comprehension of structure–function determinants in pulmonary surfactant three-dimensional

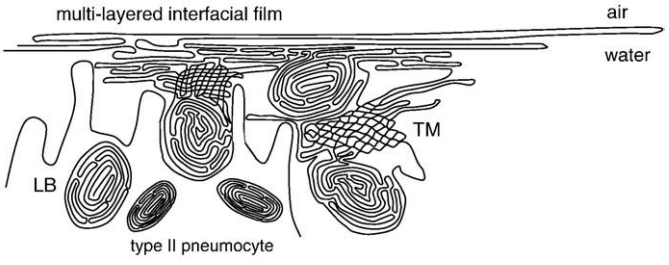

Fig. 9. Cartoon illustrating how pulmonary surfactant could be responsible for establishing a *membrane surface phase* at the respiratory surface. The thin aqueous layer covering alveoli would be saturated of multiple interconnected membrane structures, including secreted lamellar bodies, tubular myelin and multilayered films. The whole surface phase should be envisaged as a unique structure, simultaneously optimizing respiratory gas exchange, surface physical stabilization and defence against pathogens.

films is still required before therapeutic use of clinical surfactants might have reasonable chances of success in the treatment of complex pathologies associated with lung injury.

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