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Review

Structure-function relationships in pulmonary surfactant membranes: From biophysics to therapy☆

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ABSTRACT

Pulmonary surfactant is an essential lipid–protein complex to maintain an operative respiratory surface at the mammalian lungs. It reduces surface tension at the alveolar air–liquid interface to stabilise the lungs against physical forces operating along the compression–expansion breathing cycles. At the same time, surfactant integrates elements establishing a primary barrier against the entry of pathogens. Lack or deficiencies of the surfactant system are associated with respiratory pathologies, which treatment often includes supplementation with exogenous materials. The present review summarises current models on the molecular mechanisms of surfactant function, with particular emphasis in its biophysical properties to stabilise the lungs and the molecular alterations connecting impaired surfactant with diseased organs. It also provides a perspective on the current surfactant-based strategies to treat respiratory pathologies. This article is part of a Special Issue entitled: Membrane Structure and Function: Relevance in the Cell's Physiology, Pathology and Therapy.

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Contents

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1. Pulmonary surfactant

The presence of a pulmonary surfactant was directly linked with respiratory failure by Richard Pattle in England and John Clemens in the USA while studying the effects of nerve gases in the lungs [1]. A few years later, Mary Ellen Avery demonstrated that hyaline membrane disease (later known as respiratory distress syndrome, RDS) in new-borns that died after birth was caused by a lack of surfactant [2]. Consequently, hyaline membrane disease was associated with the absence of a surface-active material; under normal conditions, this material produces a surface tension of around 8 dyn/cm. In babies suffering from hyaline membrane disease, the surface tension exceeded 30 dyn/cm. The first successful animal experiments with natural surfactants were performed by Enhorning and Robertson in Stockholm, demonstrating improved survival in preterm rabbits [3,4]. Later, Adams and Fujiwara in the USA showed the same beneficial effects of natural surfactants in preterm lambs [5,6]. Once the connection between the surfactant and lung diseases in neonates was established [7–9], exogenous surfactant therapies were developed. Surfactant replacement therapy (SRT) is currently used as a prophylactic treatment in neonates of less than 35 weeks in gestational age, decreasing the mortality of premature babies up to 80%. SRT today currently enables premature babies to breathe and survive at only 25 weeks of gestational age [10]. Moreover, surfactant research has been growing and expanding to cover other lung pathologies. The primary objective of surfactant research is to understand the molecular and physical mechanisms associated with surfactant function as well as the processes interfering with the surfactant's activity and contribution to lung diseases. A better understanding of the primary or secondary implications of surfactants in respiratory pathologies is also required to facilitate the development of successful treatments and efficient clinical surfactant preparations.

1.1. Surfactant composition and structure

Pulmonary surfactant is produced in the lungs and is essential during breathing. Because it is placed at the air–liquid alveolar interface, pulmonary surfactant reduces the surface tension of the thin layer of water that covers the lung epithelium. A low surface tension reduces the work of breathing and prevents alveolar collapse. Moreover, surfactant is the first barrier that pathogens encounter within one of the largest exposed surfaces of the human body [11–13]. The lung surface area has been calculated as approximately 100 m^2 and facilitates essential gas exchange activity. For this reason, respiratory pathologies cause 13.6% of the deaths worldwide, according to the World Health Organisation (2008).

Pulmonary surfactant is a complex mixture of lipids and proteins, and lipids account for more than 90% of the surfactant by mass (see Fig. 1). The qualitative and quantitative compositions of the lipids in the surfactant vary between species and according to environmental conditions, such as body temperature [14]. Surfactant also changes according to physiological constraints, such as the breathing rate or hibernation [15], or due to pathological situations, particularly lung injury [16,17]. However, the protein composition is also critical for normal surfactant function. Surfactant proteins A (SP-A) and D (SP-D) belong to the collectin protein family. They are directly related to the innate host defence of the lung and recognise, bind and eliminate pathogens [18–20]. Surfactant proteins B (SP-B) and C (SP-C) are small hydrophobic proteins that are deeply embedded into the surfactant phospholipids; they enhance interfacial adsorption of surface active molecules into the air–liquid interface and contribute to mechanical stability of the interfacial films [21]. Animal models show that deficiencies in surfactant proteins lead to respiratory pathologies, demonstrating a direct relationship between surfactant activity and normal lung performance. Deficiencies in SP-A and SP-D are not critical at first, although animal models deficient in SP-A develop lung infections more frequently [22]. SP-D deficiency might be related to emphysema [23] and chronic obstructive pulmonary disease (COPD) [24]. On the other hand, SP-C deficiency is associated with chronic respiratory pathologies [25], and complete SP-B deficiency results in death shortly after birth [26,27].

1.1.1. Surfactant lipids

Phospholipids are amphipathic molecules that have a polar and hydrophilic moiety and non-polar or hydrophobic chains. This type of molecule adopts a particular arrangement at the air–liquid interface, minimising the contact between the hydrophobic region and water molecules. Phospholipids thus adopt an energetically favourable orientation, pointing the polar heads toward the water phase, while the non-polar chains are oriented toward the air.

As shown in Fig. 1, the most abundant component in surfactant is dipalmitoylphosphatidylcholine (DPPC), representing approximately 40% of the total surfactant mass. DPPC is essential for producing the very low surface tension observed during compression; its saturated acyl chains can adopt a highly lateral packed state. Surfactant contains other saturated phosphatidylcholines (PC), such as palmitoylmirystoyl-PC (PMPC, 16:0/14:0), and unsaturated PCs, such as palmitoyloleoyl-PC

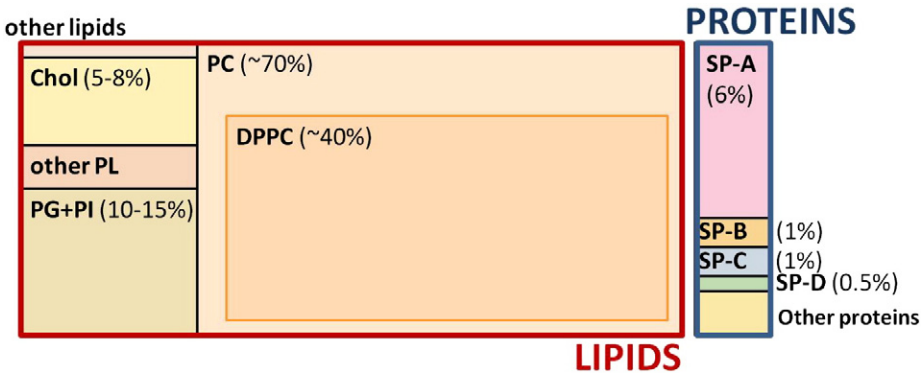

Fig. 1. Composition of lung surfactant. Proportions of the different lipid and protein components in pulmonary surfactant, represented as occupying proportional areas with respect to total surfactant mass.

(POPC, 16:0/18:1) or palmitoylpalmitoleoyl-PC (PPPC, 16:0/16:1). Other functionally important phospholipids in surfactant include a proportion (10–15%) of hydroxylated anionic phospholipids, such as phosphatidylglycerol (PG) and phosphatidylinositol (PI); these species are thought to participate in selective interactions with the cationic hydrophobic proteins [28–31]. Other phospholipids, such as phosphatidylethanolamine (PE) and sphingomyelin (SM), appear as minor components of surfactant and most likely come from other cell membranes. In addition, little amounts of lysophosphatidylcholine (LPC) can be found [32–34].

As important as the phospholipid composition is, the presence of an appropriate proportion of cholesterol in surfactant may also be key. Under normal physiological conditions, cholesterol content is approximately 3–8% by mass. The other neutral lipids present in small quantities include cholesterol esters, triglycerides, diglycerides and free fatty acids [35].

Different surfactant phospholipids offer different structural features with potentially important functional relevance. The negative charge at the head groups of anionic species, for example, permits to establish interactions with the positive charges in SP-B and/or SP-C or to interact with cations such as Ca^{2+}, which might be important for surfactant organisation. Hydroxylated phospholipids might offer unique possibilities for H-bonding among lipids or between lipids and proteins. On the other hand, the hydrophobic segments of phospholipids are defined by the different fatty acids that esterify the glycerol backbone, differing in length and the number/position of the double bonds (unsaturation). Palmitic acid (16:0) is particularly abundant in surfactant phospholipids, specifically in disaturated species, such as DPPC. Unsaturated acyl chains impose a steric hindrance that opposes highly lateral packing, strictly required to minimize surface tension during the compression of the alveolar interface (see Section 2.3).

However, spontaneous interfacial adsorption (transfer to the interface, see Section 2.2) of phospholipids is an intrinsically slow process, and desorption contributes to the loss of phospholipids from the interface. To optimise these processes, surfactant complexes contain not only phospholipids but also two small hydrophobic proteins (SP-B and SP-C) that are stably associated with phospholipids. These proteins dramatically enhance interfacial adsorption of phospholipids and help to maintain the interconnected surfactant structures.

1.1.2. Surfactant proteins

There are four surfactant proteins known to directly participate in pulmonary surfactant-associated functions. Their model structures and their organisation at the alveolar spaces are presented in Fig. 2. SP-A, SP-B and SP-C are obtained from the airways associated with surfactant phospholipids and are therefore considered apolipoproteins, while SP-D is not. SP-D could still interact with phospholipids under specific conditions, in surfactant or in other cell membranes, because interactions between SP-D and certain phospholipid species, such as glycolipids or PI, and fatty acids have been reported [36–39]. The small hydrophobic SP-B and SP-C proteins participate in surface activity of surfactant, while SP-A and SP-D play a major role in innate immune defence.

1.1.2.1. SP-B and SP-C. SP-B is a small protein with an elevated proportion of hydrophobic amino acids (approximately 40%) that adopts a mainly α-helical secondary structure (30–45%) and has a 8.7 kDa molecular mass [19,40,41]. SP-B belongs to the saposine-like family of proteins, all of them containing 6 cysteines in strictly conserved positions that establish 3 intramolecular disulphide bonds [42]. In addition, SP-B has a seventh cysteine that participates in an intermolecular disulphide bond to form a covalent homodimer. SP-B has a net positive charge that enhances its interaction with anionic phospholipids, such as PG [29]. The protein is orientated parallel to the membrane surface, establishing hydrophobic interactions between the amphipathic helical segments and the membrane surface (Fig. 2) [43]. This superficial disposition seems to be very important for the protein to promote the interconnection of membranes [44] through supradimeric oligomerisation of the protein [42,45]. Moreover, SP-B induces permeability and aggregation of phospholipid membranes [46–50]. These two processes could be essential for surface activity of surfactant, particularly regarding the ability of the protein to enhance interfacial adsorption of phospholipids. Through the connections between membranes, SP-B might facilitate refinement of the interfacial film during compression and the efficient re-extension of the material from the reservoirs during expansion (see Section 2.3). In addition, these interconnected membranes may form a continuous network that might be highly conductive for hydrophobic molecules, such as oxygen, facilitating oxygen diffusion through the lung epithelium [51].

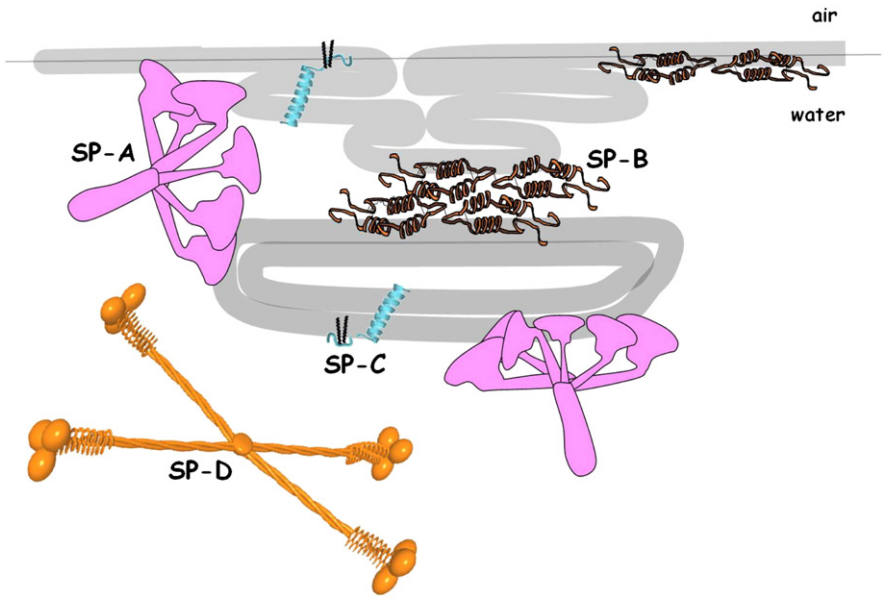

Fig. 2. Structural models of surfactant proteins and their interaction with surfactant phospholipid layers. Schematized are a structural model of octadecameric SP-A, a structural model of SP-B dimer, the resolved structure in organic solvent of SP-C [52], and a structural model of a dodecameric SP-D (modified from [149]) in lipid layers (grey bands represent monolayer/leaflets).

SP-C is another small (3.7 kDa) hydrophobic peptide with a primarily α-helical secondary structure. The N-terminal segment of SP-C has a positive net charge without a defined secondary structure and includes two palmitoylated cysteine residues; the C–terminal region is enriched in branched aliphatic residues, such as valine, forming a highly hydrophobic α-helix [52]. This helix has a transmembrane orientation and a ~70° tilt relative to the plane of the membrane [53]. Both palmitic chains play an essential role, anchoring the N-terminal segment to the membrane (Fig. 2). Consequently, non-palmitoylated versions of the protein are excluded from the interface more easily during compression of the interfacial film than the acylated forms [54,55]. The positive net charge of SP-C allows it to establish preferential interactions with anionic phospholipids [29], and the protein seems to exhibit a special behaviour in the presence of cholesterol. SP-C has an apparent protective role for surfactant in the presence of cholesterol, an activity that requires palmitoylation of the protein [56,57]. Such effect could indicate that a specific SP-C/cholesterol interaction could exist, involving the palmitoylated segment of the protein; however, this feature has not been proven.

1.1.2.2. SP-A and SP-D. These hydrophilic proteins are components of the innate immune defence system, in charge of modulating the inflammatory response while removing pathogens from the epithelial surfaces [58]. SP-A and SP-D belong to the collectin family of proteins [59]. These proteins are responsible for recognising and opsonising microorganisms, presenting them to immune cells, such as alveolar macrophages, to enhance microbial clearance [60]. They also present intrinsic antimicrobial activity in the absence of immune cells [61]. SP-A and SP-D bind numerous types of microorganisms, including viruses, bacteria, and fungi. SP-A binds lipopolysaccharides preferentially from Gram-negative bacteria, while SP-D binds also to peptidoglycans and lipoteichoic acid [62]. The structure of these proteins is optimised for their function. They have globular domains able to recognise and bind carbohydrates (CRDs, carbohydrate recognition domains) at the pathogen surface. Moreover, SP-A can bind DPPC through these domains [63]. The interaction between SP-A and the phospholipids via the CDR domain might be critical to the formation of tubular myelin [58], which is a regular network of membranes extended by the surfactant at the airways. In addition, by binding to phospholipids through this domain, it also participates in recycling and clearance of surfactant by type II cells and macrophages. As a matter of fact, SP-D is essential for regulating the pool of surfactant and surfactant homeostasis [64]. The neck and collagen domains of these collectins are critical for stabilising their oligomeric forms. Finally, the N-terminal sequences seem to be critical not only for stabilisation of oligomers but also for interaction with phospholipids and the formation of tubular myelin [65].

1.1.3. Surfactant membrane structure

1.1.3.1. Lipid phases/membrane domains. As described above, pulmonary surfactant phospholipid composition has evolved to contain approximately 50% saturated and 50% unsaturated species; special remodelling pathways in pneumocytes provide this unusual molecular composition relative to the typical phospholipid composition in any other cell membrane [66]. The coexistence of saturated and unsaturated phospholipids in surfactant responds to the simultaneous requirement of a high stability for surfactant layers at high compression rates, particularly well sustained by saturated molecules, such as DPPC, and enough fluidity and dynamics, which are facilitated by unsaturated species to permit structural transformations associated with the transference of surface active molecules from cells to the air–liquid interface.

When assembled in bilayers, phospholipids undergo a thermotropic transition between an ordered state at low temperature, typically called the *gel* (L_α) phase, and a much more disordered state at higher temperatures, the *liquid-crystalline* (L_α) phase. In the gel phase, molecules have little rotational or translational mobility and adopt a quasi-crystalline order with homogeneous well-defined intermolecular distances and extensive packing of their acyl chains. At temperatures above a certain threshold, the melting temperature, or Tm, phospholipid bilayers transition from the gel phase to a liquid-crystalline phase, gaining considerable mobility and losing their short-range organisation as well as exhibiting a high number of gauche configurations at the C – C bonds of the acyl chains. The Tm of a given phospholipid depends on the number of double bonds and length of its acyl chains, with long, fully saturated chains undergoing ordered-to-disordered transitions at much higher temperatures than shorter or unsaturated chains. Bilayers made of pure DPPC, the main surface active species in surfactant, melt from ordered to disordered phase at 41 °C, while POPC, one of the most abundant unsaturated species, melts at −2 °C. Consequently, membranes made of surfactant phospholipids exhibit a coexistence of ordered and disordered phases at a wide range of temperatures, including physiological values [45,67,68]. However, cholesterol modulates the organisation and dynamics of these phases. The rigid sterol molecules intercalate between phospholipid molecules, enhancing molecular mobility below Tm and restricting the extent of the disorder by limiting the number of gauche conformers of the acyl chains above the melting temperature. Therefore, below the Tm, cholesterol converts the gel phase into a so-called *liquid-ordered* (L_o) phase, which is ordered but fluid due to the considerable mobility gained by individual molecules. This L_o phase transforms into a *liquid-disordered* (L_d) phase at temperatures above the Tm. Recent studies have confirmed that pulmonary surfactant membranes exhibit a coexistence of ordered and disordered fluid phases at various temperatures (see Fig. 3). The lateral structure of surfactant membranes, as revealed by confocal fluorescence of giant liposomes [67,68] or by X-ray scattering of non-labelled surfactant suspensions [45], consist of L_d domains interspersed in a DPPC-enriched cholesterol-containing matrix of L_o-like phase. Surfactant proteins SP-B and SP-C partition exclusively into L_d domains, contributing to their highly dynamic character, frequently associated with protrusions and deformations that may precede three-dimensional structural re-organisations [68,69]. Removing cholesterol from surfactant membranes converts the coexistence of fluid Lo/Ld phases into a complex lateral pattern presumably composed of a solid gel-like ordered phase segregated from a disordered phase. The mobility of lipids in surfactant membranes upon cholesterol removal decreases strongly [67]; functional impact of this effect has not been fully assessed, even though most clinical surfactant preparations are fully depleted of cholesterol [70].

Once transferred into the air–liquid interface, lipid films also organise into ordered and disordered phases, depending on lipid composition, temperature and compression state. If the interfacial film contains enough disaturated phospholipid and is compressed at a temperature below its Tm, the area reduction promotes the formation of condensed domains in which the individual phospholipids organise analogously to the gel phase in bilayers with a high extent of packing and a quasi-crystalline two-dimensional organisation. Domains of the so-called *liquid-condensed* (L_c) phase are therefore formed during compression. At maximal compression, a two-dimensional solid film may form. Surfactant interfacial films with saturated and unsaturated phospholipids segregate upon compression L_c domains floating in a liquid-expanded (L_e) phase equivalent to the L_α phase of bilayers. If the interfacial film contains cholesterol, the L_c/L_e coexistence converts into a coexistence of L_o/L_d fluid phases. Films formed by the full lipid fraction of surfactant segregate small round and presumably fluid condensed domains during compression [71,72]. These domains are enriched in DPPC [73] but likely contain other saturated phospholipids and cholesterol and are partially remixed at high compression rates, a behaviour that depends on the presence of cholesterol [74–76].

1.1.3.2. Lamellar and non-lamellar lipid phases. Surfactant lipid complexes have been traditionally considered to act through the formation of a single surface active monolayer at the interface. Later, it was proposed that the interfacial monolayer is interconnected with multilayered

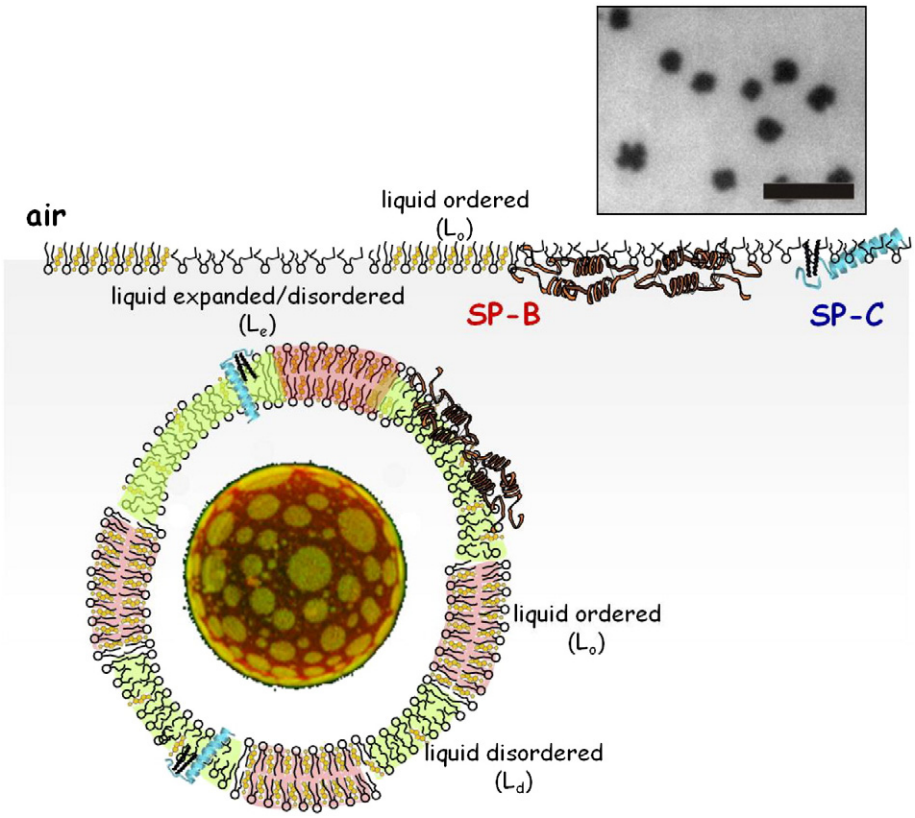

Fig. 3. Phases and lateral structures in pulmonary surfactant membranes and films. Pulmonary surfactant membranes exhibit coexistence of a DPPC-enriched cholesterol-containing liquid-ordered phase (L_o) and a liquid-disordered (L_d) phase in which surfactant proteins SP-B and SP-C selectively partition. Represented is an interpretative cartoon and a picture of a fluorescently labelled giant unilamellar vesicle (GUV) made of pulmonary surfactant (red, $diIC_{18}$; green, BODIPY-PC; diameter of the GUV, 20 μm). Interfacial films of surfactant have been shown to segregate under compression excluding DPPC-enriched ordered domains from a fluorescent (NBD-PC labelled) liquid-expanded (L_e) phase. Shown are an explicative cartoon and an epifluorescence picture (scale bar is 100 μm). Proteins SP-B and SP-C are presented as selectively interacting with disordered phases, both in bilayers and monolayers.

structures at the subphase. Current models propose that multilayers serving as reservoirs for interfacial phospholipids are interconnected through the surfactant proteins, even forming porous structures [77] or non-lamellar phases [78]. These interconnections could be important for facilitating rapid diffusion of surface active lipid species toward the interface. Porous structures also facilitate rapid diffusion of polar molecules (ions, defence proteins and peptides) through surfactant membranes [47,48], leading to the question of whether surfactant actually assembles a sort of bicontinuous phase that could facilitate a rapid and efficient diffusion of both polar and non-polar molecules along the entire liquid layer lining the respiratory epithelium [13]. The interconnected pored bilayers could contain truly non-lamellar phases, the existence of which remains to be fully explored. In this sense, it is remarkable that lipids that facilitate the formation of inverted hexagonal H_{II} phase promote surface activity [79] and that surfactant proteins SP-B and SP-C promote the formation of cubic phases in model lipid/protein systems [78,80,81]. The increasing evidence of porous structure formation by surfactant proteins lead us to believe that protein–lipid complexes of surfactant are far more complex than expected and that these poring structures may play an important role regarding surfactant function and oxygen diffusion [51].

1.1.4. Surfactant biology

During embryonic development, the lungs are the last organs to develop. At week 35 of gestation, the lungs are ready to breathe, and alveolar epithelial type II (ATII) cells have already synthesised surfactant. The lung epithelium is mainly composed of two epithelial cells, type I (ATI) and ATII. ATI cells are large cells, covering approximately 90% of the alveolar surface. The basal membrane of these cells is in close contact with endothelial cells from the capillary; therefore, through the ATI cell, gas exchange occurs from alveolar spaces to the lumen of capillaries. ATII cells are in charge of synthesising, secreting and recycling lung surfactant [82–84], making up 60% of alveolar epithelial cells. Both lipids and surfactant proteins (SP-A, B and C) are synthesised and processed in these cells.

The ATII cells are enriched in endoplasmic reticulum (ER), where surfactant lipids and proteins are synthesised [85]. Later, assembly of lipids and proteins results in a tightly packed structure that is stored in lamellar bodies, which are organelles that ultimately secrete surfactant into the alveolar spaces [86]. Once secreted, surfactant maintains a highly packed state [87,88] that partially reorganises to adopt a highly organised network known as tubular myelin [89]. Finally, phospholipids are transferred with assistance from hydrophobic proteins to occupy the entire alveolar air–liquid interface. At the interface, a phospholipid monolayer enriched in DPPC is interconnected with the rest of surfactant complexes that serve as reservoir (multilayers and multilamellar arrays) in the subphase. Hydrophobic surfactant proteins might mediate these interconnections, enhancing mechanical stability of lung surfactant at the interface [77].

The synthesis and transport mechanisms of surfactant lipids from the endoplasmic reticulum to lamellar bodies are not entirely understood. The major phospholipid in surfactant, PC, seems to be synthesised by the CDP:choline pathway that includes CTP:cholinephosphate cytidylyltransferase and cholinephosphotransferase [90]. Considering that vesicle-mediated transport plays a key role in the processing of hydrophobic surfactant proteins, this pathway may be common to lipid transport. SP-B and SP-C are also synthesised in the endoplasmic reticulum, reaching multivesicular bodies (MVB) through the Golgi apparatus. Before entering lamellar bodies (LB), they are transferred through different endosomal compartments as composite bodies (CB) [86]. However, the most recent hypothesis involving the surfactant specific

phospholipid transporter ABCA3 proposes that DPPC and PG are directly transferred from the ER to LB through a vesicle independent mechanism. This hypothesis is supported by localisation of ABCA3 at the limiting membrane of LB [91]. Phospholipid transport between membranes that remains independent of vesicles would be mediated by transport proteins and lipid diffusion within contact points between membranes. The latter mechanism requires participation of proteins that should be able to establish temporary contact with LB [66]. The ABCA3 phospholipid transporter appears to be essential because its absence leads to mortality, similar to the absence of SP-B [92,93]. ABCA3 belongs to the family of ABC transporters, which bind and hydrolyse ATP coupled to the transport of different molecules, such as phospholipids, through membranes. ABCA3 is exclusively found in ATII cells, and its expression is regulated by corticoids, being up-regulated immediately before birth [82,84,94].

The processing and maturation of hydrophobic surfactant proteins is coupled to synthesis and assembly of surfactant phospholipids. Both SP-B and SP-C are proteins synthesised as large precursors that suffer multiple proteolytic cleavage steps before reaching their mature forms (Fig. 4). The two proteins follow a parallel pathway of endosomal processing from the ER to the LB, possibly sharing processing enzymes. Although it has not been directly established, several evidences seem to indicate a coordinated processing mechanism for both proteins. The absence of SP-B alters processing and sorting of SP-C, reflecting this connection between processing and trafficking of both proteins in ATII cells [95]. In addition, genes from both proteins are regulated by the same transcription factor: TTF-1 (thyroid transcription factor 1). TTF-1 is a phosphorylated nuclear transcription factor that is essential for differentiating the lung epithelium during development. TTF-1 binds numerous factors that regulate transcription of genes, including those coding for surfactant proteins SP-A, SP-B and SP-C (*SFTPA*, *SFTPB* and *SFTPC*) [96]. TTF-1 deletion is lethal in animal models, and differential phosphorylation states allow researchers to study its involvement in lung and surfactant development [97]. Fig. 4 summarises the proteolytic cleavages and intracellular compartments involved in processing of SP-B and SP-C [98–103]. Both proteins are synthesised as much larger precursors (proSP-B and proSP-C) with N- and C-flanking domains. Although little is known about the function of these propeptide segments, they may possess a chaperone function while forming part of the precursor, facilitating proper folding of the protein and protecting the highly hydrophobic mature domains from exposure to the polar cellular environment. The C-terminal domain from proSP-C contains a BRICHOS structural domain [104] and has been related to the proper folding of the precursor protein [101]. On the other hand, the N-terminal and C-terminal propeptides of proSP-B contains saposin-like domains and may perform an antibacterial function due to their similarity to proteins with citolytic and antibacterial properties, such as amebapores, granulysin or NK lysine [42,105].

2. Surfactant activity

2.1. Surface tension

Surface tension (γ) of a liquid is the energy needed to increase its surface in a unit of area and is therefore expressed in energy per unit of area or length (J/m^2 or mN/m). Therefore, γ quantifies the energy needed to overcome the forces that minimise the area exposed to a medium other than the liquid. Surface tension is generated by the attractive forces between liquid molecules. Inside the liquid bulk phase, attractive forces act in all directions and are compensated for between molecules. However, at the interface, these attractive forces are not fully compensated, producing a net attractive force toward the interior of the liquid. For the lung epithelium, which is covered by a thin layer of water, these forces play an important role in mechanical and structural stabilisation of the lungs during the breathing process. If these forces are not minimised during exhalation, the smallest alveoli would be prone to collapse because surface tension would minimise the area exposed to the air, reducing the area available for gas exchange [106,107]. Evolution has developed a surfactant that minimises these surface forces, particularly phospholipid components placed at the interface, displacing water molecules away from the interface to compensate for the attractive forces and therefore reducing surface tension. When the interface is fully occupied by phospholipid molecules, an equilibrium surface tension (γ_{eq}) is reached. Adding more phospholipid molecules does not further decrease surface tension because adsorption to and desorption from the interface would compensate for one another,

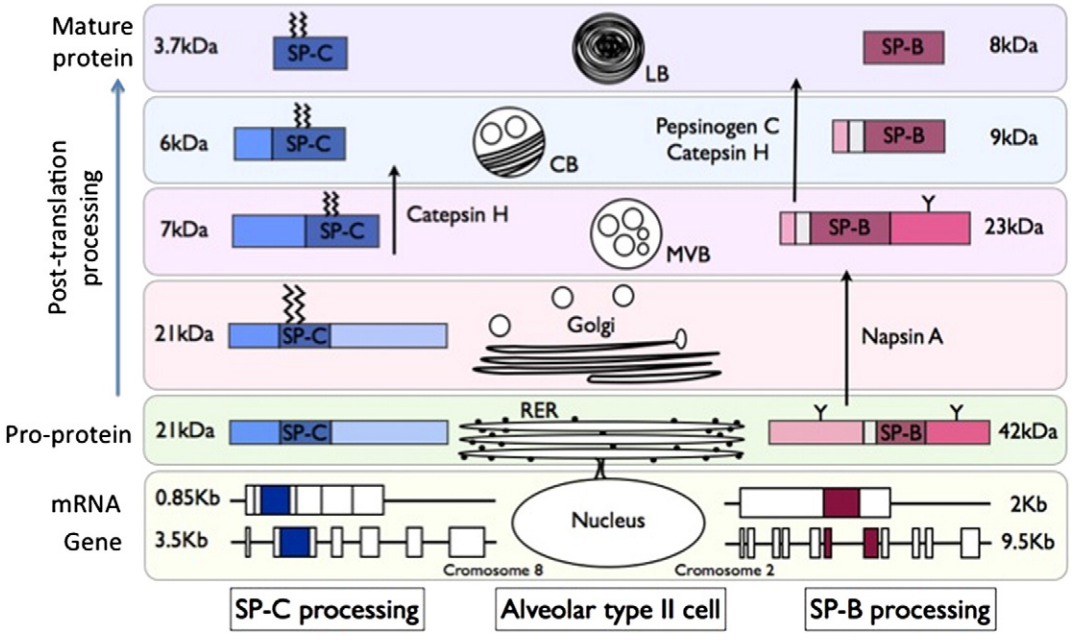

Fig. 4. Processing pathways of SP-B and SP-C in ATII cells. SP-B is synthesised as a 40–42 kDa precursor that is cleaved by Napsin A. The resulting intermediate of 23–26 kDa reaches multivesicular bodies (MVB) through the Golgi. Two enzymes, Cathepsin H and Pepsinogen C seem to be involved in the final processing of SP-B which takes place in intermediate composite bodies (CB) to LB [253]. On the other hand, SP-C is also synthesised in the ER as a 21 kDa precursor that is palmitoylated in the Golgi apparatus [103]. Unknown enzymes cleave the C-terminal domain and later, probably Cathepsin H cleaves the N-terminal domain resulting in the mature protein at the LB [99]. Figure modified from [98,99,102,103,180].

equilibrating the system. However, the area occupied by the alveolar surface varies continuously while it is compressed during exhalation and expanded during inhalation according to breathing cycles. To avoid alveolar collapse, surfactant reduces surface tension below equilibrium to a minimal value (γ_{min}) during compression due to its specific phospholipid composition, which is enriched in DPPC, allowing higher packing of its disaturated acyl chains.

2.2. Adsorption at the interface

Pulmonary surfactant should show a fast interfacial adsorption while equilibrating surface tension to stabilise the lungs. During this process, the surfactant proteo-lipid complexes reach and spread into the interface from the sub-phase, forming a surface active interfacial film. The interfacial adsorption process includes both arrival and accumulation of material near the interface, and the ultimate transfer processes that insert the molecules into the interface, forming a layer exposed to air (see Fig. 5). The interfacial adsorption of lipids is not energetically favourable because transfer of phospholipids implies a transient exposure of lipid acyl chains to the aqueous environment, making the process slow and inefficient. Hydrophobic proteins, such as SP-B and SP-C, are essential for lowering the energetic barrier for transferring material to the interface, likely protecting and stabilising the intermediates involved, making this process fast (on the scale of seconds). Surfactant proteins would therefore act as true *catalysts*, reducing the energy required to reach the "transition state" during interfacial transfer. Typically, surfactant reduces the surface tension from approximately 70 mN/m (the surface tension of the aqueous sub-phase) to 22–23 mN/m, which is the equilibrium surface tension of surfactant, in 2–3 s. This is essential to allow the opening and mechanical stabilisation of the lungs of a newborn baby.

2.3. Compression–expansion of the interfacial film

Surfactant interfacial films are constantly subjected to compression and expansion of the alveolar surface. To stabilise open alveoli, surfactant should maintain a low surface tension during successive breathing cycles. Therefore, surfactant should pack the interfacial film efficiently during compression, re-extending and re-adsorbing the interfacial film during expansion (Fig. 6).

During exhalation, the lungs lose approximately 10% of their volume, mainly translated into volume changes in the upper airways. Surfactant reduces surface tension according to the alveolar size (Laplace Law), so that volume changes are minimized at alveolar airspaces [108]. Toward this purpose, the interfacial film is laterally compacted; this process is optimised due to the presence of enough disaturated phospholipids, such as DPPC, which is able to sustain very tight packing of their saturated acyl chains. During compression, the interfacial film is thought to be further enriched in DPPC due to the exclusion of an important fraction of unsaturated phospholipids and neutral lipids to multi-layered complexes and the reservoir at the sub-phase; this process has been called the "squeeze-out" process [109]. The lower stability of the film regions that are rich in unsaturated species would facilitate this depuration once the highest lateral pressures are reached. This process may be facilitated by formation of pore-like structures or fusion/adsorption intermediates, enhancing exclusion of unsaturated phospholipids from the interfacial film [77]. The hydrophobic SP-B and SP-C proteins would take part of these structures because they are essential for minimising surface tension during compression [45,110].

Similarly, an efficient re-extension of the interfacial film upon expansion would be essential for maintaining equilibrium surface tension (22–23 mN/m) during inhalation. This re-adsorption process involves transfer of protein–lipid complexes from the interconnected material at the reservoirs back into the interface. This process is also enhanced by hydrophobic proteins, most likely through the same fusion/adsorption connecting structures already formed during compression [77] and is important for minimising the energy required to expand the alveolar interface and stabilise the lung epithelium. Good operative surfactant films exhibit reduced or no hysteresis and very limited area compression (<20%) while reaching minimum surface tension (2–3 mN/m).

3. Surfactant inactivation

Low surface tension contributes to alveolar stability according to Laplace's Law. Laplace's equation ($\Delta P = 2\gamma/R$, where P is closing pressure of an ideal spherical chamber, γ is surface tension and R is radius) establishes that two interconnected alveoli with different radii and same surface tension cannot coexist at a given pressure. The pressure in the smaller alveolus would cause it to collapse into the larger one. By variably decreasing surface tension as a function of alveolar size, surfactant plays a major role in stabilising the lungs [111]. Accordingly, loss of surface activity leading to increased alveolar surface tension is assumed to cause alveolar instability and atelectasis. Therefore, increasing surface tension may result in a marked decrease in lung compliance [112].

Surfactant inactivation refers to all processes that interfere with and decrease surface activity of surfactant. Because surface activity of surfactant is essential for breathing, surfactant inactivation is life-threatening. To date, surfactant inactivation has been tied to various respiratory diseases with different origins and outcomes. Surfactant inactivation may not be the reason for nor the consequence of the impaired lung function associated with respiratory diseases, but an increasing body of evidence

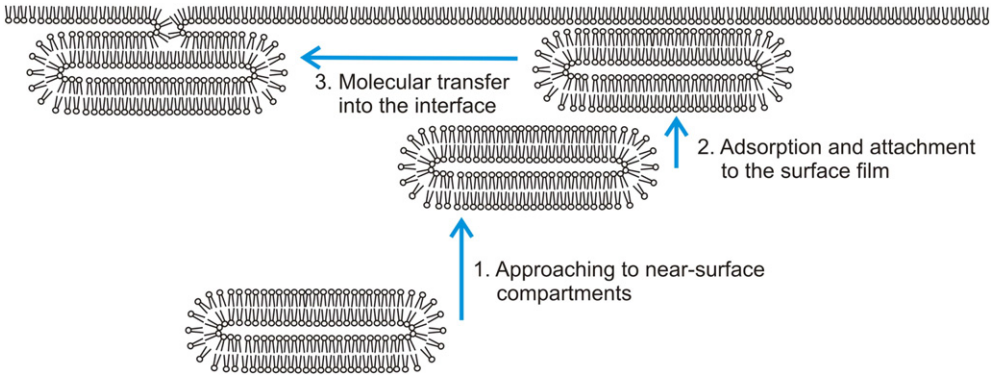

Fig. 5. Steps in interfacial adsorption of pulmonary surfactant. Adsorption of pulmonary surfactant to form surface active films at the air–water interface includes 3 main steps: 1) rapid and massive movement of large multilamellar arrays close to the interface compartment, 2) attachment and association of surfactant membranes to pre-existing films, likely promoted by protein–lipid and protein–protein interactions, and 3) Ultimate transfer of surface active lipid molecules into the air-exposed monolayer, possibly through non-bilayer intermediate structures in which surfactant proteins SP-B and SP-C likely participate.

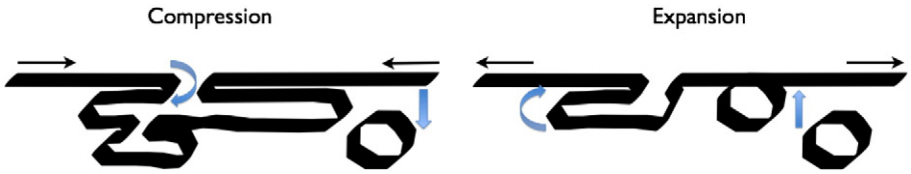

Fig. 6. Surfactant interfacial films during compression and expansion of the interface. Left: compression of the interfacial film produces a selective exclusion of mainly unsaturated phospholipids and cholesterol out of the monolayer to the interconnected reservoir by a process known as "squeeze-out". Right: expansion of the interface with re-adsorption of material from the reservoir into the interfacial monolayer. Both processes are facilitated by hydrophobic surfactant proteins [66].

indicates that surfactant dysfunction contributes to the development and outcome of these diseases [17,112–120].

A summary of the molecular mechanisms and sources of surfactant inactivation is presented in Fig. 7. Surfactant inactivation may be caused by exogenous agents, including inactivating agents that are not normally present in alveolar spaces but interfere with the surfactant after reaching the alveolar interface. This phenomenon occurs when surface-active components from serum reach the interface through a leaky epithelial–vascular membrane, as in the case of lung injury. Alternatively, direct aspiration would introduce external agents directly into alveolar spaces, making contact with surfactant complexes. For example, aspiration of meconium occurs in 2% of deliveries when meconium stains the amniotic liquid. Meconium is then mixed with surfactant at the interface, rendering it dysfunctional. Membrane-perturbing molecules, such as cholesterol, may perturb surfactant membrane properties and convert them into non-functional structures. However, the origin of surfactant inactivation can also be endogenous; ATII cells that are in charge of synthesis, assembly and storage of surfactant complexes may function abnormally. Whether there is a genetic disorder associated with mutations at genes related to surfactant, particularly those essential for surfactant function (such as *SFTPB*, *SFTPC*, *ABCA3*, or *NKX2.1*), or an impairment of surfactant metabolism by ATII cells, the result changes surfactant composition.

3.1. Exogenous inactivation

Exogenous inactivation may occur when inactivating substances reach alveolar spaces. Inactivating substances may arise from inflammation, leakage from vascular spaces and direct aspiration. Inflammation processes directly associated with lung injury serve as a mechanism for surfactant inactivation: 1) by damaging the available alveolar surfactant due to the liberation of proteases, lipases and/or free radicals; 2) by damaging ATII cells required for new surfactant synthesis, recycling, and release; and 3) by damaging the alveolar–capillary permeability barrier, leading to leakage of inactivating agents from the serum into the alveolar surface and air–liquid interface [121]. In addition, direct aspiration of exogenous substances may also impair surfactant function due to the action of inactivating agents on surfactant surface activity or membrane structure. Traditionally, two major molecular mechanisms are invoked for surfactant exogenous inactivation: i) those that are primarily related to surface-active molecules that compete with surfactant for the interface and ii) those originated by membrane-perturbing molecules that insert into and disrupt the structure of surfactant [34].

3.1.1. Surface-active molecules

Under pathological conditions, surfactant may encounter other surface-active molecules competing to reach and stably associate with the interface [122–125]. For example, albumin is one of the most abundant proteins in serum and is a surface-active protein that is able to reduce surface tension down to equilibrium values of 45–50 mN/m [126–128]. For albumin, similar to the case of detergents, compressing the interface does not decrease surface tension further. These molecules establish an equilibrium in which albumin molecules are adsorbed and desorbed to and from the interface during compression. As shown in Fig. 8, under normal conditions, surfactant can reach the interface within a few seconds, but if the interface is already occupied by an excess of other surface-active molecules, surfactant complexes cannot overcome the steric and electrostatic barrier imposed by proteins at the interface and do not form the highly surface-active film [123–125]. Therefore, in the presence of serum or albumin, the interfacial adsorption of surfactant is impaired, generating an abnormally high surface tension [125,129,130].

Acute Respiratory Distress Syndrome (ARDS) is a respiratory pathology where surface-active molecules reach the interface. ARDS is characterised by respiratory failure with many different origins, always related with severe lung inflammation [131]. The mortality associated with this syndrome is 36–44% [132]. Due to inflammation in the lung, the underlying alveolar and vascular injury leads to impaired vascular permeability, causing leakage of serum and plasmatic proteins from the blood capillaries into alveolar spaces. In addition to albumin, other

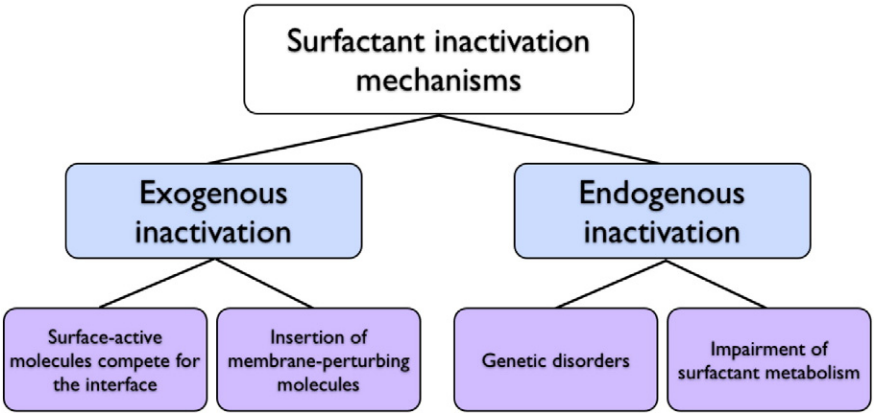

Fig. 7. Scheme of the different sources and molecular mechanisms of inactivation of surfactant under pathological conditions. Sources of inactivation (in blue) include exogenous agents (when inactivating substances reach the alveolar spaces) or endogenous mechanisms (abnormal function of ATII cells); potential molecular mechanisms of inactivation are summarized in the purple boxes.

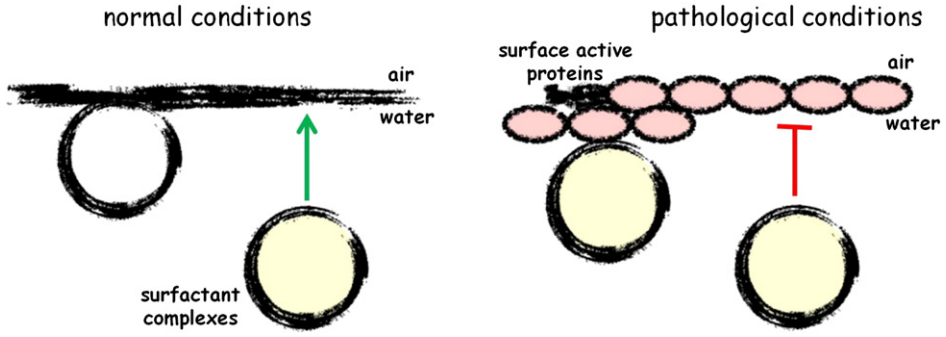

Fig. 8. Inactivation of surfactant complexes through competition for the interface with surface-active molecules. Under normal conditions, surfactant adsorption at the interface is very fast, but under pathological conditions when surface-active molecules reach the interface, surfactant is not able to overcome the steric barrier and does not reach the interface. Modified from [34].

proteins, such as fibrinogen or immunoglobulins, are strong inhibitory agents against surfactant [133–135]. The presence of surfactant producing abnormally high surface tension has already been described in patients suffering from this disease [136–139]. If the alteration in surfactant activity somehow contributes to development and outcome of ARDS, surfactant replacement therapy (SRT) should mitigate the effects of such abnormally high surface tensions [140]. To date, clinical trials have demonstrated some beneficial effects of SRT but have failed to improve patients' survival [141–143]. The problem is that clinical surfactants applied during SRT may undergo the same inactivating processes affecting endogenous surfactant, resulting in impaired function. Therefore, development of improved inactivation-resistant clinical surfactants is a crucial step.

However, it is probable that clinical research will have to solve two major problems before SRT would be a good option to ameliorate ARDS. A first problem is the poorly defined nature of ARDS itself, which makes the group of ARDS patients too heterogenous. Usually it includes both patients in which an impaired surfactant and an abnormally high surface tension is part of pathogenesis and patients in which surfactant deficiency might be a consequence rather than a cause of the pathology. The suitability of these two different types of patients for SRT could be very different, as well as the potential outcome derived from it. The determination and monitoring of a set of proper functional parameters could aid to better define the group of ARDS patients associated with a primary deficiency of surfactant function, which could be the best candidates to receive SRT [144]. On the other hand, an early intervention to treat patients at risk of severe ARDS might be crucial to have a positive output upon SRT application. Usually, the need for intubation to administer exogenous surfactant prevents approaching early treatments at the primary stages of the pathology. In this sense, the development of new SRT strategies, such as efficient administration of aerosolized surfactants (see below), could open new windows to earlier and more effective treatments, before an extensive surfactant inactivating environment would be established in a fully inflamed lung. A second important strategy will be probably the definition of good molecular markers that could allow an early detection of primary states of surfactant dysfunction and defective pulmonary mechanics. This will also contribute to make possible early SRT, which could restore surfactant activity before an intrinsically unstable respiratory surface will finally end in irreversibly damaged lungs.

3.1.2. Membrane-perturbing molecules

Under pathological conditions, surfactant complexes may also incorporate spurious components from substances reaching alveolar airspaces, such as cholesterol or free fatty acids from serum. In another example, exposure to meconium that occurs during meconium aspiration syndrome (MAS), results in incorporation of cholesterol into surfactant membranes and films [145]. Cholesterol is one of the most studied molecules with respect to membrane and surfactant structure and function [67,68,130,133]. Cholesterol content in surfactant appears to be governed by numerous factors, including diet or body temperature, and small changes in surfactant cholesterol content are associated with modulated surfactant activity [146–148]. This effect is likely related to the ability of cholesterol to modulate biophysical properties, organisation and distribution of segregating lipid phases in surfactant membranes and interfacial films [67,68]. As discussed above, cholesterol is responsible for liquefying the Lβ phases, while it condenses the liquid-crystal Lα phases. Incorporating enough cholesterol into surfactant membranes converts these phases into liquid-ordered L_o state, where phospholipids may retain ordered states, with high packing and low hydration, as occurs in L_β ordered phases, but associated with significantly higher lateral and rotational mobility [33,149–151].

The structural perturbation of lipid phases in surfactant membranes modifies their biophysical activity. Excess cholesterol generates excessively fluid membranes, leading to early collapse of the interfacial films (illustrated in the cartoons of Fig. 9); therefore, low surface tension is not reached under compression–expansion conditions [130,152,153]. In this case, there is no steric barrier against interfacial adsorption, and surfactant complexes rapidly form an interfacial film that lowers surface tension, reaching values near equilibrium surface tension. However, impairing surfactant function during compression–expansion may lead to alveolar collapse and atelectasis, similar to meconium aspiration syndrome [115]. High cholesterol content in surfactant has been related to respiratory diseases, such as MAS [115,145,154], ARDS [133], ventilation induced lung injury (VILI) [155] and idiopathic pulmonary fibrosis (IPF) [156]. All of these alterations have very different origins, yet end with an abnormally high proportion of cholesterol associated with surfactant complexes and associated impairment of surfactant function. Several studies have suggested that the tight modulation of cholesterol levels in surfactant membranes may be one of physiological mechanisms that alters surfactant to fit specific environmental demands, such as strenuous exercise, variable respiratory rates or changing body temperatures [146–148,157,158]. Therefore, exacerbated proportions of cholesterol in surfactant could be a consequence rather than a cause of the impaired respiratory function associated with pathology because incorporating cholesterol into surfactant might be part of the physiological response attempting to ameliorate dysfunctional respiratory mechanics.

Finally, not only can cholesterol interfere with biophysical activity of surfactant but so can other molecules that modify surfactant membrane and interfacial film structures, such as free fatty acids [133,134], lysophospholipids [159–161], or C-Reactive Protein (CRP) [135,162].

3.2. Endogenous inactivation

Abnormal ATII cell function regarding synthesis, assembly and secretion of surfactant is the origin of many lung disorders. Genetic disorders resulting in surfactant protein deficiency are a known origin of lung disease in neonates. Mutations in genes encoding for proteins essential for surfactant biophysical activity have fatal or chronic outcomes. In

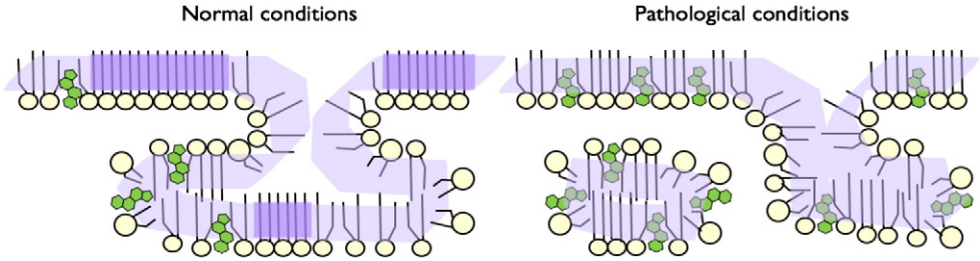

Fig. 9. Inactivation of surfactant by membrane perturbing molecules. Under normal conditions phase segregation of surfactant phospholipids is supposed to maintain the proper fluidity along the compression–expansion cycling. Ordered domains (enriched in DPPC) are represented in a dark purple colour while disordered domains are represented by light purple. Under pathological conditions, when a membrane-perturbing molecule is present, changes in phase segregation as well as an increase in fluidity render surfactant dysfunctional.

addition, alterations in surfactant metabolism leading to a deficiency in or an accumulation of the precursors and mature proteins have also been related to chronic disorders. Moreover, modifications in surfactant lipid composition caused by changes in surfactant metabolism may affect lateral structure and compressibility of membranes and interfacial films as well as surfactant surface activity. ARDS or IPF (Idiopathic Pulmonary Fibrosis) patients show significant alterations in their surfactant lipid compositions, impairing surfactant structure and surface activity [16,17,116].

3.2.1. Genetic disorders

Table 1 summarises the most important surfactant-related proteins, their function and respiratory pathologies that are associated with their deficiency or mutation. This review will mainly discuss the involvement in pathology of proteins relevant to surfactant surface activity, particularly SP-B and SP-C. SP-A and SP-D have an immunomodulatory function; therefore, their absence is correlated with lung infections. ABCA3 and TTF-1 are proteins that remain uninvolved in surfactant surface activity; rather, they participate in proper surfactant synthesis, secretion and metabolism. Therefore, their abnormal function results in impaired surfactant composition or storage within type II cells and will be assessed in a subsequent section.

3.2.1.1. Deficiencies in SP-A and SP-D. Partial or total absence of SP-A and SP-D is associated with impaired innate immunological defence functions in the lungs and exacerbated inflammation rather than affecting surface activity of surfactant. SP-A null mice show impaired clearance of pathogenic microorganisms [22], such as group B *Streptococci* [22,163,164], *Haemophilus influenzae* [64], *Mycoplasma pulmonis* [165], *Pseudomonas aeruginosa* [166] and respiratory syncitial virus (RSV) [164]. In addition, in the absence of SP-A no tubular myelin is observed [65], linking SP-A with surfactant structure. However, because surfactant biophysical function is not significantly altered in SP-A- and tubular myelin-deficient animals, participation of tubular myelin in defence, rather than remaining an intermediate during conversion of secreted lamellar bodies into the functional surface film, becomes a possibility. SP-D null mice are more susceptible to infection by RSV and IAV (Influenza A virus). However these mice exhibit a rather complex phenotype, including increased accumulation of lipids at airspaces, acute pulmonary alveolar proteinosis (PAP) [167], foamy alveolar macrophages and emphysema, contributing to development of chronic lung diseases [24]. Although the mechanisms by which SP-D regulates surfactant homeostasis remain unknown, it seems that SP-D could be involved in the conversion to and the reuptake of small surfactant aggregates [168,169].

3.2.1.2. Deficiencies in SP-B. Deficiencies in SP-B are lethal in babies with mutated *SFTPB* gene [170]. These infants show symptoms of neonatal RDS (respiratory distress syndrome) because they lack a functional surfactant [143,171] but cannot be rescued by applying an exogenous surfactant. Also SP-B knock-out mice die of severe RDS, typically within minutes of birth [26,172]. Even partial SP-B deficiency perturbs the lung function [173,174]. Genetic modifications of the SP-B gene causes SP-B deficiency as well as interrupt SP-B processing pathway, possibly leading to SP-B deficiency and impaired lung function; this situation applies to sporadic IPF, where SP-B deficiency is confirmed by bronchoalveolar lavage (BAL) from patients and the accumulation of unprocessed SP-B in the lung tissue [175,176].

Biophysical studies also reveal the essential role of SP-B during surfactant activity. Animal models with partial SP-B deficiency have an impaired surfactant with an equilibrium surface tension of approximately 50 mN/m and a minimal surface tension upon compression–expansion cycling of approximately 20 mN/m [173]. A closer look at SP-B function was accomplished through functional characterisation of lipid–protein mixtures mimicking SP-B deficient surfactant [110]. Surfactant lipids alone are not efficiently adsorbed at the interface to form surface active films; SP-B is one of the most important proteins involved in the transfer of material to the interface. When SP-B is completely absent, surfactant lipids show equilibrium surface tension above 30 mN/m and slow adsorption kinetics during expansion [110]. SP-B also enhances the efficiency of the process by decreasing the required area reduction and therefore the energy required to reach a low enough surface tension during compression. However, the impaired adsorption during expansion associated with inability to maintain equilibrium surface tension suggests that lack of SP-B also is associated with interfacial film instability. In fact, the stability of the compressed surfactant films, as tested in the CBS, demonstrated the significantly impaired ability of SP-B-deficient mixtures to maintain low surface tension in compressed films subjected to mechanical perturbations [110].

3.2.1.3. Deficiencies in SP-C. Mutations in the *SFTPC* gene are related to interstitial lung diseases in neonates, children and adults. Although

Table 1
Summary of surfactant-related proteins, their function and associated respiratory pathologies.

Protein	Function	Respiratory pathology associated to deficiency or mutation
SP-A	Pathogen binding and surfactant homeostasis [239]	Lung infections [22,163,164,167]
SP-B	Fast interfacial adsorption and interfacial film stability [13,110,240]	Neonatal respiratory distress syndrome (NRDS) [26,170,172]
SP-C	Interfacial film stability [32,151,241]	Chronic pathologies such as idiopathic pulmonary fibrosis (IPF) [175,176,182] and interstitial lung disease (ILD) [242–245]
SP-D	Pathogen binding and surfactant homeostasis [64]	Lung infections [246,247], chronic obstruction pulmonary disease (COPD) [24] and emphysema [23]
ABCA3	Lipid transporter, biogenesis of lamellar bodies [82,84,248,249]	NRDS [92,93] and ILD [186]
TTF-1	Transcription factor, development of the lung [96,250–252]	ILD [190] and development disorders in the lung [97,189]

proper SP-C function does not seem to be critical when breathing is initiated, biophysical studies indicate that SP-C could have a subtle role in modulating surfactant behaviour at the interface, helping to stabilise the lungs over the long term. Mice with a complete SP-C deficiency have an impaired surfactant that shows an equilibrium surface tension of approximately 35 mN/m and normal minimal surface tension upon compression–expansion cycling of approximately 2–3 mN/m [177]. In contrast, ablation of SP-C expression in another mice model ends in severe chronic respiratory failure [178], indicating that SP-C activity may be vulnerable to changes in the genetic background and environment, as is the case for patients suffering from SP-C deficiency [25]. A closer look at SP-C function was obtained using lipid–protein mixtures mimicking SP-C deficient surfactant [110]. When SP-C is completely absent, surfactant lipids show an equilibrium surface tension of approximately 25 mN/m with somehow slower adsorption kinetics during initial and post-expansion adsorption [110]; no effect is observed in compression–expansion dynamics because the presence of SP-B enables surfactant films to reach minimal surface tensions.

In vivo, the cause of SP-C-related pathologies frequently relies more on the accumulation of unprocessed proSP-C rather than the absence of mature SP-C [25]. For example, the unprocessed forms of SP-C with an extended N-terminus in neonatal RDS have impaired biophysical functions [179]. Phospholipids in the presence of these aberrant forms cannot lower surface tension during initial adsorption, producing equilibrium surface tension above 50 mN/m. During repetitive compression–expansion cycling, films containing these proteins showed an intermediate behaviour between phospholipids alone and phospholipids with mature SP-C [179]. Moreover, the accumulation of aberrant proSP-C forms within alveolar type II cells activates the unfolded protein response (UPR), possibly leading to ER stress and ATII cell injury. Many studies have associated ILD in new-born babies with mutations of *SFTPC* that lead to truncated or unprocessed proSP-C forms, following the same cellular damage pattern as observed in conformational disease-related proteins, such as Alzheimer's amyloid peptide or Huntington's protein huntingtin [180]. Moreover, because SP-C seems to counteract the deleterious effect of cholesterol on surfactant complexes, the production and proper processing of an adequate amount of this protein may also be important when surfactant is exposed to different inactivation agents, such as cholesterol. Specifically, proper palmitoylation of the protein through the processing pathway may be crucial for interaction with cholesterol or cholesterol-enriched membranes in surfactant layers [56].

3.2.2. Impairment of surfactant metabolism

Not only does a deficiency in mature surfactant proteins cause respiratory failure; it also occurs when there are interferences affecting processing pathways, leading to a partial deficiency in mature proteins and accumulation of unfolded precursors, generating similar outcomes. Deficiency in proteases in charge of processing essential surfactant proteins, such as SP-B, generates deficiency in the mature biophysically active protein. Decreased amounts of Napsin A (an aspartyl protease involved in SP-B processing) have already been found in patients suffering from IPF [176]. *NAPSA* null mice show impaired SP-B processing and express reduced amounts of mature SP-C, leading to a pro-apoptotic ER-stress signalling response [181]. Accumulating unfolded proteins in the ER activates the unfolded protein response (UPR), activating the apoptotic signalling pathways. For the ATII cells, these processes may cause type II cell injury and consequently lung injury [182]. In general, a lung injury of any origin affecting ATII cells alters normal cell metabolism, originates the associated partial surfactant protein deficiency and changes in the lipid profile. An ultimate reduction in proper levels of surfactant proteins B and C may lead to abnormal surfactant activity, generating high surface tension. Subsequently, high surface tension may induce mechanical stress in lung epithelium due to the repetitive alveolar collapse and forced reopening [183,184]. This mechanical stress can amplify alveolar cell damage [183] and induce fibrotic processes in the lung [185].

3.2.2.1. Deficiencies in ABCA3. The relevance of ABCA3 was confirmed by inactivating the *ABCA3* gene in mice. These mice were not able to open their lungs after delivery and died shortly after birth [92]. Moreover, lack of extracellular surfactant in these mouse lungs was associated with lack of lamellar bodies in type II cells. Therefore, mutations in the *ABCA3* gene impaired surfactant production rather than altering its function. Some of these mutations in ABCA3 are thus related with severe surfactant deficiency [93] and are tied to ILD [186] in neonates.

3.2.2.2. Deficiencies in TTF-1. As stated before, TTF-1 is the transcriptional factor that induces SP-A, SP-B and SP-C expression. Complete lack of TTF-1 results in death due to impaired lung development [187], including surfactant protein deficiency [188]. Moreover, a reduction in TTF-1 phosphorylation levels changes the regulation of cell differentiation and causes a down-regulation of SP-A, SP-B and SP-C production. In addition, Napsin A and CCSP (Clara cell secretory protein) expression can be also impaired [97,189]. Although TTF-1 mutations are not common, some have been observed in patients with ILD [190] and have been related to deficiency in surfactant proteins.

Changes in the phospholipid profile of surfactant in patients suffering from ARDS or IPF reveal dysfunction of surfactant due to an altered surfactant lipid composition. In this sense, lipid composition of surfactant from the diseased lungs has been extensively studied. In general, ARDS progress lowers the DPPC, PG and PC content, while PI, PE and SM are increased. In addition, marked reductions in the SP-A, SP-B and SP-C content have been detected. Associated with these compositional alterations, ARDS patients show a reduced proportion of the most surface active aggregates (large aggregates, LA) in surfactant; concurrently, LA fraction contains an increased amount of neutral lipids. In fact, increased amounts of cholesterol have also been reported in total surfactant from ARDS patients [133]. As an additional factor, the inflammation-induced increase in secreted phospholipases, such as sPLA2, further alters the surfactant phospholipid profile due to phospholipid hydrolysis. Changes in surfactant activity have already been correlated with sPLA2 activity in children suffering from ARDS [144]. Therefore, in ARDS, in addition to leakage of surface-active molecules competing for the interface, changes in surfactant phospholipid pattern generate an abnormally high surface tension. IPF patients also show altered surfactant compositions, not only regarding the protein content, such as decreased amounts of SP-B and SP-C in BAL, but also changes in the lipid profile, including a decrease in PG coupled with increased PI and SM [16].

4. Surfactant therapy

Due to the complex aetiology of respiratory diseases, administering a fully effective therapy remains a challenge. As stated in Section 3, different molecular mechanisms of surfactant inactivation may exist in different pathologies, and complex pathologies, such as ARDS or ILD, may have multiple inactivating mechanisms simultaneously affecting surfactant activity. Understanding molecular mechanisms behind surfactant inactivation for different respiratory pathologies would help to develop better surfactant preparations.

4.1. Surfactant replacement therapy

Surfactant is still considered to be an orphan drug by the WHO due to the small number of patients requiring it. As stated before, surfactant replacement therapy (SRT) SRT is prophylactically used in premature babies with or at risk for NRDS; no other use is currently allowed. However, surfactant administration can offer several advantages for patients with different respiratory diseases. The main problem in the use of surfactant to treat various lung diseases, particularly those affecting adults who may need larger doses than neonates, is its price and availability. Currently, natural surfactants from animal sources are produced in a few countries at a high cost

per dose. Several companies are attempting to develop new synthetic surfactants based on simple mixtures of phospholipids and recombinant human proteins or synthetic peptides — in principle, designed to mimic functional motifs of the whole proteins. These synthetic surfactants avoid the risk of introducing materials from animal sources into the human body. SP-B is essential for the biophysical function of surfactant, and its absence is life-threatening. To the knowledge of the authors, all attempts to produce recombinant versions of human SP-B have failed. Alternatively, several initiatives have attempted to develop second generation clinical surfactants by combining a lipid mixture with an SP-B-mimicking peptide, such as KL4 [191] or mini-B [192,193]. KL4 is a very simple peptide that supposedly mimics the amphipathic cationic nature of several helical segments of SP-B. Mini-B is a more elaborated SP-B-mimic, including segments from the SP-B N- and C-terminal sequences, cross-linked by disulphides, such as the N- and C-terminal ends of native SP-B [194]. While these peptides have shown some ability to promote surfactant-like behaviour in vitro, they are still unable to reproduce the full activity of native SP-B. However, recombinant human SP-C has been produced in bacteria [194,195] with similar biophysical and physiological properties as those imparted by native SP-C, at least in the absence of cholesterol [56]. Synthetic surfactants containing recombinant versions of human SP-C have been developed and tested in clinical trials. Unfortunately, the effectiveness of these entirely synthetic surfactants against ARDS could not be demonstrated [140,142,195]. Surfactant has proven to be useful in rescue of premature babies with healthy but immature lungs and babies with damaged lungs who receive high doses of clinical surfactant. The situation is very different in an adult with severely damaged lungs, where multiple factors with different origins converge to inactivate both the endogenous and exogenous surfactants. To date, several synthetic clinical surfactants have been developed, and most of them have been tested only in pre-clinical trials and animal models of RDS: Venticute® (Nycomed, Germany) [195,196], Surfaxin® (DiscoveryLabs, USA) [197] or CHF5633® (Chiesi, Italy) [198]. Surfactant therapy for adults with ARDS will only be possible after developing synthetic surfactants with enhanced inhibition resistance that can be produced at a reasonable price.

4.2. Future perspectives

As stated, development of new surfactant preparations not only pursues the production of synthetic preparations with a well-controlled composition in large amounts at a reasonable price but also surfactants with an enhanced resistance toward inactivation, improving its therapeutic efficiency during treatment of pathologies such as ARDS or MAS. However, SRT still requires the patient to be intubated; this very invasive procedure prevents surfactant administration in much earlier stages of respiratory pathologies. The development of better clinical surfactant preparations will also include the exploration of alternative application strategies, opening new opportunities for successful SRT interventions in various lung diseases. Finally, new uses for surfactant have been multiplying. Due to their optimal surface spreading properties, surfactants can be used as drug carriers that target the lung epithelium. Most respiratory pathologies are likely too complex to be solved by applying only surfactant. SRT in ARDS has not been successful in decreasing mortality most likely because, in addition to surfactant inactivation, there is also an overwhelming inflammatory response associated with lung injury from very different origins. Similarly, one cannot expect to treat on-going fibrosis by only restoring low surface tension. Combining a surfactant with anti-inflammatory, antibiotic or antifibrotic drugs may enhance the outcome of treatments for ARDS, ILD or IPF patients.

4.2.1. Inactivation-resistant surfactants

Increasing the resistance of surfactant toward exogenous inactivation may lead to new surfactant preparations, generating improved therapeutic strategies for treating ARDS or MAS; the current therapies involving supplementation with exogenous surfactant are ineffective because the exogenous surfactant becomes inactivated, similar to the endogenous surfactant. Adding polymers, such as hyaluronan (HA) or dextran, has proven to restore surfactant function in the presence of some inactivating agents under specific experimental conditions [123,199–202]. Moreover, these polymers have been tested in vitro against various exogenous inactivating agents, probing their restorative capacity. Therefore, polymers may improve the performance of surfactant complexes against various inhibitory agents through a common mechanism [129].

Adding polymers, such as HA, dextran or polyethylene glycol (PEG), enhance the activity of surfactant in vitro and in vivo [203]. It has been proposed that HA enhances surfactant adsorption through a steric barrier due to the participation of depletion forces. This entropically driven force pushes surfactant complexes toward each other, generating aggregated surfactant complexes, and toward the interface, enhancing adsorption [125] (see Fig. 10). Depletion forces have been used to explain restored surfactant activity in the presence of competing surface-active molecules at the interface. Due to these entropic forces, surfactant may overcome the steric barrier imposed by surface active proteins at the interface [124,203]. However, depletion forces do not explain why adding polymers to surfactant can also restore its activity when inhibition is associated with incorporation of membrane-perturbing agents, such as cholesterol. Recently, we have described that in addition to depletion forces, polymer-induced osmotic stress may also induce a compositional refinement, excluding cholesterol and unsaturated phospholipids out of surfactant complexes [69] (Fig. 10). This process might mimic the compression-driven compositional refinement of surfactant interfacial films, during the "squeeze-out" process [21,109], enriching the mixture in saturated phospholipid species; these species exhibit better surface tension lowering properties during compression. This process might therefore enhance resistance of surfactant toward inactivation by membrane-perturbing molecules, revealing a promising strategy for developing new surfactant preparations for ARDS or MAS.

4.2.2. Surfactant aerosolization/nebulisation

Currently approved SRT procedures involve the instillation of a bolus containing a concentrated surfactant suspension directly into the trachea, most commonly via an endotracheal tube, followed by mechanical ventilation. Intubation is an invasive procedure with some risks, including oesophageal perforation, clinical instability associated with accidental extubation and tracheal tube obstruction. Administration of intratracheal surfactant can be associated with apnoea, transient hypoxia, bradycardia, hypotension and reduced cerebral blood flow. Therefore, a non-invasive approach for effective surfactant administration is desirable. Aerosolisation or nebulisation is a common process used to deliver different drugs into the lungs. Several attempts have been made regarding surfactant nebulisation in animal and human models with interesting results [204–209]. Surfactant aerosolisation for treating ARDS in animal models has generated variable outcomes ranging from no change relative to the liquid suspensions to better survival rates in rats treated with aerosolised surfactants [210]. Even in experimental animal models, surfactant aerosolisation combined with polymers, such as Dextran [211], improved the effect of surfactant, prolonging the therapeutic response. Moreover, some pilot studies with aerosolised synthetic surfactants [197], showed beneficial effects toward preventing RDS in neonates, offering a new strategy for surfactant delivery into the lungs.

To reach distal airways, nebulised particles should be small [212]. Dispersing a concentrated lipid/protein suspension with the high viscosity typical of clinical surfactants [213] is not trivial, and development of nebulisers able to efficiently disperse nanodrops of exogenous surfactant remains a technical challenge [214,215]. Evaluating the extent to which the process of nebulisation/aerosolisation alters the structural

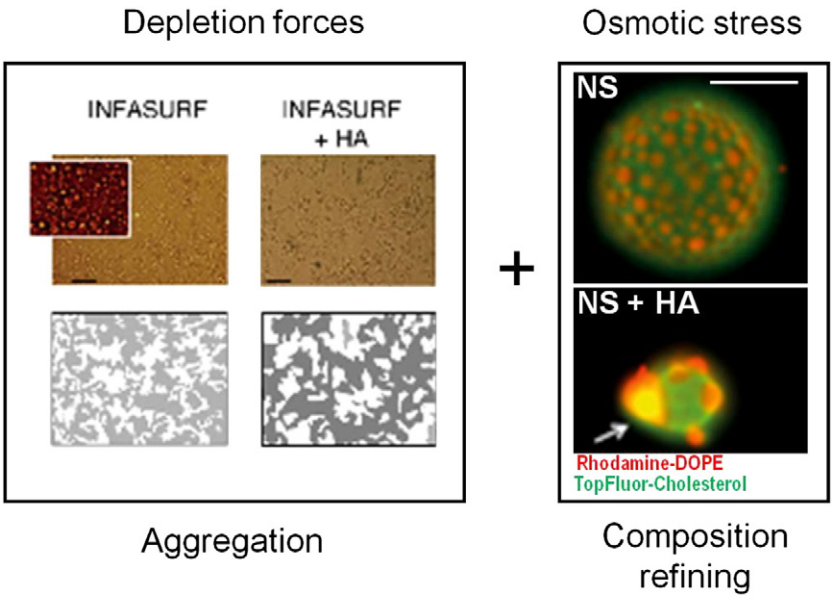

Fig. 10. Effect of polymers on surfactant structural and compositional properties. Depletion forces due to volume exclusion between surfactant complexes and polymers lead to aggregation of surfactant, increasing its local concentration. Moreover, addition of a polymer creates a higher osmotic pressure outside surfactant complexes that are now under osmotic stress, releasing its water content. Osmotic shrinkage of surfactant vesicles leads to exclusion of highly disordered lipid domains (mainly containing unsaturated phospholipids and cholesterol) leading to a refining of composition similar to that occurring during the compression driven "squeeze-out". On the left, the effect of depletion forces is illustrated on the aggregation state of surfactant [254], with addition of hyaluronan (HA) inducing aggregation of surfactant complexes. On the right, the effect of osmotic stress on surfactant composition [69], occurring upon addition of HA to the subphase, and the consequent shrinkage and exclusion of unsaturated phospholipids and cholesterol.

and physical properties of surfactants with respect to their efficient performance at the air–liquid interface is also critical [216].

4.2.3. Pulmonary drug delivery

The spreading properties and inherent therapeutic potential of clinical surfactants could be used to deliver drugs to the lung epithelium. The direct delivery of drugs targeted to airways should increase their local effectiveness while reducing their risk for systemic toxicity [217,218]. In addition, lipid membranous structure of surfactant may help solubilise hydrophobic drugs. For example, surfactant-like mixtures have been used as emulsifiers for lipophilic cancer drugs [219]. Mixing surfactant with an antibiotic (tobramycin) is more effective at protecting mice from death due to respiratory *Klebsiella pneumonia* infection than administering the antibiotic or surfactant alone [220]. The delivery of specific opsonising antibodies can also offer a new strategy for fighting infections. The incorporation of specific immunoglobulin against group B *Streptococci* (GBS) pneumonia into surfactant reduced bacterial proliferation and was more effective than using either the antibody or surfactant alone [221]. Given that the prophylactic administration of surfactant may alleviate an asthma attack [222], it has been speculated that adding bronchodilators to surfactant may improve their effectiveness [217]. In a rabbit model, using surfactant to carry corticosteroids improved delivery significantly with lower systemic side-effects [223]. Moreover, a clinical trial has already demonstrated that surfactant combined with budesonide (glucocorticoid steroid) helps to prevent chronic lung diseases in preterm babies [224]. Therefore, this strategy may be a promising tool for treating lung injury-related diseases.

Genetic lung disorders, such as the ones discussed in Section 3.2.1, cannot be treated with drugs because the genetic background is permanently modified; the possibilities include only lung transplantation or genetic therapy [225,226]. Different attempts have demonstrated that adenoviral gene transfer of SP-A or SP-B cDNA [227,228] restores the genetic deficiency of these proteins in vitro and in vivo, revealing a promising tool for treating inherited diseases. It is remarkable that the combination of adenoviral vectors with surfactant enhances delivery and distribution into the alveolar epithelium [229–231].

Even though many studies have demonstrated advantages of using a surfactant as a drug carrier, little is known about the interactions between different drugs and surfactant complexes, either in the context of the surfactant/drug complexes used as the carrier or with respect to the endogenous surfactant that might impose a barrier, preventing the drug from reaching the lung epithelium. For example, Gommers et al. [232] analysed the incorporation of immunosuppressive drugs, such as cyclosporine A (CsA) or rapamycin (RPM), into surfactant, finding no apparent effects on the surface activity of surfactant with CsA and impaired surface tension reduction with RPM. Therefore, studying the biophysical and structural properties of surfactant/drug combinations case by case is a major priority when attempting to understand and develop surfactant-based strategies for delivering substances into the lungs without interfering with the biophysical activity of endogenous surfactant.

A recent and promising extension of surfactant use as a vehicle for drug delivery is the combination of surfactant and different types of engineered nanoparticles (NPs), potentially providing new and useful properties, such as contrast imaging, hyperthermia generation or targeted drug delivery. NP-based strategies are part of a huge emerging field in nanomedicine and deserve a specific chapter, with special attention to those involving NP/surfactant integration. Again, most of the studies to date have attempted to analyse the impact of direct interaction between surfactant and NPs on the tailored properties of these particles and on functional behaviour of surfactant [233–235]. Recent work has also analysed the effect of interactions of NPs with lung cells, such as macrophages and ATII pneumocytes [236–238]. The pulmonary surfactant layer at the respiratory surface is the first system aspirated NPs meet. Studying the interaction between surfactant and a catalogue of available NPs is not only important when trying to develop new surfactant/NP materials but also when evaluating the potential impact of accidentally inhaled NPs on surfactant function and, by extension, on respiratory biophysics.

In summary, further research regarding the structure–function relationships in surfactant, particularly those focused on the role of different components and structural organisations as well as the molecular mechanisms associated with surfactant inhibition, will help to develop better clinical surfactants, particularly for the challenging conditions

imposed by an injured lung. The development of better clinical surfactant preparations should also include the exploration of alternative administration strategies, opening new opportunities for successful SRT interventions in numerous lung diseases.

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