Biomimetic Materials and Design

Biointerfacial Strategies, Tissue Engineering, and Targeted Drug Delivery

edited by
Angela K. Dillow
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Biomimetic Lung Surfactant Replacements

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I. INTRODUCTION

Pulmonary surfactant, or lung surfactant (LS), is a natural biomaterial that coats the internal surfaces of mammalian lungs and enables normal breathing. It is a complex mixture composed of about 90% lipids and about 10% surfactant proteins (SPs). Both fractions are critical for its physiological function, which is to decrease the work of breathing by regulating surface tension at the air–liquid interface of the alveoli (the network of air sacs that perform gas exchange within the lung) as a function of alveolar surface area (1,2). A deficiency of functional LS in premature infants results in the development of neonatal respiratory distress syndrome (RDS) (3), a leading cause of infant mortality. Two-thirds of infants born preterm are affected by RDS, with 60% of the incidence in infants born before 28 weeks of gestation (4). Left untreated, an infant with RDS will die. This has led to the development of exogenous lung surfactant replacements that can, if delivered within minutes of birth, either prevent RDS or mitigate its effects.

Exogenous surfactant replacement therapy (SRT) is now a standard form of care in the clinical management of premature infants with RDS. The impact of SRT on neonatal health was demonstrated by a dramatic reduction of 31% in the RDS mortality rate in the United States between 1989 and 1990 (5). In terms of the number of infants involved, another study showed that the incidence of deaths from RDS in the United States dropped from 5498 in 1979 to 1460 in 1995 (6). Each year, about 40,000 infants in the United States are afflicted with neonatal RDS (7), whereas worldwide the number exceeds 2 million (8).

Currently, there are eight different surfactant replacement formulations commercially available for the treatment of RDS (4,9–14). These formulations can be divided into two different classes: "natural" and "synthetic" LS replacements. So-called natural LS replacements are prepared from animal lungs by lavage or by mincing, followed by extraction of surfactant materials with organic solvents and purification. Synthetic surfactant replacements, on the other hand, are always protein free and are made from a blend of synthetic phospholipids with added chemical agents (generally, either lipid or detergent molecules) that facilitate adsorption and spreading of the material at the surface of the lungs.

Motivated by concerns that natural LS replacements are animal derived and hence carry risks of pathogen transmission, whereas the presently available synthetic formulations are less efficacious, extensive research has been conducted on the development of a third, not-yet-commercial class of formulations: biomimetic LS replacements. Formulations of this class are designed to closely mimic the biophysical characteristics and physiological performance of natural LS while not sharing its precise molecular composition. To date, most biomimetic LS formulations contain synthetic phospholipid mixtures in combination with either recombinantly derived or chemically synthesized polypeptide analogues of the hydrophobic surfactant proteins (8,15–33). The successful creation of a good biomimetic LS replacement will facilitate better, and safer, treatment of a medical syndrome that afflicts premature infants throughout the world. A formulation that offers the efficacy of animal surfactant, as well as the safety and relatively low cost of synthetic products, would not only improve current treatment protocols but would offer a feasible product for treating infants in nonindustrialized countries where the cost of currently available replacements remains prohibitive. In addition, there is evidence that a nonimmunogenic biomimetic LS would have applications in the treatment of other lung diseases that have surfactant dysfunction as an element of their pathogenesis, including meconium aspiration syndrome, congenital pneumonia, and acute RDS (34–36).

II. RESPIRATORY DISTRESS SYNDROME

Typically, the premature lungs of infants born after less than 32 weeks' gestation will either have insufficient amounts of, or be completely devoid of, pulmonary surfactant. This deficiency results in higher than normal alveolar surface tension and alveolar instability, factors that lead to the rapid development of respiratory distress syndrome, which is manifested as an inability to breathe and an inability to be respirated without secondary

lung trauma. RDS is a leading cause of infant mortality in the industrialized world. Since the pioneering efforts of Fujiwara et al. (9), numerous clinical trials have shown the efficacy of the administration of exogenous LS replacements for the rescue of these infants. Surfactant replacement therapy (SRT) improves lung compliance and oxygenation, and hence decreases the requirements for inspired oxygen, reduces the incidence of pulmonary complications, and, most importantly, increases the survival rate (9,37–43). Clearly, exogenous SRT is a successful means of treating premature infants at risk of developing RDS. However, there is a percentage of neonates who do not respond well to LS replacements, for reasons that we will briefly discuss and that remain poorly understood (44,45). Hence, improvements in the current therapeutic biomaterial and its method of administration are still required. Toward this end, researchers have worked on the development of a completely biomimetic LS replacement formulation that will be functional, safe, and cost effective.

III. HISTORICAL PERSPECTIVE ON THE PHYSIOLOGICAL ROLE OF LS AND THE CAUSES OF RDS

The history of LS research dates back to the late 1920s, when von Neergaard illustrated the significance of surface tension in pulmonary physiology. In his demonstration, von Neergaard showed that a greater pressure is required to expand an atelectatic (i.e., collapsed) lung with air, rather than a saline solution, and surmised that this was a result of differences in the relative magnitudes of surface tension forces on the alveoli (46). However, it was not until the mid-1950s that Pattle (2) and Clement (1,47) showed the existence of a surface-active material in the lungs that naturally reduces surface tension.

Shortly after the initial discovery of surface-active agents in the lung in 1959, Avery and Mead demonstrated that a lack of surfactant was central to the pathophysiology of RDS in neonates (3). Specifically, they showed that the deficiency or dysfunction of surfactant reduces lung compliance by increasing surface tension forces at the air–water interface of the alveoli. This knowledge led to the isolation of pulmonary surfactant from calf lung in 1961 (48). After it was recognized that dipalmitoylphosphatidylcholine (DPPC) is a major constituent of the LS mixture (48), clinical trials were conducted to test the efficacy of the first synthetic LS formulation, which was composed of DPPC and delivered as an aerosol (49,50). However, trials were unsuccessful; DPPC alone does not adequately mimic natural LS because of the rigidity of the monolayer that it forms at the air–water interface (51–53).

It was not until 1980 that surfactant replacement therapy became a reality in the treatment of neonatal RDS. That year, Fujiwara et al. successfully rescued 10 preterm infants who were suffering from severe RDS by intratracheal bolus instillation of a bovine-derived LS (9). Although these authors called their bovine surfactant replacement "semiartificial," by today's convention it would be known as natural because it was extracted from animal lungs. Since then, LS replacement treatment has become standard care for preterm infants with RDS. If infants with RDS survive surfactant replacement therapy (requiring up to 4 doses, every 6–8 h after birth), they generally begin to secrete their own pulmonary surfactant within 96 h (54,55).

Although animal-derived LS replacements have been used with success for neonate rescue, improvements to further increase survival rate and to decrease the cost per patient are still needed. Toward this end, researchers are working to develop a new class of biomimetic LS replacements that capture the advantages of both natural and synthetic formulations. In order to design a functional replacement for a complex biomaterial such as pulmonary surfactant, it is necessary to understand the properties of the natural substance and to recognize aspects of the current therapy that require improvement. Therefore, we begin with an introduction to the molecular composition and the biophysical functioning of LS, before discussing strategies for and reviewing recent progress in the development of a useful biomimetic LS replacement.

IV. BIOSYNTHESIS OF LS

Lung surfactant is synthesized in alveolar type II epithelial cells and is stored intracellularly in dense, multilayered membrane structures, referred to as *lamellar bodies* (56). The contents of the lamellar bodies are excreted into the alveoli (57), where they undergo a transformation to lattice-like, tubular double layers, referred to as *tubular myelin* (58,59), the main reservoir of surfactant (60), from which an LS monolayer at the air–liquid interface is formed (61,62) (Fig. 1). The efficient and rapid adsorption of the surfactant to the air–liquid interface imparts a dramatic reduction in alveolar surface tension, which is requisite for breathing.

V. PHYSIOLOGICAL ROLE OF LS

Pulmonary surfactant is a complex mixture of proteins and lipids that coats the internal surfaces of healthy mammalian lungs to enable normal

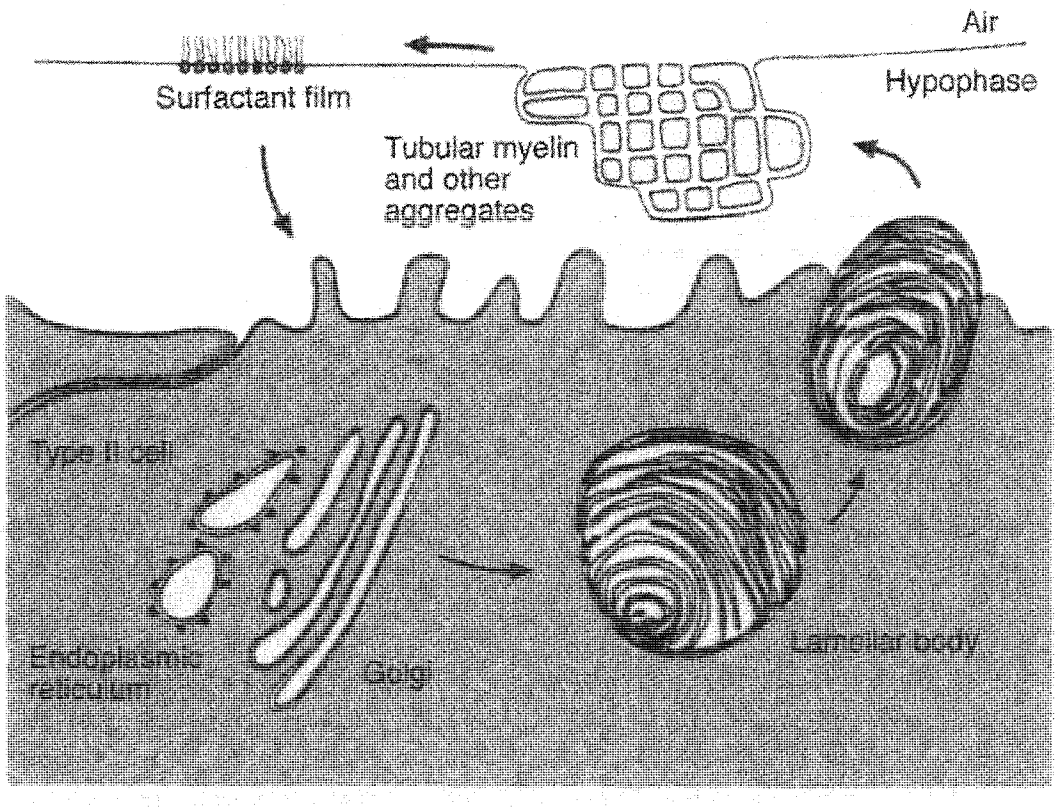

Figure 1 A schematic diagram of natural pulmonary surfactant synthesis and transport to the alveolar surface. Pulmonary surfactant is synthesized in type II alveolar cells as a complex mixture of lipids and surfactant proteins, and assembled into lamellar bodies. These organelles are secreted and transformed into tubular myelin, which then adsorbs to the air–liquid interface where it functions to control the surface tension throughout the breathing cycle. Surfactant materials are eventually taken back into the type II cells for degradation and recycling. (From Ref. 276, with permission.)

respiration (2). By virtue of its unique surface-active properties, which we will soon describe, lung surfactant reduces the pressure required for alveolar expansion and decreases the work of breathing (1,63). Lung surfactant also stabilizes the alveolar network, preventing its collapse upon exhalation (53,64–66).

VI. MOLECULAR COMPOSITION OF LS AND COMPONENT ROLES IN SURFACTANT ACTIVITY

Lung surfactant is composed of approximately 85–90% phospholipids, 5% neutral lipids, and 8–10% proteins (see Table 1) (66–70). The most abundant

Table 1 Molecular Composition of Lung Surfactant (66–70)

Components	Percentage (%)
Phospholipids	85–90
Phosphatidylcholine (PC)	68–72
Phosphatidylglycerol (PG)	8
Phosphatidylethanolamine (PE)	5
Phosphatidylinositol (PI)	3
Phosphatidylserine (PS)	Trace
Lysophophatidylcholine	Trace
Sphingomyelin	Trace
Neutral lipids	5
Cholesterol	
Cholesterol esters	
Surfactant proteins (SP)	8–10
Hydrophilic Proteins	
SP-A	5
SP-D	2
Hydrophobic Proteins	1.5
SP-B	
SP-C	

component is phosphatidylcholine (PC), which is generally dipalmitoylated and in the saturated form (DPPC). Phosphatidylglycerol (PG), an anionic lipid, accounts for another 8%. Also present are phosphatidylethanolamine (PE, about 5%), phosphatidylinositol (PI, about 3%), and trace amounts of phosphatidylserine (PS), lysophosphatidylcholine, and sphingomyelin. Some neutral lipids are also present, and include both cholesterol and cholesterol esters.

In vitro and in vivo biophysical experiments have shown that the most critical lipid molecules for reduction of alveolar surface tension are DPPC and PG. Although DPPC films are capable of reducing surface tension to near zero upon compression (i.e., DPPC monolayers can sustain high surface pressures before collapse), these phospholipids are slow to adsorb to an interface (51–53). The presence in LS of other, minor lipid components, in particular PG, has been shown to assist in the spreading of DPPC molecules at the air–water interface (52). However, such lipid mixtures alone are also ineffective as lung surfactant replacements because, under physiological conditions and in the absence of other spreading agents, DPPC and PG will not adsorb to the air–liquid interface with sufficient quickness or respread as rapidly as needed for breathing as alveolar surface

area changes cyclically (71). Instead, a unique combination of protein-based surfactants function as the necessary spreading agents.

Actually, a total of four different surfactant-specific proteins (SP) are known to be present with phospholipids on the alveolar hypophase (i.e., the aqueous lining of the lung): SP-A, SP-B, SP-C, and SP-D (72). These proteins fall into two major subgroups: the hydrophilic surfactant proteins (SP-A and SP-D), and the hydrophobic, amphipathic surfactant proteins (SP-B and SP-C). SP-A and SP-D aid in the control of surfactant metabolism and also have important immunological roles for defense against inhaled pathogens (73,74). But for therapeutic LS replacements, it is the biophysical properties of surfactant as they affect the mechanical properties of the lung that are important for the treatment of RDS. Even though SP-A is involved in the ordering of LS phospholipids in the presence of calcium, it is typically omitted from LS replacements because it does not have a significant role in reducing surface tension and is also immunogenic (75). For the same reasons, SP-D is also omitted from surfactant replacements (76).

SP-B and SP-C are required for proper biophysical functioning of LS (77), enabling attainment of low surface tensions on the alveolar hypophase and endowing proper dynamic behavior to the mixed lipid monolayers and multilayers that are found there (78–80). It has been suggested by one study that SP-B and SP-C function in a nonsynergistic manner (81); yet, considering the strict conservation of both proteins in mammals and the significant differences in their structures, which we will discuss, it seems likely that each plays a role that is important and distinct in facilitating easy breathing. However, it has been difficult to deconvolute the individual roles of SP-B and SP-C (82). Both proteins have been found to facilitate the rapid adsorption of phospholipids to an air–water interface and to allow rapid respreading of phospholipids as the alveoli expand and contract. Both have a dramatic influence on monolayer phase behavior and reduce the surface tension on alveoli upon compression of surface area (81,83). A variety of studies indicate that SP-B is more effective in enhancing the adsorption rate and dynamic surface activity of phospholipids (81,84–87), particularly in refining the films of surfactant to have enriched DPPC content (88,89). It has been suggested that SP-C is more effective at promoting respreading and film formation from the collapsed phase (88–90).

Below we briefly describe the molecular structures and biophysical properties of the SP-B and SP-C proteins. The reader is referred to recent reviews (91–94) for more comprehensive and detailed discussions of their structure–function relationships.

VII. STRUCTURAL DESCRIPTION AND APPARENT PHYSIOLOGICAL ROLE OF SP-B

SP-B is a small, hydrophobic protein, composed of 79 amino acids, that has an unusually high cysteine content (Fig. 2A) (95–97). In the native SP-B protein, seven cysteine residues form a unique disulfide pattern that includes three intramolecular bonds and one intermolecular bond, the latter of which results in the formation of SP-B dimers (98–100). The numerous positively charged side chains scattered throughout the SP-B sequence are essential for its activity (33). Electrostatic interaction of these groups with negatively charged PG molecules is known to enhance respreading of the phospholipid film, as well as to cause refinement of the monolayer by the enrichment of the DPPC content of the film through the "squeeze-out" of other lipids at the air–liquid interface (52,101–106). The hydrophobic amino acids in the SP-B sequence are known to interact with lipid acyl chains (33). Spectroscopic studies have shown that the secondary structure of SP-B is dominated by α helices, which are likely to be amphipathic given their sequence distribution. The detailed tertiary structure of the protein has yet to be determined by nuclear magnetic resonance (NMR) or crystallographic studies (20,21,107–111).

In a structural model for SP-B that was proposed by Andersson et al., four amphipathic helices are aligned in an antiparallel, left-handed hairpin motif, where one helical face is hydrophobic and the other relatively hydrophilic, as illustrated in Figure 2B (110). With this tertiary structure,

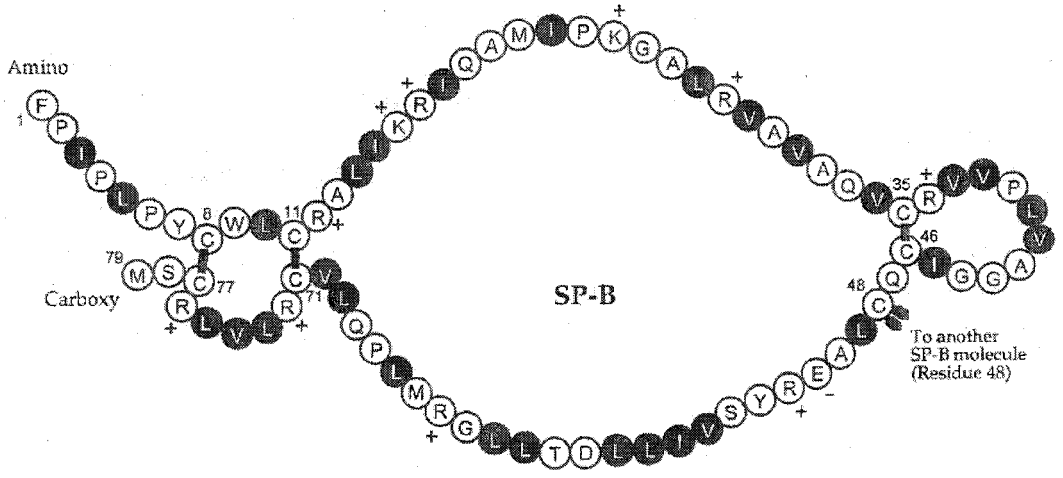

Figure 2A Primary structure of SP-B (human sequence). The identity of each amino acid is given by the one-letter code. Hydrophobic residues are shown in black, and charged residues are identified. (Adapted from Ref. 277, with permission.)

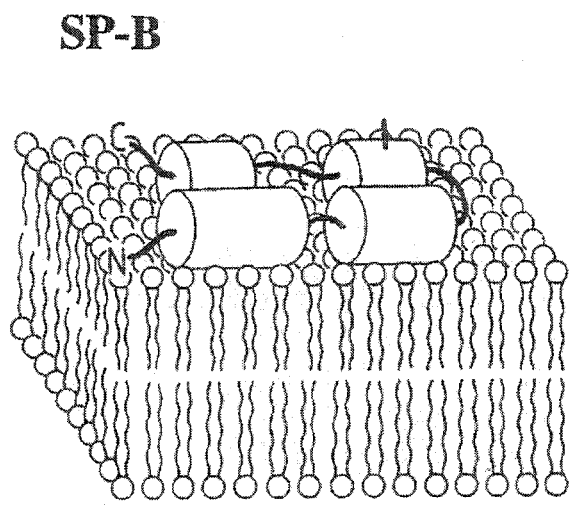

Figure 2B Hypothetical model of SP-B folded structure and its proposed mode of interaction with a phospholipid bilayer. SP-B is suggested to be a dimer of two identical 79-residue four-helix protein chains (cross-linked at Cys48, in the third helix), with the polar face of the amphipathic helix interacting with the lipid headgroups. (From Ref. 278, with permission.)

SP-B would be well suited to interact with a phospholipid monolayer or bilayer (80,112–114), with its polar (mostly cationic) faces interacting with lipid headgroups, particularly those of the anionic phospholipids (115), and the apolar faces interacting with acyl chains in the regions of the headgroup (91). Recently, another hypothetical structural model of SP-B, which reflects the homodimeric structure of native SP-B, was proposed by Zaltash et al. (116). In this model, the two SP-B monomers are linked by disulfide bond at Cys48, with the charged residues lying on one surface of the disk-like structure. The dimer is thought to be stabilized by hydrogen bonds or by ion pairs between Glu51 and Arg52 residues from each of the two monomers (116). This hinged, dimerized structure would provide correlated motion of SP-B monomers that interact with two different monolayers/bilayers, creating "cross-talk" between these organized lipid films (116).

Apparently the main physiological function of SP-B protein is to facilitate phospholipid adsorption to the air–liquid interface, thereby allowing rapid spreading and respreading of the surface tension–lowering phospholipids as alveoli expand and contract. In this way, SP-B has the effect of stabilizing the surface film. It has been shown that the ability of SP-B to induce rapid insertion of phospholipids into the monolayer is essential for the maintenance of alveolar integrity (33,84,85,96,117,118). Hence, SP-B may have a predominant role in facilitating the reduction of surface tension

in the lungs. Besides these roles, SP-B may also serve the critical functions of aiding the formation of tubular myelin structures (119–121) and inducing the calcium-dependent fusion of membranes (119,122).

In vivo rescue experiments with premature rabbits (123), in vivo blocking of SP-B with monoclonal antibodies (124), and studies with genetically engineered SP-B-deficient mice (125,126) have all confirmed the critical role of SP-B in functional LS. Furthermore, recent studies of SP-B knockout mice revealed that the presence of covalently linked homodimers of SP-B appears to be important for the optimal functioning of natural LS (127,128). These studies, in addition to the fact that an inherited SP-B deficiency in infants is lethal (129,130), provide strong evidence for the predominant importance of SP-B in LS.

VIII. STRUCTURAL DESCRIPTION AND APPARENT PHYSIOLOGICAL ROLE OF SP-C

The smaller of the two hydrophobic surfactant proteins, SP-C, is composed of 35 amino acids and has an unusual dipalmitoyl modification near the carboxy terminus (Fig. 3A) (131,132). Two-thirds of the protein consists of a long, continuous, valyl-rich hydrophobic stretch, which adopts an α-helical secondary structure as evidenced by both circular dichroism (CD) and NMR structure determination (Fig. 3B) (133–135). The length of this helix, 37 Å, is perfect for the spanning of a fluid DPPC bilayer (136). Consistent with this observation, it has been shown in other studies that the

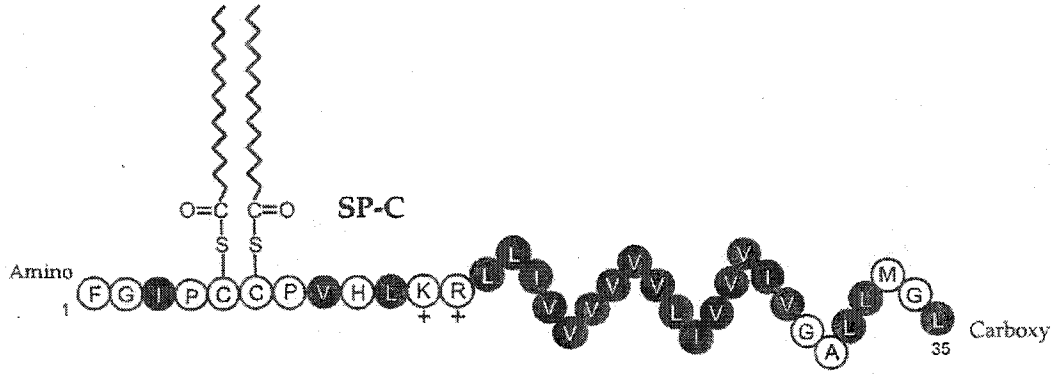

Figure 3A Primary structure of hydrophobic surfactant protein SP-C (human sequence). The identity of each amino acid is given by the one-letter code. Hydrophobic residues are shown in black, and charged residues are identified. The two cysteine residues are palmitoylated. (Adapted from Ref. 277, with permission.)

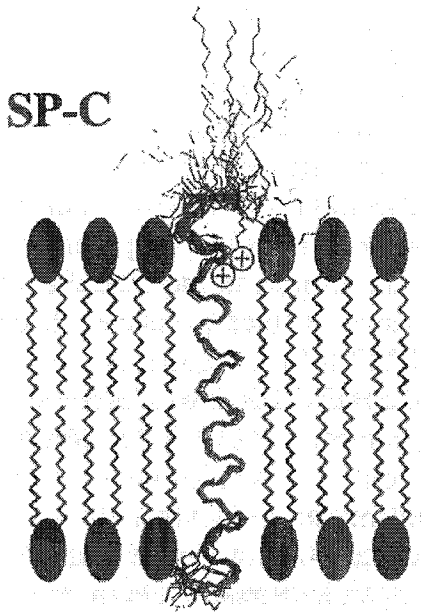

Figure 3B Schematic presentation of SP-C secondary structure and its proposed mode of interaction with a phospholipid bilayer. This SP-C structure was deduced by 2D-NMR, and in this picture is artificially superimposed on a lipid bilayer. In this transbilayer orientation, the hydrophobic part of the protein (residues 13–28) interacts with the lipid acyl chains, while the basic residues at position 11 and 12 (indicated by positive charges) interact with the polar (anionic) lipid headgroup. The two cysteine residues at positions 5 and 6 are palmitoylated; the role of these palmitoyl chains is still disputed in the literature. (From Ref. 278, with permission.)

SP-C α-helix is a transbilayer protein, with the α-helix oriented roughly parallel to the lipid acyl chains at the air–water interface (134,137). Other evidence suggests that in interactions with a DPPC *monolayer*, SP-C is situated to make a 70° tilt relative to the normal of the monolayer plane (138). The issue of whether SP-C preferentially interacts with an LS monolayer or with bilayer or multilayer structures is still under active investigation (82,114,139,140).

Palmitoylation of the two SP-C cysteines at positions 5 and 6 in the sequence has been proposed to promote protein interactions with lipid acyl chains in neighboring, stacked lipid bilayers (141), thereby facilitating SP-C binding to the bilayer (142) and/or orienting the peptide (143). However, the physiological function of the two palymitoyl chains, as well as their necessity for in vivo efficacy of LS replacements, remains to be fully understood (142,144,145). The two adjacent, positively charged lysine and arginine residues at positions 11 and 12 of SP-C most likely interact with the

phospholipid headgroups and promote binding to the monolayer or bilayer by ionic interactions (146).

Similarly to SP-B, SP-C seems to promote phospholipid insertion into the air–liquid interface (108), and thereby to enhance the rate of lipid adsorption (145,147) and the respreading of the alveolar films upon inhalation (83). SP-C also stabilizes the surfactant film during the expansion and compression phases of breathing, apparently by regulating phospholipid ordering in such a fashion as to increase the lateral pressure within the bilayer (note that increased surface pressure, Π, correlates with decreased surface tension, γ) (121,148,149). Interestingly, the results of one study have suggested that a single SP-C molecule is capable of influencing the phase behavior of 20–35 lipid molecules (135). In addition, SP-C has been found to stimulate liposomal fusion in vitro (119) and to enhance the binding of lipid vesicles to a cell membrane for endocytosis of lipids (150,151).

In vivo studies of genetically engineered SP-C knockout mice have revealed that SP-C plays an important role in endowing function to LS but is seemingly less critical for breathing than SP-B. SP-C knockout mice are viable at birth and grow normally without altered lung development or function (152,153), but lung mechanics studies reveal abnormalities in lung hysteresivity at low lung volume (153). Furthermore, studies have shown that mutations in the human SP-C gene can result in the expression of an altered proprotein, the precursor that undergoes proteolytic cleavage to yield mature SP-C, which is believed to be involved in the development of interstitial lung disease (152). The deficiency of SP-C in some Belgian blue calves has been shown to increase the likelihood of RDS (154).

IX. INTERFACIAL PROPERTIES OF LS

The physiological roles of LS require it to adsorb and respread quickly upon inhalation and to reduce surface tension upon exhalation. These requirements can be satisfied by envisioning the surface film as being composed of monolayers highly enriched in DPPC, as well as bilayers/multilayers of lipid/protein structures that remain closely attached to the film (80,88). Both selective squeeze-out and insertion of lipids has been proposed to enrich the monolayer with DPPC to enable the attainment of low surface tension observed for LS during exhalation. However, upon reexpansion, DPPC is a poor spreading material. Instead, it is the unsaturated lipids and surfactant proteins that are responsible for the rapid adsorption and respreading of LS upon inhalation (114,140,157,163). These squeezed-out components are stored in multilayers that remain closely associated with the film at the interface (80,155) and respread into the surface film upon alveolar expansion

(156). Replenishment of the surface film occurs by adsorption from the subphase, and by respreading of collapsed phases and excluded material (114,140). The transferring of the lipids to the interface and the formation of surfactant film at the air–liquid interface is enhanced by the presence of the surfactant proteins SP-B and SP-C (157), which perturb the packing of the phospholipids (85,149,158,159).

X. RECONCILIATION OF LS'S DICHOTOMY OF ROLES AS A SURFACE-ACTIVE MATERIAL

For lung surfactant to work effectively, the films that are formed must be *fluid*, so that the material adsorbs and respreads quickly and reversibly to the alveolar interface, to form a monolayer upon expansion; yet it also needs to be *rigid* as a surface film, so that it reaches near-zero surface tension during the alveolar compression accompanying exhalation (82). Hence, there is a dichotomy of the roles of LS. To reconcile the dual actions of LS, the "squeeze-out" theory was postulated (52,53). This theory states that adsorption is facilitated by the presence of the fluidizing agents, which are subsequently removed upon compression, resulting in the formation of a DPPC-enriched monolayer to promote low surface tension (52,101–104). However, this theory does not account for the presence of the surfactant proteins or for their complex roles (80,83,105,160–162).

Recent investigations of LS phase behavior and surface film morphology and 2D phase behavior of different LS components have led to the development of the "monolayer-associated" theory (82). Contrary to the squeeze-out theory, this theory states that the surfactant proteins help to retain the unsaturated fluidizing components of LS within or near monolayers at all surface pressures, even at film collapse (i.e., at high surface pressures and low surface tensions) (82,163–166). Consistent with this, experiments conducted have shown that SP-B and SP-C *prevent* the squeeze-out of unsaturated lipids by altering the film collapse mechanism from a fracturing event to a more reversible buckling or folding of the monolayer (163,164). Particularly for SP-B, it appears that these folds remain in close association with the surface film, thereby allowing facile reincorporation of the material upon expansion (164). To a greater extent for SP-C, it has been observed that the lipid components that are removed from squeeze-out upon compression (83) are stored in a multilayered phase that remains closely attached to the interface (80,155), which upon expansion respreads into the surface film (156,157).

XI. LS REPLACEMENTS FOR TREATMENT OF RDS

Clearly, a good understanding of the surfactant proteins, and their structural links to the underlying mechanisms that endow lung surfactant with its extraordinary surface-active properties, will be critical for successful bioengineering design of a functional, biomimetic LS replacement. Elucidation of the interactions between the various components of this complex protein–lipid mixture entails deconvolution of the phase behavior of both the lipid and protein components. Intense study of whole LS and various fractions thereof in recent years has afforded a number of invaluable insights into the structure–function relationships between proteins and lipids (28,33,80,82,90,112,114,139,140,157,162,166–173), and is beginning to provide enough information to guide well-informed design of novel biomimetic LS replacements.

Delivery of an exogenous LS replacement to a preterm infant is a temporary intervention, intended to maintain respiratory function and to minimize lung injury until maturation of type II cells occurs, generally within 96 hours of birth, permitting an adequate amount of endogenous LS to be produced and transported to the alveolar surface (54,55). A good replacement must capture the physiological characteristics described earlier. In vivo, the surfactant needs to be capable of improving the stability of immature fetal lungs and of providing healthy pressure–volume characteristics to the alveolar network. In terms of in vitro biophysical properties and therapeutic characteristics, this translates to (a) rapid surface adsorption of LS, to generate an equilibrium surface tension of about 25 mN/m within 1 min (60); (b) reduction of the minimal alveolar surface tension to nearly zero upon cyclic compression, to prevent alveolar collapse and to maintain the patency of terminal bronchioles at expiration (174,175); and (c) effective respreading of surfactant after compression beyond the collapse pressure, to replenish surfactant materials during alveolar expansion and to ensure that the maximum surface tension does not rise above an equilibrium level of 25 mN/m during the breathing cycle (53,65,176). LS replacements must provide these benefits and should also be pure, safe, and bioavailable (i.e., they should have no viral, protein, or chemical contamination, and should not elicit an immune response). From a production standpoint and to facilitate wide availability, the ease of surfactant manufacturing, purification, quality control, and cost must also be considered. Therefore, an ideal LS replacement would be highly similar in its properties to the natural material and also cost effective.

From a design perspective, it is not only important to understand the physiological and biophysical activities of lung surfactant but also the factors that can inhibit its performance. LS can be inactivated by the

presence of (a) plasma and blood proteins (albumin, fibrinogen, hemoglobin, etc.) (177–180); (b) unsaturated cell membrane phospholipids (178); (c) hysophospholipids (181); (d) cholesterol (182); (e) free fatty acids (183); (f) lytic enzymes (proteases and phospholipases) (184); (g) reactive radicals; and (h) meconium (first feces of a fetus) (185). Investigations of these endogenous molecules have shown that inactivation by these contaminants can, in general, be mitigated by increasing the LS concentration (177,186). This means that, potentially, a patient suffering from RDS or ARDS could be helped by the delivery of additional LS or of a functional replacement.

The design, testing, and benchmarking of any novel LS replacement necessitates in vitro characterization of the material by a number of different approaches, each of which evaluates surface activity in complementary ways. Those formulations that show promise in in vitro studies then undergo in vivo animal studies, including both pharmacological studies to determine the effectiveness of the formulation for treatment of RDS and toxicological studies to identify the proper dose regime. Only those therapeutic agents that are found to be both efficacious and safe in animals will progress to the next stage, in which human clinical trials are carried out in neonates (187).

XII. IN VITRO CHARACTERIZATION OF LS REPLACEMENTS

Three different experimental tools are used extensively to evaluate the surface-active properties of various natural and synthetic LS formulations, including (a) the Langmuir-Wilhelmy surface balance (LWSB), often used in conjunction with fluorescence microscopy (FM) to observe surface phase morphology; (b) the pulsating bubble surfactometer (PBS); and/or, (c) the captive bubble surfactometer (CBS). The Wilhelmy surface balance, first used by Clements for LS studies in 1957 (1), is designed to carry out cyclic film compression on a Langmuir trough and to allow accurate measurement of the very low surface tensions that are characteristic of LS at high levels of monolayer compression. The major utility of the LWSB in the study of LS replacements has been to allow the observation of surface pressure effects that occur within films that are spread directly onto the air–water interface (i.e., not adsorbed from the subphase), although adsorbed films are also sometimes studied. LWSB experiments allow the generation of pressure–area (Π-A) isotherms, as seen in Fig. 4, which are obtained during slow cycling of film surface area at dynamic but nonphysiological rates (188). In conjunction with the LWSB, FM can provide sensitive imaging of the phase

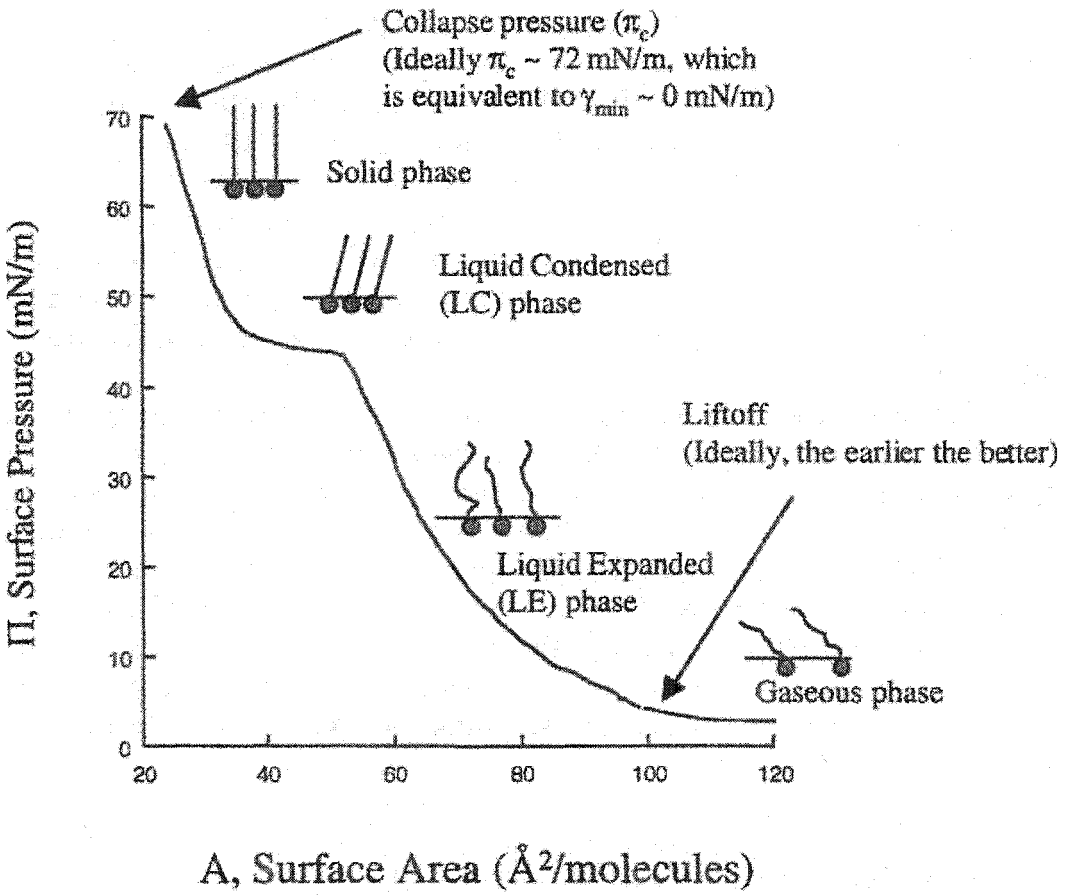

Figure 4 Surface pressure–area (Π-A) isotherm of the compression of a hypothetical surfactant film that exhibits gaseous, liquid-expanded, liquid-condensed, and solid phases. As area decreases, the surface pressure increases until the film collapses. Typical lift-off and collapse pressure values of lung surfactant are depicted. (Adapted from Ref. 279, with permission.)

morphology of LS monolayers or multilayers as they undergo compression and expansion on the trough. Interactions between different lipid components and/or between lipids and surfactant proteins, as they influence the film behavior and phase morphology, can be imaged and then correlated with other measures of performance and surface activity, especially the Π-A isotherms (155,171,189).

The PBS was developed in 1977 by Enhorning (190) and applied to the study of LS behavior with the goal of obtaining more physiologically relevant data on the surface tension-lowering ability of dispersed pulmonary surfactants. Experiments carried out on a PBS can provide information on both equilibrium adsorption and dynamic film compression and expansion

characteristics of a surfactant. Continuous measurements of surface tension are made on a cyclically expanding and contracting bubble surface covered with surfactant, and can be acquired at a physiological temperature (37°C), cycling rate (20 cycles/min), and film compression ratio (up to 50% area compression). This access to conditions mimicking those of the human lung is a major advantage of the technique. The resultant data are generally plotted as shown in Fig. 5, which shows a curve of surface tension as a function of bubble surface area (168). Particularly important are the low values of the minimal and maximal surface tensions observed during bubble compression and expansion, respectively, as well as the dramatic hysteresis

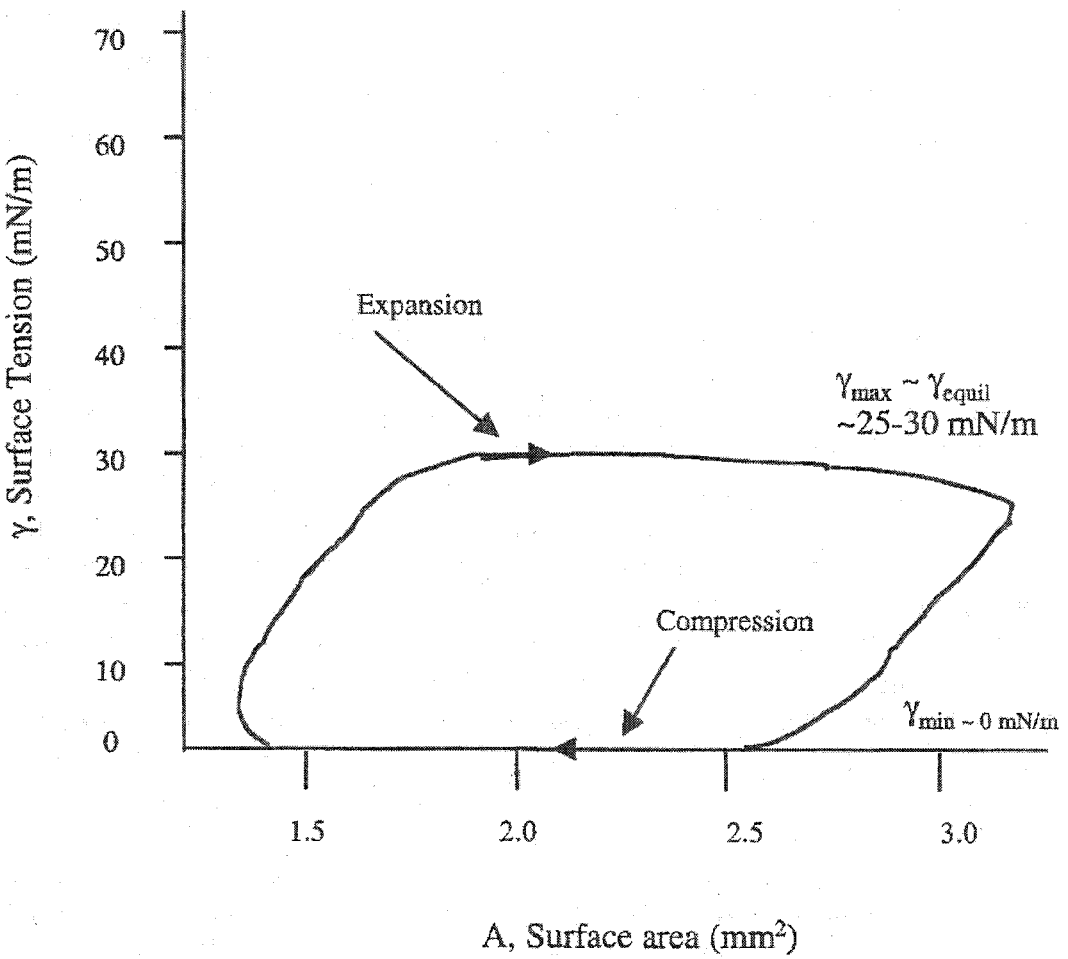

Figure 5 Schematic diagram of a typical surface tension (γ) versus interfacial bubble area (A) loop observed for calf lung surfactant (CLS) in 5 mM $CaCl_2$ and 0.15 M NaCl at 37°C, as measured during dynamic oscillations by a pulsating bubble surfactometer (PBS) at a frequency of 20 cycles/min and a bulk surfactant concentration of 1 mg/mL. (Adapted from Ref. 168, with permission.)

seen in the data curve. Here, of course, the bubble (typically ranging from about 0.8 to 1.0 mm in diameter) mimics a single alveolus. In addition to generating these curves that depict dynamic surface tension, one can also use the PBS to map out adsorption isotherms for LS (i.e., to create plots of surface tension γ versus time t), if the instrument is run in static mode (190–192).

Because of some initial concerns about a possible leakage of surfactant from the bubble surface to the capillary tube from which the bubble is suspended in a PBS, the CBS was developed in 1989 by Schürch et al. (193) to provide similar data with a lower likelihood of surfactant leakage (192,194,195). Therefore, many consider CBS data to be more reliable than PBS data (194). However, disadvantages of the CBS include its unavailability as a commercial instrument, and the time-consuming and complex nature of data analysis (192). In vitro characterization using the LWSB, PBS, and CBS can provide complimentary information. Standards for good in vitro performance have been established for these instruments and can now be used as evaluative parameters for biomimetic surfactant formulations under development.

XIII. IN VIVO CHARACTERIZATION OF LS REPLACEMENTS

Animal studies provide a necessary link between in vitro biophysical studies and clinical therapy. Multiple animal models of RDS have been established and have proven invaluable in the testing and evaluation of surfactant performance. Important evaluation parameters include (a) pressure–volume (P-V) lung mechanics (see Fig. 6); (b) lung functional parameters [i.e, arterial partial pressure of oxygen (Pa_{O_2}), arterial partial pressure of carbon dioxide (Pa_{CO_2}), and arterial/alveolar partial pressure of oxygen (a/A_{O_2})]; and (c) ventilator-associated parameters [i.e., ventilator rate, fraction of inspired oxygen (Fi_{O_2}), mean airway pressure (MAP), peak inspiratory pressure (PIP), and positive end-expiratory pressure (PEEP)]. Some common animal models used to evaluate the efficacy and safety of LS formulations include rats (196) and prematurely born rabbits (197), as well as premature lambs (198–200), baboons (201), and monkeys (202). Typically, in vivo studies evaluate lung function for either short or extended periods of time; clinically relevant procedures and manipulations are also tested.

Building on successful in vitro experiments and animal studies, the efficacy of exogenous LS replacements is evaluated in clinical trials with human infants to determine the onset and duration of the therapeutic action of the LS replacement. Incidence of mortality from RDS, typical severity of

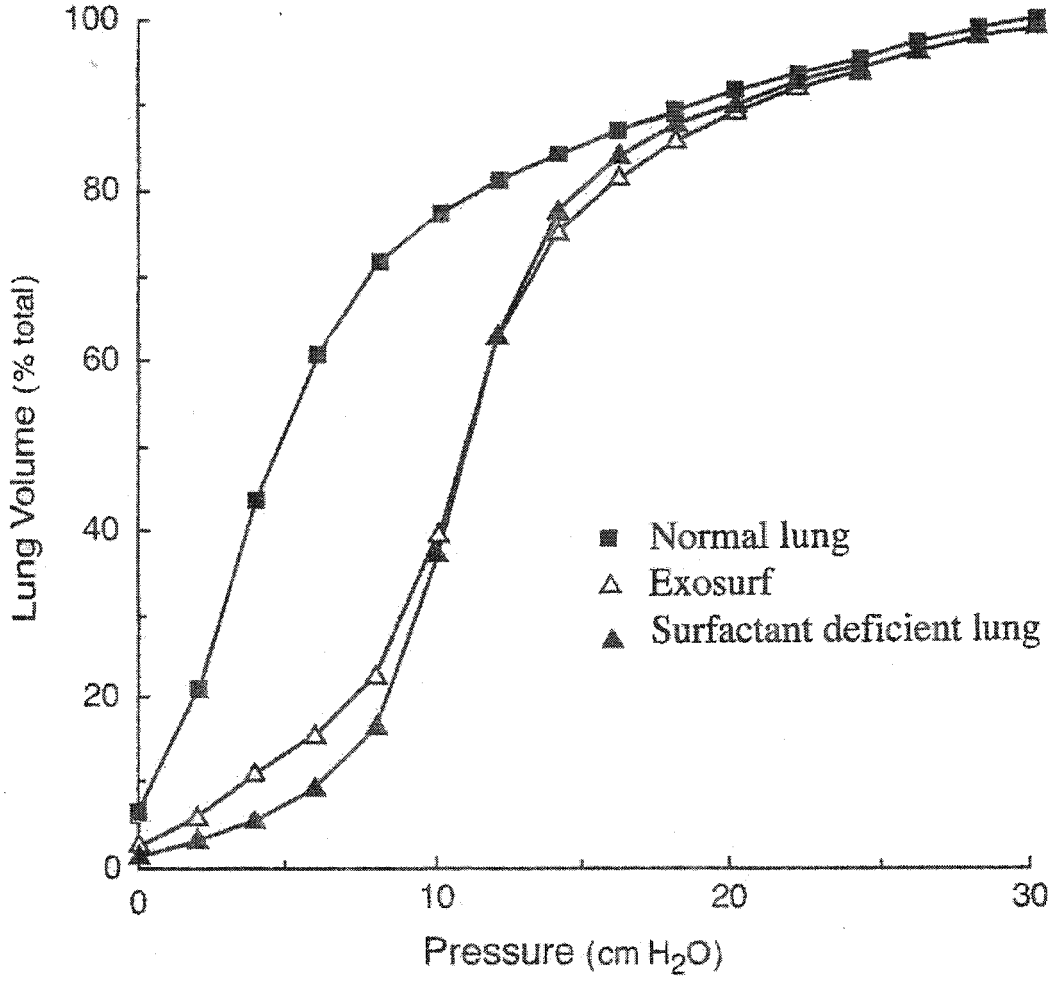

Figure 6 Pressure–volume (P–V) deflation mechanics of rat lung at 37°C: normal lungs, surfactant-deficient excised lungs, and after natural (CLSE) or synthetic (Exosurf) surfactant instillation into depleted excised lungs. Normal curve is obtained postexcision, and surfactant-deficient curve is after multiple lavages to deplete the endogenous surfactant. Exogenous surfactant is instilled at a concentration of 20 mg phospholipid/2.5 mL saline for CLSE, and 37.5 mg lipid/2.5 mL saline for Exosurf. (Adapted from Ref. 276, with permission.)

RDS, rate of recovery, and incidence and severity of bronchopulmonary dysplasias (BPD) and other chronic lung disorders, as well as any safety-related outcomes, are determined (11,203–207). The criteria assessed in human trials of LS replacements are the same as those listed above for the animal studies.

Utilizing these benchmarks for evaluation, surfactant replacement therapies have been developed and are used to effectively minimize alveolar

collapse at end-expiration and to increase lung compliance, allowing safe respiration of prematurely born infants. Current LS treatments fall into two main categories: *natural* and *synthetic*. A third, promising but not yet available class of formulations is the *biomimetic lung surfactant replacements*, which will be covered later in this chapter.

XIV. NATURAL LS REPLACEMENTS

A. Animal-Derived Replacements

Natural surfactant replacements are prepared from animal lungs, either by lavaging or mincing followed by organic phase extraction of the phosphopholipids and hydrophobic surfactant proteins. A number of different natural surfactant replacements have been commercialized (Table 2). From bovine lungs, there are surfactant TA (Surfacten, Tokyo Tanabe, Japan) (9), beractant (Survanta, Abbott Laboratories, Columbus, Ohio) (10), Alveofact (Thomae, Biberach/Riss, FRG) (11), and BLES (BLES Biochemicals, Ontario, Canada) (208,209). Both surfactant TA and beractant are obtained from minced cow lung and are spiked with added synthetic lipids [DPPC, palmitic acid (PA), and tripalmitin] to standardize the composition and to improve the physical and physiological properties of the material. From calf lungs, there is calf lung surfactant extract, CLSE (Infasurf, Forest Laboratories) (4), whereas from porcine lungs there is Curosurf (Chiesi Farmaceutici, Parma) (12). CLSE, Alveofact, and BLES are obtained by lung washing and subsequent extraction of the lavage fluid with organic solvents. Curosurf is obtained by mincing of the lung, followed by washing, chloroform-methanol extraction, and liquid–gel chromatography. As a result of this sample preparation, Curosurf is devoid of triglycerides, cholesterol, and cholesteryl esters; it is not really known to what extent this lack may change the manner in which it functions.

In vitro biophysical characterization experiments have shown that natural surfactants generally provide virtually instant surfactant adsorption, efficient surface spreading and respreading, good film compressibility, and the achievement of low surface tension during cyclic film compression and expansion (78,180,210). For example, preparations of surfactant TA and Curosurf rapidly spread to an equilibrium surface tension of 24–27 mN/m, yielding a minimal surface tension upon compression that is below 5 mN/m. (For comparison, the surface tension of a clean water surface at 37°C is about 70 mN/m.) Films of CLSE require only 20% compression to achieve a similarly low surface tension (180). Furthermore, evaluations of material performance based on animal models of RDS have shown that natural surfactants typically provide good oxygenation, pulmonary pressure–

Table 2 Commercially Available LS Replacements (Natural and Synthetic)

Product name	Company	Composition	Formulation	Performance	References
CLSE (Infasurf®)	ONY, Inc. Amherst, NY	Heat-sterilized suspension in 0.15 M NaCl at 35 mg/ml	Calf Extract (93% phospholipid, 5% cholesterol and neutral lipids, 1.5% SP-B/SP-C)	PBS at 0.2 min at 2 mg/ml: $\gamma_{ads} \sim 24$ mN/m; $\gamma_{min} \sim 0$ mN/m (reaches these values at 1 mg/ml) (180) and at 0.063 mg/ml, reduced γ_{ads} to 22 mN/m in 2.5 min (78). P-V mechanics in lavaged rat lungs: Similar to normal lung (78). Two randomized placebo-controlled clinical trials showed positive results of use of CLSE (280–282).	(4,78,180,280–282)
Surfactant-TA (Surfacten®)	Tokyo Tanabe, Tokyo, Japan	Sterile suspension in 0.15 M NaCl at 30 mg/ml	Bovine Minced Supplemented with DPPC, PA, and tripalmitin (84% phospholipid, 7% tripalmitin, 8% PA, and 1% protein) (283)	Three randomized placebo-controlled clinical trials showed positive results with the use of Surfactant-TA (284–286).	(9,283,284–286)
(SF-RI 1) Alveofact®	Thomae GmbH, Biberach, Germany	Sterile suspension in 0.15 M NaCl at 45 mg/ml	Calf Extract (99% phospholipids and neutral lipids, including 4% cholesterol; 1% SP-B/SP-C) (288)	PBS at 0.2 min at 2 mg/ml: $\gamma_{ads} \sim 24$ mN/m; $\gamma_{min} \sim 0$ mN/m (180).	(11,180,288,289)

Table 2 Continued

Product name	Company	Composition	Formulation	Performance	References
				One randomized placebo-controlled clinical trials showed positive results with the use of Alveofact (11).	
BLES®	BLES Biochemicals Inc., Ontario, Canada	Sterile suspension in 0.15 NaCl and 1.5 mM $CaCl_2$ at 25 mg/ml	Bovine Extract (98–99% phospholipids and neutral lipids and 1% SP-B/SP-C)	Two randomized placebo-controlled clinical trials showed positive results with the use of BLES (208, 290).	(208,209,290)
Beractant (Survanta®)	Ross/Abbott Laboratories, Columbus, OH Licensed from Tokyo Tanabe	Auto-claved, sterilized suspension in 0.15 M NaCl at 25 mg/ml	Bovine Minced Supplemented with DPPC, PA, tripalmitin	PBS at 0.2 min at 2 mg/ml: $\gamma_{ads} \sim 24$ mN/m; $\gamma_{min} \sim 4$ mN/m (180) and γ_{ads} reduced to 30 mN/m at 2.5 min at 0.063 mg/ml (78) P-V mechanics in lavaged rat lungs showed improved lung function (78) Seven randomized placebo-controlled clinical trials showed positive results with the use of Survanta (10,214,291–293).	(10,78,214,291–293)
Curosurf®	Chiesi Farmaceutici Parma, Italy and Dey Labs Napa, CA	Suspension in saline at 80 mg/ml	Porcine Minced (99% polar lipids and 1% SP-B/SP-C) (294)	PBS at 0.2 min at 2 mg/ml: $\gamma_{ads} \sim 24$ mN/m (180).	(12,180,294)

			One randomized placebo-controlled clinical trial showed positive results with the use of Curosurf (12).	
Colfosceril palmitate (Exosurf®)	Glaxo Wellcome NC	DPPC: hexadecanol :tyloxapol (13.5:1.5:1)	PBS at 0.063 mg/ml at 20 min: $\gamma_{ads} \sim$ 38 mN/m (78) P-V mechanics in lavaged rat lungs: Slightly improved lung function (78) Nine randomized placebo-controlled clinical trials showed positive results with the use of Exosurf (14,204,295).	(14,78,200,204,295,296)
Pumactant (ALEC®)	Britannia Pharmaceuticals Redhill, Surrey, UK	DPPC:PG (7:3)	Three randomized placebo-controlled clinical trials, with 1 showing positive results (224,297).	(13,224,297)

volume characteristics, and survival rates upon treatment (211). Finally, and more importantly, clinical trials have demonstrated the efficacy of natural surfactants to treat or prevent RDS in premature infants (12,212–215).

Although natural LS formulations are both functional and relatively safe, there are definitely a few potentially grave risks associated with sourcing a human medicine directly from animals. Because natural surfactants are extracted from animal lungs, it is impossible to eliminate the possibility of cross-species transfer of antigenic or infectious agents, such as scrapie prion (216), or other unforeseeable biological contamination (187,217). In addition, because the bovine and porcine sequences of SP-B and SP-C are only about 80% homologous to the human sequences, these animal proteins have the potential to be recognized as foreign by the human immune system (124,218,219). Antibodies developed to these homologous protein sequences could potentially inactivate the natural human proteins and lead to respiratory failure (124). This has not yet been found to occur in newborns, but for adults with ARDS, production of such antibodies could be a serious problem (216). Furthermore, the isolation of LS from animals is an expensive process that can produce variability in LS composition as a result of animal-to-animal inconsistencies. Animal-derived preparations (e.g., porcine Curosurf) are generally two to three times as expensive as some currently available synthetic surfactants (e.g., ALEC) (187,216). Finally, because of limited supply, the clinical use of natural surfactant may be restricted (34).

B. Human-Derived Replacements

As an alternative to animal-derived replacements, human lung surfactant can be harvested from the amniotic fluid of full-term pregnancies. Lung surfactant is secreted by a maturing fetus into the amniotic fluid in utero, and is present along with contaminating lipids and proteins. Whole human surfactant, obtained under sterile conditions from term amniotic fluid, has been used successfully in several studies with premature infants (212,220). However, human LS collected by this method has been shown to have reduced activity in comparison with extracts of animal lung surfactant, for reasons that are not completely understood (221). While the immunogenic risks are reduced as compared to animal-derived substances, they are nevertheless still present in human-derived LS. Furthermore, there is a possibility of disease transmission. Finally, a low supply of good-quality amniotic fluid–derived LS drastically limits its clinical use and makes it commercially unfeasible as a therapeutic replacement.

XV. SYNTHETIC REPLACEMENTS

To obviate the risks associated with natural surfactant replacements, synthetic formulations have been developed. Currently there are two synthetic products commercially available: ALEC (Pumactant, Britannia Pharmaceuticals, Redhill, UK) (13) and Exosurf (Glaxo Wellcome, Research Triangle Park, NC, USA) (14) (Table 2). The "artificial lung expanding compound," ALEC, is composed of DPPC and PG in a ratio of 7:3 and is suspended in saline at 100 mg/mL. In this formulation, PG serves the purpose of promoting the spreading of DPPC at the air–liquid interface (52). In Exosurf, hexadecanol and tyloxapol are added to DPPC to serve as spreading agents, creating a suspension consisting of 13.5:1.5:1 (DPPC:hexadecanol:tyloxapol) by weight in a saline solution, with a DPPC concentration of 13.5 mg/mL. Britannia Pharmaceutical has voluntarily suspended the marketing and distribution of Pumactant (222) pending further investigations of clinical trial results that indicated the inadequacy of this synthetic formulation in comparison with natural surfactant (223).

The advantages of synthetic surfactant replacements include their lower cost as well as a reduced potential for antigenicity, viral and protein contamination, and product variability. Clinically, some synthetic surfactants have been shown to be reasonably effective in the rescue of premature infants. For example, in a 10-center trial of ALEC, mortality was reduced from 30% in control infants to 19% in treated infants (224). However, ALEC has been shown to be less effective in the treatment of babies with established RDS, often taking several hours to produce the desired response, whereas natural surfactants take effect much more rapidly. Similarly, Exosurf also improves lung function in babies with RDS, but the therapeutic response appears to be irregular and may lag material administration by several hours (225). Hence, in comparison with natural surfactant replacements, synthetic surfactant replacements lacking surfactant proteins give inferior performance, with the reported loss of one additional infant per 42 treated (213,216). This increased mortality rate has been attributed to the absence of the surfactant proteins SP-B and SP-C, which are known to potently improve surfactant activity (211).

A meta-analysis of seven clinical trials involving more than 3000 infants was carried out to allow a comparison of the efficacy of natural surfactant replacements (mainly Survanta) with that of the synthetic surfactant replacement Exosurf. The meta-analysis revealed that natural surfactant replacements typically show a slightly superior performance, as observed in the generally lower posttreatment oxygen requirements of treated infants, and a lower risk of neonatal mortality when the natural biomaterial is administered (226,227).

XVI. BIOMIMETIC SURFACTANT REPLACEMENTS UNDER DEVELOPMENT

In light of the disadvantages present in both natural and synthetic surfactant replacements, researchers are working to develop biomimetic surfactant replacements as an improved treatment not only for neonatal RDS but for a broader class of patients and respiratory disorders. From a molecular design aspect this is a tractable bioengineering problem, as a reasonably good understanding of the composition and function of LS, as well as the deficiencies of current LS replacements, has been established. It seems clear that biomimetic LS replacements should be designed to capture the advantages of synthetic products (i.e., to be nonimmunogenic, to exclude all infectious agents and biological risks, and to be chemically pure, consistently formulated, and cost effective) while truly mimicking the performance and efficacy of natural surfactants. Up until this point, the most promising design avenues have focused on utilization of a combination of lipids with spreading agents that somehow mimic the hydrophobic surfactant proteins. Typically, the lipid fraction will include DPPC, PG, and PA. However, the selection of the lipid composition will depend on which SP-B and/or SP-C analogues are used as spreading agents in formulating the LS replacement (160).

Since the inferior performance of synthetic LS has been attributed to the absence of the surfactant proteins, various groups are working to design biomimetic versions of SP-B and SP-C. Approaches being taken range from the use of recombinant molecular biology to direct chemical synthesis of these proteins or fragments thereof, with sequences that are either similar to or completely different from the native human sequence. In all cases, present knowledge of the structure–function relationships of the proteins is taken into account in order to design SP-B and/or SP-C analogues that can serve as effective spreading agents in an LS replacement formulation.

A. Recombinant Surfactant Proteins

One approach to biomimetic LS replacement design is the development of recombinant proteins, with the goal of creating SP analogues that are highly similar to the natural proteins. However, the isolation of recombinant human SP-B and SP-C proteins from a cell culture broth is a nontrivial task. Recombinant SP-C proteins (rSP-C) with the natural human sequence have been expressed in *E. coli* (15,16,228,229,298), but recovery of the protein can be difficult, probably at least in part because of the extreme hydrophobicity and its resultant strong tendency to aggregate with itself (16). This rSP-C is currently being manufactured and researched by Scios-Nova (Sunnyvale,

CA). To increase the chances of good protein recovery, the human SP-C sequence also has been redesigned by some researchers, incorporating amino acid substitutions via standard oliogonucleotide-directed mutagenesis. In the mutant rSP-C (Cys→Ser), the cysteines at positions 4 and 5 (Fig. 3a) are replaced with serines to reduce the likelihood of protein oligomerization via the formation of disulfide bonds (15). Similarly, in the mutant rSP-C (Cys→Phe and Met→Ile), phenylalanines replace the cysteines at positions 4 and 5, whereas isoleucine replaces methionine at position 32 (16,228) to reduce the tendency to aggregate (16). This rSP-C is manufactured by Byk Gulden Pharmaceuticals (Konstanz, Germany) and is currently being investigated (16).

Biomimetic LS replacement formulations that include recombinant SP-C have shown good biophysical activity in vitro and in vivo. However, in animal studies, comparison of these biomimetic formulations, including SP-C only with natural surfactants that include both hydrophobic proteins, revealed that the recombinant surfactant formulations show poorer performance, as indicated by a lower mean P_{O_2} value and a higher mean F_{IO_2} value (15,16). It is unclear whether this drop in performance results from a lack of SP-B in the biomimetic formulation, from differences in the SP-C mimic sequence, or from a higher incidence of SP-C protein aggregation and misfolding than in the natural material.

In an alternative approach, the expression of rSP-C has been performed in eukaryotic systems such as baculovirus to enable posttranslational modification of the cysteine residues with the palmitoyl chains that are naturally present in human SP-C. Palmitoylated SP-C was expressed and purified by this method; however, the yield of the desired material was only 15% of the total product isolate (17). In addition, because of the hydrophobicity of the protein and other problems inherent to eukaryotic systems, protein expression levels were low. The activity of this palmitoylated rSP-C in comparison to nonacylated SP-C has not yet been investigated (17). Significant improvements are required to make protein production in insect cells a cost-effective means of producing SP-C for a commercial surfactant replacement. Moreover, although rSP-C-based LS is much safer than animal-derived LS replacements, there still exists the possibility of an unfavorable immune response from foreign proteins present in the vector (e.g., *E. coli* or baculovirus) used for the expression of rSP-C.

Regarding the larger and more complicated hydrophobic surfactant protein, SP-B, attempts have been made to express its mature form in *E. coli* using a truncated human SP-B cDNA (18). This recombinant protein, which ended up to be approximately eight residues larger than natural SP-B, was produced in *E. coli*, but expression levels were extremely low and it was not known to what extent the correct disulfide bonds had formed (Fig. 2a). The

limited recovery of this longer length SP-B version most likely relates to the hydrophobicity and surface activity of the protein (18,230). Future efforts to produce the correct sequence in high yield will involve the use of fusion proteins (e.g., fusion of SP-B with β-galactosidase) (18). To date, there has been no publication describing the expression of a functional, active, recombinant SP-B protein. Without a method that yields the correct sequence (with the correct fold and disulfide bonds) in sufficient quantity, the production of recombinant SP-B for therapeutic purposes will not be feasible. Therefore, the development of biomimetic SP mimics by other means is a worthwhile and important bioengineering goal.

B. Synthetic Polypeptides as Surfactant Protein Mimics

A more feasible solution for the development of biomimetic spreading agents is the use of organic chemical methods to synthesize polypeptide versions of SP-B and SP-C. Results from structural and physical experiments that provide structure–function correlations have been invaluable in the efforts to create successful SP mimics by this route (90,107,118,133–135,148,156,162,171,231). Designs for synthetic SP-B analogues have focused on mimicking the important structural features of the protein, including its amphipathic helices with opposing polar (i.e., positively charged) and hydrophobic faces (19,20,32). In the case of SP-C, designs have been created to mimic its hydrophobic helix, with attention also being paid to the two adjacent positive charges near the C terminus (146), palmitoylation of the cysteines (28,145,180), and the length of the valyl-rich helix (37 Å), which supposedly spans a lipid bilayer (27–29,232).

Synthetically engineered peptides circumvent many of the problems associated with current purification procedures for animal-derived surfactants, and should facilitate the production of sufficient quantities of material for its use as a therapeutic agent for treatment of other respiratory diseases, most particularly in children and adults. Moreover, a significant number of these synthetic, biomimetic polypeptide variants (some quite dissimilar in sequence from the natural SP-B and SP-C, as we will discuss) have been found to be biophysically functional in vitro and in vivo. A variety of synthetic polypeptide-based SP mimics have been successful to some degree in promoting the achievement of low surface tensions upon film compression, rapid respreading of surfactant lipids at an interface, and the rescue of prematurely born animals with RDS.

C. Polypeptide Analogues of SP-B

Because the SP-B protein is relatively large (with regard to what can be made on a peptide synthesizer) and structurally complex with its numerous disulfide bonds, both recombinant and chemical synthesis of the full-length human SP-B sequence are challenging endeavors. Based on a strong conservation of the SP-B sequence in various mammalian systems, peptide analogues have been templated on the human SP-B sequence, based on a desire to retain the correct structural configuration of the molecule (see Table 3 for SP-B designs). Researchers have designed, synthesized, and characterized synthetic peptide mimics of the full-length human SP-B protein, as well as truncated peptide sequences that represent different regions or domains of the protein (19–21,163,233,234). Because synthetic versions of full-length SP-B produced so far do not reproduce the three physiological disulfide bridges, heterogeneity and oligomerization of these peptides is highly likely. Nevertheless, this as well as other SP-B analogues based on synthetic peptides have been shown to have respectable biophysical activity both in vitro and in vivo. LS preparations that contain these synthetic peptides provide improved oxygenation and lung compliance in surfactant-deficient animal models (19,235).

Interestingly, the amino-terminal, amphipathic domain of human SP-B (amino acids 1–25) has been shown to adopt a helical conformation (236) and to possess many of the important surface-active properties of full-length SP-B protein, when added to biomimetic lipid mixtures such as the so-called Tanaka lipids [i.e., DPPC:PG:PA in a ratio of 68:22:9 by weight (160)]. Specifically, upon compression, an SP-B (1–25)/Tanaka lipid formulation reached low surface tension and was shown to improve oxygenation in rats in vivo (19,20,233). Interactions of this amino-terminal fragment of SP-B with anionic lipids is believed to induce the coexistence in the film of flat and buckled monolayers upon collapse, thereby reducing surface tension and improving respreading during film compression and expansion (163,164). However, the synthetic system including SP-B (1–25) in place of real SP-B protein has been shown to have a significantly slower rate of adsorption than natural surfactant, a disadvantage that reduces its therapeutic value (19,25,29).

Modification of the natural SP-B amino terminus (1–25), with substitutions of hydrophobic, charged, and/or oligomerizable residues targeted to potentially improve its surface activity, was not found to improve the adsorption rate of the molecule (20). To address this issue, peptides matching sequential, overlapping regions of SP-B were synthesized systematically, and each prepared with a lipid mixture of DPPC:PG (3:1 by weight). In vivo studies in fetal rabbit lung revealed that some of these

Table 3 Designs of SP-B Peptides

Design sequence	In vitro surface activity (mN/m)	In vivo performance	References
Full-length human SP-B (1–78) FPIPLPYCWLCRALIKRIQAMIPKGALAVA VAQVCRVVPLVAGGICQCLAERYSVILLDT LLGRMLPQLVCRLVLRCSM (note, does not have correct disulfide fold)	Both SP-B (1–78) and SP-B (1–60) confer biophysical activity to Tanaka lipids, with SP-B (1–78) having better dynamic behavior. However, both SP-B peptides show poor adsorption kinetics in comparison to Surfactant TA. LWSB at 1% (40–100% Area) SP-B (1–78): $\gamma_{min} \sim 0.2$ mN/m, $\gamma_{max} \sim 43.5$ mN/m SP-B (1–60): $\gamma_{min} \sim 2.1$ mN/m; $\gamma_{max} \sim 48$ mN/m Surfactant TA: $\gamma_{min} \sim 4.2$ mN/m; $\gamma_{max} \sim 40$ mN/m PBS at 1% SP-B (1–78): $\gamma_{min} \sim 3.3$ mN/m, $\gamma_{max} \sim 43.5$ mN/m SP-B (1–60): $\gamma_{min} \sim 22$ mN/m; $\gamma_{max} \sim 40.9$ mN/m Surfactant TA: $\gamma_{min} \sim 2.8$ mN/m; $\gamma_{max} \sim 40$ mN/m Adsorption kinetics (Note large standard deviation for SP) SP-B (1–78): $\gamma_{ads} \sim 43$ mN/m (1 min); 36 mN/m (5 min) SP-B (1–60): $\gamma_{ads} \sim 47$ mN/m; (1 min); 42 mN/m (5 min) Surfactant TA: $\gamma_{ads} \sim 30$ mN/m; (1 min); 24.6 mN/m (5 min) (22)	In vivo studies with excised rat lungs showed that SP-B (1–78) and SP-B (1–60) have similar % Total Lung Capacity values at 5 cm H_2O and 10-cm H_2O as Surfactant TA (% TLC ~ 95) (22) In vivo studies of lavaged rats showed that SP-B (1–78)-DPPC:POPG:PA improves a/A PO_2, and P-V mechanics to a slighter extent increases a/A PO_2 from 0.1 to 0.32 (235)	(22,235,238)
Truncated SP-B SP-B (1–60)			

N-terminus of human SP-B (1–25) FPIPLPYCWLCRALIKRIQAMIPKG and SP-B (49–66) LAERYSVILLDTLLGRnLL-CONH2	Studies with DPPC:POPG:PA (69:22:9) on LWSB SP-B (1–78) increases the hysteresis of compression-expansion curves when compared to lipids alone (235) The combination of SP-B (1–25) and SP-B(49–66) improves spreading of lipid mixture of DPPC:PG:PA (68:21:8). However, the SP-B peptides have slow adsorption kinetics in comparison to bovine LS. LWSB and PBS at 3 wt% B1, B2: $\gamma_{min} \sim 2$–9 mN/m; $\gamma_{max} \sim 59$–44 mN/m; $\gamma_{ads} \sim 51$ mN/m Bovine: $\gamma_{min} \sim 8$–7 mN/m; $\gamma_{max} \sim 43$–37 mN/m; $\gamma_{ads} \sim 40$ mN/m	In vivo studies with lavaged adult rats showed improvement in PaO_2 with the combo SP-B (1–25) and SP-B (49–66) after 75 min. Lipids: ~60 torr B1, B2: ~150 torr Bovine: ~250 torr	(19)

Table 3 Continued

Design sequence	In vitro surface activity (mN/m)	In vivo performance	References
Variants of N-terminal human SP-B (1–25) SP-B SP-B (C→A) SP-B (L, I→A) SP-B (R→K) SP-B (R, K→S) SP-B (R, K→E)	Adsorption and spreading of Tanaka lipids at 2 minutes were improved (slightly) with the presence of various SP-B mimics. The addition of SP-B (1–25) significantly reduces the minimum surface tension, to values that are acceptable, but the maximum surface tension is still high. SP-B: $\gamma_{min} \sim 55$ mN/m; $\gamma_{max} \sim 56$ mN/m SP-B (C→A): $\gamma_{min} \sim 54$ mN/m; $\gamma_{max} \sim 7$ mN/m SP-B (L, I→A); $\gamma_{min} \sim 51$ mN/m; $\gamma_{max} \sim 57$ mN/m SP-B (R→K); $\gamma_{min} \sim 57$ mN/m; $\gamma_{max} \sim 57$ mN/m LWSB of SP-B: $\gamma_{max} \sim 60$ mN/m; $\gamma_{min} \sim 5$ mN/m LWSB of lipids: $\gamma_{max} \sim 70$ mN/m; $\gamma_{min} \sim 55$ mN/m	Not available	(20)

N-terminus of human SP-B (1–25) FPIPLPYCWLCRALIKRIQAMIPKG	Investigation with various lipid mixtures shows that SP-B (1–25) improves surface activity by increasing the collapse pressure (i.e, reducing minimum surface tension) and altering it to be more reversible to enable respreading. Increases collapse pressure and alters the type of collapse as observed on LWSB and FM (164) 3 wt% SP-B (1–25) increases the hysteresis of P-A curve and retains this upon cycling of DPPC:PG:PA (68:21:9), liftoff ~90% trough area and π_c of 65 mN/m ~ 50% trough area (lipids alone, liftoff ~45% and π_c of 50 mN/m ~ 15% trough area) (165) 3 wt% SP-B (1–25) increases the π_c (alters it to a more reversible fold) of PA at 25°C (163, 165) 10 wt% SP-B (1–25) has activity comparable to SP-B (1–78) in conferring surface activity to DPPG:POPG (3:1): SP-B (1–25) or SP-B (1–78)–π_c ~ 60 mN/m and lipids–π_c ~ 52 mN/m (82)	Monomeric SP-B (1–25) in DPPC:POPG:PA (69:22:9) improves lung function in either premature rabbits or lavaged rats	(25,82,163,165)

Table 3 Continued

Design sequence	In vitro surface activity (mN/m)	In vivo performance	References
	However, dynamic adsorption surface tension is too high in comparison to native SP-B when added to DPPC:POPG (8:2) vesicles, as measured on CBS 1% SP-B 1–25: $\gamma_{ads} \sim 40.6$ mN/m 1% hSP-B: $\gamma_{ads} \sim 22.6$ mN/m (25)		
Fragments of human SP-B (1–81) FPIPLPYCWLCRALIKRIQAMIPKGALAVA VAQVCRVVPL_VAGGICQCLAERYSVILLD TLLGRMLPQLVCRLVLRCSMDD e.g., SP-B (59–80) SP-B (64–80) SP-B (52–81) SP-B (66–81)	Accelerated spreading of lipids: PBS; DPPG:PG (3:1) 10 mg/ml, with 10 wt% SP-B (64–80): $\gamma_{min} \sim 0$ mN/m in 5 min SP-B (59–80): $\gamma_{min} \sim 2.7$ mN/m in 5 min Native SP-B: $\gamma_{min} \sim 3$ mN/m in 15 sec	Improvement in static lung compliance in fetal lungs of rabbit with SP-B (52–81) and SP-B (66–81) Slower adsorption but shows improvement in lung compliance compared to native human surfactant for SP-B (64–80)	(21,233)
Bovine variants SP-1: LLGRLPNLVCGLRLRCSG SP-2: RLPNLVCGLRLRCSG Human variants SP-3: RMLPQLVCRLVLRCSMD SP-4: RMLPQLVCRLVLRCSM	Wilhelmy plate; DPPC:PG (3:1) SP-1: $\gamma_{ads} \sim 45$ mN/m in 5 min; $\gamma_{spread} \sim 60$ mN/m in 60 sec SP-3: $\gamma_{ads} \sim 65$ mN/m in 5 min; $\gamma_{spread} \sim 55$ mN/m in 1 min Bovine SP-B: $\gamma_{ads} \sim 30$ mN/m; $\gamma_{spread} \sim 20$ mN/m in 20 sec Crude bovine LS: $\gamma_{ads} \sim 20$ mN/m; $\gamma_{spread} \sim 15$ mN/m in 20 sec	Not available	(23)

Human SP-B bend (35–46) and variant NB: Cys_{35}RVVPLVAGGICys_{46}-$CONH_2$ MB: Ac-Cys_{35}RVVPDSerHisGGI$Cys_{46}CONH_2$	Not available	Not available	(115)
Dimeric N-terminal human SP-B (1–25) FPIPLPYCWLARALIKRIQAMIPKG FPIPLPYCWLARALIKRIQAMIPKG	CBS; DPPC:POPG:PA (7:2:1) vesicle $\gamma_{ads} \sim 40$ mN/m (compare to 23 mN/m for hSP-B); $\gamma_{min} \sim 0$ mN/m; $\gamma_{max} \sim 42$ mN/m Requires larger compression to reach zero surface tension when compared to hSP-B	Not available	(25)

synthetic peptide surfactants did provide an increase in lung compliance but that their adsorption rates were still substantially slower than that of native human surfactant (233). Other studies of the activity of different regions of SP-B peptide have revealed that peptide fragments including the carboxyl-terminal residues and composed of at least 17 amino acids accelerate surfactant spreading and improve static lung compliance in premature rabbits (21,22,233). Other modified, truncated SP-B fragments derived from both bovine and human sequences have been synthesized and characterized; the surface activities of these peptides have been studied on an LWSB. Interestingly, higher activity was observed in analogues that also showed greater overall helicity by circular dichroism (CD) spectroscopy (23).

Since the natural, dimeric form of SP-B protein is considered to be of importance for optimal function of LS replacements, a dimeric version of the *N*-terminal segment of SP-B has been synthesized. In particular, a dimeric form of SP-B (1–25) was engineered by the replacement of the cysteine at position 11 with an alanine, whereas the remaining cysteine at position 8 was used for dimerization via disulfide bonding. Comparison of monomeric and dimeric SP-B (1–25) in premature rabbit and lavaged rat models show that the dimeric form is more efficient than the monomer in improving lung function (25). However, natural SP-B is dimerized through residue 48 in its sequence, distal from the 1–25 sequence used in this mimic.

D. Polypeptide Analogues of SP-C

In contrast to SP-B, the SP-C protein is monomeric and quite small; therefore, it is feasible to chemically synthesize a full-length version of it. SP-C analogues have been designed to mimic both the sequence and the folded conformation of the human protein (see Table 4). A challenging aspect in the synthesis of human SP-C is engineering the attachment of the two adjacent palmitoyl groups at positions 5 and 6, the absence of which can lead to the formation of irreversible β-sheet aggregates of SP-C (237) and to an accompanying reduction in surface activity (142). Two different approaches that have been taken for synthetic SP-C palmitoylation are (a) use of succinylamidyl palmitate derivatives (238) and (b) formation of a thioester linkage via a palmitoyl chloride reaction (239).

Full-length and truncated *non*palmitoylated versions of human SP-C protein have already been evaluated as components of an artificial surfactant (26). These SP-C mimics, in conjunction with a DPPC:PG:PA lipid mixture (75:25:10 by weight), display promising activity in vitro on an LWSB, as well as in vivo in lung pressure–volume curves obtained for premature rabbits treated with the material. Moreover, it was shown that a nonpalmitoylated core sequence containing residues 5–31 or 6–32 of the

Table 4 Designs of SP-C

Sequence	In vitro activity	In vivo activity	References
rSP-C (Cys)2 Scios-Nova Inc, Sunnyvale, CA, USA	Not available	Good surface activity but reduced inhibition of protein plasma disruption as compared to native SP-B (298) Acylation influences physical properties (142), but does not increase physiological activity of rSP-C(Cys)2 in premature rabbits (15)	(15,298,299)
rSP-C(Cys→Phe; Met→Ile) Byk Gulden Pharmaceuticals, Konstanz, Germany Fragment of modified canine SP-C (nonpalmitoylated) IPCFPSSLKRLLAVAVAVALAVAVIAGLLMGL	Wilhelmy surface balance with DPPC:POPG (70:30): 5% PA:2% rSP-C showed good surface activity as compared to natural surfactant (16).	Improved lung function, but to lesser extent than natural surfactant in ventilated preterm lambs and rabbits (16)	(16,229,300,301)
	PBS with DPPC:PG:PA (68:22:9) with 1wt%SP-C (canine, 1–31) $\gamma_{ads} \sim 26$ mN/m (compared to bovine ~ 26 mN/m) $\gamma_{min} \sim 20$ mN/m	Not available	(238)

Table 4 Continued

Sequence	In vitro activity	In vivo activity	References
Fragments of human SP-C (nonpalmitoylated) SP-C (6–32) SP-C (5–31)	LWSB with DPPC:PG:PA (75:25:1) With SP-C: $\gamma_{spread} \sim 31$ mN/m within 30 sec; $\gamma_{ads} \sim 41$ mN/m within 60 sec Alone: $\gamma_{spread} \sim 48$ mN/m within 30 sec; $\gamma_{ads} \sim 55$ mN/m Bovine SP-C: $\gamma_{ads} \sim 37$ mN/m	Tracheal instillation in immature rabbits improved P-V characteristics to level similar to mature neonates	(26)
Variant of human SP-C Dipalmitolyated SP-C(A) FGIPC*C*PVHLKRLLAVAVAVALAVAVAVGALLMGL:	Wilhelmy balance with DPPC:POPG:PA shows that dipalmitolyated SP-C (A) confers surface activity	Improves lung function in ventilated, lavaged rats and premature rabbits (235, 240)	(24,235,240)
Variants of porcine SP-C LRIPCCPVNLKRLLVVVVVVVLVVVVIVGALLMGL SP-C (CC): C nonpalmitoylated SP-C (FF): C→F SP-C (SS): C→S SP-C (AAA): R1, L10, K11→A SP-C/BR: LRIPCCPVNLKRFYAITTLVAAIAFTLYLSLLLGY	Wihelmy balance with DPPC:PG:PA (68:22:9) Native porcine SP-C: $\gamma_{spread} \sim 37$ mN/m at 20 sec SP-C (CC): $\gamma_{spread} \sim 37$ mN/m at 30 sec	Not available	(28)

	SP-C (FF); SP-C (SS); SP-C (AAA): SP-C/BR: $\gamma_{spread} \sim 42$ mN/m at 30 sec		
Variants of porcine SP-C (nonpalmitoylated) SP-C (1–12) SP-C (1–17) SP-C (1–21)	Platinum Plate with DPPC:PG (7:3) Adsorption kinetics (π_{ads}): only SP-C (CC) reproduce native with $\pi_{ads} \sim 48$ mN/m	Not available	(232)
Variant of human SP-C SP-C (Leu) FGIPSSPVLKRLLILLLLLLLILLLILGALLMGL: Dipalmitoylated SP-C (Leu)	SP-C (Leu) has similar activity to native SP-C. However, both SP-C mimics give relatively high maximum surface tension when compared to Curosurf, which contains both SP-B and SP-C. PBS with DPPC:PG:PA (68:22:9) at 10 mg/ml SP-C (Leu): $\gamma_{min} \sim 0$ mN/m and $\gamma_{max} \sim 35$–43 mN/m Native SP-C: $\gamma_{min} \sim 0$ mN/m and $\gamma_{max} \sim 42$ mN/m	Airway instillation in preterm rabbits improved dynamic lung compliance by 30% compared with untreated controls with SP-C (Leu)	(29,239,302)

Table 4 Continued

Sequence	In vitro activity	In vivo activity	References
	Curosurf: $\gamma_{min} \sim 0$ mN/m and $\gamma_{max} \sim 30$ mN/m Wilhelmy balance (10 mg/ml) SP-C (Leu): $\gamma_{spread} \sim 25$–30 mN/m native SP-C:$\gamma_{spread} \sim 25$–30 mN/m		
Variant of human SP-C SP-C (LKS) FGIPSSPVHLKRLLILKLLLLKILLLKLGALLMGL	SP-C (LKS) improves surface activity of the lipids PBS with DPPC:PG:PA(68:22:9) or DPPC:PG (7:3) SP-C (LKS): $\gamma_{min} < 1$; mN/m; $\gamma_{max} \sim 42$ mN/m Wilhelmy balance with DPPC:PG (7:3): SP-C (LKS): $\gamma_{spread} \sim 28$ mN/m after 3 sec	Not available	(30)

total 35 amino acids seems to be sufficient to mimic virtually full biophysical activity both in vitro and in vivo (26). However, other researchers have synthesized the full-length SP-C peptide and palmitoylated it with succinylamidyl palmitate, and have reported that the hexadecyl modification of cysteine residues 5 and 6 is critical for the protein's surface activity and biophysical function (145,147). Furthermore, modified, dipalmitoylated SP-C peptide in combination with the Tanaka lipid formulation was also shown to improve lung function in lavaged rats (235) and in premature rabbits (240).

In the canine SP-C protein, a phenylalanine residue is substituted for one of the palmitoylated cysteines (28,98). Hence, another approach to the synthesis of SP-C mimics has been the replacement of palmitoylated cysteine residues with phenylalanine residues, a mimic that has been called SP-Cff (28). Other analogues have introduced serine substitutions for the two cysteine residues (28); however, in vitro results that differ substantially from natural SP-C performance have been reported. Specifically, both Ser- and Phe-substituted SP-C analogues, in combination with the Tanaka lipid mixture, were found to have inferior spreading properties in comparison to a formulation that contains natural or native SP-C (28).

Studies have shown that the α-helical conformation of SP-C protein is important for the rapid spreading and low surface tension that are exhibited by lung surfactant (28,232). Thus, the poor performance generally observed for LS replacements that contain synthetic SP-C has been attributed to a low α-helical content of the polypeptide (28) due to incorrect folding of chemically synthesized SP-C (241). In its physiological environment, SP-C protein exists in complexation with a high concentration of lipids, which enable the proper folding and subsequent structural stability of the natural chain configuration. In the absence of lipids, the native polyvaline stretch, which is extraordinarily hydrophobic, has a strong tendency to misfold into β sheets and aggregate in nonphysiological environments (241).

To overcome these challenges in synthetic SP-C production, several SP-C analogues have been designed with modified sequences in the hydrophobic stretch to maximize helicity and minimize β-sheet formation and aggregation. In one design, the polyvaline stretch was replaced with the transmembrane helical region (42–64) of bacteriorhodopsin (BR), reportedly resulting in an SP-C/BR analogue with secondary structure and spreading kinetics similar to native SP-C (28). In other SP-C analogues, replacement of the valine residues with either polyleucine or polynorleucine residues was shown to enable the rapid surface spreading activity of the natural protein in vitro, and to improve static lung compliance in preterm rabbits to levels comparable with that of natural lung surfactant (e.g., Beractant) (27). Another design approach prescribed the replacement of all

the valine residues with leucine residues, the substitution of both palmitoylcysteines with serines, and the deletion of histidine from the sequence. This SP-C (Val→Leu; Cys→Ser) analogue was determined to have a helical, transbilayer orientation by Fourier transform infrared spectroscopy (29) and reportedly to exhibit in vitro surface activity resembling that of native SP-C (29,30). In vivo studies of this SP-C mimic, in combination with the Tanaka lipid cocktail in rabbits, showed 30% improvement in dynamic lung compliance when compared with untreated premature rabbits (29). However, unlike modified natural surfactant (242), this SP-C analogue did not succeed in restoring dynamic compliance to healthy levels (29). Then again, the material that was tested lacked SP-B, having only a mimic of SP-C, so this may not have been a fair comparison to natural LS, which contains both SPs.

Part of the problem with the modified SP-C (Val→Leu; Cys→Ser) peptide may arise from its ability to aggregate via hydrophobic association of the polyleucine stretch (29). To circumvent this difficulty, one group introduced lysine residues at positions 17, 22, and 27 of this region to locate positive charges around the helical circumference and thus prevent hydrophobic aggregation by ionic repulsion. This SP-C (LKS) analogue showed good surface activity but was inferior to native surfactant, and showed a particularly high dynamic maximum surface tension ($\gamma \sim 42$ mN/m) (30).

What is striking about these studies is that many groups have designed peptide mimics of surfactant proteins B and C, and all have achieved some degree of success in creating useful LS replacements using these diverse SP mimics (Tables 4 and 5). This provides strong evidence that this biomaterial system is tolerant to modification, at least for its use in acute replacement therapy. This is perhaps to be expected, since it seems that surfactant proteins interact primarily with lipids, which is likely to be an interaction with much less specificity than many types of biomolecular associations. Researchers are also making progress in the development and characterization of simplified peptide mimics of SP-B and SP-C for use as biomimetic spreading agents in exogenous LS replacements.

E. Simplified Peptide Mimics of SP-B and SP-C

Strict sequence conservation may not be necessary to retain the proper, helical secondary structure and surface activity of the native surfactant proteins. Some groups have created simplified, amphipathic peptides and tested them as mimics of SP-B and SP-C (Table 5). Small peptides offer the advantages of being less immunogenic (243), easier to produce, and less costly than long-chain peptides.

Table 5 Simplified SP-Mimics Based on Synthetic Polypeptides

Sequence	In vitro activity	In vivo activity	References
KL4: KLLLLKLLLLKLLLLKLLLL K	PBS with DPPC:POPG (3:1):15 wt% PA: 3 WT% KL4	Increases dynamic lung compliance and oxygenation in premature rabbits and monkeys	(29,33,244,245,247,302)
	Wilhelmy with DPPC:PG:PA (68:22:9) with 2 wt% $\gamma_{spread} \sim 37$ mN/m at 60 sec (29, 244)	Improves lung expansion and gas exchange in infants: a/A $PO_2 \sim$ 0.4 by 12 h (normal is 0.4)	
KL2,3: LLLKLLKLLLKLLKLLLKLL L	Wilhelmy with DPPC:PG:PA (68:22:9) with 2 wt% $\gamma_{spread} \sim 50$ mN/m at 60 sec	Not available	(29,244)
WMAP10: Suc-LLEKLLEWLK-$CONH_2$	PBS with DPPC $\gamma_{max} > 45$ mN/m (canine LS $\sim$ 30 mN/m) and $\gamma_{min} < 4$ mN/m (canine LS $\sim$ 8)	Almost complete restoration (92%) of static pulmonary compliance in lung-lavaged rats	(29,32)
	Wilhelmy with DPPC:PG:PA Slow spreading kinetics: $\gamma_{spread} \sim 45$ mN/m at 60 s	Restore a/A PO_2 in lung-lavaged guinea pigs ($> 85\%$)	

Taking the advantages of small peptides into account, water-soluble synthetic peptides have been designed with sequences unrelated to native protein SP-B, but also coding for helical amphipathic structures, in the hope that these molecules will have suitable physical properties for an LS replacement. An amphipathic α-helical peptide called 18As (a 24-residue peptide from the lipid binding region of plasma apolipoprotein) was designed and was shown, in combination with DPPC, to be reasonably effective as an LS replacement both in vitro and in vivo (31). The success of this DPPC/18As formulation led McClean et al. (32) to develop a series of model amphipathic α-helical peptides (MAP) to be tested as potential spreading agents for DPPC. Of these, WMAP10 (succinyl-LLEKLLEWLK amide) has shown the greatest promise as a spreading agent, purportedly because it was designed to be optimally helical, by (a) facilitating the formation of salt bridges between side chains; (b) neutralizing the negative charge on the C terminus; and (c) introducing a negative charge at the *N* terminus. In vivo testing of WMAP10 function demonstrated the restoration of acceptable pulmonary compliance (32), but in vitro experiments showed slow spreading of the material at the air–liquid interface (reaching $\gamma_{spread} \sim 45$ mN/m, which is also a relatively high surface tension for an LS replacement) (29,32).

In work along the same lines, Cochrane et al. (33) developed the 21-residue peptide KL4, with a repeat sequence of lysine followed by four leucine residues. The design of KL4 was patterned after the amphipathic characteristics of the SP-B helical domain (SP-B 59–80), which had been shown in prior experiments to be biophysically active as a peptide fragment (233). KL4 is also reasonably active as an LS spreading agent. It has been proposed that the KL4 peptide associates with the peripheral regions of the lipid bilayer in such a way that positively charged residues interact with the polar lipid headgroup while the hydrophobic stretch interacts with lipid acyl side chains (33). Contrary results, which seem more likely to be correct, suggest that the KL4 peptide is in a transbilayer orientation in a lipid environment (244). Regardless of the orientation of KL4, in combination with a lipid mixture of DPPC:POPG (3:1), the peptide has been shown to create an active exogenous surfactant for the treatment of immature newborn rabbits (33) and rhesus monkeys (245,246). However, concerns about the poor spreading kinetics of the KL4/lipid mixture (to $\gamma_{spread} \sim 37$ mN/m) have been raised, and are evidenced by a reduced efficiency of this novel formulation in comparison with native SP-C and SP-C/BR in combination with a Tanaka lipid mixture (244). This may explain why, when KL4 was added to a bovine-derived LS with low SP-B content (Survanta), oxygenation was not improved in lavaged adult rats as compared to the use of Survanta alone (24). Nevertheless, and more

importantly, premature infants treated with a mixed KL4-Surfacten formulation (DPPC:POPG with a ratio of 3:1 with 15% PA and 3% KL4, by weight) do show restoration of arterial-to-alveolar oxygen tension ratios to a normal range within 12 h, suggesting a reasonably good efficacy of this surfactant replacement (247). Though promising, the clinical efficacy and safety of this formulation has yet to be fully established because of the limited number of patients in which it has been studied. Furthermore, comparisons with other commercially available surfactants are so far unavailable. Currently, this formulation is referred to as Surfaxin (U.S.-adopted name Sinapultide) and is produced by Discovery Laboratory (Doylestown, PA). Pivotal phase 3 trials are now being established to evaluate the efficacy and safety of Surfaxin for treatment of premature infants with idiopathic RDS (8). This trial will study approximately 1500 premature infants in Latin America and is designed to compare Surfaxin with currently available surfactant replacements (248).

F. Polypeptoid-Based SP Mimics

A more unusual approach to biomimicry of the SP proteins is to develop analogues based on nonnatural peptide mimics that offer greater in vivo stability and easier production while still mimicking the helical, amphipathic structure of the natural molecules. Along these lines, an alternative and novel biomimetic surfactant replacement currently under development in our laboratory is based on the use of poly-*N*-substituted glycine, or *polypeptoid*, SP mimics as additives to lipid mixtures. Polypeptoids are nonnatural, sequence-specific polymers that are based on a peptide backbone but differ from peptides in that their side chains are appended to the tertiary amide nitrogen rather than to the α carbon (Fig. 7) (249). This difference in structure has been shown to result in virtually complete protease resistance for peptoid analogues (250). Peptoids also offer the advantages of low immunogenicity (251), facile production on a peptide synthesizer (252), and a low cost relative to synthetic peptides. Peptoids with α-chiral side chains have been designed and shown to form stable, helical, secondary structures by CD (252,253), 2D-NMR (254), and molecular modeling (255). Because peptoids are N-substituted and hence lack amide hydrogen bonds, they cannot form β-sheets. Previous studies have established that polypeptoid helices are extremely stable and monomeric, with no tendency to misfold and aggregate (252,253,256). Therefore, peptoids seem to be quite promising for the development of effective spreading agents for LS formulations.

Exploiting similar strategies as have been used in the design of peptide-based SP-B and SP-C analogues, sequence-specific peptoid-based SP mimics

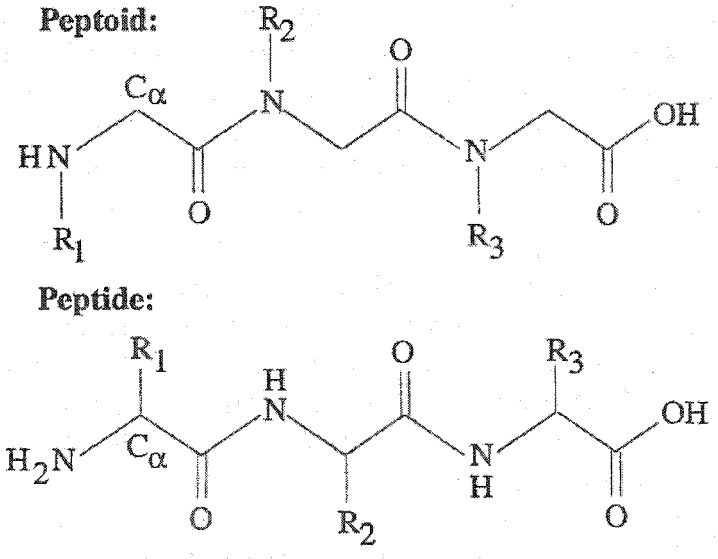

Figure 7 Comparison of the structures of peptide and peptoid trimers with arbitrary side chains R_1, R_2, and R_3. Peptoid structure differs from peptides in that the side chains are appended to the backbone nitrogen instead of the α carbon. (Adapted from Ref. 249, with permission.)

have been designed to capture both the amphipathic and three-dimensional structural characteristics of these proteins that are critical for their proper biophysical functioning. Studies so far have focused on SP-C analogues, which have been designed with a hydrophobic, helical stretch that spans a DPPC bilayer and that conserves the patterning of charged and hydrophilic residues found in natural protein. These peptoid-based SP mimics have been shown to be stably helical by CD and highly surface active by LWSB. In conjunction with various lipid mixtures (e.g., DPPC:POPG, 7:3, or Tanaka lipids), peptoid-based SP mimics have shown promise in vitro on the Langmuir-Wilhelmy surface balance and pulsating bubble surfactometer (257). Further investigations are necessary for evaluation of the efficacy and safety of these nonnatural materials, but these biostable analogues hold promise for treatment not only of RDS but of other respiratory diseases caused by the deficiency and dysfunction of LS.

XVII. FUTURE DIRECTIONS IN THE DEVELOPMENT OF BIOMIMETIC LS REPLACEMENTS

The development of a functional, reliable, safe, less immunogenic, and lower cost biomimetic lung surfactant replacement will be beneficial for the

effective prevention and improved treatment of preterm infant RDS (258). The eventual impact of such a formulation on neonatal and perinatal health could be great (34–36), as it is likely that the indications for surfactant replacement therapy in infants will expand in the future to include other lung diseases that have surfactant dysfunction as an element of their pathogenesis (259,260), including meconium aspiration syndrome (261), congenital pneumonia (262), pulmonary hypoplasia, and pulmonary hemorrhage. To make the use of surfactant replacement therapy feasible for an expanded list of infant respiratory disorders and a greater number of patients worldwide, both the immunogenicity and the cost of exogenous surfactant replacements must be minimized (259).

Adults and children will also benefit from the development of a less immunogenic and less expensive synthetic formulation (34–36). The dysfunction of lung surfactant is a major contributor to the lethal "acute RDS (ARDS)," which can occur in adults and children after shock, bacterial sepsis, hyperoxia, near-drowning, or apiration (263–265). RDS is the leading cause of death in intensive care units, and there is no generally effective and economically viable treatment for it (266–268). The dysfunction of lung surfactant in adults and children most typically results from the encroachment of blood serum or other foreign fluids into the lungs. Serum proteins can disrupt and inhibit the spreading of the natural surfactant monolayer by a number of mechanisms (71,269). There has been indication in a few studies that LS replacement therapy may be effective for treatment of adult and child ARDS (270–273). However, adult therapy requires much larger dosage, making this treatment prohibitively expensive and unfeasible (274). In addition, there are important issues of LS inactivation and immunogenicity using LS replacements in adults because of their highly developed adaptive immune systems. Recent clinical work also suggests that surfactant dysfunction may play a role in the pathogenesis of cystic fibrosis and that specially designed surfactant replacements could prove beneficial to cystic fibrosis patients (275).

XVIII. CONCLUSIONS

The design of biomaterials that effectively mimic the structure and function of natural materials but that have the advantages of low immunogenicity and good bioavailability is a challenging area of bioengineering. An important and tractable problem in biomaterials research is the need for more effective and bioavailable LS replacements for the treatment of respiratory disease.

Before venturing into the development of complex material such as a biomimetic LS replacement, we must begin by recognizing both the need for this material and the specific areas for improvement in the present therapy. An integral step in the development process is to exploit knowledge of the structure–function relationships of the natural system to provide the design criteria. In vitro and in vivo studies are providing a better understanding of the role of LS components. The saturated lipids, particularly DPPC, are involved in reducing surface tension to near zero, whereas the unsaturated lipids and proteins have a role in facilitating adsorption and respreading of the LS films. In particular, the surfactant proteins B and C are responsible for enhancing lipid adsorption, respreading, and for stabilizing the surface film.

We are beginning to understand the composition and functions of LS, allowing improved designs of novel LS replacements. Researchers are currently developing biomimetic LS replacements based on recombinant and chemical production of SP-B and SP-C analogues. Successful expression and isolation of recombinant SP offers the advantage of producing a large quantity of material, potentially at a relatively low cost. However, production of recombinant SP mimics is challenging because of the difficulty in expressing these proteins with the correct conformation and in purifying them in high yield due to their highly hydrophobic nature. An alternative approach has been to chemically synthesize full-length, truncated, and modified versions of the SP. The chemical synthesis route offers ease of production and has enabled the investigation of numerous peptide analogues. Additionally, this synthetic route offers the reduction/elimination of viral contamination and immunogenicity risks. It is chemically challenging to synthesize peptide analogues of SP-B and/or SP-C with the correct fold, but this may not necessarily be requisite for a functional LS replacement. It is interesting to note that a number of different amphipathic peptide designs have been successful in mimicking the biophysical and physiological roles of LS, to some extent. Most of the LS formulations have been based on the use of either SP-B or SP-C mimics (not both). Since both SP-B and SP-C are present in natural LS, biomimetic LS formulations most likely would be improved by the presence of both SP analogues. In addition, a better understanding of the individual roles of SP-B and SP-C would facilitate the design of improved biomimetic LS replacements.

An exciting aspect of developing these biomimetic LS replacements is the capacity to tailor their formulation to treat respiratory diseases with varying degrees of surfactant deficiency, insufficiency, and inactivation. There is an indication that LS therapy may also be effective for treating adult RDS, meconium aspiration syndrome, and pneumonia. However, the

use of current commercially available LS replacements (natural and synthetic) is not yet viable for the treatment of adults; treatment of adult respiratory dysfunction will be one major application of a biomimetic LS replacement.

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