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Delineation of the dynamic properties of individual lipid species in native and synthetic pulmonary surfactants☆

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ABSTRACT

Pulmonary surfactant (PS) is characterized by a highly conserved lipid composition and the formation of unique multilamellar structures within the lung. An unusually high concentration of DPPC is a hallmark of PS and is critical to the formation of a high surface area, stable air/water interface; the unusual lipid polymorphisms observed in PS are dependent on surfactant proteins, particularly lung surfactant protein B (SP-B). The molecular mechanisms of lipid trafficking and assembly in PS remain largely uncharacterized. Using ^{2}H and ^{31}P NMR, we characterize the dynamics and polymorphisms of the major lipid species in native PS and synthetic lipid mixtures as a function of SP-$B_{1\text{-}25}$ addition. Our findings point to increased dynamics and a departure from a lamellar behavior for DPPC on addition of the peptide, consistent with our observations of DPPC phase separation in native surfactant. The monounsaturated lipids POPC, POPG and POPE remain in a lamellar phase and are less affected than DPPC by surfactant peptide addition. Additionally, we demonstrate that the properties of a native PS can be successfully mimicked by using a fully synthetic lipid mixture allowing the efficient evaluation of peptidomimetics under development for PS replacement therapies via NMR spectroscopy. The specificity of the dynamic changes in DPPC relative to POPC suggests the importance of tuning partitioning properties in successful peptidomimetic design. This article is part of a Special Issue entitled: NMR Spectroscopy for Atomistic Views of Biomembranes and Cell Surfaces. Guest Editors: Lynette Cegelski and David P. Weliky.

1. Introduction

Mammals require high oxygen uptake, which is facilitated by the large inner surface area of the lung at 300 cm^2 per cubic centimeter [1]. A lipid/protein complex known as pulmonary surfactant (PS) lines the inside of the alveoli and reduces the work of breathing by minimizing surface tension [2]. Respiratory Distress Syndrome (RDS) occurs in premature infants, who produce inadequate amounts of PS, as well as in older children, due to low PS activity caused by lung injury [3]. PS is synthesized, processed, stored, secreted, and recycled by type II pneumocytes. It is secreted to the alveolar subphase as specialized organelles known as lamellar bodies (LB) and recycled every 5–10 h. Secreted LB can fuse to form tubular myelin (TM) while some LB maintain their packed structure; both TM and LB contribute to the in vivo formation of the interfacial film important for oxygen exchange by unraveling and adsorbing onto the air/water interface [4]. The molecular level processes by which PS lipids are trafficked to the air/water interface have been explored in several recent in vitro studies [5–9]. A fully saturated, surface stable phospholipid, dipalmitoylphosphatidylcholine (DPPC), found at high levels in PS, is thought to be the major component of the surface film; whether it is specifically trafficked to the interface or most of the non-DPPC components of PS are squeezed out during the compression/expansion cycles leaving a DPPC-rich surface film or a combination of these processes occur in the PS cycle in vivo is unknown [4]. The molecular level details of the unusual dynamics and organization of LB and TM in the aqueous phase also remain poorly characterized.

PS is an ideal system for studying the molecular basis of protein mediated lipid trafficking and/or assembly in an aqueous environment. Its simple lipid and protein composition (relative to the plasma membrane) is highly conserved among mammalian species, with lipids making up >90% of the LB and TM. A summary of the lipids found in PS is presented

Abbreviations: PS, pulmonary surfactant; CLSE, calf lung surfactant extract; SP-B, surfactant protein B; MLV, multilamellar vesicle; RDS, respiratory distress syndrome; DPPC, 1, 2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine; DPPC-d_{62}, 1,2-d_{62}-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine; P/L, peptide/lipid molar ratio; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine; POPC-d_{31}, 1-d_{31}-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylglycerol; POPG-d_{31}, 1-d_{31}-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylglycerol; POPE, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylethanolamine; POPE-d_{31}, 1-d_{31}-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylethanolamine; Chol, cholesterol

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in Table 1 [4,10–12]. Zwitterionic phospholipids (primarily phosphatidylcholines) make up ~85% of total PS phospholipids; almost half of the phosphatidylcholine component is 1, 2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC). The two saturated acyl chains of DPPC enable it to be packed tightly in the monolayer at the air/water interface; the stability of DPPC monolayers to lateral pressure during compression is thought to be critical to the integrity of this interface in the lung. The melting temperature of neat DPPC is 41 °C; the remaining PC lipids are monounsaturated and likely increase the fluidity of the DPPC rich PS, accelerating lipid trafficking and surface film formation [11]. While LB and TM are primarily lamellar, they do not have the same fluid consistency as cell membranes and their multilamellar structures are packed more tightly together than is typically observed for other lipid assemblies (e.g. intracellular organelles); it has been hypothesized that non-lamellar polymorphisms, such as the cubic or hexagonal phases, may play a crucial role in the organization and function of PS [13].

Although lipids underpin the macromolecular assemblies important to PS function, their unique properties are dependent on low levels of surfactant proteins. In particular, surfactant protein B (SP-B), which is highly hydrophobic and present at low levels (<2% by weight), is critical to LB/TM integrity and the formation of a viable air/water interface [14,15]. Humans with genetic SP-B dysfunction die soon after birth as do genetically engineered SP-B null mice. SP-B is a particularly challenging protein to produce or isolate due to its high hydrophobicity, so animal sources of PS are commonly used in treating RDS, posing a risk of infection or immune response [16]. In particular, calf lung surfactant extract (CLSE) is a surfactant replacement therapy prepared from chloroform extracts of lavaged PS from calf lungs [2]. It is commonly administered to premature infants with RDS under the name *Infasurf*. The lipids in CLSE are unusually surface active and form unique aqueous assemblies similar to native PS due to low levels of surfactant proteins SP-B and SP-C. CLSE contains 93% phospholipid, 5% cholesterol and neutral lipids, and 2% SP-B and SP-C by weight and thus closely mimics human PS.

Given the tremendous importance of SP-B to PS function, surfactant replacement methods employing simple surface-active peptide analogs of SP-B have also been investigated as they can be easily produced with high yield and purity. SP-B_{1-25} is an amphipathic peptide composed of the first 25 amino acids of the N-terminus of SP-B which retains much of the biological activity of full length SP-B and is more resistant to inhibition by plasma proteins infiltrating the lung during injury [17–22]. Understanding how it functions in the PS lipid environment would allow the development of mimetics which are even more stable. CD and FTIR indicate the presence of helical structure in the C-terminal portion of SP-B_{1-25} when it is associated with lipid monolayers [20]; the very hydrophobic N-terminal tail of SP-B_{1-25} enables rapid insertion into lipid films [8]. Previously we have shown that the first several amino acids are critical to the induction of uncommon lipid polymorphisms by SP-B_{1-25} in synthetic binary lipid systems with distinct differences between DPPC/POPG and POPC/POPG mixtures [9]. These results led us to study lipid polymorphisms in more complex lipid mixtures, including therapeutic CLSE, to gain a deeper understanding of lipid behavior in PS.

The goals of this study are threefold: 1) to individually characterize the dynamics of the major lipid species in therapeutic CLSE, 2) to compare lipid dynamics in CLSE to a completely synthetic lipid system based on PS composition, and 3) to examine how lipid dynamics and organization are affected by the addition of the PS peptide SP-B_{1-25} via ^{31}P and ^{2}H static ssNMR experiments. ^{31}P spectroscopy allows monitoring of lipid dynamics and polymorphisms for all lipid species in a given sample while ^{2}H spectroscopy allows monitoring of the dynamics and polymorphisms of individual lipid species that are deuterium enriched and in particular the dynamics of the deuterated lipid acyl chains.

Table 1
Lipid composition of native PS and lipid mixtures used in this study.

	Phospholipid composition (%total) [% disaturated]								Cholesterol
Source	PC	LPC	SM	PG	PI	PS	PE	LBPA	(%chol/PL)
Human	80.5 [47.7]	Tr	2.7	9.1	2.6	0.9	12.3	NR	7.3
Bovine	79.2 [49.9]	Tr	Tr	11.3 [33.3]	1.8	Tr	3.5	2.6	3
$CLSE^{T}$	>75 [>60]	NR	NR	NR	NR	NR	NR	NR	NR
$CSLE^{S}$	76.2 [62.5]	–	–	14.3	–	–	9.5	–	9.5

2. Materials & methods

2.1. Materials

DPPC, POPC, POPG, POPE, DPPC-d_{62}, POPC-d_{31}, POPG-d_{31} and POPE-d_{31} were purchased as chloroform solutions from Avanti Polar Lipids (Alabaster, AL) and quantified by phosphate analysis (Bioassay Systems, Hayward, CA); cholesterol in powder form was also purchased from Avanti. Research grade calf lung surfactant extract (CLSE) was generously provided as a gift from ONY, Inc. (Amherst, NY). CLSE is a chloroform extract of natural surfactant from calf lungs manufactured by ONY, Inc., as the pharmaceutical drug product *Infasurf*. Upon receipt, the chloroform was evaporated and CLSE was lyophilized for longer storage stability. CLSE contains 93–101 mg/ml of total phospholipid and ~1.3 and ~0.7 mg/ml of surfactant proteins B and C (SP-B and SP-C), respectively. Unless otherwise stated, other reagents were purchased from Fisher Scientific (Hampton, NH) and used as received.

2.2. Biochemical separation of CLSE lipids and proteins

To make samples of CLSE lacking surfactant proteins, the proteins were separated from the lipids by gel permeation chromatography with a Sephadex LH-20 (GE Healthcare) resin and 95:95:10 chloroform:methanol:0.1 N HCl (v/v/v) as the running solvent using previously established methods [23]. Eluent fractions were assayed by phosphate and protein analyses. Inorganic phosphate was liberated from the phospholipids and quantified to determine phospholipid concentration via a colorimetric assay (Bioassay systems) [24]. Protein content was assayed via the Amido Black Protein Assay [25,26]. These assays are sensitive to μg quantities and were used to monitor the separation. Fractions containing only protein or only phospholipid were separately pooled and extracted into chloroform to remove acid; fractions containing both lipids and proteins were pooled, concentrated and run over the column a second time. Phosphate and protein assays indicated successful separation of CLSE lipids and proteins after a second pass through the column. Due to the small, undetectable concentration of cholesterol in the tail end of the eluent with each run, the column was flushed with an additional volume of chloroform:methanol:0.1 N HCl at the end of each separation to recover the cholesterol. Concentrated cholesterol was identified by TLC and the collected cholesterol was added to the phospholipid fractions. The combined lipid fractions were dried with nitrogen gas and then lyophilized from cyclohexane. Actual lipid concentrations for the purpose of making NMR samples were determined after combining all lipid fractions from every pass through the column. The amount of protein isolated was too small for the purposes of this study and was not used further.

2.3. Preparation of synthetic lipid mixtures

A synthetic PS lipid system was also studied for comparison to CLSE and earlier studies of the binary mixture 4:1 DPPC/POPG [7–9]. The phospholipids were purchased as chloroform solutions and mixed after verifying their concentrations by phosphate analysis (Bioassay Systems, Hayward, CA). Cholesterol was obtained as a dry powder and dissolved in chloroform. Appropriate volumes of lipid chloroform solutions were mixed to give final lipid molar ratios of 10:6:3:2:2 DPPC/POPC/POPG/POPE/chol for the synthetic PS lipid mixture, denoted $CLSE^{S}$.