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Restoration of the interfacial properties of lung surfactant with a newly designed hydrocarbon/fluorocarbon lipid

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ABSTRACT

Serum proteins, especially fibrinogen, inactivate the lung surfactant mixture by adsorbing quickly and irreversibly to the alveolar air/aqueous interface. As a consequence of the inactivation, the surfactant becomes dysfunctional, and respiration cannot be maintained properly. Preventing the adsorption of surface active serum proteins to the air/water interface is important because this phenomenon causes fatal diseases such as acute respiratory distress syndrome (ARDS). Although some treatments exist, improvements in synthetic surfactants that can resist this inactivation are still expected. In this context, a novel ion pair lipid (IPL, $CF_3(CF_2)_7SO_3^-(CH_2CH_3)_3N^+(CH_2OCH_2)_{10}(CH_2)_{15}CH_3$) has been designed and synthesized. This surfactant reduces the inhibitory effect of fibrinogen by selectively interacting with DPPC (dipalmitoylphosphatidylcholine) and mimicking some of the interfacial properties of the pulmonary surfactant protein B (SP-B). Surface pressure–area isotherms and fluorescence microscopy images demonstrate that IPL can mix and interact synergistically with DPPC due to its unique molecular structure. Hysteresis behaviors of the monolayers, which are composed of mixtures of DPPC and IPL at different molar ratios, indicate that with increasing amounts of IPL, the lipid losses from the interface induced by the presence of fibrinogen significantly decrease. It is also found that IPL is able to adsorb to monolayers formed in the presence of fibrinogen, whereas fibrinogen cannot penetrate the monolayers formed in the presence of IPL. These results indicate that by mimicking some of the interfacial properties of SP-B, this novel hybrid molecule is promising in terms of preventing fibrinogen adsorption and therefore resisting surfactant inactivation.

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1. Introduction

Surface active agents are involved in a wide variety of applications from detergents to pharmaceuticals. Most importantly, they contribute to several critical biological processes such as breathing and digestion. Pulmonary or lung surfactant (LS) is produced as a complex mixture in the lungs by the alveolar type II cells [1–4]. During each respiration cycle, this unique mixture spreads over the alveolar air/aqueous interface, stabilizes the alveoli and reduces the work necessary for breathing by lowering the surface tension nearly to 0 mN/m through the actions of its different constituents [5,6]. Thus, the presence of this mixture is vital for mammals to sustain breathing. The functionality of LS arises from its unique composition of ~90% lipids and ~10% proteins. The lipid portion includes primarily saturated and unsaturated phosphatidylcholines and phosphatidylglycerols, with lower amounts of phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS) and others [7–9]. Among these, dipalmitoylphosphatidylcholine (DPPC) comprises approximately 40% of the lipids and is the main lipid responsible for lowering the surface tension.

Although the lipids fulfill a crucial function, they are not, by themselves, sufficient to provide a healthy respiration cycle [10,11]. Proteins also take part in the process by providing critical properties to the surface film that is formed by lipids. There are four specific surfactant proteins, which are categorized into two main groups according to their hydrophobicity. These hydrophobic proteins contribute to the preservation of the lipid stability at the interface [12–14]. Thus, an adequate decrease in the surface tension and creation of optimal conditions for respiration require the synergistic interactions of the phospholipids, neutral lipids and proteins (SP-B and SP-C) [15].

The unique composition and mechanism of LS may be corrupted or completely lost as a consequence of several conditions. Insufficient or absent LS due to preterm labor induces Neonatal Respiratory Distress Syndrome (NRDS), which is treatable. Adult

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Respiratory Distress Syndrome (ARDS) shares many symptoms with NRDS; however, it is a severe disease with high mortality rates. In addition to surfactant deficiency, this condition has several other causes including injuries, severe bleeding, trauma, infection or environmental factors such as exposure to chemicals and smoking. Under these conditions, the surface-active serum proteins leak into the alveolar subphase. Previous studies have shown that the serum protein levels are highly elevated in case of ARDS [16–20]. In vitro research studies performed using LS and surface-active serum proteins together have shown that the presence of the serum proteins also damages the functionality of LS [21]. The leakage and adsorption of these proteins to the air/aqueous interface of alveoli 'inactivates' the LS and provokes irreversible ARDS [12,16]. In order of decreasing ability to inactivate LS, the important serum proteins are fibrin monomers, fibrinogen, hemoglobin and albumin [22,23].

Currently, in contrast to ARDS, NRDS can be effectively treated with replacement surfactants. The replacement surfactants that are used in these treatments are extracted from animal lungs using expensive isolation and purification methods. In addition to the high costs, disadvantages of these agents include risks of pathogen transmission, infection and the possibility of variation in composition. These disadvantages have given rise to the idea of development and usage of synthetic surfactants as new treatment methods [24]. The surfactants currently used for ARDS treatment are adversely affected by less than 500 ppm protein. A synthetic surfactant that is able to resist the inactivating effect of the serum proteins will therefore be critical to the effective treatment of ARDS [25]. Furthermore, useful synthetic structures will need to exhibit properties such as mimicking the hydrophobic surfactant proteins and lipids, as well as having biophysical functions equivalent to the natural mixture [26].

Hydrophobic proteins provide a partial resistance to surfactant inactivation [27]; therefore, studies to develop synthetic surfactant formulations have focused particularly on capturing the behaviors of SP-B and SP-C. Although some formulations have been tested, an efficient synthetic formulation that prevents surfactant inactivation and provides the properties of a natural lung surfactant has not yet been developed. Our group has described the design and synthesis of a cationic lipid for the first time: all other synthesized surfactants are nonionic or anionic in structure. FHCL is a surfactant that can fulfill the selected features of the SP-C protein with its simple yet effective molecular structure [11].

Within the scope of this present study, the interfacial properties of a specially designed and synthesized surfactant, IPL ($CF_3(CF_2)_7SO_3^-(CH_2CH_3)_3N^+(CH_2OCH_2)_{10}(CH_2)_{15}CH_3$) (Fig. 1a), and its interactions with DPPC are investigated, and the feasibility of using this novel molecule to prevent protein adsorption is evaluated. The mechanism underlying the protein-induced LS dysfunction is thought to involve an energy barrier that is induced by the proteins as a result of their adsorption to the phospholipid monolayer [28]. With its ability to compete with DPPC for fibrinogen adsorption, the unique molecular structure and high adsorption rate of this newly synthesized molecule is proposed to minimize the damage related to protein adsorption. Moreover, IPL is expected to capture some interfacial properties of SP-B when mixed with DPPC and its presence at the interface will help to maintain a healthy respiration process.

2. Materials and methods

Brij 56 (Sigma), triethylamine (Sigma), heptadecafluorooctanesulfonyl fluoride (Fluka), diethylether (J.T. Baker, anhydrous), hexane (Sigma–Aldrich, 95%) and acetonitrile (Riedel-de-Haen, 99–99.4%) were used as received. 1,2-Dipalmitoyl-glycero-3-phosphocholine, -sn type (DPPC, 99%) and fibrinogen (type I-S: from bovine plasma, 65–85%) were purchased from Sigma–Aldrich and used without further purification. 3,6-Bis(diethylamino)-9-(2-octadecyloxycarbonyl) phenyl chloride (R18), sodium chloride (NaCl), sodium dihydrogen phosphate ($NaH_2PO_4{\cdot}H_2O$) and disodium hydrogen phosphate dodecahydrate ($NaH_2PO_4{\cdot}12H_2O$) were also obtained from Sigma and used as received. The water used in all experiments was ultrapure water with a resistivity of 18.3 MΩ cm (Millipore, USA).

2.1. Synthesis

The synthesis of IPL (Fig. 1a) was accomplished in a single step by the reaction of Brij 56, triethyl amine and heptadecafluorooctanesulfonyl fluoride in diethylether (anhydrous) in the stoichiometric ratio of 0.1:0.25:0.105, respectively. The reaction was carried at 40 °C with reflux and vigorous stirring (24 h) [29]. The ether was then removed using a vacuum evaporator. The product was obtained by washing the mixture three times with a 40:0.25 (v/v) mixture of ether and ethanol. For further purification steps, the product was dissolved in acetonitrile and extracted several times with hexane. The molecular structural characterization of the final product was performed using ^{1}H NMR and LC–MS techniques. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 3.4 (40H, OCH_2CH_2), 3.1 (6H, $N^+CH_2CH_3$), 1.5 (2H, $OCH_2CH_2CH_2$), 1.3 (9H, $N^+CH_2CH_3$), 1.2 (30H, CH_2), 0.8 (3H, CH_3); LC–MS (–): (m/z = 498.9, SO_3^- $(CF_2)_7CF_3$).

2.2. Surface pressure (π)–area (A) (Langmuir), surface pressure (π)–time (t) isotherms and hysteresis behavior

The π–A isotherms and the interfacial properties of the monolayers consisting of mixtures of IPL and DPPC were obtained by using a Langmuir–Blodgett minitrough (Kibron, Finland). The trough has a surface area of ~122 cm^2, with a subphase volume of ~80 ml. The sample solutions were spread on the interface using a Hamilton micro-syringe. n-Hexane/ethanol (9/1, v/v) was used as a spreading solvent and 20 min were allowed for the solvent to evaporate before each experiment. Each experiment was performed with both ultrapure water and PBS subphases by compressing and expanding the interface with Teflon barriers at a constant speed (93.5 mm/min) at ambient temperature (23 °C). The experiments were conducted in a temperature controlled manner using circulating water underneath the trough. The hysteresis behavior of DPPC, IPL and their mixtures at the air/liquid interface were also investigated using the Langmuir–Blodgett minitrough. To simulate the breathing cycle, after spreading the lipid solution, the interface was compressed and expanded repeatedly at a constant speed (93.5 mm/min). Five cycles were performed for each measurement set, and the percent of lipid desorbed from the interface was calculated [30]. For the cases in which the effect of fibrinogen was investigated, freshly prepared fibrinogen solutions (with final concentrations of 500 ppm and 1000 ppm) were used as subphase. After the interfacial tension had equilibrated (i.e., no variation of the interfacial tension was observed with time), DPPC, IPL or their mixtures with different molar ratios were injected onto the interface. After 20 min to allow for the solvent evaporation, cyclic compression–expansion isotherms were acquired. Representative figures are presented in Section 3. All experiments were completed within 2 h after the preparation of the fibrinogen solution to prevent protein denaturation.

To analyze the interfacial behaviors of the molecules and their interactions with one another in a time-dependent manner, the variation in the surface pressure was also measured and recorded over time without disturbing the interface. To investigate the adsorption of IPL to the DPPC monolayer under steady-state conditions, IPL was dissolved in water (0.005 mM) and injected into the subphase of a DPPC monolayer that had previously been

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