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Pulmonary surfactant layers accelerate O_2 diffusion through the air-water interface

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ABSTRACT

During respiration, it is accepted that oxygen diffuses passively from the lung alveolar spaces through the respiratory epithelium until reaching the pulmonary capillaries, where blood is oxygenated. It is also widely assumed that pulmonary surfactant, a lipid–protein complex secreted into alveolar spaces, has a main surface active function, essential to stabilize the air–liquid interface, reducing in this way the work of breathing. The results of the present work show that capillary water layers containing enough density of pulmonary surfactant membranes transport oxygen much faster than a pure water phase or a water layer saturated with purely lipidic membranes. Membranes reconstituted from whole pulmonary surfactant organic extract, containing all the lipids plus the hydrophobic surfactant proteins, permit also very rapid oxygen diffusion, substantially faster than achieved by membranes prepared from the surfactant lipid fraction depleted of proteins. A model is proposed suggesting that protein-promoted membrane networks formed by pulmonary surfactant might have important properties to facilitate oxygenation through the thin water layer covering the respiratory surface.

1. Introduction

Pulmonary surfactant is a lipid–protein complex that covers the respiratory surface and is essential to prevent its collapse at the end of expiration. To do so, surfactant reduces drastically the surface tension but, in addition to this critical feature, an effective surfactant has to adsorb rapidly onto the air–liquid interface and spread over it during inspiration [1,2]. These dynamic properties of surfactant depend critically on the presence of the hydrophobic surfactant proteins SP-B and SP-C, while DPPC, the major surfactant phospholipid species, facilitates formation of ordered tightly packed films optimized to achieve maximal surface tension reduction [3]. On the other hand, the hydrophilic surfactant proteins SP-A and SP-D are involved in the other main function of pulmonary surfactant, namely to establish a first line of defense in the large surface that the respiratory tract exposes to environment. These collectins bind to the surface of a variety of pathogens, including bacteria, virus and fungi, facilitating their clearance from the airways [4].

It has been traditionally considered that respiratory gases, and particularly molecular oxygen, diffuse freely through the respiratory epithelium, following the concentration gradient existing between the alveolar airspaces and the blood capillaries [5,6]. Specifically, the lipid-rich pulmonary surfactant layer covering the alveolar surface has never been considered an active element participating in oxygen transport. This is in part because phospholipid membranes have always been considered transparent to the diffusion of a hydrophobic small molecule such as molecular oxygen [7,8]. However, some studies suggest that oxygen diffusion through pure phospholipid membranes may be some orders of magnitude slower than expected, mainly due to the high microviscosity of bilayers [9]. As a matter of fact, an active line of research is trying to identify potential protein channels that could facilitate transport of gases through cell membranes [10,11].

In the present work, we have analyzed whether pulmonary surfactant layers, besides their surface active function, could have a role in facilitating oxygen diffusion through the air–water interface and the aqueous film covering the alveolar surface. In order to measure oxygen diffusion rates through capillary water layers containing physiologically meaningful concentrations of lung surfactant, we have developed an special setup using micrometer-sized oxygen sensors made of the luminescent probe tris(4,7-diphenyl-1,10-phenanthroline) ruthenium(II) (RDP3), synthesized in our laboratories [12]. The strong μs-lived luminescence of RDP3 at 610 nm is extremely sensitive to O_2 concentrations in the 0–8 mg L^{-1} (in water) or 0–30% (in gas phase) ranges, and has often been used to manufacture fool-proof oxygen optical microsensors for biological and environmental applications [12,13]. Using this setup, we have evaluated the kinetics of oxygen diffusion through layers of native pulmonary surfactant purified from

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animal lungs or through surfactant membranes reconstituted from the organic extract of surfactant, containing all the lipids and the hydrophobic proteins SP-B and SP-C. We have also compared the oxygen diffusion properties of preparations containing surfactant proteins with oxygen diffusion through purely lipid preparations.

2. Materials and methods

Bronchoalveolar lavages of pig lungs were made with saline and, after eliminating the cells, the supernatant was centrifuged for 1 h at 100,000×g to obtain whole native porcine surfactant, including the so-called large and small aggregates. The membranes were cleaned to remove potential cell membrane contaminants by centrifugation on a sodium bromide gradient, as previously described [14]. The hydrophobic fraction of surfactant, containing the lipids and hydrophobic proteins, was obtained by a Bligh and Dyer extraction [15]. The lipid fraction of surfactant was separated from the hydrophobic proteins by exclusion-size chromatography of the organic extract (Sephadex LH-20, from GE Healthcare). Phospholipid content of all the samples was determined by phosphorus assay [16]. In all samples prepared from organic solvent solutions, chloroform and methanol were evaporated under a nitrogen stream and organic traces were thoroughly eliminated under vacuum. The samples were then rehydrated with 5 mM pH 7 Tris buffer, containing NaCl 150 mM.

To prepare the oxygen sensors, a 200-μm thick silicone layer doped with RDP3 was sealed at the bottom of hollow glass capillaries (1.5 mm inner diameter) (see setup in Fig. 1a). The probe luminescence was monitored by a fiberoptic phase-sensitive instrument designed to carry out ultra-stable time-drive emission lifetime measurements with Ru(II) complexes. A continuous stream of water-saturated N_2/O_2 mixtures, accurately prepared by an electronic mass-flow controller and equilibrated at 37 °C, is flowed into the capillary at a 100 mL min^{-1} rate. The red emission from the O_2-sensitive film is focused with a ball lens back into the entrance of the common branch of a bifurcated fiberoptic bundle which carries also the sinusoidally modulated (blue) excitation light from a high power LED into the sample. The bifurcated bundle is connected to the dedicated phase-sensitive luminometer (OPTOSEN®, Interlab IE, Madrid, Spain). After appropriate calibration, the oxygen concentration sensed by the RDP3-loaded silicone layer can be accurately measured by following the luminescence phase shift (ϕ) change that results from the O_2 quenching on the probe emission lifetime (τ) ($\tan \phi = 2\pi f\tau$, being f the excitation LED modulation frequency) (see Fig. 1b). The sensor responds rapidly to changes in the partial oxygen pressure, exhibiting reproducible hysteresis-free signals upon exposure to increasing or decreasing oxygen levels, as illustrated by the calibration curves of Fig. 1b. Similar setups have been used, for instance, to measure oxygen diffusion through contact lenses [17], as surfaces designed to act at an epithelium/environment interface.

To monitor oxygen diffusion through different types of layers, a small volume (typically 3 μL) of 5 mM pH 7 Tris buffer in 150 mM NaCl, purified lung surfactant or phospholipid-based reconstituted membranes, was deposited on top of the oxygen-sensitive film. To monitor the gas diffusion kinetics, the capillaries were placed in the chamber over the luminescence collecting lens (see Fig. 1a), and equilibrated with a continuous flow of 100% nitrogen. Once the luminescence phase shift was equilibrated to a maximum, the gas flow was changed to 25% oxygen (in N_2, at 710 Torr) and the luminescence phase shift decrease was monitored over time.

Data presented are means with standard deviations after averaging measurements from 5 different samples, from at least two different surfactant batches, unless otherwise stated.

3. Results

As illustrated in Fig. 2, a step change in oxygen level from 0 (pure nitrogen) to 25% produces a prompt change of the luminescence

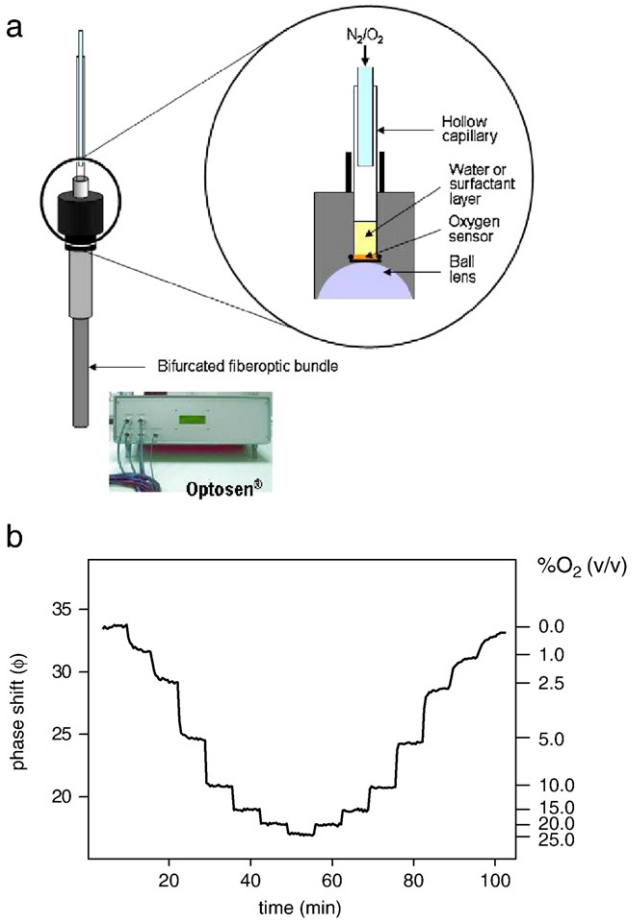

Fig. 1. Experimental setup used to monitor the O_2 diffusion through water or surfactant layers. (a) The oxygen sensor is a thin silicone layer containing the oxygen-sensitive luminescent probe RDP3, placed at the bottom of a sealed glass capillary tube, where a stream of nitrogen bearing the desired proportion of oxygen can be flowed in. (b) Calibration of the analytical signal from the capillary oxygen sensor (luminescence phase shift, left y-axis) by exposure to a water-saturated nitrogen gas stream containing increasing or decreasing proportions of oxygen (right y-axis).

phase shift (ϕ) from the fiber-optic sensor. The signal decay follows a 50-fold slower kinetics through a 3-μL aqueous buffer layer standing between the gas flow and the oxygen probe, compared to that obtained in the absence of water. Water is a physical barrier for rapid oxygen equilibration with the sensing film due to the lower solubility and diffusion of this gas in aqueous media compared to the silicone polymer. The lag time observed before the probe luminescence starts

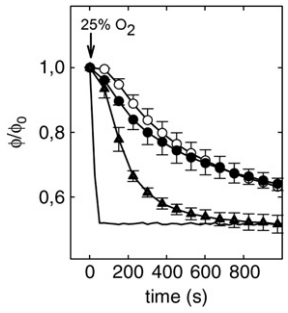

Fig. 2. Kinetics of oxygen diffusion through water or surfactant layers. O_2 diffusion through a capillary aqueous buffer solution (open circles), through a layer of pulmonary surfactant (16 mg mL^{-1}, closed circles), and through a layer of surfactant pelleted to a density of 29 mg mL^{-1} (triangles); for the sake of comparison, the practically instantaneous change of the probe luminescence upon oxygenation in the absence of water or surfactant is also shown (black line).

to decay is associated with the time required for oxygen to partition and diffuse through the still water layer. Oxygen diffusion through water layers formed by 1, 2, 3 or 5 μL of buffer yielded similar signal decays (not shown) indicating that the rate-limiting step for the oxygen diffusion to the oxygen-sensitive probe is the partition of oxygen from the gas phase into the aqueous layer, at least for the assayed range of water volumes.

We then measured the oxygen diffusion rate through a 3-μL layer of native porcine pulmonary surfactant. Fig. 2 shows that oxygen diffusion is not significantly facilitated by the surfactant at the concentration assayed (16 mg phospholipid mL^{-1}). Interestingly enough, the time elapsed before the probe starts to sense the increasing concentration of oxygen is reduced in the presence of surfactant. This suggests that the initial partition of oxygen into water may be facilitated by the surfactant film formed at the air–water interface.

It has been proposed that the actual concentration of pulmonary surfactant in the capillary water layer covering the respiratory epithelium could be in the order of 50–100 mg mL^{-1} [18]. To test the hypothesis that fast oxygen diffusion might require the presence of enough density of surfactant membranes, 5 μL of surfactant was deposited into the oxygen-sensitive capillaries, which were then centrifuged at 3000×g for 5 min to pellet the membranes. Then, 2 μL of the supernatant water was removed to let 3 μL of dense membranes, corresponding to a phospholipid concentration that was determined to be 29 mg mL^{-1}, be in contact with the oxygen-sensitive film. Fig. 2 shows that, in spite that such dense surfactant layer is still fully hydrated, it allows oxygen diffusion significantly faster than the diluted phase.

Taking into account these results, it was subsequently tested whether fast oxygen transport through surfactant membranes could be just due to the superior oxygen-dissolving properties of phospholipid membranes compared to water phases [7,8]. Fig. 3 illustrates the oxygen permeation across pure lipid membrane layers prepared under the same conditions used for the pulmonary surfactant. Pure lipid pellets led to faster oxygen diffusion in comparison to diffusion through water, with practically no lag time, but did not reach the rapid permeation rates measured through dense surfactant layers. The oxygen diffusion rates through all the assayed materials are quantitatively compared in Table 1, which summarizes the calculated values of t_{90}, defined as the time required for the sensor to reach 90% of the signal measured upon equilibration in 25% oxygen. Membranes prepared with the full lipid fraction of surfactant were more efficient in transporting oxygen ($t_{90} = 14.1 \pm 0.9$ min) than those made of pure dipalmitoylphosphatidylcholine (DPPC) (t_{90} ~21 min). Fig. 3 also shows how a pellet of membranes reconstituted from an organic solvent-extracted fraction of pulmonary surfactant containing all the lipids plus the hydrophobic surfactant proteins SP-B and SP-C, leads to the fastest oxygen transport kinetics ($t_{90} = 6.4 \pm 2.4$ min).

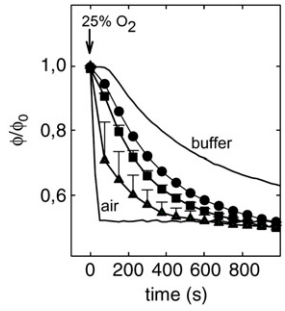

Fig. 3. Kinetics of oxygen diffusion through lipid or lipid–protein layers. O_2 diffusion through a pelleted layer of DPPC membranes (circles), of membranes reconstituted from the whole lipid fraction of surfactant depleted of proteins (squares), and of membranes reconstituted from the whole organic extract of surfactant, containing the lipids and the hydrophobic proteins (triangles). The blue and black traces are the same than above.

Table 1
t_{90} for equilibration of $[O_2]$ from 0 25%.

Medium	[PL] (mg/mL)	t_{90} (min)
Water		38.4 ± 3.1
Air		0.6 ± 0.1
Surfactant	16	23.5 ± 3.7
Surfactant (pelleted)	29	8.7 ± 1.3
DPPC membranes	29	21.3
Surfactant lipid fraction	29	14.1 ± 0.9
Surfactant organic extract	29	6.4 ± 2.4

4. Discussion

The results presented here suggest that a water layer saturated with pulmonary surfactant membranes could be optimized to transport oxygen much faster than a pure water layer or even than a layer of concentrated phospholipid membranes. It is obvious that good oxygen-diffusing properties must offer important advantages to the surface layer spread by surfactant at the respiratory epithelium. In this regard, we propose that pulmonary surfactant may have evolved to simultaneously exhibit good surface spreading activity and oxygen transport capability. Surfactant would form a membrane-based phase, covering the whole respiratory surface, with a high permeability to oxygen. Our results also show that the oxygen-diffusion properties of pulmonary surfactant complexes stand beyond the particularly high solubility of dioxygen into phospholipid membranes. A layer of isolated phospholipid bilayers would consist in a mixture of membranes containing a higher oxygen concentration than that in the water layers separating the different membranes (Fig. 4a). The rate-limiting step of the oxygen diffusion through such structure would surely be related with the partition of the hydrophobic oxygen molecule between the membrane and the bulk aqueous phase. It has been proposed that the hydrophobic surfactant proteins SP-B and SP-C promote connections between surfactant membranes that facilitate rapid transport of surface active species into the air–liquid interface [19,20]. Such molecular connections might allow favorable partition of oxygen from the interface into the membranes, and from membrane to membrane, without necessarily requiring diffusion of oxygen through water layers (Fig. 4b). We speculate that, in the absence of surfactant proteins, phospholipid membranes would be, at any density, "short-circuited" by water layers that would impose a limiting step to transport oxygen from the air phase. Surfactant proteins could then be important to build a "continuous" membrane phase, competent to move oxygen rapidly from the gas phase through the whole water layer. Congenital absence of both SP-B and SP-C proteins is lethal upon establishment of air respiration at birth, due to an impossibility of the lung to maintain oxygenation [21]. This phenotype has been interpreted as a consequence of alveolar collapse and lack of oxygenation due to reduced operative air–liquid interface. Our present results suggest that the lack of these proteins could also determine an intrinsically flawed epithelial surface unable to move oxygen fast enough from air into the underlying tissues. Moreover, patients suffering from acute respiratory distress syndrome (ARDS), a pathology associated with lung injury and leakage of serum into the alveolar spaces [22], show largely reduced blood pO_2, which has been interpreted as a consequence of collapsed injured lungs. Our results point out that an important component of the pathology could be a deficient transport of oxygen through a lung surface containing an inappropriate balance of water and surfactant membranes.

It has been recently reported that alveolar type II pneumocytes, the cells in charge of synthesizing and secreting pulmonary surfactant, express both chains of hemoglobin, being one the first non-hematopoyetic cell lines known to have this potential [23,24]. The

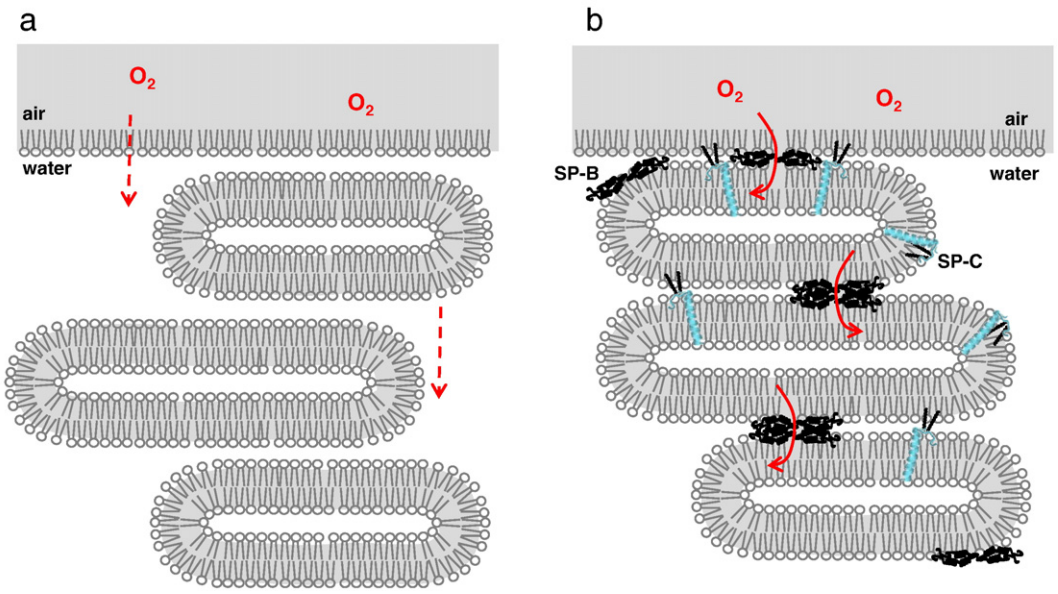

Fig. 4. Model for oxygen diffusion through surfactant films. (a) In the absence of surfactant or in the presence of surfactant-protein-free isolated phospholipid membranes, oxygen diffusion through water layers (dashed red arrows) would be limited by the poor solubility of the gas into the aqueous phase. (b) Proteins SP-B and SP-C have been proposed to form intermembrane connections that could create a continuous membrane surface phase, which could permit rapid oxygen diffusion (red arrows) through the core of membranes, with superior oxygen-dissolving properties.

meaning of the biosynthesis of hemoglobin for the different functions played by pulmonary surfactant and the alveolar epithelium is still to be elucidated. However, the present results could open a new framework to assess factors potentially modulating, sensing and/or coupling oxygen concentrations and oxygen transporting capabilities at the respiratory interface.

Pulmonary surfactant is present in the lungs of all air-breathing vertebrates. The surface active function traditionally ascribed to this lipid–protein complex is still not properly understood in the context of the varieties of architectures and environmental factors associated with the lungs of the different animal groups [25]. For instance, in birds, pulmonary surfactant is associated with the tracheoles, the gas exchange moiety of the lungs, and not that much with the air sacs, the changing volume of which is actually responsible for mobilizing air through the tubular avian lungs [26]. We think that our data open a new way to examine the potential role of surfactant along the whole phylogenetic tree as an evolutionary trait that might have been originated and evolved to maintain a proper oxygen transport through air–water respiratory interfaces.

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