Experimental report

30/01/2025

Proposal: 9-13-1109 **Council:** 10/2023

Title: Why do we sigh?: Simultaneous structural and mechanical characterization of model pulmonary surfactant interfaces

Research area: Soft condensed matter

This proposal is a new proposal

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Samples: D2O
1,2-dipalmitoyl-sn-glycero-3-phosphocholine
1-palmitoyl-2-oleoyl-glycero-3-phosphocholine
Surfactant protein B (SP-B)
Surfactant protein C (SP-C)
1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylglycerol

Instrument	Requested days	Allocated days	From	To
FIGARO User-supplied	6	5	28/02/2024	04/03/2024

Abstract:

The human lung¿s internal surface is coated by a lipidic lung surfactant (LS) interface, whose dysfunction leads to pulmonary collapse. To operate properly, LSs must respond to dilatational and compressional deformations imposed by breathing, dynamically adjusting their composition and structure through pulmonary proteins located within membrane reservoirs buried below the interface. Although LS morphology has been investigated in quiescent conditions, no studies have interrogated this aspect under physiological breathing, particularly during involuntary sighs, which are crucial to LS conditioning. We propose to interrogate LS interfaces via neutron reflectometry, to elucidate the changes in composition and thickness of the interface and associated reservoirs during dynamic conditions mimicking inhalation, exhalation, and sighing. We will perform experiments on a custom Quadrotrough, which can apply controlled compressional and dilatational deformations at physiological temperatures, strains, and frequencies. These experiments will correlate morphological information with the interface¿s mechanical properties, revealing the dynamic structural hallmarks of pulmonary physiology.

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Experimental setup at FIGARO:

The goal of this project was to nondestructively and dynamically interrogate the lung surfactant (LS) interfacial structure (top monolayer and buried multilayer reservoirs) and changes thereof, to elucidate the LS interfacial morphology and its role in determining the transient mechanical properties of the alveolar layer under physiological conditions mimicking breathing and sighing maneuvers. Experiments were conducted with a custom Quadrotrough (QT) NR environment, capable of applying controlled dilatational deformations that mimic breathing and sighing motions under physiological conditions of temperature (37 ^{o}C), strain amplitudes (5 – 10% for tidal breathing and 30 – 40% for sighing), and frequencies (0.2 – 0.33 breaths/s). Installation and mounting of the QT environment within FIGARO required customization of exiting equipment and the assembly of specific components, such as heating units to ensure that the interface and associated bulk phases (buffer and air) were maintained at 37 ^{o}C, a humidifying chamber to avoid subphase evaporation at these elevated temperatures, the installation of heating elements to prevent solvent condensation along the path of the neutrons and laser beams, and the fabrication of fluorinated neutron-transparent elastic bands to compress and dilate the interface. In addition, a specialized LabVIEW software was designed to apply the desired breathing and sighing deformations and collect the necessary surface pressure and strain data. Access to FIGARO was granted on 27/02/2024 and the equipment mounting and installation took approximately 24 hours. Images of the QT environment mounted on FIGARO are provided in Fig. 1:

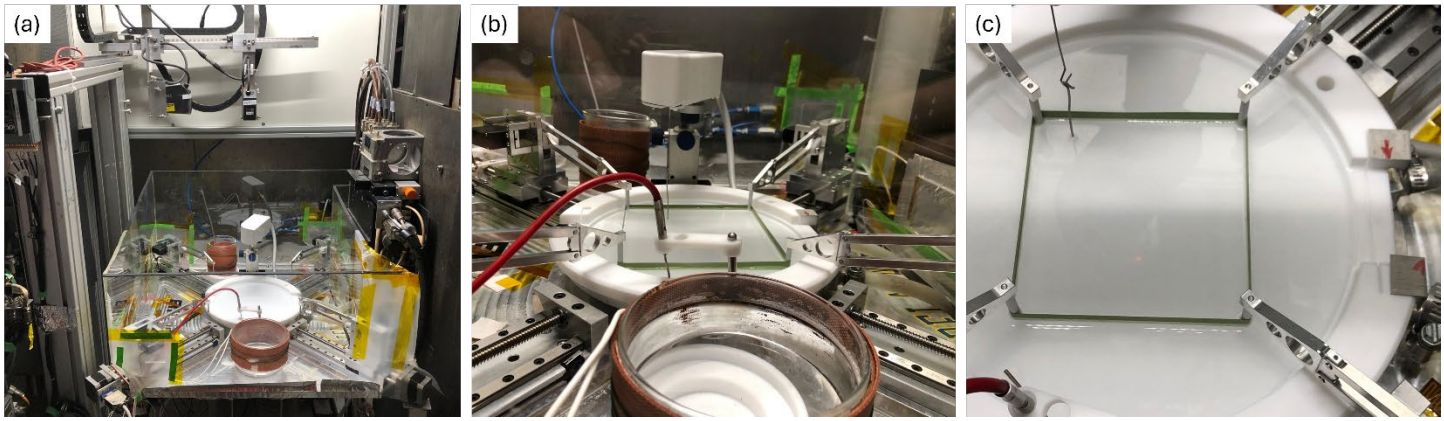

Fig. 1: **(a)** QT experimental setup, mounted inside FIGARO. A circular Teflon trough contains the desired lung surfactant solution. The QT is enclosed inside a transparent chamber that keeps the ambient air at 37 ^{o}C and 100% relative humidity. To ensure that the ambient air is sufficiently humidified, flexible resistance heaters are wrapped around a pair of beakers containing a heated buffer solution of the same composition (i.e., D_2O or ACMW) as the measured lung surfactant solution. **(b)** To compress and dilate the interface, the QT uses a set of 4 motorized fingers surrounded by a neutron-transparent elastic band. Data on the surface pressure is collected by a KSV-NIMA surface pressure sensor and Wilhelmy rod. **(c)** Close-up view of the lung surfactant interface, which is contained within the rectangular area formed by the elastic band. The Wilhelmy rod is placed outside of the neutron beam, which travels from left to right. Two openings in the trough's outer walls, such as seen at the right-hand side of the image, allow the uninterrupted passage of the neutron beam.

Experiments carried out:

To elucidate the changes in composition and thickness of the interface and associated multilayer structures during the dynamic physiological processes of breathing and sighing, the following experiments were conducted:

1. Experiments with insoluble lipid mixtures: Two of the three different insoluble lung surfactant mixtures that were described in the project proposal were examined (compositions A. and B.). Preliminary testing revealed that the pure DPPC interfaces originally outlined in the proposal were too unstable to sustain repeated breathing and sighing cycles, making them unfeasible for the proposed NR experiments, and their use was therefore discarded. Instead, mixture C., which contains an elevated protein mass fraction, was used as an alternative. Although composition C. contains a protein concentration that is 10 times larger than physiological values, preliminary interfacial rheology experiments revealed that this mixture can achieve and sustain low surface stress values characteristic of healthy lungs, and can therefore provide valuable information regarding the localization of the lung surfactant proteins within the buried multilayer structures:

A. Model lipid mixture: DPPC:POPC:POPG at 55:28:17 wt%

B. Model lipid mixture + 1% protein: Lipid mixture + SP-B and SP-C at 1% lipid:protein fraction
C. Model lipid mixture + 10% protein: Lipid mixture + SP-B and SP-C at 10% lipid:protein fraction

All lipids were used in their deuterated (d-) and hydrogenated (h-) forms while the proteins, extracted from porcine lungs, were used in their native form. The following isotopic variants were examined, as outlined in the project proposal: 1) D_2O and h-lipids; 2) ACMW and h-lipids; and 3) ACMW and d-lipids.

2. Experiments with Infasurf: In addition to the insoluble lung surfactant mixtures, experiments were conducted with the pharmaceutical lung surfactant replacement Infasurf. These experiments, not outlined in the original proposal, were introduced because results from rheological experiments conducted after the proposal deadline revealed that this mixture, containing physiological lipid:protein proportions, was better able to achieve low surface stress values compared to its analogous insoluble counterpart (composition B. above). In addition, Infasurf is available as a soluble aqueous solution, making it possible for the surfactant to adsorb directly from the subphase rather than being spread drop-wise from a chloroform solution, which we believe resembles physiological processes more closely. The native (hydrogenated) Infasurf solutions were dissolved in both ACMW and D_2O buffer subphases; however, there was no visible signal in for the ACMW solution, and thus all experimental data was only acquired for Infasurf dissolved in D_2O.

For all compositions above, the following three experimental protocols were applied:

I. Static: NR data is continuously acquired for a static (unstrained) interface, acting as a reference state.
II. Tidal breathing (TB): 5 Expansion-Compression cycles at a total area strain amplitude of 10% and a frequency of 0.2 Hz, followed by a 3-minute relaxation period during which NR data is acquired.
III. Sighing: 4 TB cycles + 1 Sigh Expansion-Compression cycle (total area strain amplitude of 35% and a frequency of 0.2 Hz), followed by a 3-minute relaxation period during which NR data is acquired.

Protocol I. was slightly adapted from what was stated in the proposal. In addition, the 1-minute data acquisition period was expanded to 3 minutes, to allow for more NR data to be collected during the relaxation process. The above processes were repeated multiple times for the same interface, until sufficient data was acquired. NR data was collected in the high-Q range, at a 4° reflection angle during the relaxation period, as outlined in the proposal. For the insoluble mixtures 1A, 1B, and 1C, only 4 – 7 repetitions could be conducted with a given sample prior to its degradation; thus, new interfaces were prepared until sufficient experimental data was acquired. On the other hand, the Infasurf mixtures were capable of sustaining >20 repetitions. All experiments were carried out from 28/02/2024 8:30am to 04/03/2024 8:30am, without any adverse incidents.

Model description and fitting of experimental data:

Infasurf model: The specular neutron reflectivity curves of Infasurf in D_2O buffer were analyzed using a multilayer slab model. For the main components, the typical Scattering Length Density (SLD) values are approximately 2×10^{-6} Å^{-2} for the lipid head groups, -0.3×10^{-6} Å^{-2} for the lipid tails, 2×10^{-6} Å^{-2} for the proteins, and 6×10^{-6} Å^{-2} for D_2O. The model categorizes the system into two types of slabs: (1) lipid heads combined with proteins, and (2) lipid tails. At the air interface, it is assumed that lipids fully cover the surface. However, within the multilayer regions, structural heterogeneity is expected. To capture this, a volume fraction relative to D_2O is introduced, enabling variations in the SLD along the z-axis. Additionally, slab thickness is allowed to vary within a range informed by known values for lipid bilayers and protein layers. The arrangement of these slabs is as follows: the topmost section is composed of lipid tails, air, and lipid heads plus proteins (referred to hereafter as the "top" layer). Beneath this, the multilayered regions are modeled as repeating bilayers, organized in the sequence: lipid heads plus proteins, lipid tails, followed again by lipid heads plus proteins (referred to hereafter as a "bilayer"). To simplify the model and reduce the number of variables, identical parameters are applied across all bilayers. The same approach is used consistently across the three experimental setups (Static, TB, and Sigh), with only the number of bilayer repeats adjusted for each case. To determine the optimal number of bilayer repeats, we began with the smallest possible configuration (the top layer plus one bilayer). The number of repeats was gradually increased until the model's chi-squared ($\chi 2$) value reached a minimum. Once the optimal number of bilayers was identified, further increases typically led to negligible improvements ($\Delta\chi 2<1$) or no meaningful change. With the final structure established, the SLD values were fixed, and a final refinement of slab thickness was performed using the DREAM algorithm to achieve higher precision.

Insoluble lipids model: The three different model systems 1.A, 1.B, and 1.C are being analyzed for each of the three contrasts (h-Lipids in D_2O, d-Lipids in D_2O and d-Lipids in ACMW). The typical Scattering Length Density (SLD) values are approximately 2×10^{-6} Å^{-2} for the h-lipid and d-lipid head groups, -0.3×10^{-6} Å^{-2} for the h-lipid tails, 4.9×10^{-6} Å^{-2} for the d-lipid tails, 2×10^{-6} Å^{-2} for the proteins in D_2O, 1.5×10^{-6} Å^{-2} for the proteins in ACMW, 6×10^{-6} Å^{-2} for D_2O, and 0 Å^{-2} for ACMW. Since different contrasts were available, a more precise model was possible, allowing a finer separation of the slabs into three types: (1) lipid heads, (2) lipid tails, and (3) proteins. The ongoing model determination will allow us to locate the proteins in relation to the lipids and to determine the effect of the protein concentration on the overall structure.

Preliminary results:

So far, only the Infasurf data has been fully analyzed. Some results are shown in Fig. 2 below:

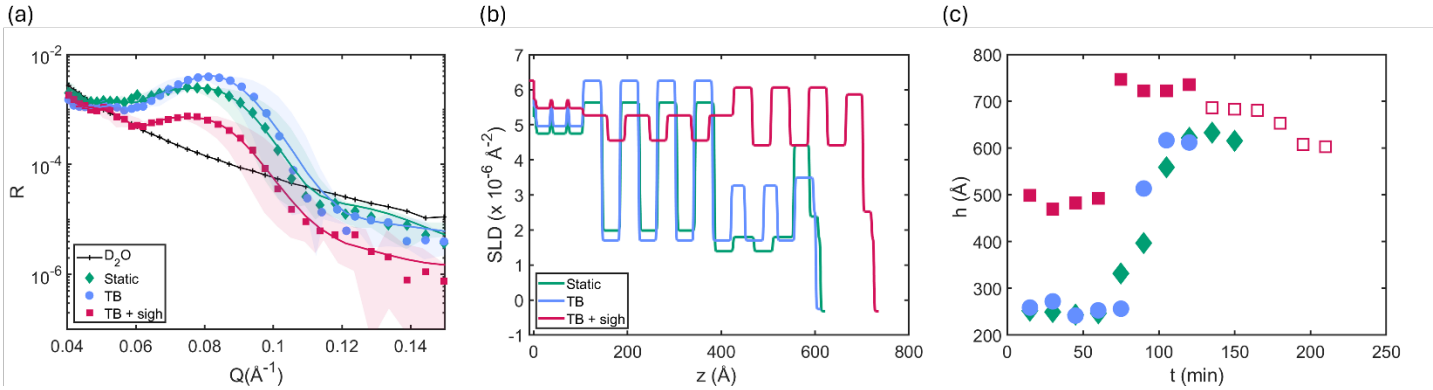

Fig. 2: (a) Example reflectivity profiles corresponding to deformation protocols I. (Static, green), II. (Tidal Breathing, blue) and III. (Sighing, magenta), taken between t = 105 – 120 min. Notable qualitative differences can be discerned between the static and TB profiles and the Sigh profile, suggesting that sighing dynamically modifies the structure of the lung surfactant interface. **(b)** SLD curves for the same datasets presented in panel (a) (t = 105 – 120 min). A position of z = 0 corresponds to the lowermost part of the interfacial structure, whereas the maximum z position corresponds to the location of the air-buffer interface. The Static and TB interfaces have a similar structure, where the bilayers close to the air-water interface have a relatively high protein and lipid fraction and the layers buried deeper into the bulk subphase gradually become less lipid and protein-rich. It is evident that the sigh interface has a different structure. Even though the limited contrasts available with the Infasurf solution make it impossible to quantitatively ascertain its composition, using the SLD values of the lipid heads, lipid tails, proteins, and D_2O we can make some qualitative assumptions about the interfacial composition. We hypothesize that one or more of the following scenarios is likely upon sighing: (i) the high SLD values suggest that upon sighing, the multilayers increase in number but decrease in density (i.e., become more water-rich); (ii) the smaller SLD variations suggest that Sigh multilayers have a more heterogeneous structure, (iii) the higher SLD values may also suggest that sigh multilayers have a higher protein:lipid ratio compared to the Static and TB cases. The analysis of the insoluble lipids data will provide further insights regarding the localization of the proteins and the multilayer architecture. **(c)** The total thickness of the LS multilayer structure increases over time and eventually reaches a plateau at around t = 1 hr for all cases studied. The multilayer structures formed upon sighing are thicker and grow faster than those formed in the Static and TB cases, suggesting the important role of sighs in establishing the LS morphology.

In addition, the rheological data acquired during the experiments reveals that there is a strong correlation between the interface's morphology and mechanical properties during breathing and sighing cycles, as shown in Fig. 3 below:

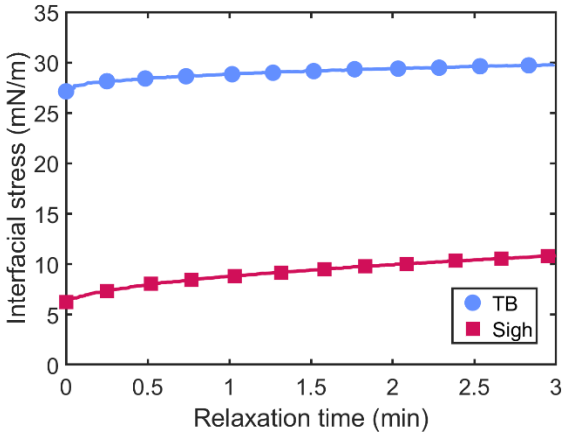

Fig. 3: Interfacial stress evolution during the 3-minute relaxation period for the TB and Sigh deformation protocols, taken at time t = 105 min, corresponding to the same time frame shown in the neutron reflectivity profiles in Fig. 2. For TB, the interfacial stress remains close to its equilibrium value at all times; however, for the Sigh protocol, the interfacial stress is reduced to values of around 5 mN/m. A quantitative analysis of the relaxation times indicates that, while interfaces subjected to TB deformations relax fully to their equilibrium (i.e., Static) state in ~2 min, LS interfaces subjected to Sigh deformations take ~ 15 minutes to relax, indicating that Sigh deformations can induce LS interfaces to achieve and maintain low surface stresses. The marked differences in the rheological properties caused by both types of deformations is strongly correlated to differences in their morphology.

At this moment, we can affirm that we have met three of the four goals that were stated in the original project proposal. Specifically, we have (1) corroborated the existence of a multilayer reservoir structure buried below the interface, (2) quantified its thickness (and changes thereof) during breathing and sighing maneuvers, and (3) found a correlation between the interfacial morphology and mechanical properties during breathing and sighing cycles. It is anticipated that the last goal, which is to elucidate the changes in composition and/or coverage if the LS interfacial layers during breathing and sighing, will be achieved once the insoluble lipids data is analyzed.