Investigating effects of hypertonic saline solutions on lipid monolayers at the air-water interface

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ABSTRACT

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More than 70,000 people worldwide suffer from cystic fibrosis, a genetic disease characterized by chronic accumulation of mucus in patients’ lungs provoking bacterial infections, and leading to respiratory failure. An employed age-old treatment to prevent the symptoms of the disease is inhalation of hypertonic saline solution, NaCl at concentrations higher than in the human body (~150 mM). This procedure clears the mucus in the lungs, bringing relief to the patient. However, the biophysical mechanisms underlying this process are not entirely clear. We undertook a new experimental approach to understand the effects of sprayed saline solutions on model lung surfactants towards understanding the mechanisms of the treatment. The surface of lungs contains mainly 1,2-Dipalmitol-sn-glycero-3-phosphocoline (DPPC). As previously assumed by others, we considered that monolayer of DPPC at the air-water interface serves as model system for the lungs surface; we employed a Langmuir-Blodgett (LB) trough and PM-IRRAS to measure surface-specific infrared spectra of the surfactant monolayers and effects on the interfacial tensions.

We investigated spraying hyper-saline solutions onto surfactant monolayers at the air-water interface in two parts: (i) validation of our methodology and techniques with stearic acid and (ii) experiments with DPPC monolayers at the air-water interface. Remarkably, when micro-droplets of NaCl were sprayed to the monolayer of stearic
acid, we observed enhanced organization of the surfactant, interpreted from the intensities of the CH₂ peaks in the surface-specific IR spectra. However, our results with DPPC monolayers didn’t show an effect with the salt added as aerosol, possibly indicating that the experimental methodology proposed is not adequate for the phenomena studied. In parallel, we mimicked respiratory mucous by preparing salt solutions containing 1% (wt%) agar and measured effects on their viscosities. Interestingly, we found that NaCl was much more effective than NaI and NaClO₄.

This thesis reports structural dynamics of monolayers of stearic acid and DPPC at the air-water interfaces and those of aqueous solutions towards understanding mechanisms underlying the most commonly employed treatment for cystic fibrosis. Our methodology has never been reported before; but requires further modifications to gain deeper insights into the effects of salt sprays on model lung systems.
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<td>AFM</td>
<td>Atomic Force Microscope</td>
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<tr>
<td>DPPC</td>
<td>1,2-Dipalmitol-sn-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>FT-IR</td>
<td>Fourier Transform Infrared Spectroscopy</td>
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<tr>
<td>LB</td>
<td>Langmuir Blodgett</td>
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<tr>
<td>PM-IRRAS</td>
<td>Polarization Modulation Infrared Reflection Absorption Spectroscopy</td>
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Measurements taken 5, 10 and 15 min after salts sprayed.
Chapter 1

Keywords


Executive summary

More than 70,000 people worldwide suffer from cystic fibrosis, a genetic disease characterized by chronic accumulation of mucus in patients’ lungs (1). The obstruction in the airway is due to high-viscosity mucous that leads to bacterial infections, lung damage and eventually respiratory failure (1-3). Common symptoms of this disease include thickness of the mucus, frequent coughing, wheezing and repeated lung infections (1, 3, 4). The treatment taken by cystic fibrosis patients involves inhalation of hypertonic saline solution, i.e. containing higher concentration of NaCl that in the human body (~150 mM) (5-7). The treatment allows the patient to breath due to mucociliary clearance. Despite the extensive studies about the disease and treatment, the physical mechanisms underlying this phenomena is not entirely clear. In response, we undertook a new experimental approach to understand the effects of micro-droplets of saline solutions on model lung surfactants towards understanding the mechanisms underlying the treatment.

The surface of the lungs is covered with pulmonary surfactants, which are mainly composed of phospholipids (8). The most abundant phospholipid present is 1,2-Dipalmitol-sn-glycero-3-phosphocoline (DPPC), which has been extensively exploited as a model phospholipid to mimic lung surfaces (9-13). For example, a variety of studies
have been conducted on DPPC monolayers deposited at the air-water interface to test the effects of common salts, such as NaCl, CaCl$_2$, MgCl$_2$, NaBr, and NaI, added to the water, also known as the sub-phase (9, 14).

Our experiments investigating sprays of hyper-saline solutions onto surfactant monolayers at the air-water interface comprised of two parts: (i) validation of our methodology and technique with stearic acid, a simple surfactant, and (ii) experiments with DPPC monolayers at the air-water interface. To this end, we exploited a Langmuir-Blodgett trough to deposit and compress lipid monolayers and Polarization Modulation Infrared Reflection Absorption Spectroscopy (PM-IRRAS) to observe orientations of the alkyl chains of the lipids at the air-water interface. Remarkably, when micro-droplets of hyper-saline solutions of NaCl were sprayed on to monolayers of stearic acid, we observed enhanced organization of the alkyl chains as interpreted from the intensities of the CH$_2$ peaks in the surface-specific IR spectra. Unfortunately, when we sprayed NaCl, NaI and NaClO$_4$ electrolytes on DPPC monolayers at the air-water interface, the signal to noise ratio was so low that we could not observe measurable changes in intensities of the asymmetric peak of CH$_2$ stretching bonds, possibly indicating that PM-IRRAS was inadequate for this system. Adopting an alternative strategy, we mimicked respiratory mucous by preparing 1% agar (%wt) in various electrolytes and measured effects of salts on their viscosities. Interestingly, we found that NaCl was much more effective at decreasing viscosity of simulated mucous compared to NaI and NaClO$_4$. 
To summarize, this thesis reports on dynamic properties of monolayers of stearic acid and DPPC at the air-water interfaces towards understanding mechanisms underlying the most commonly employed treatment for cystic fibrosis. Our strategy of spraying salt solutions in he surfactant monolayer build in pure water has never been reported before. Nevertheless, our methodology requires further modifications to gain deeper insights into the effects of salty sprays on model lung systems.

Introduction

1.1 Cystic Fibrosis

Cystic fibrosis is a progressive, genetic disease that causes persistent lung infections and limits the ability to breathe over time caused by a defective gene that enhances the production of thick build up mucus in the lungs pancreas and other organs (5, 15). The cystic fibrosis gene is in charge of the cystic fibrosis transmembrane conductance regulator protein (CFTR); a defective CFTR leads to abnormal transport of chloride and sodium ions across the epithelia, driving to excessive absorption of fluid from the airway surface layers (4, 15, 16). This increases the amount of mucus in the airway due to impairment of mucociliary clearance provoking infections (5). In the lungs, the mucus clogs the airways and traps bacteria leading to infections, lung damage and respiratory failure with time (17). More than 70,000 people worldwide live with this disease (17).
1.2 Hypertonic saline solution treatment

Currently, inhalation of a fine mist of hypertonic saline solution is one of the treatments that patients perform to control the effects of cystic fibrosis (6, 18). Hypertonic saline solution refers to any solution containing a concentration of sodium chloride of 3-7% (wt%), which is higher than the NaCl concentration physiological conditions, ~0.9% (wt%) (7). This treatment increases the mucociliary clearance ~50% (2) by reducing the thickness of the mucus (4-6) and allows patients to breath better (15).

1.3 Pulmonary Surfactants

A surfactant is by definition a surface-active agent that lowers the surface tension of the medium in which it is dissolved (19). The chemical structure of the surfactants varies within the interfacial system in which they are surface active (20); nevertheless, there are some general characteristics that a surfactant must have, such as “the head” and the “tail” (21). The “head” is the hydrophilic group and the “tail” refers to the hydrophobic group, meaning that the surfactants are amphiphilic molecules (21, 22), which have an energetic preference for an interfacial location (20). Surfactants tend to arrange at the interface in a thin surface film, affecting the surface tension (20, 21), which is directly proportional to the concentration of surfactant added to the liquid (20).

Pulmonary surfactants line and stabilize the interface of the alveoli, functioning as a first barrier between the outside air and the organism (23) allowing gas exchange during the breathing cycle (24). A major part of the work of breathing results from the expansion of the lungs against surface tension forces (20). The main function of pulmonary
surfactants is to lower the surface tension at the alveolar surface to values near zero preventing lungs to collapse, and are involved in the protection of the lungs from injury and infections (13, 25-28).

1.4 Lipid monolayers

Pulmonary surfactants are a complex and highly surface-active family of molecules composed of 90% lipids and 10% proteins found in the fluid lining of the alveolar air-water interface (8, 13, 24-29). The mechanism of pulmonary surfactant action is a great example of a problem of physiological relevance, which benefits from lipids monolayer studies (30). The advantages of lipid monolayers (Figure 1) as experimental models for biophysical studies have been well recognized with different methods ranging from electron microscopy to X-ray diffraction and different kinds of spectroscopies (13, 14, 20, 26, 31). A variety of studies conducted through decades have helped to understand molecular structures and interactions in the surfaces of monolayers and multilayers of lipids (20).
Figure 1 A) A simplified schematic of a lipid molecule where hydrophobic and hydrophilic parts of the molecule are represented. B) The deposition of the lipid molecules in the air-water interface where the hydrophilic heads are in contact with the surface of the water.

From the 90% of the lipids that compose the lung surfactant, 70-80% are phosphatidylcholines (PCs) (23) and from these, about 40% of the mammalian surfactant is composed of DPPC (Figure 2) (9, 32). This makes DPPC generally accepted to be responsible for the low (~0 mN/m) surface tension at the alveolar surface when exhalation ends (13).
Figure 2 Schematic of the DPPC molecule. Size of the molecule ~40 Å. A) Hydrophobic part of the molecule. B) Hydrophilic part of the molecule.

Until now, there have been few studies considering a mixed lipid monolayer (8, 11, 29, 33). Most of the experiments have been done with only one lipid, mainly DPPC due to its biological relevance (11, 34-36). In addition, DPPC is one of the lipids that have been best experimentally and computationally characterized (32). Even though, it is important to know that of the fact that pure lipid analysis lack of the complexity of the real lung surfactant, which means that the results observed are not realistic but give a fair approximation of the physical behavior (13, 23, 26, 28).

1.5 Electrolytes

Thermodynamic proprieties of ions in specific solutions and the relationship with their ability to work as structure inducers or breakers of the system have been well studied for several years (9, 10, 12, 14, 37, 38). For example, in an aqueous solution, a structure inducer ion refers to one that enhances the hydrogen bond strength, while the structure breaking ones weaken the hydrogen bonds (39). In the case of proteins, ions are referred as stabilizers or denaturants, meaning that there are some ions that provoke the solubilizing of the protein (stabilizers) or the precipitation of it (denaturants) (37). In
lipid monolayers, different ions studies have been tested, some of them showing induction and some other disruption of the interaction of molecules conforming the monolayer (9, 10, 14, 40, 41).

During the last ~30 years, significant research has been done to better understand specific ion effects (41). Multiple analysis of effects of ions on lipids monolayers, especially DPPC, have been made (9, 10, 12, 14, 26), discussing and proving that a correlation of disruption and organization of the lipid molecules deposited in the air-water interface exist, by using different salts as sub-phase while building a monolayer. For instance, in 2016, Adams et al. analyzed effects of various cations in the sub-phase for the formation of pressure isotherms of DPPC monolayers (Figure 3) (9). They observed that NaCl and KCl salts expanded the isotherm, while MgCl₂ could expand or compress depending on the phase of the isotherm and concentration of the salt in the sub-phase. Additionally, with CaCl₂, they observed that the DPPC monolayer got more compressed. The conclusion of this study was that monovalent ions increase disruption of the lipid packing as they expand the isotherm of the DPPC isotherm. In case of divalent cations, it cannot be assured that all of them induce organization of the lipid packing since Adams et al. show that it depends on the divalent cation as well as the concentration of it (9).

Studies of anions disrupting the order of the alkyl groups of DPPC monolayers have been done for several years. For example, in 2012, Aroti et al. showed the impact of anions on DPPC isotherms (Figure 4) using as sub-phase saline solutions at 12°C (10). In this work
they concluded that in the absence of salts, the formed DPPC monolayer behaved fairly ordered, while building the DPPC isotherm with the presence of salts in the sub-phase increased the disorder of the lipid packing. One of the theories about this behavior is that ions compete with the lipid for available space in the surface leading to less organized lipid alkyl groups (14, 41) and gives more stability to the expanded phase since more pressure is needed to have a phase change (10, 14).

In the work described in this thesis, we aimed at understanding the effect of sodium salts sprayed as aerosols on the DPPC monolayer at the air-water interface, as a model system for the lung surface. We prepared LB monolayers and analyzed them with PM-IRRAS. The main goal is to understand the effect of the organization of the molecule with the addition of the salts in the DPPC monolayer deposited in the air-water interface.
Figure 3 Surface pressure-area isotherms of DPPC monolayers effect on a) NaCl, b) KCl, c) MgCl$_2$, and d) CaCl$_2$ solutions with varying salt concentrations. NaCl and KCl salts expand the DPPC monolayer, while MgCl$_2$ condenses or expands depending on phase or concentration. CaCl$_2$ condenses the monolayer. (9)
Figure 4 (a) Pressure–area isotherms of DPPC monolayers at 12°C on (a) NaSCN solutions of various concentrations, where can be seen that at 12°C the DPPC isotherm lacks of the expanded-condensed phase and by having NaSCN in the sub-phase it reappears proportionally to the concentration of the sub-phase, meaning destabilization of the monolayer. (b) 0.75 M solutions of different sodium salts where can be seen that the bigger the anion of the salt, the more effect in destabilization does to the isotherm. (14)
Chapter 2

2.1 Objectives

The main objective of this work is to study the molecular effect of the hypertonic saline solution of NaCl 0.5 M sprayed as aerosol in in DPPC monolayer deposited in the air-water interface by LB isotherms and PM-IRRAS. Four goals were proposed:

1. Analyze the effect of hypertonic saline solution in DPPC monolayers by LB and PM-IRRAS.
2. Extend the study on the impact of other salts, such as NaClO₄ and NaI, in order to understand the effect at a molecular level.
3. Study the effect of hypertonic saline solution on a highly viscous solution to mimic the mucus in order to understand the effect on the mucus of the patients with cystic fibrosis.
4. Verify the effect of the salts studied in point 2 in the mucus solution’s viscosity.

2.2 Hypothesis

When applying hypertonic saline solution to a lipid monolayer, we are expecting the salt to induce structural modifications, such as a better organization of the lipid molecules in the monolayer phase, which may cause a modification in the monolayer phase induced by the presence of the salt ions. In the same way, we are expecting that applying a high concentration of sodium salts (NaClO₄ and NaI) will organize the structure of the monolayer in a way that the bigger the anion, the more organized the structure will be.
This means the lipid molecules have less freedom of movement and will arrange in a more static phase. In addition, we expected to watch a decrease in the viscosity of an agar solution prepared to mimic the mucus of cystic fibrosis patients. This solution will be later on used as sub-phase for the monolayer to model the alveolar system of the patients’ lungs.
Chapter 3

Materials and methods

3.1 Chemicals

1,2-Dipalmitol-sn-glycero-3-phosphocoline (DPPC) (Sigma-Aldrich, ≥99%) was used as received and dissolved in chloroform (HPLC grade, Fisher Scientific) to make a 1 mM solution. The salts used for this experiments where sodium chloride (NaCl) (Sigma-Aldrich, ACS certified, ≥99%), sodium iodide (NaI) (Alfa Aesar, ACS certified, ≥99.5%), and sodium perchlorate (NaClO₄) (MP Biomedical, ACS certified, ≥99%). All salts were dissolved in ultrapure water with a resistivity of 18.2 MΩ•cm.

3.2 Surface pressure and surface tension

Surface tension is a thermodynamic quantity related to the work needed to expand the surface area of the system (42). At the air-liquid interface, the molecules of the liquid interact with higher attraction with each other since they don’t have any other similar molecules to bond to, provoking the effect of the surface tension (20, 42, 71). For example, the surface tension of the air-water interface at 20°C is 72.8 mN/m.

Changes in the surface tension of a surfactant film are often described in terms of the surface pressure, which is the amount by which the surface tension of a liquid is lowered by the presence of a surfactant film. The surface pressure, \( \pi \), is defined by Eq. 1 where \( \gamma^o \) is the surface tension of the pure liquid sub-phase and \( \gamma \) is the surface tension with surfactant (20).
\[ \pi = y^o - y \]  \hspace{1cm} \text{Equation 1}

The surface tension of surfactants films is usually reported as isotherms of surface pressure vs. surface area. The isotherm indicates how surface tension varies with interfacial concentration at a certain temperature (20, 26, 36).

### 3.3 The Langmuir-Blodgett technique

In the early 1920’s Katherine Blodgett and Irving Langmuir established the LB film technique by analyzing the transfer of monolayers onto substrates (43). Even nowadays this is one of the few methods of building organized molecules (mono)layers, making it possible the analysis of thin organic films (43). The LB technique measures the surface tension between air and water with or without a surfactant, by a plate attached to sense surface pressure (Figure 5) while the surfactant film is being compressed or expanded and allows the recovery of the surface pressure (13, 43). One of the major advantages of the system is to allow the study of air-water interface experimental models, such as the studies of phospholipid monolayers, fabrication of functional coatings of nanotubes, nanowires, and graphene, among other molecules monolayers formation (27, 34). This technique has been extensively used to study the interaction of different molecules and nanoparticles with monolayers of different lipid compositions (9-14, 26, 31-33, 36, 41, 44-49). For example, Leontidis analyzed the interactions of soft matter (DPPC) with hydrophobic ions (NaNO₃, NaClO₄, NaSCN, NaPF₆ and NaTPB); where he saw that when the surface did not reach the monolayer phase, the ions push surface molecules aside as they competed for interfacial sites (40). Griffith et al., analyzed the
effect of L-phenylalanine, an essential amino acid, in the DPPC monolayer, and observed that L-phenylalanine had a preference for the surface, which ended up in surface competition with the phospholipid molecules and modified the organization of the DPPC monolayer in the air-water interface (32).

**Figure 5** A schematic of the Langmuir-Blodgett technique, showing the parts of the LB trough to build monolayers, such as the Teflon barriers, the surface pressure sensor, and the Teflon trough. The isothermal compression fixes the molecules of the amphiphilic substance and forms a monolayer.
Langmuir monolayers containing either pure surfactants or mixtures of lung surfactant components have been widely and successfully used as model systems in fundamental research of the lungs surfactant system (13). Furthermore, monolayers of zwitterionic phospholipids, such as DPPC, have been used as model systems to understand specific anion effects in physicochemical and biological systems (14), where it was observed that low temperatures (<15°C), the monolayer improves stabilization. This effect was contradicted, meaning that the monolayer got disordered, specially with big anions such as I⁻, ClO₄⁻, and SCN⁻ (14). Based on the concern about the increasing concentration of carbon nanotubes in the air, Melbourne et al., analyzed the effect of these in the lipid interface of pulmonary surfactant and found that the carbon nanoparticles affect negatively the packing ability of pulmonary surfactant and DPPC (35).

The organization of the DPPC monolayer in the air/water interface has been well described (13, 20, 48). During the isotherm formation, several surface phase transitions are observed and are ideally described as gas, expanded and condensed phases (Figure 6 and Figure 7). In the “gas” state the film molecules have minimum or no interaction. In the “expanded” state, the film molecules have some degree of interaction. In the “condensed” state, the film molecules have closer molecular packing and the chains of the molecules are fully extended (20). During these transitions phase coexistence occurs, gas-liquid and liquid-solid, which means that both phases occur at the same time (9, 11, 20, 26).
Figure 6 Surface phase transitions of an amphiphilic molecule isotherm. In the “gas” state the film molecules have minimum or no interaction. In the “expanded” state, the film molecules start to interact. In the “condensed” state, the film molecules have closer molecular packing; the chains of the molecules are fully extended. Figure adapted (20).

In the work described in this thesis Surface pressure vs. molecular area isotherms were obtained on a computer controlled LB trough (Large trough, KSV instruments, Helsinki, Finland; $A_{\text{total}} = 841 \text{ cm}^2$) with a surface made of Teflon with two compression barriers. A monolayer was built by measuring surface pressure, which was measured with a
Wilhelmy plate of filter paper (KSV, Biolin Scientific). Before any measurement, the trough and the barriers were cleaned with ethanol and ultrapure water. After filling the trough with ultrapure water as the sub-phase, the water surface was further cleaned by sweeping the barriers to a smaller exposed surface area and aspirating the surface. The aqueous surface was considered clean when the surface pressure ($\pi$) of pure water was lower than 0.2 mN/m. Monolayers were formed by spreading a known number of the molecules solution of interest (stearic acid and DPPC) at the surface, before starting compressions. After 10 minutes to allow evaporation of the spreading solvent, we started compression at a constant rate of 5 mm/min/barrier. We repeated the pressure isotherms at least three times to ensure reproducibility.
Figure 7 A representative isotherm of DPPC molecules, surface pressure vs. mean molecular area, at the air-water interface. (I) Gas and expanded phase in coexistence; (II) Expanded phase; (III) Expanded and Condensed phase in coexistence; (IV) Condensed phase.

3.4 Polarization Modulation Infrared Reflection Absorption Spectroscopy

Polymerization Modulation Infrared Reflection Absorption Spectrometry (PM-IRRAS) (Figure 8) aids the investigation of chemical composition and molecular orientation of interfacial films on liquid and solid substrates (46, 50-52). The major advantage of this technique is that it conserves the IRRAS assets of electric field enhancement and the surface selection rule, and adds high sensitivity in absorption detection and the ability to make in situ experiments, this allows to resemble “real life” situations (53). The surface specific Fourier transform infrared spectroscopy (FT-IR) spectra of materials that the PM-IRRAS technology allows, is done by the differences in the reflection of p- and s-
polarized light from the interface (43, 53). It helps to understand better the bands of the spectra since it eliminates the background signals coming from the surrounding environment (43, 46, 52, 54).

One of the main drawbacks with the conventional spectroscopy (FT-IR) is the absorption of light by water, which gives a non-accurate spectrum as a result (55). This problem forces to add dry nitrogen gas or use high vacuum in order to avoid the signal of water vapor (commonly used in experiments with only IRRAS) (43).

![PM-IRRAS Machine Schematic](image)

**Figure 8** Schematic of PM-IRRAS machine. On the left side, the spectrometer and polarization modulation units are mounted, on the right side a highly sensitive detector is mounted. Adapted figure (43).

PM-IRRAS has been used with different lipid monolayers obtained by LB technique, such as 1,2-dimystoyl-sn-glycerol-3-phosphocholine (DMPC) (50), 1,2-di[cis-9-octadecenoyl]-sn-glycerol-3-[phosphor-L-serine] (DOPS) (51), dimyristoyl-phosphatidyl-glycerol (DMPG) (44), and DPPC (30). As an example, PM-IRRAS has being used to study the hydrolysis
reaction in DPPC monolayers, concluding that PM-IRRAS is a versatile method to study the hydrolysis of long-chain lipids deposited at the air-water interface (56). PM-IRRAS technique gives accurate results in environments or experiment set-ups that can be manipulated and see the effect of the environment in the monolayer of interest.

In this thesis, PM-IRRAS was performed using KSV NIMA PM-IRRAS (KSV Instrument Ltd., Helsinki, Finland). The LB trough is positioned in a way that the beam light reaches the monolayer at an incidence angle of 76° (43). The beam is continuously modulated by s-polarization and p-polarization, allowing simultaneous measurement of both polarizations. Calibration of the equipment was made following manufacturer recommendations (43). Measurements were performed at 23°C and were taken as specified in the results and discussion chapter in this thesis. After obtaining a spectrum of DPPC with water as sub-phase to be used as background for the following measurements, salt solutions, all of them with a concentration of 0.5 M, were sprayed (~0.5 mL) and new spectra were collected at defined time points.

3.5 Viscosity measurements

To model the lungs mucus that lines the alveolar surface, 1% agar (Spectrum Chemical) solutions were used. Agar was dissolved with ultrapure water with a resistivity of 18.2 MΩ•cm at 70°C, for 30 min. After the 30 min, the solution was cooled down at room temperature until it reached equilibrium and the viscosity was measured using Brookfield viscometer DV1 (Ametek Brookfield Engineering Laboratories, Middleboro,
USA). After the first measurement, 1mL of salt solution (0.5 M) was added and the viscosity was assessed again to observe the effect of the salt on this parameter.
Chapter 4

Results and discussion

4.1 LB and PM-IRRAS

Langmuir isotherms (surface pressure vs. mean molecular area) are a conventional way to understand the different phase behavior of any molecule (13, 47). In order to validate the proposed experimental methodology, experiments were first conducted with stearic acid (Figure 9), a fatty acid with an 18 carbons chain. Stearic acid was chosen since it has a simpler chemical structure than DPPC. Stearic acid was dissolved in chloroform at a concentration of 1 mg/mL and 80 µL spread at the water surface. After 10 minutes to allow evaporation of the spreading solvent, the compression started at a constant rate of 5 mm/min/barrier up to 15, 30 and 35 mN/m. Figure 10 shows the increase of molecular organization by increasing surface pressure, meaning that the molecules organize in a more compact way and the tails of stearic acid can be more easily detected with the PM-IRRAS technique. Further stearic acid experiments were done at 35 mN/m, at the isotherm condensed phase region and knowing that the monolayer is not collapsed since it has not reached ~50 mN/m, which was determined to be the collapse pressure for stearic acid monolayer at the used experimental conditions used. Stearic acid isotherms and PM-IRRAS spectra (Figure 11) gave us similar results to the ones reported in the literature (43, 44). These preliminary experiments allowed us to validate our methodology and design the next experiments.
Figure 9  Stearic acid schematic of the molecule. Size of the molecule ~28 Å.

Figure 10  Spectra of stearic acid (1 mg/mL) collected by PM-IRRAS at 15, 30 and 35 mN/m. Spectra shows the asymmetric and symmetric CH$_2$ bands. The symmetric bond requires less energy to stretch reflected in a lower wavenumber (~2850 cm$^{-1}$) and the asymmetric bond requires more energy to stretch and is reflected in a higher wavenumber (~2916 cm$^{-1}$).
Figure 11 (A) Isotherm of stearic acid (1mg/ml) with pure water as sub-phase. The state transition of the molecules is shown I) gas, II) expanded, and III) condensed. (B) Stearic acid PM-IRRAS spectra at the condensed phase (35mN/m). Symmetric and asymmetric CH$_2$ is shown at ~2850 and ~2916 cm$^{-1}$ respectively and asymmetric CH$_3$ at ~2960 cm$^{-1}$. 
After obtaining the stearic acid PM-IRRAS spectrum, a hypertonic saline solution (NaCl 0.5 M) was sprayed on to the monolayer at the air-water interface. The experimental methodology of spraying the salts in the surface of the monolayer has never been done before. Previous experiments analyzing the effect of ions in soft matter were executed using the salts in the sub-phase of the monolayer. Subsequently, we developed three measurements with PM-IRRAS at different time points, 5, 10, and 15 min. We analyzed the effect on the PM-IRRAS spectra (Figure 12) and noticed an increase in the signal intensity at the characteristic bands of CH$_2$ asymmetric and symmetric stretching (2800-3000 cm$^{-1}$). We have reproduce this experiments three times, and in all the times the results are in concordance. The spectra obtained, suggest that NaCl sprayed from the air has an organizing effect on the stearic acid monolayer deposited in the air-water interface, obtaining higher characteristic peaks’ intensity of CH$_2$ by spectroscopy. This result is in accordance with the main hypothesis of this thesis, since increasing the organization of the alkyl chain would induce the decrease of surface tension. This phenomenon could be the reason why after breathing hypertonic saline solution, cystic fibrosis patients experiment a higher expansion of the lungs and a relief in the respiratory function.
Figure 12 (A) Stearic acid PM-IRRAS spectra before spraying NaCl 0.5 M (red line) and 10 min after spraying (black line) in the stearic acid monolayer deposited on the air-water interface held at 35 mN/m; (B) Stearic acid PM-IRRAS spectra before spraying NaCl 0.5 M (red line) and 15 min after spraying (black line) in the stearic acid monolayer deposited on the air-water interface held at 35 mN/m. Spectra show the asymmetric and symmetric CH$_2$ as well as the asymmetric CH$_3$. 
Our speculations of the system’s behavior with the experimental methodology proposed are that the salty drops (~0.3 mm) of NaCl sprayed at the surface of the stearic acid monolayer bring some of the stearic acid molecules that formed the monolayer to the sub-phase. This will induce the formation of micelles and the remaining molecules in the surface will rearrange to form the monolayer and it will become a more organized monolayer (Figure 13). Cl⁻ is proved to don’t disturbed the soft matter monolayer (14). The effect of organization in our results can be due to the chloride low surface competition and allowing the surfactant molecules to get closer.

![Image](image.png)

**Figure 13** A) Addition of sprayed salt to the surface of a compressed lipid monolayer at the air-water interface. B) Addition of salty drops releases ions in the water. Some of the drops could carry lipids from the monolayer into bulk water, which C) might form micelles, The ions spread in bulk water, and compete with the surfactants to appear at the air-water interface and/or fit between lipid molecules, which changes the packing arrangement of the compressed monolayer.

After confirming our hypothesis with stearic acid, we moved on to DPPC monolayers that have a higher biological relevance for lungs as stated in chapter 1 section 1.4. In addition, to understand the physical phenomena at the monolayer with sprayed salt solutions, we decided to do experiments with other sodium salts, NaI and NaClO₄. We
hoped that such a comparison would help us understand effects of various properties of ions, such as size, polarizability, and hydration sizes, on the organization/disorganization of lipids at the model lung surfaces. To eliminate possible experimental errors, the order of the experiments was chosen randomly and we decided to use the ratio of maximum intensity of the CH₂ asymmetric stretching peak, since it is the highest peak shown in the IR spectra, to have better data comprehension and be able to compare the diversity of experiments.

We collected DPPC isotherms at the air-water interface to observe the phase behaviors of the monolayers and to identify reasonable surface pressure to perform the spray experiments. Figure 14 shows DPPC isotherm, at room temperature, where the different phases and phase coexistence are highlighted in Figure 7 (gas-expanded, expanded, expanded-condensed, and condensed phases). As can be seen between Figure 10 A and Figure 14, the isotherms formed by stearic acid compared to the ones made by DPPC comprise of some differences in the phases. DPPC has transition phases while stearic acid doesn’t; this is due mainly to the geometry of the molecule and the composition, meaning that almost all the molecules that have a single alkyl chain will build isotherms similar to the stearic acid. We chose to work with a surface pressure at 35 mN/m where the monolayer is already in the condensed phase and has not reached the collapse surface pressure (~42 mN/m). At this phase, it can be assumed that the phospholipid molecules are organized in a less flexible conformation, without significant internal degrees of freedom (48). In the past years, several authors have proved that isotherms
behave differently according to the experimental conditions. For example, Christoforou et al. and Toimil et al. observed that at lower temperatures (<15°C), the expanded phase in coexistence with the condensed phase disappears from the isotherm formed with DPPC deposited in the air-water interface (Figure 15), which suggest a more stable liquid condensed phase \( 14, 57 \). Additionally, another study demonstrates a dependency between the expanded-condensed phases with pH; at higher pH this phase takes longer to change to the condensed phase \( 45 \). Thus, we consider that specifics of the experimental conditions crucially influence the properties of these soft systems.

**Figure 14** Isotherm of DPPC molecules (1 mM), surface pressure vs. mean molecular area. (I) Gas and expanded phase in coexistence; (II) Expanded phase; (III) Expanded and Condensed phase in coexistence; (IV) Condensed phase.
Figure 15 Langmuir π-A isotherm of DPPC monolayers at different temperatures. Range 8-25°C.
At temperatures lower than 15°C, the expanded-condensed phase disappears from the isotherm, suggesting a more stable condensed phase. (14)

After obtaining the DPPC isotherm, PM-IRRAS spectra of DPPC monolayer with a sub-phase of water was measured. The known FT-IR spectrum of DPPC (Figure 16) shows the characteristic bands for C-H stretching bond (2800-3000 cm\(^{-1}\)), the carbonyl-stretching bond (~1700 cm\(^{-1}\)), and the band due to the phosphate group (1000-1300 cm\(^{-1}\)). In our results (Figure 18), the PM-IRRAS spectra exhibited clear bands due to the hydrocarbon asymmetric and symmetric stretching, between 2800 and 3000 cm\(^{-1}\), as reported before in the literature (9, 14).
Three different salt solutions at a concentration of 0.5 M were sprayed (in separate experiments) on the surface of DPPC monolayers deposited in the air-water interface and the PM-IRRAS spectra were recorded.

Previous results obtained by G. Ma and H.C. Allen, Aroti et al., Leontidis et al., and Avila et al., conclude that by adding disrupting salts, such as NaClO$_4$ and NaSCN, to the sub-phase the isotherm tends to expand suggesting interfacial competition and disorganization of the alkyl chains (10, 26, 40, 41, 44). Based on these results, we expected to see a similar effect by adding the salts as spray in the surface of the DPPC monolayer deposited in pure water. The experiments were done at constant pressure (35 mN/m), as explained before in chapter 3 section 3.4, being our blank the measurement before the salt addition. Then, we sprayed the salt solution in the DPPC monolayer deposited in the air-water interface and took 3 measurements at different
time intervals, one at 5 min, 10 min and 15 min. These experiments were repeated 6 times for each sodium salt and the ratio of maximum intensity was determined. According to Adams et al., the presence of NaCl in the sub-phase induces a shifting to a lower wavenumber in CH$_2$ stretching bonds in the coexistence phase, expanded-condensed, compared with the spectrum where the sub-phase is pure water, which suggests a better organization of the molecules but at the condensed phase, this effect is not observed (Figure 17) (9). Figure 18 depicts a comparison between PM-IRRAS spectra of DPPC monolayer in the condensed phase before and after the application of sprayed NaCl 0.5 M in the surface of the monolayer showing no shifting in the wavenumber for the CH$_2$ symmetric and asymmetric stretching bonds.
**Figure 17** IRRAS spectra of DPPC monolayers in the LE-LC and LC phases on (a) water and (b) 2.0 M NaCl. Vertical lines indicate the peak positions of CH vibrational modes of DPPC on water in the Expanded-condensed phase. Shifts to lower wavenumbers relative to water indicate that DPPC alkyl chains are more ordered on NaCl and CaCl$_2$ solutions. (9)
Figure 18 DPPC monolayer at constant surface pressure of 35 mN/m PM-IRRAS spectra. DPPC monolayers are recorded at the condensed phase. The black line spectrum is measured before applying 0.5M NaCl sprayed in the surface of the monolayer, and the red line is after spraying ~0.5mL of NaCl 0.5M. No shifting in the wavenumber of asymmetric and symmetric CH₂ stretching bonds is observed.

We compared ratios of maximum intensities of CH₂ stretches after and before spraying electrolytes, but found that the signal to noise ratio was so high that effects were within error bars (Figure 19). We speculate that DPPC monolayers behave so differently from stearic acid monolayers because the former are constituted of molecules significantly more hydrophobic than the latter. This issue requires further investigation by theoretical chemists.
Figure 19 Ratio of maximum intensity of CH\textsubscript{2} stretching peak spectrum for DPPC monolayer deposited in the air-water interface after addition of sodium salts (black square) NaI 0.5M; (red circle) NaCl 0.5M; and (blue triangle) NaClO\textsubscript{4} 0.5 M. Measurements taken 5, 10 and 15 min after salts sprayed.

These results suggest that the methodology used to study stearic acid is not appropriate for more complex systems, such as DPPC. This is probably due to the presence of more complex functional groups in the DPPC molecule, such as phosphate and amine groups. The addition of such groups in the molecule moiety implies new interactions between salts and the DPPC molecules, creating a more dynamic system. To overcome this issue, new experimental methodologies must be tested. For example, the first and significant experimental change should be the reduction of temperature. The decrease in
temperature will induce a more static monolayer that can induce a more detailed measurement from the equipment used.

4.2 Viscosity

One of the biggest challenges of cystic fibrosis is to understand why after taking the hypertonic saline solution medication, the viscosity of the mucus in the lungs reduces. Viscosity experiments were made with the different sodium salts used in the previous experiments at the same concentration. Agar was chosen in order to simulate the natural viscosity found in the mucus of the lungs. It was observed that sodium salts have an effect in lowering the viscosity of a 1% agar solution. NaCl is the salt that lowers the viscosity of the most, followed by NaI and then NaClO₄. Nevertheless, all of the salts presented higher impact than pure water (Table 1), suggesting that the effect on decreasing the viscosity is correlated with the proprieties of hydrated ions and their interactions with the polymer. For instance, Saito and co-workers investigated effects of salts on viscosity of aqueous solutions of poly(vinyl)alcohol (0.6% by weight), and found that NaI decreased the intrinsic viscosity of PVA more than NaClO₄ (58). They concluded that hydrated ions decreased interactions between polymer chains, thereby decreasing viscosity (58, 59). We consider our agar gel to be similar to theirs, wherein hydrated ions of Cl⁻ can partition more or less equally everywhere, whereas the larger ClO₄⁻ ions tend to not fractionate at hydrophilic interfaces. We derive this analogy based on the fact that larger anions have been demonstrated to speciate significantly more at water-hydrophobe interfaces than smaller ones (60).
**Table 1** Difference of viscosity obtained after the addition of 1 mL sodium salt to a 1% agar solution.

<table>
<thead>
<tr>
<th>Solution added</th>
<th>Δμ (cP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>4800</td>
</tr>
<tr>
<td>NaI</td>
<td>3600</td>
</tr>
<tr>
<td>NaClO₄</td>
<td>3160</td>
</tr>
<tr>
<td>Water</td>
<td>2850</td>
</tr>
</tbody>
</table>
Chapter 5

Conclusion

The main goal of this thesis was to understand the molecular interactions that occur in the lungs system of patients with cystic fibrosis when they breathe mist of hypertonic saline solutions. To explore this question, we decided to work with two main techniques, LB and PM-IRRAS. LB allowed us to build monolayers in an aqueous subphase, while PM-IRRAS allowed us to analyze the presence of different functional groups and the molecular orientation of interfacial films on a liquid substrate (46, 50-52) without having a negative impact on the surface of interest. These techniques allowed us to see the effects of adding hypertonic saline solution (NaCl 0.5 M) in the aerosol form to DPPC monolayers deposited in the air-water interface.

In order to understand and validate our techniques and methodology, we started experiments with stearic acid, which is a well-known fatty acid that is structurally similar, yet simpler, than DPPC. These experiments were done building a stearic acid monolayer and analyzing the orientation of the molecules with and without the effect of NaCl sprayed at the lipid-air interface. Such experimental methodology has never been reported before. We demonstrated that the spray organized the stearic acid monolayer, as evidence by enhancement in the CH$_2$ stretching peaks in the PM-IRRAS spectra. With these promising results, we move investigated DPPC molecules, which is the main lipid found in the pulmonary surfactant. In addition, we decided to analyze the effect of
other sodium salts such as NaI and NaClO₄ to compare the effect of the presence of different anions. We had expected similar trends in the behaviors of DPPC and stearic acid monolayers at the air-water interface, but were wildly surprised to find an inconsistency.

Intriguingly, our experiments with aqueous sprays on DPPC monolayers had such a low signal-to-noise ratio that we could not resolve the effect of salty droplets on orientations of monolayers. We speculated that this difference in signal-to-noise ratio between DPPC and stearic acid monolayers is due to the higher hydrophobicity of the DPPC molecules. We recommend that experimental setup with PM-IRRAS requires further optimization, such as reduction of temperature of the sub-phase, to be able to observe significant signal-to-noise ratio.

Simultaneously, we measured effects of the same sodium salts on viscosity of a 1% agar solutions. The salt that had the highest impact was NaCl, followed by NaI and the one that had the less impact was NaClO₄. Although NaI and NaClO₄ are not recommended for human consumption, they were compared against NaCl; we found that their effect on reducing the viscosity was lower than that of NaCl.

With the results described in this thesis, it is possible to conclude that the sprayed salt solution in stearic acid induces disturbances on molecular orientation but the same effect is not observed by the same methodology with DPPC monolayers. It is particularly important to note that the aerosol salt solution of NaCl induces an increase of the molecular organization of the alkyl chains of stearic acid and that the viscosity of the
solution decreases with the addition of NaCl. The combination of these two phenomena could be the reason why cystic fibrosis patients find it easier to breathe after the inhalation of highly concentrated NaCl solutions.
Chapter 6

Future work

As a continuity for the project and to better understand the phenomena of hypertonic saline solution on the lungs surfactants, the following points would bring important insights:

• Perform a rational study on the impact of several temperatures in the stability and equilibrium of DPPC monolayers.

• Carry out a similar study on the effect of hypertonic saline solution on the monolayers maintaining the anion and changing the cation of the salts studied.

• Study the impact of hypertonic saline solution on DPPC monolayer build on top of a viscous sub-phase (e.g. agar 1%).

• Increase the complexity of the monolayer composition, by using a mixture of lipids like DPPC, DMPC and DOPS among others, to resemble the lungs lipid composition.


56. Q. He, J. B. Li, Hydrolysis characterization of phospholipid monolayers catalyzed by different phospholipases at the air-water interface. *Adv Colloid Interfac* 131, 91-98 (2007).


