Self-Assembly in Lipid-Protein Systems
Lung Surfactant, Stratum Corneum and Model Membranes

Jenny M. Andersson

Lund University
Self-Assembly in Lipid-Protein Systems: Lung Surfactant, Stratum Corneum and Model Membranes

Abstract
This thesis explores lipid self-assembly and aims to give a broad picture of self-assembly structures in simple and complex lipid-protein systems. The systems studied are lung surfactant, *stratum corneum* and simple model membranes. The lung surfactant mixture lines the alveoli in our lungs and stabilises the air-tissue interface. The lung surfactant lipid phase behaviour was here investigated with respect to the effects of cholesterol concentration and changes in the external conditions of temperature and a water gradient. Taken together the studies of lung surfactant give a comprehensive picture of the phase behaviour of a clinical lung surfactant extract, showing the importance of cholesterol and non-equilibrium conditions. It is a recurrent observation for all studies that the addition of physiologically relevant levels of cholesterol to the clinical lung surfactant forms a single robust liquid ordered phase under both equilibrium and non-equilibrium conditions.

Conditions such as draught, high salinity or freezing, exerts the lipid systems to osmotic stress, which can lead to phase changes between different self-assembled structures. The outer layer of the skin, the *stratum corneum* is most of the time exposed to osmotic stress from dry air in the environment. The *stratum corneum* contains small polar molecules, to counteract phase changes due to osmotic stress. We study how the self-assembly in *stratum corneum* and model membranes are affected by the presence of osmolytes under conditions of osmotic stress. It is shown that these compounds under dry conditions act to replace the water in both *stratum corneum* and model membranes and they may stabilize the fluid lipid phases at lower humidities.

In plants, there are several strategies for protection against osmotic stress. One strategy involves the expression of specific proteins. We have studied how one such protein from the family of dehydrins, influences lipid self-assembly, aiming at molecular understanding of how these proteins can protect membranes against osmotic stress. The dehydrin protein, Lti30, is shown to stabilise the liquid crystalline lamellar phases over a large range of hydration conditions, preventing phase transitions at low water contents, and extensive swelling of the lamellar phase at high water contents.

Key words
Lipid self-assembly, lung surfactant, stratum corneum, dehydrin, cholesterol, osmolytes, urea, TMAO, ssNMR, cSAXS
Self-Assembly in Lipid-Protein Systems
Lung Surfactant, Stratum Corneum and Model Membranes

Jenny M. Andersson
“There is no sense crying over every mistake. You just keep on trying till you run out of cake”

– GLaDOS
Contents

List of Papers i

List of Author Contributions ii

Publications not included in this thesis iii

List of Abbreviations v

Populärvetenskaplig Sammanfattning vii

1 Aims of this thesis 1

2 Lipid self-assembly 3
   2.1 Self-assembly structures ............................. 3
      2.1.1 Molecular properties that influence self-assembly structure .............................. 3
      2.1.2 Lamellar phases .................................. 5
      2.1.3 Cholesterol in PC bilayers .......................... 6
   2.2 Phase transitions ................................... 8
      2.2.1 Gibb’s phase rule .................................. 8
      2.2.2 Phase transitions between different self-assembly structures ............................. 8
      2.2.3 Mixed lipid systems ................................ 9
   2.3 Interplay between phase behaviour and membrane material properties .......................... 10

3 Lung surfactant 11
   3.1 Lung surfactant related diseases .......................... 11
   3.2 Aims of this chapter .................................. 12
      3.2.1 Model systems of the lung surfactant ............... 13
      3.2.2 Experimental approach ............................. 14
   3.3 Lung surfactant composition ............................ 16
      3.3.1 Lipid composition .................................. 16
      3.3.2 Surfactant proteins ................................. 16
      3.3.3 Lung surfactant extracts ........................... 17
   3.4 Self-assembly in lung surfactant ........................ 17
3.4.1 Self-assembly of lung surfactant extracts in bulk conditions

Method 2: R-PDLF and Order parameters

3.4.2 Interfacial multilayers in non-equilibrium conditions

Method 3: Determination of water content from IR

3.4.3 Interfacial layers under compression-expansion cycles

Tubule formation at the multilayer-bulk interface

Conclusions and outlook

Acknowledgements

References

Appendix

Paper I: Effect of cholesterol on the molecular structure and transitions in a clinical-grade lung surfactant extract

Paper II: Cholesterol induces homogeneity in non-equilibrium lung surfactant multilayers

Paper III: Interfacial multilayers of lung surfactant observed by neutron reflectometry under compression-expansion cycles

Paper IV: Dehydrin Lti30 stabilizes lipid lamellar structures at varying hydration conditions

Paper V: Stratum corneum molecular mobility in the presence of natural moisturizers
List of Papers

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals. The papers are appended at the end of the thesis.

I Effect of cholesterol on the molecular structure and transitions in a clinical-grade lung surfactant extract  
Jenny Marie Andersson, Carl Grey, Marcus Larsson, Tiago Mendes Ferreira and Emma Sparr  
Proceedings of the National Academy of Science 2017, 114(22), E3592–E3601.

II Cholesterol induces homogeneity in non-equilibrium lung surfactant multilayers  
Jenny Marie Andersson, Kevin Roger, Marcus Larsson and Emma Sparr  
Submitted

III Multilayers of Lung Surfactant at the Air-Liquid Interface under Compression-Expansion Cycles Observed by Neutron Reflectometry  
Jenny Marie Andersson, Maximillian W.A. Skoda, Marcus Larsson, Emma Sparr and Tommy Nylander  
Manuscript

IV Dehydrin Lti30 stabilizes lipid lamellar structures at varying hydration conditions  
Jenny Marie Andersson, Quoc Dat Pham, Helena Mateos Cuadros, Sylvia Eriksson, Pia Harrysson and Emma Sparr  
Manuscript

V Stratum corneum molecular mobility in the presence of natural moisturizers  
Sebastian Björklund, Jenny Marie Andersson, Quoc Dat Pham, Agnieszka Nowacka, Daniel Topgaard and Emma Sparr  
Author Contributions

I I, ES, TF and ML designed the study. I performed all experiments and performed data analysis together with ES and TF. I, ES and TF wrote the paper with contributions from the other authors.

II I, ES, KR and ML designed the study. I performed the IR and Raman microscopy, polarized light microscopy and bulk SAXS/WAXS experiments. I, ES and KR performed the cSAXS experiments. I, ES and KR performed the data analysis. I, ES and KR wrote the paper with contributions from the other authors.

III I, TN, ES and ML designed the study. I, TN and MS performed the neutron reflectivity measurements. I performed the BAM and SAXS/WAXS experiments. I, TN and MS performed the data analysis. I wrote the manuscript with contributions from the other authors.

IV I, ES, PH and SE designed the study. I and ES selected the lipid model systems. I developed the lipid-protein sample preparation protocol. I, HMC and QDP performed the SAXS/WAXS experiments. I and QDP performed the NMR experiments. I performed the sorption balance experiments. I and HMC did the SDS-PAGE and protein concentration experiments. I, ES and QDP analyzed the data. ES wrote the manuscript with contributions from me.

V ES and SB designed the study. I, QDP, SB and AN performed the experiments. I, ES, SB and QDP analyzed the data. ES and SB wrote the paper with input from me and the other authors.
Publications not included in this thesis

1. **Pressurised hot water extraction with on-line particle formation by supercritical fluid technology**
   Jenny Marie Andersson, Sofia Lindahl, Charlotta Turner and Irene Rodriguez-Meizoso
## List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPC</td>
<td>1,2-dipalmitoyl-sn-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>DMPC</td>
<td>1,2-dimyristoyl-sn-glycero-3-phosphocholine - (sodium salt)</td>
</tr>
<tr>
<td>DMPG</td>
<td>1,2-dimyristoyl-sn-glycero-3-phospho-(1’-rac-glycerol)</td>
</tr>
<tr>
<td>DOPC</td>
<td>1,2-dioleoyl-sn-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>DOPE</td>
<td>1,2-dioleoyl-sn-glycero-3-phosphoethanolamine</td>
</tr>
<tr>
<td>DOPS</td>
<td>1,2-dioleoyl-sn-glycero-3-phospho-L-serine (sodium salt)</td>
</tr>
<tr>
<td>POPC</td>
<td>1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>ssNMR</td>
<td>solid state nuclear magnetic resonance</td>
</tr>
<tr>
<td>INEPT</td>
<td>insensitive nuclei enhanced by polarization transfer</td>
</tr>
<tr>
<td>CP</td>
<td>cross polarization</td>
</tr>
<tr>
<td>DP</td>
<td>direct polarization</td>
</tr>
<tr>
<td>R-PDLF</td>
<td>R-type proton-detected local field</td>
</tr>
<tr>
<td>cSAXS</td>
<td>coherent small angle x-ray scattering</td>
</tr>
<tr>
<td>WAXS</td>
<td>wide-angle x-ray scattering</td>
</tr>
<tr>
<td>RH</td>
<td>relative humidity</td>
</tr>
<tr>
<td>RDS</td>
<td>respiratory distress syndrome</td>
</tr>
<tr>
<td>SP</td>
<td>surfactant protein</td>
</tr>
<tr>
<td>TMAO</td>
<td>trimethylamine oxide</td>
</tr>
<tr>
<td>NMF</td>
<td>natural moisturising factor</td>
</tr>
<tr>
<td>LEA</td>
<td>late embryogenesis abundant</td>
</tr>
<tr>
<td>MD</td>
<td>Molecular dynamics</td>
</tr>
</tbody>
</table>
Populärvetenskaplig sammanfattning


Yttersta lagret av huden, stratum corneum, består även den av lipider och proteiner som utgör vår kropps största barriär mot omgivningen. Hudens innehåller också små polära molekyler, så kallade osmolyter, för att skydda denna barriär mot torka, vilket kan göra huden bräcklig. För att förstå mekanismen bakom hur dessa osmolyter skyddar huden mot torka har vi undersökt hur dessa små molekyler påverkar lipider och proteiner i stratum corneum vid olika fuktighetsförhållanden. Osmolyter återfinns även i andra organismer och växter för att skydda dessa mot uttorkning och kyla. I växtriket finns även andra mer komplicerade strategier som involverar specifika proteiner för att skydda...
växterna mot extrema väderförhållanden. Vi har studerat hur ett sådant pro-
tein, från familjen dehydriner, påverkar modellmembran designade för att likna
strukturer relevanta för växter, och vi har jämfört effekterna av dehydrin pro-
teinet med hur osmolyter påverkar dessa membran för att få en bättre bild av
olika skyddsmechanismsers roll i naturen.

Tillsammans ger dessa studier en bred bild över strukturer i olika lipid-
protein system och hur dessa påverkas av sammansättningen och hur övergång
mellan olika strukturer i reaktion på yttre stress kan förhindras i dessa system.
Chapter 1

Aims of this thesis

The outer barrier between the air we breathe and the blood stream consists of a thin film composed of lipids and proteins, commonly referred to as the lung surfactant layer. The lung surfactant film stabilises the alveolus in our lungs and facilitates breathing. Lack of the lung surfactant can lead to severe syndromes. Prematurely born children can suffer from lung surfactant deficit due to that the body have not yet had time to produce enough surfactant to make a stable film. The treatment of these states involves the addition of an external source of surfactant derived from animals. The extracts used in clinical treatments, lacks some of the components that are naturally present in the endogenous lung surfactant. Some open questions in the field concern what structure the lipid-protein mixture adopts at the air-liquid interface in the alveoli, and how the individual components affect this structure.

Lipids are amphiphilic molecules that organise themselves into self-assembled structures in systems that contain water. There is a huge variety of lipids and depending on their molecular properties they will form different structures in solution. One aim of this thesis have been to study how the different lipid components in the lung surfactant mixture affects the self-assembled structure (Paper I-III). Another central question concerns the structure of the lung surfactant film at the non-equilibrium conditions when it is present at the air-liquid interface and how that differ from the equilibrium structure that forms in bulk solution (Paper I-II). We have also studied how external conditions such as temperature and dry air affects the self-assembly of the lipids (Paper II-III).

Conditions such as draught, high salinity or freezing, exerts the lipid membrane to osmotic stress. Such stress can make the lipid undergo phase changes between different self-assembled structures. The outer layer of the skin, the stratum corneum is often exposed to osmotic stress from dry air in the environment. Stratum corneum is made up of lipids and proteins and contains small polar molecules, referred to as osmolytes, which can protect the skin from phase transitions that might make it brittle. We have studied the mechanisms behind how such osmolytes protect the lipids and proteins in stratum corneum against osmotic stress (Paper V).
The production of osmolytes in response to osmotic stress is a common protective strategy not only in skin, but also in plants and other organisms. In plants, there are also other, more complicated strategies, involving the expression of specific proteins. We have studied how one such protein from the family of dehydrins, influences lipid self-assembly, aiming at molecular understanding of how these proteins can protect membranes against osmotic stress (Paper IV).

In this thesis, we investigate a variety of lipid-protein systems. The different studies in Paper I-V give a broad picture of self-assembly structures in simple and complex lipid-protein systems, and how these are affected by the addition of other molecules, including cholesterol, osmolytes and dehydrin protein. We also study how the self-assembly is affected by changes in the external conditions of temperature and osmotic pressure (relative humidity). One important aspect of the work in this thesis, is the correlation between self-assembly in equilibrium bulk conditions and in non-equilibrium conditions, where the self-assembly structures are present in several composition gradients. The lipid-protein systems are studied both at the mesoscopic level, determining the self-assembly structure, and on the molecular level, investigating the effects of the individual molecules on specific components of the lipid-protein systems.

Outline
This thesis consists of a summary of the work of five papers. In addition to this short introduction, the thesis contains another three chapters:

- Chapter 2, introduces some basic concepts and gives a general description of lipid self-assembly and lipid phase transitions.

- Chapter 3, gives a general introduction to the system of the lung surfactant together with a description of the major findings of Paper I-III.

- Chapter 4, describes the effects of dehydrin proteins and osmolytes on lipid-self-assembly in model lipid systems and intact stratum corneum under conditions of osmotic stress. The chapter gives a summary of the major findings of Paper IV & V.
Chapter 2

Lipid self-assembly

Lipids are amphiphilic molecules, they have one part that is hydrophilic and one part that is hydrophobic. The hydrophobic part is made up of hydrocarbon chains with varying length and saturation, whilst the hydrophilic part is a polar or charged headgroup. The amphiphilic property of the lipids causes them to spontaneously self-assemble into well-defined structures with distinct properties in aqueous solutions. This is widely exploited in nature, where such lipid structures constitute the core of all biological membranes.

2.1 Self-assembly structures

2.1.1 Molecular properties that influence self-assembly structure

The self-assembly of the lipids is a competing process between two opposing forces. The hydrophobicity of the chain drives the self-assembly due to the hydrophobic effect where, the interaction between the water molecules through hydrogen bonds are more favourable than interaction between water and the hydrophobic chains, which causes the hydrophobic content to cluster together to reduce contact with water. For molecules only consisting of the hydrocar-

![Figure 2.1](image-url):
Cartoons of self-assembly structures; A) Lamellar phase, B) normal hexagonal phase and C) bicontinuous cubic phase
Lipid self-assembly

bon chains, macroscopic segregation is thus expected. In case of amphiphilic molecules, segregation is opposed due to the repulsive interactions between the lipid headgroup. Depending on the molecular properties and shape of the lipid molecule the self-assembly can take on a vast variety of structures. The size of the hydrophobic part can vary depending on the length of the acyl chains, the number of chains and the number of double bonds in the chains. The interfacial curvature of the self-assembled structures depends on the effective area of the headgroup with respect to the length of the acyl chain in a given molecular volume, from which a packing parameter, $N_s$, can be defined predicting the preferred shape of the aggregate.\(^1\)\(^2\)

$$N_s = \frac{V}{a l}$$

Where $V$ is the volume of the hydrophobic part of the molecule, $a$ the effective area of the headgroup and $l$ the length of the acyl chain. If there is a large difference in cross-sectional area between headgroup and tail so that $N_s < \frac{1}{3}$, then spherical micelles are expected. If the cross sectional area of the headgroup and tail closely match, planar structures are preferred and if $N_s > 1$ reversed structures are formed. The value of $N_s$ may therefore be used as a guide to predict the aggregate structure. Here, one should bear in mind that the cross sectional area of the headgroups will change with total lipid concentration and with the addition of salt. The latter aspect is particularly important for ionic lipids. Below follow a short description of the general structures that can be found in amphiphile-water solutions.

The planar bilayer structures arrange themselves in stacks, called lamellar phases (Figure 2.1a). They can also be dispersed into multi- or uni-lamellar vesicles. The normal or reversed hexagonal phases can be described as elongated

![Chemical structures of glycerophospholipid headgroups treated in this thesis with, A) phosphatidylcholine (PC) with the glycerol backbone shown in green. B) Phosphatidylethanolamine (PE). C) phosphatidylinositol (PI), D) phosphatidylserine (PS) and E) phosphatidylglycerol (PG). R1 and R2 are acyl chains with varying length and saturation.](image)

Figure 2.2: Chemical structures of glycerophospholipid headgroups treated in this thesis with, A) phosphatidylcholine (PC) with the glycerol backbone shown in green. B) Phosphatidylethanolamine (PE), C) phosphatidylinositol (PI), D) phosphatidylserine (PS) and E) phosphatidylglycerol (PG). R1 and R2 are acyl chains with varying length and saturation.
Lipid self-assembly

(normal or reversed) micelles forming narrow channels of either lipid tails or water (Figure 2.1b). The channels are arranged in a hexagonal pattern. In the bicontinuous cubic phases (Figure 2.1c), the bilayer are arranged with saddle like interfacial curvature, with a non-zero mean curvature. There are also micellar cubic phases, which are characterised as micelles with a long ranged order, arranged in cubic arrays. The cubic phases can have different geometry and belong to different space groups. Among the bicontinuous cubic phases, the gyroid space group is the most common one, which is made up of two continuous channels of water separated by a bilayer. The different self-assembled structures are often described in terms of curvature of the plane/interface. Here, curvatures are defined as the mean curvature at each point of the interface, determined by the radii of curvature. Molecules with $N_s$ around 1 typically gives rise to planar structures with curvature close to zero. Both the packing parameter and the preferred curvature depend on the molecular properties of the lipids, as well as on solution and external conditions. Changes in conditions such as concentration, temperature, pH and pressure, may cause transitions between different self-assembled structures.\textsuperscript{1,2}

There is a large variety of lipid species forming a range of self-assembly structures, however, this thesis will mainly focus on the lamellar phases formed by phospholipids. The phospholipid is the most common lipid class in cell membranes, and it is also abundant in other lipid-rich structures in our body, for example, at the alveolar interface in our lungs. Phospholipids typically contain two hydrocarbon chains attached to a glycerol backbone and with either a charged or zwitterionic headgroup containing a phosphate group. Figure 2.2 show some chemical structures of the phospholipid classes treated in this thesis.

2.1.2 Lamellar phases

Most of the common phospholipids form lamellar phases when dispersed in aqueous solution. The basic building block of the lamellar phase is the lipid bilayer, and this is also the core element of most biological membranes. The plasma membranes in cells typically consist of a single bilayer that separate two

Figure 2.3: Schematic cartoons of the lamellar phases mentioned in this thesis; A) Lamellar gel phase ($L_{\beta}$), B) Rippled lamellar gel phase ($P_{\beta}$), lamellar liquid crystalline disordered phase ($L_{\alpha(d)}$) and lamellar liquid crystalline ordered phase ($L_{\alpha(o)}$), where the presence of cholesterol is indicated in orange.
Lipid self-assembly

liquid solutions, creating a barrier with low permeability for small hydrophilic molecules and ions. Bigger molecules like proteins need active transport mechanisms to be able to pass the membrane. The barrier function is a crucial property for the cell membrane as many cellular functions rely on the build up of ionic gradients. The bilayer membrane also generates an intra cellular structure to organise processes in the living cell. The bilayer can also act like a two dimensional solvent for membrane proteins. The barrier properties of the lipid membrane depend on the phase of the lamellar bilayer. The single bilayer makes up the cell membrane, however, there are many other lipid membranes in our body that are made up of multilayer arrangements, e.g. the lung surfactant film, the lipid tear film in our eyes and the skin.\textsuperscript{3–5} In many of these, the bilayer units builds up a multilayer stacks similar to the lamellar phase.\textsuperscript{6}

The lamellar phase consists of stacks of bilayers, where the properties of the single bilayers may vary depending on composition and external conditions. A good way of distinguishing between different lamellar phases is by the order of the acyl chains. The lamellar liquid crystalline phase (L\textsubscript{α}) (Figure 2.3c) have disordered acyl chains with a large fraction of gauche conformations and a fast lateral diffusion. The lamellar gel phases, on the other hand, consist of bilayers with solid acyl chains with an all-trans conformation, which are usually arranged in a hexagonal or distorted hexagonal packing. The acyl chains of the lamellar gel phase are usually either non-tilted (L\textsubscript{β}) (Figure 2.3a) or tilted (L\textsubscript{β}'). In certain conditions, non-planar lamellar rippled gel phase (P\textsubscript{β}) (Figure 2.3b) can also form.\textsuperscript{7} The P\textsubscript{β} phase is a gel phase with solid acyl chains and periodic undulations. The P\textsubscript{β} phase is observed e.g. in the DPPC system at full hydration\textsuperscript{8} and the formation of the rippled structure have been observed to be sensitive to the presence of other lipid species, as well as "contamination" with other small hydrophilic and hydrophobic molecules.\textsuperscript{9–13} The ripple phase can also form in PC bilayers that contain rather high amounts of cholesterol, and addition of cholesterol lead to an increase in the ripple periodicity.\textsuperscript{14–16}

\subsection{Cholesterol in PC bilayers}

The incorporation of cholesterol in saturated PC systems (e.g. DMPC or DPPC) leads to an interesting phase behaviour.\textsuperscript{17,18} At low concentrations of cholesterol (<5\%) the phase behaviour of the PC lipid is similar to the cholesterol-free system, indicating that cholesterol has similar solubility in lamellar phases with solid and fluid acyl chains. At higher cholesterol content (ca. 25 - 30 \%), a new lamellar phase is formed that is commonly referred to as the liquid ordered phase, L\textsubscript{α(o)}\textsuperscript{17,18} (Figure 2.3d). This is a liquid crystalline phase that has more ordered acyl chains compared to the common L\textsubscript{α} phase formed from the single

Lyotrophic phases are denoted by a capital letter signifying the aggregate structure e.g. L for planar lamellar phases or P for the rippled lamellar phase, while the subscript denotes the acyl chain conformation e.g. \textalpha{} for liquid acyl chains and \textbeta{} for solid acyl chains with the ’ signifying tilted chains.
lipid species. In order to distinguish between the different $L_\alpha$ phases, we here use the notations $L_\alpha^{(d)}$ and $L_\alpha^{(o)}$, denoting a lamellar liquid crystalline phase with disordered and ordered chains, respectively. When cholesterol is incorporated in the $L_\alpha^{(d)}$ phase, the internal molecular mobility of the acyl chains are constrained favouring a trans over a gauche conformation, inducing order. In the $L_\beta$ phase, the incorporation of cholesterol causes disorder in the crystalline packing of the acyl chains.

The different lamellar phases are characterised by their translational and rotational motion. One way of making a quantitative comparison of the different lamellar phases on a molecular level is to compare the so-called order parameters. They can be determined from e.g. NMR or WAXS or derived from MD simulations. A common way of measuring order parameters are by the use of $^2\text{H}$ NMR, however this requires deuterated samples, which in more complex samples might be difficult to attain. In recent years the use of R-PDLS ssNMR have been used to derive order parameters from samples with natural abundant isotopes. The $L_\alpha^{(d)}$ phase is disordered both dynamically translational and internally in the molecule, while the $L_\alpha^{(o)}$ phase has high lateral diffusion, but is internally ordered and has order parameters of the acyl chains closer to a $L_\beta$ phase. Comparison of order parameter profiles between a PC lipid acyl chain in the $L_\alpha^{(d)}$ phase and PC with \sim 30 mol% cholesterol show that the entire chain experiences an increase in order when going from the $L_\alpha^{(d)}$ to the $L_\alpha^{(o)}$ phase. However, the effect is most pronounced for the C-H segments close to the lipid headgroup. This can be explained by that the cholesterol molecules are situated in the bilayer with the OH group anchored in the the polar headgroup region. It should be noted that the definition a liquid ordered phase can vary somewhat in the literature. Throughout the work presented in this thesis when referring to the $L_\alpha^{(o)}$ phase we are referring to the $L_\alpha^{(o)}$ phase defined by Ipsen et al, i.e. a liquid lamellar with high conformational order in the acyl chains and a greatly reduced membrane area compressibility.

Many biological membranes of our body contains a considerable amount of cholesterol and its presence is highly connected to the so called lipid rafts in the cell plasma membranes. The self-assembly structure of the rafts are believed to be similar to the $L_\alpha^{(o)}$ phase and to be associated with important functions of membrane proteins. Cholesterol can also be found in other membranes, such as the lung surfactant lining the alveolar interface and the outer layer of the skin.
2.2 Phase transitions

2.2.1 Gibb’s phase rule

The macroscopic ordering in concentrated amphiphile systems can lead to the formation of different self-assembled structures that can change with variations in molecular composition and external conditions. The presence of different phases at equilibria can be described by Gibbs phase rule, with the number of phases $P$, that can exist in a system with $C$ components and $F$ degrees of freedom.

\[ P + F = C + 2 \]  

(2.2)

The degrees of freedom are the independent intensive thermodynamic variables that describe the system, which include temperature, pressure and composition.\(^2\) Alterations of the variables of the system can lead to changes in the properties of the phase or it can lead to the transition into another phase.

2.2.2 Phase transitions between different self-assembly structures

In self-assembled systems, one can distinguish between solid-solid, solid-liquid or liquid-liquid phase transitions. For solid-solid phase transitions, the change can be between crystalline and gel phases (e.g. $L_c$ to $L_{\beta}$) or between two different gel structures (e.g. $L_{\beta}$ to $P_{\beta}$). The liquid-liquid transitions involve transformations between two liquid crystalline phases (e.g. $L_{\alpha(d)}$ to $L_{\alpha(o)}$, or $L_{\alpha(d)}$ to $H_{II}$) or transitions between liquid crystalline phases and isotropic solutions containing (normal or reversed) micelles. The solid-liquid transition involves melting of the acyl chains, which involve a change from chains with an all-trans conformation to chains with a high fraction of gauche conformations. The solid-liquid transition is often referred to as the main transition. For phospholipid systems in water, the main transition is typically a first order transition and involves a discontinuous change between two states. This can be compared to continuous phase transitions (or a second order), which are characterised by a continuous change in properties between two phases.\(^2\)

A phase transition can be triggered by alterations in either one of a number of conditions, including water content, ion composition, pH, temperature and pressure. The length of the acyl chains and the number of double bonds will have a large influence on the phase behaviour. The longer the chains are the stronger is the total attractive van der Waals attraction between the chains, and the more energy it takes for the chains to rotate, and adopt a gauche conformation. Therefore, the attraction is strongest for the longer and saturated chains and more energy is needed to induce the solid-fluid transitions in systems containing these chains, which in turn lead to higher melting temperatures, $T_m$. For example, the disaturated 16 carbon long DPPC at full hydration melts ($P_{\beta}$ to $L_{\alpha}$), at 41 °C, while the shorter 14 carbon long disaturated DMPC has a main transition at 23 °C at full hydration. The incorporation of a cis-double bond into the
Lipid self-assembly

acyl chain, creates a kink in the chain and greatly reduces the attractive forces between the chains. As an example, the mono unsaturated POPC has a $T_m$ of -2 oC.\(^8\)

The melting temperatures listed above all refer to temperature-induced transitions in excess solution conditions. For the present thesis, it is important to note that melting transitions as well as other phase transitions, can also be induced at a constant temperature by changes in the water chemical potential ($\mu_w$), or equivalently, the osmotic pressure of water ($\Pi_{osm}$), which can be directly related to the relative humidity (RH) in the vapour phase:\(^1\)

$$
\Pi_{osm} = -\frac{1}{V_m} \Delta \mu_w = -\frac{RT}{V_m} \ln\left(\frac{RH}{100}\right)
$$

where $V_m$ is the molar volume of water.

### 2.2.3 Mixed lipid systems

Biological membranes and lipid mixtures (including the ones presented in this thesis) contain more than one lipid species. The phase behaviour in mixed lipid systems depends on the miscibility of the lipid species in different conditions. The incorporation of more than one lipid species to a system may induce segregation for certain conditions in terms of temperature or water content. In these conditions, more than one phase are present in the system. Phase co-existence between different bilayer phases can lead to domain formation in the lateral plane, while phase co-existence between liquid crystalline phases with distinctly different geometries typically leads to more macroscopic segregation.

At cholesterol levels in PC systems between ca. 10 - 20 mol%, there is phase segregation and domain formation in the bilayer systems. At temperatures above $T_m$ of the PC, The $L_{\alpha(d)}$ and $L_{\alpha(o)}$ phases coexists.\(^17,18\) At lower temperatures, one can have co-existence of $L_{\beta^-L_{\alpha(o)}}$ phases\(^17,18\) or incorporation of $L_{\alpha(o)}$-like domains in the $P_{b2}$ phase.\(^14-16,27\) At even higher cholesterol concentrations a single $L_{\alpha(o)}$ phase is formed. The $L_{\alpha(o)}$ phase is stable over a large temperature interval and the gel - $L_{\alpha(d)}$ phase transitions observed in cholesterol-free PC systems are abolished by the addition of these amounts of cholesterol.\(^17,18\)

For many practical applications as well as functional biological systems, the presence of a single phase over a large range of temperatures and water contents\(^17,27\) can be important to sustain a robust membrane system that is not too sensitive to changes in the external environment. This is of particular interest for membranes that are exposed to conditions with large fluctuations in temperature or humidity. Here, the cholesterol may act to make the system more robust, maintaining its properties also in varying conditions.\(^28\) It is here noted that some reptiles, who are exposed to cold conditions, have been found to have a high concentration of cholesterol in the lung surfactant layer.\(^29\) Furthermore, membranes that are exposed to atmosphere with varying temperature and humidity, for example the skin and the alveolar interface in lung, are rich in cholesterol.
2.3 Interplay between phase behaviour and membrane material properties

The properties of a lipid membrane are strongly related to its self-assembly structure. Here, important "material" properties of the membrane include permeability as well as mechanical properties. The lipid bilayer constitutes a barrier for polar solutes as the lipid acyl chains create a hydrophobic region, where the polar solutes have very low solubility. The permeability ($P_i$) of a bilayer to a certain diffusing species, $i$, depends on the partitioning of $i$ between the bilayer and the surrounding solution, $K_i$, as well as on the diffusion coefficient of $i$ in the bilayer, $D_i$, and the bilayer thickness, $L$. The steady-state flux, $J_i$, of $i$ across the bilayer can be related to the permeability by:

$$J_i = -\frac{D_i K_i}{L} \Delta c_i = P_i \Delta c_i$$  \hspace{1cm} (2.4)

where $\Delta c_i$ is the concentration gradient of $i$ across the bilayer. The diffusion coefficient in the interior of the liquid bilayer is similar for all small (hydrophobic and hydrophilic) compounds and the permeability of the bilayer to a certain compound is mainly determined by the partitioning of the diffusing compound between the membrane and the surrounding.\textsuperscript{31, 32} A multilamellar arrangement with alternating layers of bilayers and water makes a more efficient barrier compared to a simple bilayer as it has low effective permeability to both hydrophilic and hydrophobic substances.\textsuperscript{28}

The permeability of the $L_\beta$ phase is much lower compared to the $L_{\alpha(d)}$ phase. The diffusion coefficient of the solute is lower in the $L_\beta$ phase due to the low mobility in the solid bilayer interior.\textsuperscript{33, 34} Furthermore, the partition coefficient of the solute is also much lower for the gel phase compared to the $L_{\alpha(d)}$ phase. However, the flexibility of the bilayer is greatly reduced when going from $L_{\alpha(d)}$ to gel phase, and a solid layer likely has the risk of getting brittle and break. The $L_{\alpha(o)}$ phase forms a bilayer with lower permeability compared to the $L_{\alpha(d)}$ phase due to a decreased diffusion rate as well as a lower solubility of the solutes in the ordered bilayer interior, while it still provides a more flexible layer compared to the $L_\beta$ phase.

The segregation of different bilayer structures within the lateral plane of the layer have been shown to cause very high membrane permeability.\textsuperscript{22} This observations can be related to defects along the boundaries of the segregated domains where the diffusing solutes may have a higher solubility. For some lipid system, segregation and domain formation may be circumvented by the addition of cholesterol, which may lead to the formation of a single $L_{\alpha(o)}$ phase. It has also been shown that ethanol, which has a strong effect on biological membranes, has a much lower tendency to partition in to DMPC model membranes containing 35 % cholesterol as compared to the phase segregated $L_\beta - L_{\alpha(d)}$ membrane in the cholesterol-free DMPC membrane at temperatures close to the chain melting transition.\textsuperscript{28}
Chapter 3

Lung surfactant

When breathing, the exchange of oxygen to our blood stream takes place in the alveolus of our lungs. The alveolus are \( \sim 100 \ \mu\text{m} \) in diameter and the interface is covered by a thin film estimated to be a few \( \mu\text{m} \) thick.\(^{35}\) This film is made up of lipids and proteins and is commonly referred to as the ‘lung surfactant’. It is vital for proper lung function and without it the alveolus would collapse. Functional properties ascribed to the lung surfactant includes stabilising the interface by lowering the surface free energy, enabling rapid area compression-expansions involved during the breathing cycle, and controlling diffusional transport between the outside air and the blood stream.\(^{3, 36-38}\)

The field of the lung surfactant is reportedly born in 1929 when K. von Neergaard, a tuberculosis physician hypothesised of a monolayer of unknown composition lowering the surface tension in the lung.\(^{39}\) However, it took until the 1950’s for the field to get real scientific attention with physiologists focusing on a DPPC-rich monolayer and its connection to ailments.\(^{40-44}\) Since then, hundreds of papers regarding the lung surfactant have been published, yet numerous questions still remain open. In this chapter, questions regarding interfacial structure and composition-structure relation in the lung surfactant will be discussed. These are also the topics of Paper I-III.

3.1 Lung surfactant related diseases

Despite the emphasis of this chapter being the study of the biophysical properties of the lung surfactant film, I want to start with a section on its medical importance. With such vital functional properties as the lung surfactant film posses, it is not hard to understand that deficiencies in the lung surfactant or an imbalance of its constituents can lead to a range of life threatening conditions.\(^{45}\) RDS is an umbrella term for a range of conditions all leading to impaired breathing and the cause of the syndrome can have a range of reasons, including physical trauma, infections in the lung or sepsis.\(^{46, 47}\) The latter is responsible for the majority of the fatalities of the syndrome, which lies at \( \sim 35 - 40 \%\).\(^{48}\) Premature birth, before gestational age of 35 weeks, may lead to neonatal RDS occurring
because the lung surfactant is not yet fully developed or completely missing. In severe cases, the syndrome can be fatal without treatment. Neonatal RDS is treated by intratracheal administration of an exogenous source of lung surfactant, most often consisting of an animal derived extract of mainly bovine or porcine origin. The clinical extracts used in lung surfactant therapy are extracted by organic solvents and they are therefore depleted from the hydrophilic content present in the endogenous lung, for example hydrophilic proteins. Further, in the extraction process cholesterol is also removed and the remainder of the extract consists of mainly phospholipids and a few percent of small hydrophobic proteins. Even though the composition of the extracts differs from that of the endogenous lung, the therapy is successful in increasing the survival rate of the pre-term infants. However, despite the turnover effect of the introduction of the lung surfactant therapy in the late 1980’s which dramatically increased the possibility to rescue extreme pre-term babies, there is still a significant acute mortality, and late-effect diseases (e.g. bronchopulmonary dysplasia) remain high with the present treatments. Furthermore there is no effective corresponding treatment for the acute RDS inflicted in adult lungs.

### 3.2 Aims of this chapter

Two major biophysical questions dealt with in this thesis regarding the lung surfactant are: 1) What are the self-assembly structures that forms in a long surfactant mixture at an air-liquid interface? (Paper II & III) and 2) how does the different components of the lung surfactant mixture affect its self-assembly structure? (Paper I & II).

1) The lung surfactant self-assembly structure at the air-liquid interface has long been a debate for which consensus still does not exists. An early model of the lung surfactant was a single monolayer, as introduced by Clemens. However, several studies have suggested multilayer structuring at the interface. The distinction between the monolayer and multilayer interfacial structures will have major functional consequences. For example, a multilayer structure provides a reservoir of material allowing for area expansions during a breathing cycle. It may also reduce the surface free energy below the value set by surface tension of the monolayer through mechanical forces acting through the structure. Furthermore, a multilayer structure will affect transport properties of the interfacial layer.

One question that is addressed in this thesis concerns whether the lung surfactant spontaneously form a multilayer at the air-liquid interface (Paper II & III). As the lung surfactant film is situated at the air-tissue interface, there will be a small difference in chemical potential of the water between the tissue on the lower side, and the humid vapour phase in the lung. As a consequence, there will be a small water gradient across the lung surfactant film. This gradient likely has structural consequences for an interfacial film self-assembly. It is also likely that other gradients in composition are also built up in the non-
equilibrium conditions in the interfacial layer. Such non-equilibrium conditions are investigated for a lung surfactant extract interfacial film in Paper II. Another non-equilibrium aspect relevant to the lung is that the alveolar interface is highly dynamic, and it is repeatedly exposed to changes in interfacial area due to inhalation and exhalations. The effect of compression-expansion cycles on an interfacial lung surfactant film is studied in Paper III.

2) The lung surfactant is a complex multicomponent system and the full structure-composition relation is not fully elucidated. Specifically, one controversy has revolved around the role of cholesterol in the surfactant layer. Cholesterol is removed from the extracts used in clinics to treat neonatal RDS, while it is present in the endogenous lung. Cholesterol has major impact on the phase behaviour of simple lipid systems (e.g. DPPC), where the inclusion of \( \sim 25\% \) creates a liquid ordered phase, \( L\alpha(\omega) \), over a large range of temperatures. The effect of a physiological relevant amount (10 wt%) cholesterol on the structure of a lung surfactant extract in bulk conditions at varying composition and temperature conditions is studied in Paper I. The effect of the same amount of cholesterol on multilayer interfacial films of a lung surfactant extract is studied in Paper II.

### 3.2.1 Model systems of the lung surfactant

The biophysical characterisations of lung surfactant aimed at in this thesis are not possible to do in vivo. Therefore, one needs to create a model system of the endogenous lung surfactant that is relevant in composition, structure and conditions, but still simple enough to allow for molecular interpretations. The lung surfactant is a complicated system and one needs to make a choice of which samples to use as model systems. Animal derived extracts are used in the majority of the biophysical studies in literature concerning lung surfactant. For the work presented in this thesis, we chose to work with a clinical extracts of porcine origin developed for treatments of neonatal RDS. These extracts are very similar to the endogenous lung surfactant in phospholipid content and contain small hydrophobic proteins. However, the extracts lack the bigger hydrophilic proteins present in lung surfactant, and they have considerable reduced levels of cholesterol. The lung surfactant extracts are considered suitable for biophysical studies of the lipid components and its lamellar phases. In order to investigate the effects of cholesterol on the lung surfactant self-assembly, we add cholesterol in varying amounts to the clinical extract samples. To make an extract completely free of cholesterol, the small fraction remaining, can be removed using β-cyclodextrin. The phase behaviour of the lung surfactant extracts is also compared to simple lipid model systems with well characterised phase behaviour in the relevant temperature and hydration ranges (e.g. DPPC and DPPC:cholesterol).

During the studies presented in this chapter, two different extracts have been used. Both extracted from minced lung tissue of porcine origin and they have similar composition. The major difference between the two extracts lies in the formulation. The extract HL-10 (Paper I) was chosen due to that it is supplied as a freeze-dried powder in flame sealed vials and was ideal for the ssNMR
Lung surfactant experiments were the presence of excess solution leads to loss in signal. The other extract used, Curosurf ® (Paper II & III), is commercially available and used in clinics for surfactant therapy of neonatal RDS. This sample is supplied as an aqueous dispersion of multilamellar vesicles.

3.2.2 Experimental approach

To elucidate the phase behaviour of the lipids in lung surfactant in bulk conditions, a lung surfactant extract was studied with the combination of ssNMR and SAXS/WAXS (Paper I). The polarisation transfer solid-state NMR (PT ssNMR) is a powerful tool for studying the dynamics in soft matter, with co-existing solid and liquid parts. SAXS and WAXS are excellent tools for studying the structure of the sample, i.e. Bragg peaks show if a lamellar phase is present and a distinct peak in the WAXS spectra signifies solid acyl chains. However, in the $L_\alpha(d)$ phase the acyl chains are disordered and therefore does not give rise to a peak in the WAXS spectra. This makes the determination of co-existing $L_\beta$ and $L_\alpha(d)$ difficult to do with only SAXS/WAXS, and it is good to combine with, e.g., PT ssNMR.

The PT ssNMR method is a combination of two $^{13}$C NMR techniques, IN-EPT$^{66,67}$ and CP$^{68}$ which are commonly used for liquid and solid samples, respectively (Method 1). By combining the two methods one gets one spectrum which has a high signal for fluid/disordered segments and another spectrum with a high signal for solid/ordered segments. Furthermore, the peaks from C-H segments in the acyl chain shifts in chemical shift when the structure changes from liquid to solid states due to different chain conformation. From this combined information, it is possible to detect small amounts of of co-existing $L_\beta$ and $L_\alpha(d)$ phases. The $L_{\alpha(o)}$ phase is a liquid crystalline phase with ordered chains, these properties makes the determination of the phase non-trivial. The acyl chains do not give a distinct peak in the WAXS spectra, and the PT ssNMR spectra is not typical of the common $L_{\alpha(d)}$ phases without cholesterol, or the solid $L_{\beta}$ phase. The PT ssNMR spectra from the $L_{\alpha(o)}$ phase has similarities with both the spectra from the $L_{\beta}$ and the $L_{\alpha(d)}$ phases. One common way of characterising the $L_{\alpha(o)}$ phase is to determine order parameters of the acyl chains by the means of deuterium NMR. A deuterated biological lung surfactant sample is, however, not readily accessible. We utilised a NMR technique, R-PDL$^{69}$ to determine the order parameters of the chains from natural abundant $^{13}$C. From the combined data of the order parameters, SAXS/WAXS spectra and PT ss-NMR we could draw conclusions of the presence of the $L_{\alpha(o)}$ phase in the lung surfactant extract (Paper I).

To enable detailed characterisation of the self-assembly of the interfacial layer of a lung surfactant extract under non-equilibrium conditions we utilised a home-built sample cell, first described by Roger et al.$^{70}$ The sample cell consists of a rectangular boro-silicate capillary that is open to the atmosphere in one end and connected to a reservoir of bulk solution in the other end (Figure 3.1). Due to capillary forces, the sample solution goes to the capillary edge that is
open to the atmosphere. Because there is a difference in chemical potential of water between the bulk solution and the vapour phase, water will evaporate and there will be a gradient in water chemical potential close to the air-liquid interface at the edge of the capillary. The outside relative humidity of the atmosphere can be controlled to achieve a high humidity similar to the conditions in the endogenous lung, and defining the conditions for both boundaries of the film. In Paper II, a vesicular dispersion of lung surfactant extract was placed in the capillary cell. Even at high RH (97 %RH), the difference in chemical potential is enough to drive the spontaneous formation of an interfacial layer of lung surfactant extract. Due to the small volumes in the capillary, convection in the sample is limited and steady-state conditions are not reached within the time of the experiment. The interfacial films can therefore grow to become several hundreds of μm allowing for adequate spatial resolution. In the capillary set-up the interfacial layer can be visualised with cross polarisation microscopy, showing the build up of the film over time. In order to monitor the self-assembly of the layer in the water gradient coherent X-ray scattering (cSAXS) can be used recording SAXS and WAXS spectra at different positions going stepwise from the interface and in to the bulk solution. Using an instrument with a small beamsize (1.4x5 μm), we thus obtain structural characterisation with adequate resolution at different positions in the interfacial layer. The water content of the film at different positions from the interface can be determined by IR spectroscopy.71

Figure 3.1: Schematic over capillary sample cell with an image of a lung surfactant extract taken with polarised light microscopy and an illustration of the multilamellar vesicles in the bulk solution, migrating to the interface forming lamellar layers.
In order to study the interfacial film of the lung surfactant under the non-equilibrium situations resembling the compression-expansion cycles of the alveolar interface during breathing, a lung surfactant extract was spread on the air-liquid interface in a Langmuir trough (Paper III). By moving the teflon barriers the surface area can be controlled to simulate the breathing cycle. To investigate the structure of the interfacial film neutron reflectometry was used as it probes directly the distribution of matter in the normal of the interface. By using $D_2O$ as a solvent the measurements are really sensitive to structuring beneath the surface due to the high contrast between the lipids and the $D_2O$. By combining the neutron reflectivity measurements with Brewster angle microscopy we also get an image of the surface of the interfacial film.

### 3.3 Lung surfactant composition

#### 3.3.1 Lipid composition

The lung surfactant is a complex multicomponent lipid-protein system with more than 90 different lipid species. However, the overall composition can be simplified to 80% phospholipids, 10% non-charged lipids and 10% surfactant proteins. The main phospholipid class is PC with the disaturated DPPC making up about half of the total amount of phospholipids. Such high concentrations of saturated phospholipids is rarely found in other tissues of the body. Other common phospholipid classes in the lung surfactant are PE, PG and PI, while PS is only present in minor concentrations. The total amount of charged lipid ranges between 8-15%, and is mostly PI and PG, while the ratio of the two differs between species and age. The non-ionic lipid fraction contains a small amount of mono-, di-, and triacylglycerol and $\alpha$-tocopherol together with a larger fraction of un-esterified cholesterol (8-15% of total lung surfactant weight). Although the ratio between the different lipid classes varies between species and age and even individuals, the overall composition is remarkably similar between different mammalian species with only small variations in the distribution of lipid species.

#### 3.3.2 Surfactant proteins

The studies described in this chapter mainly focus on the self-assembly of the lung surfactant lipids. In addition to lipids, the lung surfactant also includes proteins. The major fraction of proteins found in the lung surfactant is composed of the so-called surfactant proteins SP-A, SP-B, SP-C and SP-D. These four proteins can be divided in to two main categories: the small hydrophobic proteins SP-B and SP-C and the bigger hydrophilic proteins SP-A and SP-D.

The hydrophobic proteins SP-B and SP-C are both transmembrane proteins and mainly $\alpha$-helical in its secondary structure. While SP-C is mainly found as a monomere of 4.2 kDa, and SP-B is present as a dimer with 8.7 kDa per monomer. SP-B constitutes $\sim 0.7$ wt% and SP-C $\sim 0.5$ wt% of the total dry
mass of isolated lung surfactant.\textsuperscript{79} SP-B has been suggested to be the only surfactant protein vital for survival and the lack of the protein in the lung surfactant leads to respiratory failure.\textsuperscript{3, 80–82}

The bigger hydrophilic proteins SP-A and SP-D belong to the protein family collectins, and assemble in large macromolecular arrangements. SP-A is \(\sim 26\text{-}38\,\text{kDa per monomer with a quaternary structure of trimers assembling to a hexamer.}^{78, 83}\) SP-A is able to bind to \(\text{Ca}^{2+}\) and phospholipids, allowing it to bind to the surface of pathogens.\textsuperscript{84} SP-A makes \(\sim 3\text{-}5\%\) of the total mass of the lung surfactant.\textsuperscript{85} The SP-D monomer is 43 kDa and assemble into trimers which in turn assemble into larger structures of four trimers.\textsuperscript{78} SP-D makes \(\sim 0.5\%\) of the total lung surfactant mass and is the only of the SPs not found associated with the lipids.\textsuperscript{52}

### 3.3.3 Lung surfactant extracts

The commonly used lung surfactant extracts are either extracted from minced lung tissue by chloroform:methanol, according to the protocol of Bligh and Dyer,\textsuperscript{86} or by bronchoalveolar lavage (BAL), in which the extract is produced by rinsing the lung with saline solution. Usually the BAL samples are further purified by extraction with chloroform:methanol. The unpurified sample is usually referred to as BAL fluid (BALF). Due to the use of organic solvents both types of extract (BAL and minced lung) are relatively similar in content.\textsuperscript{87} Neither extract contain the hydrophilic proteins SP-A and SP-D and in the extraction process non-ionic lipids like cholesterol are also actively removed by acetone. Furthermore, both types of extracts have a slightly lower concentration of hydrophobic proteins SP-B and SP-C compared to the endogenous lung surfactant.\textsuperscript{87}

The lipid content of a clinical extract from minced porcine lung was analysed by mass spectrometry (MS) and found to be very similar to other extracts of porcine origin (\textit{Paper I}). The charged lipid content was determined to \(\sim 13\%\) with PI as the major charged phospholipid species and the cholesterol level was determined to \(\sim 1.5\%.\textsuperscript{21}

### 3.4 Self-assembly in lung surfactant

The self-assembly of the lung surfactant will have major consequences on the functional properties of the interfacial film. To characterise the interfacial self-assembly of the lung surfactant can pose several challenges. It can be a challenge to find a technique with sufficient resolution that can determine the mesoscopic structures at the air-liquid interface. Some techniques that can distinguish between a multilayer and monolayer structure are, e.g. gracing incidence SAXS (GI-SAXS) or neutron reflectometry and studies using these techniques show clear indications of multilayers at the interface,\textsuperscript{60, 62} which is also shown in \textit{Paper III}, where the effects of compression-expansion cycles are investigated.
Method 1: PT ssNMR

The following section will briefly describe the PT ssNMR technique, assuming basic NMR understanding. The $^{13}$C nucleus accounts for only $\sim 1\%$ of the carbon nuclei in the sample, which results in weak NMR signal. To increase the signal, polarisation can be transferred from more abundant nuclei in the vicinity of the $^{13}$C, namely from the $^1H$ nucleus. There are several different methods for polarisation transfer. In the PT ssNMR technique, two very common methods are employed, CP$^{68}$ and INEPT.$^{66,67}$ The CP and INEPT experiments are used for polarisation transfer in solid and liquid samples, respectively. The terminology PT ssNMR is used for the experiment sequence DP-CP-INEPT, which has been successfully used in studies of soft matter.$^{65}$ The effectiveness of the polarisation transfer, and hence the signal intensity, in each of the INEPT and CP experiments depends on the rotational correlation time, $\tau_c$, and the order parameter of the C-H bond, $S_{CH}$.

- In the INEPT experiment the transfer of the polarisation is through the scalar couplings (through the bonds) and reaches through a couple of bonds becoming weaker with distance. In rigid segments, with slow motion and/or high order, the relaxation rate is fast, and the signal dies before it can be recorded, making INEPT inefficient for such segments. However, for isotropic segments with fast motion, the relaxation rate is longer and INEPT provides an efficient signal enhancement, for a more detailed description of the dependence of the CP and INEPT signal intensities refer to Nowacka et al.$^{65,88}$ and Warchawski et al.$^{67}$

- In the CP experiment the transfer of polarisation is through space. The efficiency of the transfer depends on the orientation of the coupling in relation to the external magnetic field, and with fast isotropic re-orientational motion the dipolar couplings average out. This makes CP inefficient for fast isotropic segments, while with slow oriented segments the technique is much more efficient.

- The DP experiment provides signal from all $^{13}$C in the sample without the dependency on efficient polarisation transfer, and it can thus be used as a reference to the other two experiments.

By comparing the signal intensities of each segment in the different experiments, one can elucidate the change in order/mobility of the individual segments with changing conditions. The PT ssNMR technique is also very powerful in detecting small variations in mobility in samples with a small fraction of fluid in an otherwise solid sample, which is a non-trivial task with many other techniques.

Figure 3.2: Example spectra for A) the $L_{\beta}'$ phase with all-trans conformation of the acyl chains with the crowded spectral region from the middle of the acyl chains at 31-33.5 ppm (seen in only the CP spectra). B) The $L_{\alpha(d)}$ phase with trans/gauche conformation of the acyl chains with the crowded spectral region at 29-31 ppm (seen in both the CP and INEPT spectra). C) the $L_{\alpha(o)}$ phase where the crowded spectral region closer in ppm to that of the AT conformations of the acyl chains (seen in both the CP and INEPT spectra). The maxima of the peak from the AT acyl chains and the TG acyl chains are indicated by a blue and red dashed line, respectively.
Another major challenge is to make a detailed characterisation of the self-assembly on a molecular level in a complex biological sample with many different components. The structures formed in bulk is believed to be similar to the structures in a multilayer interfacial film. However, the conditions set at the interface creates non-equilibrium conditions, which have structural consequences for the interfacial self-assembly, giving rise to structures that are not present in bulk conditions (Paper II). The most commonly reported structures in suspensions of lung surfactant extracts are multilamellar vesicles, so called lamellar bodies, which can form spontaneously from different types of lamellar phases at various conditions.\(^3,36,52\) In the lung, the LBs are secreted from the Type II pneumocyte cells into the alveolar subphase.\(^3\) In addition, one can find so called tubular myelin in lung surfactant samples. The latter structure consists of grid-like structures only observed in the presence of SP-A, SP-B and \(Ca^{++}\) ions, where oligomers of SP-A is believed to scaffold the grid.\(^78,89\) As SP-A is not present in most extracts used in biophysical studies, and the focus of the lung surfactant field has very much been laid on the lamellar phase, which is also the case for this thesis (Paper I & II).

### 3.4.1 Self-assembly of lung surfactant extracts in bulk conditions

There are several studies showing co-existence between different segregated lamellar phases at physiologically relevant conditions in bulk (37 °C and close to full hydration). The segregated phases have been assigned to either lamellar gel (\(L_β\)) and lamellar liquid crystalline (\(L_α\)) phases,\(^90\) or two lamellar liquid crystalline phases with ordered (\(L_α(o)\)) and disordered chains (\(L_α(d)\))\(^91,92\) The precise phase behaviour of the lung surfactant extracts will depend on several parameters, including the composition of the extracts, water content, solution conditions, temperature or pressure, and variations in the experimental conditions may explain the apparent discrepancies between different published studies. In particular, phase behaviour will depend on the exact cholesterol concentration in the extract, which is studied in Paper I & II.

In Paper I, we investigate the self-assembly in lung surfactant extract. Instead of fixing the conditions to one temperature we wanted to elucidate the accessible phases in the lung surfactant extract in and around physiologically relevant temperatures and cholesterol concentrations. The precise phase behaviour at a specific temperature might vary depending on the exact composition of the sample, solvent conditions or water content, as well as on other external conditions. Small variations in any of these parameters might shift the phase boundaries. Instead of fixing the conditions, we take the approach to study lung surfactant phase behaviour at varying relevant conditions, in order to draw more general conclusions.
Method 2: R-PDLF and Order parameters

In ssNMR the spectral resolution is achieved by magic angle spinning (MAS) to reduce the broadening effects of the chemical shift anisotropy (CSA) and the dipolar couplings. The CSA and the dipolar couplings arise from anisotropic orientations and interactions in the sample, and are dependent on the orientation relative to the external magnetic field. Because of their orientational dependence, structural information can be derived from the spectra, which results from these interactions.\(^9^3\) Regrettfully, this is lost when MAS is applied. R-PDLF is a NMR technique which is able to bring back this information by introducing a recoupling step in the pulse sequence before acquisition.\(^6^9\) In this recoupling step the dipolar splittings, \(\nu^{R-PDLF}\), of the peaks in the 2D R-PDLF NMR spectra which arises from the dipolar couplings can be recorded. The dipolar splittings in the R-PDLF 2D spectra can then be used to retrieve the order parameter magnitudes, \(|S_{CH}|\) by:

\[
|S_{CH}| = \frac{\Delta \nu^{R-PDLF}}{0.351d_{max}^{CH}}
\]

(3.1)

\(d_{max}^{CH}\) is the maximum dipolar splitting of a rigid C-H segment, which is 22 kHz.\(^9^4\) Either CP or INEPT are used as polarisation transfer techniques depending on the nature of the sample. The order parameters derived in this thesis are all from the lamellar liquid crystalline \(L_\alpha(d)\) and \(L_\alpha(o)\) phases. It was not possible to obtain the corresponding information for the the lamellar gel phase because the relaxation rate for this phase is too fast for the R-PDLF technique to be efficient.

The order parameters \((S_{CH})\) describe the local order of each C-H segment in the acyl chain where each of the order parameters for the different segments is related to the angle between the C-H bond, \(\theta_{CH}\), and the bilayer normal.

\[\text{Figure 3.3: A) 2D R-PDLF spectra of the acyl chain region from lung surfactant extract (LSE) (black) and LSE with cholesterol (red). B) Dipolar splittings from the 2D spectra for selected segments in the acyl chains for LSE (black) and LSE with cholesterol (red).}\]

where \(d_{max}^{CH}\) is the maximum dipolar splitting of a rigid C-H segment, which is 22 kHz.\(^9^4\) Either CP or INEPT are used as polarisation transfer techniques depending on the nature of the sample. The order parameters derived in this thesis are all from the lamellar liquid crystalline \(L_\alpha(d)\) and \(L_\alpha(o)\) phases. It was not possible to obtain the corresponding information for the the lamellar gel phase because the relaxation rate for this phase is too fast for the R-PDLF technique to be efficient.

The order parameters \((S_{CH})\) describe the local order of each C-H segment in the acyl chain where each of the order parameters for the different segments is related to the angle between the C-H bond, \(\theta_{CH}\), and the bilayer normal.

\[\text{Figure 3.4: Schematic drawing of the angle (\(\theta_{CH}\)) between the }^{13}\text{C-}^1\text{H bond and the bilayer normal (n) and illustration of increasing order when going from a }L_{\alpha(d)}\text{ to }L_{\beta}\]
The phase behaviour of a clinical lung surfactant extract was studied by the means of ssNMR and SAXS/WAXS at a range of temperatures (30-46 °C) and conditions close to full hydration. Starting at 30 °C and increasing the temperature in steps of 5 °C the PT ssNMR spectra follow the gradual melting of the acyl chains of the lung surfactant extract. The phase behaviour goes from clear co-existence of \(L_\beta\) and \(L_\alpha(d)\) at 30 °C to a single \(L_\alpha(d)\) phase at 40 °C. The presence of solid acyl chains is further confirmed by WAXS, showing a distinct peak at 15.2 \(^{-1}\) signifying hexagonal packing of the chains at 30 °C. No sharp WAXS peak was detected at 40 °C.

The phase behaviour of lipid systems is strongly affected by the addition of cholesterol. By varying the amount of cholesterol, we were able to follow the transition from co-existing \(L_\beta\) and \(L_\alpha(d)\) phases to one single \(L_\alpha(o)\) phase when varying the cholesterol content between 0 and 10 wt%. The different liquid crystalline phases were further characterised using the R-PDLF NMR method (see Method 2), which makes it possible to determine the order parameters for the acyl chains.

The order parameters \((S_{CH})\) describe the local order of each C-H segment in the acyl chain where each of the order parameters for the different segments is related to the angle between the C-H bond, \(\theta_{CH}\), and the bilayer normal (see Method 2) through:

\[
S_{CH} = \frac{1}{2} (3 \cos^2 \theta_{CH} - 1) \tag{3.2}
\]

![Figure 3.5](image)

**Figure 3.5:** Order parameter profiles of A) a \(L_\alpha(d)\) phase of lung surfactant extract (△) and a \(L_\alpha(o)\) phase of lung surfactant extract + 10 wt% cholesterol (△) at 30 °C. B) At 40 °C the order parameter profile of the \(L_\alpha(d)\) phase in the lung surfactant extract (○) is unchanged compared to the order parameter profile at 30 °C. The lung surfactant extract + 10 wt% cholesterol (○) forms a \(L_\alpha(d)\)-like phase at 40 °C.
Lung surfactant

At 30 °C the order parameter profile for the acyl chain of the lung surfactant extract closely resembles order parameters measured for acyl chains in the $L_{\alpha(d)}$ phase in simple one-component PC systems$^{19,24}$ (Figure 3.5a). When the surfactant extract is supplemented with 10 wt% cholesterol, the values of the order parameters increases for almost all carbons in the chain, and the profile closely resemble that of the $L_{\alpha(o)}$ phase measured for binary PC-cholesterol mixtures.$^{17,24}$ The order parameter profile for the lung surfactant extract with 5wt% cholesterol is in between that of the clinical lung surfactant extract and the extract with 10 wt% cholesterol, strongly implying co-existing $L_{\alpha(d)}$ and $L_{\alpha(o)}$ phases with fast exchange. When the temperature increases with a few degrees, the order parameters for the lung surfactant extract with 10 wt% cholesterol becomes smaller and approaches the values measured for the $L_{\alpha(d)}$ phase (Figure 3.5b). These data imply that cholesterol-containing lung surfactant mixtures continuously changes from a $L_{\alpha(o)}$ phase to a $L_{\alpha(d)}$-like phase.

3.4.2 Interfacial multilayers in non-equilibrium conditions

For a clinical lung surfactant extract in a capillary cell (Paper II), multilayers are spontaneously formed at the air-water interface, which can be observed by the birefringent phase in a cross-polarised light microscope. The water content at different positions in the film was monitored by IR spectroscopy (see Method 3) showing a close to linear increase in concentration from the interface to the bulk. The phase behaviour of the film was determined by cSAXS measured at intervals stepwise from the interface and in to the bulk solution. When moving from the interface towards more hydrated conditions in the inner layer of the multilayer interfacial film, the Bragg peaks from the lamellar gel phases gradually loses intensity until the system becomes one single $L_{\alpha(d)}$ phase. In the layer closest to the interface we detect three co-existing lamellar phases. The phases are assigned to planar lamellar gel phase ($L_{\beta}$), rippled lamellar gel phase ($P_{\beta}$) and a lamellar liquid crystalline phase with disordered chains ($L_{\alpha(d)}$). The interfacial structure was compared to that formed in corresponding bulk conditions (lung surfactant extract equilibrated at 97 %RH) and only $L_{\beta}$ and $L_{\alpha(d)}$ phases were observed. Previous characterisation of the rippled phase in DPPC or DMPC model systems have shown this structure to be sensitive to the presence of other lipid species.$^{9,10}$ This led to the hypothesis that DPPC, which is the major component in lung surfactant, is enriched in the outer layers of the film. Indeed, Raman spectra of the interfacial film taken at different positions in the capillary show a higher level of saturated chains at the very outer layer of the film as compared to the layers close to the bulk solution. The combined cSAXS, IR and Raman microscopy studies thus imply that more than one gradient might build up within the interfacial layer, leading to structural differences between equilibrium bulk samples and samples under non-equilibrium conditions.
Method 3: Determination of water content from IR

The water content of the lung surfactant film at each position in the water gradient could be determined with IR spectroscopy, by recording a spectrum at regular intervals in a line from the interface to the bulk. The peak from the OH bend-stretch of water appears in the spectra at 4800-5300 cm$^{-1}$ and the water content can be determined from integration of this peak. A calibration curve of isotropic surfactant solutions with known compositions are used to convert the peak area to concentration.

![Plot showing IR spectra with marked peak at 4800-5300 cm$^{-1}$](image)

**Figure 3.6:** IR spectra of a lung surfactant film at the air-liquid interface showing the peak that appears from the OH bend-stretch of water.

Method 4: Neutron Reflectometry

In neutron reflectometry the intensity of a neutron beam after reflection at the sample surface is measured and the (absolute) reflectance is given by the ratio between the intensity of the reflected and incident beam. One particular strength of using neutrons lies in the possibility to contrast match the sample. The scattering of neutrons is described by the scattering length density (SLD) which differs widely between the $^1H$ and $^2H$ isotopes. By performing a set of measurements with selectively deuterated solvent and samples one can also determine the chemical composition of a surface layer. Lipids with hydrogenated acyl chains gives a high contrast in $D_2O$. Thus under these conditions the measurements are very sensitive to structures at the interface. This was taken advantage of for detecting multilayer structures of lung surfactant at the air-liquid interface. The lung surfactant extract was spread on the interface in a Langmuir trough and the teflon barriers were moved to control the surface area in the trough and simulate the compression cycles that occurs during breathing.

![Schematic drawing of a neutron reflectivity measurement on a liquid surface and an illustration of the compression cycles used to simulate the breathing cycle in the lung](image)

**Figure 3.7:** Schematic drawing of a neutron reflectivity measurement on a liquid surface and an illustration of the compression cycles used to simulate the breathing cycle in the lung.
When cholesterol is added to the same system, a far less rich phase behaviour is observed. In the presence of cholesterol, only a single $L_{\alpha}(o)$ phase is present over the whole range of water contents at all positions in the capillary. The interfacial $L_{\alpha}(o)$ phase show very shallow swelling profile throughout the film as compared to the cholesterol-free sample, again implying the formation of a more homogenous film when cholesterol is added. The observed swelling profile in the water gradient is also consistent with previous bulk studies on simple model systems showing that the $L_{\alpha}(o)$ phase is rather resilient to changes in water activity.  

### 3.4.3 Interfacial layers under compression-expansion cycles

In Paper III, we wanted to investigate the overall structure of the interfacial film of a clinical lung surfactant extract and mimic the breathing cycles, by continuously compress and expand the interfacial layer. A multilamellar vesicular dispersion of the clinical extract was spread at the air-liquid interface of a Langmuir trough and the barriers controlling the interfacial area were compressed and expanded in cycles. The interfacial structure was evaluated by neutron reflectometry, (see Method 4) showing a distinct Bragg peak for all samples of lung surfactant extracts studied. The experiments clearly demonstrate the formation of multilayer structures at the air-liquid interface. The lamellar repeat distance measured with the reflectometry is smaller than corresponding distance measured for bulk samples at full hydration, which further supports that the multilayer aggregates are actually at the air-water interface where the water chemical potential in the vapour phase is slightly lower compared to the excess solution conditions. This is also consistent with the observations for the multilayers at the interface in the capillary cell where measured lamellar repeat distances are smallest close to the air-liquid interface. Furthermore, the neutron reflectometry experiments show that the thickness of the multilayers increases with repeating compressions until a steady-state thickness is reached. The model best fitted to the reflection curves is the combination of a reflectivity curve from a monolayer and a reflectivity curve of a multilayer with the overall coverage of the multilayer as a fitting parameter. This model agrees with the proposed structure of the lung surfactant film as a monolayer with connected multilayer aggregates.
3.5 Tubule formation at the multilayer-bulk interface

The interfacial films in the capillary cells are formed from a bulk solution containing dispersed multilamellar vesicles. During the build-up of the film the lipids are transferred from the vesicles to the interfacial layer through fusion processes, giving rise to a highly dynamic interface of the fully swollen inner layer of the interfacial film. It is a recurring observation for all systems investigated that the fusion of vesicles with the interfacial film is associated with the formation of tubular structures (Figure 3.8). The tubules are highly dynamic and extend from the film-bulk interface into the bulk solution, that is against the direction of the flow in the capillary. The mesoscopic structure of the lipids building up the tubules was determined by SAXS, showing a lamellar phase with similar repeat distance as what is found in the inner parts of the fully swollen multilayer film. The tubules were shown to have multilayer structure where (determined from the 2D SAXS spectra) the bilayers orient with the curling of the tubules. Similar tubular structures have previously been observed for a similar lung surfactant extract, however under different experimental conditions. The mechanism behind the tubule formation in the lung surfactant film is not fully understood. However, it is possible that the tubulation is a consequence of slow diffusion in the plane of the lamellar structure and that tubules are formed to adopt for the increase in area due to the addition of new material during the fusion process.

![Air-liquid interface](image)

*Figure 3.8: Cross polarised microscopy image of a lung surfactant extract film at the air-liquid interface displaying tubule formation. The scale bar is 100 μm*
3.6 Conclusions and outlook

The self-assembly of clinical lung surfactant extracts were studied at the mesoscopic and molecular level, determining the phase behaviour in bulk solutions in equilibrium conditions, and at the air-water interface under non-equilibrium conditions. The phase behaviour was investigated at varying temperatures, cholesterol content and for different composition gradients. The interfacial structure of the lung surfactant extract film under compression cycles were also studied.

Multilayer structure spontaneously form at the air-liquid interface in the capillary cell. Also when the the lung surfactant film is situated at a large interface and exposed to repeated area compression-expansion cycles, multilayer structures are detected. From the data fitting to the neutron reflectometry data, the assumed model for the interface is a monolayer with multilayer aggregates that build up under the compressions.

It is a recurrent observation for all studies (Paper I - III) that the addition of 10 wt% cholesterol to the clinical lung surfactant forms a single robust $L_{\alpha(o)}$ phase under both equilibrium and non-equilibrium conditions. The clinical extract without cholesterol transitions from co-existing $L_{\beta}$ and $L_{\alpha(d)}$ phases to one $L_{\alpha(d)}$ phase in the temperature interval 30 - 40 °C. When a similar clinical extract is situated in non-equilibrium conditions at an interface at 37 °C an even more complicated phase behaviour is observed. At the very outer layers of the film $L_{\beta}$, $P_{\beta}$ and $L_{\alpha(d)}$ co-exists that with increasing water content transitions into a single $L_{\alpha(d)}$ phase with increasing water content closer to the bulk solution. When a physiological relevant amount of cholesterol is added to the extracts, the complicated phase behaviour is abolished, and the system forms a single homogenous $L_{\alpha(o)}$ phase, resilient to changing conditions. Such phase behaviour could have important biological function. With cholesterol the system forms a stable membrane over a large range of temperatures and water content. Phase segregated membranes are generally more permeable to small molecules and some compounds, for example, ethanol, show higher partitioning to segregated membranes compared to bilayers composed of one single phase.

The different approaches taken in this chapter all have their advantages and disadvantages. The best resolution of the techniques is achieved with ssNMR, where we can get detailed information on the molecular level of the changes in dynamics of the segments in the lipids. However, these studies can only be performed in bulk conditions. In the capillary system with limited convection the interfacial film is allowed to grow to several hundreds of $\mu m$, enabling a detailed characterisation of the phase behaviour with enough spacial resolution, to make it possible to distinguish different phases present in the gradient between the interface to the bulk. The endogenous layer, however is estimated to be a few $\mu m$. The system measured with neutron reflectivity in the Langmuir through, with a large surface-to-bulk ratio, would in that case closer resemble the endogenous alveolar interface. However the resolution of the neutron measurements are limited, and we can only get information on a mesoscopic level.
Taken together the studies in Paper I - III give a comprehensive picture of the phase behaviour of a clinical lung surfactant extract, showing the importance of cholesterol and non-equilibrium conditions.

Outlook
In this thesis, animal derived extracts are studied, which do not contain the full repertoire of lipids and proteins as the endogenous human lung surfactant. It would be immensely interesting to study human lung surfactant samples (with the full endogenous composition) both at the high detail level of molecular dynamics achieved by the ssNMR techniques and the mesoscopic structure at air-liquid interface, elucidated by the capillary cell and cSAXS. Such human extracts can be achieved by the extraction of the lung surfactant from the amniotic fluid collected during planned caesareans. As the amniotic fluid washes the lungs of the baby in the sac, the fluid contains the full lung surfactant and the purification of such extracts results in the so-called "first-breath extract". One intriguing question that could potentially be answered by the use of the capillary cell is wether the TM structures observed in lung surfactant samples containing, SP-A, SP-B and Ca$^{+2}$, forms at the outer layer of the film at the air-liquid.

The complete role of the surfactant proteins in the lung surfactant interfacial film is not yet fully understood. I believe that studying the individual proteins in simple model system in the capillary cell and with the use of cSAXS could provide novel, information on the effect of the individual proteins on the structure of the lipids under non-equilibrium conditions. The small hydrophobic proteins have a high degree of $\alpha$-helical structures, which give rise to peaks in the WAXS spectra, enabling us to follow the distribution of the proteins in the interfacial film.

The formation of the tubular structures in the film-to-bulk interface is an intriguing observation. Some simple model systems have already been studied in order to further characterise this phenomena, however, the tubule formation phenomena was not reproduced in these systems. Future investigations could involve other model lipid systems to systematically study, for example, the effects of mismatch between the acyl chains and the effects of including lipids species that are prone to form non-planar cubic or hexagonal structures in solutions (e.g. DOPE). To test if the tubular formation is an effect of the remaining lung surfactant proteins, one could remove or denature the proteins in a lung surfactant extract.
Chapter 4

Lipid membranes under osmotic stress

In nature plants, simple organisms and higher animals are regularly exposed to osmotic stress through dry air, high salt concentrations or freezing. The osmotic stress can affect the macromolecular organisation, which in lipid systems can lead to swelling of multilayer structures, membrane fusion and phase transitions. Such structural changes may, in turn, influence important membrane functions. For example, phase segregation and domain formation, as well as transitions between different self-assembly structures, will influence the permeability of the membrane.

Nature has found several different ways to counteract osmotic stress and protect biomolecular systems against dehydration induced injury. One common strategy is to introduce small polar molecules, often referred to as osmolytes. Examples of such polar molecules are sugars, amino acids, glycerol and urea, which are found in plants, simple organisms as well as in higher animals.98–102 One interesting example is found in the outer layer of the skin, the stratum corneum, which is almost constantly exposed to dry conditions. The osmolyte compounds present in stratum corneum are generally referred to as the natural moisturising factor (NMF) and the same compounds are common ingredients in formulations for skin care.103–106

Plants can also be exposed to rather extreme conditions, and they have different strategies to respond to osmotic stress. Many plants have several different protective systems that may be activated in parallel.107 In addition to osmolytes, many plants produce specific proteins, for instance heat shock proteins and some late embryogenesis abundant (LEA) proteins. These proteins are found in plants and simple organisms, and they are believed to protect membranes and other proteins against dehydration and freezing.108–114 While extensive literature exists on the effects of osmolytes, LEA proteins and heat shock proteins on protein stability, less is known about their effects on lipid self-assembly under osmotic stress conditions.
4.1 Aims of this chapter

Two major questions dealt with in this thesis regarding the lipids systems in osmotic stress conditions concerns: 1) What are the effects of osmolytes in drying lipid systems? (Paper IV & V) and 2) how does the LEA dehydrin protein Lti30 influence lipid self-assembly in dry conditions? (Paper IV)

1) We have investigated the effects of osmolytes in lipid model systems for which changes in the osmotic pressure leads to phase transitions from the $L_\alpha(d)$ phase, to either reversed hexagonal phases, $H_{II}$, (Paper IV) or to lamellar phases with solid chains (Paper V). The transitions from planar to non-planar structures is relevant to, for example, membranes in leaves and seeds for which, such a transition may lead to cell injury due to massive leakage of the membrane.\textsuperscript{115–117} The transition between fluid and solid lipid structures is highly relevant the barrier function of the stratum corneum.\textsuperscript{118}

2) Dehydrin proteins constitute a class of LEA proteins that are over-expressed in seeds and plants under conditions of environmental stress like drought, salt or low temperature. The mechanism of how dehydrin proteins protect plants from acute stress is still not clear. In Paper IV, we investigated how the LEA dehydrin protein Lti30 influences lipid self-assembly in varying hydration conditions, and we compare the effects of Lti30 to the effects of some simple osmolytes.

4.1.1 Model systems

The model lipid systems were chosen on basis of their phase behaviour at varying osmotic pressure, and the main goal is not to mimic certain lipid membranes in nature. As model systems we use mixtures of phospholipids with different hydration-induced phase transitions;

- The transition between bilayer structures with solid and fluid chains ($L_\beta$ to $L_\alpha$) was studied in systems composed of DMPC or DMPC:DMPG.

- The transition between non-lamellar phases ($H_{II}$ or bicontinuous cubic) and a liquid crystalline lamellar ($L_\alpha$) phase was studied in model systems composed of DOPE:DOPC:DOPS at various ratios.

- In addition, we study the effect of hydration on the swelling of charged lamellar $L_\alpha$ phase in a model system composed of POPC:POPG.

The association of the dehydrin protein and the lipid membrane is driven by electrostatic attraction, and therefore a fraction of charged lipids is needed in the model system for the lipid-protein complex to form. In Paper IV and V the osmolytes chosen were; urea, glycerol, PCA and TMAO. In Paper IV, the dehydrin protein Lti30, extracted from seed was studied. In addition, the effects of small polar molecules were studied in samples of intact stratum corneum and isolated corneocytes.
4.1.2 Experimental approach

As described in chapter 3, PT ssNMR is a powerful technique to observe small changes in molecular dynamics in liquid crystalline phases, and to detect minor fractions of liquid components in solid samples. This is taken advantage of in the studies of intact stratum corneum, which have large fraction of solid lipid and protein components at all hydration conditions at ambient temperatures. The PT ssMNR technique was also shown to be very useful in the studies of dehydrin Lti30 binding to lipid membranes, in order to visualise which particular segments in the lipid molecules that are affected by the binding. From these data, it was possible to draw conclusions about the localisation of the protein in the lipid-protein complex. SAXS and WAXS provides good tools for determining the phase behaviour of the lipid system as the different phases gives rise to a distinct Bragg peak pattern, that for lamellar phases is given by an equidistant repeating peaks and for a hexagonal phase the peaks repeat by $q_1$, $q_1\sqrt{3}$, $q_1\sqrt{4}$, $q_1\sqrt{7}$. From the Bragg peaks the dimensions of the self assembly structures can be determined, where the lamellar repeat distance is given by; $d = \frac{2\pi}{q_1}$ and for hexagonal; $a = \frac{4\pi}{q_1\sqrt{3}}$, where a is the distance between the centre of two water channels in the hexagonal array. Sorption microbalance was also used to determine the water uptake of the lipid and lipid-protein systems at different RH.

4.2 How small polar molecules protect membranes

4.2.1 Osmolytes in nature

The use of small polar molecules to protect lipid membranes during osmotic stress is a strategy found in a large variety of species, from plants to simple organisms to higher animals. Sugars are common osmolyte molecules that can be found in a wide range of organisms, including bacteria, fungi, yeast, insects and invertebrates. In anhydrobiotic organisms that can survive states of extreme dehydration upon which they assume a dormant state until more favourable conditions are found, a strong correlation between trehalose content and the ability for the organism to survive have been shown. Similar to the mechanism of the anhydrobiotic organisms, the so called resurrection plants have shown a high accumulation of soluble sugars upon dehydration. For instance, the production of sucrose in Craterostigma plantagineum leaves to a concentration of 40% compared to the total dry weight has been observed after exposure to desiccation. Other compounds found in plants during osmotic stress from high salt levels are proline, glycine betaine, and pinitol. High concentrations of urea and TMAO is found in deep sea elasmobranchs, such as sharks and rays, that live under conditions of high salinity, pressure and low temperatures. Urea is commonly known to denature proteins, while TMAO has been shown to stabilise proteins. Glycerol is found in e.g. seaweed and marine alga to make them tolerant to stress due to the saline water. High levels of glycerol is also found in amphibians. For instance, the Siberian salamander,
which can survive for many years at temperatures as low as -45 °C has high levels of glycerol.\textsuperscript{100,126} In human skin the so called NMFs comprises a mixture of free amino acids, amino acid derivatives, lactic acid, urea and glycerol.\textsuperscript{103–105}

4.2.2 Osmolytes in model lipid systems

The DMPC-water binary system is well characterised and has a phase transition between $L_\beta$ and $L_\alpha$ phases in accessible hydration and temperature intervals.\textsuperscript{127,128} The phase behaviour of the DMPC model lipid system was investigated using PT ssNMR (described in Method 1) showing a phase transition from a solid lamellar phase at 84 %RH to a $L_\alpha$ phase at at 96 %RH at 27 °C. When polar solutes, urea or glycerol, are added to the DMPC system, the $L_\alpha$-$L_\beta$ melting transition takes place at lower relative humidities compared to the neat lipid system, demonstrating that these molecules can protect the fluid lamellar structure in dry conditions.\textsuperscript{129,130} Similar results were obtained when urea and another polar solute, TMAO, were added to the charged lipid lamellar system composed of DMPC:DMPG (95:5)(\textit{Paper IV}). This lipid system forms a $L_\beta$ phase at 84 %RH, while a $L_\alpha$ is present at higher RH (27 °C). When 5 wt% urea or TMAO are added to this lipid system, the $L_\beta$-$L_\alpha$ phase boundaries are shifted to lower RH. The DOPE:DOPC:DOPS lipid systems forms $L_\alpha$ phases at high RH, while non-lamellar $H_{II}$ or bicontinuous cubic structures are present at lower RH (Figure 4.2). When urea is added to the model lipid systems composed of these lipids, the non-lamellar structures are abolished at all RH studied (\textit{Paper IV}).

In all lipid system studied here and in previous publications (\textit{Paper IV-V} and,\textsuperscript{129,131}) urea and glycerol stabilises the $L_\alpha$ phase at reduced RH. Still the water contents in the pure lipid system and the lipid-solute system were shown to be approximately the same. The proposed mechanism is that the polar solutes with low vapour pressure can stabilise the self-assembled structures that are otherwise only present at higher RH by simply substituting for the water in dry conditions.\textsuperscript{129} TMAO shows a different behaviour in that it favours the $H_{II}$ phase at higher RH compared to the pure lipid system (\textit{Paper IV}). TMAO has previously been shown to be expelled from the region close to the polar phosphatidyl choline headgroup.\textsuperscript{131,132} This will lead to a less hydrated headgroup layer, which may in turn explain that the $H_{II}$ structure with negative curvature is favoured above the planar $L_\alpha$ phase.

In summary, the studies of osmolytes in model lipid systems clearly show that the polar small molecules can influence lipid self-assembly and melting of hydrophobic chains (Figure 4.2). Many osmolytes show similar effects, preventing dehydration-induced phase transitions with a close to ideal mechanism where the polar solutes exchange with water.\textsuperscript{13,129,133} When comparing different osmolytes, we show that, for example, TMAO differ from urea and glycerol. This can be explained by repulsive interactions between the solute and the headgroups.\textsuperscript{131,132}
4.3 Dehydrin stabilise lipid bilayers

4.3.1 Dehydrin proteins

The dehydrin proteins belong to a family called LEA (late embryogenesis abundant) proteins, which were first found accumulating in seeds just before desiccation and in other plant tissue after osmotic stress. The LEA proteins are divided into different classes depending on sequence similarity. Even though the sequences differ between the different classes of LEA proteins, they have in common that they are very hydrophilic with a high content of charged amino acids. Dehydrins belong to group 2 of the LEA proteins, which is exclusively found in plants. They are characterised by short conserved repeating amino acid sequences, and in particular so-called the K-segments, which is highly enriched in lysine. In this study we are working with a dehydrin protein called Lti30 which is found in *Arabidopsis thaliana* vascular tissue and anthers after stress by low temperature. It is comprised of 6 K-segments distributed in the sequence and it is intrinsically disordered in solution.

4.3.2 Dehydrin Lti30 in lipid systems

The dehydrin Lti30 protein have previously been shown to bind to charged phospholipid membranes and to aggregate charged phospholipid vesicles in solution. The binding is sensitive to the charged residues on the Lti30, which to a large extent comes from histidine side chains. The histidine side chains have intrinsic $pK_A$ values around 6.5, making the charges on the Lti30 tunable to the pH value of the solution. It has also been shown that the association of Lti30 to anionic lipid membrane may lead to reduction in the $L_\beta$-$L_\alpha$ melting transition temperature in excess solution conditions.

The transition from lamellar to hexagonal structures has previously been investigated in relation to dehydration-induced injury in rye leaves. To study the effect of Lti30 on corresponding phase transitions, we used two model systems composed of DOPE:DOPC:DOPS at different molar ratios, and the self-assembly at varying RH was studied by the means of SAXS (Paper IV). Both systems go from planar bilayers in lamellar phases at high RH, to closely packed rods or folded bicontinuous layers at reduced RH. When Lti30 is added to either of these systems, the $L_\alpha$ phase is stabilised at lower RH where the system otherwise would form a non-lamellar phase. The preservation on the lamellar phase at dryer conditions might be crucial to maintain the membrane integrity and prevent leakage or cell injury.

To investigate the effect of Lti30 on dehydration induced phase transition between $L_\alpha$ and $L_\beta$, the model system composed of DMPC:DMPG with and without protein was studied by SAXS/WAXS and PT ssNMR at varying RH. The pure lipid system goes from $L_\beta$ at 84 %RH to co-existing $L_\beta$ and $L_\alpha$ phases at 93 and 97 %RH. When Lti30 is added to the lipid system, the $L_\beta$ phase is stabilised up to 93 %RH. At 97 %RH the $L_\beta$ and $L_\alpha$ co-exists, which is easily recognised.
Lipid membranes under osmotic stress

from the ssNMR spectra. In other words, dehydrin Lti30 stabilises the solid bilayer lamellar phase at higher RH compared to the neat lipid system. From PT ssNMR experiments, it was also shown that the association of Lti30 with lipid membranes causes reduced mobility in the lipid molecules in the glycerol backbone and segments close to the bilayer interface. The strong association between the protein and the lipid headgroups likely lead to a more stable bilayer headgoup layer, which may in turn explain why the more condensed $L_{\beta}$ phase is favoured.

4.3.3 Dehydrin Lti30 effect on swelling in a lamellar bilayer

In a system that contain charged lipids, the multilayer stack in the lamellar phase will exhibit strong swelling with increasing amount of water due to electrostatic repulsion between the charged headgroup layers. We wanted to investigate the effect of Lti30 on stabilising the lamellar phase, making it less sensitive to variations in water content compared to the neat lipid system. In Paper IV, we show that the addition of Lti30 dehydrin indeed limits the swelling of the lamellar phase, which reaches a maximum with a lamellar repeat distance of $\sim 90$ Å, whereas the lamellar phase formed in the pure lipid system can swell up to at least 200 Å (Figure 4.1a). On the other hand, at water contents below the swelling limit of the lipid-protein system, the lamellar phase composed of the neat lipid system has smaller repeat distances compared to the lipid-protein lamellar phase. From this we conclude that the protein makes the lamellar system less sensitive to variations in water content compared to the protein-free system.

The dehydrin protein used in this study, Lti30, consists of six charged K-segments, which have been shown to form $\alpha$-helical structures upon binding.

---

**Figure 4.1:** A) Lamellar repeat distance ($d$) between the bilayers in the POPC:POPG ($\triangle$) system with increasing amount of water, showing a large swelling at high water contents. Lamellar repeat distance in the lipid-dehydrin ($\circ$) system reaching maximum swelling at $\sim 60$ wt% water. Cartoon illustrating the mechanism of dehydrin reducing swelling in charge lipid membrane by bridging between the bilayers.
with the lipid membrane.\textsuperscript{136} The residues in between the K-segment are likely unstructured and can either span the interbilayer space to bridge between the bilayers, or fold back to the same bilayer (Figure 4.1b). The longer unstructured segments are similar in length to the maximum interbilayer separation observed in the X-ray measurements for the lipid-protein system. This is consistent with the picture that the longer unstructured segments bridge between the bilayers and the shorter segments fold back to the same bilayer. The bridging hypothesis was further tested in experiments with two model peptides that are smaller segments of the Lti30 protein (\textbf{Paper IV}). Analogues bridging behaviour has previously also been described for polymer-surfactant systems with flexible hydrophilic chains flanked with segments that associate with the bilayer either through electrostatic or hydrophobic interactions.\textsuperscript{137–140}

### 4.3.4 Comparison between osmolytes and dehydrin in drying lipid systems

During osmotic stress the plant has several defensive mechanism that might be activated in parallel with each other. In \textbf{Paper IV}, we compare the effects of dehydrin Lti30 on the lipid self-assembly in dry conditions, to the effects of urea and TMAO on the same lipid systems. We notice both similarities and differences between all three additives, which can be related to widely different

![Figure 4.2: Phase behaviour of the DOPE:DOPC:DOPS 60:35:5 system, with the pure lipids going from L\textsubscript{α} (red) at high humidities to co-existing L\textsubscript{α}/H\textsubscript{II} (red and black stripes) approaching 84 \%RH. At 80 and 75 \%RH the pure lipid system is in a bicontinuous cubic phase (grey). The dashed lines represent measured data points for which the phase behavior was characterized, and the one and two-phase regions indicated in between the measurement points are deduced from thermodynamic arguments to fulfill the phase rule. Adding Lti30, the system remains in a single L\textsubscript{α} phase to humidities as low as 80 \%RH at which it forms co-existing L\textsubscript{α}/H\textsubscript{II} phases. Upon adding Urea the system remains in a single L\textsubscript{α} phase for all humidities measured. Adding TMAO favours the formation of the H\textsubscript{II} phase, the system becomes co-existing L\textsubscript{α}/H\textsubscript{II} phases already at 93 \%RH. At 80 and 75 \%RH the system with TMAO is in a H\textsubscript{II} phase (black).](image-url)
molecular mechanisms. Dehydrin acts through direct interactions with the lipid headgroup layer, and the solutes are acting as being part of the aqueous domain in the self-assembled structures. Dehydrin has a remarkable effect of making the lamellar bilayer phase robust towards changes in water content, preventing the formation of non-lamellar phases. The Lti30 dehydrin also has the effect to prevent extensive swelling at high water contents, which is possible thanks to the direct molecular interactions of the protein with the lipid bilayer interface together with protein bridging between adjacent bilayers. Small polar solutes that are not depleted from the bilayer interface, like urea and glycerol, can show similar effects at low water contents where they may act to prevent dehydration-induced transitions. However, the polar solute only affects the self-assembly though the aqueous solution. They will not prevent extensive swelling in humid conditions, unless they are completely depleted from the liquid crystalline phase, which has been shown for TMAO in excess solution conditions.\textsuperscript{141}

4.4 How small polar molecules protect skin against drying

4.4.1 Stratum corneum

The skin is the biggest organ of the body and the outermost barrier to the outside environment. The outer layer of the skin, the \textit{stratum corneum}, separates the water rich epidermis from the dry atmosphere and protects us from uptake of foreign molecules or microbes. The \textit{stratum corneum} is often exposed to osmotic stress from dry air. \textit{Stratum corneum} is comprised of ca. 80-90\% (dry weight) corneocytes, which are embedded in an extracellular lipid matrix,\textsuperscript{142} and it is often envisioned as a brick and mortar wall, with the corneocytes making up the bricks and the lipid matrix making up the mortar.\textsuperscript{4} The entire \textit{stratum corneum} is ca 20 $\mu$m thick.\textsuperscript{143} The lipids present in the lipid matrix is ceramide, fatty acids and cholesterol in a ca. 1:1:1 ratio, which is a lipid composition that differs from most other biomembranes.\textsuperscript{142} The main fraction of the lipid components are solid at ambient temperatures.\textsuperscript{144,145} The lipid matrix makes the only continuous route through the SC\textsuperscript{146} and any molecule passing the skin barrier must pass through the extracellular lipids. Since the permeability is much lower for solid lipids than for fluid lipids, the state of the lipids will greatly affect the barrier properties of the SC.\textsuperscript{147} The corneocytes are flat differentiated dead epidermal cells with a diameter of ca. 30 $\mu$m and 0.3 $\mu$m high filled with keratin filaments. The corneocytes are surrounded by the corneocyte envelope which is described as a lipid monolayer, mainly fatty acids and ceramides, covalently bound to the proteins.\textsuperscript{148} The \textit{stratum corneum} has an overall low water content and the main water uptake takes place in the corneocytes.\textsuperscript{149,150} Still, both lipids and protein self-assembly is clearly affected by variations in the RH in the environment.\textsuperscript{118}

The \textit{stratum corneum} contain a mixture of small polar molecules, commonly referred to as the natural moisturising factor, NMF. It comprises a mixture of
free amino acids, amino acid derivatives, lactic acid, urea and glycerol.\textsuperscript{103–105} The NMF is considered important for skin softness and flexibility, and lack of NMF has been associated with skin diseases and states of very dry skin.\textsuperscript{151, 152} Some of the compounds from the NMF are also used in skin care formulations and treatments of dry skin and some skin diseases.\textsuperscript{106, 153}

### 4.4.2 Osmolytes in intact stratum corneum

In Paper V, PT ssNMR was used to evaluate the effects of osmolytes and hydration on the order and dynamics of the individual C-H segments of the lipids and proteins in intact \textit{stratum corneum} as well as in isolated corneocytes. The PT ssNMR experiment provide information about the overall state (solid or fluid), and it also gives molecularly resolved information on which lipid or protein segments in the samples that are affected when adding osmolytes or changing RH. The assignment of the individual amino acid and lipid segments were done previously for intact \textit{stratum corneum} and model lipids.\textsuperscript{118} From the ssNMR spectra the increased mobility in the sample upon hydration can be visualised by the increase in INEPT signal compared to the DP signal (Figure 4.3). At 80 %RH the spectra show almost no INEPT signal for any lipid or protein component, signifying that almost the entire sample is solid. The broad peak centered around 57 ppm in the CP spectra of both intact \textit{stratum corneum} and isolated corneocytes are assigned to the $C_\alpha$ of the peptide backbone (except for Glycine residues), indicating that the entire keratin filament is completely rigid. However small amount of lipids with fluid acyl chains are detected around 29-31 ppm. When the same samples are hydrated (96 %RH), we observe strong INEPT signal from the serine and glycine amino acids in the keratin filaments. Furthermore, there is a gradual increase in the mobility in the lipid acyl chains when RH is increased.

Figure 4.3: PT ssNMR $^{13}$C spectra from intact stratum corneum and corneocytes at 32 °C, 80 %RH and 96 % RH. Spectra with added urea to stratum corneum and corneocytes at 80 % RH is shown to the right. The peaks from the AT and TG conformations of the acyl chains are indicated in the spectra. ‡ Data from Björklund et al.\textsuperscript{118} Reproduced from Ref. 130 with permission from the Royal Society of Chemistry.
When 20 wt% urea or glycerol is added to the stratum corneum at 80 %RH, there is a strong increase in INEPT signal as compared to pure stratum corneum at the same conditions. There is a clear increase in INEPT signal for the serine and glycine, as well as in the lipid chains. It is noted that the spectra from stratum corneum with 20 wt% urea or glycerol at 80 %RH has striking similarities with the spectra from pure stratum corneum at 96 %RH. It is also noted that the water content of the sample with stratum corneum +solute is almost the same as the water content in neat stratum corneum at the same RH (80 %RH). In other words, the addition of urea or glycerol lead to melting of both lipids and protein, but it does not really influence the water content in the sample. We therefore conclude that the observed effects are not due to higher water content but likely related to the amount of polar components (water + solute) in the stratum corneum samples (see Table 2, Paper V).

4.4.3 Comparison of effects of osmolytes in intact stratum corneum and model lipid systems

In paper V, we show that the addition of urea or glycerol to stratum corneum lead to an increased fluidity in both lipid and the protein components, and the effect of these solutes is very similar to the effect of adding more water. The increased fluidity in the stratum corneum samples containing osmolytes without an increase in water content indicates that urea and glycerol replaces water as a polar solvent for the stratum corneum instead of increasing the water uptake. The osmolytes have a low vapour pressure and stays in the skin when water evaporates, and they thereby can protect the stratum corneum against osmotic stress. The observed effect of the osmolytes in the intact stratum corneum is very similar to the effect seen for the simple model systems described in section 4.2.2. Also in model system, it was shown that urea and glycerol stabilises the fluid phase at dryer conditions compared to the pure lipid systems, without increasing the water uptake.

4.5 Conclusions and outlook

We show individual effects of osmolytes and dehydrin proteins on lipid self-assembly in dry conditions. We also demonstrate similar effects of osmolytes in simple model mixtures and the stratum corneum. Urea and glycerol are polar compounds with low vapor pressure, and the addition of these compounds leads to a larger total volume of polar (water + solute) components in both the lipid systems and the stratum corneum. Thereby, these compounds act to replace the water and they may stabilize the fluid lipid phases at lower humidities. The dehydrin protein, Lti30, is shown to stabilise the $L_\alpha$ phase over a large range of hydration conditions, preventing phase transitions at low water contents, and extensive swelling of the lamellar phase at high water contents.
Outlook. If the Lti30 bridges the bilayers with the unstructured segments located in between the charged K-segments, this hypothesis could be tested by using model peptides with varying length of the unstructured segment and comparing the lamellar repeat distance in the bilayers. One could also compare the lamellar repeat distance in the same system where the electrostatic repulsion is screened by the addition of salt. It would also be interesting to investigate the \( \alpha \)-helical structure in more dehydrated lamellar systems and in the reversed hexagonal phase. Here, one open question concerns whether the \( \alpha \)-helix is incorporated to these self-assembled structures with narrow water channels or if the K-segments unfold, loosing the secondary structure. We here study the effect of urea and glycerol on intact stratum corneum. These compounds are both part of the NMF. However there are many more small polar compounds that belong in the NMF, for example, lactate, free amino acids and salts, for which, the effect on stratum corneum under dry conditions has not been studied. It would be interesting to apply a similar approach as taken in Paper V to investigate the effect of other compounds of the NMF on stratum corneum and be able to compare the observed effects of the different compounds.
I would like to thank everyone who has in any way contributed to the work of my thesis. Above all, I would like to thank;

Emma, I have been very privileged to get to do my phd under your supervision, I could not think of a better supervisor. You have been very inspiring and have taught me very much. You set a very good standard for what an excellent researcher should be, both scientifically and as a person. Thanks for all the help over the years and thank you for your patience.

I have also had some great co-workers in Kevin and Tiago, whom I have to thank for two very good papers. I have really enjoyed your company during beamtimes and conferences.

I would also like to thank Tommy, for all the help and good food over the years and for introducing me to neutrons and an endless string of people.

Marcus, thank you for introducing me to the world of lung surfactant, it is a truly intriguing field and thank you for your enthusiasm and knowledge.

Pia and Sylvia, thank you for a very interesting project and for all the discussions.

Per Persson and César, thank you for your generosity and help with the IR and Raman microscopes.

Chris and Maria, thank you for making our work easier.

Ricardo, Ilaria and Julien, thank you for sharing the office with me over the years and for filling it with happiness, music, pink posters and cheese (even the smelly ones).

Sofi, Emelie, Jessie, Dat, Maria V. and Tommy G. thank you for all the help and all the fun.

I would like to thank all friends and co-workers at the physical chemistry division over the years for a warm atmosphere and good discussions. I have been GLaD to work at a place where the cake is not a lie.
Acknowledgements

Irene, thank you for being my friend. You were the one who got me really interested in research to begin with and I would probably not have done a phd without you.

To all my friends and family, thank you for being a great support.

Manja, Elsa och Heléne, tack för att ni alltid finns där och för att ni gör mitt liv så ofantligt mycket roligare.

Tack Mamma för ett otroligt stöd, utan dig hade inte den här boken varit möjlig.

Björn, tack för allt. Jag älskar dig.
References


REFERENCES


REFERENCES


REFERENCES


REFERENCES


