Foreign molecules in biomembranes

Molecular effects on intact stratum corneum and model lipid systems

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QUOC DAT PHAM

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Quoc Dat Pham

LUND
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Abstract

This thesis mainly explores how the molecular mobility of lipid and protein components in the outermost layer of the skin, the stratum corneum (SC), varies with different conditions and upon adding foreign compounds. These studies require methods that makes it possible to detect and characterize the minor amount of fluid components in the highly ordered solid SC sample with resolved molecular information. ^{13}C polarization transfer solid-state nuclear magnetic resonance (PT ssNMR) is well suited for this task, and was employed as the main method through this thesis. The aims of this thesis were achieved by studies on well-controlled systems of intact SC, extracted corneocyte, and model lipid systems, and by combining PT ssNMR with different complementary biophysical techniques.

A general conclusion from the presented studies of different foreign compounds is that apolar compounds mainly affect SC lipids, while more polar compounds affect both SC lipid and protein components. The effects strongly depend on the identity of the additive, and on the hydration conditions, and they vary with concentrations and temperatures. One interesting finding is the essential role of water in the mobility of keratin filaments. Apart from the effects on SC components, one can also simultaneously characterize the molecular mobility of the additves that are incorporated in the complex SC matrix by using PT ssNMR.

We also demonstrate a clear correlation between the molecular mobility in SC lipids and proteins and the skin permeability upon additives, which forwards the understanding of the skin macroscopic barrier function at a molecular level. The findings of this research can be applied in topical formulations for, e.g., drug delivery and skin care products.

Key words

Stratum corneum, lipid, keratin filament, protein, DMPC, penetration enhancer, osmolyte, solvent, urea, glycerol, monoterpene, fatty acid, hydration, mobility, fluidity, permeability, PT ssNMR, diffusion cell

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Molecular effects on intact stratum corneum and model lipid systems

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Coverphoto by Quoc Dat Pham. A foreign object approaching an interface.

Content

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. **Cyclic and linear monoterpenes in phospholipid membranes: Phase behavior, bilayer structure, and molecular dynamics**
    Quoc Dat Pham, Daniel Topgaard, and Emma Sparr
    Langmuir **2015**, *31*, 11067−11077

II. **Hydration interactions in lipid bilayer systems in the presence of polar solutes urea and TMAO**
    Quoc Dat Pham, Anirudh Gupta, Alexander Schlaich, Emanuel Schneck, Roland R. Netz, and Emma Sparr
    Manuscript

III. **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
    Soft Matter **2014**, *10*, 4535-4546.

IV. **Chemical penetration enhancers in stratum corneum - Relation between molecular effects and barrier function**
    Quoc Dat Pham, Sebastian Björklund, Johan Engblom, Daniel Topgaard, and Emma Sparr
    Journal of Controlled Release **2016**, *232*, 175−187

V. **The effects of transcutol and dexpanthenol on molecular mobility, permeability, and electrical impedance of the stratum corneum**
    Sebastian Björklund, Quoc Dat Pham, Louise Bastholm Jensen, Nina Østergaard Knudsen, Lars Dencker Nielsen, Katarina Ekelund, Tautgirdas Ruzgas, Johan Engblom, and Emma Sparr
    Journal of Colloid and Interface Science **2016**, *479*, 207−220

VI. **Tracking solvents in the skin - Molecular mobility of solvents, lipids and proteins in intact stratum corneum**
    Quoc Dat Pham, Daniel Topgaard, and Emma Sparr
    Submitted

VII. **Characterization of the ceramide EOS induced fluid fraction in stratum corneum lipid model membrane**
    Quoc Dat Pham, Enamul H. Mojumdar, Gert S. Gooris, Joke A. Bouwstra, Emma Sparr, and Daniel Topgaard
    Manuscript

List of Author Contributions

I. I, ES and DT designed the study. I performed all experiments and analyzed the data together with ES and DT. I wrote the paper with contributions from ES and DT.

II. I and ES designed the experimental part of the study. I performed all experiments and related data analysis. I and ES wrote the first draft of the paper.

III. ES and SB designed the study. Experiments were performed by JMA and me. The data were analyzed by JA, SB and me. The paper was written by ES and SB with inputs from me and the other authors.

IV. I, ES and SB designed the study. I performed all experiments and analyzed the data together with ES and DT. I wrote the paper with contributions from the other authors.

V. SB, ES, JE and I designed the study together with scientists from LEO Pharma A/S. I performed all the NMR experiments. SB and I analyzed the NMR data. SB and ES wrote the paper with contributions from the other authors.

VI. I, ES and DT designed the study. I performed all experiments. I analyzed the data together with ES and DT. I wrote the paper with contributions from ES and DT.

VII. I, ES and DT designed the study. GSG, I and EHM prepared the samples. I performed all NMR experiments. I analyzed the data together with ES, DT and EHM. I, ES and DT wrote the manuscript with contributions from the other authors.

List of publications not included in this thesis:

1. **Controlling water evaporation through self-assembly**
   Kevin Roger, Marianne Liebi, Jimmy Heimdal, Quoc Dat Pham, and Emma Sparr
   Proceedings of the National Academy of Sciences of the United States of America **2016**, in press.

2. **Kinetic influence of siliceous reactions on structure formation of mesoporous silica formed via the co-structure directing agent route**
   Ruiyu Lin, Göran Carlström, Quoc Dat Pham, Michael W. Anderson, Daniel Topgaard, Karen J. Edler, and Viveka Alfredsson
   The Journal of Physical Chemistry C **2016**, *120*(7), 3814-3821.

3. **Controlling interfacial film formation in mixed polymer-surfactant systems by changing the vapor phase**
   Tahereh Mokhtari, Quoc Dat Pham, Christopher Hirst, Benjamin M. D. O'Driscoll, Tommy Nylander, Karen J. Edler, and Emma Sparr
   Langmuir **2014**, *30*, 9991-10001.

4. **Impact of oxidized phospholipids on the structural and dynamic organization of phospholipid membranes: a combined DSC and solid state NMR study**
   Marcus Wallgren, Lenka Beranova, Quoc Dat Pham, Khanh Linh, Martin Lidman, Jan Procek, Konrad Cyprych, Paavo K. J. Kinnunen, Martin Hof, and Gerhard Gröbner
   Faraday Discuss **2013**, *161*, 499-513; discussion 563-89.

5. **The oxidized phospholipid PazePC modulates interactions between Bax and mitochondrial membranes**
   Marcus Wallgren, Martin Lidman, Quoc Dat Pham, Konrad Cyprych, and Gerhard Gröbner
   Biochimica et Biophysica Acta (BBA) - Biomembranes **2012**, *1818*, 2718-2724.

List of Abbreviations

AT	All-trans
CP	Cross polarization
DMPC	1,2-dimyristoyl-sn-glycero-3-phosphocholine
DP	Direct polarization
DSC	Differential scanning calorimetry
EOS	Esterified omega-hydroxy sphingosine
ESR	Electron spin resonance
INEPT	Insensitive nuclei enhanced by polarization transfer
IR	Infrared spectroscopy
$K_{o/w}$	Octanol-water partition coefficient
NMR	Nuclear Magnetic Resonance
MAS	Magic angle spinning
POPC	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
PT ssNMR	Polarization trasnfer solid-state NMR
RH	Relative humidity
SAXD	Small-angle X-ray diffraction
SAXS	Small-angle X-ray scattering
SC	Stratum corneum
TEWL	Transepidermal water loss
TG	Trans-gauche
WAXD	Wide-angle X-ray diffraction

Popular Science Summary

The skin is our largest organ and its main function is to protect us from uptake of foreign chemicals and from desiccation. The barrier function of the skin is mainly assured by its outermost layer, the stratum corneum (SC). When one wants to deliver a compound into or through the skin, for example in (trans)dermal drug delivery and cosmetics, one generally needs to make the skin more permeable. Therefore, it is desirable to control the skin barrier function. The purpose of this thesis is to deepen the understanding of the mechanisms that determine the skin permeability at a molecular level. The focus is laid on the molecular mobility and fluidity of components in SC, which play crucial roles in the barrier property. One major goal was to characterize the fluid SC fractions and how they vary with different conditions, for example, hydration, temperature, addition of moisturisers and penetration enhancers, solvents and in diseased skin, for example, psoriasis. The molecular information can be related to other macroscopic properties of the skin, for example, water-holding capacity, elasticity, softness, dryness, and barrier function. The findings of this research can be applied in topical formulations for drug delivery and skin care products as well as for treatment of diseased skin. These results were achieved by performing experiments on intact SC, extracted corneocyte and model lipid systems and by combining different complementary techniques like NMR (nuclear magnetic resonance), scattering, calorimetry, sorption microbalance and diffusion-cell systems.

1 Objectives of this thesis

The skin is one of the largest organs of the body, and it comprises many vital functions, for example, thermoregulation, synthesis of vitamin D and mechanical protection in that it tolerates deformation from physical strain and stress. One of the most important functions of the skin is its ability to serve as a large interfacial barrier film that protects our body from evaporation and uptake of hazardous chemicals from the environment. Furthermore, the skin is highly attractive for drug therapy because it offers an easily accessible route without first-pass metabolism. Transdermal drug delivery is also associated with high patient compliance and through the site of application, drug delivery can be locally directed. Taken together, skin is an interface that tempts to be controlled to allow for molecular transport of active molecules and still remain an efficient protective barrier.

The barrier function of the skin is mainly assured by its outermost layer, the stratum corneum (SC)[1] that is comprised of lipids and proteins. The purpose of this PhD thesis is to deepen the molecular understanding of how the mobility/fluidity of different SC lipid and protein components varies with the addition of various foreign molecules (Fig. 1.1), and with changes in the environment conditions, like temperature and hydration. The molecular information can then be related to macroscopic properties of the skin, for example, water-holding capacity, elasticity, softness, dryness, and barrier function. Many of the additives that have been studied in this thesis have been described as moisturizers and penetration enhancers in the literature, and one major goal is to

forward the established understanding with novel molecular characterization of how these compounds influence the relatively unexplored components of the SC that is its minute fluid fraction. In previous literature, the molecular characterization of the fluid SC lipids and proteins in intact SC is rather incomplete. One major complication in the characterization of the fluid SC components is that it is difficult to find methods with high enough molecular resolution and sensitivity to the minor disordered fluid fraction in the complex and highly ordered solid SC sample. In this thesis, we employ natural abundance ^{13}C polarization transfer solid-state nuclear magnetic resonance (PT ssNMR)[2] to obtain molecular resolution on the dynamics in different segments of the SC components with high sensitivity to small changes in the fluid protein and lipid components. We illustrate how this NMR method can provide completely new information on the fluid fraction in SC treated in different conditions, and we believe that this information is important for the molecular understanding of SC. Experiments were performed on intact SC, extracted corneocytes, and model lipid systems by combining PT ssNMR and complementary biophysical techniques, including scattering, calorimetry, sorption microbalance, diffusion-cell systems as well as other NMR methods.
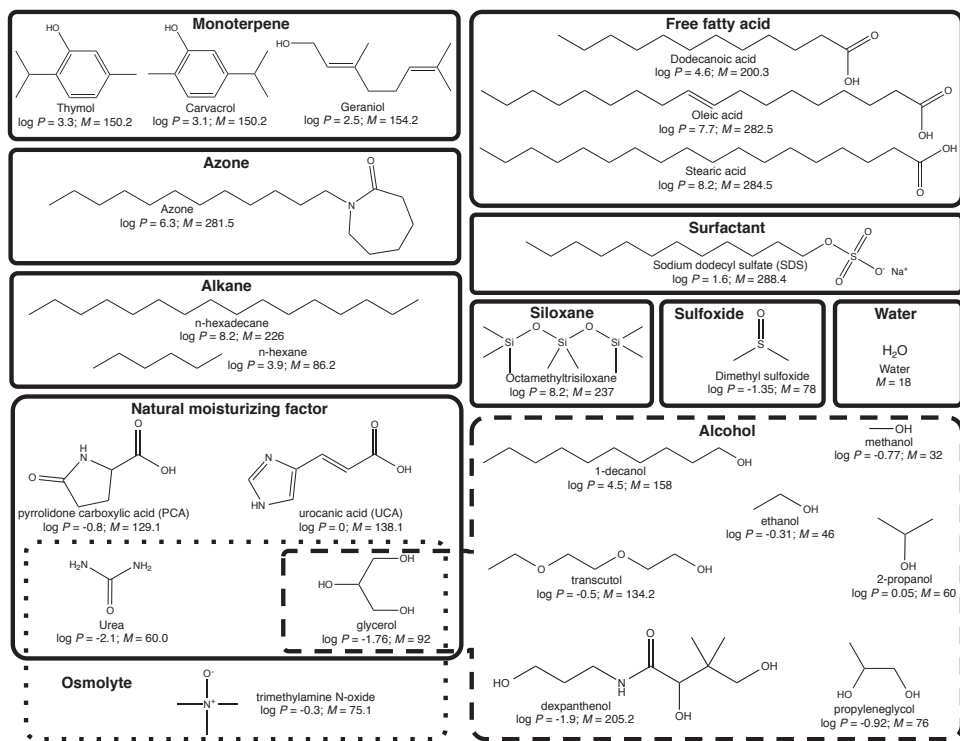
Fig. 1.1 Chemical structures of different additives studied in this thesis.

2 Basic concepts

2.1 Lipid self-assembly

Lipids, as other amphiphiles, spontaneously self-assembly to form different structured aggregates with different topology, including the planar lamellar phases (Fig. 2.1A), as well as normal and inverted cubic, hexagonal and micellar phases. The precise structure depends on the type of lipid and on the sample conditions, including temperature, hydration, pressure, pH, and presence of, e.g., ions and co-surfactants. The most common lipid self-assembled structure in biological membranes is the planar bilayer, while for SC lipids also other more complicated structures have been suggested, including lamellar phases consisting of stacked bilayers[3] or trilayers.[4]

2.2 Phase transitions

Lipid self-assembly may change between phases with different topology and/or different acyl-chain packing. The transitions between these phases can involve melting of acyl-chains and/or changes in mobility of lipid headgroups. As an example, different lamellar phases are shown in Fig. 2.1A including $L_{\beta'}$ (planar gel phase with ordered tilted chains), P_{β} (rippled gel phase with ordered chains) and L_{α} (lamellar liquid crystalline phase with disordered chains). The term "gel phase" is

used to describe a system in which one constituent is immobile and another constituent is mobile.[5] In a lipid gel phase, for example, $L_{\beta'}$ and P_β, lipids have crystalline chain packing and liquid water in the gap between the lamellae is mobile. One type of phase transition that is essential to the work in this thesis is the transition between solid phases with crystalline packing and conformationally ordered hydrocarbon chains, and fluid liquid crystalline phases with conformationally disordered hydrocarbon chains. This transition is generally referred to as chain melting, and it can be induced by, e.g., changes in temperature (thermotropic phase transition, solid red arrow in a phase diagram in Fig. 2.1B), or changes in hydration (solid blue arrows in the phase diagram in Fig. 2.1B and in the sorption isotherm of DMPC at 27 °C in Fig. 2.1C). Although a given phase exists over a range of temperatures and water contents, its properties gradually change when moving within the one-phase region in the phase diagram by changing water content or temperature. As an example, the swelling of the L_α phase occurs over a broad range of water content (dashed blue arrows in Fig. 2.1B-C), where the water gap between the bilayers in the lamellar structure is enlarged. Swelling might also lead to small changes in the area per lipid headgroup and bilayer thickness in the L_α phase.[6] The term "phase transition" signifies the situation where a small step in composition/ environmental condition (temperature, osmotic pressure etc.) leads to a dramatic change in structure and property.
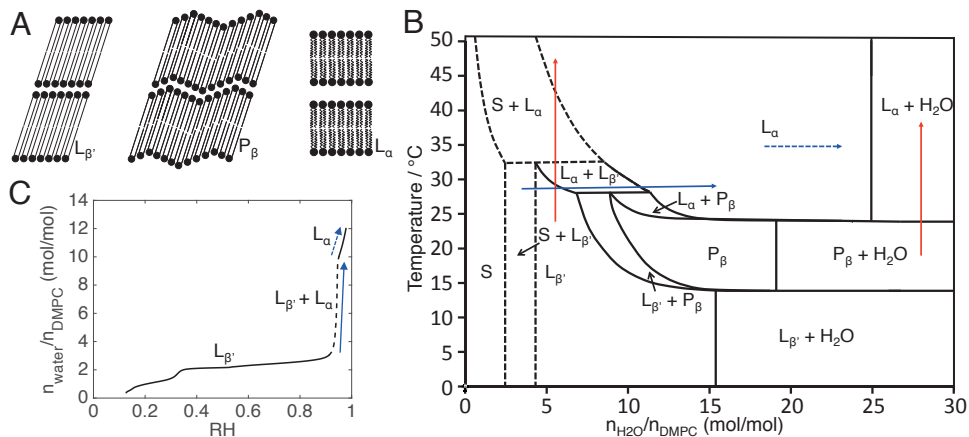
Fig. 2.1 (A) Schematic representation of some lipid bilayer structures. $L_{\beta'}$ (planar gel phase), P_β (rippled gel phase) and L_α (lamellar liquid crystalline phase). (B) Temperature-composition phase diagram of DMPC (paper I and [7-8]). The exact compositions at the dotted phase boundaries were not determined. S: solid crystalline phase. The solid red and blue arrows indicate phase transitions induced by temperature and hydration, respectively. The dashed blue arrows indicate swelling of the L_α phase. (C) Sorption isotherm of DMPC at 27 °C. The dashed line shows the phase transition at constant RH (i.e. constant water chemical potential). The solid and dashed blue arrows indicate phase transitions induced by hydration and swelling of the L_α phase, respectively.

At equilibrium, a phase transition occurs when the chemical potentials of all components are the same in all phases.[5] Consequently, in a binary lipid-water system at a constant temperature, the phase transition induced by hydration occurs at a constant chemical potential of water even though it often occurs over a range of water contents (Fig. 2.1C).

2.3 Hydration

Hydration can refer to the amount of water in a sample, as expressed in terms of concentration. Hydration can also refer to the thermodynamic concept of water activity a_{H2O}, chemical potential of water $\Delta\mu_{H2O}$, relative humidity (RH), and osmotic pressure, Π_{osm}, which are all related as:

$$\Delta\mu_{H2O} = -V_{H2O}\Pi_{osm} = -RTln(\text{RH}) = -RTln(a_{H2O}) \qquad (2.1)$$

where V_{H2O} is the molar volume of water.

For most systems, there is no trivial relation between the water content and the water activity. Their relation depends on, for example, the swelling characteristics of different phases, charges, self-assembled structures, and phase transitions. In experimental studies, the relation between the water content and the water activity can be obtained from sorption isotherms. The example sorption isotherm in Fig. 2.1C clearly shows a non-linear relation between water content and RH. Dehydration can be caused by drying, freezing or by exposure to aqueous systems with high osmotic pressure due to the presence of water-soluble components, e.g., ions, like in saline water, polar solutes and polymers.[5]

2.4 Partitioning - Solubility - Permeability

Partitioning of a compound between two phases is based on its solubility in the different phases. For a heterogeneous medium that consists of different regions with different polarity, like the lamellar phases, a foreign compound may partition between the polar aqueous regions, apolar regions of lipid acyl-chains and the bilayer interfacial headgroup layer. If the added compound also influences the phase behaviour, the solubility and the partitioning of the compound in the self-assembled structure will also be altered. As an example, the addition of a hydrophobic "contamination" to a solid bilayer may lead to melting point depression and formation of more fluid structures. This can have large impact on the partitioning of the added compound, as the solubility is generally much higher

in a fluid phase compared to a solid phase. The octanol/water partition coefficient $K_{o/w}$ of a compound is often used when predicting partition coefficients of a certain compound. This value can be useful for predicting the properties of hydrophobic and hydrophilic compounds, while for amphiphilic molecules it is a facile reference value since it does not account for the surface activity.

Solubility and partitioning are important concepts when predicting permeability of a barrier membrane, as the SC or a single lipid bilayer membrane. The permeability P_i of a molecule i in a membrane depends on its partition coefficient, K_i, the diffusion coefficient, D_i, as well as the membrane thickness L

$$P_i = K_i D_i / L \tag{2.2}$$

Here, K_i is the partition coefficient between the surrounding solution and the membrane. The diffusion coefficients of small molecules in the membrane interior are similar for most polar and apolar molecules, and the key parameter to determine whether the permeability is high or low is the partition coefficient, which is directly related to solubility. In complex membrane structures, one can as a first approximation replace the single permeability constant with an effective permeability constant that takes into account heterogeneous membrane structures. In many self-assembled membrane structures, the permeability may further change due to interactions with the diffusing compounds, and the permeability can then not be treated as a single constant. This is treated in models describing responding lipid membranes.[9]

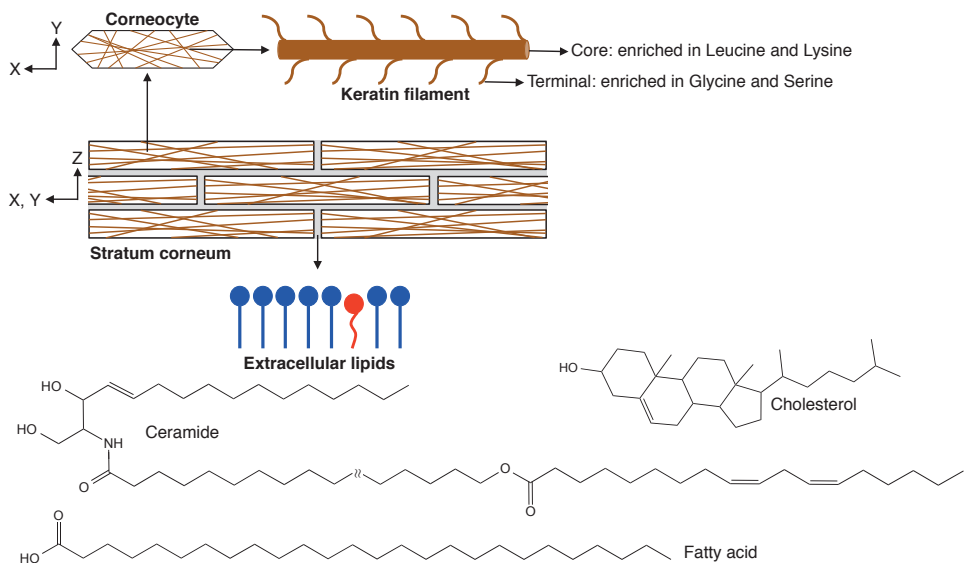# 3 Stratum corneum - the main barrier of the skin

Fig. 3.1 Schematics illustrate the brick and mortar model of SC with corneocytes filled with keratin filaments, surrounded by a multilamellar lipid matrix. Chemical structures of ceramide (here illustrated with ceramide EOS (esterified omega-hydroxy sphingosine) linoleate), cholesterol and fatty acid (here illustrated with cerotic acid) are also shown.

Stratum corneum, the main barrier function of the skin, is typically only ca. 20 µm in thickness and has very low water content.[1] This thin and dry layer is composed of 10–15 layers of anucleated dead epidermal cells (corneocytes), which are filled with keratin filaments and embedded in a continuous multilamellar lipid matrix in an array similar to 'bricks and mortar' (Fig. 3.1).[10]

3.1 SC lipid matrix - a crucial role in SC barrier function

The extracellular lipids play a decisive role in the SC barrier function since they constitute the only continuous regions of the SC[11] (Fig. 3.1) and molecules passing the skin barrier must be transported through them. The SC lipid composition is clearly different from most other biological membranes in that it includes basically no phospholipids and has longer and more saturated lipids.[12] The main components of the SC extracellular lipid system are ceramides, fatty acids and cholesterol in a relatively equal molar ratio.[13] At ambient temperatures, the majority of the lipid components are solid.[14-15] Several studies have demonstrated that SC lipids are arranged in lamellar structures with two typical repeating units of about 130 Å and 60 Å, which are often referred to as the long periodicity phase (LPP) and short periodicity phase (SPP), respectively.[16-17] Within these lamellae, the chains of the lipids form different crystalline arrangements, where orthorhombic packing coexists with hexagonal packing at ambient temperature.[14, 17] The multilamellar arrangement of the lipids represents an almost ideal barrier towards strongly polar as well as strongly non-polar substances, while those of intermediate polarity may penetrate the SC more readily. This can be related to the fact that the relatively few transdermal delivery drugs that are currently on the market have log $K_{o/w}$ around 1-3.[18]

The water content in SC lipids is very low. Still sorption studies reported that extracted SC lipids can take up substantial amounts of water at high RH.[19] Furthermore, SC lipid self-assembly responds to changes in water content. Most importantly, changes in hydration may induce changes in lipid crystalline packing[20-21] and lipid fluidity.[22-23] Some SAXS studies have also shown swelling of the SC lipids in water from an increase in the lamellar repeat distance.[17, 24-25] However, in other studies,[16, 26-27] no swelling was detected. The contradictory results may be due to overlapping peaks in the SAXS spectra, which might not resolve minor changes. The discrepancies might also be explained by different experimental conditions and different sources of SC.

The amount of cholesterol in SC lipid mixtures is quite high. Segregated crystalline cholesterol has indeed been shown in previous X-ray studies of SC lipid model mixtures and intact SC.[28-29] There are indications in the literature that the segregation of cholesterol depends on the sample preparation and sample

history,[29-30] implying that the concentration of cholesterol is close to a solubility limit. Cholesterol acts as a fluidizer when added to a solid lipid phase since it disturbs the solid lipid packing.[31-32] On the other hand, in fluid lipid systems, cholesterol acts as a stabilizer as it may induce increased acyl-chain ordering.[31] SC lipids are mainly solid at ambient condition, and the cholesterol is therefore expected to act as a fluidizer in the extracellular lipid matrix.

3.2 SC keratin filaments in corneocytes - where water is mainly hold

The major components of SC are the corneocytes, which make up ca. 85 % of the total weight of dry SC.[13] These are flat hexagonal-shaped dead cells with diameter of ca. 30 μm and thickness of ca. 0.3 μm. The corneocytes are joined together by corneodesmosomes. Each corneocyte is filled with keratin filaments (Fig. 3.1) and surrounded by a cornified cell envelope. The envelope has been described as a lipid monolayer comprised of mainly fatty acids and ceramides, especially hydroxyceramide, and covalently bound to the corneocyte protein.[33] The keratin filaments are built up from protein chains that consist of an α-helical rod domain flanked by two disordered N- and C- terminal domains. The monomer protein units associate to form dimers, which go on to form tetramers, protofilaments and finally keratin filaments.[34] The core of the keratin filament is enriched in leucine and lysine while its protruding terminal chains are rich in hydrophilic amino acids, i.e., glycine and serine (UniProt ID P04264 and P13645).[35] At ambient temperatures, the highly structured keratin filament core is solid while the mobility of the unstructured terminal domains varies with water content.[23] The corneocytes can be seen as rather large polar regimes in SC. The corneocytes can also take up substantial amounts of water.[20] The swelling of the corneocytes is mainly responsible for the water-holding capacity of SC at ambient conditions.[19-20] Furthermore, the swelling of corneocytes implies that their walls are permeable to water. The corneocytes are therefore expected to contribute to the transport route of water and most likely also other small polar compounds.

The literature on biophysical and structural characterization of SC lipids is much more extensive than that on characterization of corneocytes and keratin filaments. This may be due to the role of the lipid matrix as a continuous route through SC. On the other hand, one cannot neglect the importance of corneocyte as another contribution to the transport route and as the major fraction of SC. In addition, corneocytes are likely crucial to determine SC material properties, including water-holding capacity, elasticity, and softness.

3.3 Skin hydration and pH

The water content of SC is very low compared to the rest of the epidermis. The water content is determined by the water-holding capacity of SC, and by the water activity in the surrounding regions. The boundary on the inner side of the SC, in the viable epidermis, corresponds to the water chemical potential of physiological saline solution. The boundary condition on the outer side of the SC is determined by the RH in air. This boundary condition may vary significantly, and this is crucial in determining the SC water content.[19] When the SC is exposed to a formulation, the water activity in the outer surface of the skin is instead determined by the properties of the (drying) film of the formulation[36] and so is the skin hydration.

Another aspect of SC that is often discussed in the literature is skin pH. There are several studies showing that the pH varies with occlusion[37] and SC depth.[38-39] In evaluating these data, it is important to be aware of that it is difficult to define SC pH due to its very low water content. The variation in pH with SC depth and SC treatment may alter the degree of ionization of SC fatty acids. The fatty acids are assumed to be in the deprotonated form at neutral pH (the intrinsic pK_a of the free fatty acid is ca 4.8).[40] Self-assembly as well as variation in water content may influence the apparent pK_a and the degree of fatty acid protonation. The change in water activity can alter the local electrostatic interactions and the dissociation equilibrium of the charged components, which can lead to variations in the apparent pK_a.[41]

3.4 SC is a heterogeneous, non-equilibrium and responding system

SC is a heterogeneous membrane with a co-existence of different solid lipid phases[14, 17] and a small fraction of fluid lipids.[19, 23, 42-43] In addition, SC separates regions with completely different properties, implying several different gradients in, e.g., water activity, temperature, and pH across SC.[38] All these gradients may influence SC structure at different depth, which in turn may alter its overall function with respect to, e.g., permeability and water-holding capacity.[9, 22, 44] The gradients also imply several simultaneous transport processes across SC. One important example is the non-negligible transepidermal water loss (TEWL) that reaches about 100–150 ml per day and square meter for intact healthy skin.[45] In addition, other studies have suggested gradients in lipid composition[46] and ion concentration[47] across SC.

The fact that the structure and function of SC are influenced by the conditions in the surrounding environment demonstrates that SC has the properties of a responding membrane system. One important example of this is the reduced SC permeability to water and other small compounds when the outside environment becomes more dry.[22, 44] This responsive behavior was previously modelled by Sparr et al.[9] based on the response in self-assembly structures in a multilayered array of lipid bilayers.

3.5 Importance of the fluid fractions in SC

At ambient temperatures, the main part of both the lipid and the keratin components are solid. In hydrated conditions, minor fractions of the SC lipid and protein components become fluid (mobile disordered).[23, 48-50] The tiny fluid fraction is likely crucial for macroscopic material properties of SC, including barrier function, elasticity, softness and water-holding capacity.[22, 51-52] Increasing fluidity or increased size of the fluid fraction is expected to lead to higher solubility and higher effective permeability to both polar and apolar compounds.[53-54] The existence of a minor fluid fraction at ambient conditions may account for the non-negligible TEWL through the intact healthy skin, the high elasticity of the skin and the enzyme activity in the SC intercellular space that is unlikely to take place in a solid phase.

3.6 Open questions in skin research addressed in this PhD thesis

There has been extensive research over many years focusing on the barrier function of the skin and how it can be affected. The research field is interdisciplinary covering skin biology, dermatology, chemistry, formulation, and cosmetics. A large fraction of these studies focuses on how macroscopic properties of skin, mainly the barrier function, can be altered by additives like moisturizers, penetration enhancers, humectancts, as well as to changes in hydration and temperature.[55-56] From these studies, it can be concluded that a certain formulation or condition leads to increased permeability to certain drugs or active molecules. However, as the formulations often have complex compositions, it is usually difficult to distinguish the effect of a certain compound. The observation may be related to combination of molecules in a formulation, and to variations in the water activity of the formulation.

There are numerous biophysical studies of SC and model mixtures using, e.g., calorimetry, scattering, diffraction, infrared spectroscopy (IR), electron spin resonance (ESR) and NMR techniques. Differential scanning calorimetry (DSC) is powerful to detect temperature-induced transitions, and this method has in particular provided information on thermal transitions in SC components at elevated temperatures.[21] On the other hand, DSC is not a sensitive method to resolve small and gradual changes in SC with temperature.[23] Most studies using diffraction, scattering and IR methods on SC and SC model lipid systems focus on detailed molecular characterization of the solid lipid domains, while information on the minor coexisting fluid fractions is generally not resolved in greater detail.[42, 57] In most studies based on IR spectroscopy, there are detailed conclusions based on the vibrations of CH_2 and CH_3 groups in hydrocarbon chains and of amide groups,[57] which correspond to a few segments of the lipid and protein molecules. Other experimental techniques are more sensitive to the minor fluid SC components. ESR studies have provided indirect information on fluid components in complex SC mixtures based on the partitioning of probes.[22] Still this method only reports on a fraction where the probe partitions. Deuterium NMR studies on model lipid systems have provided resolved molecular information on the coexisting solid and fluid lipid fractions[50, 58-59] for the specific segments that are deuterated.

In summary, previous molecular characterization of the fluidity in SC lipid and protein components in intact SC is not yet complete. It is therefore highly desirable to develop methods that allow for detecting and characterizing the minor amount of fluid components in the highly ordered SC solid sample under near-native conditions, without using probes or selective labelling. It is of particular relevance to characterize how the fluid fraction changes in response to the external conditions or additives. PT ssNMR is a very powerful technique to characterize mobility of ^{13}C segments with resolved molecular information on both the coexisting solid and fluid fractions in intact SC.[23] In this PhD project, we demonstrated the applicability of this NMR tool to skin research, and we believe that the use of this technique together with well-characterized systems and conditions can significantly advance molecular understanding on lipid and SC systems.

4 Approaches and considerations

4.1 Samples: from model systems to pig and human skin

Different model systems, including model lipid mixtures as well as porcine and human SC, were studied in this thesis. The studies using model lipid mixtures were designed to investigate molecular effects of different additives in different hydration conditions. The model mixtures are generally not chosen as mimics of SC lipids. We used simple systems composed of phopsholipids: DMPC (1,2-dimyristoyl-sn-glycero-3-phosphocholine) and POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine) (papers I and II). These are not skin lipids. The model systems were chosen as well-characterized lipid systems that are relevant to biological membranes. DMPC undergoes a phase transition between solid and liquid crystalline lamellar phases in accessible hydration and temperature intervals,[60-61] which is similar to the observed phase changes in SC lipids at increasing hydration and temperature.[23] Still the extrapolation to SC must be carried out carefully. We also used more complex model systems, composed of mixture of fatty acids, ceramides and cholesterol, which are close approximation to the *in situ* SC lipid composition (paper VII).[13] The use of model mixtures allows for studies of the lipid components without interference from overlapping signal from the corneocyte components. It also makes it possible to vary the lipid composition in systematic ways, which is an advantage if one want to examine the role of individual lipid components.

Porcine skin has frequently been used in experimental studies because of its availability in large quantities and its similarity to human skin.[62-64] There are still some differences in chemical composition and structure between human and porcine SC.[65] For example, the orthorhombic chain packing is more common in human SC than in porcine SC.[17] We have used porcine SC (papers III-VI), as well as extracted corneocytes from porcine skin (papers III and VI). Previous studies on extraction of SC lipids have shown complications related to contamination and degradation of the lipids,[23, 66] and we therefore did not focus on extracted lipids in the present studies.

4.2 Experimental set-up

In most parts of this thesis we focus on understanding equilibrium systems of a complex and heterogeneous sample of intact SC under controlled conditions. In these studies, we do not mimic practical situations of complex and non-equilibrium SC systems with non-uniform distribution of water and other diffusing species. In papers I and IV, hydrophobic additives were uniformly mixed with SC/lipids and water. With this approach, we aim to study the effect of added compounds with no interference from other solvents.

When choosing experimental conditions for studies at different hydration conditions, one can choose to add a known amount of water to SC, or to equilibrate SC samples at controlled RH/water activity. Controlling the water activity is relevant to many practical situations. In cases when one or more components in the system are volatile, it is not possible to equilibrate samples in controlled RH without also loosing the evaporating component. This complication was considered in the studies of volatile monoterpenes in SC and model systems (papers I and IV).

The equilibration of solid phases, which make up the major fraction of SC, is usually much slower than the equilibration of fluid phases, which are the parts of the sample where molecular transport mainly occurs. One should therefore be aware of potential complications related to metastability when working with solid phases.[67-68] On the other hand, too long equilibration times may cause problems with degradation of the biological SC sample. In this thesis, samples were equilibrated for 1-2 days. For some cases, experiments were performed on the same sample at different days to confirm that one has reached a stable state, representing close to equilibrium conditions. In some studies in this thesis, intact SC was made into a fine powder to make the equilibration processes faster. Previous studies have shown that there are no detectable differences in the measured properties of molecular mobility between samples of sheets or powder from intact SC.[23]

4.3 PT ssNMR: a molecular mobility approach

In this section, PT ssNMR method is briefly described together with some highlight and considerations based on a basic NMR user's understanding.

4.3.1 PT ssNMR method

Natural abundance ^{13}C solid-state NMR with ^{1}H→^{13}C polarization transfer using CP (cross polarization)[69] and INEPT (insensitive nuclei enhanced by polarization transfer)[70] (Fig. 4.1A) equipped with high-power proton decoupling and magic-angle spinning (MAS) was employed to obtain atomically resolved qualitative information on molecular dynamics in model lipid systems (papers I, II and VII) and the intact SC (papers III-VI). DP (direct polarization) spectrum was also recorded together and acts as a reference. The acronym PT ssNMR has been previously introduced to denote the DP-CP-INEPT set of NMR measurements for brevity.[71] A theoretical model for calculating the CP and INEPT intensities as a function of rotational correlation time τ_c and C–H bond order parameter S_{CH} was developed by Nowacka et al.[2] (Fig. 4.1B). τ_c is a measure of the rate of the C-H bond reorientation and $|S_{CH}|$ is the measure of the anisotropy of the C-H bond reorientation. $|S_{CH}|$ ranges from 1 for ordered and rigid segments to 0 for segments with isotropic reorientation. Example spectra for DMPC-water binary systems are shown in Fig. 4.2. The ^{13}C spectrum is the sum of resonance lines from magnetically inequivalent ^{13}C sites, each of which being characterized by different factors: chemical shift, lineshape and signal intensity.
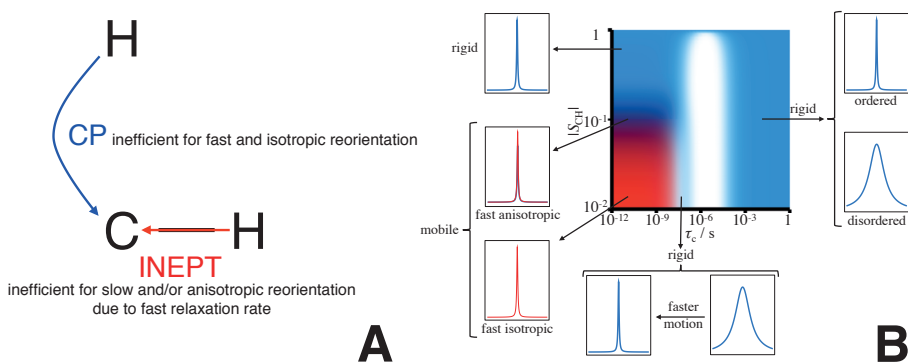
Fig. 4.1 (A) Illustration of ^{1}H→^{13}C polarization transfer through-space (blue arrow, CP) and through-bond (red arrow, INEPT). (B) Theoretical CP (blue) and INEPT (red) signal enhancement in PT ssNMR experiment as a function of τ_c and $|S_{CH}|$ for a CH$_2$ segment at 11.72 T magnetic field and 5 kHz MAS (adapted from Nowacka et al.[2]). White indicates the absence of signal for both CP and INEPT. Corresponding lineshapes and intensities of CP (blue) and INEPT (red) signals in different regimes are also shown.
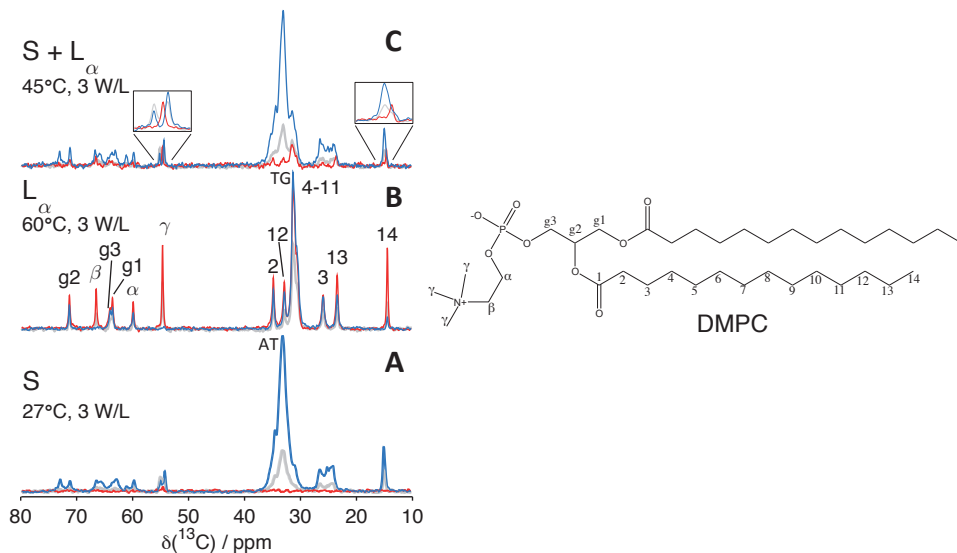
Fig. 4.2 ^{13}C MAS NMR spectra (DP: gray; CP: blue; INEPT: red) of DMPC at different temperatures and water contents corresponding to different phases. (A) S (solid crystalline phase), (B) L_α (lamellar liquid crystalline phase), and (C) coexistence of S and L_α phases. Chemical structure of DMPC with numbered segments is also shown. The insets in (C) show the magnification of the spectral region of Cγ and C14.

As seen in Fig. 4.2, different molecular segments can be distinguished in the spectra as they resonate at different chemical shifts. Even the same molecular segment may resonate at different chemical shifts depending on molecular conformation and dynamics. This fact can refer to solids where exchange between the various conformations is slower than the chemical shift time scale. The majority of the lipid acyl-chain $(CH_2)_n$ resonates within a range of 30 to 34 ppm, and this resonance is usually used as a fingerprint to distinguish solid and fluid phases since the AT (all-trans signifying solid acyl-chains) and TG (trans-gauche, a sign of mobile disordered acyl-chains) conformations resonate at 33 and 30 ppm, respectively.[72] This difference in the chemical shifts of $(CH_2)_n$ has been extensively used through all the studies to detect the fluid lipid fraction. Another example is the end segment of the hydrophobic tail, which has different chemical shifts in the solid and fluid lipid phases (see Fig. 4.2).

The value of the observed chemical shift represents an average over all the molecular conformations sampled on the relevant NMR time scale, typically milliseconds. The spreading of the chemical shift of each resonance affects its lineshape. This spreading can be due to the timescale of molecular reorientation[73-74] and/or a range of molecular conformations and microenvironments with different chemical shifts[75] (Fig. 4.1B). Under the experimental conditions used in all papers, the line narrowing effect is expected to be observed at $\tau_c \sim 0.1$ µs and τ_c

16

~ 0.1 ms, the latter is only observed in an rigid ordered system (Fig. 4.1B, more details in paper VI). An example is the CP signal of Cγ nuclei of DMPC in the solid phase at 54.5 ppm appears as a well-defined doublet in the CP spectra corresponding to two distinct conformations of Cγ methyl groups with slow motion (paper I) (Fig. 4.2A, C) rather than a broad distribution for a rigid disordered segment (Fig. 4.1B).

The ^{13}C signal intensities obtained with the CP and INEPT polarization transfer schemes yield further information on molecular dynamics (Fig. 4.1B). Polarization transfer from the neighboring ^{1}H can be considered as a filter and an amplifier for the chemically identical segments on different molecules with different mobilities. The selective signal enhancement is related to how the magnetization is transferred from ^{1}H nuclei to neighboring ^{13}C and the anisotropy and dynamics of a carbon segment (Fig. 4.1). The basis is to compare the signal intensities acquired from DP, CP and INEPT.

The differences between DP, CP and INEPT can be described as follows:

• The DP spectrum generally shows resonances from all carbons in the sample and acts as a reference. Under the experimental setup in this thesis, the DP signal was previously shown to be quantitative only if it is accompanied by INEPT, i.e., for fast isotropic and fast anisotropic motions.[2]

• For CP the polarization is transferred from ^{1}H→^{13}C by using heteronuclear through-space dipolar couplings[69] (Fig. 4.1A) which are averaged to zero by fast isotropic reorientation ($|S_{CH}| < 0.01$ and $\tau_c < 10$ ns) and therefore CP is efficient in boosting the signal of segments with slow (with $\tau_c > 0.1$ ms) and/or anisotropic motions.

• In the INEPT experiments, on the other hand, there is through-bond polarization transfer[70] (Fig. 4.1A), and INEPT yields signal enhancement as long as the ^{1}H and ^{13}C transverse relaxation times are longer than the time required for ^{1}H-^{13}C polarization transfer (typically a few milliseconds). Therefore INEPT will yield no signal for rigid molecules with slow motion ($\tau_c > 0.1$ μs) and/or highly anisotropic reorientation ($|S_{CH}| > 0.5$) due to fast relaxation rate resulted from non-averaged ^{1}H-^{1}H and ^{1}H-^{13}C dipolar interactions. On the other hand, the signal from mobile segments ($\tau_c < 10$ ns) is selectively enhanced in INEPT spectra.

It is also noted that around $\tau_c = 1$ μs neither CP nor INEPT yield signal on account of fast relaxation (white region in Fig. 4.1B).[2] To facilitate the presentation of the data in the present thesis and included papers, we here define "mobile" and "rigid" segments according to the rate and anisotropy of C-H bond reorientation as shown in Fig. 4.1B. The INEPT signal indicates a "mobile" segment, which can be "fast isotropic" or "fast anisotropic". In the latter case, the INEPT signal is accompanied by a sharp CP signal having the same line shape and chemical shift, thus showing that these peaks originate from molecular segments experiencing anisotropic reorientation. The term "rigid" is defined by the conditions when only CP signal is detected and there is no INEPT signal.

4.3.2 PT ssNMR on SC

Despite the complexity of the NMR spectra from intact SC, essentially all of the resonances from lipid and protein components were assigned in a previous study[23] (Fig. 4.3A) through comparisons with isolated SC components, model systems and databases. Using the combination of experiments in PT ssNMR, it is thus possible to distinguish molecular mobility in acyl-chains, ceramide headgroups and cholesterol. Furthermore, molecular mobility in SC proteins in the keratin filaments can be resolved, and we distinguish the amino acids that are enriched in the terminal domains (rich in serine and glycine) or in the core (rich in leucine and lysine) within the keratin filaments (UniProt ID P04264 and P13645).[35]
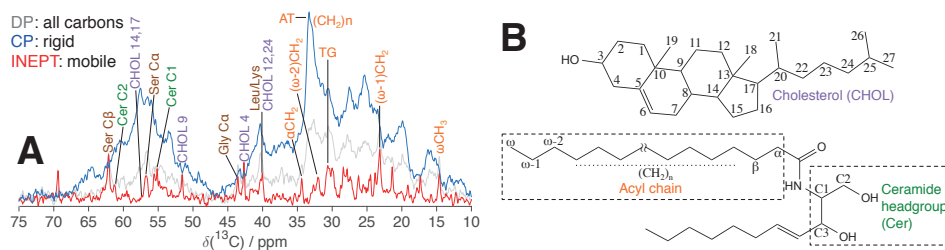
Fig. 4.3 (A) ^{13}C MAS NMR spectra (DP: gray; CP: blue; INEPT: red) of SC with 40 wt% water at 32 °C. (B) Chemical structures with numbered segments of cholesterol and relevant lipid carbons (here illustrated with ceramide non-hydroxy sphingosine) are also shown.

Fig. 4.3A shows PT ssNMR spectra for SC with 40 wt% water to illustrate the information obtained from such experiments. A first observation is that the majority of SC components are rigid, as implied from the dominance of the CP signal for most of the spectral range. The main contribution to the CP signals comes from the keratin filaments as they are the main component of SC.[13] Moreover, the rigid Cα resonances from all amino acid residues (except glycine Cα) are observed as the broad and dominating CP peaks around 57 ppm. On the other hand, the rigid lipids with AT conformation are probed at the sharp and most prominent CP $(CH_2)_n$ AT peak at 33.4 ppm.[72] Still there is a minor co-existing fraction of fluid SC components, which can be seen in the INEPT signal of both lipid and protein segments.

In a rigid disordered system like SC, a segment with $\tau_c > 0.1$ ms would give rise to a broad signal (Fig. 4.1B) since the motion is not fast enough to average the resonance frequencies.[75] Therefore the line narrowing effect due to faster motion in these systems is expected to be observed only at $\tau_c \sim 0.1$ μs and this has been

18

taken advantage to monitor the changes in motion of solvent molecules present in SC systems (paper VI).

4.3.3 Advantages of PT ssNMR in studies of intact SC

The PT ssNMR measurements are sensitive to small changes in the minor fluid fraction of SC lipids and proteins in the rigid SC material. This is possible as the fluid fraction is detected separately in the INEPT spectrum. Similar sensitivity and molecular resolution for fluid phases are more difficult to reach with, e.g., X-ray, diffraction, and IR techniques. Simultaneously with the detection of fluid components, one can obtain information on the major fraction of solid SC components. In addition, based on different "detectors" and "filters" including CP and INEPT intensities, chemical shift and lineshape, one can detect different fractions of the same molecular components. Finally, it is a non-invasive method on natural abundance ^{13}C sample, and it does not rely on partitioning of labelled probes.

4.3.4 Additional remarks on the PT ssNMR method

The PT ssNMR method can determine whether the sample is solid or liquid crystalline, but it cannot be used to distinguish between different anisotropic liquid crystalline structures. The latter can be done by other complementary measurements for example scattering/diffraction. In addition, this NMR method can provide detailed information about the molecular consequences of adding foreign compounds to SC, while it is not possible to pinpoint the location of the added compound or draw any conclusions about direct contacts between the added compound and, for example, certain lipid species. It is also noted that neither CP nor INEPT yields truly quantitative data. Comparison between the areas of CP and INEPT peaks gives qualitative information on the solid and fluid fractions, and growth of one signal at the expense of the other indicates a transformation between these fractions. When comparing the changes in signal intensities in different samples, one therefore needs a good internal reference. More detailed discussion about the reference in studies of SC samples can be found in paper IV.

In a lamellar phase, a segment with a preference for orientations close to the magic-angle 54.74° with respect to the bilayer normal will have $|S_{CH}|$ close to zero, although it may not experience isotropic reorientation. An example is C-H bond of Cg1 segment of the glycerol backbone carbons of DMPC in paper I. Different segments of the same lipid molecule can have quite different values of order parameter and dynamics. As shown in paper I, the segment in the end of the lipid

chain has lower order parameter and faster motion than the one in the beginning of the tail.

The anisotropy obtained from PT ssNMR is orientational anisotropy, which is different from translational/diffusion anisotropy. Nowacka et al.[76] reported a fast isotropic reorientation of glycerol that is present in the aqueous gap between lipid bilayers, while glycerol is expected to have anisotropic translational motion/diffusion as water. The orientational order in this case can be affected by, e.g., anisotropic interactions and thickness of the aqueous gap.

4.4 Calorimetry

Calorimetry is a method where the change in heat (calori-) is measured (-metry). This method can provide both thermodynamic and kinetic data and is useful for a quick scan of a system at different conditions, for example, temperature and concentration, depending on the type of calorimeter. One common application of calorimetry in colloidal science is to construct phase diagrams. DSC can detect thermal transitions in the system when changing the temperature. On the other hand, sorption calorimetry[60, 77] - a type of isothermal calorimetry - monitors changes in the system during water uptake at a constant temperature. The latter conditions are relevant for systems that experience a range of low water contents, like SC. The sorption microcalorimetry method used in this thesis allows for simultaneous measurement of partial molar enthalpy of sorption, and chemical potential of water as a function of water content,[60] thus providing an almost complete thermodynamic description of the hydration process.

It is noted that calorimetry only pinpoints endothermic/exothermic events in the system, while it does not provide the molecular origin or further characteristics of this event. Consequently complementary techniques are needed to fully characterize the changes. In addition, an event that involves no or small heat effects, and changes that occur over a broad range in temperature or water content, will not be easily detected. As an example, DSC could not detect the transition from L_β to P_β of DMPC-thymol-water system in paper I.

5 Learning from model lipid systems

Lipid phase transition can be induced by changes in the external conditions, for example, temperature, hydration, and osmotic pressure.[7] One common way to affect the fluidity of lipid at ambient temperature that is employed both in nature and in technical applications is to add small compounds that alter the phase equilibria. The added compounds can be, for example, osmolytes, natural moisturizing factor (NMF), humectants, emollients, or penetration enhancers.[55-56, 78-79] In papers I-III, we study the effects of different small molecules, including both polar and apolar molecules, on phospholipids with a focus on detailed molecular characterization of the effects in dehydrated conditions. This was achieved by combining several complementary techniques, including PT ssNMR (papers I-III), calorimetry (papers I-III), X-ray scattering (papers I-II), and molecular dynamics simulations (paper II). One main conclusion from these studies is that the addition of small compounds can alter the phase equilibrium in lipid systems by stabilizing more fluid phases, although the molecular mechanisms differ between apolar and polar additives. The insights from studies of mechanisms in model systems can be very useful to deepen the understanding of the same mechanisms also in more complex systems, for example, the SC.

5.1. How small polar molecules affect lipid self-assembly in dehydrated conditions

When small polar molecules are added to phospholipid lamellar phase, they are mainly present in the aqueous layers in between the bilayers (Fig. 5.1). This is expected on grounds of solubility, and in our studies in papers II-III, it is inferred from the fast isotropic reorientation of carbon segments of these molecules, as observed from PT ssNMR (papers II-III), and from the density profiles in the simulations (paper II).

One of the prerequisites for a polar additive to affect lipid phase behaviour is that this compound dissolves in the self-assembled lipid phases. This becomes quite important in systems with low water content and mainly solid phases. The partitioning of the added compound into the bilayer system depends on the interaction between the added compound and the lipid components. The solubility limit can be shifted towards lower RH if dissolution is more favoured in the lipid-containing phase compared to the neat water solution, as demonstrated for urea (paper II) and PCA (paper III). On the other hand, if the added compound is repelled from the lipid bilayer interface, the added compound does not partition into the lipid phase at low water content. This behavior was shown for TMAO in paper II. It was shown that, as TMAO is repelled from the lipid bilayer interfaces, it only partitions into fluid lipid systems when the water gap is large enough to avoid close contact between TMAO dissolved in the water phase in the gap and the lipid headgroups.

Being present in the aqueous layers, the added small polar molecules increase the amount of polar components (water + polar molecules) in the system. It may also substitute for water in lipid bilayer systems under dehydration. In this way, small polar molecules with low vapor pressure can retain the lipid fluidity at dehydrated conditions, which otherwise would lead to solid lipid phases (papers II-III).[76, 80] This was illustrated in previous studies of the DMPC-urea-water systems[80] showing that the lipid phase behavior is basically determined by the amount (volume) of polar solvents, irrespective of the composition of that solution. In this thesis, we further characterized the effects of urea on the phase transition by means of sorption microcalorimetry and simulations (paper II).

Adding polar compounds to a lipid system may affect the bilayer fluidity, and it is also expected to influence the properties of the aqueous regions in the lamellar system. Fluid lipid bilayer phases generally have stronger ability to swell in water than their solid counterparts (paper II). The swelling of the fluid lipid lamellar phase in the presence of urea or TMAO were extended to thermodynamic characterization in paper II. In the same study, it was also concluded that the addition of the solutes to the lipid-water system also leads to a broadening of the two-phase regions.[5] In the two-phase coexistence region one can envision the

formation of segregated domains with different composition and structures,[81] which will also have impact on the membrane properties in terms of partitioning and permeabilty of added compounds.[82]
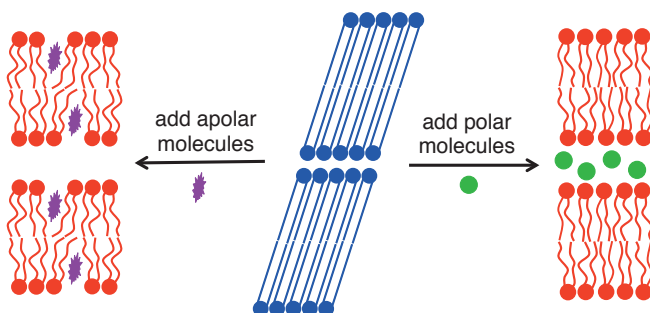

Fig. 5.1 Schematic illustration of the effects of adding polar and apolar molecules to solid bilayers. Blue represents rigid segments (as seen from the (blue) CP spectra), while red represents mobile segments (as seen from the (red) INEPT spectra).

5.2 Melting point depression by adding apolar molecules

Melting point depression caused by the addition of small amounts of apolar compounds or impurities to solid bilayer systems has been well-characterized in the literature.[31, 83] The molecular basis for this phenomenom is the higher solubility of the apolar contaminant in the fluid bilayers compared to the solid bilayers (Fig. 5.1).[5] This effect is also observed upon adding monoterpenes to different DMPC solid phases (paper I). The effect is much stronger at lower hydration conditions.[5] We also demonstrated a concentration dependence of monoterpenes in lipid systems, i.e., the addition of the first mole fractions of monoterpenes has a very strong effect on the melting transition, while further addition of monoterpenes has less effect counted per added molecule. The same behavior was not observed for polar compounds in DMPC (paper II and [76, 80]), which rather showed gradual changes in melting point depression with increasing concentration.

Thymol was shown to dissolve in the lipid phase at temperatures much lower than the melting temperature of pure thymol (paper I). Another associating effect is broadening of the two-phase coexistence region in a concentration dependent manner (paper I). It is finally noted that while monoterpenes fluidize the solid DMPC lipid bilayers, their presence in a fluid DMPC lipid bilayer has an ordering effect on the lipid acyl-chains. Similar but more pronounced ordering effect was previously also shown for, e.g., cholesterol and benzyl alcohol.[31, 84-88]

5.3 Ripple phase - an intriguing structure

The ripple P_β gel phase is an intriguing structure with its undulations and well-defined interlamellar distance, ripple periodicity and the tilt angle (Fig. 5.2A) which change with temperature.[89] At the molecular level, the chains of the lipid molecules in this phase are solid, while the headgroups are mobile (Fig. 5.2B) (paper I). The molecular understanding of the formation of this phase is still not complete, although several models have been proposed suggesting that the P_β phase is a consequence of the imbalance between the expansive forces associated with the hydration of the headgroups, and the cohesive forces due to the chain-chain interactions.[90-91] The ripples may also result from periodic changes in the local spontaneous curvature in the bilayer.[92-94] Although the P_β phase formed in the binary lipid-water system is thermodynamically one phase, it is obviously heterogeneous in terms of different lipid molecules, i.e., the lipids along the curvature of the rippled bilayer have different interactions depending on their positions. Previous experimental and simulation studies have suggested that this phase can be compared to the situation of nano-scale domain coexistence of gel-like and fluid lipids.[95-99] The P_β phase is generally not observed upon contamination of the bilayer with some molecules[100-103] including urea,[80] while this phase is still formed in the presence of cholesterol and monoterpenes with a modification of interlamellar distance and ripple period.(unpublished data and [88, 104-105])
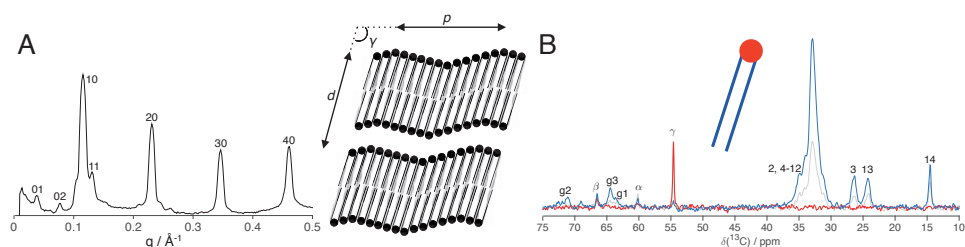
Fig. 5.2 SAXS scattering pattern (A) and ^{13}C MAS NMR spectra (DP: gray; CP: blue; INEPT: red) (B) of DMPC ripple phase at 13 W/L and 18 °C. Schematic illustration of the ripple structure (A) and the mobility in the lipid molecules (B) are also shown. Blue represents rigid segments (as seen from the (blue) CP spectra), while red represents mobile segments (as seen from the (red) INEPT spectra). The SAXS peaks are indexed according to the reciprocal lattices calculated from the lamellar repeat distance (d), the ripple period (p) and the angle (γ) between d and p.[68]

6 Controlling fluidity in SC lipids - effects of hydration and additives

6.1 Changing SC lipid phase behavior by adding foreign molecules

Similar to the model lipid systems, SC lipid phase behavior can be altered by changes in hydration and temperature. This response in SC lipid self-assembly has been extensively studied using different techniques, including IR, diffraction, calorimetry, and spectroscopy methods.[17, 21, 106] In a previous NMR study, Björklund et al.[23] characterized the molecular mobility of different segments in SC lipids. It was shown that heating has stronger effect on melting SC lipids than increasing hydration at constant temperature. Considering that the SC is mainly composed of lipids with long and saturated chains,[13, 107] it is expected that it is difficult to melt these lipids by adding water at ambient temperatures. In order to melt SC lipids at ambient temperatures, one can therefore add other molecules, including NMF components,[108-109] humectants, emollients, or penetration enhancers.[55-56] Many of these additives have been extensively studied in SC by a variety of techniques due to their practical relevance in pharmaceutical and cosmetic formulations. Several of these compounds have been shown to have the ability to increase permeability of the skin.[110] Still, the molecular characterization of how these different compounds affect the fluidity of SC lipids in the intact SC

is rather incomplete. In papers III-VI we study the effects of different molecules on intact SC using PT ssNMR. With this experimental approach, it is possible to reach novel molecular insight into how various classes of additives influence the balance between mobile and rigid components in the very same sample of intact SC.

6.1.1 Addition of small polar and apolar compounds can alter SC lipid phase behavior

A general conclusion from studies of many different compounds in this thesis (papers III-VI) is that both apolar and polar compounds can melt SC lipids, provided that they are dissolved in the SC matrix (Fig. 6.1). This is very similar to the observations of melting of lipids in model lipid systems upon addition of apolar and polar compounds, as described in sections 5.1 and 5.2. The extracellular SC matrix is very hydrophobic, containing long chain lipids and very low water content.[3] Apolar compounds partition more readily into the extracellular lipid regions compared to the water-rich regions inside the corneocytes. These compounds may therefore mainly influence the properties of the SC lipids, for example, inducing mobility in certain lipid segments (papers IV and VI). Small polar compounds are expected to partition into the polar domains in the SC, which is mainly the keratin-filled corneocytes. However, there are also the narrow aqueous layers within the extracellular lipid matrix where the polar compounds can dissolve and thereby influence the properties of the SC lipids. The most obvious example of this is found for water, which has been shown to induce mobility and lower melting point temperatures both in SC lipids and in SC model lipid systems.[20, 23, 27] The partition of apolar compounds into the hydrophobic region makes it likely that these compounds have stronger effect on the acyl-chain packing compared to polar compounds (paper IV). Also a few amphiphilic molecules were investigated in this thesis (paper IV), and the strongest effect of these compounds was observed on the extracellular lipids (paper IV and [111]).

In analogue to the model lipid systems (paper I), the dissolution of thymol in SC leads to fluidizing of SC lipids, and this is observed at temperatures much lower than the melting points of pure thymol (paper IV).

Fig. 6.1 Schematic illustration of the effects of adding polar and apolar molecules to SC lipids. Blue represents rigid segments (as seen from the (blue) CP spectra), while red represents mobile segments (as seen from the (red) INEPT spectra).

6.1.2 Combined effects of hydration and additive on SC lipids

A general conclusion from several studies in this thesis is that the molecular consequences of adding foreign compounds to SC strongly depend on degree of SC hydration. This is also highly relevant to many practical applications as the skin is generally present at temperatures around 32 °C, and varying RH. It was shown that the relative increase in mobility of SC lipids upon the addition of foreign compounds is more pronounced at dehydrated conditions compared to at high water contents (paper IV). At high water content the addition of urea and glycerol has only minor influence on the mobility of the SC lipid acyl-chains compared to SC at the same water content. The suggested mechanism is that the polar compounds basically act to replace water (paper III),[80] and the additional effect upon adding these compounds is minor when the polar fraction is already high. The change in hydration would also lead to a change in relative amount of water in the SC lipid and protein domains,[19] which is expected to affect the partitioning of the added compounds in these SC domains.

Another general observation is that a higher number of different molecular segments of the SC lipids are affected by the addition of various foreign compounds at higher water content (papers III, IV and VI). In the hydrated conditions, mobile lipids are already present in neat SC without added compounds,[23] and the addition of foreign molecules can more easily dissolve the molecules in the fluid regions to further shift the balance between fluid and solid phases. There may be also other effects of hydration on, for example, the ionization of fatty acids and pH.[40-41]

When a compound is added to lipid systems with a given concentration of water, which is common in many *in vitro* studies (including papers IV and VI), one can draw conclusions about the systems with a certain concentration. However, the molecular mechanisms for observed changes may be due to combined effects of the interactions with the added compound and the effects of reduction of the water activity caused by adding this compound to the formulation of study. This complication is most relevant for the cases when the added

27

compound has high water solubility and thereby stronger ability to reduce the water activity at increasing concentration. In neat SC without added compounds, the reduction in water activity leads to increased rigidity of the SC lipids.[23] In paper IV, urea and glycerol were added to SC and compared to reference SC samples at the same water content. To be able to distinguish the effects of water activity and the added compounds, we also performed studies (papers III and V) where effect of a certain compound was investigated at a fixed water activity, as controlled by RH or the water activity in an excess aqueous solution. In paper V, SC was soaked in solutions of PBS with added transcutol or dexpanthenol (a_w = 0.936-0.986) and compared to SC soaked in neat PBS solution with similar water activity (a_w= 0.992). In paper III, urea and glycerol were added to intact SC equilibrated at RH = 80% and compared to SC equilibrated at the same RH. The main conclusion from papers III and V is that these polar compounds can act to maintain the fluidity of SC lipid chains at dehydrated conditions. The compounds with low vapor pressure remain in the SC membrane system also at reduced RH when water evaporates. The suggested mechanism is that these compounds replace water at dehydrated conditions rather than soaking more water into the membrane. These findings have strong practical relevance to skin formulations and they provide molecular explanations for the effects of humectants and NMF compounds in SC.

6.1.3 Concentration dependence of the effect of additive on SC lipids

In paper VI, different types of solvents were added to dry SC at varying concentrations. For several solvents investigated, the fluid SC lipid fraction increases with increasing solvent concentration. Simultaneous to this, we also observe a change in the molecular dynamics of the solvent molecules themselves, indicating changes in the solvent-SC interactions. A common characteristic for most of the solvents investigated is that the first solvent molecules added are strongly affected when incorporated into SC. At higher solvent concentrations, SC appears "saturated" with tightly associated solvent; and above this limit, additional solvent molecules are present in an isotropic microenviroment. Still, the addition of solvent may lead to further increase in the fluidity in SC lipids also above the saturation limit, as shown in paper VI. At very high concentration of solvents, there is an additional effect that lipids may be extracted from the SC sample.[112] This effect seems to be more pronounced for the fluid lipid fractions than the solid ones (paper VI).

6.2 Detailed characterization of fluid SC lipids

The fluid SC lipids apparently play a crucial role in SC structures, properties and functions.[22, 51-52] This section is devoted to the fluid SC lipid fraction based on our observations on intact SC in papers III-VI and SC model lipid mixtures in paper VII.

A main observation in all PT ssNMR spectra of intact SC (papers III-VI and [23]) and SC model lipids (paper VII) is that none of the INEPT peaks from mobile SC lipids are accompanied by a CP peaks. An example is shown in Fig. 4.3A. These observations imply that the reorientation of C-H bonds is fast isotropic (Fig. 4.1B), corresponding to the fluid lipids being located in a nearly isotropic microenvironment. An anisotropic liquid crystalline phase would give rise to INEPT and CP resonances with identical line shapes and comparable amplitudes (Fig. 4.1B),[71] for example the L_α phase in Fig. 4.2B. Our observation of fast isotropic reorientation is also in agreement with previous studies using ^{1}H NMR on extracted SC lipids[19] and ^{2}H NMR on model mixtures of SC lipids.[50, 58-59]

In order to further characterize the isotropic fluid lipids, we investigated composition, and molecular dynamics of the fluid lipid fractions in SC model lipid mixtures composed of ceramides, cholesterol and fatty acids (paper VII). An important finding is the essential role of ceramide EOS linoleate (Fig. 3.1), which induces the isotropic fluid lipid phase at ambient temperatures. The fluid phase was not observed in the reference SC model lipid mixtures without the ceramide EOS linoleate. These observations can also be related to the presence of the fluid lipid phase in intact SC at 32 °C even at very low water contents (papers IV and VI).[23] The similarity between the PT ssNMR spectra for the ceramide EOS linoleate-containing sample and lipids in the intact SC shows that the model lipid mixture that contains ceramide EOS linoleate indeed matches the property of the SC lipids quite well.

The composition of the fluid isotropic phase in the model lipid sample that contains ceramide EOS linoleate was further characterized to consist of segments in the middle and the terminal of the acyl-chains and segments of the conjugated C=C double bonds of the ceramide EOS linoleate (paper VII). On the other hand, the mobility in the chain segments closest to the lipid interface (αCH_2 and βCH_2) and ceramide headgroups are not observed. In previous studies, it has also been shown that ceramide EOS is needed for the formation of the long periodicity phase in SC lipids.[42, 113] From these data one can envision an isotropic domain consisting of dangling linoleate tails embedded and anchored in the long periodicity phase of the solid lamellar structure. This picture is in line with previous neutron diffraction studies, suggesting that the linoleate chain can wiggle around and also partly fold back.[114]

Cholesterol is a main component of the extracellular SC lipids. It is therefore reasonable to ask whether a liquid-ordered phase similar to what is seen in other lipid systems, including fatty acids and phospholipids,[31-32, 87, 115] is also present in SC lipids. The NMR data from all different SC systems and SC model lipids throughout this thesis (for example, Fig. 4.3A) show no signature of the liquid-ordered lamellar phase, which would give rise to high and sharp CP signal and low or no INEPT signal for the lipid acyl-chain carbon resonances.[84] In addition, ceramide has been shown to displace cholesterol from ordered lipid domains in model lipid system composed of phospholipid-cholesterol-ceramide,[116-117] while we observed a simultaneous increase in mobility of ceramide and cholesterol in most cases (papers IV-VI). This might indicate ceramide and cholesterol are present in the same phase.

7 Influencing keratin filaments

7.1 Solid core and responding terminals as revealed from PT ssNMR

Based on the resolution of different amino acids in the ^{13}C spectra (Fig. 4.3A), PT ssNMR can be used to simultaneously track the mobility of amino acids in the N- and C-terminal domains and the coiled-coil cores of the keratin filaments. One general conclusion is that the majority of SC amino acid segments are rigid in all conditions investigated with the present NMR method ([23, 29] and papers III-VI). This is clear from the large and broad CP resonances in the protein Cα region from all amino acid residues (except glycince Cα) centred around 57 ppm (Fig. 4.3A). In particular, the core of the keratin filaments remains rigid. In some conditions, as will be described in more detail below, the terminal domains may undergo a transition to become more mobile.
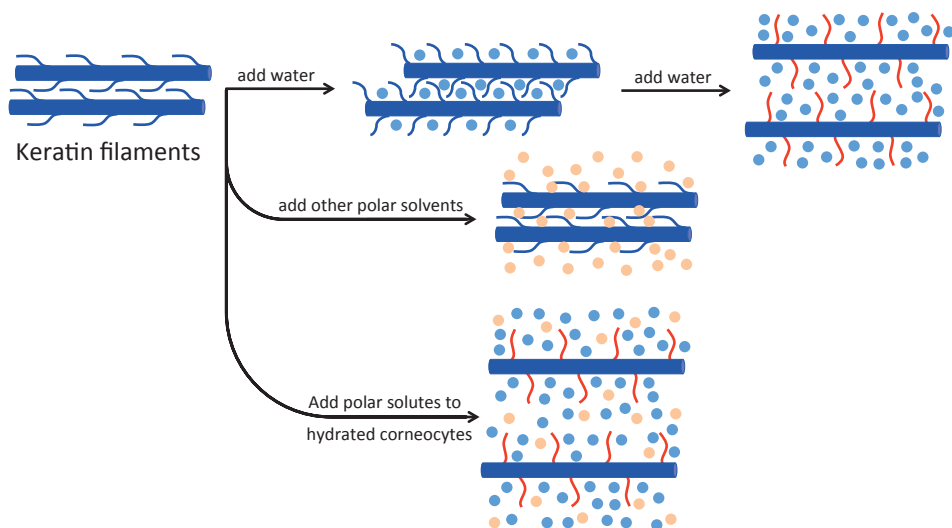
Fig. 7.1 Schematic interpretations of the effects of added compounds on the keratin filaments. Blue represents rigid segments (as seen from the (blue) CP spectra), while red represents mobile segments (as seen from the (red) INEPT spectra).

7.2 Water is critical for SC protein mobility

At ambient temperature the mobility in the terminal segments of the keratin filaments is only detected in the presence of relatively high amounts of water (paper VI) (Fig. 7.1), except for SC samples at high temperature (> 60 °C) where mobility was also seen for dry SC samples.[23] In paper VI, it was shown that the addition of any of the other polar solvents investigated does not show any effect on SC protein mobility (Fig. 7.1). This indicates that water is an essential solvent for SC protein mobility. Still, the experimental data clearly suggest that the polar solvents are associated with the corneocytes, as inferred from reduced molecular mobility of solvent molecules also in samples only consisting of isolated corneocytes (paper VI).

The special effect of water on mobility in the terminal segments may be explained by that water is a good solvent for the hydrophilic amino acids in the terminals (papers III-VI). It is also noticed that although there is a gradual uptake of water in the corneocytes,[19] the induced mobility effect of water is only detected above a certain relative humidity (ca. 80% RH, which corresponds to ca. 24 wt% water[23]) (Fig. 7.1). This indicates that a certain volume of water is required for the side-chains of the polypeptide backbones in the terminal domains to be free to explore isotropic reorientations.

32

7.3 How different additives affect hydrated keratin filaments

The protruding terminal chains of keratin filament are rich in hydrophilic amino acids, for example, serine and glycine. The interiors of the corneocytes are therefore seen as relatively polar regions of SC that can take up substantial amounts of water.[20] The expected partitioning of added compounds between the lipids and the corneocytes is based on their solubility in intracellular regions of the corneocytes compared to the extracellular apolar lipid regions. Throughout the studies on different additives (papers III-VI), it was concluded that the addition of hydrophobic compounds have very minor or no effects on the SC proteins, while hydrophilic compounds have strong effects on the mobility in the terminal amino acids in cases when water is also present. The few amphiphilic compounds investigated showed little or no effect on the amino acids in the keratin filaments.

When polar compounds are present in the SC, the transition between mobile and rigid protein structures occurs at lower RH or water activity compared to the neat SC sample with no additives. This is comparable to the effects of the same compounds on SC lipids (papers III-VI). In other words, the molecular mobility in the SC treated with these compounds can remain similar to the hydrated SC even at dehydrated conditions. This might be highly relevant to the evaluation of the effect caused by skin creams or formulations that have low water activity due to water-soluble excipients. It is not necessary so that it is more efficient to use high concentrations of these polar compounds in a "moisturizing" formulation. Adding more solute will also decrease the water activity, and there is always a competition between the effects of the added polar compound and the effect of dehydration (papers III-V).

In papers IV and VI, it was shown that the effects caused by polar compounds on SC protein mobility strongly depend on the SC hydration conditions. When propylene glycol and glycerol are added to dry SC, the motion of these molecules appears slower compared to the neat solutions, implying strong interaction with SC components. No mobility in the protein segments was detected in any of these cases (paper VI) (Fig. 7.1). However, when the same compounds were added to hydrated SC (> 20 wt% water), the addition of these molecules caused increased molecular mobility of SC proteins (papers III, IV and VI) (Fig. 7.1). It is here an interesting finding that propylene glycol has stronger effect on inducing mobility in the amino acids in the terminal domains (paper VI) compared to all other solvents or solutes investigated in papers III-VI.

Urea is known to weaken the hydrophobic interactions and it is commonly used for protein denaturation.[118] However, the addition of urea does not lead to solubilization or disruption of the solid core of the protein keratin filaments or the phase behavior in the extracellular lipids at concentrations used in the present

studies. This is concluded from the observations that there are no major changes in the protein mobility of the filament cores (papers III and IV) upon the addition of urea. It is an interesting observation that the mobility in the keratin protein side chains is very similar in SC samples with urea at ambient RH (80%) and almost fully hydrated SC in the absence of urea (paper III). Urea is naturally present in SC at high concentration as a part of the NMF.[119] The mixture of NMF also comprises sugars and histidine,[119] which were previously shown to have stabilizing effect on folded protein structures.[120-121] It is possible that nature takes advantage of the use of a mix of small polar compounds in the NMF mixture to both protect SC lipids and proteins from osmotic stress caused by dehydration, and to avoid protein denaturing caused by NMF components like urea. Similar combined effect of urea and TMAO is also discussed in paper II.

7.4 Swelling of corneocyte

The corneocytes have been shown to swell substantially in water, mainly in the vertical dimension.[20, 122] The keratin filaments with mobile terminal chains can be seen as solid rods covered by brushes (Fig. 7.1). The swelling of corneocyte is determined by the repulsion between the keratin rods, which is expected to be more long-ranged when the terminal brushes are mobile due to long-range steric entropic repulsive forces.[5] From comparison between NMR studies of SC protein molecular mobility at different RH,[23] and sorption isotherms of intact SC and isolated corneocytes,[19] it is concluded that the onset of the mobility in the terminals (around RH=80%) coincides with the RH region where there is a strong increase in water uptake. The swelling of corneocytes in water has been correlated with SC mechanical properties[22] that are important to, for example, tolerance of physical stress, and SC is non-elastic and brittle in the dry state.[123] These characteristics are likely influenced by addition of other compounds that affect SC protein mobility and interaction forces between the solid rods.

It has previously been shown that the ability of corneocytes to take up water decreases with increasing amounts of ethanol, and that the uptake of neat ethanol is negligible compared to that of neat water.[124] These observations are in agreement with the fact that ethanol is not a good solvent for keratin filament and has no effect on the molecular mobility in any protein segment (paper VI). Polar compounds such as urea and glycerol, on the other hand, may cause increased swelling of the corneocytes at slightly dehydrated conditions (paper III). It was shown that when these compounds are added to SC, mobility in the terminal segments was retained also at lower RH compared to the neat SC. One can therefore expect long-ranged repulsion,[125] as compared to the neat SC at the same

RH. The corneocytes are in these cases filled with aqueous solution that contains also the polar solute (paper III).

8 Coupling between SC barrier function and molecular changes in SC components

One essential aspect in the development of skin formulations is to understand the mechanisms behind different excipients in different solution conditions. This understanding can be deepened by combining permeation studies with molecular studies of self-assembly of the SC lipid and protein components in the conditions of interest. The barrier function of the skin has been widely studied in many different applications, e.g., (trans)dermal drug delivery and pharmaceutical and cosmetic formulations. This has often been accomplished by using diffusion cell systems to study molecular transport across the skin membrane (examples in references [30, 51], papers III-IV) (Fig. 8.1), or by studying transepidermal water loss (TEWL) *in vivo* or *in vitro*.[126]
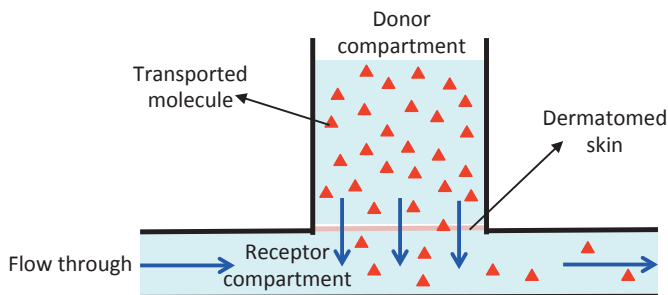
Fig. 8.1 Schematic of the diffusion cell set-up employed for permeation studies.

Diffusional transport of a molecule *i* across SC is driven by the gradient in chemical potential $\Delta\mu_i$ (or activity a_i as $\Delta\mu_i = -RT\ln a_i$) of the diffusing molecule. In the simplest case, the ability of the transported molecule to cross SC can be characterized by permeability constant, P_i (compare Eq. 2.2). The flux of molecule *i*, J_i, is then calculated as

$$J_i = P_i \Delta a_i \tag{7.1}$$

The effective SC permeability to a certain molecule depends on the solubility and diffusion coefficient of the transported molecule (Eq. 2.2) in different regions of SC, and the interaction of these molecules with SC components.

8.1 Coupling of molecular mobility and macroscopic permeability

The mobility or fluidity of the molecular components in a membrane may be related to the macroscopic permeability of the membrane. The permeability of solid membranes is generally much lower compared to fluid membranes.[53-54] This difference can be attributed to higher solubility and diffusivity of the diffusing compounds in the fluid structures. The correlation between fluidity and permeability has been illustrated in the literature and in the present thesis by the combination of data obtained from, e.g., PT ssNMR and flow-through diffusion cells studies (examples in references [23, 30, 51] and papers III-V). In most cases it is difficult to make quantitative comparisons between molecular characterization studies and diffusion-cell studies as the experiments are generally performed in different conditions. For example, the molecular characterization studies in this thesis are done in conditions that are close to equilibrium, while the transport studies are done in non-equilibrium conditions with different boundary conditions on each side of the membrane. Still, qualitative trends can be revealed from

comparisons. Previous studies by Björklund et al.[51] demonstrated that changes of a gradient in water activity across the skin can be used as a switch to regulate the skin permeability to different model drugs in a reversible manner. This effect was later correlated to reversible structural and dynamical changes of the SC lipid or protein components upon changes in water activity.[23] It was found that increased SC hydration leads to higher mobility in the non-aqueous SC molecular components, associated with increased solubility and lowered diffusional resistance to external chemicals.[9, 13, 23, 51] Correlation between fluidity and permeability was also observed in SC after the addition of other compounds than water. The combination of molecular and transport studies (paper III and reference[30]) have shown that urea and glycerol can retain the molecular mobility in SC components at reduced hydration conditions. It was also shown that these compounds can retain high permeability of skin membranes to a model drug at dehydrating conditions,[30] which otherwise would decrease the permeability due to dehydration.[51] In paper V, the same mechanism was proposed for skin membranes treated with transcutol and dexpanthenol, resulting in a retained and high skin permeability correlated with a maintained molecular mobility of SC at dehydrating conditions. Clear correlation between permeability and mobility in SC components was also shown for hydrophobic and amphiphilic compounds in paper IV. We confirmed increased skin permeability for monoterpenes and dodecanoic acid that cause increased mobility in SC components. On the other hand, no changes in skin permeability were shown for stearic acid, which was also shown to have very minor effects on the SC molecular dynamics.

In paper IV, thymol shows much stronger penetration enhancement compared to all other compounds investigated. This may be related to that thymol has very strong effect on the mobility in SC lipids, and in particular in the lipid acyl-chains. On the other hand, if the added compound only has the effect to induce mobility in the polar headgroups, the expected effect on the overall permeability is less prominent as the main resistance to the flux lies in the rigid hydrocarbon chains.

8.2. Transport routes

One crucial factor for predicting SC permeability is to consider different possible transport routes. This will depend on the arrangement of different solid and fluid regions in SC, the nature of the transported molecules, and the effects of the transported molecules on the SC self-assembly. Based on the brick and mortar structure of SC, the transepidermal transport may follow either the intracellular (transcellular) or the tortuous extracellular routes (Fig. 8.2), of which the latter is the only continuous pathway.[10] With this view, any small molecule passing the skin barrier must be transported through the extracellular matrix. In the SC

extracellular lipid matrix there is furthermore a coexistence of solid and fluid domains,[127] and transport preferentially occurs in the fluid domains that have lower diffusional resistance.[53-54]

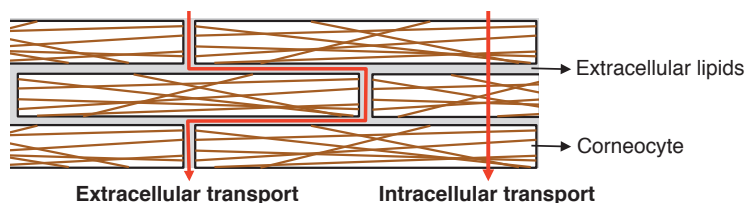**Extracellular transport**   **Intracellular transport**

Fig. 8.2 Schematic illustration of transepidermal transport routes.

The hydrophobicity of the transported molecule is crucial in determining the partitioning between lipid and corneocyte regions in SC, and consequently the transport routes in the skin. Hydrophilic compounds are expected to pass both lipid and corneocyte domains, while hydrophobic compounds are expected to avoid the polar regions of corneocytes and only travel through the continuous lipid matrix. Unlike the SC lipids, the corneocytes can take up substantial amounts of water,[20] indicating the partitioning of water into the corneocytes. Therefore the corneocytes should be considered as a relevant transport route for the added compounds, depending on their partition coefficients.
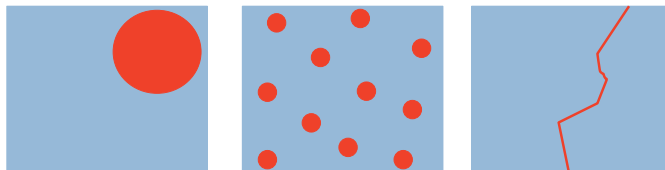

Fig. 8.3 Schematic illustration of different arrangements of the fluid fraction (red) in the solid material (blue).

The arrangement of the segregated fluid and solid domains in SC is crucial to determine the diffusional pathways (Fig. 8.3). The pathway and overall permeability would be very different for macroscopic and microscopic segregation of fluid and solid domains. Previous studies have demonstrated anomalous high permeability in segregated bilayer membranes, and this has been attributed to the domain interfaces.[53] From the present PT ssNMR measurement we only see changes in the balance between fluid and solid domains, and we cannot draw any conclusions about their internal arrangements. Previous studies have shown reversibility in SC molecular mobility with temperature scans (32–60 °C),[23] which indicates that the fluid domains are integrated in the solid structure rather than segregated to a separated macroscopic phase. Extraction of certain components in SC due to additives, for example, solvents in excess solution (paper VI) may lead

40

to defects in the structure, and/or re-assembly of remaining components to other structures. This will also influence transport routes and overall permeability.

8.3 Impact on drug delivery and skin formulations

In the evaluation of the effects of skin formulations, it is crucial to distinguish the effects caused by individual components, for example, solvents (paper VI), hydration/water activity and other components (papers I-VI) as well as the types of interactions in complex formulations, for example non-interactive, additive, synergistic and antagonistic interactions.[128-129] Considering a compound as a chemical penetration enhancers should not be based solely on its hydrophobicity and partition coefficients.[130] A key aspect to the penetration enhancement efficiency is the phase changes promoted by the added compounds when incorporated into the SC at a certain condition, e.g., concentration and hydration. When applying a formulation on the skin, the composition in the formulation will also change due to evaporation and skin absorption, and skin hydration will depend on the occluding properties of the film of cream as well the environment humidity.[19, 36]

The partitioning of the added compound between lipid and protein regions in SC depends on its hydrophobicity. This is highly relevant in relation to targeting effect on certain SC components. Adding compounds that induce fluidity in SC lipids (including polar, apolar and amphiphilic compounds) are expected to increase SC permeability to both hydrophilic and hydrophobic active (small) compounds. For hydrophilic drugs, additional penetration enhancement might be achieved by adding excipients that also induce mobility in the protein components, like polar compounds.

9 Skin diseases that affect molecular mobility in SC components

In papers III-VI, we demonstrated that PT ssNMR is a powerful tool to study molecular mobility in the complex system of SC with molecular resolution. One interesting route for the future is to take advantage of this tool to also study molecular mobility in diseased skin. There are many skin diseases including psoriasis, eczema, lamellar ichthyosis and netherton that have been shown to correlate with changes in SC composition and organization as well as phase behavior, particularly SC lipids.[28, 131] Pharmaceutical formulations have been developed for the treatment of these diseases, while they are often not able to cure the diseases. With the presented PT ssNMR tool, it is possible to characterize changes in molecular material properties (or the fluid protein and lipid fractions) in diseased SC and how it responds to changes in, e.g., hydration or treatment with certain compounds, providing new insights on possible treatments of the diseased skin.
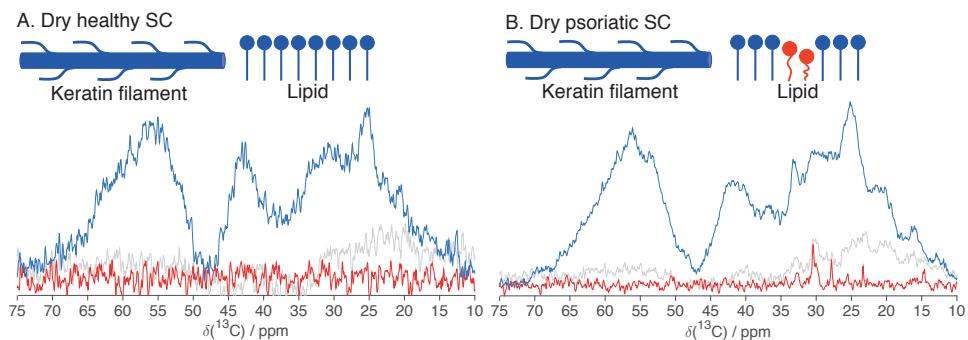
Fig. 9.1 ^{13}C MAS NMR spectra (DP:grey, CP: blue, INEPT: red) of dry healthy SC (A) and dry psoriatic SC (B) at 32 °C obtained from human heel. Schematic illustrations of the molecular dynamics of SC lipids and proteins are also shown.

PT ssNMR has been applied on psoriatic skin, and preliminary data are shown in Fig. 9.1. Reduced levels of ceramide EOS[132-133] and filaggrin[134] have been proposed for psoriasis. Ceramide EOS is important for the formation of the long periodicity phase in SC lipids,[42, 113] and ceramide EOS linoleate was shown to induce fluidity in SC model lipid mixture at ambient temperature (paper VII). Filaggrin influences keratin filament formation. The reduction in ceramide EOS and filaggrin likely influences SC material and molecular properties. Preliminary results obtained in collaboration with the Department of Clinical Sciences, Lund University indeed showed the presence of fluid lipids in dry psoriatic SC obtained from human heel and elbow, while the fluid lipids were not detected in dry healthy SC (Fig. 9.1). The samples of psoriatic and healthy SC were collected after removal the top SC layers by tape-stripping. In this way, the sebacuous lipids were removed, and the observed fluid lipids in Fig. 9.1 could be confirmed to be present inside psoriatic SC. In addition, triglycerol, a main component of sebacuous lipids,[135] is not detected in the NMR spectra. The high fraction of fluid lipids in psoriatic SC can be related to changes in psoriatic SC lipid organization reported by van Smeden et al.,[131] which may partly caused by the change in the levels of Cer EOS[133] and are relevant to the defective barrier function.[132] The dry state of psoriatic skin and its low water-holding capacity[136] can probably be related to the defective corneocyte. This aspect will be further studied in detail in relation to hydration.

10 Future prospects

The PT ssNMR method has proven to be a useful tool to study molecular mobility of both SC components and additives. This method can be used to further study the effects of other additives or physical treatment such as solar UV radiation on skin. It also opens up a new way to study diseased skin, e.g., psoriasis, eczema, lamellar ichthyosis and netherton, and to evaluate new treatments based on recovering similar mobility of SC components as in healthy SC, and consequently similar macroscopic properties of the skin in terms of barrier function (section 8), water-holding capacity, elasticity, etc.[22]

There are still many unsolved questions in the fields of skin research that are waiting to be explored. The organization of the SC lipids have been suggested according to different models, including domain mosaic,[137] sandwich,[138] and single gel phase[139] models. In papers III-VII, we showed the existence of the fluid isotropic lipids in intact SC, and in paper VII we characterized this fluid fraction in terms of composition in SC model lipid mixtures. Unresolved questions for the future include: How are the fluid lipid fractions arranged together with the solid lipid domains in the SC lipid matrix? How is the diffusivity of the fluid components in SC? How does the arrangement of fluid and solid domains vary with hydration and added molecules, and what is the impact of this on SC barrier function?

The corneocytes can swell substantially upon hydration but mainly in the vertical dimension.[20, 122] This opens up for questions around the differences in organization and interactions between the keratin filaments in the same horizontal

plane or vertical plane. How is the response of the cornified envelope to the shrinking and fully swelling of the cornecoyte? Does the cornified envelope have very high elasticity? Is the swelling limit set by the interactions between the keratin filaments or by the cornified envelope? Some studies have suggested a change between α-helix and β-sheet structures in the keratin filament core,[140-142] but it is not clear how this will affect the larger-scale morphology filament arrangements in different conditions.

To be able to answer these questions, one need to take a multi-technique approach using both intact SC and model systems and the combined information to build up new knowledge around this self-assembled complex system that is the SC. NMR methods can provide information at a molecular level on fluid components (PT ssNMR), self-diffusion and size of the domains (NMR diffusion). Scattering and diffraction are useful to characterize SC organization. NMR imaging and different microscopic methods with high resolution can also be essential tools to solve some of these questions.

The studies on SC at different conditions and in the presence of additives can be forwarded to more detail. One can get quantitative information on the additive, for example, order parameter (by deuterium NMR, for instance) and reorientational correlation time (as shown in paper I). The present PT ssNMR data showed the consequence of adding foreign compounds to the skin. One can use nuclear overhauser effect (NOE) in NMR or develop new methods to study the location of the additives in the skin at a molecular scale to forward the understanding. Based on the knowledge of individual additive on SC, further studies on more complicated systems with different additives can proceed.

There are also other remaining questions related to the studies on the lipid model systems. In paper II, we showed the effects of urea and TMAO separately on lipid systems. One obvious question based on this study concerns the combined effect of these compounds on lipids and proteins in model systems and in complex biological systems.

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