Stratum corneum model membranes : molecular organization in relation to skin barrier function

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Chapter 1

Introduction

1. The skin barrier function

The natural function of the skin is to act as a barrier against unwanted influences from the environment. The skin consists of three layers, from the superficial to the innermost layer: the epidermis, the dermis and the hypodermis (subcutaneous fat tissue) (1), see figure 1. The epidermis also consists of various layers, of which the stratum corneum (SC) is the uppermost nonviable layer. This very thin layer is only 15 to 20 µm thick and acts as the main barrier against permeation of substances (2, 3).

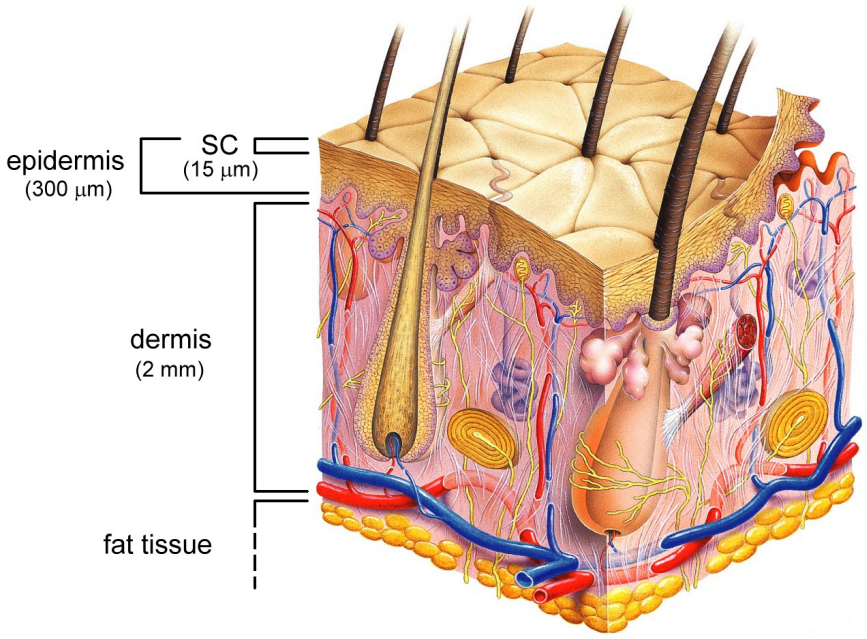

Figure 1: Schematic overview of the different tissue layers in the skin. This figure is adapted from Benjamin Cummings, Pearson Education Inc. copyright 2009.

The excellent barrier properties of the SC can be ascribed to its unique structure and composition. The SC is generated by the underlying viable epidermis. As the structure of the SC is very different from that of the viable epidermis, many events occur during SC formation within a very short time period. The viable epidermis is densely populated with keratinocytes that are generated in its basal layer. During the formation of the SC, keratinocytes move into the direction of the skin surface. When passing the viable epidermis-SC interface, the cells transform into dead flattened cells (corneocytes). Simultaneously, the cornified envelope, a densely cross linked protein layer surrounding the corneocytes, is formed by sequential deposition of various proteins. Subsequently, a monolayer of non-polar lipids is esterified to the cornified envelope. Besides the formation of the corneocytes, an intercellular lipid matrix is formed, composed of non-polar lipids.

1.1 The structure of the SC

As described above, the SC consists of corneocytes (dead flattened cells) surrounded by the highly impermeable cornified envelope. The corneocytes are embedded in a lipid matrix as “bricks in mortar”, see figure 2. The lipids in the intercellular regions form crystalline lipid lamellae. The corneocytes and the lipid lamellae are oriented approximately parallel to the skin surface. As the lipid lamellae form a continuous pathway for diffusion of substances across the SC, the lipid domains are considered to play a dominant role in the skin barrier function (12). Furthermore, the orientation of the lamellae as well as the lipid organization is suggested to contribute largely to the excellent barrier function of the SC.

1.1.1 Permeation pathway through the SC

Although hair follicles, sweat glands and sebaceous glands are potential routes of compound penetration, the total surface covered by these appendages is only around 0.1%. For this reason compounds applied onto

the skin are considered to penetrate primarily via the transepidermal route into the deeper regions of the skin. During this penetration process, compounds may follow the intercellular or transcellular pathway, depicted in figure 2.

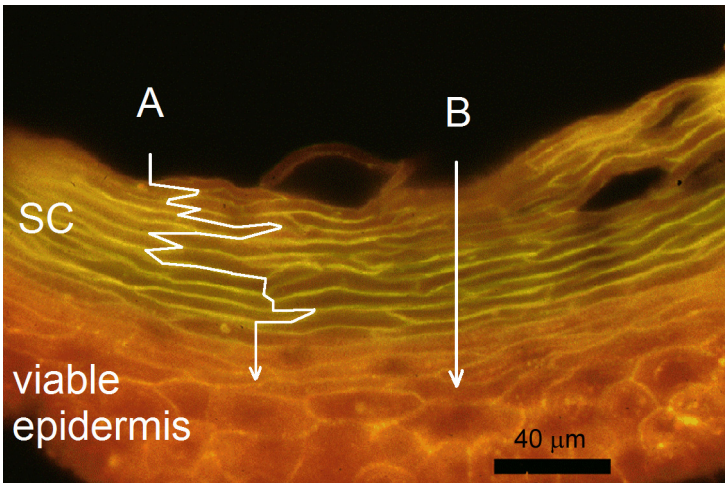

Figure 2: Fluorescence image of Nile red-stained human SC, presented in (9). Depicted are two possible transepidermal penetration pathways: The intercellular route (A) only involves transport along the lipid lamellae, whereas the transcellular route (B) directly crosses the corneocytes and intervening lipids. This figure is reprinted by permission from the American Association of Pharmaceutical Scientists, copyright 2001.

Because of the highly impermeable character of the cornified envelope, the tortuous intercellular pathway has been suggested to be the preferred route for most drug molecules (13). Although this is still a subject of debate, several studies have indeed reported transport mainly along the intercellular space in the SC (14-16). Moreover, it has been demonstrated that drug permeation across the SC increases many folds after lipid extraction (17), again demonstrating an important function of the lipids for a proper skin barrier. Hence, knowledge of the structure and biophysical properties of the intercellular lipid matrix is crucial for understanding the skin barrier function.

1.1.2 Intercellular lipid composition and organization in the SC

The lipid matrix in the intercellular region is mainly composed of ceramides (CER), cholesterol (CHOL) and free fatty acids (FFA), in an approximately equimolar ratio (18-22). However, there is a high inter-individual variability in this lipid composition (23). The CER consist of two long saturated hydrocarbon chains and a small polar headgroup. Each of the twelve CER subclasses identified in human SC contains a sphingoid base and a fatty acid, which are linked by an amide bond between the carboxyl group of the fatty acid and the amino group of the base (18, 21, 22, 24). The sphingoid moiety can be sphingosine (S), phytosphingosine (P), 6-hydroxysphingosine (H) or dihydrosphingosine (dS), whereas the fatty acid moiety is non-hydroxylated (N) or α-hydroxylated (A) with chain lengths of predominantly 24 to 26 carbon atoms. The most remarkable CER are the acylceramides. These CER consist of an unusual long ω-hydroxy fatty acid of 30 to 34 carbon atoms to which an unsaturated linoleic acid is ester-linked (EO). In figure 3 the molecular structure of the main CER subclasses in human SC are presented. The FFA fraction in SC mainly consists of saturated hydrocarbon chains with the prevalent chain lengths being C22 and C24 (25).

In 1987 the use of ruthenium tetroxide as a post-fixation agent made it possible to visualize the unique lamellar arrangement of the intercellular lipids in an electron microscope (7, 26). Multiple lamellae, consisting of a broad-narrow-broad sequence of electron lucent bands were observed demonstrating an unusual arrangement, see figure 4. A few years later small-angle X-ray diffraction (SAXD) measurements on human, pig and mouse SC revealed the presence of a long periodicity phase (LPP) with an approximately 13 nm repeat distance (27-32). Besides the LPP, another lamellar phase was observed using X-ray diffraction. The periodicity of this phase is approximately 6 nm and it is therefore referred to as the short periodicity phase (SPP). In addition to the lamellar phases (LPP and SPP),

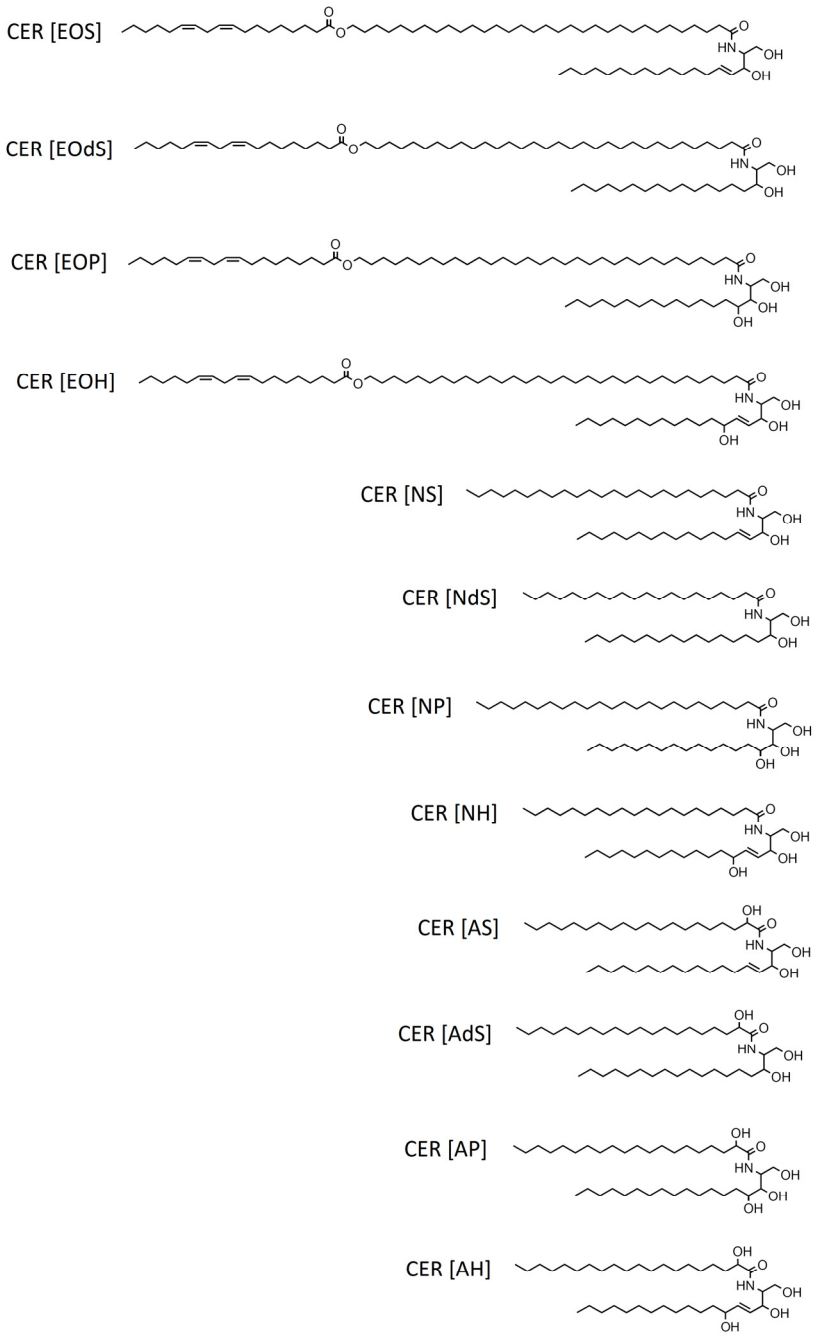

Figure 3: Molecular structure of the CER present in human SC.

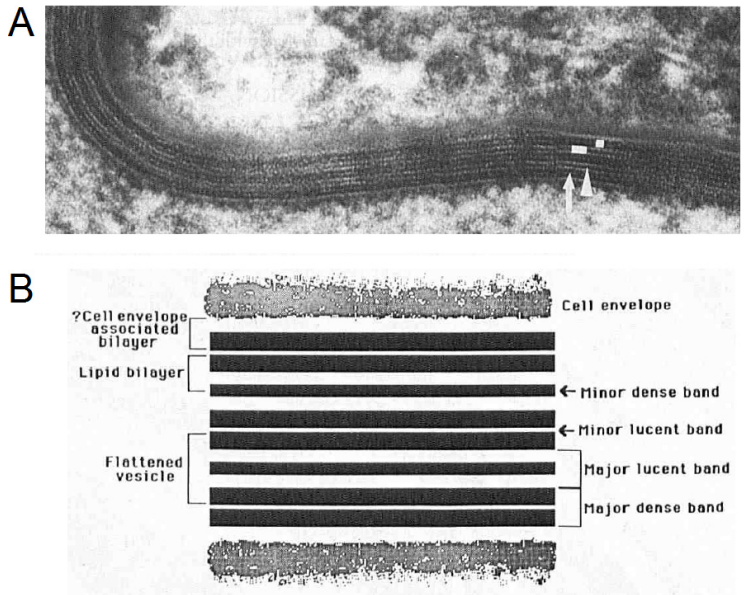

Figure 4: A) Electron micrograph of lamellar lipid structures in the intercellular space of the outer SC of neonatal mouse skin B) Schematic diagram of the intercellular space in figure A. Both figures are from (7), reprinted by permission from MacMillan Publishers Ltd: JID, copyright 1987.

the presence of phase separated crystalline CHOL is also observed in SC (33, 34). In subsequent studies using model lipid mixtures it was found that the presence of CER EOS (see figure 3), is a prerequisite for the formation of the LPP (35-37). Although over the years, a lot of information has been gathered on the SC lipid organization and the role the various lipid classes play in this organization (26, 33, 35, 37), until now the detailed molecular structure of the LPP is not known. In previous studies, several attempts have been made to determine an electron density profile of the LPP, using SAXD. White et al. performed the first calculations using a block shaped electron density profile (27). Our group performed electron density calculations in which the electron density profiles were simulated by Gaussian curves (28, 29). However, both studies suffered from the fact that no swelling of the

lamellae could be induced and therefore no unique electron density profile could be determined. More recently, McIntosh used a mixture of isolated pig CER, CHOL, and palmitic acid and performed X-ray diffraction studies (38). The results indicated a repeating unit consisting of two layers. However, due to the low resolution of the electron density profile the lipid organization in the unit cell could not be unraveled.

Concerning the molecular structure of the SPP, in several other studies progress has been made on the fundamental interactions between the lipid classes using simplified ternary or quaternary lipid mixtures. The mixtures included mainly CER AP with a short acyl chain length of 18 carbon atoms, CHOL and cholesterol sulfate. In these studies the neutron scattering length density profile was determined of CER rich phases with a short periodicity (39-41). However, the phases formed in these mixtures all contain repeat distances much smaller than observed for the SPP in SC. Because the model mixtures described above do not closely resemble the composition in SC, the molecular structure of these CER rich phases might be different from the structure of the SPP (42, 43).

Besides the lamellar organization, the packing of the lipids within these lamellae is also of importance for the barrier function of human skin. The packing density decreases in the order orthorhombic>hexagonal>liquid, see figure 5. Wide-angle X-ray diffraction studies revealed that, at a skin temperature of around 32°C, the lipids in human SC are predominantly forming an orthorhombic packing, although a subpopulation of lipids form a coexisting hexagonal packing (44, 45). It is almost impossible using X-ray diffraction to determine whether a liquid phase coexists with the crystalline phases, as its broad reflection in the diffraction pattern is obscured by the reflections attributed to keratin present in the interior of corneocytes.

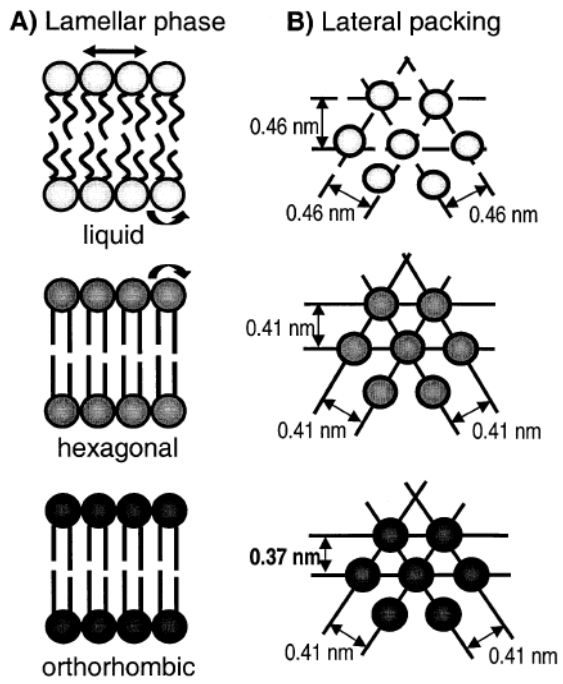

Figure 5: Schematic overview of the types of lateral lipid packing A) Side view showing the lamellae B) Top view showing the lateral packing of the hydrocarbon chains. This figure is from (11), reprinted by permission from MacMillan Publishers Ltd: JID, copyright 2001.

2. Lipid organization in SC of diseased skin

Diseased skin generally displays defects in the SC structure which may lead to a reduced skin barrier function. For example, in lamellar ichthyosis (LI) patients the activity of one of the enzymes involved in the formation of the densely packed cell envelope is reduced, most probably rendering a more permeable cornified envelope (46, 47). Not only enzymes involved in the synthesis of protein structures in SC are impaired in diseased skin. In several skin diseases the activity of enzymes involved in the synthesis of the lipids may be altered as well. For example, in type 2 Gaucher's disease

patients, the level of glucocerebrosidase is strongly reduced, resulting in a strong increase in the ratio of glucosylceramides/CER, leading to an altered lipid organization and a subsequent reduction in the skin barrier function (48-50). This demonstrates that the CER are very important for a proper barrier function. In LI skin, in addition to small changes in CER composition, the level of FFA is strongly reduced compared to that observed in healthy SC (51). Furthermore, SAXD studies with SC of LI skin revealed an altered lamellar organization, as the diffraction peaks in the scattering profile were located at higher scattering angle revealing smaller spacings than in SC of healthy skin (51). Freeze fracture electron microscopy and electron diffraction techniques have also been used to study the lipid organization in LI patients (11). The results showed that the lamellae in LI skin exhibit strong undulations compared to normal skin, confirming an altered lamellar organization. Another example is atopic eczema (AE), which is frequently observed in children especially in the industrialized countries. There is increasing evidence that the impaired skin barrier function is causative for AE. A defect barrier facilitates the transport of allergens and irritants into the skin resulting in skin inflammation. As this disease is a major problem for the western society, many research groups devoted on the skin barrier are now focusing their research on AE. However, there is only limited information about the lipid composition and organization in SC of AE skin. It has been reported that in the SC of AE patients the enzymes sphingomyelin deacylase ceramidase and glucosylceramide deacylase are increased in activity, resulting in a decreased CER level (52-60). Although the CER composition and the ratio between the FFA, CHOL and CER are reported, almost no information is available on the changes in the SC lipid organization in AE patients. In 2001 the lipid organization in three AE patients was investigated with freeze fracture electron microscopy and electron diffraction (ED) in a limited number of patients (11). In these patients the frequency of the hexagonal lateral packing was significantly increased compared to that in normal skin. In the 1980s, the lipid organization in essential fatty acid

deficient (EFAD) SC has also been elucidated (26, 61). It appeared that elimination of linoleic acid from the diet of pigs resulted in a progressive increase in the oleate content in CER EOS at the expense of the linoleate content. This increase in CER EOS-oleate content was accompanied by a strong reduction in the skin barrier. This change in lipid composition is of interest to study, as in normal skin the CER EOS-oleate/CER EOS-linoleate ratio increases dramatically during the winter season and is also observed to be one of the characteristics of cosmetically dry skin (62, 63).

Furthermore, in psoriasis skin the keratinization process is deranged and the barrier function decreased (64). Analysis of CER from the psoriatic scale, compared to those from normal human SC, revealed a reduction in the CER EOS levels and in the CER containing the phytosphingosine base (65, 66). Recessive X-linked ichthyosis skin is also characterized by an impaired skin barrier function (67). As far as the lipid composition is concerned, a strongly increased level of cholesterol sulphate has been reported which also accounts for the pathological scaling (67-69). In previous studies it was shown that an increase in cholesterol sulphate level resulted in a change in the lipid organization. However, whether this change in lipid organization is responsible for the skin barrier impairment is not yet known.

As shown above, diseased skin often parallels with an altered SC lipid composition, which may lead to a change in the lipid organization. This in turn may be an important factor for the impaired skin barrier function. In order to understand the effects of an aberrant lipid organization in dry or diseased skin, information on the relation between lipid composition, lipid organization and barrier function is crucial. However, this is difficult to obtain with diseased human skin, as this is almost not available and in addition SC has a very complex structure. This makes it difficult to delineate the link between lipid organization and lipid composition in diseased human skin. Furthermore, as it is impossible to modulate systematically the lipid composition in SC, the use of lipid membranes in which the lipid composition can be varied on demand, offers an attractive alternative.

3. SC lipid models

3.1 Ternary and quaternary lipid mixtures

These mixtures generally incorporate one or two individual CER, a fatty acid and CHOL. As the focus is usually on only one CER class, the advantage of these simple systems is that the interpretations of the lipid organization are more detailed than in multi-component systems. Therefore, these mixtures are well suited to investigate fundamental interactions between different lipid classes (70-73). For example, It has been shown that, regardless of the type of lateral packing, hydrogen bonds between headgroups play an important role in the order-disorder transition temperature (70).

However, the limited chain length variation in the ternary and quaternary systems renders them more crystalline and due to a mismatch between the CER and FFA chain lengths the systems often exhibit phase separation (41, 74, 75). In human SC up to 12 CER classes and multiple FFA are present exhibiting a broad variation in chain lengths. This variation in chain length and headgroup architecture increases the ability to form solid mixtures and reduces the formation of separate phases (76). Another difference between single component CER mixtures and the more complex mixtures is the inability to form the LPP in the former. Because of the high crystalline character, the absence of the LPP and the tendency towards phase separation, the ternary and quaternary mixtures are less suited as models to mimic the lipid phase behaviour in SC.

3.2 Multi component lipid mixtures

Before the synthetic CER classes became available, models mimicking the SC lipid composition were prepared using CER isolated from native SC. In 2001 we observed for the first time that mixtures prepared from CHOL and isolated human CER form two lamellar phases with periodicities of 5.4 and 12.8 nm, closely mimicking the lipid phase behavior in human SC (36). In

these mixtures, however, the lipids form a hexagonal lateral packing independent of the CER:CHOL ratio. With these mixtures, studies were also performed focusing on the role of CER EOS. Phase behavior studies with equimolar CHOL:CER mixtures lacking CER EOS revealed that the LPP was only weakly present, indicating that CER EOS plays a prominent role in the formation of the LPP (35, 77).

Furthermore, studies with lipid mixtures containing CHOL, CER and FFA have also been performed. To mimic the FFA composition in SC, a FFA mixture containing predominantly long chain FFA (C22 and C24) has been used. In the presence of FFA the formation of the SPP was promoted and two lamellar phases were formed with periodicities of 13.0 and 5.5 nm, mimicking even more closely the lipid organization in intact SC. Furthermore, the addition of long chain FFA induced a phase transition from a hexagonal to an orthorhombic lattice and therefore increased the lipid density in the structure (78).

In contrast to the above studies in which solely mixtures prepared with CER isolated from native tissue were used, in more recent studies the lipid organization in mixtures prepared with various synthetic CER with a defined acyl chain length was also investigated (79). The lipid organization in equimolar mixtures of CHOL, synthetic CER and FFA closely resembled that in SC, as both LPP (12.2 nm) and SPP (5.4 nm) were present and the lateral packing of the lipids was orthorhombic. Furthermore, CHOL was also observed in phase separated crystalline domains, similarly as in SC. No additional phases could be detected. Interestingly, only in the presence of FFA a dominant formation of the LPP was observed. This observation is different from that made with mixtures prepared with natural CER. This difference in phase behavior might be related to the limited acyl chain length variation in the synthetic CER mixtures.

The above studies demonstrate that mixtures with CHOL, FFA and synthetic CER can offer an attractive tool to unravel the importance of individual CER for a proper SC lipid organization. Up to now, only the relation between lipid

composition and organization was discussed. However, it is also of importance to correlate the lipid composition and organization to the barrier function. Therefore, to examine whether mixtures applied on an appropriate substrate could be used to replace native SC in permeation studies, a SC substitute (SCS) was developed (43). The barrier properties of the SCS were evaluated in a series of in vitro passive diffusion studies, using three structurally related compounds; p-amino benzoic acid (PABA), ethyl-PABA and butyl-PABA (80). Of these 3 model drugs PABA is the most hydrophilic compound and the lipophilicity increases with increasing ester chain length. The diffusion profiles of all 3 model compounds across 12 µm thick lipid membranes closely resembled those of human SC. Furthermore, exclusion of CER EOS from the lipid mixture revealed a reduced barrier function of the SCS, demonstrating that CER EOS is not only very important for the proper skin lipid phase behavior, but also for the skin barrier function.

Although a lot of information has been gathered on the SC lipid organization and the role the various lipid classes play in this organization, a detailed molecular structure of the LPP and SPP has not yet been presented. More knowledge on these molecular structures can be gained by performing additional X-ray and neutron diffraction studies using mixtures with CER, CHOL and FFA.

3.3 Molecular models for the SC lipid organization

In literature, several molecular models for the lipid organization in SC have been proposed: The stacked monolayer model (8), the domain mosaic model (4), the single gel phase model (6) and the sandwich model (10, 37). The stacked monolayer model presented in 1989 describes the molecular arrangement in the LPP for the first time. Based on the broad-narrow-broad pattern obtained after ruthenium tetroxide fixation of pig skin, a trilayer model was proposed. In this model the CER are arranged in a planar arrangement and the linoleic moiety of CER EOS is randomly distributed in the two broad layers adjacent to the narrow central layer in the repeating unit, see figure 6.

Furthermore, in the stacked monolayer model the CHOL interfacial area is assumed to be similar to that of CER in a planar alignment. However, this is not in agreement with the interfacial areas as reported for CHOL (0.37 nm^2) and CER in a planar (0.25 nm^2) alignment by Dahlén and Pascher (81).

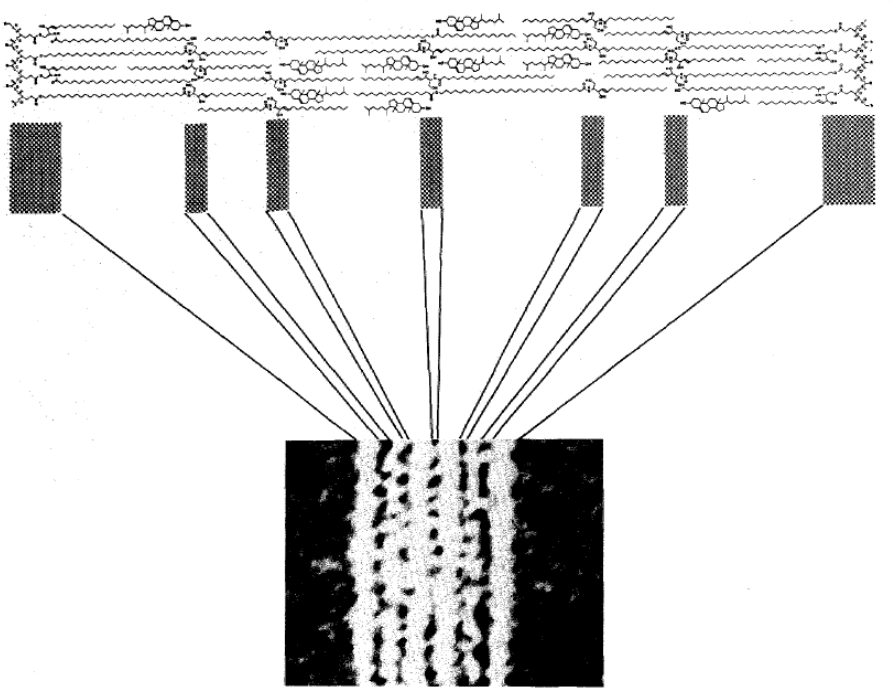

Figure 6: The stacked monolayer model as proposed in (8). A proposed molecular arrangement of one Landmann unit and two corneocyte lipid envelopes with associated monolayers, explaining the pattern of intercellular lamellae where the lucent bands are broad-narrow-broad-broad-narrow-broad. This figure is reprinted by permission from MacMillan Publishers Ltd: JID, copyright 1989.

More recently, Hill and Wertz presented a follow up of the stacked monolayer model again based on the same ruthenium tetroxide data (5), but now including more detailed knowledge on the chemical reaction of ruthenium tetroxide fixation. In this model the linoleate of CER EOS is

located in the central layer, but the CHOL interfacial area is again assumed to be similar to that of CER in a planar arrangement, see figure 7.

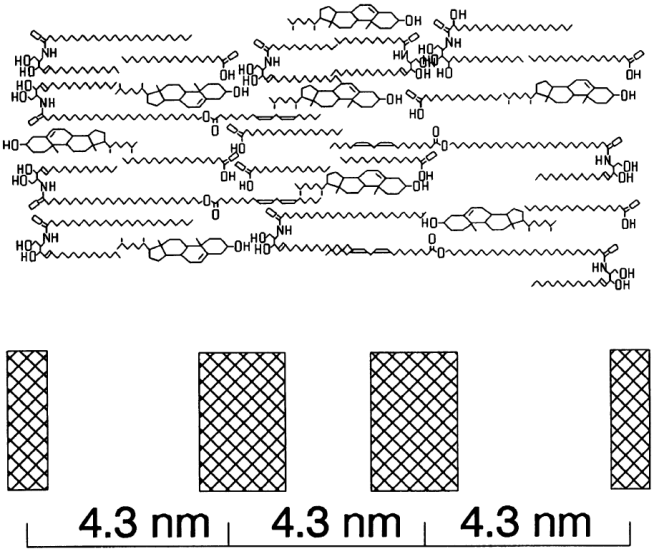

Figure 7: Proposed molecular model as presented in (5), also based on the broad-narrow-broad pattern in ruthenium tetroxide fixed pig SC. Shaded boxes represent the pattern of reduced ruthenium on a corresponding portion of a transmission electron micrograph. This figure is reprinted by permission from Elsevier, copyright 2003.

In 1993 Forslind proposed for the first time a model that incorporates the presence of a liquid phase in the SC. This model postulated the presence of a continuous liquid phase from the superficial layers of the SC down to the viable epidermis, the so-called domain mosaic model, see figure 8. Although this was the first model including the presence of a liquid phase in SC lipid structures, until now no experimental data are available to verify this model.

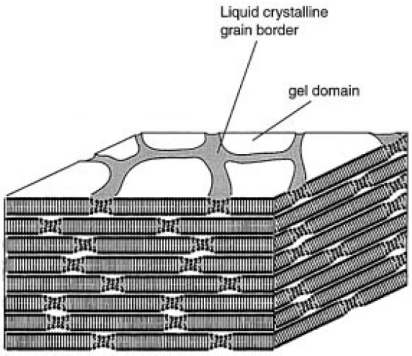

Figure 8: The domain mosaic model as presented in (4). Reprinted by permission from Acta Dermato-Venereologica, copyright 1994.

In a more recent paper, another model has been proposed for the SC lipid organization, called the single gel phase model, see figure 9. According to this model the intercellular lipids within the SC exist as a single and coherent lamellar gel phase without domain boundaries. In this gel phase the hydrocarbon chains are packed simultaneously in a hexagonal (close to hydrocarbon chain ends) and an orthorhombic ordering (close to polar headgroups). If this is the case, the orthorhombic and hexagonal phases should always coexist. This has not been observed in electron diffraction studies, in which diffraction patterns were measured attributed either to only a hexagonal or to only an orthorhombic phase (45, 82). Furthermore, in proposing the single gel phase model no attention has been paid to the role the individual lipids play in the lipid organization. For example, the crucial role CER EOS plays in the formation of the 13 nm lamellar phase and the presence (or absence) of long-chain FFA that facilitate the formation of the orthorhombic packing in vitro as well as in vivo, was not taken into consideration.

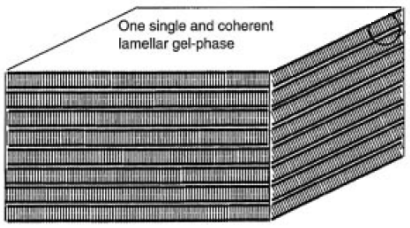

Figure 9: The single gel phase model as presented in (6). Reprinted by permission from Macmillan Publishers Ltd, JID, copyright 2001.

Finally, the sandwich model proposed by our group suggests that the lipids within the LPP are organized in a tri-layer structure: two broad layers with a crystalline (orthorhombic) structure are separated by a narrow central lipid layer with fluid domains, see figure 10.

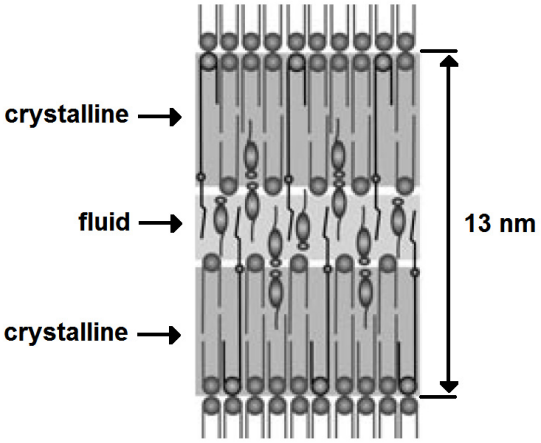

Figure 10: The sandwich model as presented in (10). Reprinted by permission from Acta Dermato-Venereologica, copyright 2000.

This broad-narrow-broad pattern of hydrocarbon chains corresponds to the images obtained with electron microscopy of the SC intercellular lamellae (figure 4). CHOL and the linoleic acid moieties of the acylceramides CER

EOS, CER EOH and CER EOP are proposed to be located in the central narrow layer, whereas crystalline packed CER are present on both sides of this central layer (83). Due to their unusual long structure, the acylceramides are able to span a layer and extend into another layer. The acylceramides are therefore thought to contribute to the stability of the 13 nm phase. The central, non-continuous fluid phase may be of importance for proper elasticity of the lamellae and for the enzyme activity in the SC, as enzymes are unlikely to be active in crystalline phases. In the sandwich model the CHOL is suggested to be located near the linoleate chains in the central layer. More recent data, however, revealed that the CHOL molecule prefers to arrange with saturated hydrocarbon chains rather than with unsaturated chains (84-86). Therefore it is more likely that CHOL is located in the crystalline layers adjacent to the narrow central layer than in the central layer with unsaturated linoleate chains. Furthermore, as Kessner et al recently suggested, the CER molecules in the sandwich model are arranged in the hairpin conformation while it can not be excluded that the CER molecules are arranged in a fully extended conformation (72).
Several models for the molecular structure of the intercellular lipids were discussed above. In conclusion, there is no consensus on the best model for the intercellular lipid arrangement in SC and therefore continuing research is necessary to gain more insight into the molecular organization of the SC lipids.

4. This thesis

4.1 Aim of this thesis

As described above, the SC forms the main barrier function of the skin and the lipid domains in the SC are considered to play a dominant role in this barrier function.
In previous studies a synthetic SC substitute (SCS) was developed to replace native SC in permeability studies. The SCS consists of synthetic SC

lipids (CER, CHOL and FFA) casted on a porous substrate. One of the advantages of the SCS is that its lipid composition can be modified to mimic that in SC of dry or diseased skin. This modified SCS can subsequently be assessed on its barrier function in permeation studies. Furthermore, the unique SC lipid organization can be investigated using SC lipid models in FTIR, SAXD and neutron diffraction studies.

The objectives of this thesis are:

1) To improve the preparation method of the SCS to obtain a lower lipid loss during preparation, a more uniform lipid layer thickness and a better reproducibility in terms of thickness and lipid organization.

2) To investigate the lateral and lamellar lipid organization in SC in more detail, using the SCS in permeation studies and SC lipid models in FTIR and X-ray and neutron diffraction studies. Concerning the lamellar organization, more knowledge can be gained especially on the molecular structure of the LPP in SC and on the role of CER EOS in the formation of this phase.

3) To evaluate the barrier properties of SCS mimicking the SC lipid composition in dry or diseased skin, to assess whether or not the altered SC lipid organization in diseased skin results in a decreased barrier function.

4.2 Outline of this thesis

In the studies described in **Part I** of this thesis the SCS is used as a tool to study the relation between lipid composition, organization and barrier function in one model. In chapter 2 we describe two new methods to prepare the SCS, in order to improve reproducibility and to increase the efficiency of the preparation method. Subsequently the properties of the SCS prepared by the different methods are investigated and the most optimal preparation method is selected for future studies. In the studies described in chapter 3 we use the SCS to determine whether a change in the lateral lipid organization affects the permeability of the SCS. We examine the effect of the orthorhombic to hexagonal phase transition on the barrier function of SCS and compared it with human SC. In the studies described in chapter 4

we examine SCS that mimic selected changes in lipid composition reported for dry or diseased skin.

In the studies described in **Part II** the molecular organization in the repeating units of the SC lamellar phases is investigated. As CER EOS plays an important role in the formation of the LPP, in the studies described in chapter 5 we investigate whether CER EOS in the absence of the other CER subclasses, mixed with CHOL and FFA, forms similar phases as observed in SC. In the studies described in chapter 6 the molecular structure in the unit cell of the LPP present in SC is investigated into detail. This characteristic LPP is suggested to be very important for the barrier function of the skin. To gain more insight into the molecular organization of this lamellar phase, we perform SAXD studies using various lipid mixtures mimicking the lipid composition in SC, with a slight variation in repeat distance of the LPP. Finally, in the studies described in chapter 7 the molecular structure of the SPP, also present in SC, is investigated into detail. To gain more insight into the molecular organization of the SPP we perform neutron diffraction studies on a mixture that incorporates a deuterated CER subclass.

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