A model membrane approach to elucidate the molecular organization in the skin barrier

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CHAPTER 1

General Introduction, Aim and Outline of this Thesis

The skin: function and structure

The skin is the largest organ of the human body and has various biological functions. The paramount functions of the skin are i) to provide the essential physical barrier against the external hazardous environment [1], ii) to protect the body from desiccation [2] and iii) to maintain temperature homeostasis [3]. The skin consists of three morphological layers, starting from outside to inside; the epidermis, dermis and the hypodermis (subcutaneous fat layer) (figure 1A) [4]. The epidermis is approximately 50 to 150 µm thick and consists of various layers. The outermost layer of the epidermis is referred to as the *stratum corneum* (SC) and this layer plays a key role in the skin barrier function [2].

The SC: structure and function

The SC is a non-viable layer consisting of corneocytes (dead cells filled with keratin and water) embedded in a highly ordered lipid matrix. It is about 10-15 µm thick and consists of 10-20 layers of corneocytes [5, 6]. The corneocytes have a diameter of around 20-30 mm and a thickness of around 0.5 mm. The corneocytes are oriented parallel to the skin surface. By nature, the corneocytes have a limited permeability of many compounds due to the cornified envelop (densely cross-linked layer of proteins) wrapped around these cells [7]. The intercellular space in between the corneocytes are filled by a crystalline lipid matrix. Due to the low permeability of the cornified envelop, topical applied molecules preferably diffuse along the intercellular lipid matrix [8, 9]. The architecture of the SC therefore is often described as a simplified '**brick and mortar**' structure with the corneocytes being the bricks and the lipids being the mortar [10] (figure 1B). Although the corneocytes in the SC are dead flattened cells filled with keratin and water, the SC is a dynamic tissue because of its high enzymatic activity which can regulate i) the desquamation process of the outermost corneocytes, ii) the formation

of a matured cornified envelop and iii) the hydration level in the SC. The hydration level is affected by the formation of the natural moisturizing factor (NMF) composed of e.g. (chemically-modified) amino acids, glycerol, urea and salts. The SC serves as the main barrier against the intruding pathogens and the penetration of substances across the skin [11-14].

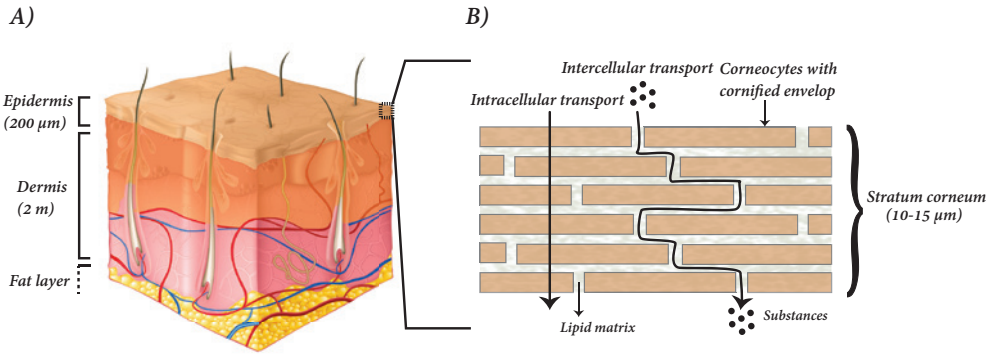

Figure 1: *A) Schematic drawing of the skin structure presenting the various layers. B) Schematic drawing of the stratum corneum structure showing different penetration routes. The intercellular pathway is an important pathway for drug penetration.*

Drug transport and permeation pathway through the skin

A drug compound may take two different routes to penetrate through the skin; the transappendageal route (transport via the sweat glands, hair follicles and sebaceous glands) and the transepidermal route. The transappendageal route accounts for 0.1 % of the total skin surface area [15], although there is much debate in literature about the contribution of hair follicles to the permeation of substances, it is considered to be less important when it comes to skin permeation. The transepidermal route may follow either the intercellular or the intracellular (transcellular) pathway depending on the nature of the molecules. As the cornified envelope minimizes the uptake of most compounds into corneocytes, the intracellular pathway may be less prominent. For this reason it has been thought that the intercellular tortuous pathway along the intercellular lipid matrix is the main route of compound penetration through the skin [9, 16-18] (figure 1B). Studies have shown that after lipid extraction, the permeability of the SC has increased several folds [19], which may suggest that indeed the intercellular lipid matrix plays a pivotal role in the skin barrier.

Lipid composition and organization in the SC

Composition

The intercellular lipid matrix present in the SC consists of three main lipid classes; ceramides (CERs), cholesterol (CHOL) and free fatty acids (FFAs). The ratio between these lipids in the human SC is approximately equimolar [20-25]. The CERs in the SC are of

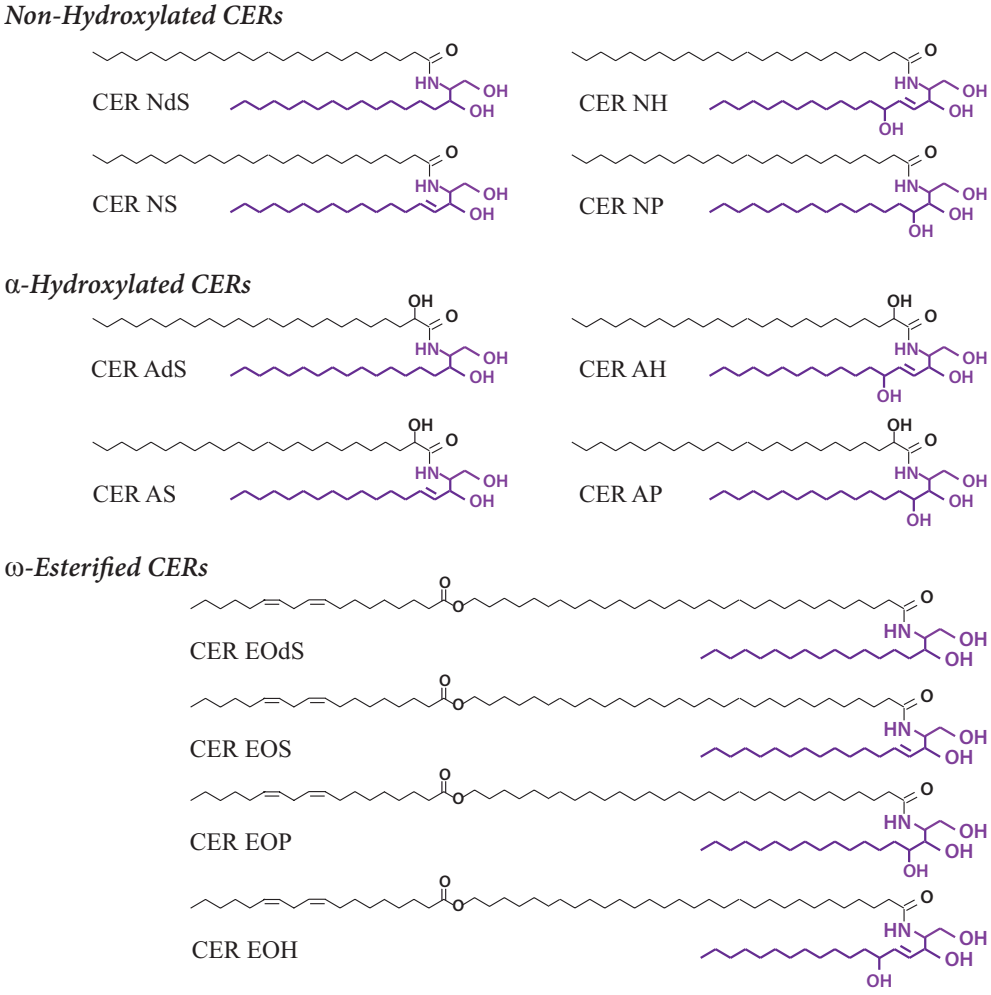

Figure 2: *Molecular structure of the CERs present in human SC. The CERs can be sub-grouped based on their hydroxylation and esterification in the fatty acid and sphingoid moiety as presented in the figure.*

exceptional molecular architecture, see figure 2. They are composed of a long hydrocarbon acyl chain linked to a sphingoid base through an amide linkage. The head-group moiety of the sphingoid base can vary in structure; sphingosine (S), phytosphingosine (P), 6-hydroxysphingosine (H) or dihydrosphingosine (dS) are the four different sphingoid bases. The acyl chain moiety also varies. The acyl chains are either non-hydroxylated (N), α-hydroxylated (A) or ω-hydroxylated (O). The latter can be linked to another fatty acid chain (e.g. linoleate (C18:2)) through an ester linkage referred to as esterified ω-hydroxy (EO) fatty acid. The EO subclass also known as acyl CER has an exceptionally long acyl chain which is extraordinary in the SC lipids. The different combinations of both the variation in acyl chain architecture and the variation in sphingoid head-group lead to 12 different CER subclasses. Two new subclasses have also identified recently and are referred to as 1-O-acylceramides [26]. These CERs consist of saturated long acyl chains in both N- and O- position, hence named as 1-O-ENS and 1-O-EAS CERs respectively (the letter E in the acronym describes the additional FFA esterified to the CER). Therefore, to date, 14 different subclasses of CERs have been identified in the human SC [20, 23, 25-27] (figure 2). Furthermore, apart from variation in the subclasses, the CERs exhibit a wide distribution in their total chain length, i.e. the total chain length of the acyl chain + the sphingoid have been reported to be between 34 and 72 carbon atoms in healthy individuals [23, 28].

When focusing on the FFAs, a wide variation in the chain length has also been observed. The FFAs reported in the human SC are mainly saturated and ranges in chain length between C14 to C34 with an average chain length of C20 to C22 [29-33]. In addition to the saturated FFA, low levels of unsaturated FFA (e.g. oleic acid, linoleic acid) and hydroxy FFA are also present in SC [32-34].

Apart from CERs and FFAs, the other third lipid class is CHOL. Some other derivatives of CHOL, CHOL sulphate, CHOL esters are also in trace amounts present in the SC [35-37].

Organization

The extracellular lipid matrix present in the SC is very unique and shows an exceptional three dimensional ordering of the lipids. The lipids are organized in stacks of lamellae referred to as the lamellar organization. The lamellae are oriented approximately parallel to the skin surface. Two lamellar phases coexist in the lipid matrix of human SC. The length of the repeating unit of these lamellar phases as examined by X-ray diffraction is about 13 nm and 6 nm, being referred to as the long periodicity phase (LPP) and the short periodicity phase (SPP), respectively [38-41] (figure 3 top). Unlike the SPP, the LPP is only detected in SC. This may suggest that the LPP plays a key role in the skin barrier function [42]. The presence of lamellar phases in SC was first monitored by freeze fracture electron microscopy [43]. Subsequently the broad-narrow-broad electron translucent bands were reported using electron microscopy with RuO_4 post-fixation [39, 44]. Using small angle X-ray diffraction (SAXD), the presence of the LPP with a periodicity of ~13 nm has been detected in human, pig and mouse SC [38, 40, 45-48].

The lipid arrangement perpendicular to the basal layer of the lamellae is referred to as the lateral organization. Not only the lamellar phases, but also this lateral organization in the lipid matrix is of crucial importance for the skin barrier function. Depending on the lipid composition, the lateral packing can be either orthorhombic (dense packing), hexagonal (less dense packing) or liquid (loose packing), figure 3 bottom part. Wide angle X-ray diffraction (WAXD) and Fourier transform infra-red (FTIR) studies revealed that close to skin temperature (~32 °C), in human SC, the lipids prevail mainly an orthorhombic lateral packing with a small subpopulation of lipids forming a hexagonal packing [49, 50]. Whether the liquid lateral packing coexist with the crystalline packing is difficult to detect with X-ray diffraction as the broad reflection of the liquid packing is obscured by the reflections from the keratin present in the corneocytes [51].

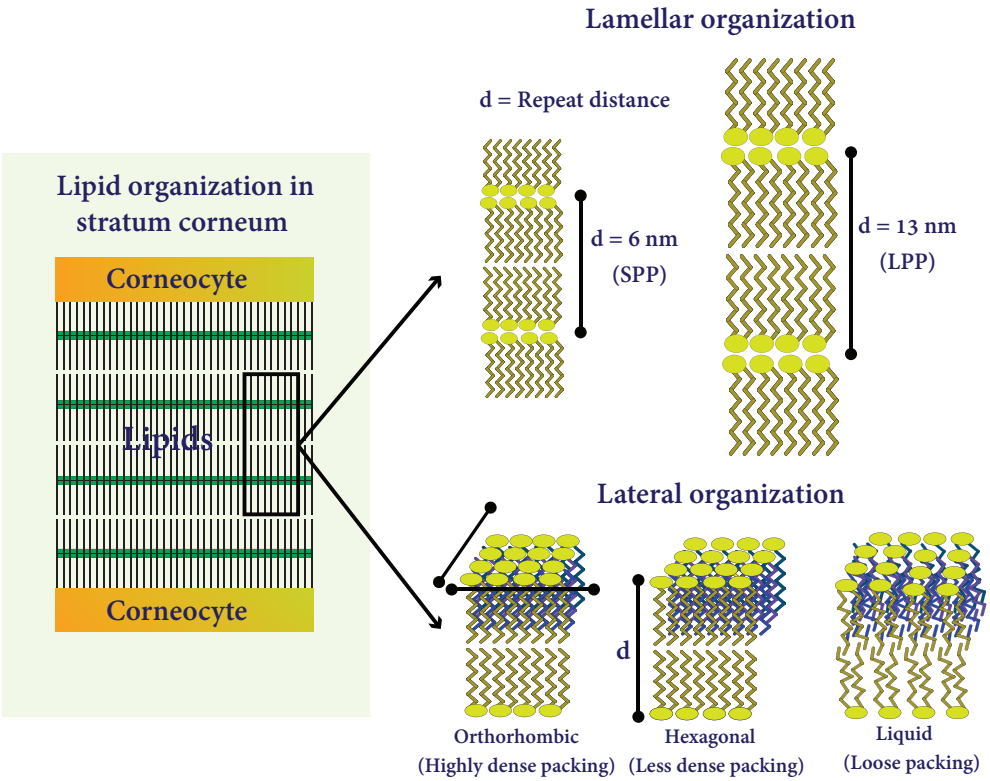

Figure 3: *SC lipid organization. The lamellar organization (on top) can be two different types; long periodicity phase (LPP) with a repeating unit (d) of ~13 nm and short periodicity phase (SPP) with a repeating unit of ~6 nm. The lateral organization (bottom) describes the crystallinity of the lipids in the lipid matrix. The lateral packing can be orthorhombic (highly dense), hexagonal (less dense) or liquid (loose packing) as depicted in the figure.*

Lipid composition and organization in the diseased skin

In several skin diseases (atopic dermatitis, Netherton syndrome, psoriasis, lamellar ichthyosis etc.), the skin barrier function is significantly reduced [30, 52-57]. Besides other factors that may play a role in this reduced barrier function, the lipid composition in the SC of these patients show significant deviations from that of healthy skin. **Atopic dermatitis** is a prevailing inflammatory skin disease with an impaired skin barrier function. The SC of patients suffering from atopic dermatitis is marked by a significant reduction in the level of CER EO subclasses in the lipid composition [57, 58]. The reduction in the level of CER EO together with the increased level of the short chain CERs resulted in a reduction in the mean CER chain length in SC of atopic dermatitis patients, which in turn resulted in an increased level of lipids forming a hexagonal lateral packing. The reduced chain length and increased presence of the hexagonal lateral packing both correlated with the impaired skin barrier function in atopic dermatitis [59, 60]. Besides the contribution of CER EO to the increased formation of the hexagonal lateral packing, the level of CER EO subclasses also contribute to the formation of the LPP. As the LPP has been shown to be important for the skin lipid barrier, this may also contribute to the impaired skin barrier in these patients [31, 60]. Apart from the reduction in the CER EO level and increase in the level of short chain CERs, an increased level of short chain FFAs, a change in the CER/CHOL/FFA ratio and an increased level of monounsaturated fatty acids (MUFAs) are also reported in the SC of these patients [59-63]. The elevated level of MUFAs is also observed in another skin disorder known as **Netherton syndrome**. A very high variation in lipid composition has been reported in the SC of Netherton syndrome patients. In some patients the level of MUFA can approach up to 80-90 % of the total FFAs [64]. In addition, a decrease in FFA chain lengths, an increase in the level of short chain CERs, an increased levels of unsaturated CERs and a reduction in the level of acyl CERs are also observed in Netherton patients [64]. Consequently an altered lipid organization in the SC is characteristic in these patients [60, 64-66].

In **lamellar ichthyosis**, the overall level of FFAs is significantly reduced compared to the healthy SC [67]. This may enhance the increased presence of lipids adopting the less dense hexagonal lateral packing in this disease [58]. However, as no information on chain length of the FFA has been reported, it is not known whether altered chain length distribution of FFA or CERs may also play a role. X-ray diffraction studies revealed changes in the lamellar organization compared to the healthy SC, in which an altered CER composition as well as a reduction in chain length may play a role [67]. **Psoriasis**, another prevalent inflammatory skin disorder, the level of CER EOS is drastically reduced and the sphingosine containing CERs are present at higher levels at the expense of phytosphingosine containing CERs [68, 69]. Furthermore, transmission electron microscopy studies reveal an aberrant SC lipid structure in the psoriatic skin [70]. No information is available on chain length distribution of CERs and FFAs. Another skin disease, **recessive X-linked ichthyosis** suffers from the elevated levels of CHOL sulphate in the SC due to the deficiency of the enzyme CHOL sulphatase [71, 72]. An increment in CHOL sulphate resulted in a fluid phase formation in the lipid model mixtures and therefore elevated cholesterol sulphate levels may contribute to a reduced skin barrier in this disease [73].

The SC models

Lipid models

In order to obtain information on the role the lipids play in the lipid organization, SC lipid models have been developed. This allows studying of the lipid properties without interference of the corneocytes, also present in SC. Furthermore, the lipid composition can be changed on demand, which is impossible in studies using intact SC. Therefore, these models facilitate the interpretation when focusing on the lipid phase behavior and hence can provide novel information on the role of the lipid classes and subclasses in the lipid phase behavior. The most simplistic models of this category are mono, di, ternary or quaternary lipid mixtures of CERs, CHOL and FFA [74-80]. These models are particularly useful to study the basic interactions between different lipids. Although these models provide important information regarding lipid phase behavior, nonetheless, they often form phases different from those in SC. Furthermore, neutron diffraction studies reveal that these model lipid mixtures often lack the ability to form the LPP and SPP. This makes the simplified model systems less suitable for identifying the role of various lipid subclasses in the lipid phase behavior in the SC [78, 79].

As opposed to simple lipid mixtures, the CERs isolated from human or pig SC have also been used together with CHOL and FFA in order to examine the lipid phase behavior [34, 51]. The mixtures of isolated human CERs and CHOL revealed the presence of two lamellar phases with repeat distances of 5.4 and 12.8 nm lamellae, closely mimicking the lamellar phases present in human and pig SC [51]. However, there was one clear difference. These lipid mixtures exhibited hexagonal lateral packing instead of orthorhombic which is dominantly present in the SC [49, 50]. The addition of FFA mixture with variable chain lengths resulted not only in the formation of the two prominent lamellar phases, but also induced an orthorhombic lateral packing [81]. The addition of FFA also increase slightly the repeat distance of the LPP to 13 nm. Further studies revealed that the absence of CER EOS in the isolated CER mixture dramatically reduced the formation of the LPP, signifying the impact of CER EOS for proper lipid organization in the SC [51, 82, 83]. The effect of the degree of unsaturation of the fatty acid which is ester bound to the 30 C acyl chain of CER EOS was also investigated using equimolar FFA, CHOL and isolated human CERs. In these studies the CER EOS was replaced by synthetic CER EOS linoleate (contains double unsaturation in the linoleate moiety), CER EOS oleate (single unsaturation) or CER EOS stearate (saturated) [84]. These studies revealed that the degree of unsaturation in the fatty acid chain highly affects the lipid lamellar organization. The lipid mixtures prepared with CER EOS linoleate forms dominantly the LPP lamellae compared to the CER EOS oleate. In case of CER EOS stearate the LPP was even absent [85].

As human skin is not readily available and isolation of CERs from SC is a time consuming process, as an alternative synthetic CERs can be used [86]. The advantage of synthetic CERs is that the composition can be changed on demand, facilitating the role the various lipid subclasses play in the lipid organization. The lipid mixtures of equimolar synthetic CER, CHOL and FFA results in the formation of LPP and SPP similar to that present in the SC, but with slightly shorter periodicities of 12.2 and 5.4 nm along with the orthor-

hombic lateral packing [86]. This phase behavior is very similar to that obtained with the isolated CERs. In the lipid mixtures, the phase separated crystalline CHOL domains are also observed similar to that in the SC. Point to be noted here, in case of synthetic CERs, the LPP formation is only possible in the presence of FFA which contrast with the observations made from natural CER mixtures. The difference in phase behavior between natural and synthetic CER mixtures might be due to the limited acyl chain length variation in the latter case.

The SCS as a tool to investigate the lipid barrier function

As described above, the lipid mixtures prepared from synthetic CERs, CHOL and FFA can be used to study the lipid phase behavior and offers an excellent tool to investigate the role of individual CER subclasses or lipid classes in the SC lipid organization. Using these lipid mixtures so far, the relation between the lipid composition and organization has been examined. However, in order to understand the skin barrier function, the effect of lipid composition and organization on the lipid barrier function need to be established. For this reason a lipid membrane device was developed, referred to as stratum corneum substitute (SCS) [87, 88]. The SCS was prepared by casting lipids (mixtures of synthetic CERs, CHOL and FFAs) on a porous support (for example, spraying lipids on top of polycarbonate filter membrane). It was observed that when selecting the proper composition, the SCS mimics the SC lipid composition and organization very closely [87, 89]. To examine the barrier functionality and to relate the lipid barrier to the lipid composition and organization, the SCS can be clamped in a diffusion cell, which allows to examine the transport of (model) compounds by performing *in vitro* permeation studies (figure 4). Various model drug compounds have been selected to perform permeation studies. These compounds varied in the physical properties; benzoic acid, para amino benzoic acid (PABA), ethyl PABA, butyl PABA, hydrocortisone [88, 90-92]. Among these benzoic acid is the least lipophilic drug compounds. The permeation profile of all these compounds across the SCS revealed a similar flux as observed in human SC, demonstrating the suitability of the SCS model to investigate the lipid barrier when comparing with SC.

As mentioned earlier, the reduction in the level of CER EO and the increased level of short chain CERs and FFAs have been reported in SC of diseased skin, *in vitro* permeation studies were performed using SCS model membrane where the lipid composition was changed to mimic various aspects of the lipid composition in SC of atopic eczema [92, 93]. Studies performed with lipid mixtures in the absence of CER EOS have reported reduce lipid barrier, indicating the importance of CER EOS not only in the lipid phase behavior but also for proper skin lipid barrier function [90]. In other studies, the effect of reduced chain length of FFAs has also been examined using benzoic acid as model drug. A reduction in the FFA chain length impairs the lipid barrier compared to the SC, demonstrating the importance of FFA chain length in the skin barrier [91].

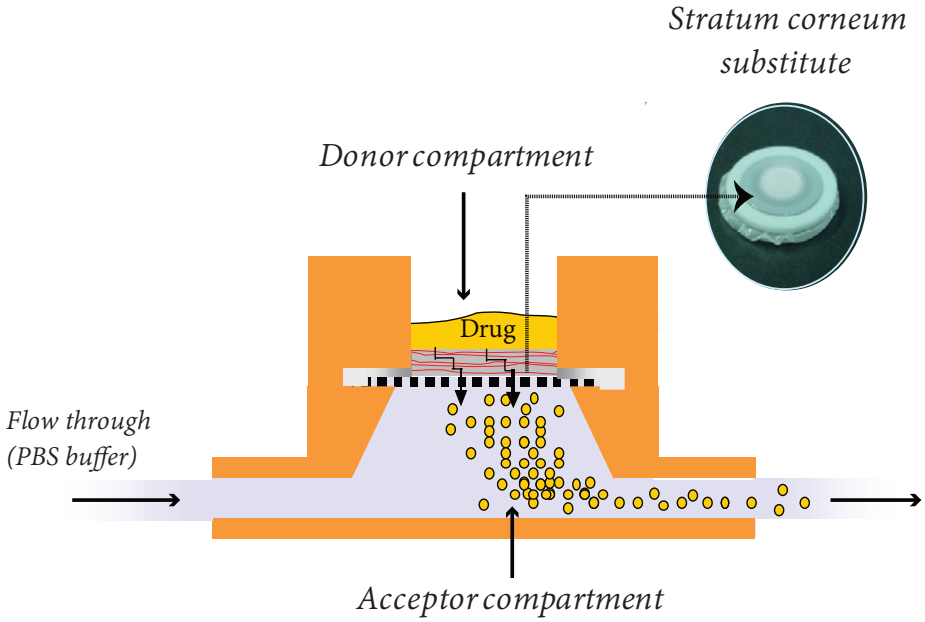

Figure 4: *Schematic of the diffusion setup used to perform the permeation studies in order to study the barrier functionality of the SCS model lipid membrane. Inset the SCS prepared on polycarbonate filter disk.*

In addition of permeation studies, another method of detecting the barrier functionality is to measure the trans-epidermal water loss (TEWL) which detect the inside-outside skin barrier using water as the permeating compound: The TEWL measure the amount of water evaporation from the skin per time unit and per skin area. Therefore it is another indicator for the skin barrier function.

This thesis

Aim of this thesis

The aims of the research describes in this thesis are two folds: First, to gain insight in the role of different lipid classes and subclasses play in the lipid organization and barrier function of healthy and diseased skin and second, to obtain insight in the molecular arrangement of the lipids in the unit cell of the two lamellar phases (SPP and LPP) in the SC. To achieve our goals, we performed the following studies:

1. The effect of MUFAs on the SC lipid barrier were examined. As tool the SCS was used.
2. The effect of CER chain length distribution on the lipid organization and SC lipid barrier has been studied using SCS.
3. The role of CHOL on the formation of each of the lamellar phases and the lateral packing was examined using lipid mixtures.
4. The molecular arrangement of CHOL and FFA C24 in the unit cell of the SPP was determined using model lipid mixtures. Based on these studies a molecular model has been proposed for the unit cell of the SPP.
5. The localization of CER NS and FFA C24 was elucidated along with water distribution profile in the unit cell of the LPP.
6. The localization of CER EOS and CHOL in the unit cell of the LPP was determined. Based on the localization of CER NS, FFA C24, CHOL and CER EOS, a molecular model for the unit cell of the LPP has been proposed.

Outline of this thesis

The thesis is divided into two parts which will address the various objectives mentioned above.

PART I (chapters 2-4) describes studies performed with SCS model membrane and lipid mixtures to examine which elements in the deviation in lipid composition and phase behavior play a prominent role in the impaired skin barrier in inflammatory skin diseases.

In PART II (chapters 5-7) describes studies to elucidate the molecular arrangement in the unit cell of the SPP and LPP. For both lamellar phases, a molecular model has been proposed.

Chapter overview

In Chapter 2 the effect of MUFAs on the lipid organization and the skin lipid barrier are reported. To study this saturated FFAs were replaced by their unsaturated counterparts. The lipid organization was examined by FTIR and SAXD. Permeation studies and TEWL measurements were performed to study the changes in lipid organization on the permeability of hydrocortisone across the SCS.

Chapter 3 describes studies examining the role of CER chain length distribution on the lipid organization and permeability of hydrocortisone through the SCS. SCS were prepared either from isolated pig CERs, in which each of the CER subclasses show a wide chain length distribution or from synthetic CER subclasses with only one or two chain lengths. The CER subclass composition of the isolated and synthetic CERs was very similar. The lipid organization was examined by means of SAXD and FTIR.

In Chapter 4 the role of CHOL in the lipid phase behavior of each of the lamellar phases are described. To examine this the CHOL levels were varied systematically in the lipid mixtures and their effect on the formation of the lipid lamellar and lateral organization was determined by SAXD and FTIR.

In Chapter 5 the molecular arrangement of CHOL and FFA C24 in the unit cell of the SPP is reported. These studies were performed using neutron diffraction together with D_2O/H_2O contrast variation. To localize the lipid molecules in the unit cell of the SPP, partly deuterated CHOL and perdeuterated FFA C24 were used. A molecular model for the SPP has been proposed based on these lipid arrangements.

In Chapter 6 studies are described to elucidate the molecular localization of CER NS and FFA C24 in the unit cell of the LPP. Using isotopic H/D substitution combining with neutron diffraction, the perdeuterated acyl chains of CER NS and FFA C24 are located in the trilayer unit cell of the LPP.

In Chapter 7 the molecular arrangement of CER EOS and CHOL in the unit cell of the LPP is reported. To examine these location, neutron diffraction in combination with D_2O/H_2O and CER EOS contrast variation method were used. The perdeuterated linoleate chain of CER EOS and selectively deuterated CHOL were used to determine their location in the unit cell of the LPP. A molecular model for the LPP has been developed based on these and previously determined lipid arrangements.

Chapter 8 sums up the results of this thesis, provides an overall conclusion and focuses on future research perspectives.

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