Review
The skin barrier in healthy and diseased state

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Abstract
The primary function of the skin is to protect the body for unwanted influences from the environment. The main barrier of the skin is located in the outermost layer of the skin, the stratum corneum. The stratum corneum consists of corneocytes surrounded by lipid regions. As most drugs applied onto the skin permeate along the lipid domains, the lipid organization is considered to be very important for the skin barrier function. It is for this reason that the lipid organization has been investigated quite extensively. Due to the exceptional stratum corneum lipid composition, with long chain ceramides, free fatty acids and cholesterol as main lipid classes, the lipid organization is different from that of other biological membranes. In stratum corneum, two lamellar phases are present with repeat distances of approximately 6 and 13 nm. Moreover the lipids in the lamellar phases form predominantly crystalline lateral phases, but most probably a subpopulation of lipids forms a liquid phase. Diseased skin is often characterized by a reduced barrier function and an altered lipid composition and organization. In order to understand the aberrant lipid organization in diseased skin, information on the relation between lipid composition and organization is crucial. However, due to its complexity and inter-individual variability, the use of native stratum corneum does not allow detailed systematic studies. To circumvent this problem, mixtures prepared with stratum corneum lipids can be used. In this paper first the lipid organization in stratum corneum of normal and diseased skin is described. Then the role the various lipid classes play in stratum corneum lipid organization and barrier function has been discussed. Finally, the information on the role various lipid classes play in lipid phase behavior has been used to interpret the changes in lipid organization and barrier properties of diseased skin.

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Abbreviations: CERs, ceramides; SAXD, small angle X-ray diffraction; WAXD, Wide angle X-ray diffraction; ED, electron diffraction; λ, wavelength of X-rays; d, periodicity; FFAs, free fatty acids; CHOL, cholesterol; SPP, short periodicity phase; LPP, long periodicity phase; CER1-ol, ceramide 1 oleate; CER1-lin, ceramide 1 linoleate; CER1-ste, ceramide 1 stearate; FFEM, freeze fracture electron microscopy; EFAD, essential fatty acid deficient; LI, lamellar ichthyosis

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1. Introduction

The skin is composed of several morphologically distinct layers, see Fig. 1. The protection of the skin is provided primarily by the stratum corneum. Underlying the stratum corneum is the viable epidermis (50–100 μm thick), which is responsible for generation of the stratum corneum. The viable epidermis consists of various layers. From the inside to the outside these layers are the stratum basale, the stratum spinosum and the stratum granulosum. The epidermis is a dynamic, constantly self-renewing tissue, in which a loss of the cells from the surface of the stratum corneum (desquamation) is balanced by cell growth in the lower epidermis. Upon leaving the basal layer, the keratinocytes start to differentiate and during migration through the stratum spinosum and stratum granulosum they undergo a number of changes in both structure and composition. The keratinocytes synthesize and express numerous different structural proteins and lipids during their maturation. The final step in keratinocyte differentiation is associated with profound changes in their structure resulting in their transformation into corneocytes. The corneocytes are flat dead cells filled with keratin filaments and water, which are surrounded by a densely crosslinked protein layers, the cell envelope. One of the enzymes involved in the formation of the densely packed cell envelope is transglutaminase 1, the activity of which is reduced in lamellar ichthyosis (LI) patients [1,2]. A lipid envelope is chemically linked to this densely packed cell envelope. This lipid monolayer serves as an interface between the hydrophilic corneocytes and the lipophilic extracellular non-polar lipids, which are surrounding the corneocytes. Furthermore, corneodesmosomes are interconnecting the corneocytes and are important for the stratum corneum cohesion.

Late in the process of differentiation, characteristic organelles (lamellar bodies) appear in the granular cells and play a crucial role in stratum corneum formation. Lamellar bodies enriched mainly in polar lipids and catabolic enzymes serve as carriers of precursors of stratum corneum barrier lipids. They consist mainly of glycosphingolipids, free sterols and phospholipids. In the uppermost granular cells the lamellar bodies move to the apical periphery of the uppermost granular cells and fuse with the plasma membrane. After the extrusion of lamellar bodies at the stratum granulosum/stratum corneum interface their content is secreted into the intercellular spaces. There, the polar lipid precursors are enzymatically converted into nonpolar products and assembled into lamellar structures surrounding the corneocytes. The intercellular lamellae are orientated approximately parallel to the surface of the cell [3–9]. In this orientation process, the lipid envelope [10–12] most probably acts as a...

Fig. 1. A schematic drawing of a skin cross-section. The skin is composed of a dermis and an epidermis. In the basal layer of the epidermis cells proliferate. Upon leaving the basal layer cells start to differentiate and migrate in the direction of the skin surface. At the interface between stratum granulosum–stratum corneum terminal differentiation occurs, during which the viable cells are transformed into dead keratin filled cells (corneocytes). The corneocytes are flat dead cells filled with keratin filaments and water, which are surrounded by a densely crosslinked protein layers, the cell envelope. One of the enzymes involved in the formation of the densely packed cell envelope is transglutaminase 1, the activity of which is reduced in lamellar ichthyosis (LI) patients [1,2]. A lipid envelope is chemically linked to this densely packed cell envelope. This lipid monolayer serves as an interface between the hydrophilic corneocytes and the lipophilic extracellular non-polar lipids, which are surrounding the corneocytes. Furthermore, corneodesmosomes are interconnecting the corneocytes and are important for the stratum corneum cohesion.
template. During this extrusion and fusion process hydrolysis of glycolipids generates ceramides (CERs), while phospholipids are converted into free fatty acids (FFAs). In diseased state often the activity of one or more enzymes involved in the synthesis of the barrier lipids is altered compared to that in normal skin. For example, in type 2 Gaucher disease patients, the level of glucocerebrosidase is strongly reduced, inducing a strong increase of the ratio of glucosylceramides/CERs. This is accompanied by an altered lipid organization and a reduction in the skin barrier function [13–15] demonstrating that CERs are very important for a proper barrier function. Another example is atopic dermatitis: the enzymes sphingomyelin deacylase and glucosylceramide deacylase are increased in activity resulting in an altered CER profile in stratum corneum of these patients [16–23].

The change in lipid composition and cell structure during terminal differentiation results in the formation of a very densely packed structure in the stratum corneum. It is the impermeable character of the cornified envelope that conduct the penetrating substances along the tortuous pathway between the corneocytes as revealed by confocal laser scanning microscopy and X-ray microanalysis studies [24,25]. It is for this reason that the lipids play an irreplaceable role in the skin barrier. This makes their mutual arrangement in the lamellar domains a key process in the formation of the skin barrier.

2. Stratum corneum lipid composition and organization in normal skin

2.1. Lipid composition in stratum corneum

The major lipid classes [26,27] in stratum corneum are CERs, cholesterol (CHOL) and FFAs. The CERs head groups are very small and contain several functional groups that can form lateral hydrogen bonds with adjacent CER molecules. The acyl chain length distribution in the CERs is bimodal with the most abundant chain lengths being C24–C26. Only a small fraction of CERs has an acyl chain length of C16–C18. The chain lengths of C24 and C26 are much longer than those in phospholipids in plasma membranes. In human stratum corneum [28–30] 9 subclasses of CERs have been identified, see Fig. 2. These CERs, referred to as CER 1 to 9, differ from each other by the head-group architecture (sphingosine (S), phytosphingosine (P) or 6-hydroxysphingosine (H) base) linked to a non-hydroxylated fatty acid (N) or an ω-hydroxylated fatty acid (EO) with a chain length of approximately 30–32 C-atoms. In this respect the CERs are different from CERs isolated from pig stratum corneum (pigCERs), in which only pigCER1 has this exceptional molecular structure [31]. In both species the FFA fraction consists mainly of saturated acids. The major portion of FFAs has an acyl chain length of C22 and C24. Another important lipid in stratum corneum is cholesterol sulfate. Although cholesterol sulfate is present in small amounts (typically 2–5% w/w), this lipid plays an important role in the desquamating process of stratum corneum [32].

2.2. Lipid organization in stratum corneum isolated from normal skin

Among many other biophysical techniques, such as Fourier transformed infrared spectroscopy and differential scanning calorimetry, X-ray diffraction is a very powerful technique to study the lipid organization in stratum corneum. First a brief explanation of the X-ray diffraction technique will be provided.

In case of X-ray diffraction, a source produces X-rays. These produced X-rays are transported across the sample of interest and is referred to as the primary beam. However, a small part of the X-rays of this primary beam is scattered by the sample. The intensity of the scattered X-rays are measured as a function of θ, the scattering angle, see Fig. 3A. When the sample is composed of lipids or proteins and are arranged in a repeating structure the intensity of the scattered X-rays in the diffraction plane is characterized by a series of peaks (intensity maxima of scattered X-rays determined by the so called structure factor). Often, the scattered intensity is plotted as a function of Q (the scattering vector), which is directly related to the scattering angle by Q=4π sin θ/λ, in which λ is the wavelength of the X-rays. In case of small angle X-ray diffraction the scattered intensity is measured as low angle, typically between 0 and 5° and provides information about the larger structural units in the sample, such as the repeat distance of a lamellar phase (see Fig. 3B). Wide angle X-ray diffraction provides information about the scattered intensity at higher angle (see Fig. 3A). At higher angle the X-rays contain information about the smaller structural units in the sample, such as the lateral packing in a lamellar phase (see Fig. 3C).

2.2.1. Small angle X-ray diffraction

The X-ray diffraction pattern of a lamellar phase is characterized by a series of maxima in scattered intensity, of which the sequential peaks are positioned at equal interpeak distance. The position of the sequential peaks is referred to as 1st order (positioned at Q1), 2nd order (Q2), 3rd order (Q3), etc. The repeat distance (da) of a lamellar phase A can directly be calculated from the peak positions da=2π/Q1=4π/Q2=6π/Q3, etc. A larger repeat distance in the structure results in a series of peaks with a smaller interpeak distance at the scattered X-ray curve (compare da and db in Fig. 3B). If two lamellar phases are present in the sample (lamellar phases A and B with repeat distances da and db) the X-rays scattered by these structures are additive.

2.2.2. Wide angle X-ray diffraction

Wide angle X-ray diffraction provides information about the localization of the lipids in the lamellae. For SC lipid
organization 3 classes of lateral packing are important. In the liquid phase the distance between the molecules is not well defined, which results in an X-ray pattern with a very broad peak at around 0.46 nm. If the lipids in membrane are in a liquid phase, the membrane is a highly permeability for most substances. In the hexagonal lateral packing, which is a much denser structure than the liquid packing with a strongly reduced permeability, the distance between the neighboring molecules is equal in the $x$–$y$ plane. Lipids still can rotate along their longest axis. This diffraction pattern of this packing is characterized by one strong reflection at approximately 0.41 nm spacing and a series of low intensity reflections at well-defined spacings. Often this phase is also referred to as a gel-phase. Finally, the orthorhombic lateral packing is very densely packed with a very low permeability. The neighboring molecular distance is not equal in the $x$ and $y$ direction. This results in two strong diffraction rings at 0.37 and 0.41 nm spacing. The molecules are even not able to rate along their longest axes. The relation between the unit cell and the diffraction pattern is given schematically in Fig. 3C. In all examples in Fig. 3C it is considered that many small crystals are exposed to the X-ray beam. Due to a variation in orientation of these crystals, the X-ray pattern is characterized by diffraction rings in stead of diffraction spots, which are observed when dealing with single crystals. In an electron diffraction experiments due to a very small cross section of the electron beam, it is also possible to expose one or a few single crystals to the electron beam. In these cases spots in stead of rings are observed.

At the end of the 1950s and in the early 1960s, the lipid organization in human stratum corneum [33,34] has been investigated using X-ray diffraction. The excellent measurements revealed a similar pattern as observed nowadays with the synchrotron facilities. Since at that time no information was available about the lipid organization at the ultrastructural level, the X-ray diffraction patterns were interpreted as being from lipids organized in tubes surrounding keratin filaments. At least 10 years passed before important additional information became available that provided completely new insights in the lipid organization. Freeze fracture electron microscopy revealed the presence of intercellular lipid lamellae between the cells [35,36]. This was a big step forward in understanding the structure of the stratum corneum.

For transmission electron microscopic studies the key problem in visualizing lipid lamellae was the saturated nature of the stratum corneum lipids, which made the fixation with osmium tetroxide impossible. In 1987, Madison et al. used ruthenium tetroxide as a post-fixation agent to preserve the saturated lipids in the stratum corneum during the embedding procedure [37] and demonstrated that the lipid lamellae are arranged in a repeating pattern with electron translucent bands in a broad-narrow-broad sequence [38–41]. At the end of the 1980s White et al. [42] performed X-ray diffraction studies with mouse stratum corneum. Using small angle X-ray diffraction (SAXD) they observed a diffraction pattern of a series of sharp peaks indicating the presence of a lamellar phase with a periodicity of approximately 13 nm, further referred to as the long periodicity phase (LPP) [43,44] with a transition to
a hexagonal lateral packing occurring between 30° and 40 °C. In an additional series of studies with human and pig stratum corneum synchrotron facilities have been used [45,46]. As the lipid organization in pig stratum corneum is very similar to that of human stratum corneum, only the studies performed with human stratum corneum will be discussed. The SAXD curves of human stratum corneum revealed the presence of very broad partly overlapping peaks. For this reason additional information was required for proper interpretation of the obtained data. This was achieved in SAXD experiments with stratum

Fig. 3. (A) A schematic presentation of the X-ray diffraction technique. A source produces X-rays that are partly scattered by the sample. The scattered intensity is measured as a function of, the scattering angle \( \theta \). When the scattered intensity is measured at low angle, the technique provides information about the larger structural units in the sample. In that case the technique referred to as small angle X-ray diffraction (SAXD). When the scattered intensity is measured at higher scattering angle, the technique provides information about the smaller structural units such as the lateral packing of the lipids in the lamellae. Often the scattered intensity (I) is plotted as function of Q, the scattering vector. The scattering vector is related to \( \theta \) as \( Q = \frac{4 \pi \sin \theta}{\lambda} \). (B) Small angle X-ray diffraction. The intensity of the scattered X-rays is plotted as function of Q. The diffraction pattern of a lamellar phase consists of a series of peaks, referred to as the 1st order located at \( Q_1 \), 2nd order located at \( Q_2 \), 3rd order located at \( Q_3 \), etc. From the positions of these peaks the periodicity \( d_a \) (in the 3 dimensional structure in \( z \) direction) of the lamellar phase can directly be calculated by \( d_a = \frac{2 \pi}{Q_1} = \frac{4 \pi}{Q_2} = \frac{6 \pi}{Q_3}, \) etc. When the repeat distance of the lamellar phase is larger, the distance between the sequential peaks is smaller (compare \( d_a \) (LP) with \( d_a \) (SP)). If two lamellar phases (LP + SP) are present in the sample the diffraction peaks of the two phases are additive. This often results in a formation of a broader peak with a shoulder (see e.g. Fig. 4). (C) Wide angle X-ray diffraction: a schematic presentation of the positions of the alkyl chains in liquid, hexagonal and orthorhombic phases parallel to the basal plane (that is in \( x–y \) direction perpendicular to the \( z \) direction) of the lamellae and their corresponding diffraction patterns. In a liquid phase (high permeability) the distances between the hydrocarbon chains is not very well defined resulting in a broad reflection at approximately 0.46 nm. In a hexagonal packing (medium permeability) the hydrocarbon chains of the lipids are equally distributed in the structure at interchain distances of 0.48 nm (spacing 0.41 nm). This results in a strong reflection at approximately 0.41 nm spacing. The orthorhombic phase (low permeability) is a very dense structure of which the hydrocarbon chains are not equally distributed in the lattice. This results in a diffraction pattern with two reflections at 0.41 and at 0.37 nm, respectively.
corneum in which the lipids were re-crystallized from 120 °C to room temperature, see Fig. 4A. The diffraction curves revealed the presence of a series of sharp peaks, indicating that after recrystallization the lipids in human and pig stratum corneum were organized in a LPP (=long periodicity phase) with a periodicity of approximately 13 nm. Comparing the peak positions in the diffraction patterns obtained prior and after recrystallization revealed the presence of at least two lamellar phases: one lamellar phase with a periodicity of approximately 6 nm referred to as SPP (=short periodicity phase), and the LPP (=long periodicity phase) with a periodicity of approximately 13 nm [43,44]. As the LPP has been found to be present in all species examined until now, and has a very characteristic molecular organization (see below), it has been suggested that the presence of this phase plays an important role in skin barrier function.

More detailed information on the stratum corneum lipid organization can be obtained when changes in diffraction pattern as a function of temperature are monitored. Such experiments revealed that up to 60 °C the lipid lamellae are still present and disappear thereafter within a temperature range of approximately 10 °C (see Fig. 4B).

Besides the lamellar organization, also the lateral packing is crucial for proper barrier properties of the stratum corneum. The lateral packing discloses information about the density of the lipids within the lipid lamellae. WAXD studies revealed the presence of orthorhombic lateral packing [45–48], which is in agreement with the earlier infrared spectroscopy results. The presence of the orthorhombic packing indicates that the lipids within the lamellae are very densely packed. This is considered to be very important for a proper skin barrier function. It remained unclear whether a liquid phase coexisted with the orthorhombic lateral packing, as the broad reflection of the liquid phase in the diffraction pattern was obscured by the reflections based on soft keratin present in the corneocytes (see Fig. 5A). In addition, it could not be concluded whether a hexagonal lateral packing is present either, as the reflections of the orthorhombic packing obscure the reflections based on the hexagonal lateral packing [48]. Furthermore, frequently the CHOL phase separates from the lamellar phases [43,44,48]. In contrast to mouse and human stratum corneum, surprisingly, in pig stratum corneum no orthorhombic lateral packing was present, but a hexagonal lateral packing prevailed.

More recently using the electron diffraction (ED) technique [49] more detailed information has been obtained on the lateral organization in human stratum corneum. With this method a small area with a diameter as small as 1 μm² can be selected for exposure to the electron beam and thereby allows to acquire reflections only from one or a few crystals. Due to this small area of exposure ED provides information on the lateral packing of stratum corneum lipids that is supplementary to WAXD. Another advantage of ED is the possibility to obtain diffraction patterns from stratum corneum strips as a function of depth. This allows not only examination of stratum corneum lateral packing as a function of stratum corneum depth in vitro, but also to perform similar measurements in vivo.

The diffraction pattern of an orthorhombic single crystal is characterized by 2 paired strong reflections at a spacing of approximately 0.406 nm and 1 pair of strong reflection at a 0.367 nm spacing. Besides these strong reflections higher order reflections could also be detected. The strong reflections are...
separated by angles that are close but not equal to 60°. A single crystal of a hexagonal sublattice is characterized by 3 paired diffraction spots separated by angles of 60° at a spacing of 0.41 nm. As in these patterns spots are detected instead of rings, it is usually possible to establish whether the hexagonal sublattice coexists with the orthorhombic one. Furthermore, a number of reflections can be attributed to phase separated CHOL. The position of the reflection in the pattern indicates that CHOL crystals have a preferred orientation in a similar direction as the lipid lamellae. The strong broad reflections at 0.46 and 0.92 nm can be attributed to soft keratin. (B) One-dimensional WAXD pattern of the equimolar CHOL:CER mixture plotted as function of Q (see Fig. 3). A broad reflection at 0.415 nm spacing is attributed to the presence of the hexagonal lattice. A large number of sharp reflections is based on phase separated CHOL indicated by *, which obscures the possible presence of a broad reflection (at a spacing of 0.46 nm) attributed to a liquid lateral packing. (C) One-dimensional WAXD pattern of the equimolar CHOL:CER:FFA mixture. Two strong sharp reflections indicate the presence of an orthorhombic lateral packing. In addition a broad peak is observed at a spacing of approximately 0.46 nm indicative for a liquid lateral packing.

Stratum corneum lateral packing as a function of stratum corneum depth was studied both in \textit{in vivo} and in \textit{ex vivo} human skin. Some characteristic ED patterns are shown in Fig. 6. The diffraction patterns consisted of concentric rings as in WAXD or of opposite arcs/spots at both 0.41 and 0.37 nm, especially when smaller areas were selected for ED. These latter ED patterns make it possible to establish whether only the orthorhombic packing is present or the hexagonal packing is present too. Furthermore, faint reflections at 0.22 and 0.25 nm were recorded occasionally. These reflections can be attributed to other sets of lattice planes of the same crystal. The spacings are in agreement with calculated values and with the spacings observed by WAXD.

In order to estimate the frequency the various lattices are present in human stratum corneum, the frequency of occurrence of the various ED patterns were quantified. The relative distribution of the ED patterns in stratum corneum depth was similar for \textit{ex vivo} and \textit{in vivo} skin (not shown) and a transition was observed from an orthorhombic to a hexagonal phase between 30 and 40 °C, similar as observed with WAXD [45].
From our studies it became very clear that throughout the stratum corneum the orthorhombic packing prevailed, however, in the upper part of the stratum corneum the hexagonal packing could occasionally be detected as well. These observations do not confirm the single-phase model proposed by Norlen et al. [50]. He suggested the presence of a single phase in a gel-phase packing.

Furthermore, from the ED studies it was concluded that ED patterns attributed to the hexagonal packing were recorded mainly in the superficial layers of the stratum corneum at room temperature and at 32 °C. However, in vivo one would expect to observe the more frequent presence of hexagonal lateral packing in the lower part of the stratum corneum where the temperature approaches 37 °C, but this was not the case. Therefore, there should be another explanation for this phenomenon, which might be the presence of sebaceous lipids that are secreted on the surface of the skin and partly penetrate the intercellular matrix of the stratum corneum [51,52]. These sebaceous lipids may form a hexagonal packing or create a phase transition within the endogenous lipids from orthorhombic to hexagonal. Fourier transformed infrared spectroscopic studies by Bommannan et al. [51] showed a fluidization of the upper stratum corneum lipids, while Golden et al. [53] have shown that the unsaturated fatty acids might function as penetration enhancers. As sebum mainly consists of glycerides, squalene, wax/sterol esters as well as (short chain) free fatty acids, it is very likely that they may alter the endogenous lipid structure by increasing alkyl chain mobility.

Another striking observation was that the ED patterns frequently displayed reflections forming 3 pairs of double arcs at 0.41 and 0.37 nm. These reflections can be assigned to the orthorhombic lateral packing only by rotating three successive orthorhombic crystals over an angle of 60° relative to each other (Fig. 6). These three orientations may be present within the same lamellae or superimposed on top of each other. The latter possibility could be in good agreement with a recently proposed molecular model for the LPP [54], and with the broad-narrow-broad (Landmann-unit) sequence visible in ruthenium tetroxide-stained stratum corneum sections [37–41], which suggest that these lamellae consist of three lipid layers. The alignment of the lipid layers is probably of importance for a proper barrier function, because mismatches between crystallites, occurring when the lateral or lamellar organization is not maintained, are sites where permeability for compounds may increase.

WAXD and ED patterns obtained with stratum corneum revealed spacings corresponding to 0.406 and 0.367 nm reflections, which are slightly shorter than observed in orthorhombic and hexagonal lattices in phospholipid based systems. Possibly, due to a strong attractive van der Waals interaction between hydrocarbon chains of the very long hydrocarbon chains of the CERs and FFAs, the hydrocarbon chains are slightly denser packed as compared to that formed in phospholipid based systems [55]. Furthermore, already two decades ago it has been shown by Pascher [56,57] that an extensive network of hydrogen bonds exists between the head groups of CERs, which promotes the formation of a very dense lattice. The surface area per acyl chain in the structure corresponding to hexagonal and orthorhombic sublattice in human skin appears to be approximately 0.190 nm² and 0.179 nm², respectively.
2.3. Lipid organization in stratum corneum isolated from dry or diseased skin

In order to determine whether an altered lipid composition results in an altered lipid phase behavior studies have been carried out with stratum corneum derived from dry and diseased skin (LI, atopic dermatitis and type 2 Gauser disease). In the 1980s, the lipid organization in essential fatty acid deficient (EFAD) stratum corneum [58,38] has been elucidated. It appeared that elimination of linoleic acid from the diet resulted in pig stratum corneum in a progressive increase in the olate content in CER1 at the expense of the linoleate content. This increase in CER1-oleate content was accompanied by a strong reduction in the skin barrier. However, both electron microscopy and SAXD studies revealed no drastic changes in the stratum corneum lipid lamellar organization in spite of that there was clear evidence that in EFAD skin the lamellar bodies contain amorphous rather than lamellar material. In EFAD animals only a great variability in the number of intercellular lipid lamellae was reported. It seems that other factors, such as a change in lateral packing, may also play a role in the formation of competent skin barrier (see below). The effect of the CER1-oleate/CER1-linoleate ratio on barrier properties and lipid organization is of interest, as in normal skin this ratio decreases dramatically during the winter season [59]. This increased ratio in CER1-oleate/CER1-linoleate is one of the characteristics of cosmetically dry skin.

Dry skin was selected because of its low content of CER1 [60]. Examination of SAXD patterns in volunteers participating in the dry skin study revealed that the only feature that is uniquely related to the 13 nm phase is its 3rd order diffraction peak (see Fig. 4A); in stratum corneum samples in which the 3rd order peak was absent the contents of both CER1 and CER4 were also reduced [62], see Table 1. This relation between CER1 and CER4 contents and the presence of the 3rd order diffraction peak suggests that at low level of CER1 and CER4 the formation of the LPP is reduced in the in vivo situation. However, it cannot be excluded that the decrease in intensity of the 3rd order diffraction peak is caused by a dramatic altered lipid organization within this LPP. It should be noted that in the absence of the 3rd order peak the reduction in CER1 content is more pronounced than the reduction in CER4 content. In contrast to studies of Imokawa et al. on the stratum corneum of the flexor forearm of three healthy volunteers was compared to that of LI patients [63]. It was very obvious that in LI patients the lipids formed a hexagonal lateral packing coexisting with only a small number of crystals forming an orthorhombic packing. Fig. 8 shows some characteristic ED patterns that have been recorded in the stratum corneum of LI patients. The appearance of orthorhombic ED patterns differed from control samples as it mainly appeared as small faint spots next to the hexagonal lattice and a fluid phase was clearly observed already at physiological temperature. In fact, these differences in lateral packing were much more pronounced than those observed in stratum corneum of atopic dermatitis patients. The reduced FFA levels in LI patients (Table 2) form the most likely explanation for this observation. However, as a reduction in the chain length of either CERs or FFAs might also occur, it cannot be excluded that this may play a role as well. To unravel whether changes observed in lipid organization in diseased and dry skin can be explained by an altered lipid composition or whether other factors also play a role can be obtained by systematic phase behavior studies with mixtures containing major stratum corneum lipids.

The changes in the lateral packing and in the lamellar organization observed in the stratum corneum of LI patients may account for the aberrations in stratum corneum barrier function. Several studies on lipid phase behavior in both phospholipid membranes [65] and in stratum corneum [66] have shown that coexisting phases (e.g. during phase transitions) lead to increased permeability of certain compounds. The reason for this may be an increase in alkyl chain mobility as a result of which compounds can cross lipid layers more readily. Furthermore, lipid layers may show leakage between grain
mosomes was altered [64]. The lamellar body extrusion process lamellar organization; moreover the morphology of corneodesmosomes was shown that aberrations are present with respect to the be possible as well. Using freeze fracture electron microscopy, it could be observed in the same ED patterns.

Increased levels [14]. Not only the lipid lamellar phases were distorted as in stratum corneum [43,45]. In CHOL:CER mixtures, the lipids form a hexagonal lateral packing (Fig. 5B and C) independent of the lamellar body extrusion process appears normal in LI skin although the reorganization of the lipid stacks into lamellar sheets seems to be incomplete [68,69,63]. Other studies on LI skin reported changes in the cornocyte envelope [1,2,70–72] as well as defects in the gene coding for the enzyme transglutaminase. The latter is involved in the cornified envelope formation by catalyzing the cross-linking of precursor proteins such as involucrin [73]. These findings may also contribute to the impaired stratum corneum barrier function. Thus, it seems likely that stratum corneum barrier function depends on both lipid organization and protein structures.

Changes in barrier function and lipid composition and organization in stratum corneum of Gauser Disease patients has also been reported. Infants with type 2 Gauser disease revealed an abnormal lipid organization in stratum corneum and an increased level of glucosylceramides compared to the CER levels [14]. Not only the lipid lamellar phases were distorted as revealed by electron microscopy and SAXD, but also the lateral packing was drastically changed to a more fluid one (unpublished results). The increased glucosylceramide/CER ratio is most likely responsible for the abnormal lamellar organization in these patients, confirming that the conversion from glucosylceramides to CERs at the interface between stratum granulosum and stratum corneum is very important for a proper establishment of a competent skin barrier.

3. Relation between lipid composition and organization

3.1. Mixtures based on isolated human CERs

Due to the complexity of the native tissue it difficult to delineate the link between the lipid organization and lipid composition. Such studies with native tissue are often hampered by the low availability of the required material especially when it is derived from the diseased skin. As it is impossible to modulate systematically lipid composition in the stratum corneum, the use of lipid mixtures isolated from stratum corneum offers an attractive alternative. In our initial first series of studies mixtures of CHOL, FFAs and pig CERs were used, as pig stratum corneum is readily available and its lipid organization is very similar to that of human stratum corneum [74]. However, in this paper we will mainly review the studies with human CERS as these are more relevant in explaining the results obtained with stratum corneum isolated from diseased, dry and reconstructed skin.

In mixtures prepared from CHOL and CERS two lamellar phases with periodicities of 5.4 and 12.8 nm were formed, mimicking lipid phase behavior in stratum corneum [75]. The presence of LPP was dominant and remained unchanged over a wide range of CHOL:CER molar ratios (between 0.2 and 1) indicating that the formation of the lamellar phases is insensitive towards changes in CHOL:CER molar ratio. At CHOL:CER molar ratio between 0.6 and 1 also the presence of crystalline CHOL is observed. The high insensitivity of the lipid organization towards changes in the CHOL:CER molar ratio suggests that in the in vivo situation a variation in CHOL:CER molar ratio will not lead to a substantial change in lipid phase behavior, but that CHOL will form separate crystalline domains when exceeding the amount necessary to saturate the lamellae. The presence of phase separated CHOL is indeed also observed in stratum corneum [43,45]. In CHOL:CER mixtures, the lipids form a hexagonal lateral packing (Fig. 5B and C) independent

<table>
<thead>
<tr>
<th>LI</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak positions</td>
<td>5.3–5.9 nm</td>
</tr>
<tr>
<td>CHOL/CER</td>
<td>0.59</td>
</tr>
<tr>
<td>FFA/CHOL</td>
<td>0.18</td>
</tr>
<tr>
<td>FFA/CER</td>
<td>0.12</td>
</tr>
</tbody>
</table>

In stratum corneum of LI skin the lipid composition and lipid organization are different from that in stratum corneum of normal skin.
of the CHOL:CER molar ratio. In this respect the phase behavior in CHOL:CER mixtures is different from that observed with CHOL:dipalmitoylphosphatidylcholine mixtures, in which a hexagonal lateral packing is observed at low CHOL content, but at increased CHOL content a phase transition from a hexagonal to an ordered fluid phase has been observed \[76–78\] demonstrating the very important role the CERs play in the skin barrier function.

As FFAs also belong to the major stratum corneum lipid classes, experiments with lipid mixtures containing CHOL, CERs and FFAs have also been performed. To mimic the FFA composition in intact stratum corneum, a FFA mixture containing predominantly long chain fatty acids (C22 and C24) has been used. In the presence of FFA the formation of the short periodicity phase 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 stratum corneum. Furthermore, the addition of long chain FFAs induced a phase transition from a hexagonal to an orthorhombic lattice and therefore increased the lipid density in the structure \[79\]. This phase transition was not observed with mixtures containing predominantly short chain (C16 and C18) fatty acids \[74\]. In the presence of long-chain FFAs besides an orthorhombic and/or hexagonal phase also a liquid phase was formed, as the broad reflection at 0.46 nm (indicative for a liquid phase) was clearly present.

As often a deviation in CER composition and in lipid organization occurs in diseased skin \[14–23,60,61,80\], individual CER subclasses may play an important role in stratum corneum lipid phase behavior. Using CER isolated from human stratum corneum, we predominantly focused on the role of CER1. Phase behavior studies with equimolar CHOL:CER mixtures lacking CER1 clearly revealed the LPP was only weakly present, indicating that the CER1 plays a crucial role in the formation of the LPP \[81–83\].

To elucidate the role of fatty acid linked to \(\omega\)-hydroxy acid in stratum corneum lipid organization, natural CER1 was replaced by either synthetic CER1-linoleate (CER1-lin), by CER1-oleate (CER1-ol) or by CER1-stearate (CER1-ste) \[84\]. Interestingly, when CER1 was substituted by CER1-ste, no liquid phase could be detected, while substitution by either CER1-ol or CER1-lin revealed the presence of the liquid phase. As far as the lamellar phases are concerned, the LPP was not present in CER:CHOL mixtures in which CER1 was substituted for CER1-ste, while the LPP was dominantly present in mixtures in which CER1 was substituted by either CER1-lin or CER1-ol. The results of these studies indicate that for the formation of the LPP a certain (optimal) fraction of lipids has to form a liquid phase.

3.2. Lipid mixtures based on synthetic CERs

In contrast to the previous investigation, in which solely mixtures prepared with CERs isolated from native tissue were studied, in a very recent study \[85\] the lipid organization in mixtures prepared with various synthetic CERs with defined acyl chain length was investigated. The initial CER mixture comprised 15% CER1(C30), 51% CER2(C24), 16% CER3 (C24), 4% CER4(C24), 9% CER3(C16) and 5% CER6(C24),
resembling the pigCER composition in pig stratum corneum, having either a fatty acid chain length linked to the (phyto)sphingosine base of 30 (C30), 24 (C24) or 16(C16) carbon atoms. Subsequently, the composition of the CER mixture was gradually modified to elucidate the role various synthetic CERs play in the formation of the LPP. As the synthetic counterpart of natural CER5 is not available, the CER mixtures were prepared with CER3(C16).

The lipid organization in equimolar mixtures of CHOL, synthetic CERs and FFAs closely resembles that in stratum corneum, as both LPP (12.2. nm) and SPP (5.4 nm) are present and the lateral packing of the lipids is orthorhombic. Furthermore, a minor fraction of CHOL phase separates into crystalline domains, and no additional phases can be detected. Interestingly, FFAs are required for proper lipid organization, as only in their presence a dominant formation of the LPP could be detected. This observation is different from that made with mixtures prepared with natural CERs, in which the two lamellar phases are already formed when mixtures contain only CERs and CHOL. This difference in phase behavior might be related to the limited acyl chain length variation in the synthetic CER mixtures.

The results described above unequivocally demonstrate that mixtures of CHOL, FFAs and synthetic CERs containing CER1, CER2, CER3, CER4 and CER6 offer an attractive tool to unravel the importance of individual CERs for proper stratum corneum lipid organization. The results show that the presence of CER1 and a variation in acyl chain length distribution are crucial for the formation of the LPP, but reduction in the levels of other CER subclasses does not affect the formation of the lamellar phases. These results are very similar to those observed with mixtures containing natural pigCER [81].

To examine whether mixtures of CHOL, FFAs and CERs applied on an appropriate substrate can be used to replace native stratum corneum, recently a stratum corneum substitute has been developed. The barrier properties of the stratum corneum substitute were evaluated in a series of in vitro passive diffusion studies, using three structurally related compounds, namely p-aminobenzoic acid (PABA), ethyl-PABA and butyl-PABA. PABA is the most hydrophilic compound and its lipophilicity increases with increasing ester chain length. The diffusion profiles of all three model compounds across 12 µm thick lipid membranes closely resemble those of human stratum corneum. Exclusion of CER1 from the lipid mixture revealed a reduced barrier function of the stratum corneum substitute, demonstrating that CER1 is not only very important for the proper skin lipid phase behavior, but also for the skin barrier function. This indicates that the reduced barrier function in psoriatic and LI skin might partially be caused by a reduction in CER1 in stratum corneum [86].

4. A unique molecular arrangement in the long periodicity phase

In 1989 a model that describes the molecular arrangement in the LPP was proposed for the first time [87]. Based on the broad-narrow-broad pattern obtained after fixation of pig skin with ruthenium tetroxide, a trilayer model was proposed. In this model the CERs are arranged in a planar arrangement and the linoleic moiety of CER1 is not located in the narrow layer, but is randomly distributed in the two broad layers adjacent on both sides of the narrow layer. Furthermore, the CHOL interfacial area is assumed to be similar to that of the CERs in planar alignment. Based on more recent knowledge about the lipid phase behavior of CHOL:pigCER and CHOL:CER mixtures another trilayer model has been proposed for the molecular arrangement of the LPP [82,88,89] see Fig. 9. In this model the CERs are either partly interdigitating (the broad low-electron density layers) or fully interdigitating (the narrow low-electron density layer in the center of the lamellae) and are arranged tale to tale in a double arrangement in the lipid layers. The two broad low-electron density regions are formed by CERs with the long-chain fatty acids (predominantly C24 to C26) linked to the (phyto)sphingosine backbone and by CHOL, while the narrow low-electron density region is formed by CHOL and the unsaturated C18 acyl chain linked to the ω-hydroxy fatty acid. The proposed molecular model is based on the electron density profile of the repeating unit calculated from the intensities of the 1st to 10th order of the lamellar pattern attributed to the LPP in CHOL:pigCER mixtures [82] resulted in a sequence of broad-narrow-broad low electron density regions with in between the higher electron density regions corresponding to the head groups. However, it has to be noticed that the electron density profile of the trilayer arrangements is not a unique solution for the intensities of diffraction peaks of the LPP. Therefore this trilayer arrangement is also proposed based on other observations, such as a) the crucial role of CER1 in the formation of the long periodicity phase and [81,82], b) the bimodal fatty acid chain-length distribution of the CERs, c) the observed phase separation in a mixture containing CERs with long acyl chains and short acyl chains [90]. The LPP consists of three different regions in which the liquid sublattice is located in the central lipid layer. There, mainly unsaturated linoleic acid and CHOL are present. Adjacent to this central layer two regions are located in which a gradual change in lipid mobility occurs in the direction perpendicular to the central plane is proposed. Very recently FTIR studies were performed of lipid mixtures in which the Linoleate moiety of CER1 was deuterated (unpublished results). These studies revealed the presence of linoleate in a liquid state, which is in agreement with the sandwich model. The decreased mobility in these adjacent layers can be attributed to the presence of less mobile long saturated hydrocarbon chains. As only a small fraction of lipids forms a fluid phase in the stratum corneum, it is assumed that this central lipid layer is not a continuous one. Because the lipid lamellae are mainly oriented parallel to the surface of the corneocytes, substances always have to pass the crystalline lipid lamellar region and partly diffuse through the less densely packed lipid regions parallel to these regions. In this way an excellent barrier is maintained, even when a fluid phase is present. When comparing our model with the molecular model proposed by Swartzendruber et al. [87], our model suggests an approximately equal interfacial area of CHOL and of CERs having a tail to tail arrangement, while Swartzendruber’s model suggests an equal interfacial area of CHOL and CERs in a
planar alignment. Dahler and Pascher reported an interfacial area of CERs in planar arrangement of approximately 0.25 nm² [91]. This is different from that of CHOL (0.37 nm²). The interfacial area of CERs having a tail to tail double arrangement is predicted to be approximately 0.40 nm² [90], which is indeed a value close to that of CHOL. Furthermore, in molecular model proposed by Swarzendruber et al. [87] the presence of a liquid layer is not possible, due to the random distribution of the linoleate of CER. It is a task for future studies to validate whether the proposed “sandwich model” correctly represents the lipid organization of the in the stratum corneum. In 2003 McIntosh [92] studied CHOL:pigCER:palmitic acid mixtures with X-ray diffraction. In order to calculate electron density profiles swelling of the LPP was induced by adding cholesterol sulphate to very high ratios or by a pH shift up to a value of 8.5. This method results in a unique solution of the electron density profile based on the intensities of the diffraction peaks of the LPP and revealed electron density profile of the LPP indicative for a repeating unit containing two asymmetric bilayers, which is not in agreement with the trilayer arrangement. However, the studies of McIntosh et al. were performed with lipid compositions and environmental conditions not mimicking the natural situation in stratum corneum. Furthermore only 3 orders of the diffraction peaks attributed to the LPP were used for the electron density calculations. This makes an extrapolation to the situation in stratum corneum uncertain. Therefore more studies have to be carried out in order to determine whether a trilayer or a double layer arrangement of the lipids is present in the repeating unit of the LPP.

In 1993 Forslind proposed for the first time a model [93] for the presence of liquid phases in the stratum corneum. This model postulated the presence of a continuous liquid phase from the superficial layers of the stratum corneum down to the viable epidermis, the so-called mosaic model. Although this was the first model including the presence of a liquid phase in stratum corneum lipid structures, until now no experimental data are available to verify this model. In a recent paper, another model has been proposed for the stratum corneum lipid organization, called the Single Gel Phase Model. According to this model the intercellular lipids within the stratum corneum exist as “a single and coherent gel phase” and “this structure has virtually no boundaries” [50]. This gel phase is defined as a “crystalline lamellar lipid structure that usually has a hexagonal hydrocarbon chain packing”. Although this model is similar to our sandwich model with respect to the absence of grain boundaries, the Single Phase Model proposes in the deeper layers of the stratum corneum either the hexagonal phase or a coexistence of the hexagonal and orthorhombic phase such that the orthorhombic phase is located close to the head-group region and changes gradually into a hexagonal phase in the central region of the lamellae. If this is the case the orthorhombic and hexagonal phase should always be present simultaneously. However, as already mentioned in Section 3, no evidence has been found for the presence of the hexagonal phase in the lower stratum corneum regions. In addition, the orthorhombic phase was frequently observed without simultaneous presence of the hexagonal phase. Therefore, the “single gel phase model” is not confirmed by experimental data. Finally, in discussing “the
single gel phase model” no attention has been paid to the role individual lipids play in the lipid organization, this includes (i) the crucial role of CER1 in the formation of the 13 nm lamellar phase and its influence on the phase behavior; (ii) the presence (or absence) of long-chain free fatty acids that facilitate the formation of the orthorhombic packing in vitro as well as in vivo, as shown in diseased skin.

5. Extrapolation of the in vitro finding to the lipid composition and organization in diseased and in dry skin

In atopic dermatitis patient [94,60] a reduced CER1 content alters the organization of lamellar phases [95], which reflects the observations made with lipid mixtures. There, in the absence of CER1 the formation of the 12–13 nm lamellar phase was reduced, while the formation of the 5–6 nm lamellar phase was strongly promoted. In addition, in dry skin, in skin during the winter season and in essential fatty acid deficient skin the CER1-ol content is increased at the expense of CER1-lin [59]. One can expect that changes in lipid phase behavior might also occur [84], as in CER1-ol containing lipid mixtures the presence of the liquid phase is increased as compared to the CER1-lin containing mixtures. In fact, in essential fatty acid deficient and dry skin a reduced skin barrier has been observed, while the ultrastructural appearance of the lipid lamellae was still similar to that of the normal skin [38,58]. It can be speculated that fraction of lipids forming a fluid phase increases. Although the presence of a subpopulation of lipids forming the fluid phase might be required for a proper functioning of the stratum corneum, an excessive presence of a fluid phase may lead to the reduction of the barrier function. Furthermore, we have also noticed that in CHOL:CER mixtures a hexagonal lateral packing is formed and that the presence of FFAs facilitates transformation of the hexagonal into an orthorhombic lateral packing. The prominent presence of the hexagonal lateral packing in LI skin [63] might be caused by the observed reduced content of FFAs in this skin disease. Finally, cholesterol sulfate was found to promote the formation of a fluid phase in mixtures prepared with pigCER [79]. An increase in cholesterol sulfate content, as observed in recessive x-linked ichthyosis skin [96], may further lead to reduction of the lattice density and consequently to an increase in the stratum corneum permeability and in this way can account for the observed reduction of barrier function in this skin disease.

6. Conclusions

Although impressive progress has been made in elucidating the lipid organization in stratum corneum, essential information is still missing. In fact even the lipid lamellar organization is still under debate as the localization of the lipids within the LPP and SPP is not fully understood. It is expected that by using lipid mixtures based on synthetic CERs, the role the individual subclasses of lipids play in the stratum corneum lipid organization will be elucidated in more detail. Furthermore, only limited information is available on the lipid organization and composition in stratum corneum of diseased skin. However, this unequivocally stresses that that there is a need to explore this scientific field in more details.

References


