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

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Chapter 8

Summary

The stratum corneum (SC), the thin uppermost layer of the skin, consists of dead flattened skin cells (corneocytes) embedded in a lipid matrix. The lipid matrix is considered to play a crucial role in the skin barrier function. It consists of ceramides (CER), cholesterol (CHOL) and free fatty acids (FFA) forming crystalline lipid lamellae. From studies with native SC and SC lipid models much information has been gained on the phase behavior of the SC lipid matrix. However, little is known about the correlation between SC lipid organization and the permeability of the SC. This is difficult to investigate using native SC, due to its complex structure. Therefore SC lipids were casted on a porous membrane, resulting in a lipid organization and lamellar orientation similar to that in SC. This lipid membrane is referred to as the stratum corneum substitute (SCS) (1, 2). The SCS can be used to perform diffusion studies. Therefore, when modifying the lipid composition and thus the lipid organization in the SCS, it is possible to study the relationship between lipid organization and permeability.

The main objectives of this thesis are 1) to investigate the influence of lipid organization on the barrier function in the SCS and 2) to obtain insights in the molecular organization within the unit cell of the lamellar phases in SC.

Part I: SCS as a tool to study the relation between lipid composition, organization and barrier function in one model

In previous studies the SCS was developed. However, the preparation method of the SCS was suboptimal. For this reason in chapter 2 two new methods were introduced to prepare the SCS, to improve the reproducibility and to increase the efficiency of the preparation method. Subsequently, the properties of the SCS prepared by the three methods, i.e. the manual airbrush method, the rotor airbrush method and the linomat method, were

investigated. The results show that the SCS prepared with the various methods share the properties of a uniform lipid composition and a homogeneous distribution of these lipid components over the substrate. Furthermore, irrespective of the preparation method, the lipids form two crystalline lamellar phases, mimicking the lipid organization and orientation in human SC very closely. In subsequent studies permeation profiles of benzoic acid through SCS were measured. These permeation profiles were very similar to that across human SC. The rotor method increases the efficiency and reproducibility compared to the manual airbrush method, while the linomat method reduces the lipid loss during preparation and results in SCS with a more uniform membrane thickness. Based on these results, the linomat method was selected as the preferred method for preparing the SCS.

After having optimized the quality and the preparation method of the SCS, in subsequent studies the SCS was used to determine the effect of lipid organization on the permeability of the SCS. These studies are described in chapter 3. We examined the effect of the orthorhombic to hexagonal phase transition on the barrier function of SCS and compared that with human SC. This was performed by monitoring the permeability to benzoic acid as function of temperature. Arrhenius plots were constructed. As the slope of the Arrhenius plots below and above the transition temperature was very similar, it was concluded that the orthorhombic to hexagonal phase transition does not affect the diffusivity of benzoic acid across the SCS. The benzoic acid flux as function of temperature across human SC and the SCS was very similar over a temperature region between 31 and 43°C. From the slopes the activation energies were calculated. The activation energy for the diffusivity of benzoic acid appeared to be very similar in SC and SCS. This confirms that the lipids form the main barrier for diffusion in human SC. In subsequent studies the SCS composition was modified by reducing the FFA chain length distribution from around 24 carbon atoms to around 18 carbon atoms in the fatty acid chain. This resulted in a hexagonal packing and a perturbed

lamellar organization. These changes in lipid organization resulted in a significant increased permeability to benzoic acid, which was related mainly to its perturbed lamellar organization. Thus, a proper lamellar organization is more crucial for a competent barrier function than the presence of an orthorhombic lateral packing.

In the studies described in chapter 4 we used the SCS to investigate the effect of changes in lipid organization on the barrier function, again using benzoic acid as model compound. First, in preparing the SCS we increased the level of one of the three major lipid classes (CER, CHOL or FFA) keeping the ratio between the other lipid classes constant. An increased CHOL level induced a higher amount of phase separated CHOL and a reduction in the permeability. An increase in CER or FFA level to twice the original level resulted in the formation of additional phases, but had no significant influence on the permeability. We also examined models that mimic selected changes in lipid compositions reported for dry or diseased skin. In seasonally dry skin (winter xerosis) elevated levels of CER EOS-oleate have been reported. This change in composition was induced in the SCS by replacing 50% of the CER EOS-linoleate by CER EOS-oleate. This change in lipid composition did not induce changes in the lipid organization. Permeation studies revealed a very similar barrier as in the normal SCS. A SCS was also prepared based on an altered CER profile observed in SC of involved psoriasis skin. Its lipid organization and barrier properties were again similar to normal SCS. However, a SCS that mimics an important aspect of the composition in recessive X-linked ichthyosis skin, namely an excess of cholesterol sulfate, displayed a twofold higher permeability as compared to normal SCS. This increase in permeability is possibly related to the formation of an additional, less ordered lipid phase in this model.

It is for the first time that a SC model is used to investigate not only the effect of the lipid composition on the lipid organization, but also to study the relationship between lipid organization and barrier function, which is a very relevant and unique feature of the SCS.

Part II: The molecular organization in the repeating units of the SC lamellar phases

From X-ray diffraction studies using isolated SC it is well known that two lamellar phases are present in the lipid matrix of the SC. One with a unique long periodicity of about 13 nm (the long periodicity phase or LPP) and another with a shorter periodicity of around 6 nm (the short periodicity phase or SPP). However, although a lot of information has been obtained on the lipid phase behaviour of the lipid classes in SC, the molecular arrangement of the CER, CHOL or FFA classes in the unit cell of the lamellar phases is largely unknown. Several studies revealed that CER EOS plays an important role in the formation of the LPP. Therefore, in the studies described in part II of this thesis, we investigated the different lamellar phases in mixtures with CER EOS as the only CER component, in mixtures with all major CER classes present (including CER EOS) and in mixtures with the major CER classes present except for CER EOS.

Firstly, we investigated whether CER EOS in the absence of the other CER subclasses mixed with CHOL and FFA forms similar phases as observed in SC. These studies are described in chapter 5. The phase behaviour was examined using small angle X-ray diffraction (SAXD) and Fourier transformed infrared spectroscopy (FTIR). Our SAXD studies reveal that an equimolar ratio of EOS, CHOL and FFA forms a lamellar phase with an unusual long repeat distance of approximately 14.7 nm, different from that observed in SC. When focusing on the CH_2 stretching frequencies that provide information on the conformational disordering of the lipid chains, an exceptional thermotropic response was measured. The FFA and the CER chains undergo an order-disorder transition in different temperature ranges, indicating that at least a fraction of the FFA and CER do not mix. However, we also noticed by measuring the scissoring vibrations in the FTIR spectrum that a part of the hydrocarbon chains of CER and FFA are mixing in the orthorhombic lattice. Based on these observations, the molecular structure of the CER and the length of the unit cell, a molecular model for the 14.7 nm

lamellar phase has been proposed. This model is composed of three different lipid layers forming a symmetric arrangement in the unit cell. Based on this model and on the periodicity of 14.7 nm of the lamellar phase, it was concluded that the arrangement in the repeating unit is different from that proposed in chapter 6 for the LPP, suggesting that indeed additional CER subclasses are required to form the LPP.

In chapter 6 the molecular structure of the unit cell of the LPP in SC is investigated in 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 unique lamellar phase, we performed SAXD using various lipid mixtures containing all CER subclasses, mimicking the lipid composition in SC. These lipid mixtures formed the LPP with a slight variation in repeat distance. In the SAXD pattern of each mixture at least 6 diffraction orders were observed, attributed to the LPP with a repeat distance ranging from 12.1 to 13.8 nm. Using Shannon's sampling theorem we determined phase angles for the 6 structure factors associated to the first 6 diffraction orders of the LPP. By Fourier synthesis, using the 6 structure factors and phase angles, for the LPP a high resolution electron density distribution could be constructed. The density distribution suggests a unit cell with three lipid bilayers of 4.5, 4.0 and 4.5 nm in width. Subsequently, from SAXD patterns of isolated SC the electron density distribution of the lamellar phase was also constructed and appeared to be very similar to that in the lipid mixtures. This demonstrates that the lipid mixtures serve as an excellent model for the lipid organization in SC, not only with respect to the repeat distance of the LPP, but also in terms of the molecular arrangement within the unit cell.

In chapter 7 the molecular structure of the SPP is investigated into detail. To gain more insight into the molecular organization of the short periodicity lamellar phase we performed neutron diffraction studies on a mixture with all CER subclasses except EOS. In the diffraction pattern, five diffraction orders were observed attributed to the SPP with a repeat distance of 5.4 nm. Using

contrast variation by changing the H_2O/D_2O ratio, the scattering length density profile could be calculated. This density profile suggests a typical bilayer arrangement. To obtain information on the arrangement of the CER in the unit cell, a mixture that included a partly deuterated CER was also examined. The scattering length density profile of the 5.4 nm phase containing this deuterated CER demonstrated a symmetric arrangement of the CER with interdigitating acyl chains in the center of the unit cell.
The lamellar phases play a crucial role in the barrier function of the SC and the studies described above reveal new insights into their molecular structures. It is for the first time that the molecular structure in the lamellar phases is described with this level of detail.

Conclusions

In our first studies, the preparation procedure of the SCS was optimized while a proper lipid organization was maintained. In previous studies it was shown that the SCS closely mimics the human SC concerning lipid organization and barrier function. In the subsequent studies described in this thesis we have shown that the SCS is also a very suitable model for studying the relation between lipid organization and barrier function. Interestingly, based on the results obtained in these studies it appears that the crystalline lateral packing in the SC lipids is of less importance for the barrier function than the presence of the correct lamellar phases. Furthermore, as the formation of the proper lamellar phases is of crucial importance for the skin barrier function, the molecular organization within their repeating units was investigated into more detail. We studied the different lamellar phases present in mixtures with EOS as the only CER, in mixtures with all CER subclasses present and in mixtures with all CER subclasses except EOS. These studies showed a unique very long periodicity phase in the mixture with only CER EOS, of which the molecular arrangement in the unit cell appears to be different from that in the unit cell of the LPP. Studies on mixtures with all CER subclasses revealed the LPP with a trilayer unit cell

and studies on mixtures in absence of EOS demonstrated a SPP with a symmetric ceramide arrangement in the unit cell. Furthermore, based on X-ray studies with native SC, the molecular arrangement in the unit cell of the LPP in the models appears to be very similar to that in native SC. As the LPP is a unique lamellar phase in the SC and is considered to play an important role in the skin barrier function, the latter observation confirms that the mixtures form very relevant models for studying the SC lipid organization.

Perspectives

Although the studies presented in this thesis demonstrate that the SCS is an excellent model for studying the SC lipid organization and barrier function, the permeation studies have been performed with only one model drug (benzoic acid). Benzoic acid was chosen for its medium lipophilicity (logP = 1.9), low molecular weight (mw = 122 Da) and medium water solubility (3.4 g/l), making it a suitable molecule for skin permeation studies. In previous permeability studies similar compounds with a variation in logP value between 0.6 and 2.6 were also used to compare the flux profile across SCS and human SC, exhibiting an excellent correlation (2). However, very hydrophilic and very lipophilic drugs have not yet been assessed in permeability studies to compare the barrier function of SCS with human SC. This is of interest to perform in future studies. From our studies we may conclude that the SCS is an attractive tool to study the effect of changes in lipid organization on the barrier function. In the studies presented in this thesis models mimicking the composition in SC of winter xerosis, psoriasis and x-linked ichtyosis were evaluated. However, other skin diseases in which the SC lipid composition is affected (and quantified by chromatography methods) can be mimicked as well, by adapting the lipid composition in the SCS. For example, an altered SC lipid composition was also reported for type 2 Gaucher's disease (3-5), lamellar ichthyosis (6) and atopic eczema (7-15). The consequences of such changes in lipid

organization to the contribution of an impaired skin barrier function can in principle be studied using the SCS. Of course one should keep in mind that this is only one aspect of a reduction in skin barrier function. Also changes in the barrier proteins have been reported that affect for example the structure of the cornified envelope or shedding of the corneocytes. These changes are likely to influence the skin barrier function as well. Besides mimicking the composition in diseased skin it is also of interest to use the SCS to investigate the effect of penetration enhancers and moisturizers on the lipid organization and permeability of the SC lipid lamellae.

Concerning the permeation pathway of a permeant molecule through the SC, although the tortuous intercellular pathway has been suggested to be the preferred route for most drug molecules (16), detailed information on the penetration route within a stack of SC lipid lamellae is not available. The permeation through stacks of lamellae within the intercellular spaces in SC itself may also follow a tortuous pathway, effectively lengthening the total permeation pathway through SC or the SCS. By fitting the permeation data of a passive diffusion experiment to the diffusion formula (17), see figure 1A, in theory both the length of the permeation pathway (L) as well as the diffusion constant (D) of the permeant molecule can be calculated. These parameters could lead to new insights on the permeation pathway. However, to successfully determine L and D, it is necessary to accurately determine the partition coefficient (K) by assessing the solubility of the model compound in the donor solution and in the lipids of SC or SCS. Since the SC is a heterogeneous membrane with lipid domains and corneocytes, it is a challenge to obtain a partition coefficient between donor solution and SC lipids. To determine the partition coefficient, the SCS can also replace the SC. For example, with the SCS using benzoic acid as permeant, preliminary measurements revealed a partition coefficient between the SCS lipid film and the donor solution (2 mg/ml benzoic acid in PBS 7.4) of K = 5.3 (unpublished data).

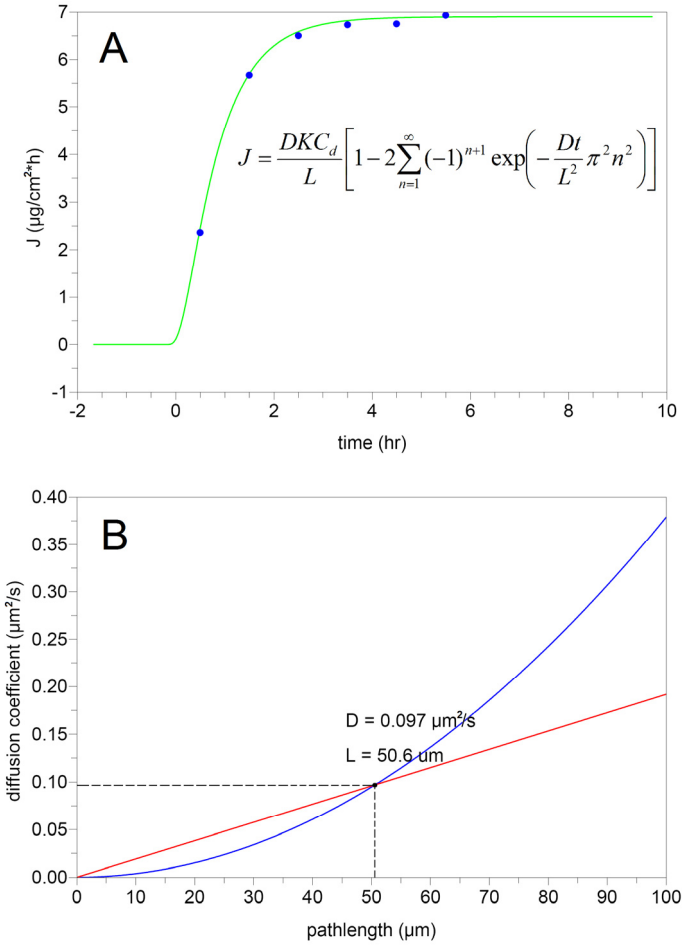

Figure 1: A) Plot of the benzoic acid flux trough a SCS model membrane composed of CER, CHOL and FFA in molar ratios of 1:2:1. The dots denote the measured flux values while the solid line denotes the fit of the diffusion formula (also shown in figure A) with the measured flux values. B) Solutions for D and L from the fit of the diffusion formula with the flux data in (A). The blue line depicts all possible values for D/L resulting from a fit with the steady state part of the flux data and the red line depicts all values for D/L^2 resulting from a fit with the up-going part of the flux data. A unique solution for both D and L is found when the two lines (denoting both fits) coincide. These values are also depicted in figure B.

In a first attempt, fitting the diffusion equation to permeation data of benzoic acid through a SCS (with CER:CHOL:FFA composition of 1:2:1) resulted in approximate values for L of 51 μm and D of 0.1 $\mu m^2/s$ (see figure 1B, unpublished data). When combining these values with the thickness of the lipid membranes, being around 13 μm, we may conclude that the diffusion occurs partly parallel to the basal plane of the lipid lamellae.

Regarding the molecular structure in the lamellar phases of SC, neutron and x-ray diffraction experiments using SC lipid models can be used for unraveling the lipid arrangement in the LPP and in the SPP. By specifically deuterating one of the major components (CER, CHOL or FFA) more insights can be gained on the location of each of these components, or even on the location of their headgroups or tails in the unit cell. When focusing on the molecular arrangement of the LPP, according to the x-ray results presented in this thesis, the electron density profile of the repeating unit is symmetric and therefore in principle it is possible to perform neutron diffraction studies using H_2O/D_2O contrast to resolve the density pattern and to locate the position of deuterated (parts of) molecules in the LPP unit cell. However, recent preliminary results suggest that the water in the LPP unit cell is not (or not only) located at the borders of the unit cell (in the headgroup regions, as is normally the case in lipid bilayers), but (also) at specific positions inside the unit cell. Therefore, as the method for resolving the density profile using H_2O/D_2O contrast is based on the location of the water molecules being only at the borders of the unit cell, it will be a challenge to resolve the molecular structure of the LPP in more detail and to localize several subclasses of ceramides within this repeating unit.

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