Systematic investigations into the role of ceramide subclass composition on lipid organization and skin barrier

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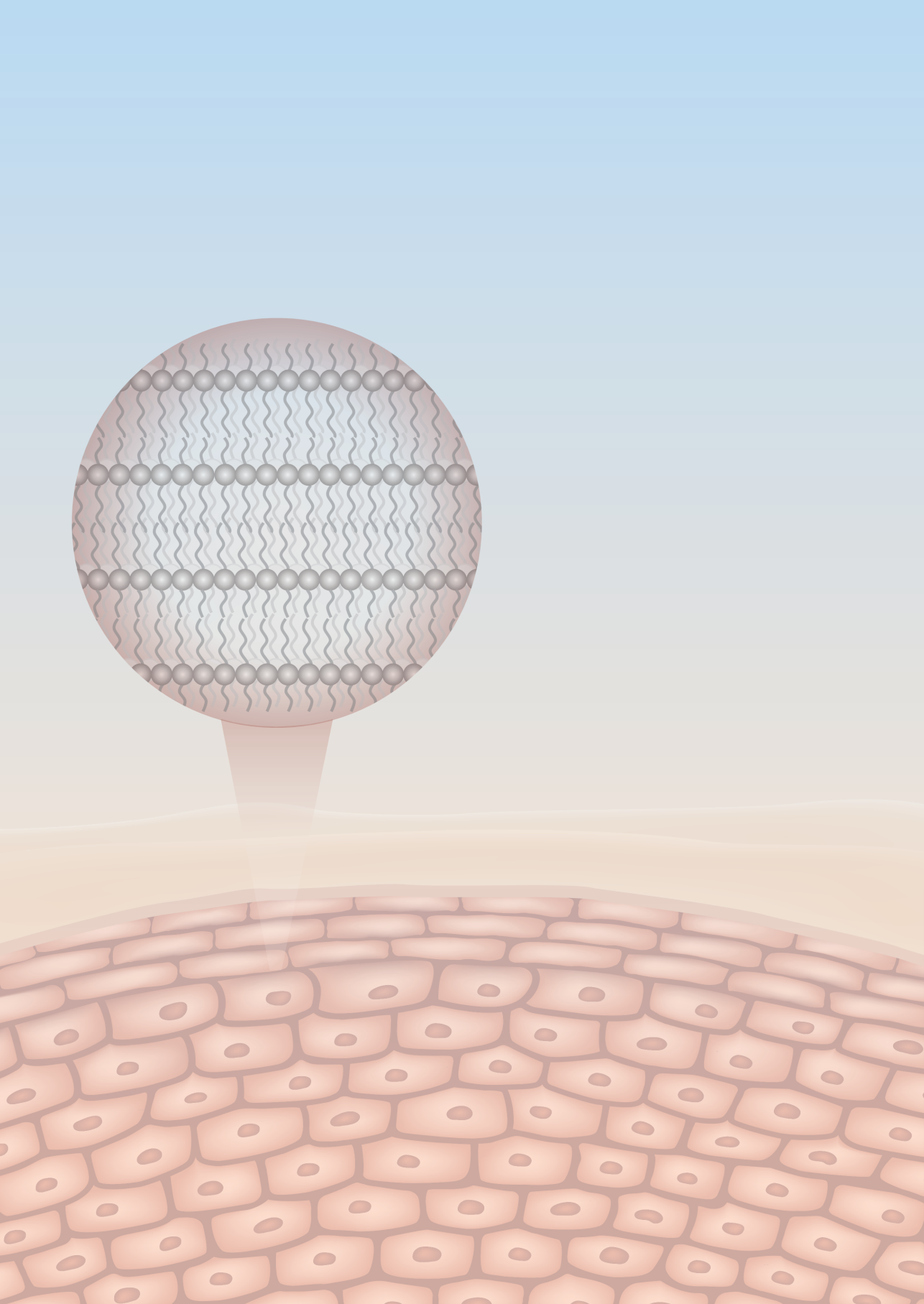

CHAPTER 7

Summary and perspectives

SUMMARY

The barrier function of the skin is attributed to the outer-most layer, the stratum corneum (SC), that protects the body from pathogens from the external environment and prevents water loss from the body (1, 2). The SC comprises of corneocytes (keratin-rich dead cells), embedded in a lipid matrix. The latter represents an important pathway for compounds permeating through the skin. The main SC lipid classes are ceramides (CERs), cholesterol (CHOL) and free fatty acids (FFAs), present in the SC in an approximately equimolar ratio (3).

X-ray diffraction investigations revealed that the SC lipids form two distinct crystalline lamellar phases, identified as the short periodicity phase (SPP) and long periodicity phase (LPP), each characterized by a repeat distance of approximately 6 and 13 nm, respectively (4, 5). The LPP has a trilayer structure (one central layer and two outer layers), is exclusively found in SC and is reported as crucial for maintaining the skin barrier function (6). Within the lamellar structure, most of the SC lipids are organized in a dense orthorhombic lateral packing (at skin temperature, ~32°C), while a fraction of the lipids adopts a less dense hexagonal organization (7, 8).

In inflammatory skin diseases, such as atopic dermatitis, psoriasis or Netherton syndrome, the SC lipid composition is different compared to healthy SC and the barrier function of the skin is impaired (9-18). Some of the lipid changes commonly reported for these three skin diseases are: an increased fraction of unsaturated CERs and FFAs, shorter carbon chain length of the CERs and FFAs and altered CER subclass ratios. In clinical studies these alterations were previously correlated with the barrier function, measured by trans-epidermal water loss (TEWL). However, as these lipid compositional changes occur simultaneously in diseased SC, the primary factor responsible for the increased TEWL cannot be identified in clinical studies. Lipid models represent an excellent alternative for studying systematically the role of lipid characteristics on the skin barrier function. This is explained in **Chapter 1** of this thesis.

The studies described in this thesis focused on understanding the effect of the CER subclass composition on the lipid organization and barrier function. Firstly, the CER composition of the inflammatory skin disease seborrheic dermatitis (SD) was examined in a clinical study. Subsequent studies using lipid model systems aimed to investigate the influence of changes in the CER subclass composition as observed in SD on the lipid organization, the lipid barrier function and the conformation of CER NS and CER NP.

In **Chapter 2**, skin barrier impairment, changes in the microbiome, and CER compositional changes in the skin of seborrheic dermatitis (SD) patients are described. The main characteristics for this inflammatory skin disease are an imbalanced immune system resulting in inflammation, cutaneous microbial dysbiosis and an impaired skin barrier function. The aim of the study was to characterize the CER composition of lesional and non-lesional SC of 37 patients and relate that to the impaired barrier function. The results

showed a significantly impacted barrier in lesional SC as measured by TEWL and a significant correlation between TEWL values and several CER compositional changes: i) increased molar ratio of CER NS:CER NP, ii) elevated fraction of CER NS with a short chain length (C34), iii) increased degree of unsaturated CER NS and iv) decreased average total chain length of CERs (acyl chain + sphingosine base) in lesional SC. This indicates the interdependence of the impaired barrier function and the CER compositional changes in SD. The influence of the CER chain length, degree of unsaturation and increased level of CER NS with a short chain length have already been studied and these factors affect the lipid barrier in model systems. However, no information was reported so far about the effect of an altered CER NS:CER NP molar ratio.

Chapter 3 aimed to examine the location of CER *N*-(tetracosanoyl)-phytosphingosine (CER NP C24) in the unit cell of the LPP using neutron diffraction and compare its location with CER *N*-(tetracosanoyl)-sphingosine (CER NS C24). CER NP was selected as this CER subclass is the most abundant in the human SC and its location in the LPP unit cell was not determined yet. As deuterated lipids are required and interpretations in simple systems can be very detailed, the lipid model consists of only CER EOS, CER NS, CER NP, CHOL and FFA C24 (CER NS: CER NP ratio 1:1). The detailed analysis was performed by using Fourier transform infrared (FTIR) spectroscopy and neutron diffraction. The study showed that this lipid composition formed the LPP trilayer structure, with the repeating unit cell consisting of an inner layer and two outer layers. Within the LPP unit cell, CER NP adopts a similar location as CER NS, with the acyl chains of CER NP being localized predominantly in the inner layer of the LPP unit. FTIR studies showed that the acyl chains of CER NP and CER NS and the FFA C24 chains are neighboring and are all located primarily in the inner layer of the LPP unit cell. This also suggests that CER NP adopts a linear conformation, similar to that of CER NS. The results of this study were a starting point for understanding the importance of the CER NS:CER NP lipid ratio, which is further described in the next chapter.

The effect of an altered CER NS:CER NP ratio on lipid organization and lipid barrier was the focus of the research described in **Chapter 4**. This ratio is changed not only in SD, but also in other inflammatory skin diseases such as atopic dermatitis and psoriasis. The lipid models were prepared with the CER NS:CER NP ratio of 1:2 (mimicking the ratio in healthy SC) and 2:1 (mimicking the ratio in SC of inflammatory skin). Subsequently, the lipid organization and barrier function were examined. The lipids in both compositions formed the LPP. A combined approach using neutron diffraction and FTIR showed that within the LPP unit cell the position of CER NS and CER NP is very similar to that of the CER NS:CER NP 1:1 ratio. Both of these acyl chains were mainly positioned in the inner layer, but with a minor part located in the outer layers of the LPP. TEWL, a measure of barrier functionality, was significantly elevated in the CER NS:CER NP 2:1 model (mimicking the ratio in inflammatory skin diseases) compared to the CER NS:CER NP 1:2 ratio (representing healthy skin), while the flux of the model drug ethyl-p-aminobenzoic acid (E-

PABA) was not different in the two models. These findings offer a more in-depth understanding of lipid organization in both healthy and diseased skin, implying that in clinical studies, the molar ratio between CER NS:CER NP entailing the same chain length, contributes to barrier impairment, but it might not be the primary factor.

In the studies described in **Chapter 5** a lipid model forming exclusively the SPP was prepared to examine whether the change in CER NS: CER NP molar ratio has a similar effect on the lipid organization as that observed in the LPP. Simple lipid models were prepared with CER NS and CER NP (ratio 1:2 mimicking healthy SC ratio and 2:1 mimicking inflammatory skin diseases), mixed with CHOL and FFA C24. The results suggest that the acyl chains of CER NS, CER NP and FFAs are neighboring in the SPP unit cell and that the CER NS and CER NP adopt an extended CER conformation in the SPP unit, similar to the arrangement in the inner layer of the LPP models described in the previous chapter. The SPP NS:NP 1:2 model is characterized by the presence of a lipid domain containing phase-separated CER NP. Unlike the SPP model, phase separation is not observed in the LPP models and this suggests that the addition of CER EOS improves the miscibility of the lipids in the model. The two SPP compositions displayed a different hydrogen bonding network, as the CER NS:CER NP 1:2 model showed a stronger hydrogen bonding than the CER NS:CER NP 2:1 model, which might be determined by the intermolecular hydrogen bonding between the CER NP head groups.

In the previous chapters simple systems were used with only two or three CER subclasses. In **Chapter 6** studies are described in which the number of CER subclasses is expanded, to examine whether more complex mixtures affect the lipid organization, barrier function and the location of the acyl chains of CER NS and CER NP in the unit cell. First, CER compositions were examined with four CER subclasses, where either *N*-(2R-hydroxy-tetracosanoyl)-sphingosine (CER AS C24), *D*-(2R-hydroxy-tetracosanoyl)-phytosphingosine (CER AP C24) or CER *N*-(tetracosanoyl)-dihydrosphingosine (CER NdS C24) were used as an additional CER subclass. The lipid organization, lipid molecular arrangement and permeability of the LPP models were examined. The results show that the inclusion of an additional CER subclass did not impact the locations of the acyl chains of CER NS and CER NP in the LPP trilayer unit cell. Then finally, a complex lipid model mimicking the composition of the human SC CER subclass was examined. Again, the two acyl chains of CER NS and CER NP maintained their positions in the inner layer of the unit cell. This suggests a remarkable insensitivity of the location of the acyl chains of CER NS and CER NP in the LPP unit cell with respect to variations in CER subclass composition. However, the different head group structures of CER AS, CER AP and CER NdS had an effect on the hydrogen bonding network in the lipid models, as the addition of CER AP (with the most hydroxyl groups in the structure) resulted in a stronger hydrogen bonding in comparison with the other two models.

In summary, seborrheic dermatitis lesions are characterized by skin barrier dysfunction and an altered lipid composition and organization of the SC. The observed

changes in lipid composition and barrier perturbation are similar to other inflammatory skin diseases including atopic dermatitis, psoriasis and Netherton syndrome. Previously, the effect of some lipid compositional changes, such as the reduction of the level of CER EO subclass, lower chain length of the FFAs and CERs and the degree of unsaturation of the FFAs, were investigated using lipid models.

Reducing the concentration of the CER EO subclass (either CER EOS alone or a combination of CER EOS, CER EOH, CER EOP, and CER EOdS) in lipid models corresponds to a lower lipid fraction forming the LPP (6, 19, 20). This had an effect on the flux of model drugs such as indomethacin and E-PABA, which was increased by ~2-fold when the CER EO concentration was reduced. Another CER compositional change, the increased fraction of short chain CERs, also affected the barrier function, as the addition of CER NS C34 and CER AS C34 resulted in a higher E-PABA flux in a model mimicking the human CER composition (~2-fold increase compared to control) (20).

Changes in the FFA composition have a considerable impact on the barrier function. When FFA C24 was replaced by a mix of FFA with different chain lengths (FFA_{mix}, including the short chains of FFA C16 and C18), the fluxes of the model drugs indomethacin, theophylline and E-PABA were increased 3, 4 and 7-fold, respectively (21, 22). Increasing the fraction of short chain FFAs in the FFA_{mix}, determined a 3-fold higher flux of E-PABA in a model mimicking the human CER composition (20). An elevation of the level of mono-unsaturated free fatty acids (muFFAs) in the lipid composition, affected the lateral lipid organization by increasing the disordering in lipid chain conformations and it influenced the barrier function, as the flux of hydrocortisone was 5-fold higher, while the TEWL had approximately a 3-fold increase (23). However, these models contained a larger degree of unsaturation than detected in skin diseases, like atopic dermatitis.

In this thesis, translational studies are described focusing on the importance of the CER subclass composition on the barrier function. As presented in **Chapter 4** in the LPP models, there was a 1.5-fold increase of the TEWL values for the CER NS:CER NP 2:1 ratio (diseased SC) compared to the CER NS:CER NP 1:2 ratio (healthy SC), while no significant differences were observed for the diffusion of the model drug E-PABA. The studies described in **Chapter 4 and 5,** the effect of the CER NS:CER NP ratio in LPP and SPP models, and in **Chapter 6,** focusing on the CER subclass composition, result in a better understanding of the influence of this CER ratio on the lipid organization and barrier function. These studies show that the lamellar organization does not change when the CER head group composition is different (with the exception of CER EOS) and only minor changes are detected in the lateral packing.

Concluding, the results of this thesis show that a change in CER subclass composition contributes to the skin barrier disfunction and that the lamellar organization is very flexible when the CER subclass composition is altered. The results also suggest that CER NS:CER NP ratio might not be a major factor in the barrier impairment. Previous studies

show that the chain length reduction (primarily of FFA, but also of CERs) has more impact on the barrier function, compared to the CER subclass compositional changes.

The studies outlined in this thesis additionally demonstrate that simplified lipid model systems, when prepared with selected CER subclasses, can resemble key aspects of the human SC lipid organization. Simple models prove to be effective for conducting a more thorough investigation and gaining a deeper understanding of the lipid-related alterations observed in inflammatory skin diseases.

PERSPECTIVES

Perspectives for repairing the skin barrier

Restoring the skin barrier function through the application of topical formulations including emollients designed to enhance the compromised barrier has been a focal point in the treatment of patients with atopic dermatitis (24, 25). Future studies should aim to develop formulations including lipid subclasses that can restore the barrier in diseased SC. Regarding the CERs, these formulations should contain especially phytosphingosine-based CERs (CER NP and CER AP with a total chain length of at least 42 carbon atoms) and CER EO-subclasses (CER EOS, CER EOP), the latter having a total chain length of at least 66 carbon atoms. These total CER chain lengths of at least 42 and 66 carbon atoms are close to the most abundant chain lengths in normal SC. However, applying a topical cream with these long-chain CERs will not immediately repair the lipid compositional disbalance in the SC of inflammatory skin. First, the CERs need to partition in the SC lipid matrix and secondly, if successful, the short chain lengths of CERs (especially CER NS C34), high levels of CER NS subclass and the disbalance between in CER/FFA ratio are not yet corrected. However, during long-term topical treatment, the inflammation might be reduced, thereby maybe normalizing lipid synthesis in the viable epidermis. Therefore, in the long-term, topical treatment might improve lipid composition, lipid organization and the skin barrier function.

It is vital to comprehend the mechanism of the topical application, its interaction with SC lipids, and its impact on permeability when developing an effective topical formulation. In literature there are hardly any reports on the interactions of the formulations with the skin. A recent study showed that a topical cream, containing CER NP, CER AP, CER EOP and CHOL improved the lipid organization (measured using FTIR) after a 4-week application on eczema-prone skin, in comparison with a ceramide-free reference cream (26). Although the authors reported an improved lipid organization after the treatment, the conclusions were only based on the stretching vibrations. Moreover, there is no information on the partitioning of the lipids into the lipid matrix and no information about the altered lipid profile (CERs or FFAs) that might be caused by the treatment. Therefore, future studies should also include a thorough lipidomic analysis to elucidate the uptake of the topically

applied lipids into the lipid matrix of the SC. This can be investigated by including deuterated CERs in the formulation, which would allow a more detailed lipid analysis and lipid organization.

Previous studies mainly focused on the CER composition in the SC of inflammatory skin diseases, whereas there are only limited reports on the FFAs composition in (diseased) SC (16, 18). In future, more focus should be put on both the ratio between the CER:FFA and the FFA chain length distribution in diseased skin compared to controls. FFAs are crucial for the formation of the orthorhombic packing of the lipids (21, 27), while CERs usually have a less important effect on this packing. In atopic dermatitis, the hexagonal packing is increased at the expense of an orthorhombic packing, and the FFA/CER ratio seems to be lower (16). Especially longer chain length of FFAs (typically FFA C24) will be more effective in enhancing an orthorhombic packing compared to shorter-chain FFAs. Furthermore, it was previously shown that FFAs topically applied on skin can be intercalated in the lipid lamellae in the lipid matrix in SC (28). Therefore, including the FFAs with long chain lengths in a formulation could enhance barrier repair.

Some recent approaches are shifting from symptom control to using biologics to target individual inflammatory pathways. It was reported that, among others, interleukin (IL)-4 and IL-13 are the major cytokines involved in atopic dermatitis, as they interfere with the lipid synthesis in the skin by inhibiting the expression of elongases (29). Dupilumab, an antibody to the IL-4 receptor, has been approved as a treatment of atopic dermatitis and showed significant improvement in the severity of the disease (30). The effect of dupilumab on the SC lipid composition has not been thoroughly investigated, but an important first step has been reported by Berdyshev et al. (31). They reported an increase in the amount of CER EOS and a decrease in the fraction of CER NS in lesional and non-lesional skin already after 2 weeks treatment. The change in the amount of CER NS correlated with an improved skin barrier function as monitored by TEWL. A more detailed lipid analysis should be performed to further examine the entire CERs subclass composition, as well as the CER chain length distribution, and the CER:CHOL:FFA molar ratio, in comparison with control SC. As FFAs play an important role in the lateral packing, in future studies it would also be of interest to examine the FFA chain length distribution in lesional and non-lesional skin. Apart from the lipid composition, the lipid organization should also be examined after the treatment, to investigate whether the lamellar organization and the lateral packing are improved in the SC. This will result in a better understanding of the barrier repair mechanism.

Future studies using lipid model membranes

The studies performed in this thesis showed that when selecting the proper CER subclasses, the complexity of the CER subclass composition of a lipid model does not influence the lamellar phases and only minor changes are noticed in the lateral packing of the lipids. This was shown when comparing the LPP NS:NP models (with different CER

NS:CER NP molar ratio) to a complex model resembling the human CER subclass composition (Chapters 4 and 6). In all these compositions, a certain level of CER NS is present. These results are also in agreement with a very simple LPP model composed of only CER EOS and CER NS (mixed with FFAs and CHOL) (32). This CER composition (CER EOS/CER NS) could be used to further study the impact of the CER chain length changes on the barrier function in diseased skin. As CER NS with a large variety of chain lengths is commercially available (Avanti Polar Lipids, U.S.A.), this allows a systematic study to examine the chain length reduction of CER NS.

Previous studies revealed the important role of FFAs on the lipid organization and barrier function using lipid models. However, until now the molar ratio between CER:FFA was not the focus of lipid model studies. When performing clinical studies to determine the CER:FFA ratio in the different inflammatory skin diseases as suggested in the previous section, the effect of the altered CER:FFA molar ratio can be investigated using lipid models to examine its role in skin barrier impairment.

Lipid model systems can also be used to understand the mechanism of action of topical formulations. As discussed above, lipids that are lacking in the diseased SC lipid composition (CERs, FFAs) can be incorporated in various formulations aimed at repairing the barrier. A further investigation of their efficacy and mechanism of action could be performed using lipid model systems. Deuterated lipids of interest should be included in the formulation, which can be applied on the surface of lipid model systems. The uptake of the lipids can be examined by investigating the lateral and lamellar lipid organization and molecular arrangement of the lipid models. This will provide information about the mechanism of action of these formulations. For example, when measuring the scissoring frequencies using FTIR it can be examined whether deuterated lipids (could also be moisturizers) form mixed crystalline lattices with the SC lipids, or that they form separate domains. The barrier improvement could also be examined using TEWL measurements and permeability studies of these lipid models.

In addition to lipid compounds employed in topical formulations, the incorporation of moisturizers is a common practice to enhance the skin barrier. Lipid models can be used to examine the effect of lipophilic moisturizers on the skin barrier. X-ray diffraction and FTIR can offer insights into the influence of moisturizers on lamellar phases and lateral organization of lipid models after exposure to these compounds, while neutron diffraction can be employed to unravel the molecular interactions of the lipids with these molecules. The observations from these studies could potentially influence the development of methods aimed at repairing the skin barrier.

Molecular simulations of lipid models represent a valuable tool for validating experimental hypothesis and exploring structural features that are inaccessible to experimental methods. These simulations provide atom-level information that can contribute to a better understanding of lipid organization and barrier function. Further research should first aim to solve some of the current issues associated with the long-time

scale required for the simulation of large lipid systems and high computational cost (33). Future studies should focus on developing methods for the simulation of diffusion and permeability of compounds through the lipid systems. To predict the flux of chemical compounds though the skin, it is essential to integrate multiscale simulations with multiphasic diffusion models resembling a brick-and-mortar structure (the structure of the native SC), which account for the microscopic heterogeneity within the lipid matrix. Combining both experimental and simulation techniques would offer a comprehensive description of the lipid membrane structure and behavior.

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