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Modelling the stratum corneum lipid organisation with synthetic lipid mixtures: the importance of synthetic ceramide composition

Miranda W. de Jager[a], Gert S. Gooris[a], Igor P. Dolbnya[b], Maria Ponec[c], Joke A. Bouwstra[a,*]

[a] *Leiden/Amsterdam Center for Drug Research, Gorlaeus Laboratories, Department of Drug Delivery Technology, University of Leiden, P.O. Box 9502, 2300 RA Leiden, The Netherlands*

[b] *Netherlands Organisation for Scientific Research, DUBBLE CRG/ESRF, European Synchrotron Radiation Facility, Grenoble, France*

[c] *Department of Dermatology, Leiden University Medical Center, Leiden, The Netherlands*

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Abstract

Cholesterol (CHOL), free fatty acids (FFA) and nine classes of ceramides (CER1–CER9) form the main constituents of the intercellular lipid lamellae in stratum corneum (SC), which regulate the skin barrier function. Both the presence of a unique 13-nm lamellar phase, of which the formation depends on the presence of CER1, and its dense lateral packing are characteristic for the SC lipid organisation. The present study focuses on the lipid organisation in mixtures prepared with CHOL, FFA and a limited number of *synthetic* CER, namely CER1, CER3 and bovine brain CER type IV (ΣCERIV). The main objective is to determine the optimal molar ratio of CER3 to ΣCERIV for the formation of the 13-nm lamellar phase. CER3 contains a uniform acyl chain length, whereas ΣCERIV contains fatty acids with varying chain lengths. Using small angle X-ray diffraction (SAXD), it is demonstrated that the CER3 to ΣCERIV ratio affects the formation of the 13-nm lamellar phase and that the optimal ratio depends on the presence of FFA. Furthermore, the formation of the 13-nm lamellar phase is not very sensitive to variations in the total CER level, which is similar to the in vivo situation.

Keywords: Skin barrier; X-ray diffraction; Lipid organisation; Synthetic ceramide

1. Introduction

The barrier properties of mammalian skin can be ascribed to the unique composition and structure of its most apical layer, the stratum corneum (SC). The SC consists of flat corneocytes, embedded in an intercellular matrix of lipids. As these lipid domains form the only continuous structure in the SC, they are considered to play a crucial role in the maintenance of the skin barrier. Therefore, detailed information on the structure and organisation of the SC lipids is essential for a better understanding of the skin barrier function.

The main constituents of the intercellular lipid matrix are ceramides (CER), cholesterol (CHOL) and long-chain free fatty acids (FFA). Nine different extractable CER have been detected in human SC [1–4], which are classified as CER1 to CER9. The CER can be subdivided into three main groups, based on the nature of their head group architecture (sphingosine (S), phytosphingosine (P) or 6-hydroxysphingosine (H)). Through an amide bonding, long-chain non-hydroxy (N) or α-hydroxy (A) fatty acids with varying acyl chain lengths are chemically linked to the sphingosine bases. CER1 (EOS), CER4 (EOH) and CER9 (EOP) are unique in structure as they contain linoleic acid linked to an ω-hydroxy fatty acid (EO) with a chain length of 30 to 32 carbon atoms. The molecular structures of the CER together with the two nomenclatures are illustrated in Fig. 1A.

X-ray diffraction studies on native SC have demonstrated that the SC lipids are organised in two coexisting crystalline lamellar phases: the short periodicity phase (SPP) with a periodicity of approximately 6 nm and the long periodicity phase (LPP) with a periodicity of approximately 13 nm [5–7]. The 13-nm lamellar phase and its predominantly orthorhombic lipid packing are considered to be crucial for the skin

* Corresponding author. Tel.: +31-71-527-42-08; fax: +31-71-527-45-65.
E-mail address: bouwstra@chem.leidenuniv.nl (J.A. Bouwstra).

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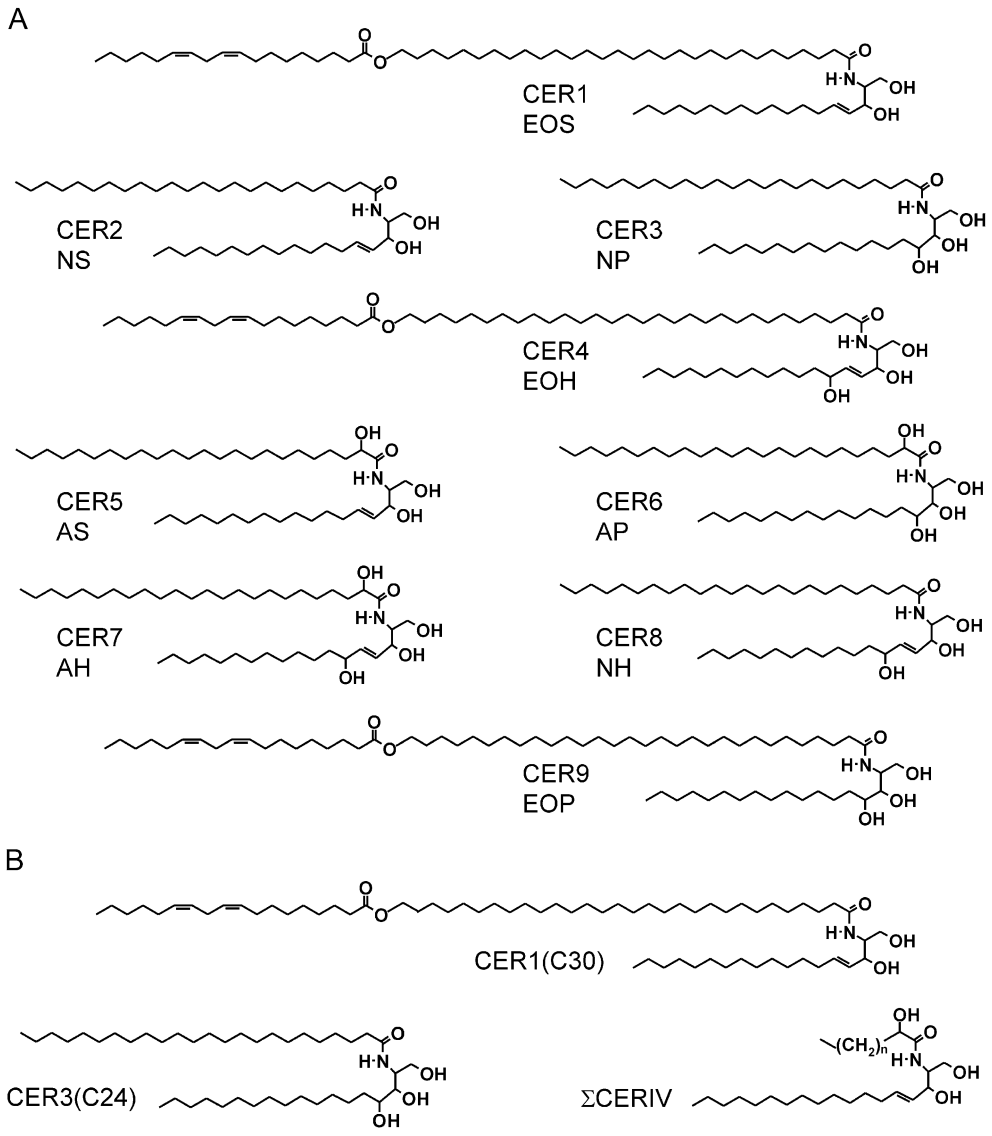


Fig. 1. The molecular structures of the CER present in human SC (A) and the molecular structures of the synthetic CER used in the study (B). The acyl chain lengths of CER1(C30) and CER3(C24) are very well defined, whereas bovine brain CER type IV (ΣCERIV) shows a distribution in chain lengths with C18 and C24 as the most abundantly present.

barrier function. X-ray diffraction studies on lipid mixtures prepared with isolated pig or human CER, CHOL and FFA revealed that the lipid organisation in these mixtures is similar to that observed in native SC. The main advantage of these SC lipid models is the possibility to selectively exclude particular lipid classes from the mixtures, which is not possible with SC. Therefore, these studies greatly contributed to our present knowledge on the role of individual SC lipid classes in the SC lipid organisation. Interestingly, the formation of the characteristic LPP depends on the presence of CHOL and specific CER, in particular CER1, whereas FFA are required for the crystalline (orthorhombic) character of the lateral lipid packing [8–11].

The use of synthetic lipids can overcome problems related to isolated SC lipids, such as variability of the native tissue and the laborious isolation and separation of the SC lipids. In a previous study it was shown for the first time that the SC lipid phase behaviour can already be reproduced to a high extent with a synthetic lipid mixture consisting of only three synthetic CER [12]. In that study a fixed CER composition was used, namely CER1, CER3 and bovine brain CER type IV (referred to as ΣCERIV) at a molar ratio of 1:7:2. In synthetic CER1 and CER3 a fatty acid with a well-defined acyl chain length is bound to the sphingoid backbone, whereas in ΣCERIV fatty acids with varying acyl chain lengths are present (Fig. 1 and Table 1). Both an appropriately chosen equilibration temperature during the sample preparation and the presence of FFA in the mixture are crucial to achieve a similar lipid organisation as found in native SC [12]. In spite of great similarities in lipid phase behaviour between mixtures prepared with natural CER (natCER) and synthetic CER (synthCER), some differences

Table 1
α-Hydroxy acyl profile in two batches of ΣCERIV given as percentages of total α-hydroxy acids

Acyl chain length α-hydroxy fatty acid	Batch 1 (%)	Batch 2 (%)
C18	18.0	22.2
C20	1.7	0.5
C22	8.0	7.9
C23	11.1	10.6
C24	44.0	41.8
C25	11.3	11.5
C26	5.9	5.5

have also been disclosed. The main difference is the presence of either one or two additional phases in mixtures prepared with synthetic lipids. These additional phases, with repeat distances of respectively 3.7 and 4.3 nm, can be ascribed to the presence of crystalline CER3 in a V-shaped conformation [13–15].

In the study presented in this paper, we further optimised the composition of the synthetic lipid mixtures to accomplish higher resemblance to the SC lipid organisation. To diminish the formation of the additional crystalline phases, which are not present in native SC, the amount of CER3 in the mixture was gradually reduced in favour of ΣCERIV. The main objective of the present study was to establish the optimal molar ratio of CER3 to ΣCERIV for the formation of the characteristic LPP and to determine the sensitivity of the lipid organisation to this ratio. In addition, the sensitivity of the lipid organisation to changes in the total synthCER level was studied. This was performed by varying the composition of the CHOL/synthCER/FFA mixture over a wide range of molar ratios, thereby maintaining the CHOL/FFA ratio constant.

2. Materials and methods

2.1. Synthetic lipids used in the study

Palmitic acid, stearic acid, arachidic acid, behenic acid, tricosanoic acid, lignoceric acid, cerotic acid, cholesterol and bovine brain ceramide type IV were purchased from Sigma-Aldrich Chemie (Schnelldorf, Germany). *N*-(30-Linoleoyloxy-triacontanoyl)-sphingosine (synthetic ceramide 1(C30)-linoleate) was a gift from Beiersdorf AG (Hamburg, Germany). *N*-Tetracosanoyl-phytosphingosine (synthetic ceramide 3 (C24)) was generously provided by Cosmoferm B.V. (Delft, the Netherlands). Fig. 1B shows the ceramides used in this study. All organic solvents used were of analytical grade and manufactured by Labscan (Dublin, Ireland).

2.2. Preparation and analysis of fatty acid methyl esters (FAMEs)

To establish the fatty acid profile in ΣCERIV, the lipid was dissolved in 100-μl toluene and transmethylated in 1-ml boron trichloride in methanol (10%) using microwave irradiation, which was carried out at the lowest power setting (85 W) for 4 h. FAMEs dissolved in hexane and purified on a silica gel column were separated and analyzed on a Vega GC 6000 gas chromatograph (Carlo Erba Instruments, Italy) using capillary column CP Wax 52 (Chrompack, The Netherlands). An initial temperature of 80 °C was increased to 160 °C with a rate of 40 °C/min followed by a 4 °C/min increase to 250 °C that was maintained until all peaks had eluted. The peaks were identified by comparison with FAME standards (Sigma-Aldrich Chemie). Integration of peak areas and calculation of relative percentages were performed by Baseline 810 integrator. Heptadecanoic acid was used as an internal standard.

2.3. Preparation of the lipid mixtures

For the preparation of all CHOL/synthCER and CHOL/synthCER/FFA mixtures, a synthetic CER mixture was used that consisted of CER1, CER3 and ΣCERIV at a molar ratio ranging from 1:1:8 to 1:8:1. For the preparation of the CHOL/synthCER/FFA mixtures, the following fatty acid mixture was used: C16:0, C18:0, C20:0, C22:0, C23:0, C24:0 and C26:0 at molar ratios of 1.3, 3.3, 6.7, 41.7, 5.4, 36.8 and 4.7, respectively. This ratio is similar to that found in native SC [16]. Appropriate amounts of individual lipids dissolved in chloroform/methanol (2:1) were combined to yield lipid mixtures of approximately 1.5 mg dry weight at the desired composition with a total lipid concentration of 7 mg/ml. A Camag Linomat IV was used to apply the lipid mixtures onto mica. This was done at a rate of 4.3 μl/min under a continuous nitrogen flow. The samples were equilibrated for 10 min at the appropriate elevated temperature that varied from 70 to 90 °C and subsequently hydrated with an acetate buffer of pH 5.0. Finally, the samples were homogenised by 10 successive freeze–thawing cycles between −20 °C and room temperature, during which the samples were stored under gaseous argon.

2.4. Small angle X-ray diffraction (SAXD)

All measurements were performed at the European Synchrotron Radiation Facility (ESRF, Grenoble) using station BM26B. A more detailed description of this beamline has been given elsewhere [17]. The X-ray wavelength and the sample-to-detector distance were 1.24 Å and 1.7 m, respectively. Diffraction data were collected on a two-dimensional multiwire gas-filled area detector. The spatial calibration of this detector was performed using silver behenate. The samples were mounted in a specially designed sample holder with mica windows. All measurements were recorded at room temperature for a period of 10 min.

SAXD provides information about the larger structural units in the sample, namely the repeat distance of a lamellar phase. The scattering intensity I (in arbitrary units) was

measured as a function of the scattering vector q (in reciprocal nm). The latter is defined as $q=(4\pi\sin\theta)/\lambda$, in which θ is the scattering angle and λ is the wavelength. From the positions of a series of equidistant peaks (q_n), the periodicity, or d-spacing, of a lamellar phase was calculated using the equation $q_n=2n\pi/d$, n being the order number of the diffraction peak.

3. Results

3.1. Fatty acid profile in ΣCERIV

To assess the fatty acid profile in ΣCERIV, two batches of ΣCERIV were subjected to transmethylation with boron trichloride in methanol followed by purification of the FAME by column chromatography. In this way, fractions containing methyl esters of α-hydroxy fatty acids were collected and subsequently analysed by gas chromatography. The results of the FAME analysis summarised in Table 1 show low batch-to-batch variation in the composition and the relative amounts of the various α-hydroxy fatty acids. All lipid mixtures used in our studies were prepared with batch 2. There, the most abundantly fatty acids were C18 (22%) and C24 (42%).

3.2. Mixtures prepared with equimolar ratios of CHOL and syntCER

The main objective of these studies is to determine the optimal molar ratio between CER3 and ΣCERIV for the formation of the LPP. All syntCER mixtures contained the same relative amount of CER1 (10%m/m), as earlier studies revealed that this amount is sufficient for the formation of the LPP. The relative content of CER3 and ΣCERIV in CER1/CER3/ΣCERIV mixtures was varied between a molar ratio of 1:1:8 and 1:8:1. During preparation, the dry lipid mixtures were equilibrated at either 80 or 90 °C. These temperatures were chosen, as use of a higher temperature results in melting of the CER1/CER3/ΣCERIV mixture with a molar ratio of 1:1:8, while use of a lower equilibration temperature results in reduced LPP formation [12]. As no clear differences in the formation of the lamellar phases have been observed between the lipid mixtures prepared at 80 or 90 °C, only the results of the mixtures equilibrated at 90 °C will be presented.

The diffraction pattern of the equimolar CHOL/synthCER mixture in which the CER fraction was prepared by mixing CER1/CER3/ΣCERIV to achieve a molar ratio of 1:1:8 is shown in Fig. 2A. A lamellar phase with a repeat distance of 5.5 nm (SPP) is indicated by the presence of a strong reflection at $q=1.14$ nm^{-1} and two weak reflections at 2.29 and 3.43 nm^{-1}. The diffraction pattern further depicts three weak diffraction peaks at 0.48, 0.97 and 1.45 nm^{-1}, respectively. Taking into account the diffraction patterns depicted in Figs. 2 and 3 (see below), it is conceivable to ascribe these reflections to the 1st, 2nd and 3rd order maxima of the LPP with a repeat distance of approximately 13 nm. In addition, crystalline CHOL in separate domains is present, deduced from the sharp peaks at 1.87 and 3.74 nm^{-1}.

A rise in the CER3 content at the expense of ΣCERIV to reach a CER1/CER3/ΣCERIV molar ratio of 1:3:6 was accompanied by a slight increase in peak width (Fig. 2A). This results in a partial overlap of the peaks located at 0.98 and 1.16 nm^{-1}. In addition, a new structure with a repeat distance of 3.7 nm is present in the mixture, suggested by two reflections at respectively 1.69 and 3.40 nm^{-1}. This phase can be ascribed to crystalline CER3 in a V-shaped structure [13–15].

Fig 2B shows the diffraction pattern of the CHOL/synthCER mixture prepared at a 1:5:4 ratio of CER1, CER3 and ΣCERIV. Compared to the diffraction patterns obtained at lower CER3 content (1:1:8 and 1:3:6), the peak intensities of the LPP are considerably increased compared to the reflections attributed to the SPP, indicating that the formation of the LPP is promoted at increased CER3 content. Similarly as in the 1:3:6 mixture, only one reflection of the SPP can be detected. In addition, crystalline CER3 and crystalline CHOL are present.

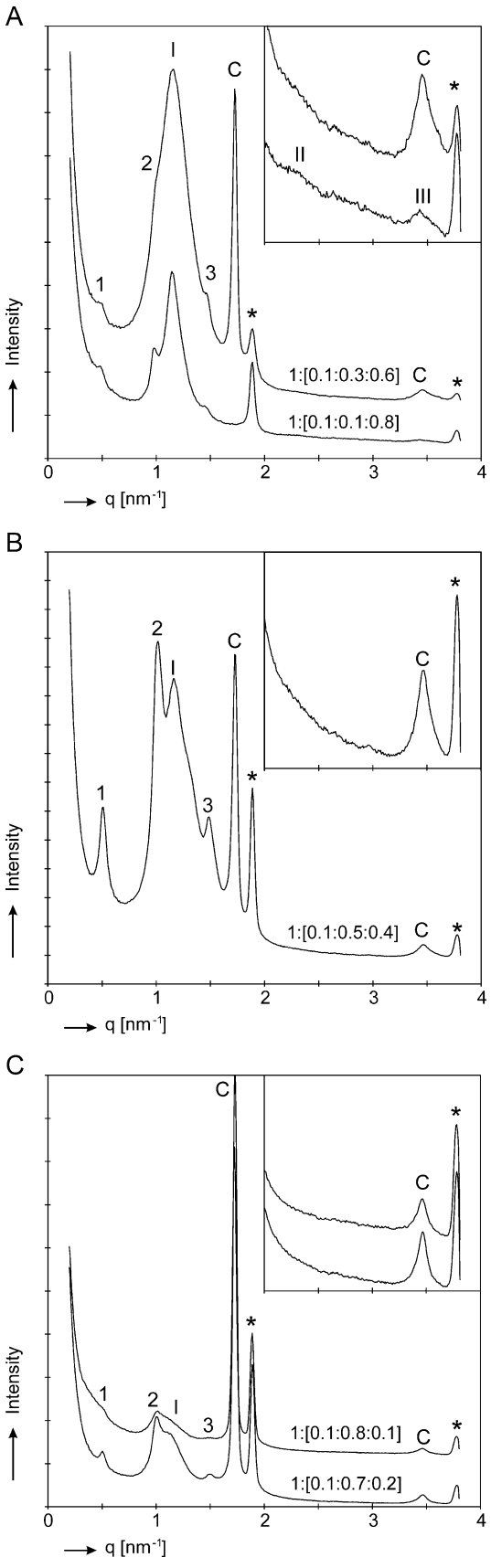
A further rise in CER3 content to a CER1/CER3/ΣCERIV ratio of 1:7:2 results in the diffraction pattern illustrated in Fig. 2C. Obviously, the reflection at $q=1.71$ nm^{-1} is the most prominent peak. This suggests that a considerable amount of crystalline CER3 phase-separates into a 3.7-nm phase. The LPP and SPP are still present in the mixture, but compared to Fig. 2B, the intensities of the reflections attributed to both phases are weaker than the peak intensities of CHOL and CER3.

At a CER1/CER3/ΣCERIV ratio of 1:8:1, a marked reduction of LPP formation is observed. Only a small lipid fraction forms a lamellar phase with a periodicity of 12.4 nm, whereas substantial amounts of crystalline CER3 are present in separate domains.

3.3. Mixtures prepared with equimolar ratios of CHOL, synthCER and FFA

The lipid organisation of mixtures containing FFA has also been examined, as FFA represent the third major lipid species in the SC. In a previous study it was observed that dry CHOL/synthCER/FFA mixtures melted at a lower equilibration temperature than CHOL/synthCER mixtures [11]. The dry lipid mixtures in the present study were therefore equilibrated at slightly lower temperatures, namely at 70 or 80 °C. As no obvious differences could be observed in the formation of the lamellar phases between mixtures equilibrated at 70 or 80 °C, only the results obtained at an equilibration temperature of 80 °C will be reported.

The phase behaviour of the CHOL/[CER1/CER3/ΣCERIV]/FFA mixture prepared at a molar ratio of



1:[0.1:0.1:0.8]:1 is depicted in Fig. 3A. The diffraction pattern reveals the presence of a strong reflection at $q = 1.05$ nm^{-1} and two weak reflections at 2.09 and 3.15 nm^{-1}. These peaks are located at positions at which the 2nd, 4th and 6th order diffraction peaks of the LPP should be expected. However, as no 1st and 3rd order reflections of the LPP are present, the reflections most likely relate to another phase with a periodicity of 6.0 nm. In addition, the SPP with a periodicity of 5.4 nm is present, deduced from the presence of a shoulder at the right-hand side of the strong peak (1st order) and a weak reflection at 3.50 nm^{-1} (3rd order). A small fraction of CHOL phase separates into crystalline domains, indicated by one weak reflection at 1.87 nm^{-1}.

A rise in CER3 content to reach a CER1/CER3/ΣCERIV ratio of 1:3:6 results in a clear appearance of the reflections attributed to the LPP. At this increased CER3 level, crystalline CER3 is present in separate domains as indicated by the two peaks at 1.71 and 3.43 nm^{-1}. In addition, the SPP and small amounts of crystalline CHOL are present in the lipid mixture.

The diffraction pattern of the mixture at a CER1/CER3/ΣCERIV molar ratio of 1:5:4 is presented in Fig. 3B. The increased CER3 level obviously results in increased intensities of the peaks attributed to the LPP compared to those attributed to the SPP. Five diffraction peaks of the LPP with a periodicity of 12.0 are present and two reflections of a 5.4-nm phase (SPP) can be detected. Crystalline CER3 and crystalline CHOL can also be detected in the mixture.

A further rise in the CER3 content to a molar ratio of 1:7.2 promotes the formation of the LPP even more. The presence of the LPP, with a slightly decreased repeat distance of 11.9 nm, is indicated by six reflections, whereas the SPP is indicated by two reflections. Weak reflections designate small amounts of crystalline CHOL, whereas strong peaks at 1.71 and 3.43 nm^{-1} indicate that

Fig. 2. The effect of the CER3 to ΣCERIV molar ratio on the phase behaviour of equimolar CHOL/synthCER mixtures. The inset is a magnification of the reflections in the q-range between 2 and 4 nm^{-1}. The Arabic numbers indicate the diffraction orders of the LPP, whereas the Roman numbers indicate the diffraction orders of the SPP. The letter C refers to the reflections of crystalline phase of CER3 at 1.71 and 3.42 nm^{-1}. The asterisk (*) indicates the reflections of crystalline CHOL located at 1.87 and 3.74 nm^{-1}. (A) Diffraction patterns of CHOL/[CER1/CER3/ΣCERIV] mixtures at a molar ratio of 1:[0.1:0.1:0.8] and 1:[0.1:0.3:0.6]. The various orders of the LPP are located at $q = 0.48$ nm^{-1} (1st), 0.97 nm^{-1} (2nd) and 1.45 nm^{-1} (3rd). The various orders of the SPP are located at $q = 1.14$ nm^{-1} (1st), 2.29 nm^{-1} (2nd) and 3.43 nm^{-1} (3rd). (B) Diffraction pattern of 1:[0.1:0.5:0.4] mixture. The various orders of the LPP are located at $q = 0.50$ nm^{-1} (1st), 1.01 nm^{-1} (2nd) and 1.49 nm^{-1} (3rd). The only reflection attributed to the SPP is located at $q = 1.16$ nm^{-1} (1st). (C) Diffraction patterns of 1:[0.1:0.7:0.2] and 1:[0.1:0.8:0.1] mixtures. The various orders of the LPP are located at $q = 0.50$ nm^{-1} (1st), 1.01 nm^{-1} (2nd) and 1.49 nm^{-1} (3rd). The only reflection attributed to the SPP is located at $q = 1.14$ nm^{-1} (1st).

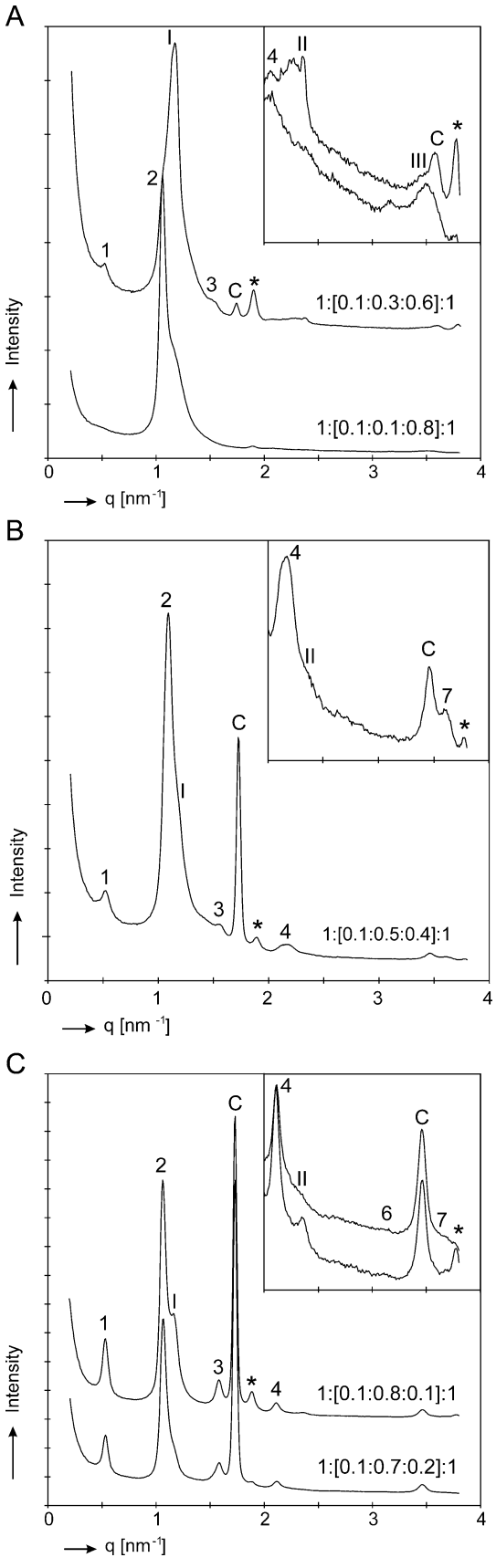


Fig. 3. The effect of the CER3 to ΣCERIV molar ratio on the phase behaviour of equimolar CHOL/synthCER/FFA mixtures. The inset is a magnification of the reflections in the q-range between 2 and 4 nm^{-1}. The Arabic numbers indicate the diffraction orders of the LPP, whereas the Roman numbers indicate the diffraction orders of the SPP. The letter C refers to the reflections of crystalline phase of CER3 at 1.71 and 3.42 nm^{-1}. The asterisk (*) indicates the reflections of crystalline CHOL located at 1.87 and 3.74 nm^{-1}. (A) Diffraction patterns of CHOL/[CER1/CER3/ΣCERIV]/FFA mixtures at a molar ratio of 1:[0.1:0.1:0.8]:1 and 1:[0.1:0.3:0.6]:1. The various orders of the LPP in the latter mixture are located at $q=0.52$ nm^{-1} (1st), 1.03 nm^{-1} (2nd), 1.52 nm^{-1} (3rd) and 2.06 nm^{-1} (4th). The various orders of the SPP are located at $q=1.16$ nm^{-1} (1st), 2.31 nm^{-1} (2nd) and 3.48 nm^{-1} (3rd). (B) Diffraction pattern of 1:[0.1:0.5:0.4]:1 mixture. The various orders of the LPP are located at $q=0.52$ nm^{-1} (1st), 1.05 nm^{-1} (2nd), 1.56 nm^{-1} (3rd), 2.11 nm^{-1} (4th) and 3.64 nm^{-1} (7th). The various reflections attributed to the SPP are located at $q=1.16$ nm^{-1} (1st) and 2.33 nm^{-1} (2nd). (C) Diffraction patterns of 1:[0.1:0.7:0.2]:1 and 1:[0.1:0.8:0.1]:1 mixture. The various orders of the LPP are located at $q=0.52$ nm^{-1} (1st), 1.05 nm^{-1} (2nd), 1.58 nm^{-1} (3rd), 2.11 nm^{-1} (4th), 3.12 nm^{-1} (6th) and 3.65 nm^{-1} (7th). The various orders of the SPP are located at $q=1.17$ nm^{-1} (1st) and 2.34 nm^{-1} (2nd).

a substantial amount of CER3 phase separates in a crystalline phase.

The diffraction pattern of the lipid mixture prepared with a 1:8:1 composition of CER1/CER3/ΣCERIV is plotted in Fig. 3C. Compared to the diffraction pattern of the 1:7:2 mixture, the relative intensities of the reflections of both LPP and SPP do not change significantly. However, the number of higher order reflections, which can be detected for both LPP and SPP, is reduced.

3.4. CHOL/synthCER/FFA mixtures with a variation in synthCER content

In order to determine whether the lipid organisation is sensitive to the synthCER content, the composition of the CHOL/synthCER/FFA mixture was varied over a wide synthCER level, thereby maintaining an equimolar CHOL to FFA ratio. The synthCER fraction was prepared with a 1:7:2 molar ratio of CER1, CER3 and ΣCERIV, as this appeared to be optimal for the formation of the LPP in the presence of FFA. At a CHOL/synthCER/FFA molar ratio of 1:2:1 and 1:1.5:1, the majority of the lipids form the LPP with a repeat distance of 12.0 nm (Fig. 4A). Reducing the synthCER content to a CHOL/synthCER/FFA ratio of 1:0.75:1 does not affect the formation of the LPP or SPP dramatically. A further decrease in the synthCER content to 1:0.5:1 is accompanied by a slight reduction in the fraction of lipids forming the LPP (Fig. 4B). These findings indicate that the formation of the lamellar phases is not very sensitive towards substantial changes in the relative synthCER content.

Concerning phase-separated CHOL, we observe that an increased relative amount of CHOL results in increased intensities of the reflections attributed to phase-separated CHOL, whereas almost no phase-separated CHOL is present at a CHOL/synthCER/FFA ratio of 1:2:1. This is in

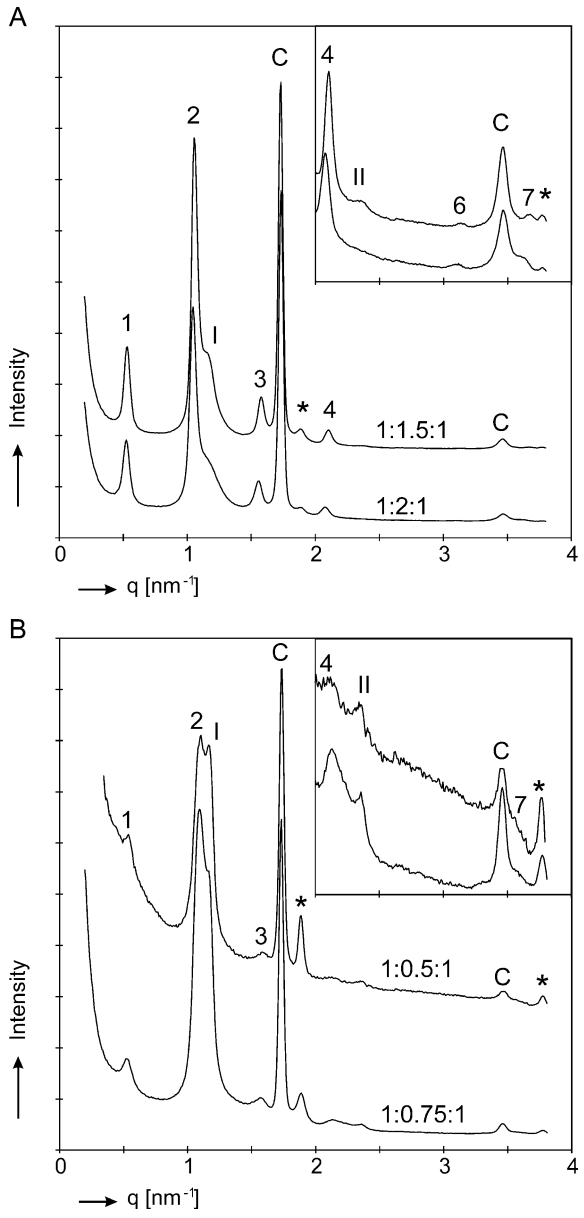


Fig. 4. Effect of changing the CER molar ratio, thereby maintaining an equimolar CHOL to FFA ratio. The inset is a magnification of the reflections in the q-range between 2 and 4 nm^{-1}. The Arabic numbers indicate the diffraction orders of the LPP, whereas the Roman numbers indicate the diffraction orders of the SPP. The letter C refers to the reflections of crystalline phase of CER3 at 1.71 and 3.42 nm^{-1}. The asterisk (*) indicates the reflections of crystalline CHOL located at 1.87 and 3.74 nm^{-1}. (A) Diffraction patterns of CHOL/synthCER/FFA mixtures at a molar ratio of 1:2:1 and 1:1.5:1. The various orders of the LPP are located at $q=0.52$ nm^{-1} (1st), 1.03 nm^{-1} (2nd), 1.56 nm^{-1} (3rd), 2.08 nm^{-1} (4th), 3.09 nm^{-1} (6th) and 3.60 nm^{-1} (7th). The various orders of the SPP are located at $q=1.16$ nm^{-1} (1st) and 2.32 nm^{-1} (2nd). (B) Diffraction patterns of 1:0.75:1 and 1:0.5:1 mixtures. The various orders of the LPP are located at $q=0.49$ nm^{-1} (1st), 0.99 nm^{-1} (2nd), 1.48 nm^{-1} (3rd), 1.99 nm^{-1} (4th) and 3.49 nm^{-1} (7th). The various reflections attributed to the SPP are located at $q=1.17$ nm^{-1} (1st) and 2.34 nm^{-1} (2nd).

agreement with the findings of McIntosh et al. [11,18], who do not observe phase separation of CHOL at a CHOL/CER/FFA ratio of 1:2:1.

4. Discussion

Previous studies revealed that mixtures prepared with CHOL, FFA and CER isolated from pig or human SC mixed in a proper molar ratio mimic the SC lipid phase behaviour very closely [8–10]. These observations offered an attractive opportunity to study the role these lipids play in the SC lipid organisation. Furthermore, due to its lipophilic nature, CER1 could be separated from the remaining CER and isolated in sufficient large quantities. This offered the possibility to examine in detail the role CER1 plays in SC lipid organisation. As each subclass of natural CER consists of ceramides with varying acyl chain lengths [4], the study on the effect of the variation in acyl chain length on the SC lipid phase behavior is difficult to perform with natCER. Therefore, the use of synthCER with a well-defined acyl chain length and head group architecture may overcome this problem. In a previous study, a first step has been made to investigate this by selecting a synthCER mixture consisting of CER1 and either synthetic CER3 with a uniform acyl chain length of 24 carbon atoms or semisynthetic ΣCERIV containing fatty acids with varying acyl chain lengths. We demonstrated that equimolar CHOL/synthCER mixtures prepared with CER1 and ΣCERIV do not form the LPP, while in mixtures prepared with synthetic CER1 and CER3 a small lipid fraction forms a 11.6-nm lamellar phase [13].

In a very recent study, we studied the phase behaviour of CHOL/synthCER and CHOL/synthCER/FFA mixtures prepared with a 1:7:2 molar ratio of CER1, CER3 and ΣCERIV [12]. The results of that study reveal that the LPP is formed in the lipid mixtures, provided that an optimal equilibration temperature is used during the preparation procedure. The phase behaviour of equimolar CHOL/synthCER/FFA mixtures equilibrated at 80 °C resembled to a high extent the lamellar and lateral lipid organisation found in native human or pig SC. However, the presence of one or two additional crystalline phases was noticed, which are not present in SC and can be ascribed to crystalline CER3. Furthermore, the relative intensities of the various diffraction peaks attributed to the LPP were slightly different from those observed with mixtures prepared with isolated CER.

In the present study, the CER3 to ΣCERIV ratio in the synthCER fraction was varied to enhance the formation of the LPP and to reduce the formation of additional crystalline phases. The results clearly demonstrate that in the absence of FFA, a CER1/CER3/ΣCERIV molar ratio of 1:5:4 is optimal for the formation of the LPP. In the presence of FFA, the optimal CER composition shifts to a CER1/CER3/ΣCERIV molar ratio of 1:7:2. Furthermore, the peak width at half maximum of a number of reflections is much smaller in the diffraction patterns of the mixtures prepared with FFA compared to those of the CHOL/synthCER mixtures. This indicates that FFA increase the ordering of the lipid organisation. This has also been observed in our previous study [12]. Although the optimal synthCER compositions could be selected for the formation of the LPP, it is obvious from

our data that the lipid organisation is not extremely sensitive towards variations in relative amounts of CER3 and ΣCERIV in the lipid mixture. This is similar to the observations made with equimolar CHOL/natCER mixtures, which were remarkably insensitive towards changes in the natCER composition [19]. Only a strong reduction in the level of natCER1 decreased the formation of the LPP.

In equimolar CHOL/synthCER/FFA mixtures, the intensities of the reflections attributed to crystalline CHOL are much weaker than in the CHOL/synthCER mixtures. This indicates that FFA increase the solubility of CHOL into the lamellar phases, which is consistent with observations made with mixtures based on natCER [8–10,18]. Furthermore, the diffraction patterns of the synthCER mixtures do not show reflections ascribed to FFA in a separate phase (Figs. 3 and 4). This suggests that FFA are completely intercalated in the lamellar phases and their presence is required for the formation of an orthorhombic sublattice within the lipid lamellae [8,10,12].

The synthetic lipid mixtures also contain an additional 3.7-nm phase, which is not present in native SC and can be ascribed to crystalline CER3. A rise in ΣCERIV content at the expense of CER3 increases the acyl chain length variation in the synthCER mixture (Table 1) and reduces the formation of phase-separated crystalline CER3. For a complete elimination of the 3.7-nm phase, the relative CER3 level should be below 3 (CER1/CER3/ΣCERIV molar ratio of 1:3:6). However, this increased ΣCERIV level is accompanied by a reduction in LPP formation, as is clearly demonstrated by the results of the present study. Partial replacement of CER3 by CER4 with known acyl chain lengths (e.g. C18 and C24 at a ratio of 1:2, mimicking the distribution in the ΣCERIV batch used in the present study) might offer a possibility to reduce the amount of phase-separated CER3.

The present study further demonstrates that a reduction in LPP formation is only observed when the total synthCER content is reduced to a CHOL/synthCER/FFA molar ratio of 1:0.5:1. This low sensitivity towards changes in the CHOL/synthCER/FFA molar ratio is in agreement with previous studies performed with mixtures prepared with natCER [8,10]. When extrapolating these findings to the in vivo situation, one can state that small variations in the molar ratios of the SC lipids will not lead to a substantial change in lipid organisation. Most phase behaviour studies performed with either isolated or synthetic lipids have been performed with equimolar lipid mixtures. A recent study on tape-stripped SC shows that CHOL, CER and FFA are present at a molar ratio of approximately 1:1.5:1 [20], rather than the generally assumed equimolar ratio. However, inspection of literature data shows that there is a high interindividual variability in SC lipid composition. This high variability together with the finding that the LPP has been detected in all SC samples studied so far can be ascribed to the fact that the LPP is formed in CHOL/CER/FFA mixtures even when the composition varies over a wide molar range.

In conclusion, the results of the present study and previous studies demonstrate that one can generate lipid mixtures with limited number of synthCER that mimic closely the lipid phase behaviour of native SC. The presence of FFA, the temperature used for equilibrating the mixtures and the CER3 to ΣCERIV ratio are important parameters for a proper lipid organisation.

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References

[1] P.W. Wertz, M.C. Miethke, S.A. Long, J.S. Strauss, D.T. Downing, The composition of the ceramides from human stratum corneum and from comedones, J. Invest. Dermatol. 84 (1985) 410–412.
[2] K.J. Robson, M.E. Stewart, S. Michelsen, N.D. Lazo, D.T. Downing, 6-Hydroxy-4-sphingenine in human epidermal ceramides, J. Lipid Res. 35 (1994) 2060–2068.
[3] M.E. Stewart, D.T. Downing, A new 6-hydroxy-4-sphingenine-containing ceramide in human skin, J. Lipid Res. 4 (1999) 1434–1439.
[4] M. Ponec, A. Weerheim, P. Lankhorst, P. Wertz, New acylceramide in native and reconstructed epidermis, J. Invest. Dermatol. 120 (2003) 581–588.
[5] J.A. Bouwstra, G.S. Gooris, J.A. van der Spek, W. Bras, The structure of human stratum corneum as determined by small angle X-ray scattering, J. Invest. Dermatol. 96 (1991) 1006–1014.
[6] J.A. Bouwstra, G.S. Gooris, J.A. van der Spek, W. Bras, Structural investigations of human stratum corneum by small angle X-ray scattering, J. Invest. Dermatol. 97 (1991) 1005–1012.
[7] J.A. Bouwstra, G.S. Gooris, W. Bras, D.T. Downing, Lipid organization in pig stratum corneum, J. Lipid Res. 36 (1995) 685–695.
[8] J.A. Bouwstra, G.S. Gooris, K. Cheng, A. Weerheim, W. Bras, M. Ponec, Phase behavior of isolated skin lipids, J. Lipid Res. 37 (1996) 999–1011.
[9] J.A. Bouwstra, G.S. Gooris, F.E.R. Dubbelaar, A.M. Weerheim, M. Ponec, pH, cholesterol sulfate, and fatty acids affect the stratum corneum lipid organization, J. Invest. Dermatol. 3 (1998) 69–73.
[10] J.A. Bouwstra, G.S. Gooris, F.E.R. Dubbelaar, M. Ponec, Phase behavior of stratum corneum lipid mixtures based on human ceramides: the role of natural and synthetic ceramide 1, J. Invest. Dermatol. 118 (2002) 606–617.
[11] T.J. McIntosh, M.E. Stewart, D.T. Downing, X-ray diffraction analysis of isolated skin lipids: reconstruction of intercellular lipid domains, Biochemistry 35 (1996) 3649–3653.
[12] M.W. de Jager, G.S. Gooris, I.P. Dolbnya, W. Bras, M. Ponec, J.A. Bouwstra, Novel lipid mixtures based on synthetic ceramides reproduce the unique stratum corneum lipid organization, J. Lipid Res. 45 (2004) 923–932.
[13] M.W. de Jager, G.S. Gooris, I.P. Dolbnya, W. Bras, M. Ponec, J.A. Bouwstra, The phase behaviour of skin lipid mixtures based on synthetic ceramides, Chem. Phys. Lipids 124 (2003) 123–134.
[14] B. Dahlen, I. Pascher, Molecular arrangements in sphingolipids. Ther-

motropic phase behaviour of tetracosanoylphytosphingosine, Chem. Phys. Lipids 24 (1979) 119–133.
[15] S. Raudenkolb, W. Hubner, W. Rettig, S. Wartewig, R.H. Neubert, Polymorphism of ceramide 3: Part 1. An investigation focused on the head group of *N*-octadecanoylphytosphingosine, Chem. Phys. Lipids 123 (2003) 9–17.
[16] P.W. Wertz, D.T. Downing, Epidermal lipids, in: L.A. Goldsmith (Ed.), Physiology, Biochemistry and Molecular Biology of the Skin, 2nd edition, Oxford Univ. Press, Oxford, 1991, pp. 205–235.
[17] W. Bras, A SAXS/WAXS beamline at the ESRF and future experiments, J. Macromol. Sci., Phys. B 37 (1998) 557–566.
[18] T.J. McIntosh, Organization of skin stratum corneum extracellular lamellae: diffraction evidence for asymmetric distribution of cholesterol, Biophys. J. 85 (2003) 1675–1681.
[19] J.A. Bouwstra, F.E.R. Dubbelaar, G.S. Gooris, A.M. Weerheim, M. Ponec, The role of ceramide composition in the lipid organisation of the skin barrier, Biochim. Biophys. Acta 1419 (1999) 127–136.
[20] A. Weerheim, M. Ponec, Determination of stratum corneum lipid profile by tape stripping in combination with high-performance thin-layer chromatography, Arch. Dermatol. Res. 293 (2001) 191–199.