Development of a vernix caseosa substitute : a novel strategy to improve skin barrier function and repair

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Long periodicity phase in extracted lipids of vernix caseosa obtained with equilibration at physiological temperature

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Abstract
The outermost layer of the skin, the stratum corneum, comprises the main barrier function between body and environment. The stratum corneum features a highly structured lipid organization: a short periodicity phase and a long periodicity phase with a repeat distance of 6 and 13 nm, respectively. Like stratum corneum, vernix caseosa, the creamy white skin-surface biofilm of the newborn, also contains barrier lipids, i.e. ceramides, cholesterol and free fatty acids. Aim of this study was to investigate whether isolated vernix caseosa lipids also form the characteristic long periodicity phase. Several preparation methods were examined and only when the solution of the lipid mixture, isolated either from vernix caseosa or stratum corneum, was dried under nitrogen at 37°C and subsequently spread onto a support, the long periodicity phase was formed. When barrier lipids of vernix caseosa were first exposed to elevated temperatures and subsequently cooled down, the LPP was formed at around 34°C, which is much lower than observed with the lipids in stratum corneum. In conclusion, we showed for the first time that depending on the preparation method, I) vernix caseosa lipids also form the long periodicity phase and II) the long periodicity phase in vernix caseosa lipids and stratum corneum lipids was obtained after at a low equilibration temperature mimicking the physiological temperatures.

Introduction
Vernix caseosa (VC) is the creamy white skin-surface biofilm which covers the skin of the third trimester fetus and the newborn. It exhibits various biological functions such as facilitating skin development under aqueous conditions as well as hydrating and protecting the skin of the newborn [1-4]. VC is composed of about 80% water, 10% proteins (mostly keratin originating from corneocytes) and 10% lipids [4, 5]. Unlike the upper layer of the human skin, the stratum corneum (SC), VC displays a wide range of different lipid classes. VC lipids are composed of high levels of sebaceous gland-derived, nonpolar lipids such as sterol esters (SE), wax esters (WE) and triglycerides (TG) [6]. SC derived barrier lipids - cholesterol (CHOL), free fatty acids (FFA) and ceramides (CER) - are also present with approximately 10-30% of the total lipid mass of VC [5, 7]. Due to the difference in lipid composition, the lipid organization in VC is different from that in human SC. In previous studies, it was reported that VC lipids form a long range ordering, but no well defined phases could be identified [8]. In contrast to that, in human SC the intercellular lipids form two coexisting lamellar phases: the short periodicity phase with a repeat distance of 6 nm and the long periodicity phase (LPP) with a repeat distance of around 13 nm [9]. The latter is only identified in SC and is considered to be of major importance for the barrier function of the skin [10-12]. Lipid mixtures prepared from native human CER,

CHOL and FFA also form these two lamellar phases [13, 14]. Until now, however, a crucial step for the formation of the LPP is equilibration of the lipid mixtures at high temperatures, typically 60 to 80°C [13, 14] with subsequent repetitive freeze-thawing cycles.
As the main classes of SC barrier lipids are also present in VC [5], the question arises whether VC lipids are also able to form the LPP. In order to investigate this, the phase behaviour of VC lipid mixtures prepared by various methods was studied by small-angle X-ray diffraction. To mimic the physiological situation as closely as possible, no equilibration step at elevated temperatures was included. In subsequent studies the best performing preparation method for the formation of the LPP was selected to examine whether lipid mixtures prepared from isolated CER, CHOL and FFA and SC lipid extracts can form the LPP without an equilibration step at elevated temperatures.

Materials & Methods

Materials

All organic solvents were of analytical grade and provided by Labscan Ltd. (Dublin, Ireland). Silica gel with a pore size of 60 Å and 10x20 cm high-performance thin layer chromatography (HPTLC) plates were manufactured by Merck KGaA (Darmstadt, Germany). The synthetic ceramides were kindly provided by Cosmoferm B.V. (Delft, The Netherlands). All other synthetic lipids were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands).

Collection and preparation of vernix caseosa and stratum corneum

VC was scraped off gently immediately after vaginal delivery or caesarean section of healthy term neonates. The samples were directly transferred in sterile plastic tubes, and stored at 4ºC until use. The collection of VC was approved by the ethical committee of the Leiden University Medical Center.
Abdomen or breast skin was obtained after cosmetic surgery from local hospitals. Subcutaneous fat tissue was removed, after which the skin was carefully cleaned with tissue paper, which was prior soaked in 70% ethanol. After cleaning the skin, it was dermatomed to a thickness of approximately 300 µm (Padgett Electro Dermatome Model B, Kansas City, USA). After overnight incubation with the dermal side on a trypsin soaked filter paper at 4ºC and subsequently 1 h at 37˚C (0.1% trypsin buffer in phosphate buffer saline without calcium or magnesium, pH 7.4), the SC was peeled off from the underlying epidermis and dermis. Remaining trypsin activity was blocked by incubating the SC for 1 h in a 0.1% trypsin inhibitor solution in PBS. The SC was subsequently washed several times with water and then stored in a silica gel containing desiccator under gaseous nitrogen.

Extraction, separation and identification of lipids

VC and SC samples were extracted according to a modified method of Bligh & Dyer [15] as described recently [7]. The extracts were then dried under a continuous flow of nitrogen at 37˚C. Then the lipid extracts were stored at 4˚C until further use.

Individual VC lipid fractions were isolated by means of column chromatography. 32 g of Lioprep 60R (15-25 µm) were dehydrated for 1 h at 130°C after which 40 ml chloroform was added and poured into a glass column with a diameter of 20 mm and a length of 420 mm. A pre-column consisted of 4 g dehydrated silica gel (43-60 µm). Subsequently, the column was eluted and packed with 100 ml chloroform/hexane 1:1 (v/v). The dry lipid sample was dissolved in the first eluent and carefully applied. After discarding the dead-volume of ~55 ml, fractions of 3.8 ml were collected during the elution with following eluents: hexane/chloroform/diethylether/isopropanol/ethylacetate/methylethylketone/ acetone/methanol/acetic acid/water. The different eluents were applied with increasing polarity. The lipid fractions were subsequently dried under a gentle stream of nitrogen at 40°C and analyzed qualitatively by means of HPTLC in order to pool the individual fractions.

Analysis of lipid fractions obtained from the column chromatography and lipid mixtures was performed by using a one-dimensional HPTLC as described earlier [5, 16]. Briefly, 5-50 µg of lipid samples were applied on a rinsed and dehydrated 10x20 cm HPTLC plate by means of the sample applicator Linomat IV (Camag, Muttenz, Switzerland). For separation and evaluation of the fractions obtained previously from the column chromatography, a HPTLC plate was developed sequentially as reported earlier [16]. A synthetic standard was co-migrated and was composed of squalene, cholesteryl oleate representing SE, oleyl oleate representing WE, glycerol trioleate representing TG, CHOL, palmitoleic acid representing FFA, cholesterol sulphate and synthetic CER, consisting of the acyl-CER (EOS(C30)linoleate), two non-hydroxy-CER (NS(C24) and NP(C24)) and the α-hydroxy-CER (AS(C24), AS(C16) and AP(C24)) according to the terminology of Motta *et al.* [17]. After charring at 170°C the HPTLC plate was scanned with the Bio-Rad GS-710 Calibrated Imaging Densitometer (Hercules, USA) and analyzed with the Bio-Rad software Quantity One. According to the analysis of the fractions the pooling was performed by combining the dissolved fractions, i.e. SE/WE or TG or barrier lipids (CHOL + CER + FFA).

Preparation of the lipid mixtures

Lipid specimen were prepared by the following procedures. First, the lipid extracts were dissolved in chloroform/methanol 2:1 (with a final lipid concentration of approximately 5 mg/ml). Subsequently the samples were processed in three different ways as described below:

Linomat spraying - The solution containing 1.5 mg lipids (~300 µl) was sprayed on mica by the Linomat under continuous nitrogen flow and with a solvent flow rate of 8 µl/min.

Direct spreading - The lipid solution was dried at 37˚C under a continuous nitrogen flow in a curved test-tube. 1-2 mg of dry lipids were directly applied on mica by spreading with a spatula at room temperature.

Freeze-drying - 1.5 mg of the dried lipids were redissolved in cyclohexane/isopropanol 9:1 (v/v), shock-frozen in liquid nitrogen and subsequently freeze dried with a VaCo I (Zirbus, Bad Grund, Germany) at -55°C and a pressure of ~0.2 mbar overnight. In the final step, the lipid samples were applied on mica by means of a spatula.

Small-angle X-Ray Diffraction

SAXD provides information about larger, organized structures in the sample such as the repeating distance of a lamellar phase. The scattering intensity (in arbitrary units) is measured as function of the scattering vector q (in reciprocal nm). This scattering vector is defined as $q=(4\pi\sin\theta)/\lambda$ where θ and λ represent the scattering angle and the wavelength, respectively. The spacing was determined from the q-value corresponding to the position of the diffraction peak and is equal to $2\pi/q$. The periodicity was calculated from the positions of the various diffraction peaks attributed to a lamellar phase using the equation $d=2n\pi/q_n$ with n being the order of the diffraction peak. The measurements were conducted at station BM26B at the European Synchrotron Radiation Facility in Grenoble, France [18] and were carried out as described earlier [11]. Briefly, the mica with the lipid specimens were transferred in a special sample holder which was subsequently mounted in the X-ray beam. The diffraction data were collected on a two-dimensional gas-filled area detector with a 1.5 m sample-detector distance. The spatial calibration of this detector was performed using silver behenate and CHOL. The diffraction pattern at room temperature was obtained for a collection period of 5 min. Temperature-dependent measurements were performed with a heating rate of 0.5°C/min and cooling rates of 5°C/min (VC lipid extract) and 2°C/min (barrier lipids).

Results

Long range ordering of VC lipids is dependent on preparation method

The long range ordering of VC lipids after the various preparation methods has been investigated by SAXD. The following results are obtained:

Linomat sprayed method: The SAXD patterns of VC lipids of three different donors exhibit a long range ordering as revealed by a single diffraction peak located at a spacing of around 4.5 nm ($q=1.40\ nm^{-1}$; Fig. 1A). This long range ordering is very similar to that observed in the diffraction pattern for fresh VC [7]. No higher order diffractions were detected, which hampers a more detailed analysis.

Spreading method: VC lipids applied by *direct spreading* on mica showed a completely different diffraction pattern as those prepared by the *Linomat sprayed* method. In the diffraction patterns of each lipid extract the 1st ($q=0.49\ nm^{-1}$), 2nd ($q=0.99\ nm^{-1}$) and 3rd order diffraction peak ($q=1.48\ nm^{-1}$) are observed, attributed to the LPP with a periodicity of 12.8 nm (Fig. 1A, donor B). Furthermore, the diffraction patterns of donor A and C exhibit a sharp peak with a spacing of 4.3 nm ($q=1.45\ nm^{-1}$), while the diffraction curves of donor B and C revealed an additional broad peak with a spacing around 5.0 nm ($q=1.26\ nm^{-1}$). The origin of the latter diffraction peaks is not clear.

Freeze-drying method*:* VC lipid samples of two other donors are depicted in figure 1B and are compared to the *spreading* method. The diffraction patterns are characterized by a very broad and a single sharp peak at a spacing of 4.6 nm ($q=1.37\ nm^{-1}$; Fig. 1B). This pattern is very different from that of VC lipid mixtures, which were *spread directly,* but shows great similarities to the *Linomat sprayed* samples (Fig. 1A). The lipid mixtures prepared by the *spreading* method form the LPP (Fig. 1B), similarly as observed for the lipid mixtures of the other donors in figure 1A.

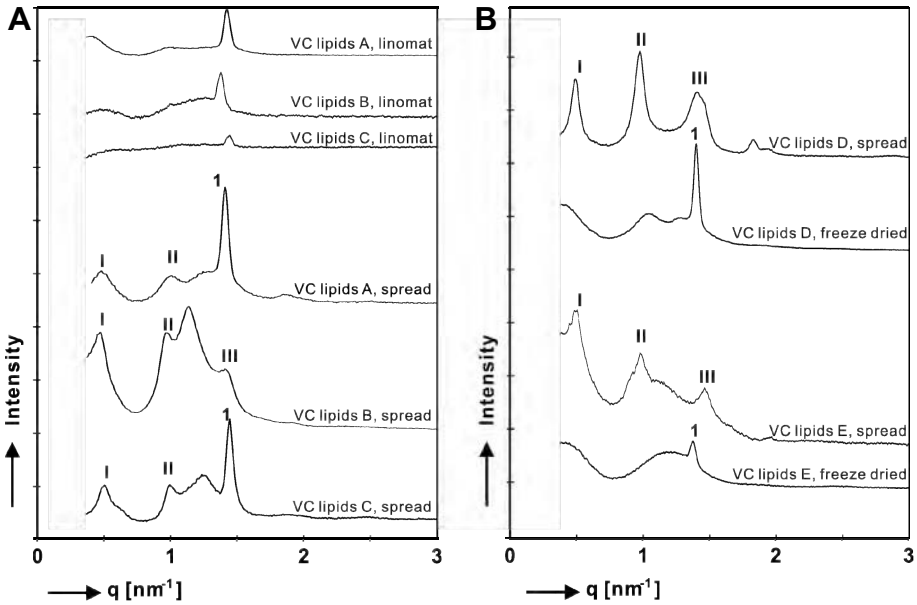

Figure 1. The small angle X-ray diffraction patterns of VC lipid extracts (donors A, B, C, D and E) obtained with 3 different preparation methods are presented. The upper three patterns show the sample prepared with the *Linomat*, whereas the lower three were obtained by *spreading* the sample directly on mica (A). Two different donors are displayed in panel B: The upper and lower two patterns show differences in the *spreading* and the *freeze dried* preparation method for the individual donor, respectively. The roman numerals indicate the various orders of the LPP (q-values and d-spacings given for donor B): 1st order (I) located at q=0.49 nm^{-1} (d=12.8 nm), 2nd order (II) at q=0.99 nm^{-1} (d=6.3 nm) and 3rd (III) order at q=1.45 nm^{-1} (d=4.3 nm). The Arabic number designates a sharp peak at q=1.45 nm^{-1} (d=4.3 nm) for donors A, C and at q=1.37 nm^{-1} (d= 4.6 nm) for donors D and E.

Individual VC components exhibit different long range ordering

In order to assign the observed diffraction peaks in the VC lipid extracts to individual lipid classes, the respective lipid classes of VC extracts were isolated and analyzed. Isolation of the lipids was performed by column chromatography [8] and the composition was determined by HPTLC. The results are provided in figure 2A. Besides the standard mixture with a known lipid composition (lane 1) and the entire VC lipid extract (lane 2) the various collected column fractions were applied (lane 3-31). The fractions containing major lipid classes of VC were combined, namely SE/WE (lane 3), TG (lane 6 and 7) and VC barrier lipids which consist of CHOL (lane 12-14), FFA (lane 15-20) and the different CER subclasses (lane 21-31). These three lipid fractions of VC were applied by the *spreading* method on mica and subsequently measured by SAXD. The obtained diffraction patterns are shown in figure 2B. The diffraction pattern of the SE/WE fraction exhibited a sharp diffraction peak with a spacing of d=4.3 nm ($q=1.45\ nm^{-1}$), similar to the spacing observed with the *Linomat sprayed* samples of VC lipids (Fig. 1A). The diffraction pattern of the isolated TG fraction shows weak, very broad peaks with spacings of around 5.3 nm ($q=1.19\ nm^{-1}$) and 4.7 nm ($q=1.34\ nm^{-1}$; Fig. 2B). The peaks cannot be assigned to a particular phase, but the pattern shows that some long range ordering is present in this sample. Finally, the reconstituted barrier lipids of VC form the characteristic LPP with a repeat distance of 13.2 nm as the 1st ($q=0.46\ nm^{-1}$), 2nd ($q=0.99\ nm^{-1}$) and 3rd order ($q=1.43\ nm^{-1}$) diffraction peaks are clearly discernable (Fig. 2B). These positions are very similar to those observed in the pattern of VC lipids applied by *direct spreading* indicating that the barrier lipids are responsible for the formation of the LPP in VC lipids.

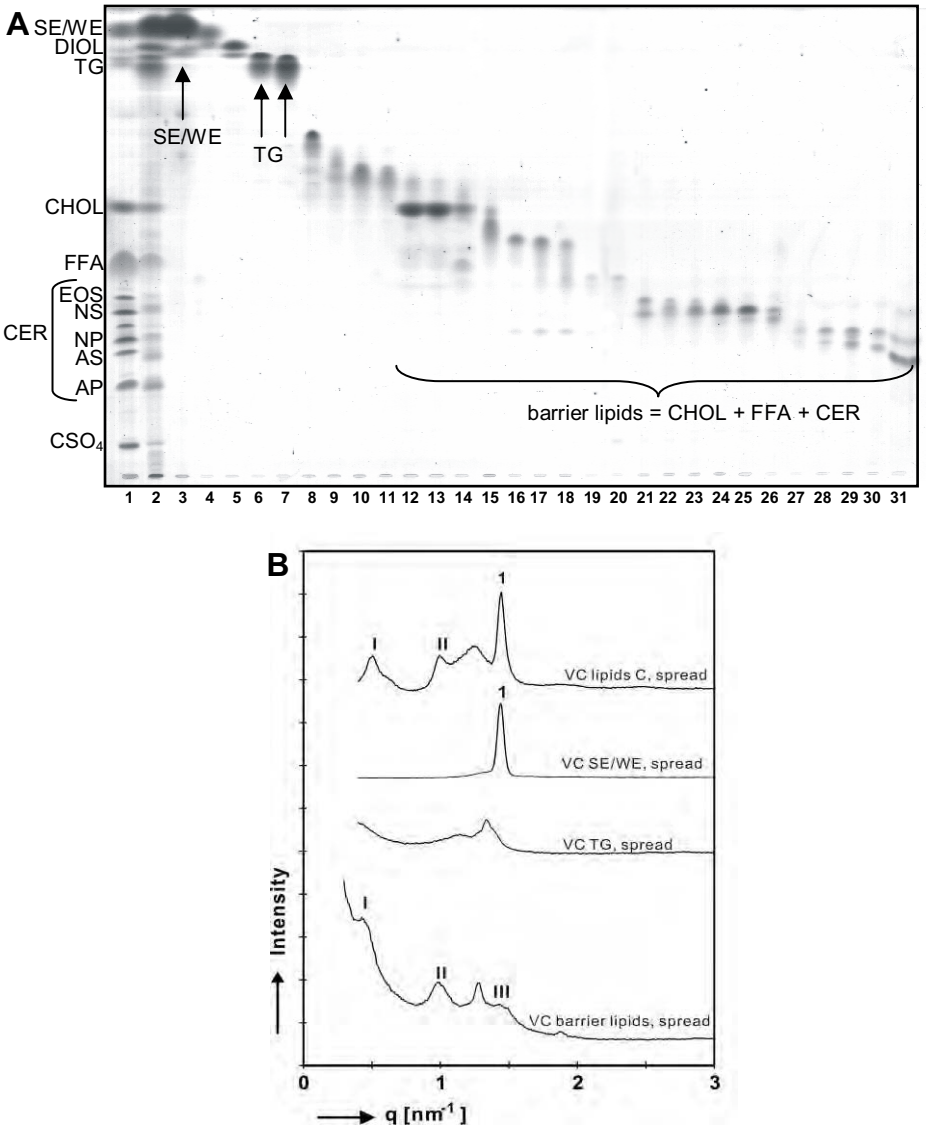

Figure 2. HPTLC of synthetic standard mixture (lane 1), a VC lipid extract (lane 2) and the various lipid fractions of VC lipids obtained after column chromatography is depicted in panel A. Fractions were collected and combined in the SE/WE fraction (lane 3), TG (lane 6 and 7) and barrier lipids (lane 12-31) comprising CHOL, FFA and different CER. Legend on the left indicates the R_f values of different compounds.The scattered intensity (arbitrary units) is plotted as function of scattering vector (q) in panel B. VC lipids and its major fractions, i.e. SE/WE, TG and CHOL, FFA, CER (barrier lipids), were applied using the spreading method. The roman numerals indicate the various orders of the LPP: 1st order (I) located at q=0.46 nm^{-1} (d=13.7 nm), 2nd order (II) at q=0.99 nm^{-1} (d=6.3 nm) and 3rd order (III) at q=1.43 nm^{-1} (d=4.4 nm) for VC barrier lipids. The Arabic number at q=1.45 nm^{-1} (d=4.3 nm) designates a sharp peak (VC lipids donor C and VC SE/WE).

Long range ordering of VC lipids disappears within physiological temperature range and the transition is reversible

The diffraction patterns of the VC lipid mixtures prepared by the *Linomat sprayed* method were also measured as a function of temperature. A typical example is presented in figure 3. At 16°C the diffraction pattern of the VC lipids is characterized by a sharp peak with a spacing of 4.4 nm ($q=1.42\ nm^{-1}$). Furthermore, a weak, broad ordering with a spacing of 5.1 nm ($q=1.23\ nm^{-1}$) is also observed. Upon heating, the sharp diffraction peak decreases in intensity and finally disappears at 30°C. The diffraction pattern remains unchanged upon heating to 36°C (dashed line). Subsequent cooling of the sample showed the reappearance of the diffraction peak with a slightly elongated spacing of 4.5 nm ($q=1.39\ nm^{-1}$) between 30°C and 20°C which is similar to fresh VC [19].

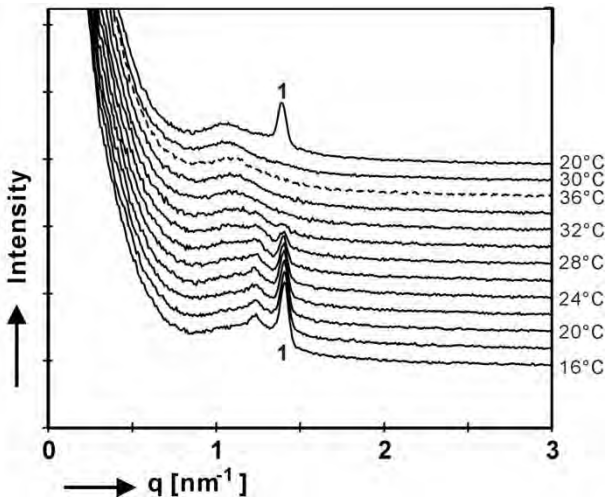

Figure 3. Scattered intensity (arbitrary units) plotted as function of scattering vector (q). Sequential SAXD patterns are plotted of a VC lipid extract Linomat sprayed during heating from 16 (bottom curve) to 36°C (dashed curve) and cooling down to 20°C (top curve). The Arabic number 1 designates a sharp peak at $q=1.42\ nm^{-1}$ (d=4.4 nm) before heating.

During cooling LPP appears at 34°C in VC barrier lipid mixtures

To study the thermotropic behaviour of barrier lipids of VC, the lipids were applied by the *Linomat sprayed* method and equilibrated for 5 min at 68°C. This is the equilibration temperature used in previous studies for mixtures which contain synthetic CER, CHOL and FFA [20]. The sample was cooled to 18°C and the monitored diffraction patterns are presented in figure 4. At 68°C the diffraction pattern of the mixture is characterized by two diffraction peaks with a spacing of 4.9 nm (q=1.27 nm^{-1}) and 3.4 nm (q=1.86 nm^{-1}). The latter is attributed to crystalline CHOL. During cooling with 2°C/min the intensity of the peak with d=4.9 nm increases gradually and no further changes are observed until a temperature of 34°C is reached. At this temperature, three peaks appear at the diffraction pattern, which are identified as the 1st (I), the 2nd (II) and the 3rd order (III) of the LPP (d=12.8 nm). Upon further cooling to 18°C the intensity of these peaks increases gradually.

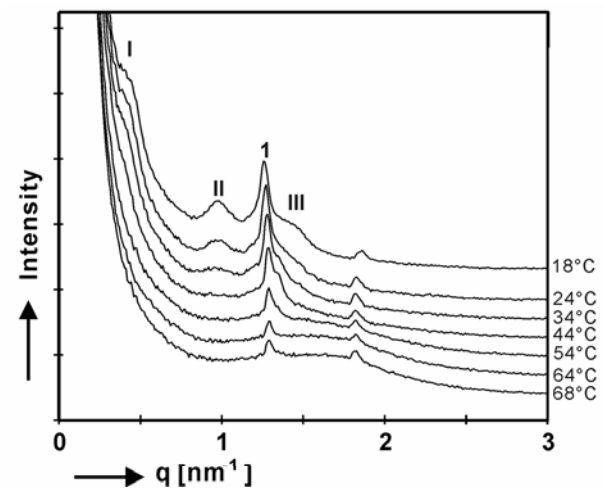

Figure 4. Scattered intensity (arbitrary units) plotted as function of scattering vector (q). Sequential SAXD patterns are plotted of VC barrier lipids Linomat sprayed during cooling from 68°C (bottom curve) to 18°C (top curve). The roman numerals indicate the different orders of the LPP with a periodicity of approximately 12.7 nm: 1st order (I) is located at q=0.49 nm^{-1} (d=12.8 nm), the 2nd order (II) at q=0.99 nm^{-1} (d=6.3 nm) and the 3rd order (III) is represented by the shoulder at q=1.48 nm^{-1} (d=4.2 nm). The Arabic number points towards a sharp peak at q=1.27 nm^{-1} (d=4.9 nm). During cooling a peak at q=1.86 nm^{-1} (d=3.4 nm), presumably CHOL attributed, is observed.

Long range ordering in SC lipids obtained by equilibration at 37°C

As the VC lipids and VC barrier lipids both form the LPP without equilibration at elevated temperatures, the question arises whether human SC lipids can also form the LPP at 37°C without pre-equilibration at elevated temperatures. Therefore, SAXD patterns of human SC lipid extracts prepared by the *spreading* method were also examined. The diffraction profile in figure 5 (central pattern) revealed the LPP with a repeat distance of 13.4 nm, exhibiting a weak 1st ($q=0.47\ nm^{-1}$), 2nd ($q=0.94\ nm^{-1}$) and 3rd order ($q=1.40\ nm^{-1}$). However, when preparing the lipid mixture by the *Linomat sprayed* method, a very different diffraction pattern was observed: a broad peak with a spacing of 5.5 nm ($q=1.14\ nm^{-1}$) was present whereas no LPP-attributed diffractions peaks could be detected (lower pattern). The mixture of CHOL, FFA and isolated human CER (referred to in the figure as SC barrier lipids) applied by *direct spreading* also showed the presence of the LPP with a periodicity of 12.6 nm although only the 2nd ($q=1.03\ nm^{-1}$) and 3rd order ($q=1.50\ nm^{-1}$) reflections were discernable (Fig. 5, upper pattern). In all diffraction patterns of figure 5 a structure with a spacing of 3.4 nm is observed. This peak is very broad and might be assigned to crystalline CHOL.

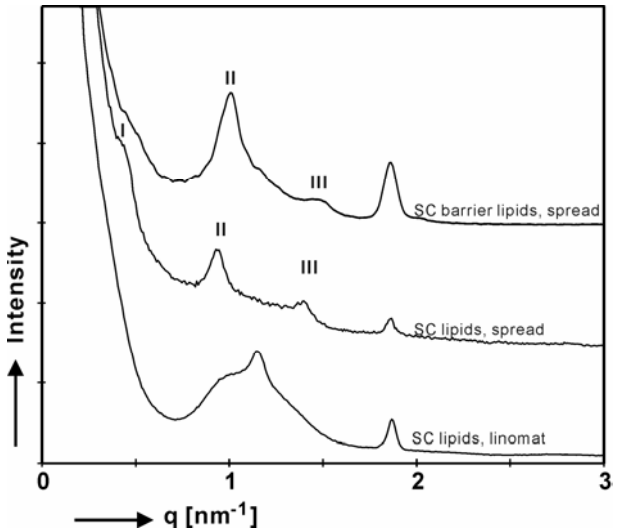

Figure 5. SAXD patterns of SC barrier lipids (upper trace) and SC lipid extract with two different preparation methods. The middle trace is obtained by *spreading* the sample directly on mica; the lower trace was acquired by the *Linomat spraying* method. The roman numbers above the middle trace indicate the orders of the LPP: 1st order (I) at $q=0.47\ nm^{-1}$ ($d=13.4$ nm), 2nd order (II) at $q=0.94\ nm^{-1}$ ($d=6.7$ nm) and 3rd order (III) at $q=1.40\ nm^{-1}$ ($d=4.5$ nm). In all patterns a peak at $q=1.86\ nm^{-1}$ ($d=3.4$ nm) is observed attributed to crystalline CHOL.

Discussion

In human, pig and mouse SC the lipids form two lamellar phases: the short periodicity phase and the LPP with repeat distances of approximately 6 and 13 nm [9, 21, 22]. As the LPP is very characteristic for the SC lipid organization and it has been observed in the SC of various species, this ordered structure is considered to be important for the skin barrier function. Its crucial role for the barrier function was demonstrated in recent studies, in which in the absence of the LPP, the penetration through the SC barrier lipids increased by a factor of two [11]. The skin barrier lipids are not only present in SC, but are also discerned in VC [5]. In previous studies the lipid organization and thermotropic behaviour of VC were elucidated [7, 8, 19]. These studies revealed that in VC and in isolated VC lipids the LPP was not formed. Although in lipids prepared from isolated or synthetic CER, FFA and CHOL the LPP is formed, an equilibration step at elevated temperatures is required to mimic the lipid organization in SC [13, 23]. As these equilibration temperatures, typically between 60 and 80°C, are far from physiological conditions, in the present study we focused on the formation of the LPP at room temperature or close to body temperature in isolated VC and SC lipid mixtures.

The formation of the LPP depends on the application method of the lipid mixture

Extracts of VC applied with the *Linomat sprayed* method and *freeze dried* method, showed a single, sharp peak with a spacing around 4.5 nm in the corresponding diffraction patterns (Fig. 1), clearly demonstrating that no LPP is formed. However, when the same lipid mixture is applied by *direct spreading*, the LPP could be detected. This shows for the first time that VC lipid mixtures are able to form the characteristic LPP without an equilibration step at elevated temperatures and shows furthermore, that the preparation method is of crucial importance. The shock freezing in combination with freeze drying procedure removes the organic solvent from the solid state by sublimation. During this process it is presumed that reduced segregation of lipids takes place compared to drying the lipids at 37°C. Furthermore, in case of the *Linomat spraying*, very small droplets are formed initially, followed by the evaporation of the organic solvent. This procedure also reduces the segregation and domain formation of the lipids. This is in contrast to the *spreading* method, where a variation in lipid solubility of respective components may lead to a variation in the lipid composition and precipitation, possibly resulting in lipid domains in which the barrier lipids are present at relatively high levels.

Role of VC compounds in the formation of the LPP

As the LPP was observed in VC lipids applied by *spreading*, it was of interest to determine the role the various lipid fractions play in LPP formation. For this reason the phase behaviour of the various lipid classes applied by *direct spreading* was examined. It appeared that only the VC barrier lipids are able to form the

LPP. Therefore, although the SE/WE fraction is the major lipid class of VC lipids [7], the VC barrier lipids are most likely responsible for the formation of the LPP. The crucial importance of acyl-CER, especially EOS with a linoleic acid moiety, for the formation of the LPP had been demonstrated in previous studies [10, 24]. In VC lipid mixtures, 24% of ester-linked and 11% of the amide-linked fatty acids of the acyl-CER are branched [25] whereas in SC almost no branched chains are observed [26, 27]. However, the most prominent ω-hydroxy-linked fatty acid of EOS in VC lipids is the linoleic acid moiety (C18:2) which is similar to SC. Moreover, comparable amounts of the amide-linked triacontanoic moiety (C30:0) were detected for both specimen [25, 27]. This may explain the formation of the LPP in VC lipids and the similarity in the repeat distance of approximately 12.8 nm in VC (barrier) lipids (Fig. 1A and 2B), in SC lipids (Fig. 5) and in isolated SC [9].

Temperature dependence in LPP formation

In this paper, we show for the first time that the LPP can be formed already at body temperature and that the thermal equilibration step at higher temperatures, as used in the previous studies, is not required. In CER containing SC lipid mixtures LPP (de-) formation is observed at approximately 65°C which coincides with the transition from hexagonal lateral packing to the liquid phase [14]. These earlier studies were conducted using equimolar mixtures of CER/CHOL/FFA containing CER isolated from native human SC and typically the equilibration temperature was chosen just below this phase transition. However, in the barrier lipid mixtures of VC, after equilibration at around 68°C, the LPP is formed between 44°C and 34°C (Fig. 4) during cooling which is a much lower temperature range than observed for the SC barrier lipid mixtures. This might be due to the higher content of branched fatty acids present in the CER and FFA of VC [25]. These branched fatty acid chains are packed less densely and consequently, a lower temperature for the lipid order-disorder transition is observed, as demonstrated by Fourier transform infrared spectroscopy [8]: at 40°C VC lipids are already in a fluid phase. This difference might also explain the different temperatures for LPP formation, since a certain degree of mobility of the lipids seems to be crucial to form the LPP. In conclusion, although the repeat distance is similar, the temperature required for the LPP formation is different for VC and SC barrier lipids, and can be most likely attributed to the different chain composition and the resulting difference in physicochemical properties.

Formation of LPP in extracted SC lipids

As it is possible to form the LPP in VC lipid samples at 37°C, the question rises whether the LPP can also be formed in SC lipid extracts under the same conditions. Our present studies reveal that the LPP can also be formed in total lipid extracts of human SC without equilibration at higher temperature (Fig. 5). This is only observed when using the *spreading* method, which might promote

the formation of domains of barrier lipids in the lipid sample. Besides the barrier lipids, these investigated SC lipid extracts contained also low amounts of polar phospholipids and sebum components, i.e. SE and TG (data not shown). The latter components have been suggested to reduce crystallinity [28] which may facilitate the formation of the LPP in these samples (see above). As the equilibration temperature is 37°C, these studies demonstrate for the first time that the LPP can be formed at body temperature. Even when we use mixtures prepared from isolated human CER, CHOL and synthetic FFA, the formation of the LPP is frequently observed at 37°C. The formation of the LPP in these mixtures indicates that the application of shear stresses during the *direct spreading* method facilitates this process of formation. In a very different field, it has been reported that shear and sample history affect the crystallization of the polymorphic, triglyceride-rich chocolate [29]. The process of controlled crystallization of an individual, stable polymorph of the chocolate is called tempering and involves a characteristic time-dependent and temperature-controlled cooling procedure as well as application of distinct shear-rates [29, 30]. The effect of shear stresses on the formation of the LPP will be subject of future studies.

In conclusion, we showed for the first time that VC lipids are able to form the LPP with similar spacing as observed in human SC. Like in SC, the LPP is formed by VC barrier lipids. In addition, the formation of LPP in SC and VC lipid extracts at physiological temperatures was observed.

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