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

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# Lanolin-derived lipid mixtures mimic closely the composition and organization of vernix caseosa lipids

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**Abstract**
The aim of the present study was to use semi-synthetic lipid mixtures to mimic the complex lipid composition, organization and thermotropic behaviour of vernix caseosa lipids. As vernix caseosa shows multiple protecting and barrier supporting properties before and after birth, it is suggested that a vernix caseosa substitute could be an innovative barrier cream for barrier deficient skin. Lanolin was selected as the source of the branched chain sterol esters and wax esters – the main lipid class of vernix caseosa. Different lipid fractions were isolated from lanolin and subsequently mixed with squalene, triglycerides, cholesterol, ceramides and fatty acids to generate semi-synthetic lipid mixtures that mimic the lipid composition of vernix caseosa, as established by high-performance thin-layer chromatography. Differential scanning calorimetry and Fourier transform infrared spectroscopy investigations revealed that triglycerides play an important role in the (lateral) lipid organization and thermotropic behaviour of the synthetic lipid mixtures. Excellent resemblance of vernix caseosa lipids was obtained when adding unsaturated triglycerides. Moreover, these lipid mixtures showed similar long-range ordering as vernix caseosa. The optimal lipid mixture was evaluated on tape stripped hairless mouse skin *in vivo*. The rate of barrier recovery was increased and comparable to vernix caseosa lipid treatment.

**Introduction**
The creamy white skin-surface biofilm vernix caseosa (VC) which covers the skin of the fetus and the newborn is known for its multiple biological functions. *In utero* it might act as waterproof film and facilitate the formation of the skin [1]. During delivery it acts as a lubricant and reduces the friction, while it exhibits anti-infective [2], anti-oxidant [3], skin hydrating [4] and skin cleansing properties postnatally [5]. These multiple biological functions and unique properties of natural VC suggest that the generation of a synthetic VC equivalent could lead to a new generation of biofilms. These might be applicable for extremely low birth weight infants with deficient barrier-function and absence of VC [5, 6] but may also be beneficial for adult skin to enhance wound healing [7].
Besides approximately 80% water, VC is composed of about 10% proteins (mostly keratin originating from corneocytes) and 10% lipids [6]. VC displays a wider range of different lipid classes compared to stratum corneum (SC). The most abundant lipids in VC are sebaceous gland-derived nonpolar lipids such as sterol esters (SE), wax esters (WE) and triglycerides (TG) [8]. In addition, SC derived barrier lipids, cholesterol (CHOL), free fatty acids (FFA) and ceramides (CER) are also present but in lower amounts than in SC, amounting approximately 10-30% of the total lipid mass in VC [6, 9]. An overview of the

individual lipid classes is provided in table 1A. In all major lipid classes straight, methylbranched, saturated and unsaturated fatty acids have been identified [8, 10]. For example the SE fraction of VC lipids is composed of about 50% branched fatty acids [9]. In contrast to that, in human SC mainly straight saturated fatty acids are present [11, 12], although branched chains are also observed in sebum of human skin [13]. Recently it has been reported that these FFA in VC exhibit antibacterial activity [14].

Because of the excellent properties of VC in facilitating SC hydration [4], it is suggested that a VC substitute could be an innovative barrier cream for immature skin of preterm infants. Our present study aims to generate a lipid mixture with a very similar composition and organization as found for VC lipids. As a source of lipids, commercially available CHOL, synthetic CER, FFA, TG and squalene (SQ) have been used. However, the nonpolar branched lipids (SE/WE) are scarcely commercially available. Therefore natural sources had to be selected, such as spermaceti wax from the whale or wool fat from the sheep – referred to as lanolin. Fawaz and co-workers [15-17] reported that lanolin consists of 43.7% (hydroxy-) SE and WE and 7.5% dihydroxy wax esters (DIOL), the lipid classes that are also present in VC. Besides these lipid classes, lanolin contains 4.1% free sterols, 6.5% free alcohols and triterpene esters (35.4%). CER also have been reported to be present up to 2.3% [18]. In addition, lanolin also contains branched fatty acids, which amount to 54% of the fatty acid chains in the wool wax [19]. For this reason, lanolin was chosen as a source for the branched SE/WE. The fractions containing SE, WE and DIOL were isolated from lanolin, using column chromatography, and subsequently mixed with SQ, barrier lipids and TG. The lipid organization and thermotropic behaviour of extracted VC lipids, isolated lipids (i.e. fractions from lanolin) and lipid mixtures (i.e. isolated fractions from lanolin + barrier lipids + SQ + TG) were investigated. From these studies the optimal lipid composition mimicking most closely the lipid organization in VC was selected. The biological effect of this lipid mixture was evaluated on barrier recovery of tape stripped mouse skin and the results were compared to the effects of VC lipids and Vaseline.

**Table1**

**A.** Composition of natural VC lipids* (w/w)%

| | SQ | SE/WE | DIOL | TG | CHOL | FFA | CER |
|---|---|---|---|---|---|---|---|
| natural VC lipids | 6,4 | 42,0 | 6,0 | 35,7 | 3,5 | 1,5 | 4,9 |

**B.** Composition of semi-synthetic lipid mixtures (SSLM, weight ratios)

| | SQ | LanSE | LanX | LanDI | TG§ | TGa§ | CHOL | FFA§ | CER§ | lanolin |
|---|---|---|---|---|---|---|---|---|---|---|
| SSLM | 6,4 | 42,0 | 48,0 | 6,0 | 35,7 | 35,7 | 3,5 | 1,5 | 4,9 | 48,0 |
| SE | + | + | | | + | | + | + | + | |
| DI | + | + | | + | + | | + | + | + | |
| X | + | | + | | + | | + | + | + | |
| SEa | + | + | | | | + | + | + | + | |
| DIa | + | + | | + | | + | + | + | + | |
| Xa | + | | + | | | + | + | + | + | |
| Xa 2barrier | + | | + | | | + | ++ | ++ | ++ | |
| La | | | | | | + | | + | + | + |

* adapted from [9]
§ refer to Materials & Methods section for detailed composition
LanSE, LanX, LanDI represent the different isolated fractions from lanolin
++ doubled amount present

## Materials & Methods

*Collection and lipid extraction of vernix caseosa*

VC was scraped off gently immediately after vaginal delivery or caesarean section of healthy term neonates. The samples were transferred into sterile plastic tubes and stored at 4ºC until use. For the experiments, various samples from different donors were used. The collection of VC was approved by the ethical committee of the Leiden University Medical Center and informed consent was given by the parents. To isolate VC lipids, extraction of VC was performed by using a modified method of Bligh and Dyer [20]. Briefly, the VC samples were extracted by mixtures of chloroform/methanol as has been described previously [9].

*Materials*

All organic solvents were of analytical grade and provided by Labscan Ltd. (Dublin, Ireland). Hydrous lanolin was purchased from Caesar & Loretz (Bonn, Germany). The synthetic CER were kindly provided by Cosmoferm B.V. (Delft, The Netherlands). The different TG and FFA were purchased from Sigma

(Schnellendorf, Germany). Silica gel with small (15-25 μm) and larger (43-60 μm) particles with a pore size of 60 Å was manufactured by Merck KGaA (Germany). Furthermore, 10x20 cm high-performance thin-layer chromatography (HPTLC) plates were obtained from Merck KGaA (Germany). 6-8 weeks old male hairless mice (SKH-rh1) were purchased from Charles River (France). The D-squame tape strips for the *in vivo* experiments were obtained from Cuderm (Dallas, USA). Vaseline (petrolatum) is an occlusive, oil-based ointment and was purchased from Elida Fabergé (London, United Kingdom).

*Column chromatography of lanolin*

Prior to usage, 1 g of hydrous lanolin was dispersed in 4 ml chloroform/methanol 2:1 (v/v) and dehydrated at 40°C under a gentle stream of nitrogen. The nonpolar lipids from (anhydrous) lanolin were isolated by means of column chromatography. 32 g Lioprep 60R was dehydrated for 1 h at 130°C, after which 40 ml chloroform was added. The mixture was then poured into a glass column with a diameter of 20 mm and a length of 420 mm. The pre-column was packed with 4 g dehydrated silica gel. 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 (see below) and carefully applied on the column. After discarding the dead-volume of ~55 ml, fractions of 3.8 ml were collected during the elution with the following eluents: 150 ml hexane/chloroform/ diethyl ether 96:4:0.5 (v/v), 100 ml hexane/chloroform/diethyl ether 90:6:1 (v/v), 100ml hexane/chloroform/diethyl ether/dioxan 88:10:2:0.5 (v/v). The lipid fractions were subsequently dried under a gentle stream of nitrogen at 40°C and analyzed qualitatively by means of HPTLC.

*High-performance thin-layer chromatography*

Analysis of the various lipid fractions obtained from the column chromatography was performed by using a one-dimensional HPTLC as described earlier [6, 21]. Briefly, 5-50 μg of lipid samples was applied on a rinsed and dehydrated HPTLC plate by means of a Linomat IV (Camag, Muttenz, Switzerland). Two different separation protocols were used: I) For separation and evaluation of the nonpolar lipids (mainly SE, WE and DIOL), HPTLC plates were developed with hexane/chloroform/diethyl ether/ ethyl acetate 48:48:4:1 (v/v) for 95 mm. II) A more optimal separation of all lipid classes in VC and lanolin was achieved by using a more complex and sequential developing protocol as described by Ponec *et al.* [21]. 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.

*Preparation of lipid mixtures*

The individual lipid components (Table 1) were dissolved in chloroform/methanol 2:1. The lipid composition of VC lipids has been reported in literature [9] and is provided in table 1A. The composition of the semi-synthetic lipid mixtures were chosen as presented in table 1B with the composition closely mimicking the one of VC lipids. The terminology of the semi-synthetic lipid mixtures (SSLM) was chosen according to their main nonpolar lipid component: the SE/WE fraction (referred to as LanSE) is present in SSLM-SE and together with the DIOL fraction (LanDI) in SSLM-DI, whereas SSLM-X represents a miscellaneous mixture of SE/WE and DIOL (LanX) as main nonpolar component.

All molecular structures of the synthetic (skin) CER, which resemble pig CER and closely mimic human CER, were reported by de Jager *et al.* [22]. CER subclasses are denoted by the letter-based system introduced by Motta *et al.* [23] with the number of carbon atoms (C) in the acyl-chain being either C16, C24 or C30. The samples consisted of synthetic CER mixture, which is composed of EOS(C30)linoleate, NS(C24), NP(C24), AS(C24), AS(C16) and AP(C24) at weight ratios of 14.6, 20.8, 8.3, 8.3, 16.7 and 31.3%, respectively. Unlike native CER, the synthetic CER are characterized by a uniform chain length. As this might affect the lipid phase behaviour, it was decided to compensate for this by selecting TG mixtures of different composition. The first TG mixture tested, mimics the TG composition in VC most closely according to our analysis published previously [9] and is composed of TG16:0, TG16:1, TG18:0, TG18:1 at weight ratios of 30.4, 39.7, 5.6 and 24.3, respectively. In addition, an adjusted triglyceride mixture (TGa) with only unsaturated fatty acid chains was prepared, which consisted of TG16:1, TG18:1, TG24:1 at weight ratios of 1:1:1. This replacement with unsaturated TG was denoted e.g. SSLM-Xa (adjusted mixture). The simplified FFA mixture, as opposed to VC, consisted of fatty acids C16:0, C16:1, C18:0 and C18:1 with weight ratios of 53.3, 20.0, 6.7 and 20.0, respectively. Lipid mixtures were prepared by pipetting the solution of the individual component into a glass tube. Subsequently, the mixture was dried under a stream of nitrogen at 40°C.

*Differential scanning calorimetry*

The thermotropic behaviour of isolated VC lipids and semi-synthetic lipid mixtures of varying composition was examined by DSC. The measurements were carried out on a Q-1000 calorimeter (TA Instruments, New Castle, Delaware, USA). Dry lipids (1-5 mg) were transferred into an aluminium pan. Subsequently, the pan was hermetically sealed. After 5 min equilibration at 5°C, DSC was

performed with a heating rate of 2°C/min and a modulation of ±1°C/min up to 50°C.

*Fourier transform infrared spectroscopy*

The dried lipids (~1.5mg) were applied on a ZnSe window and then sandwiched between a second window. Subsequently, the windows were mounted into a special designed heating/cooling cell. FTIR was performed on a Bio-Rad Excalibur FTS 4000 XM (Bio-Rad Laboratories Inc., Cambridge, Massachusetts, USA), equipped with a SHA 10 FTIR air purifying system (Hitma BV, Uithoorn, the Netherlands) and a mercury–cadmium–telluride detector which was cooled with liquid nitrogen. The IR spectra in the frequency range of 600-4000 $cm^{-1}$ were collected during 8 min at 2°C intervals between 10 and 50°C as a function of temperature (heating rate of 0.25°C/min). Each spectrum resulted from the co-addition of 128 scans with a nominal resolution of 1 $cm^{-1}$.

*Small-angle X-ray diffraction*

The SAXD measurements were conducted at station BM26B at the European Synchrotron Radiation Facility in Grenoble, France [24]. Lipid samples (~1.5 mg) were smeared on mica windows. In order to obtain the same diffraction patterns as VC, VC lipids were dissolved in chloroform/methanol 2:1 and sprayed onto mica windows by a Linomat IV (Camag, Muttenz, Switzerland). Subsequently, the samples were transferred into a special sample holder which was mounted into the X-ray beam. The diffraction data were collected by a two-dimensional gas-filled area detector with a 1.5 m sample-detector distance. The X-ray wavelength was 1.24 Å. The diffraction pattern was acquired for 10 or 15 min at 15°C. The spatial calibration of this detector was performed using silver behenate and CHOL.

*In vivo study on hairless mice*

All animal experiments were conducted in conformity with the Public Health Service Policy on use of laboratory animals and were approved by the ethical committee for animal studies of Leiden University (Leiden, The Netherlands). In order to acclimatize the animals, the hairless mice were left after arrival in our animal facilities (Gorlaeus Laboratories, Leiden, The Netherlands) for at least one week before starting the experiments. All measurements were conducted in rooms with controlled temperature and relative humidity. A mixture of ketamine/xylazine/physiologic salt solution 2:1:1 (v/v) was injected i.p. to anesthetize the mice for approximately 1 h. After washing the skin of the back with water, two sites (each 1 $cm^2$ in size) on the upper back were marked and sequentially tape stripped until a very red and shiny skin was obtained. The

direction of tape stripping was alternated. Transepidermal water loss (TEWL) was measured with a Tewameter TM 210 (Courage & Khazaka, Cologne, Germany) prior to disruption (TEWL ~10g/m²/h), directly after disruption (TEWL 70-85 g/m²/h) and 6 and 48 h after treatment with 5 mg Vaseline, isolated VC lipids or (semi-) synthetic lipid mixture. The disrupted, untreated site was used as negative control. At each time point the baseline TEWL was determined at a lower site of the back.

**Results**

*Isolation of nonpolar lipid fractions from lanolin*

VC major lipid fractions are SE, WE and TG [8, 9]. Column chromatography was used to isolate similar lipid classes from lanolin. As shown in figure 1., the various fractions of lanolin were pooled in three major fractions, a SE/WE fraction (LanSE, lane 2-4), a DIOL fraction (LanDI, lane 12-20) and a miscellaneous fraction consisting of SE/WE and DIOL (LanX, lane 5-11). These fractions were characterized and compared with the properties of VC lipids (lane 23) and used as a basis for the generation of the synthetic VC lipid mixtures.

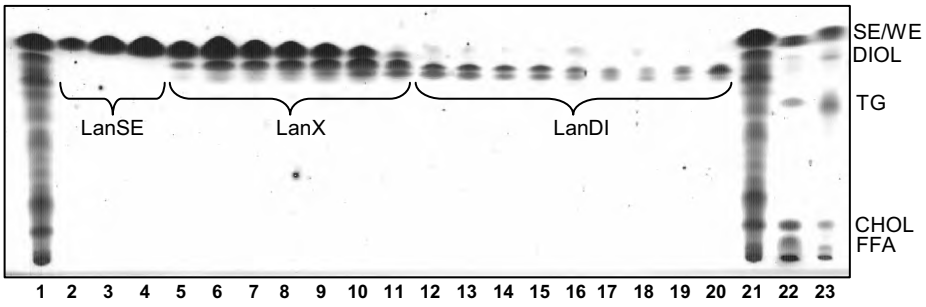


**Figure 1.** Isolation of nonpolar lipids from lanolin. A HPTLC of the nonpolar lipid fractions of lanolin obtained after column chromatography is depicted. Fractions were reunited in the SE/WE fraction (LanSE, lane 2-4), DIOL (LanDI, lane 12-20) and a miscellaneous fraction (LanX, lane 5-11). Lanolin lipids are depicted on lane 1 and 21, a synthetic standard mixture and a VC lipid sample on lane 22 and lane 23, respectively.

*Thermotropic behaviour of isolated lanolin fractions resemble that of VC lipids*

In order to characterize the obtained lanolin fractions, their thermotropic behaviour was investigated and compared to that of the VC lipids in the temperature range between 5 and 50°C. As already observed for fresh VC samples [25], VC lipids showed the presence of two overlapping transitions with onset temperatures at 20.5 and 24.3°C (Fig. 2A). The enthalpies yield -0.6 J/g for the exothermic and 1.2 J/g for the endothermic transition. The thermotropic behaviour of lanolin revealed also two transitions, an endothermic and an exothermic transition. The onset temperature of the 2nd transition is 28.4°C, which is at a slightly higher temperature than observed for the VC lipids (24.3°C). In order to select the most appropriate fraction to simulate the lipid organization and thermotropic behaviour of VC lipids, the various fractions isolated from lanolin were measured. The thermotropic behaviour of LanSE and LanDI are both characterized by an exothermic and an endothermic transition (Fig. 2A), similarly to VC lipids. The exothermic transition has onset temperatures between 18.6°C and 20.6°C for LanDI and LanSE, respectively.

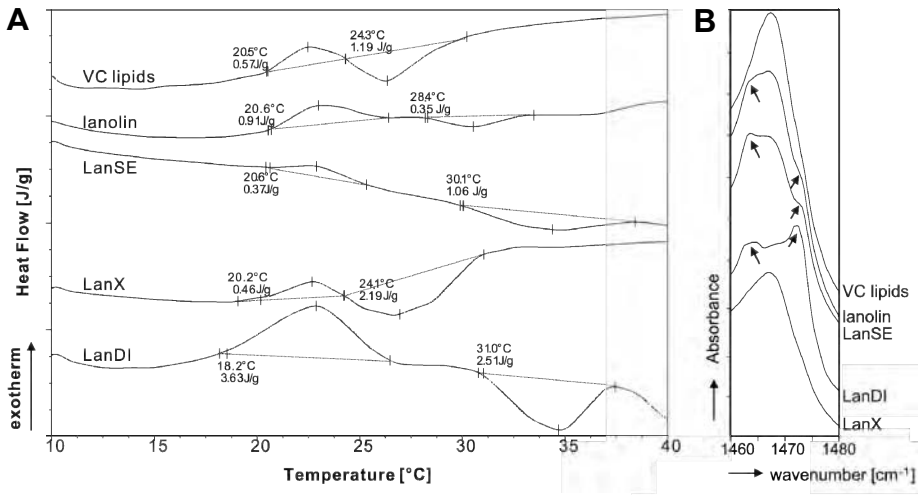


**Figure 2.** Thermotropic behaviour and lateral packing of isolated lipid fractions and VC lipids.

**(A)** DSC thermograms of VC lipids and lanolin show two overlapping transitions. The different isolated, nonpolar lipid fractions of lanolin and synthetic VC lipids are also characterized by two events. An exothermic peak is visible with an onset of 18.5-20.7°C whereas a second, endothermic peak is characterized by a higher variability in onset temperatures (24.2-30.5°C).

**(B)** Lipid samples exhibit different lateral organization as monitored by the methylene stretching mode at 15°C: VC and LanX are characterized by the presence of a single peak whereas lanolin, LanSE and LanDI exhibit a doublet, which is indicative for a different (orthorhombic) phase behaviour.

The endothermic events of these fractions are also characterized by similar onset temperatures being 30.1°C (LanSE) and 31.0°C (LanDI). These onset temperatures, however, are approximately 5°C higher than observed for VC lipids but are still in the physiological temperature range. LanX exhibits two overlapping transitions with onset temperatures of 20.2°C and 24.1°C, very similar to those in VC lipids. The transition enthalpies are -0.5 J/g and 2.2 J/g for the exothermic and endothermic transition, respectively. To summarize, all lipid fractions show some similarities in thermotropic behaviour to VC lipids but LanX mimics VC lipids the closest concerning transition onset and enthalpies.

*Different lanolin fractions are characterized by different lateral packing*
The lateral packing and conformational ordering of the isolated lipid fractions, lanolin and VC was examined by FTIR. We focused on the methylene scissoring mode, which provides information on the lateral packing of the hydrocarbon chains in the lipid mixture [26]. A doublet contour is caused by short range coupling between the protonated chains in the lattice, which is indicative for an orthorhombic lateral packing. A singlet band represents a hexagonal or a fluid phase. As depicted in figure 2B, the methylene scissoring mode of VC lipids at 15°C shows a singlet at 1467 $cm^{-1}$, which indicates the absence of an orthorhombic phase. In the spectrum of lanolin, besides the singlet at 1467 $cm^{-1}$, a weak doublet contour at 1472 $cm^{-1}$ and 1463 $cm^{-1}$ (arrows) is also noticed suggesting that a small population of lipids forms an orthorhombic lateral packing. The spectrum of LanSE and LanDI features also a doublet (arrows) being most pronounced in the spectrum of LanDI (arrows). The spectrum of LanX is only characterized by a singlet band at 1467 $cm^{-1}$, very similar to the spectrum of VC lipids. Therefore, LanX resembles most closely the lateral packing of the VC lipids. LanX seems the most promising candidate for the SSLM. However, as all three isolated lanolin components exhibit transitions within physiological temperature range, these three fractions were included in the studies described below to select the optimal synthetic VC lipid mixture.

*SSLM mimic lipid composition and thermotropic behaviour of VC lipids*
To mimic the lipid composition and organisation of VC (Table 1A) more closely, SQ, TG, CHOL, FFA and CER were added to the three different nonpolar lanolin fractions. These fractions are referred to as the SSLM. The composition of the SSLM was systematically varied and is provided in table 1B. HPTLC was performed (Fig. 3) in order to compare VC (lane 2), lanolin (lane 3) and the various SSLM (lane 4-7). In general, the lipid profile of lanolin derived lipid mixtures showed great resemblance with VC lipid profile.

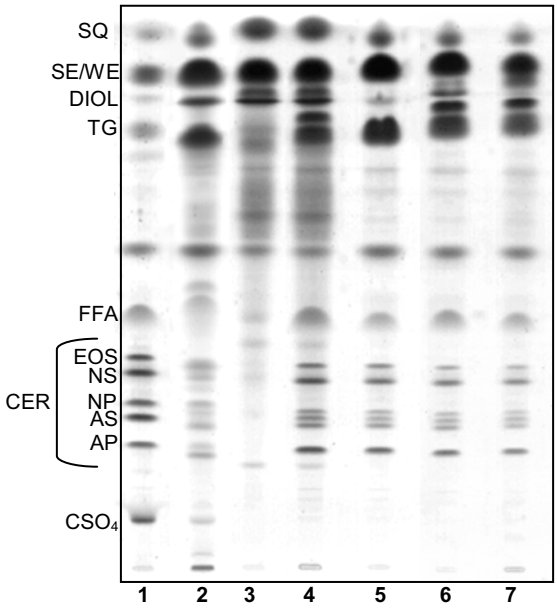


**Figure 3.** Composition of semi-synthetic lipid mixtures (SSLM). An HPTLC showing a lipid standard (lane1), VC lipids (lane 2), lanolin (lane 3), different, representative (semi-) synthetic VC lipid mixtures: lane 4 – SSLM-La, lane 5 – SSLM-SE, lane 6 – SSLM-DIa, lane 7 – SSLM-Xa.

Initially the thermotropic behaviour was studied of the various lipid mixtures, in which the TG most closely mimics the TG composition in VC. As depicted in figure 4A, the addition of SQ, barrier lipids and TG to the nonpolar lanolin fractions altered the thermotropic behaviour of the three mixtures SSLM-SE, SSLM-DI and SSLM-X. The exothermic peaks of synthetic lipid mixtures show similarities to VC lipids. In contrast, the enthalpy of transition of the endothermic peak is much higher than in VC lipids. This increase in enthalpy of transition is most dramatically in the SSLM-X and SSLM-SE mixtures. Furthermore, the transition occurs over a much larger temperature range. The end temperature of the endothermic transitions are 39.2°C, 33.3°C and 40.7°C for SSLM-SE, SSLM-DI and SSLM-X, respectively. In VC lipids the transition completes at 30°C.

*Adjusting the TG mixture composition in SSLM to resemble thermotropic behaviour of VC lipids more closely*

As the endothermic transition of the lanolin fractions mimics the endothermic transition of VC lipids more closely, the composition of either the barrier lipids or TG in the SSLM should be adjusted. For this reason TG of VC were isolated by column chromatography and compared for thermotropic behaviour to the synthetic TG mixture. The thermogram of the natural TG shows an exotherm with a peak maximum at 22.9°C and an endotherm at 35.1°C (Fig. 4B). However,

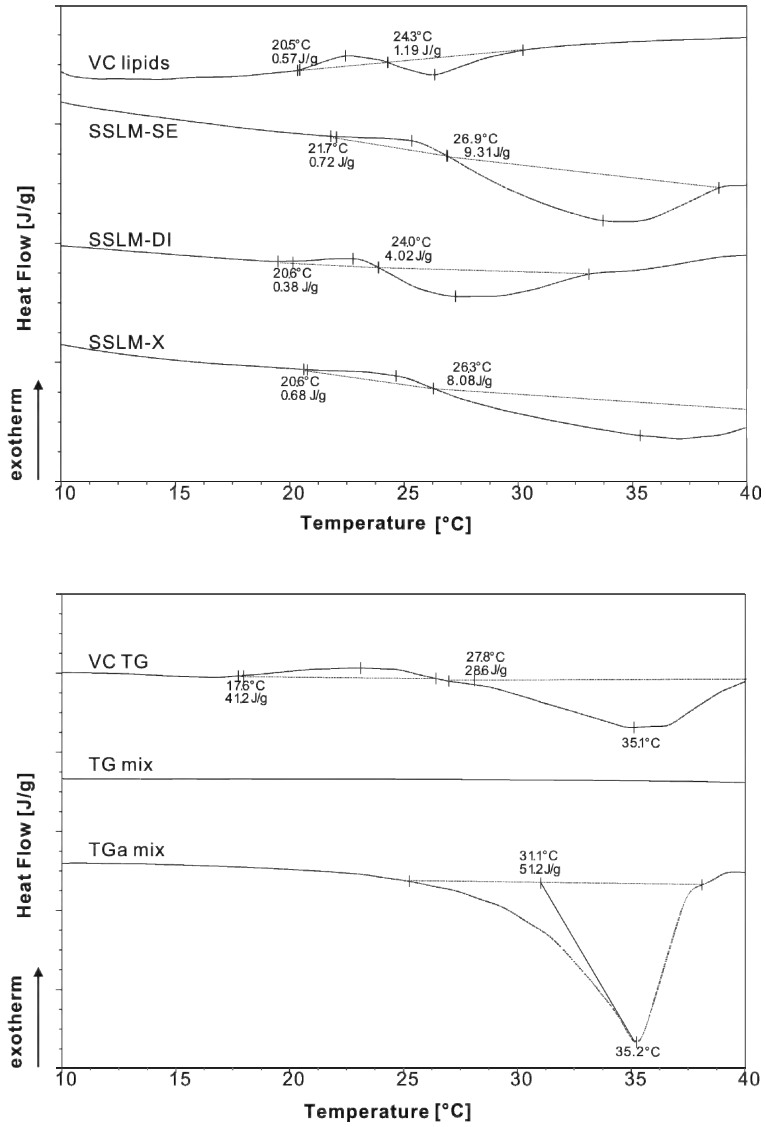


**Figure 4.** Thermotropic phase behaviour of VC lipids and synthetic lipid mixtures with the first (simplified VC) TG mixture. The DSC thermograms show two overlapping phase transitions in VC and SSLM-SE, SSLM-DI and SSLM-X (A). DSC thermograms of isolated VC TG, synthetic TG and the adjusted TG mixture (TGa) are depicted (B).

the synthetic TG mixture, representing a simplified TG mixture of VC, shows no transitions in this temperature range (Fig. 4B). As TG represent the largest fraction of lipids added to the lanolin-derived lipids, it was decided to use a TG mixture composed of only unsaturated triglycerides (TGa) as this might reduce the crystallinity of the mixture. This resulted in a substantial change in the thermotropic behaviour of the TG. An endothermic transition with a peak maximum at 35.2°C was detected resembling the endothermal transition temperature of the natural TG mixture (Fig. 4B). Although the transition enthalpies are different with 28.6 J/g and 51.2 J/g for the natural TG and for synthetic TGa, respectively, we decided to select this mixture for our further studies. TGa was mixed with one of the lanolin fractions (LanX, SE, DI), and SQ, CHOL, FFA and CER. In all three SSLM differences were observed: I) the endothermic transitions were characterized by smaller melting enthalpies, e.g. $\Delta H$=8.08 J/g for SSLM-X (Fig. 4A) versus $\Delta H$=0.73 J/g for SSLM-Xa (Fig. 5) and II) the temperature range of endothermic transition changed. The end temperature of the endothermic transition was reduced, e.g. from 40.7°C for SSLM-X to 32.5°C for SSLM-Xa. When comparing the thermotropic transitions of the SSLM, the SSLM-Xa most closely mimics the phase transitions of the VC lipids. Increasing twofold the barrier lipid content (SSLM-Xa 2barrier) did not affect the thermotropic phase behaviour (Fig. 5).

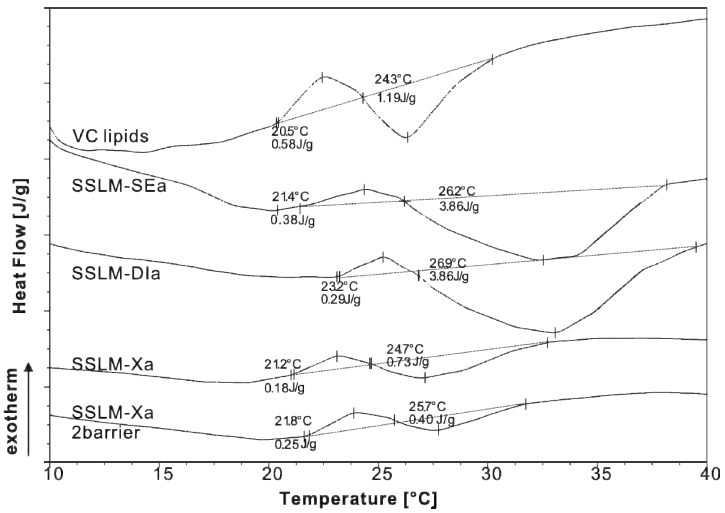


**Figure 5.** Optimized thermotropic behaviour of adjusted semi-synthetic lipid mixtures. DSC thermograms of VC lipids and different synthetic lipid mixtures with the adjusted TG mixture are shown.

*SSLM show similar thermotropic response to VC lipids as monitored by FTIR*

In order to obtain more detailed information on the similarities between the SSLM and VC lipids, the lipid organization of the SSLM and VC lipids was studied by FTIR. In figure 6 the symmetric $CH_2$-stretching frequencies are shown, which provide information about the conformational order–disorder transitions [27]. VC lipids are characterized by a steady, gradual increase from 2850.6 $cm^{-1}$ at 10°C to 2853.1 $cm^{-1}$ at 50°C. This shift in frequency represents an ordered-to-disordered transition over a wide temperature range. A representative synthetic mixture with the TG mixture, SSLM-X, and with the adjusted TG mixture, SSLM-Xa, is presented in figure 6 as well. The latter shows a higher symmetric $CH_2$-stretching frequency at 10°C compared to VC and SSLM-X. This indicates a slightly more conformational disordering than observed in the VC mixture at 10°C. In the temperature range between 10 and 50°C, the symmetric $CH_2$-stretching frequencies of SSLM-Xa increase gradually to 2853.7 $cm^{-1}$, slightly higher frequency than observed in the VC samples. However, the shape of the curve and the total shift in $CH_2$ stretching frequency are very similar to that in VC. For comparison, lanolin is also depicted in figure 6. At 10°C the symmetric methylene stretching mode is 2849.8 $cm^{-1}$, which is characteristic for an ordered all trans conformation of the hydrocarbon chains. When the temperature is increased, a small shift in the $CH_2$ symmetric frequency is observed. A further increase in temperature does not change the symmetric $CH_2$-stretching frequencies until a temperature of about 22°C is reached. Then a gradual shift in frequency is seen reaching a value of 2854 $cm^{-1}$ at 42°C. This displays a more pronounced order-disorder transition than observed in VC lipids and the SSLM mixtures.

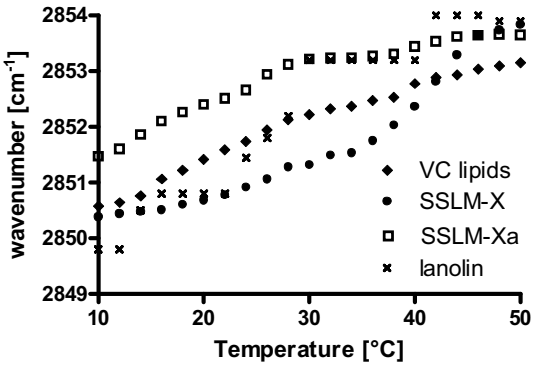


**Figure 6.** Lipid order – disorder transitions as observed by FTIR. The spectra of VC lipids (♦), lanolin (x) and the lipid mixtures SSLM-X (●) and SSLM-Xa (□) depicting the thermotropic response curves of the symmetric methylene stretching mode are shown.

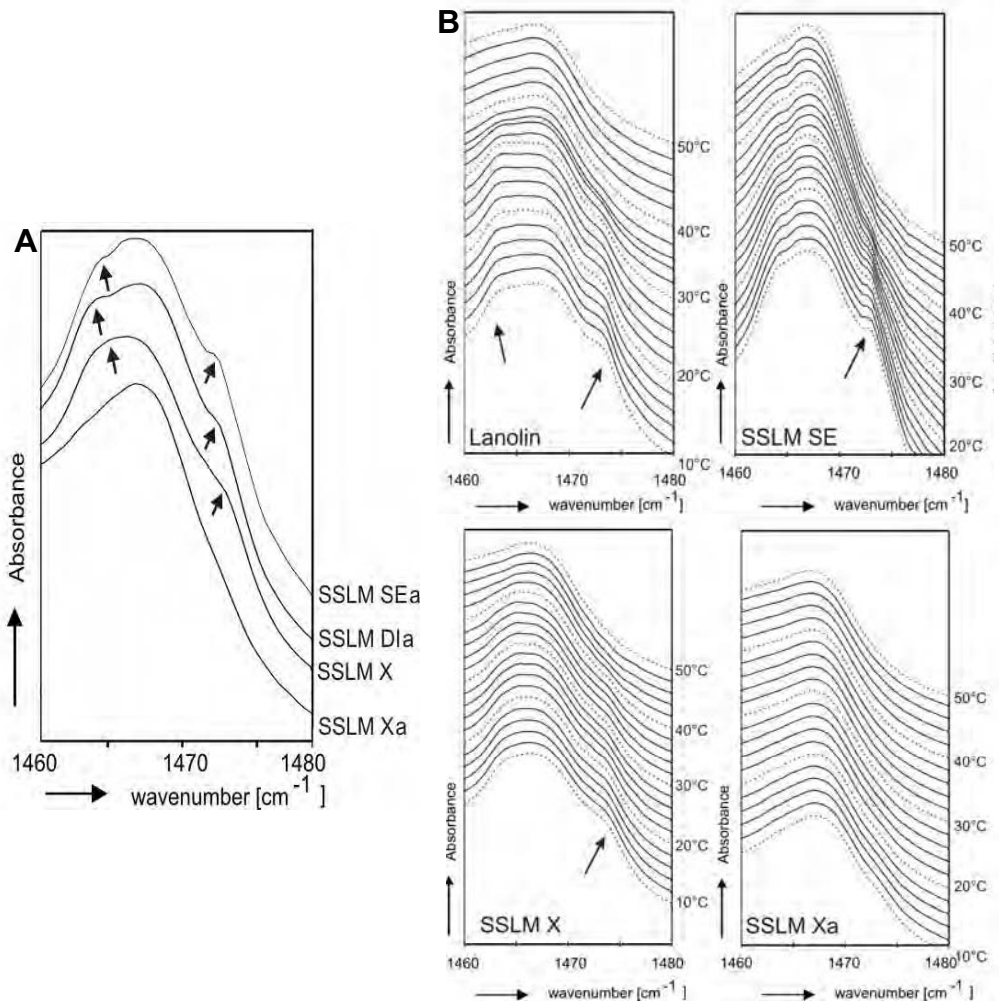


**Figure 7.** Lateral organisation of lipid mixtures. FTIR spectra of different synthetic lipid mixtures at 15°C (panel A) and of lanolin and representative SSLM as a function of temperature (panel B).

*Lateral lipid organization of the SSLM*

At 15°C, the $CH_2$ scissoring contours in the spectra of SSLM-SEa and SSLM-DIa are characterized by a singlet at 1467 $cm^{-1}$ and two weak shoulders representing a doublet at 1472 $cm^{-1}$ and 1463 $cm^{-1}$ (arrows in Fig. 7A). The presence of the doublet suggests that a small population of lipids is in an orthorhombic phase. The spectrum of SSLM-Xa exhibits only a singlet at 1467 $cm^{-1}$, which is very similar to the spectrum of VC lipids (Fig. 2B). Without the adjustment of TG to a complete unsaturated chain content, the doublet is also present in the spectrum of SSLM-X, indicating the importance of TG for the lateral packing.

The thermotropic behaviour of the scissoring modes is depicted in figure 7B. In lanolin, SSLM-SEa and SSLM-X, the weak shoulders (arrows) dissolve at around 38°C whereas in SSLM-Xa no doublet is discernable. This is similar to VC lipids, which are characterized by a singlet at 1467 $cm^{-1}$ at 15°C (Fig. 2B) and throughout the entire temperature range up to 50°C (data not shown).

*SSLM-Xa shows best match to VC lipids concerning long range ordering*

To obtain information on the long range ordering of the SSLM with the TGa mixtures, SAXD measurements were conducted. To avoid interference with the phase transitions, the studies were performed at 15°C, which is below the phase transitions. Figure 8A shows the small-angle diffraction patterns of extracted VC lipids and of fresh VC samples. Both the fresh samples and the extracted VC lipid are characterized by two diffraction peaks with very similar spacings. Comparison of various SSLM mixtures revealed that SSLM-Xa most closely mimics the long range ordering observed in VC (Fig. 8B). Mixture of lanolin with barrier lipids and TGa (SSLM-La) did not show any long range ordering.

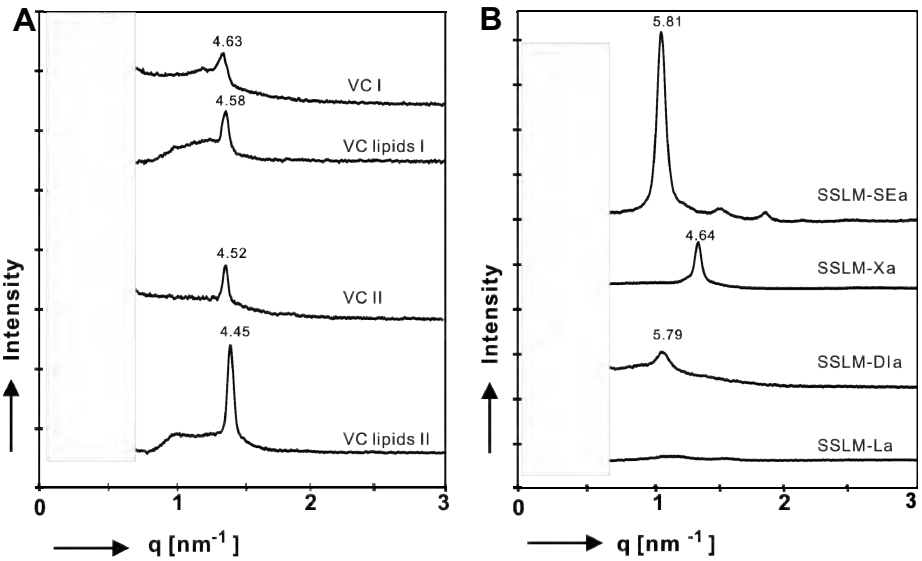


**Figure 8.** Long-range ordering of VC (lipids) and SSLM. SAXD measurements showed, that fresh VC samples are characterized by two diffraction peaks: at q=1.36 $nm^{-1}$ (d=4.63 nm) and q=1.39 $nm^{-1}$ (d=4.52 nm), respectively (A). Isolated lipids exhibited similar spacings. Data of two different representative donors are presented (I and II). SSLM exhibited long range ordering at q=1.08 $nm^{-1}$ (d=5.81 nm), q=1.09 $nm^{-1}$ (d=5.79 nm) for SSLM-SEa and DIa, respectively (B). SSLM Xa shows a similar long-range ordering as VC lipids, exhibiting a peak at q=1.35 $nm^{-1}$ (d= 4.64nm) whereas SSLM-La did not show any long range ordering.

*Synthetic lipid mixtures accelerate barrier recovery on mouse skin*

To evaluate the *in vivo* effect of the lipid mixtures, the skin barrier recovery after disruption by tape stripping was measured. The TEWL at different time intervals after disruption is depicted in figure 9. The baseline TEWL of the untreated and undisrupted skin only shows little variation ~10 g/m$^2$/h (diagonal striped bars). Immediately after disruption, the skin is characterized by a TEWL of 70-85 g/m$^2$/h (data not shown). 6 h after disruption, the TEWL is still 75.1 g/m$^2$/h (grey bars) whereas after 48 h a decrease to 61.1 g/m$^2$/h was observed. The Vaseline treated site (black bars) showed a similar TEWL value at 6 h (74.4 g/m$^2$/h) and a lower value at 48 h (46.1 g/m$^2$/h). However, after treatment with extracted VC lipids (horizontal striped bars) a reduction of the TEWL was already observed after 6 h (54.9 g/m$^2$/h), which decreased further after 48 h (26.8 g/m$^2$/h). The effect of the optimized synthetic lipid mixture (white bars) is comparable to the effect of VC lipids. In general, the barrier recovery facilitating effect of natural VC and the SSLM is evident in this murine model.

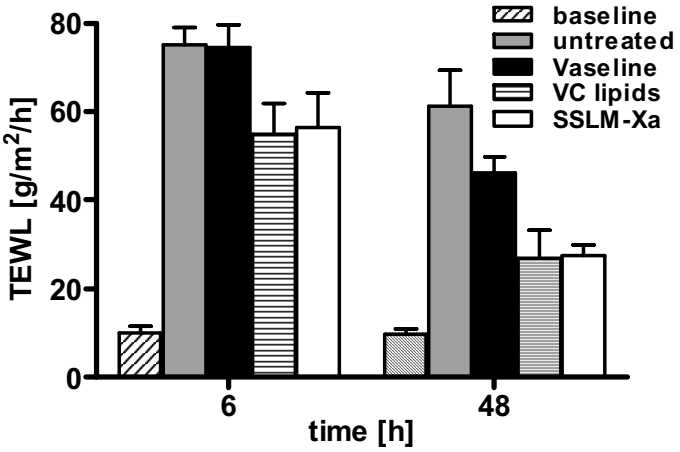


**Figure 9.** Barrier recovery with topical application of VC and SSLM. The effect of VC lipids, the optimized (semi-)synthetic lipid mixture (SSLM-Xa) and Vaseline on the barrier recovery of tape strip disrupted, hairless mouse skin was evaluated. The transepidermal water loss (TEWL) is depicted at 6 and 48 h. Baseline (diagonal striped bars), disrupted, untreated (grey bars), Vaseline treated (black bars), VC lipid treated (horizontal striped bars) and the adjusted synthetic lipid mixture (SSLM-Xa, white bars) treated sites are shown as mean + SD (n=6).

**Discussion**

In the first step to generate a (semi-)synthetic equivalent of the natural biofilm VC, the composition and organization of the synthetic biofilm lipids should resemble the natural counterpart as closely as possible.

*Why is it so important to mimic VC lipid organization?*

VC is characterized by its various and highly complex functions [7]. One of those features is the strong temperature-dependence of the dehydration property which enables VC to hydrate the newborn's skin in a sustained manner [4, 25]. It was speculated that the lipid-derived endothermal phase transition between 24 and 32°C plays a role in the dehydration rate of VC which is 4 times increased at 37°C compared to room temperature. Furthermore, the temperature-dependent order-disorder transition and the long-range order might be of benefit for VC as a barrier cream. Therefore, when mimicking VC, one of the important features is to design lipid mixtures which resemble the thermotropic behaviour of VC lipids.

*Selection of the lipid source for designing lipid mixtures mimicking closely lipid composition, lipid organization and thermotropic behaviour of VC lipids*

Nonpolar unsaturated branched chain SE, WE and DIOL are hardly commercially available, but these branched chain esters are present in lanolin. However, lanolin does not show the lipid transitions and lateral packing of the VC lipids (Fig. 2). Besides SE, WE and DIOL lipid fractions, other lipid classes are also present in lanolin; therefore it was decided to separate the nonpolar lipid fractions (SE, WE and DIOL) from the polar fractions. Separation of lanolin by column chromatography led to the isolation of three major fractions containing the nonpolar lipids (Fig. 1): LanSE, LanDI and LanX. These three fractions were selected for further studies to determine the most appropriate fraction for generation of the SSLM that would mimic the properties of the VC lipids. Our studies revealed that - from the isolated, nonpolar lanolin fractions – LanX itself already mimics the thermotropic behaviour and lipid organization of VC rather closely (Fig. 2). This was shown by a similar thermotropic behaviour and the absence of orthorhombic domains.

In order to approach the lipid composition, organization and thermotropic behaviour of VC mixtures even more closely, the isolated fractions, LanSE, LanDI and LanX were mixed with commercially available lipids (CER, TG, SQ, CHOL and FFA). To substitute the scarcely available natural CER, mixtures of synthetic CER was used. The composition of this CER mixture was similar as used in previous studies, which revealed that lipid organization in mixtures prepared of synthetic CER, CHOL and FFA mimicked to high extent the lipid organization in human SC [22]. It should be noted that one important difference

exists between synthetic and native CER. While in native CER wide acyl chain length distribution has been observed, synthetic CER mixtures contain CER with acyl chain length of either C30, C16 or C24. This difference may cause a difference in crystallinity. To substitute the TG of VC, a simplified mixture was prepared from TG with acyl chains of C16:0, C16:1, C18:0 and C18:1.This resulted in preparation of different semi-synthetic lipid mixtures (SSLM, lanes 4-7 in Fig. 3) with similar lipid composition as observed in VC lipids (lane 2).

Investigation of the several SSLM by DSC displayed similarities in the basic profile of the transitions, but the completion temperatures of the endothermal peak were shifted to higher values and the endothermal transition enthalpies were increased (Fig. 4A) compared to respective lanolin fractions. We speculated that the reason for this difference can be found in the composition of the TG fraction and DSC confirmed differences in thermotropic behaviour of natural and synthetic TG (Fig. 4B). After adjustment of the TG mixture, the thermotropic behaviour of SSLM prepared with TGa showed a lower completion temperature of the endothermal peak and thus the desired shift of the endotherm to lower temperatures (Fig. 5). It seems that especially the endothermal transition is very susceptible to this variation of TG composition. The barrier lipids have less influence, since SSLM prepared with a twofold increased barrier lipid content did not affect the endothermal transition (Fig. 5). When comparing the observed transitions with other lipid specimen, similarities to CER-rich internal wool lipids can be found, as an endothermic transition occurs at 21°C [28]. In contrast, no exothermic transition was reported. Unlike in our SSLM, SC exhibits major lipid derived transitions only at much higher temperatures but a small lipid transition occurs also at 35°C [29].

Of interest is whether the thermotropic transitions are correlated to the changes in the lipid organisation, as in all our mixtures a similar thermotropic behaviour has been observed. Important is that the exothermal transition is not sensitive to the lipid composition, while the endothermal transition depends strongly on the composition of the mixture. For this reason the main focus will be on the endothermal transition. Initially lanolin will be discussed, as in this mixture the changes in lipid organisation are most clearly detected. The endothermal phase transition of lanolin is observed between 28 and 33°C and is very small. By using FTIR and SAXD the following observations are made: I) The $CH_2$ symmetric stretching vibrations reveal a clear transition from an ordered (crystalline) to a disordered state between 22 and 30°C, but there is a further increase in symmetric stretching vibration until a temperature of 40°C. II) In the FTIR spectrum the scissoring band reveals a doublet of the orthorhombic packing and a singlet denoting either a hexagonal and/or liquid packing. As the $CH_2$

stretching vibrations indicate mainly an ordered state (wavenumber 2850.8 $cm^{-1}$), at 22°C the lipids most likely mainly participate in hexagonal and orthorhombic domains. III) The doublet of the orthorhombic packing disappears between 26 and 36°C (Fig. 7B). These observations suggest that in lanolin the endothermal transition might be due to both a melting of lipids and a disappearance of the orthorhombic packing into a hexagonal and/or liquid phase. The coexistence of different crystalline domains (orthorhombic, hexagonal and even liquid domains) demonstrates the heterogeneity of the mixture and therefore the thermotropic transitions are difficult to assign to a particular change in the lipid organisation. In case of lanolin, the SAXD curves do not reveal any long range ordering (data not shown), therefore a change in long range ordering is not involved in this transition. This is different from VC (lipids), in which the disordering of the long range ordering coincides with the 2nd endothermal transition [25]. Now the question arises whether these observations can be extrapolated to the synthetic lipid mixtures. When focussing on the SSLM mixtures, in general the shifts in the $CH_2$ symmetric stretch vibrations are less clearly detected. However, when comparing the shift in $CH_2$ symmetric stretch vibrations in e.g. the SSLM-X and SSLM-Xa, a gradual shift is detected between 10 and 30°C in the spectrum of the SSLM-Xa mixture, which is at a lower temperature region compared to that in the spectrum of the SSLM X mixture (approximately 25 to 45°C; Fig. 6). The endothermal transitions are located between 25 and 33°C and 26-41°C for SSLM-Xa (Fig. 5) and SSLM-X (Fig. 4A), respectively. Furthermore, the orthorhombic packing in the SSLM-X mixtures disappears between 30 and 38°C (Fig. 7B), while no orthorhombic lateral packing is observed in the spectrum of SSLM-Xa. Therefore, changes in the lipid organisation occur during the endothermal phase transitions, but these thermal transitions cannot be assigned to a particular change in organisation, as it is also observed for the lanolin mixture. Similar observations have been made for the other SSLM mixtures. In conclusion, the endothermal transition of lanolin and the SSLM may be correlated to both a shift in $CH_2$ symmetric stretching vibration rendering a more disordered structure and a disappearance of the orthorhombic lateral packing (except for SSLM-Xa). However, as the mixtures are very heterogeneous, a very detailed correlation is difficult to obtain and therefore other unknown changes in lipid organisation may also play a role.

In order to test the biological efficacy of the *in vitro* optimized lipids mixture, *in vivo* studies were conducted. It has already been shown that lipid mixtures which contain barrier lipids enhance the recovery of the mouse skin after tape stripping [30, 31]. VC lipids showed a significant reduced evaporative water loss after 6 and 48 h (Fig. 9), which is indicative for an increased barrier recovery rate. Our

optimized mixture (SSLM-Xa) showed comparable TEWL values as observed with VC lipids at these time points. The occlusive ointment Vaseline (petrolatum) already has been speculated to accelerate barrier recovery in mice [32]. In our model Vaseline also shows slight facilitation of the barrier recovery but to a much lesser extent than VC lipids and our synthetic lipid mixture (Fig. 9). Lanolin itself also had been reported to facilitate barrier recovery in mice [33], whereas internal wool lipids showed their protective function in application on human skin [34]. In a pilot study it was observed that lanolin was less effective than the synthetic VC lipids.

In conclusion, an excellent resemblance was achieved in lipid composition, lipid organization and thermotropic behaviour between lanolin-derived lipid mixtures and the natural VC lipids. These results demonstrate that the SSLM-Xa most closely resembles the properties of VC lipids. Furthermore, the beneficial effect of VC lipids and SSLM-Xa upon application of disrupted mouse skin was demonstrated.

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