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Stratum corneum hydration : mode of action of moisturizers on a molecular level

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Citation

Caussin, J. (2009, June 17). *Stratum corneum hydration : mode of action of moisturizers on a molecular level*. Retrieved from <https://hdl.handle.net/1887/14739>

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CHAPTER 7

FTIR STUDIES SHOW LIPOPHILIC MOISTURIZERS TO INTERACT WITH STRATUM CORNEUM LIPIDS, RENDERING THE MORE DENSELY PACKED

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1. SUMMARY

Lipophilic moisturisers are widely used to treat dry skin. However, their interaction with the lipids in the upper layer of the skin, the stratum corneum (SC), is largely unknown. In the present study this interaction of three moisturisers, isostearyl isostearate (ISIS), isopropyl isostearate (IPIS) and glycerol monoisostearate (GMIS), has been elucidated using lipid mixtures containing isolated ceramides (CER), cholesterol (CHOL) and free fatty acids (FFA), mimicking the lipid composition and organization in SC. The conformational ordering and the lateral packing of the lipid mixtures were examined by Fourier transformed infrared spectroscopy.

CER:CHOL:FFA mixtures show an orthorhombic to hexagonal phase transition between 22 and 30°C and an ordered-disordered phase transition between 46 and 64°C. Addition of 20% m/m ISIS or IPIS increased the thermotropic stability of the orthorhombic lateral packing, while GMIS had no influence. Furthermore, small amounts of all three moisturizers are incorporated into the CER:CHOL:FFA lattice, while the majority of the moisturizer exists in separate domains.

Especially the thermotropic stabilisation of the orthorhombic lateral packing, which might reduce water loss from the skin, is considered to contribute to the moisturising effect of IPIS and ISIS in stratum corneum.

2. INTRODUCTION

Dry skin, the most common symptom of dermatological diseases, is caused by a shortage of water in the stratum corneum (SC), the uppermost layer of the skin. Only 15 to 20 μm thick, the SC protects the body from water loss and invasion of chemical and biological substances. Although approx. 90% of the SC's weight is provided by large, flat cells called corneocytes, the barrier function is provided mainly by the intercellular lipid matrix that surrounds these cells (Jackson et al., 1993; Potts and Francoeur, 1991). This lipid matrix consists of ceramides (CER), cholesterol (CHOL) and free fatty acids (FFA) in an approx. equimolar ratio (Lampe et al., 1983; Weerheim and Ponc, 2001; Wertz, 1991). In human skin the SC lipids form two lamellar phases with repeat distances of approx. 6 and 13 nm. These lipid lamellae are oriented parallel to the surface of the skin. Within the lamellae, the lipids are highly organised, mostly in an orthorhombic organization, which is converted into the more penetrable hexagonal organization around 40°C, slightly above skin temperature. The presence of this tightly packed orthorhombic structure is considered to be crucial for the low penetration of water across the skin (Pilgram et al., 2001b).

Dry skin has long been treated by the application of hydrophilic and lipophilic moisturizers. Hydrophilic moisturizers are usually small hygroscopic molecules, such as glycerol and urea. After penetration into the SC they attract water thereby increasing the water levels in the SC (Sagiv and Marcus, 2003). Many of the lipophilic moisturizers act by forming an almost water-impenetrable layer on the surface of the SC, thereby reducing water evaporation from the SC. Such moisturizers are referred to as occlusive moisturizers and usually do not penetrate the SC (Loden, 2003; Raney and Hope, 2006). However, some moisturizers appear to increase water levels in the SC without the formation of a water impenetrable layer on the surface of the SC (Wiechers, 1999). As yet, the mode of action of such non-occlusive lipophilic moisturizers is unclear. An interesting hypothesis is that non-occlusive lipophilic moisturizers penetrate into the SC, thereby changing the lipid organization of the SC and thus increasing the barrier function. This may reduce the trans epidermal water loss (TEWL) and consequently result in

increased water levels in the SC. In this study, the possibility of this mechanism of action is investigated.

The SC lipid organization can be reconstructed in vitro by mixing ceramides, cholesterol and free fatty acids in the appropriate molar ratios (Bouwstra et al., 1996a; de Jager et al., 2004b; Gooris and Bouwstra, 2007). By adding moisturizers to these mixtures, it is possible to study their effect on the SC lipid organization.

In the present study, the three selected moisturizers are non-occlusive low molecular weight isostearate derivatives chosen because of their systematic change in molecular architecture and their ability to increase the water level in the skin in humans as monitored by corneometry measurements (Wiechers, 1999). Isostearyl isostearate (ISIS, $C_{17}H_{35}COOC_{18}H_{37}$) is an ester of isostearic acid and isostearic alcohol, while isopropyl isostearate (IPIS, $C_{17}H_{35}COOC_3H_7$) is an ester of isostearic acid and isopropanol. Glycerol monoisostearate (GMIS, $C_{17}H_{35}COOC_3H_4(OH)_3$) is a mono-ester of glycerol and isostearic acid, combining a long, branched carbon chain with a hydrophilic head group. All three moisturisers are liquid at room temperature. Very recently several studies have been undertaken to unravel the underlying mode of action of the selected moisturisers with the following results. i) In vitro studies using human dermatomed skin revealed that the three lipophilic moisturisers show the tendency to increase the hydration level of the central part of the SC more efficiently than the superficial and deeper layers in the SC (Caussin et al., 2007b). ii) In vivo studies in humans revealed that the moisturisers do not disorder the lipid lamellae in the SC and form partly separate domains in the intercellular regions (Caussin et al., 2007a). iii) In vitro studies using isolated lipid mixtures have shown that the three moisturizers promote the formation of the long periodicity phase (Caussin et al., 2007a). This lamellar phase is very characteristic for the lamellar organization of SC lipids and has been shown to be essential for the skin barrier function (de Jager et al., 2006). These studies may already partly explain the moisturizing capabilities of ISIS, IPIS and GMIS. However, also the lateral packing of the SC lipids is speculated to be of crucial importance to the barrier function, especially as it has been shown that phospholipids membranes are less permeable to water than those with hexagonal packing (Abrahamsson et al., 1978). It is therefore crucial to study the effect of the selected moisturizers on the lateral packing of the SC.

SC lipids with and without moisturizer were studied using Fourier transform infrared spectroscopy (FTIR), a method which can provide two types of information on the

organization of a lipid and moisturizer sample (Gooris and Bouwstra, 2007; Mendelsohn et al., 2000; Moore et al., 1997a; Moore and Rerek, 2000). Firstly, the methylene (CH_2) scissoring and stretching modes can provide information on the lateral organization and conformational ordering of the lipids in the sample. Secondly, by using perdeuterated moisturizers, the perdeuterated methylene (CD_2) stretching modes can be used to determine the mixing properties of the lipid sample, by comparison of the ordered to disordered transition temperatures of the lipids (CH_2 stretching) and the moisturizer (CD_2 stretching) and by comparison of the CH_2 scissoring modes. From these and previous studies (Caussin et al., 2007a) a mechanism of action will be proposed for these moisturizers.

3. MATERIAL AND METHODS

CHEMICALS

The perdeuterated FFA (DFFA) with chain length of C16:0 and C22:0 were obtained from Larodan (Malmö, Sweden). The DFFA with chain length of C18:0 and C20:0 were purchased from Cambridge Isotope laboratories (Andover, Massachusetts), while DFFA with chain length of C24:0 was obtained from ARC laboratories (Apeldoorn, The Netherlands). Cholesterol, protonated fatty acids and deuterated and protonated acetate buffer salts were purchased from Sigma-Aldrich Chemie GmbH (Schnelldorf, Germany). Protonated and perdeuterated lipophilic moisturizers were kindly provided by Uniqema (Gouda, The Netherlands).

ISOLATION AND SEPARATION OF PORCINE CERAMIDES

Fresh pig skin was obtained from a slaughter house. Upon arrival the SC was isolated as described elsewhere (Tanojo et al., 1997). SC lipids were extracted using the method of Bligh and Dyer (Bligh and Dyer, 1959). The extracted lipids were applied on a silicagel 60 column and the various lipid classes were eluted sequentially using various solvent mixtures as published previously (Bouwstra et al., 1996a). The lipid composition of the collected fractions was established by one dimensional high performance thin layer chromatography, as described earlier (Bouwstra et al., 1996a). For quantification, authentic standards were run in parallel. The quantification was performed after charring, using a photodensitometer with peak integration (Biorad, GS 710). The fractions containing ceramides (CER) were pooled and the resulting CER mix had a CER EOS (Motta et al., 1993) content of approx. 12%, which is similar to the acylceramide content of human SC (Ponec et al., 2003a; Wertz et al., 1985). The remainder

of the mix primarily consisted of CER NS (64%) with CER NP, AS (long chain FFA), AS(short chain FFA) and AP in concentrations of 8, 6, 4 and 6%, respectively.

PREPARATION OF SC LIPID SAMPLES AND SAMPLES WITH LIPOPHILIC MOISTURIZERS

The isolated CER were mixed with CHOL and FFA or with CHOL and DFFA. The FFA mixture contained C16:0, C18:0, C20:0, C22:0, C23:0, C24:0 and C26:0 in a ratio of 1.3%, 3.3%, 7%, 41.7%, 5.4%, 36.8%, and 4.7% (w/w). This composition is based on the FFA composition reported by Wertz and Downing (5). Because not all of the FFA are available in a perdeuterated form, the DFFA mixture contained C16:0, C18:0, C20:0, C22:0 and C24:0 in a ratio of 1.3%, 3.3%, 7%, 47%, and 41.4% (w/w). To prepare samples with moisturizer, either perdeuterated or normal moisturizer was dissolved in chloroform/methanol (2:1, v/v) and added to the mixture in the required molar percentage, while maintaining the equimolar SC lipid composition. In most cases, 20% moisturizer was added, which is comparable to the molar ratio in the superficial SC layers after application of approx. 25 g/cm² moisturizer on the skin, which is similar to the amount of moisturiser on human skin in vivo in previously reported in vivo studies (Caussin et al., 2007a).

Samples were prepared by dissolving 1.5 mg of the lipids (and moisturizer, if applicable) in the desired ratio's in 200 μ l of chloroform/methanol (2:1, v/v). The mixture was sprayed over an area of 1 cm² on a ZnSe window. This was performed at a very low spraying rate (4.2 μ l/min) under a gentle stream of nitrogen gas using a sample applicator (CAMAG LINOMAT IV (Muttentz, Switzerland). The sample applicator was adapted by constructing an extra axis (Y-direction) of spraying perpendicular on the existing axis (X-direction), allowing application in two directions simultaneously.

The ZnSe window with applied lipids was then heated to a temperature of 60°C, covered with 40 μ l of a perdeuterated acetate buffer at pH 5.0 (50 mM), closed with a 2nd ZnSe window and equilibrated for 10 minutes at 60°C. Perdeuterated buffer was used in order to avoid interference of the broad hydroxyl vibration peak with the CH₂ stretching vibrations. The sample was then cooled slowly to room temperature. To equilibrate and protonate the acid groups of the lipid mixture 10 freeze-thawing cycles were carried out between -18°C and room temperature.

FOURIER TRANSFORM INFRARED SPECTROSCOPY (FTIR)

All spectra were acquired on a BIORAD FTS4000 FTIR spectrometer (Cambridge, Massachusetts) equipped with a broad-band mercury cadmium telluride detector, cooled with liquid nitrogen. The sample was under continuous dry air purge starting 30 minutes before and during the data acquisition. The spectra were collected in transmission mode, as a co-addition of 256 scans at 1 cm^{-1} resolution during 4 minutes. In order to detect phase transitions, the sample temperature was increased at a heating rate of 0.5°C/min resulting in 2°C temperature rise during each measurement. The lipid phase behaviour was examined between -10°C and 90°C. The software used was Win-IR pro 3.0 from Biorad (Cambridge, Massachusetts).

4. RESULTS

In figure 1a through 1d, a frequency graph and representative spectra of CER:CHOL:(D)FFA mixtures are shown. In figure 1a the frequencies of the CH_2 symmetric stretching vibrations of an equimolar CER:CHOL:FFA sample are plotted in a temperature range of -10°C to 90°C. The stretching vibrations provide information on the conformational disordering of the lipids. At -10°C the symmetric stretching frequency is 2848.9 cm^{-1} , indicating an ordered all trans solid state at this temperature. An increase in temperature results in a gradual increase in the symmetric stretching frequency until a temperature of 22°C is reached. Between 22 and 30°C a small but clearly steeper increase in symmetric stretching frequency is observed, indicating an orthorhombic-hexagonal phase transition (see arrow in figure 1a). A further temperature rise results in a 2nd clearly visible transition from a hexagonal (wavenumber 2850.1 cm^{-1}) to a disordered liquid state (wavenumber 2853.1 cm^{-1}) between 46 and 64°C. The transition from orthorhombic to hexagonal lateral packing is more clearly visible in figure 1b, in which infrared spectra of the CH_2 scissoring band of this equimolar CER:CHOL:FFA sample are shown at temperatures from 0°C to 60°C. The contours of this band provide information on the packing of the lipid chains perpendicular to the basal plane. In a crystalline orthorhombic lattice adjacent chains in a scissoring mode interact via a short-range coupling resulting in a broadening or splitting of the contours. The presence of an orthorhombic packing is indicated by a doublet at 1462.8 cm^{-1} and 1473.5 cm^{-1} that disappears at approx. 30°C to form a singlet at approx. 1468 cm^{-1} . This demonstrates the transition from orthorhombic to hexagonal lateral

packing. A magnitude of the splitting of 10.7 cm^{-1} as seen in figure 1b at 0°C indicates large domains of lipids forming the orthorhombic packing.

Figure 1c shows spectra of the CD_2 scissoring band of an equimolar CER:CHOL:DFFA mixture (substitution of FFA by DFFA). The degree of splitting of the CD_2 scissoring band, which in pure DFFA mixtures can be as large as 8 to 9 cm^{-1} , is reduced and only a splitting of approx. 5 cm^{-1} remains. This is the result of the participation of DFFA in the same orthorhombic lattice as the protonated CER, as DFFA mixture itself results in a clear splitting until a temperature of around 60°C is reached (Gooris and Bouwstra, 2007). The almost complete elimination of splitting indicates that no short-range coupling of the vibrations occurs, demonstrating that protonated and deuterated lipid chains mix in one lattice. The remaining slight splitting indicates that a very small population of DFFA forms separate domains. Preparation of a CER:CHOL:FFA mixture with a FFA mixture matching the exact composition of the DFFA mixture showed that the slight difference in composition between DFFA and FFA does not affect the thermotropic behaviour of the CER:CHOL:FFA lipid mixture (not shown).

To investigate the role of CER in the SC lipid mixture, a sample with a CER:CHOL:FFA 0.5:1:1 molar ratio was also examined (not shown). Although using this mixture a transition from an orthorhombic to hexagonal phase is noticed at around 30°C , the doublet band of the CH_2 scissoring mode is clearly visible, also above 40°C indicating an orthorhombic packing at higher temperatures (not shown). To determine whether this orthorhombic packing is due to phase separated FFA, a CER:CHOL:DFFA mixture in a 0.5:1:1 molar ratio was prepared. Spectra of the CD_2 scissoring band are shown in figure 1d. At approx. 22°C , part of the orthorhombic lattice that is represented by this splitting is converted into a hexagonal lattice. However, the remaining splitting persists up to temperatures higher than 60°C . This indicates that two types of domains of the DFFA with an orthorhombic packing are present: domains with a phase transition to a hexagonal phase between 20 and 30°C and domains in an orthorhombic lattice until at least 60°C (see arrows figure 1d). To study the effect of the moisturizers on the lipid organization of the CER:CHOL:FFA mixtures, 20% m/m moisturizer was added to the samples, resulting in a CER:CHOL:FFA:moisturizer ratio of 1:1:1:0.75. Figures 2a, 2b and 2c show spectra of the CH_2 scissoring modes of mixtures with 20% ISIS, IPIS and GMIS, respectively. The spectra are very similar to those of CER:CHOL:FFA mixtures (figure 1b), but differ in two important aspects. Firstly, an extra, small weak peak at around 1467 cm^{-1} is present in all three spectra (see arrow pointing up), most clearly at temperatures around 20°C , although it is

slightly less visible in figure 2a. As the moisturizers are in a liquid, disordered state, this peak most probably represents the fluid, phase separated domains of the moisturizer.

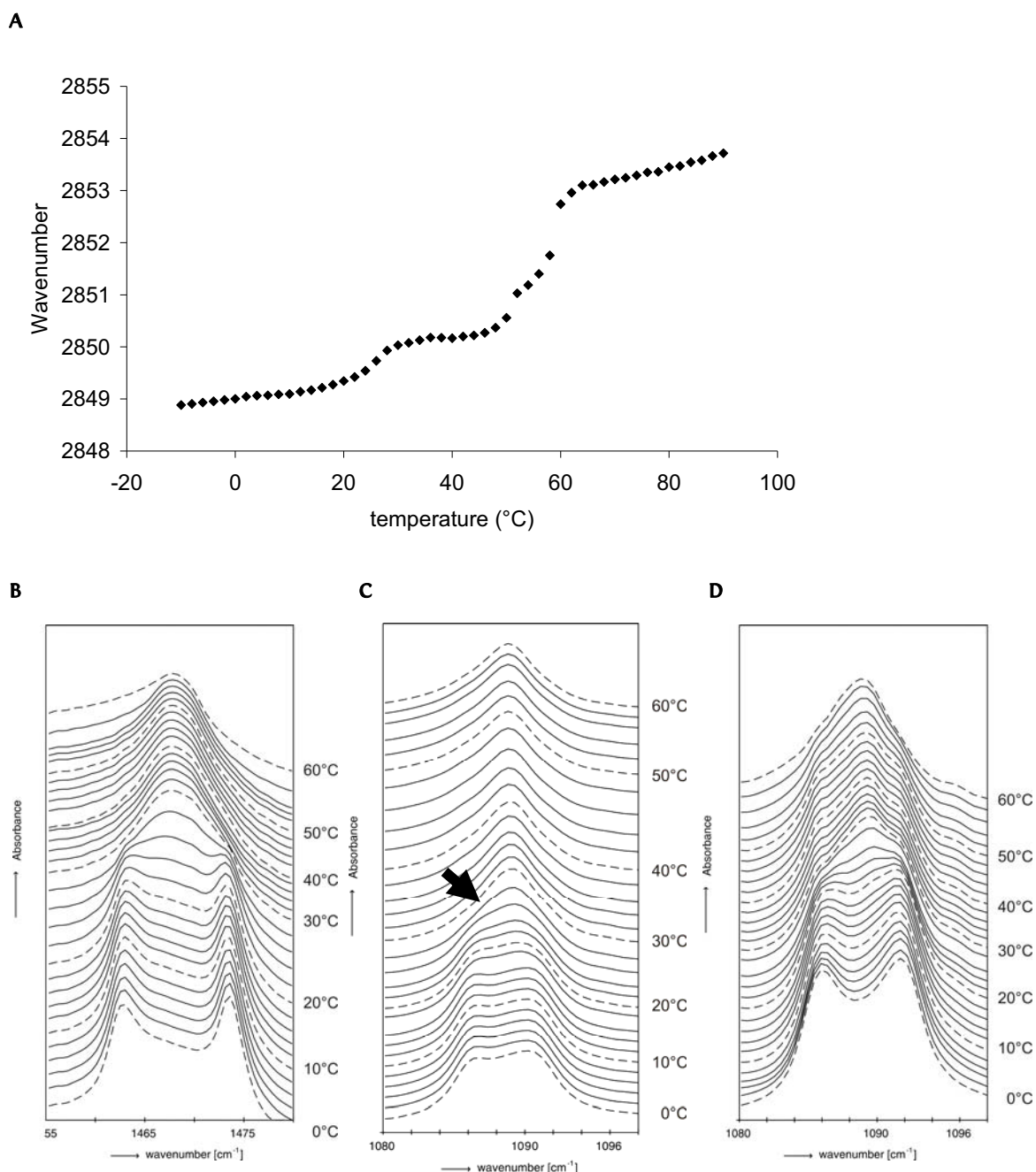


FIGURE 1. (a) Frequencies of the symmetric CH_2 stretching bands in an equimolar CER:CHOL:FFA sample at different temperatures between -10 and 90°C . Two phase transitions are visible. The first shift between 22 and 30°C indicates a transition from an orthorhombic to hexagonal packing (arrow). A second shift between 46 and 64°C represents a hexagonal to liquid state phase change. (b) Spectra of the CH_2 scissoring band of an equimolar CER:CHOL:FFA mixture in a temperature range of 0 and 60°C . A transition from orthorhombic (doublet) to hexagonal packing (singlet) is noticed in a temperature range of 22 to 34°C . (c) Spectra of the CD_2 scissoring bands of an equimolar CER:CHOL:DFFA mixture in a temperature range of 0 and 60°C . Only a slight broadening of the CD_2 scissoring band is observed, indicating that DFFA and CER participate in the same orthorhombic lattice. The

slight splitting indicates that a small fraction of DFFA phase separates, forming an orthorhombic packing. The phase separated DFFA transforms into a hexagonal lateral packing between 20 and 28°C. (d) Spectra of the CD₂ scissoring bands of a 0.5:1:1 CER:CHOL:DFFA mixture in a temperature range of 0 and 60°C. A strong doublet of the CD₂ scissoring vibration indicates that the FFA have formed large, separate domains. Between 22 and 30°C, part of the orthorhombic lattice that is converted into a hexagonal lattice, although splitting persists up to temperatures higher than 60°C (arrows). This indicates that two types of orthorhombic DFFA domains are present.

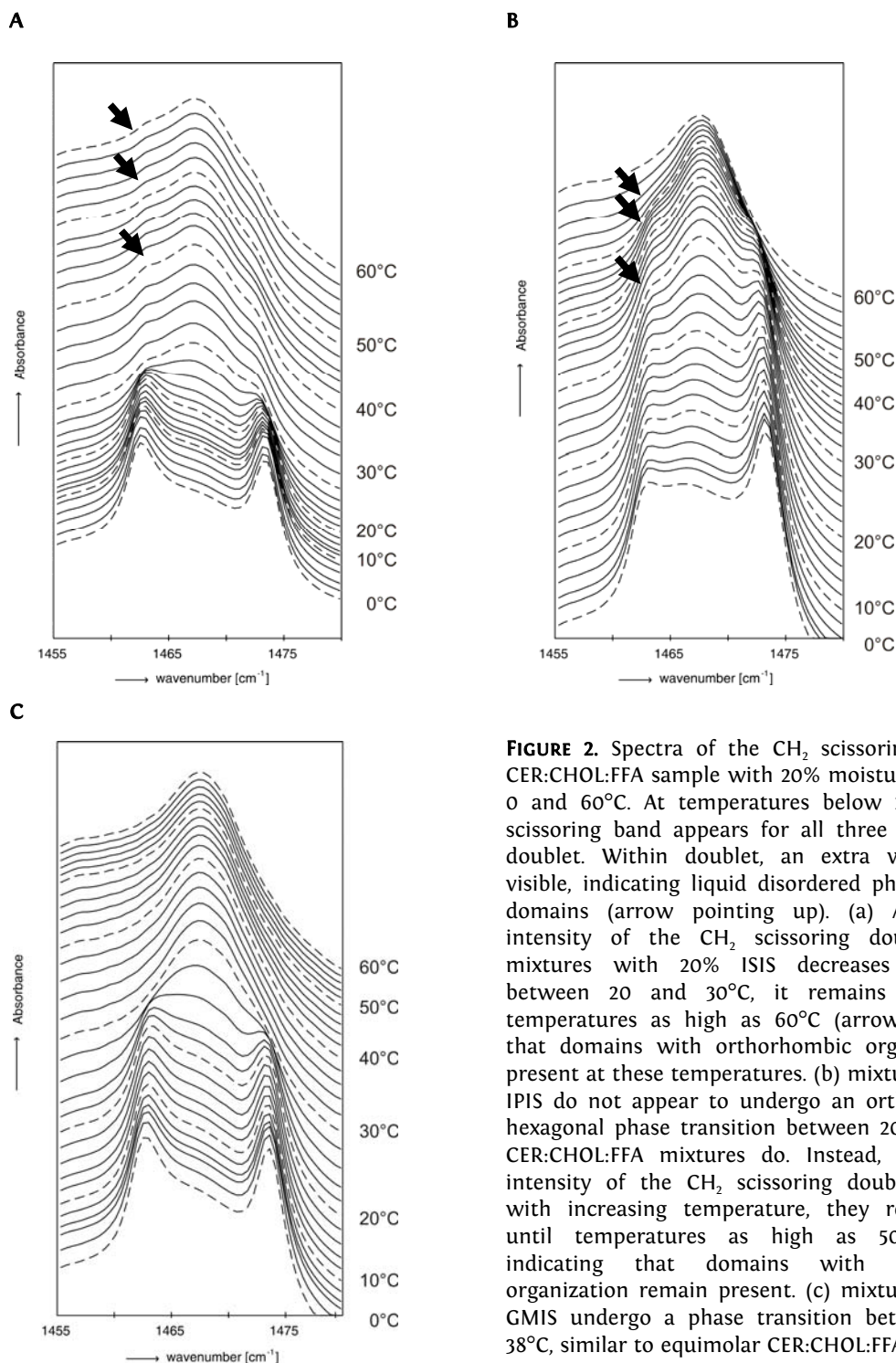


FIGURE 2. Spectra of the CH₂ scissoring band in a CER:CHOL:FFA sample with 20% moisturizer between 0 and 60°C. At temperatures below 20°C the CH₂ scissoring band appears for all three samples as a doublet. Within doublet, an extra weak peak is visible, indicating liquid disordered phase separated domains (arrow pointing up). (a) Although the intensity of the CH₂ scissoring doublet in the mixtures with 20% ISIS decreases dramatically between 20 and 30°C, it remains visible until temperatures as high as 60°C (arrows), indicating that domains with orthorhombic organization are present at these temperatures. (b) mixtures with 20% IPIS do not appear to undergo an orthorhombic to hexagonal phase transition between 20 and 30°C as CER:CHOL:FFA mixtures do. Instead, although the intensity of the CH₂ scissoring doublet decreased with increasing temperature, they remain visible until temperatures as high as 50°C (arrows), indicating that domains with orthorhombic organization remain present. (c) mixtures with 20% GMIS undergo a phase transition between 20 and 38°C, similar to equimolar CER:CHOL:FFA mixtures.

Secondly, it is clearly visible that in the spectra of the samples with ISIS and IPIS the splitting of the CH₂ scissoring doublet remains visible until approx. 50°C, which is at much higher temperatures than in the CER:CHOL:FFA samples. This suggests that in the mixtures with added ISIS or IPIS, the orthorhombic packing is present at much higher temperatures than in the mixtures with SC lipids only (arrows). In contrast, the doublet band of the CH₂ scissoring vibration of samples containing GMIS disappears between 20 and 30°C, very similar to the equimolar CER:CHOL:FFA mixtures. Additionally, especially in figure 2b, a marked asymmetry in the intensity of the CH₂ scissoring doublet is observed, not visible in the contours of the equimolar CER:CHOL:FFA mixtures. The distance between the bands remains unchanged after addition of ISIS and GMIS, and is only slightly decreased after IPIS addition (10 cm⁻¹), suggesting that the size of the orthorhombic domains is virtually unchanged. The results described above reveal that at least two of the moisturizers stabilize the orthorhombic organization of the SC lipids. This suggests an interaction of SC lipids with the moisturizer. To unravel the increased presence of the orthorhombic phase at elevated temperatures in the presence of IPIS or ISIS, several additional studies were performed.

First, to determine whether the moisturizer is present in the same lattice as the CER and FFA, experiments similar to those presented above were performed, but now with 20% perdeuterated moisturizers. Figure 3 shows spectra of the CH₂ scissoring mode of CER:CHOL:FFA mixtures to which 20% perdeuterated ISIS, IPIS or GMIS has been added. After addition of perdeuterated ISIS or IPIS (figures 3a and 3b, respectively), the splitting of the doublet (1464 and 1474 cm⁻¹) and the temperatures at which the doublet is observed (arrows) indicates the presence of large domains of an orthorhombic lattice that remains present at much higher temperatures compared to the orthorhombic packing in the CER:CHOL:FFA samples. This was also observed for the CER:CHOL:FFA samples with added protonated ISIS or IPIS (figures 2a and 2b). The scissoring bands after addition of perdeuterated GMIS (figure 3c) are similar to those in figure 2c, suggesting no stabilization of the orthorhombic packing compared to the equimolar CER:CHOL:FFA mixtures. An additional observation is the strong peak asymmetry in the spectra of the samples with perdeuterated moisturizer, in which the peak at approx. 1474 cm⁻¹ is much stronger than the peak at 1464 cm⁻¹, compared to CER:CHOL:FFA mixtures without moisturizer.

A

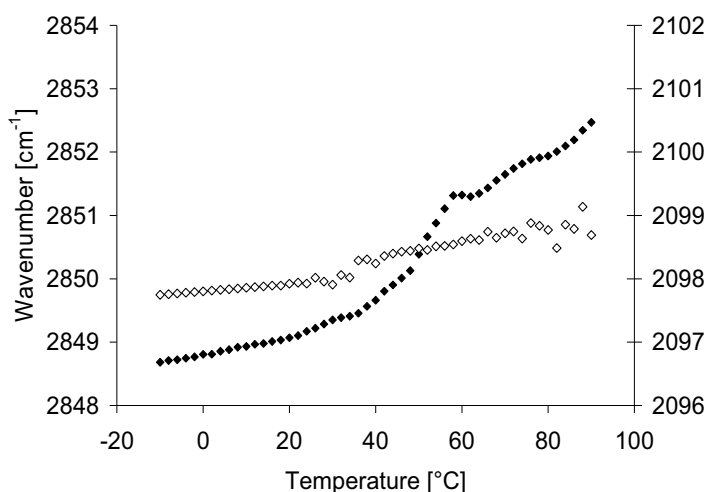
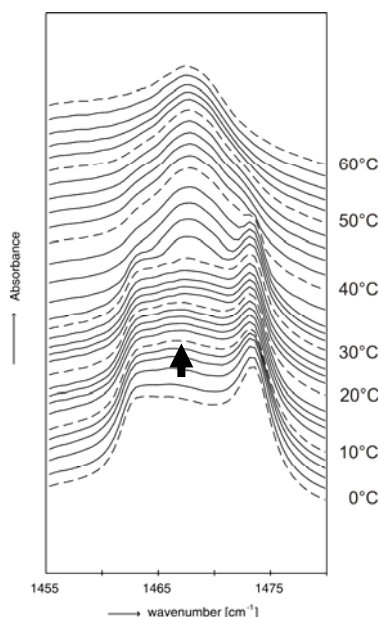
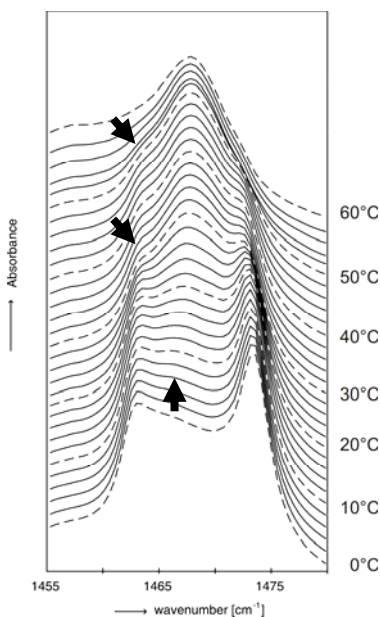


FIGURE 3A. Spectra of the CH₂ scissoring band in a CER:CHOL:FFA mixture with 20% perdeuterated moisturizer at temperatures between 0 and 60°C. (a) After addition of perdeuterated ISIS the CH₂ scissoring mode is a doublet, representing an orthorhombic packing of the protonated SC lipids. A transition to a hexagonal packing (singlet) is observed between 26 and 30°C, while a minor fraction of lipids still forms an orthorhombic phase (see arrows) that persists until 60°C. Additionally, a weak peak at 1466 cm⁻¹ is observed (arrow pointing up) at temperatures below the orthorhombic-hexagonal phase transition.

B



C



D

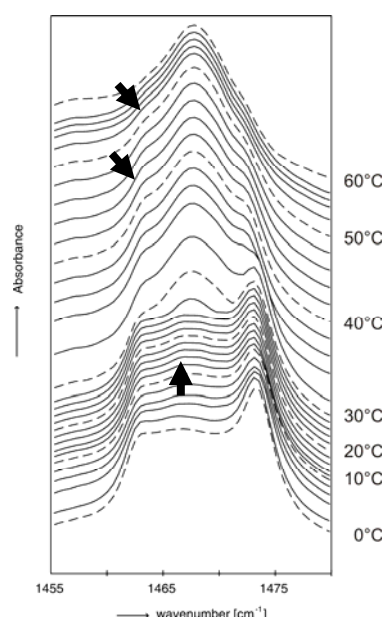


FIGURE 3. Spectra of the CH₂ scissoring band in a CER:CHOL:FFA mixture with 20% perdeuterated moisturizer at temperatures between 0 and 60°C (b) In the presence of perdeuterated IPIS the CH₂ scissoring mode is a doublet, representing an orthorhombic packing of the protonated SC lipids. A transition to a hexagonal packing resulting in a singlet is observed between 20 and 30°C. A minor fraction of lipids forms an orthorhombic phase (see arrows) that persists until 60°C. Additionally, a weak peak at 1466 cm⁻¹ is observed (arrow pointing up) at temperatures below the orthorhombic-hexagonal phase transition. (c) In the presence of GMIS, the doublet of the scissoring mode is also observed. At temperatures higher than 30°C, the doublet dissolves and a singlet representing hexagonal packing is observed at temperatures above 36°C. A very weak peak is present at 1466 cm⁻¹, (see arrow pointing up) at temperatures below the orthorhombic-hexagonal phase transition. (d) Wavenumbers of the symmetric CH₂ stretching band of SC lipids (filled diamonds) and the symmetric CD₂ stretching band of ISIS (open diamonds) in a sample of CER:CHOL:FFA with 20% perdeuterated ISIS at temperatures between 0 and 60°C. The SC lipids, represented by the CH₂ stretching mode, undergo two phase transitions. No shifts in the symmetric CD₂ stretching bands are observed.

Most interesting is the band around 1467 cm^{-1} at temperatures below 25°C in the presence of perdeuterated ISIS or IPIS (see arrow pointing up in figure 3). This weak band, which is not present in the absence of ISIS or IPIS (figure 1b), indicates that in a small population of lipids the short-range CH_2CH_2 interactions have been substituted by CH_2CD_2 interactions between CER, FFA and perdeuterated ISIS or IPIS. This is referred to as the decoupling of the scissoring vibrations. This observation suggests that in these domains perdeuterated IPIS or ISIS participates in the same lattice as CER and FFA. The decoupling is less pronounced, but also slightly visible, after perdeuterated GMIS addition. Conversely, the weak peaks may also indicate that, in presence of ISIS, IPIS or GMIS, a small proportion of the CER:CHOL:FFA domains display hexagonal packing at low temperatures. However, the latter is very unlikely, as in that case one substance promotes orthorhombic as well as the hexagonal lateral packing. In figure 3d the frequencies of the protonated, as well as those of the CD_2 stretching mode of a CER:CHOL:FFA sample with 20% perdeuterated ISIS are plotted against the temperature. For the protonated lipids, an ordered-disordered transition is visible between 38 and 62°C , similar to that observed in the absence of moisturizer (figure 1a). Such a transition is absent for the CD_2 stretching bands, which indicates that the majority of the perdeuterated ISIS is present in separate domains. Similar observations were made of samples with perdeuterated IPIS and GMIS (not shown).

In order to examine whether the stabilization of the orthorhombic phase can also be achieved by adding a smaller fraction of moisturizer, only 5% (m/m) perdeuterated ISIS was added to the equimolar CER:CHOL:FFA mixtures. Spectra of the CH_2 scissoring bands of such a sample are shown in figure 4. The phase behaviour appears to be very similar to that of control CER:CHOL:FFA samples. Clearly, addition of only 5% perdeuterated ISIS results in very little promotion of the orthorhombic packing. Also, no additional peak at 1467 cm^{-1} indicating that ISIS is almost not participating in the same lattice as CER and FFA. Examination of the symmetric CH_2 and CD_2 stretching bands of SC lipids and ISIS, respectively, shows that the majority of the moisturizer exists in phase-separated, liquid domains (not shown).

To rule out that the splitting of the CH_2 scissoring bands at higher temperatures observed in CER:CHOL:FFA mixtures with 20% IPIS or ISIS is caused by phase separated FFA instead of newly formed, thermotropically more stable CER:CHOL:FFA orthorhombic domains, a CER:CHOL:DFFA sample with 20% moisturizer was prepared. Spectra of the CD_2 scissoring band are shown in figure 5. Compared to the same band in the spectrum of a sample with SC lipids

only (figure 1c), which showed a slight splitting, in the presence of the moisturizer the splitting of this peak seems to be even less pronounced (approx. 3 cm^{-1}) and only indicated by a broadening of the peak towards 1088 cm^{-1} . The broadening disappears almost completely at approx. 20°C (in figure 1c at higher temperatures), at which temperature the peak is located at 1088.7 cm^{-1} . The contour might indicate that there are only some very weak shoulders still present. This suggests that there is no additional FFA phase separating due to the presence of ISIS.

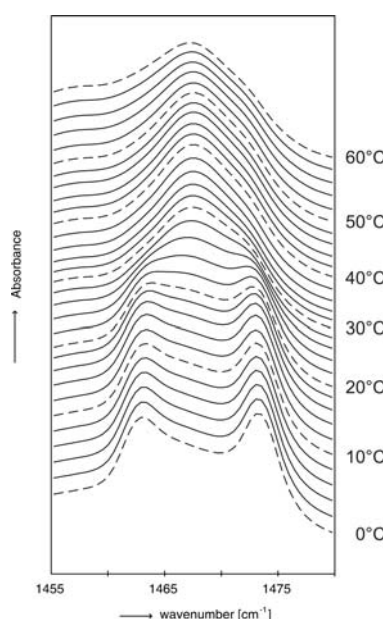


FIGURE 4. Spectra of the CH_2 scissoring mode in a CER:CHOL:FFA sample with 5% perdeuterated ISIS between 0 and 60°C . The doublet of the CH_2 scissoring vibration is visible until approx. 28°C , after which only very slight broadening of the resulting singlet.

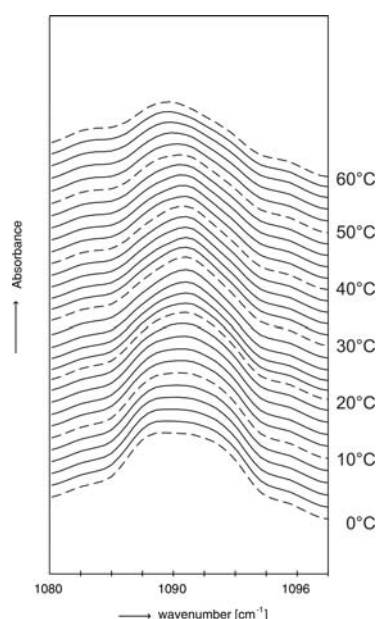


FIGURE 5. Spectra of the CD_2 scissoring band in a $1:1:1:0.75$ CER:CHOL:DFFA:ISIS mixture between 0 and 60°C . The broadening of the contour at approx. 1089 cm^{-1} below 18°C indicates the presence of small DFFA domains. However, these are no longer orthorhombically packed at physiological temperatures.

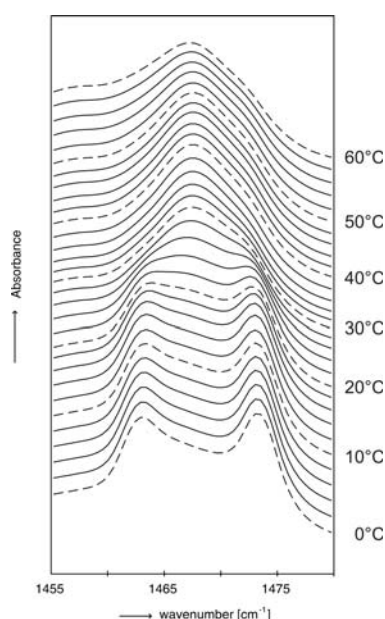


FIGURE 6. Spectra of the CH_2 scissoring band in a FFA:perdeuterated ISIS sample between 0 and 60°C . The contours of the CH_2 scissoring peak show a doublet suggesting that ISIS and FFA exist in separate domains. The doublet remains present until 60°C , where the transition to a liquid, disordered phase starts.

Lastly, to determine whether ISIS and FFA can participate partially in one lattice, FFA and perdeuterated ISIS were mixed in a $1:0.75$ molar ratio. The spectra of the CH_2 scissoring mode are shown in figure 6. The contour of the doublet shows no sign of decoupling of short-range interaction between FFA and is completely split with an interpeak distance of 9.8 cm^{-1} . This value is similar to the value found in samples of pure FFA, indicating short-range coupling of

the FFA with (almost) no incorporation of ISIS into this lattice. This suggests that ISIS and FFA exist entirely in separate domains.

5. DISCUSSION

Dry skin has traditionally been treated with lipophilic moisturizers, most of which have been assumed to occlude the SC, thereby preventing evaporation of water from the skin surface. However, some lipophilic moisturizers have been shown to be non-occlusive. This observation raises the question how these moisturizers prevent water from escaping the skin. Very recent in vivo studies have shown that the three selected isostearate derivatives penetrated the upper layers of the SC already after 3 hours of application (Caussin and Bouwstra, unpublished data) and increase the hydration level in human SC (Wiechers, 1999). Therefore, one intriguing possibility is that they change the organization of the intercellular lipids in such a way that the lipid regions become less permeable to water, which may result in higher water levels in SC. In recent studies it has been shown that all three moisturizers promote the formation of the long periodicity phase (Caussin et al., 2007a). However, this is not the only determining factor for increasing the skin barrier function. An increase in the formation of the orthorhombic packing at the expense of a hexagonal lateral packing may also play a role in their moisturizing capabilities. In healthy skin, this packing is mostly only present in the top layers of the SC, whereas in diseased skin it is found frequently also deeper in the SC (Pilgram et al., 1999). The lateral packing in dry skin has not been reported yet. As a means to investigate whether the three moisturizers increase the packing density of the lipids, mixtures of isolated porcine CER, CHOL, FFA and moisturizers were selected.

LATERAL PACKING AND CONFORMATIONAL DISORDERING OF CER:CHOL:FFA MIXTURES

The lateral packing in equimolar CER:CHOL:FFA mixtures undergoes two major transitions; the first transition changes the orthorhombic organization into a hexagonal packing and takes place between 22 and 30 °C. The second one is a conformational transition from ordered to disordered between 46 and 64°C. Substitution of FFA with DFFA results in decoupling of the short range interaction, as is indicated by a singlet of the CD₂ scissoring mode at approx. 1089.5 cm⁻¹. This suggests that the DFFA are evenly distributed among the CER in the same lattice. However, the slight splitting of the contours of the CD₂ peak around 1090 cm⁻¹ indicates that FFA form separate domains in the mixture. This suggests that in the equimolar CER:CHOL:FFA mixtures a small excess of FFA is present, as has also been seen in mixtures of

human CER (Gooris and Bouwstra, 2007). Interestingly, the separate domains formed by the FFA are no longer detectable at temperatures above 30°C, even though FFA without CER or CHOL under normal circumstances does not undergo a transition from an orthorhombic to a liquid organization until approx. 60°C (Gooris and Bouwstra, 2007). This observation suggests that the orthorhombic-hexagonal phase transition is influenced by the presence of CER and indicates that separate domains of FFA are located within the lamellae in which CER and CHOL also participate. As expected, in the 0.5:1:1 CER:CHOL:FFA mixture, the phase separation has been intensified and is still present at higher temperatures (above 40°C). This suggests that at higher FFA levels, besides being incorporated into the lamellar phases, FFA also forms separate domains.

ADDITION OF ISIS AND IPIS PROMOTES THE FORMATION OF THE ORTHORHOMBIC PACKING

Addition of 20% ISIS or IPIS to the CER:CHOL:FFA mixture was marked by a prolonged splitting of the scissoring contours of the 1470 cm⁻¹ region, indicating that these two moisturizers thermotropically stabilise the orthorhombic packing present in this mixture. Addition of IPIS and ISIS resulted in the presence of orthorhombic domains above 30°C, whereas this is barely observed in the equimolar CER:CHOL:FFA mixtures. This observation is very interesting, as the increased presence of orthorhombic domains may lead to a lower permeability to water. The increased presence of the orthorhombic phase was not seen for samples with 20% GMIS, the phase behaviour of which were very similar to that of the control. Addition of either the deuterated moisturizers does however result consistently in asymmetry of doublet of the CH₂ scissoring band. Asymmetry in the two scissoring vibrations has been described before and has been attributed to a distorted orthorhombic packing (G_d or L), in which the hydrocarbon chains are tilted with respect to the basal plane of the lamellar phase. (Snyder et al., 1996). However, in the present mixtures an increase in temperature does not lead to a shift in the positions of the doublet band, which is in contrast to the observations in the spectrum of the phospholipids in G_d packing (23). Furthermore, a tilt in the hydrocarbon chains would result in a reduction in the repeat distance of the lamellar phases. This has not been observed in our previous studies (Caussin et al., 2007a). Therefore, although the asymmetry indicates a phase change, it is not obvious that a distorted orthorhombic packing has been formed, but it indicates that there is indeed an interaction between the moisturisers and the SC lipids within the orthorhombic lattice.

To determine the difference in mode of action of ISIS and IPIS on the one hand and GMIS on the other hand, additional studies were performed.

MOISTURIZERS ARE AT LEAST PARTIALLY INCORPORATED INTO THE SC LIPID DOMAINS

By replacing ISIS, IPIS and GMIS in the CER:CHOL:FFA mixture with their perdeuterated counterparts, it is possible to discern between the moisturizers and the SC lipid components within one mixture. From these studies it has been established by comparison of the symmetric CH₂ and CD₂ stretching vibrations of the moisturizers that the majority of the 20% ISIS, IPIS or GMIS added to CER:CHOL:FFA mixtures exists in separate domains that are in a disordered state (figure 3d). It is however unclear whether they form separate domains only, or are at least incorporated to a certain degree, which was suggested by earlier X-ray diffraction experiments (Caussin et al., 2007a). In order to clear this matter, the scissoring contours in the spectrum of the CHOL:CER:FFA:moisturizer spectra should be evaluated. These spectra show the appearance of a weak, broad peak at 1467 cm⁻¹ in between the CH₂ scissoring doublet indicating orthorhombic packing. This broad peak at 1467 cm⁻¹ was not observed in the CER:CHOL:FFA spectra and is indicative of decoupling of the short-range interaction between protonated chains by the presence of perdeuterated chains participating in the same lattice. This observation suggests participation of the moisturizer in the same orthorhombic phase as the SC lipids. As the weak, broad band of the CH₂ scissoring contours is less pronounced present in the spectrum in CER:CHOL:FFA:GMIS mixture, it can be concluded that GMIS is incorporated to a lesser extent in the orthorhombic lattice. This difference between ISIS and IPIS on the one hand and GMIS on the other may be explained their difference in chemical structure. ISIS and IPIS are branched ester, whereas GMIS has a branched carbon backbone with a hydrophilic head group and is consequently surface active and a spontaneous vesicle or emulsion former (Caussin et al., 2007a). Incorporation may therefore be more difficult or less favourable energetically for GMIS, and therefore occurs to a lesser extent than incorporation of ISIS and IPIS. A possible difference in amount incorporated and difference in head group architecture might also explain why ISIS and IPIS promote the orthorhombic organization, whereas GMIS does not.

To determine whether the formation of the orthorhombic lateral packing is also promoted if only little moisturizer is added to the mixture, 5% perdeuterated ISIS was added to SC lipids. Only very slight broadening of the singlet CH₂ scissoring band was observed above 30°C. No peak at 1466 cm⁻¹ was observed, indicating no decoupling of short-rang interaction, as can be

seen in figure 3a, where ISIS is at least to some extent incorporated in the CER:CHOL:FFA matrix when an excess of ISIS is added to the CER:CHOL:FFA mixtures. However, only a very small fraction may be incorporated after a 5% addition, resulting in only a very slight effect, and too little incorporation to cause decoupling of the short-range interaction between lipid tails in detectable amounts.

Although there is strong evidence of interaction between the SC lipids and the moisturizers, it cannot be excluded that the moisturizer also extracts lipids out of the CER:CHOL:FFA domains, thereby changing the lateral packing of the remaining CER:CHOL:FFA domains. To determine whether extraction might play a role in the increased orthorhombic packing in the samples, several additional experiments were performed.

DO THE MOISTURIZERS EXTRACT LIPIDS FROM THE SC LIPID?

There are three possible extraction hypotheses. Firstly, CHOL may be extracted. However, it has been reported (Bouwstra et al., 2001) that changes in the fraction of CHOL have no influence on the lateral packing in SC lipid mixtures, and therefore extraction of CHOL would not lead to stabilization of the orthorhombic packing. Secondly, it is possible that FFA are extracted from the lipid domains. However, earlier studies (Bouwstra et al., 2001) showed that this would promote the formation of the hexagonal lateral packing at the expense of the orthorhombic one. Nevertheless, we cannot exclude that in presence of moisturizer, FFA form separate orthorhombic domains instead of mixing with CER and CHOL in one orthorhombic lattice. In order to unravel the mechanism for the formation of the orthorhombic phase in the presence of ISIS, a mixture with perdeuterated FFA and protonated ISIS was prepared. In this mixture extracted FFA forming separate domains would result in contour splitting at 1090 cm^{-1} . However, this was only very slightly the case (figure 5), indicating that very little FFA are separated, even less than in the absence of ISIS (compare figures 1c and 5). Furthermore, it was also established that FFA do not mix with ISIS (figure 6), which might cause further decoupling of the short-range interaction of the FFA. It therefore seems that FFA are dissolved to a higher extent into the orthorhombic lattice in the presence of the moisturizer, than in its absence.

Thirdly, it is possible that CER are extracted, resulting in a CER:CHOL:FFA mixture that is essentially enriched in FFA. This should induce increased phase separation of FFA. However, as can be inferred from figure 5 showing at elevated temperatures almost only a singlet of the

CD₂ scissoring mode of DFFA at 1090 cm⁻¹, this is not the case and CER extraction is therefore unlikely.

LIPHILIC MOISTURIZERS MOST LIKELY INCREASE THE ORTHORHOMBIC ORGANIZATION IN SC LIPID SAMPLES BY INCORPORATION

When large amounts of lipophilic moisturizer are added to SC lipid samples, small amounts of these moisturizers are incorporated into the equimolar CER:CHOL:FFA matrix. This is in agreement with previous results of SAXD experiments (Caussin et al., 2007a). For some moisturizers, this incorporation results in increased orthorhombic organization in these samples, even though the overall composition of the domains remains very similar to CER:CHOL:FFA mixtures. The molecular mechanism that causes the lipids in the lamellar domains to become more orthorhombic is as yet unclear. Possibly the answer lies in the physicochemical characteristics of the moisturizers that influence the degree of incorporation into the SC lipid matrix, although it is also possible that ISIS in some way facilitates the incorporation of FFA into the lamellar domains, rendering them more orthorhombic.

The present and previous studies provide insight into the mode of action of the non-occlusive lipophilic moisturizers to increase the SC hydration levels observed in vivo. In related studies it has already been shown that all three moisturisers penetrate into the human SC in vivo (Caussin, J. and Bouwstra, J.A., unpublished data) and increase especially the hydration levels in the central part in the SC (Caussin et al., 2007b). The studies presented in this paper suggest that addition of ISIS and IPIS increases the thermotropic stability of the orthorhombic organization, which might play an important role for the high permeation barrier formed by the SC lipids. Application of ISIS and IPIS may therefore reduce the permeability of SC lipids at physiological temperatures and hereby decrease transepidermal water loss (TEWL). In turn, this would result in increased water levels in the SC. Previously, it has been shown that addition of either of the three moisturizers examined in this paper results in a promotion of the LPP, at expense of the short periodicity phase (SPP) (Caussin et al., 2007a). This change may also have a marked influence on the barrier function, as it has been shown that the LPP is essential for the barrier function (de Jager et al., 2006). IPIS and ISIS may therefore increase water levels in the SC by increasing both LPP formation and the orthorhombic organization, while GMIS works via the former mechanism. However, GMIS has a glycerol head group, which may in addition attract water molecules leading to higher water levels in SC.

6. CONCLUSION

It was established that small amounts of the lipophilic moisturizers ISIS, IPIS and GMIS can be incorporated into equimolar SC lipid samples. Incorporation of ISIS and IPIS results in promotion of the formation of domains with an orthorhombic packing. This may explain the moisturizing capabilities of non-humectant, non-occlusive moisturizers.

7. ACKNOWLEDGEMENTS

The authors wish to acknowledge Uniqema, The Netherlands for provision of the (perdeuterated) moisturizers, Dr Johann Wiechers of Uniqema, The Netherlands for valuable discussion and Miss Christel Slot for technical assistance during the start-up of these studies. This project was partly financed by ICI Plc, London.

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