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

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CHAPTER 5

LIPID ORGANIZATION IN HUMAN AND PORCINE STRATUM CORNEUM DIFFERS WIDELY, WHILE LIPID MIXTURES WITH PORCINE CERAMIDES MODEL HUMAN STRATUM CORNEUM LIPID ORGANIZATION VERY CLOSELY

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1. SUMMARY

The conformational disordering and lateral packing of lipids in porcine and human isolated stratum corneum (SC) was compared using Fourier transform infrared spectroscopy (FTIR). It was shown that SC of both species differ markedly, porcine SC lipids being arranged predominantly in a hexagonal lattice while lipids in human SC are predominantly packed in the denser orthorhombic lattice. However, the lipid organization of equimolar ceramide:cholesterol:free fatty acid (CER:CHOL:FFA) mixtures prepared with isolated porcine CER or human CER are very similar, only the transition temperatures differed being slightly lower in mixtures with porcine CER. Therefore, the difference in lateral packing between human and porcine stratum corneum is not due to the difference in CER composition. Furthermore, it is possible to use more readily available porcine CER in model lipid mixtures to mimic lipid organization in human SC.

As the equimolar porcine CER:CHOL:FFA mixtures closely mimic the lipid organization in human SC, both human SC and this mixture were selected to examine the effect of glycerol on the lipid phase behaviour.. It was found that high concentrations of glycerol change the lamellar organization slightly, while domains with an orthorhombic lateral packing are still observed.

2. INTRODUCTION

The body's skin barrier against influences from the environment is essentially formed by its uppermost layer, the stratum corneum (SC). The SC consists of corneocytes embedded in a continuous lipid matrix. When applying substances onto the skin in order to reach the underlying viable epidermis, these substances always have to pass the SC lipid regions. Consequently, the lipid matrix provides the main barrier of the skin.

The lipids in human SC are organized in two lamellar phases with repeat distances of approximately 6 and 13 nm, referred to as the short periodicity phase (SPP) and the long periodicity phase (LPP), respectively (Bouwstra et al., 1991). In human SC the lipids in these lamellar phases mainly form an orthorhombic lateral packing.

Not only the lipid organization, but also the lipid composition is exceptional. No phospholipids are present and the main lipid classes in SC are cholesterol (CHOL), ceramides (CER) and long chain free fatty acids (FFA). In human SC the CER consist of a hydrophilic head group that can be a sphingosine (S), phytosphingosine (P) or a 6-hydroxysphingosine (H) base linked with a long saturated carbon chain that can be non-hydroxylated (N) or α -hydroxylated (A) fatty acids (Ponec et al., 2003b; Robson et al., 1994; Stewart and Downing, 1999; Wertz et al., 1985) with a chain length of mostly 22 to 26 hydrocarbon atoms. Additionally, three acylCER in which a linoleic acid is ester-linked to a long chain ω -hydroxy fatty acid (EO) have been identified. These acylCER are EOS, EOP and EOH.

In recent years, many new insights into the role the various lipid classes play in the SC lipid organization have been gained by the use of lipid mixtures prepared from CER, CHOL and FFA. The most important observations are that: i) acylCER are of great importance for the formation of the LPP (Bouwstra et al., 1996b; Bouwstra et al., 1998), ii) long chain FFA are crucial for the formation of the orthorhombic lateral packing (de Jager et al., 2004a; de Jager et al., 2006; Mendelsohn et al., 2000), iii) some papers report that synthetic CER head group architecture affects the lateral packing, while other papers report that a variation in CER

composition does not affect the lateral packing (Bouwstra et al., 1999; Rerek et al., 2001; Thewalt et al., 1992), iv) lipid mixtures prepared from a single subclass of CER with uniform chain length, CHOL and a single FFA form often phase separated domains of FFA and CER (Chen et al., 2001; Chen et al., 2007; Moore et al., 1997c), while in mixtures with a chain length distribution and head group heterogeneity CER and FFA participate in the same orthorhombic lattice (Chen et al., 2007; Gooris and Bouwstra, 2007). From these studies it can be concluded that when aiming to create a model for SC lipids having similar phase behaviour as in stratum corneum, a certain degree of heterogeneity in the lipid mixture should be created.

Most free fatty acids are easily purchased from a variety of vendors; however, reproduction of the CER mix is very costly and difficult to achieve. Human CER (HCER) can be isolated from human skin. However, human donor skin is sparse, making isolation of large batches of HCER nearly impossible. This is not the case for porcine CER (PCER). Pig skin is easily obtained and has been widely used as a replacement for human skin in drug penetration studies, due to the fact that its global characteristics such as epidermal thickness ratio and turnover are very similar (Vardaxis et al., 1997; Weinstein, 1966). Also, the lamellar organization of the SC of the two species is similar, both having a SPP and a LPP (Bouwstra et al., 1991; Bouwstra et al., 1995). There is however no consensus as to the nature of the lateral packing of the lipids in porcine SC. While some studies indicated that, the lipids in porcine SC form an orthorhombic organization (Ongpipattanakul et al., 1994), other studies reported a predominantly hexagonal organization (Bouwstra et al., 1995). It has also been reported that HCER and PCER differ in composition and chemical structure (Ponec et al., 2003a; Wertz and Downing, 1983a; Wertz and Downing, 1983b). Most notably, only one of the PCER is an acylCER, the largest fraction is formed by CER NS, the CER AS has a fatty acid chain length of only approximately C16, and no 6-hydroxysphingosines have been identified.

In this study, firstly the lateral packing of porcine and human SC is examined by Fourier transform infrared spectroscopy (FTIR). FTIR provides more detailed information on the lateral organization and the conformational ordering in the lipid phases (Gooris and Bouwstra, 2007; Krill et al., 1992; Moore et al., 1997a; Moore and Rerek, 2000). Secondly, lipid mixtures prepared with PCER are studied by FTIR to provide more detailed insight in the role the lipids play in the lateral packing and to ascertain the possibility to use the lipid mixtures to model the lateral packing in human SC. Thirdly, the effect of glycerol on the lipid organization is studied in human SC sheets and lipid mixtures using FTIR and small angle X-ray diffraction (SAXD).

SAXD has been used extensively to determine the lamellar organization of SC lipid mixtures (Bouwstra et al., 2002b; Hatta et al., 2006). Glycerol is one of the most widely used moisturizers to treat dry skin. It penetrates the skin, where it acts as a humectant, i.e. attracts water, thereby increasing water levels in the SC (Sagiv and Marcus, 2003). In the past, it has been speculated that glycerol increases the hexagonal packing in the SC at the expense of the orthorhombic packing (Froebe, 1990). As this may reduce the skin's barrier function this is of great interest.

3. MATERIAL AND METHODS

CHEMICALS

Perdeuterated FFA (DFFA) with chain lengths of C16:0 and C22:0 were obtained from Larodan (Malmö, Sweden). DFFA with chain lengths 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 acetate buffer salts were purchased from Sigma-Aldrich Chemie GmbH (Schnelldorf, Germany). Glycerol and glycerol-1,1,2,3,3-d₅ were purchased from Sigma Aldrich (Zwijndrecht, The Netherlands).

EXTRACTION AND ISOLATION OF PCER

Fresh pig skin was obtained from a slaughter house and 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 PCER were pooled and the resulting PCER mixture had a CER EOS (Motta et al., 1993) content of approx. 12% (weight), which is similar to the acylCER content of the human CER mixture (see below). The remainder consisted of CER NS (64%) with CER NP, AS (long chain), AS (short chain) and AP in concentrations of 8, 6, 4 and 6%, respectively.

EXTRACTION AND ISOLATION OF HCER

Human skin was obtained from cosmetic surgery. The skin was dermatomed to a thickness of 250 μm approx. and the SC was isolated by trypsin digestion, dried at the air and kept over silica under argon at room temperature as described previously (Tanojo et al., 1997). For HCER isolation, the lipids were extracted from the SC as described above. The resulting HCER mixture consisted of 5% CER EOS, 28% NS, 28% NP, 4% EOH, 18% AS/NH, 9% AP, 5% AH and 3% EOP.

PREPARATION OF CER:CHOL:FFA MIXTURES FOR FTIR MEASUREMENTS

Isolated CER, CHOL and (D)FFA were dissolved in chloroform/methanol (2:1, v/v) and mixed in the desired ratio. The (D)FFA 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). This composition was based on the FFA composition reported by Wertz and Downing (5). 1.5 mg of the dissolved lipids were sprayed on an area of 1 cm^2 of a ZnSe window at a very low spraying rate (4.2 $\mu\text{l}/\text{min}$) under a gentle stream of nitrogen gas using a sample applicator (CAMAG LINOMAT IV (Muttenez, Switzerland)). The applicator was adapted by constructing an extra axis (Y-direction) perpendicular to 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 μl of a perdeuterated acetate buffer at pH 5.0 (50 mM), closed with a 2nd ZnSe window and equilibrated for 10 minutes. Perdeuterated buffer was used in order to avoid interference of the broad OH vibration peak closely located to the CH_2 stretching vibrations in the spectrum. The sample was then cooled slowly to room temperature. To homogenize the sample 10 freeze-thawing cycles were carried out between -18°C and room temperature.

PREPARATION OF CER:CHOL:FFA MIXTURES FOR SAXD MEASUREMENTS

Samples for SAXD were prepared using the method described above, except that they were sprayed on mica on a surface of 1.5 mm^2 and that for equilibration the samples were completely submerged in a protonated 50 mM citrate buffer.

PREPARATION OF GLYCEROL CONTAINING LIPID MIXTURES FOR FTIR AND SAXD MEASUREMENTS

Two methods were used to add glycerol to the lipid mixture. In the first method, 35% glycerol was added to the pH 5.0 perdeuterated acetate equilibration buffer, while the pH was kept constant and the equilibration time at 60°C was increased from 10 to 60 min to allow the

glycerol to penetrate into the lipid mixture. In the second method, perdeuterated glycerol and the lipids were dissolved in chloroform/methanol (2:1, v/v) to achieve a mixture with molar ratio of CHOL:PCER:FFA:glycerol of 1:1:1:0.75, resulting in a mixture containing 20% glycerol. The subsequent steps of the preparation method are described above. The control sample is the equimolar composition in the absence of glycerol.

TREATMENT OF SC SAMPLES FOR FTIR MEASUREMENTS

A single approx. 1 cm² sheet of SC was submerged in water, 35% w/v glycerol or pure glycerol for a period of 24 h at 32°C, before enclosure between a pair of ZnSe windows.

TREATMENT OF SC SAMPLES FOR SAXD MEASUREMENTS

A SC strip of 70 by 5 mm was rolled up and pressed to flatten, resulting in a tight 'stack' of approx 45 SC layers. The stacks were stored under argon on silica gel. 24 hours prior to the SAXD measurement, the dry SC stacks were completely immersed in either water or 35% w/v glycerol in water at 32°C.

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 the phase transition the sample temperature was increased at a heating rate of 0.25°C/min resulting in 1°C temperature raise 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).

SMALL ANGLE X-RAY DIFFRACTION (SAXD)

All measurements were performed at the European Synchrotron Radiation Facility (ESRF, Grenoble) using the Dutch-Belgian beamline (BM26B). A more detailed description of this beamline has been given elsewhere (Bras, 1998). The X-ray wavelength and the sample-to-detector distance were 1.24 Å and 1.7 m, respectively. Diffraction data were collected on a two-dimensional multiwire gas-filled area detector. The spatial calibration of this detector was performed using silver behenate and cholesterol. The samples were mounted in a specially

designed sample holder with mica windows. All measurements were recorded at room temperature for a period of 10 min.

SAXD provides information about the larger structural units in the sample, such as the periodicity of a lamellar phase, which is the distance over which the structure is repeated. The dimensionless scattering intensity (I) is measured as a function of the scattering vector q (in nm^{-1}). The latter is defined as $q = 4\pi \sin \vartheta / \lambda$, in which ϑ is the scattering angle and λ is the wavelength of the X-rays. From the positions of a series of equidistant peaks (q_n), the periodicity d of a lamellar phase is calculated using the equation $q_n = 2n\pi/d$, n being the order number of the diffraction peak.

4. RESULTS

CONFORMATIONAL DISORDERING AND LATERAL PACKING IN ISOLATED HUMAN AND PORCINE SC

As a first step to assess the possibility of using PCER in lipid mixtures to model the lipid organization of dry human and porcine SC were studied using FTIR. Figure 1a and 1b depict curves of the CH_2 scissoring bands of the lipids in human and porcine SC, respectively, at temperatures between 20 and 60°C. By its contour, the scissoring band provides information on the lateral packing of the lipid mixture. In human SC (figure 1a), the contours of the CH_2 scissoring band display a doublet at temperatures below 40°C, with peak positions at approx. 1463 and 1473 cm^{-1} . This doublet is caused by short-range interaction between the CH_2 in the lipid tails (Davydov or factor-group splitting) and indicates that a large population of lipids are arranged in an orthorhombic lattice (Mendelsohn et al., 1995; Moore et al., 1997c). Within the doublet, a weak third peak is visible at approx. 1467 cm^{-1} , which may be attributed to small domains of a hexagonal or liquid lateral packing. From 40°C upwards, this peak increases in intensity, whereas the doublet reduces in intensity indicating the transition from an orthorhombic to hexagonal phase. At approximately 48°C, only one CH_2 scissoring band is observed suggesting the presence of mainly a hexagonal lateral packing.

The CH_2 symmetric stretching vibration (figure 1e) provides information about the conformational disordering. At 0°C, the CH_2 stretching vibration has a frequency of 2849.1 cm^{-1} . In the temperature region lower than the temperature of the first transition, the frequency of the stretching vibration is below 2850 cm^{-1} indicating an all-trans conformation of the hydrocarbon chains. The transition from an orthorhombic to hexagonal packing is visible as a

small but steep increase in wavenumber from 2849.8 to 2850.5 cm^{-1} that occurs between 38 and 50°C. At 66°C it is followed by the transition to a conformational disordered phase with a large increase in wavenumber from 2850.7 until it is completed at approx. 2854.2 cm^{-1} at 94°C. Spectra of the CH_2 scissoring mode of porcine SC (figure 1b) are markedly different from those observed in human SC. Although some asymmetry of the singlet at 1466.5 cm^{-1} suggests some lipids are in an orthorhombic lateral packing, the majority of the SC lipids in porcine SC appears to be packed in a hexagonal lattice. This organization is stable, with frequencies of the symmetric stretching mode increasing from 2850.0 cm^{-1} at 0°C to 2850.7 cm^{-1} , until 50°C (figure 1e). At this temperature an increase in frequency to 2853.9 cm^{-1} at 85°C marks the transition from ordered hexagonal packing to a liquid disordered phase.

CONFORMATIONAL DISORDERING AND LATERAL ORGANIZATION IN LIPID MIXTURES PREPARED WITH PCER

To determine whether PCER can be used in SC lipid model systems mimicking the lateral organization in human SC, lipid mixtures containing PCER were examined. Figure 2a shows the relationship between the frequency of the stretching mode of an equimolar mixture of PCER and CHOL and the temperature. Only one transition can be observed. Conformational ordering is indicated by a CH_2 stretching frequency. At 0°C this frequency is 2850.5 cm^{-1} and gradually increases until approx. 60°C, when a sharp rise in the frequency of the symmetric stretching mode from 2851.0 to 2853.3 cm^{-1} at 80°C indicates a transition from an ordered to a disordered state. Figure 2b depicts spectra of the CH_2 scissoring band of the same mixture. At 20°C the scissoring band appears as a singlet at 1467.6 cm^{-1} , indicating a hexagonal packing. However, a slight asymmetry of the singlet suggests that a very small portion of the lipids is organized in an orthorhombic lattice.

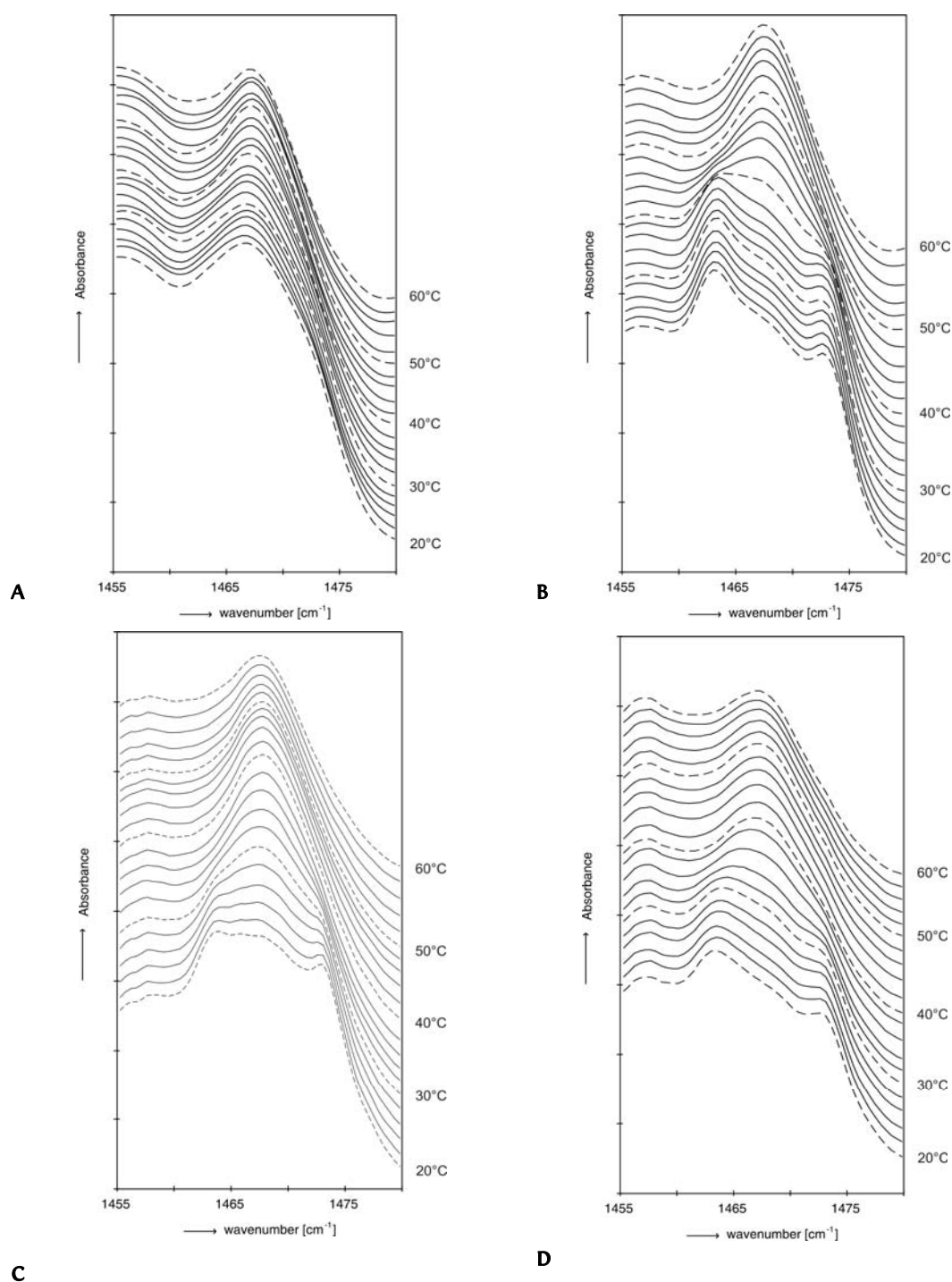


FIGURE 1A-D. Spectra of the CH₂ scissoring band of SC in a temperature range of 20 and 60°C. Each successive spectrum represents a temperature shift of 2°C. (a) CH₂ scissoring bands of dry human SC. At low temperature, a doublet is observed at 1463 and 1473 cm⁻¹. In between the doublet a singlet is observed at 1467 cm⁻¹. At 40°C the doublet starts to dissolve into a singlet indicating an orthorhombic-hexagonal phase transition. At 48°C only a singlet remains. (b) CH₂ scissoring contours of dry pig SC. A singlet is observed at 1467 cm⁻¹. (c) CH₂ scissoring contours of fully hydrated human SC. A doublet is present at 1463.9 and 1472.9 cm⁻¹. In between the doublet a singlet is observed at 1467 cm⁻¹. An increase in temperature weakens the doublet until it dissolves at 32°C. (d) CH₂ scissoring bands of 35% glycerol treated human SC. A doublet is present at 1463.1 and 1472.6 cm⁻¹. In between the doublet a weak singlet is observed at 1467 cm⁻¹. An increase in temperature weakens the doublet until it dissolves at 32°C.

Besides the PCER:CHOL mixture, also an equimolar PCER:CHOL:FFA mixture was examined using FTIR. In figure 2a the CH₂ symmetric stretching vibration frequencies of this mixture are plotted against temperature. Two conformational transitions are visible: at 0°C, the stretching vibration in this mixture is observed at 2848.6 cm⁻¹ and increases slightly to 2849.0 cm⁻¹ at 22°C, after which the stretching vibration immediately increases to approx. 2849.7 cm⁻¹ at 32°C, indicating a transition from an orthorhombic to a hexagonal organization. The stretching vibration remains at this frequency until a temperature of 50°C is reached. At this temperature, the frequency starts to increase sharply until 2853.5 cm⁻¹ at 70°C, indicating a second transition, from a hexagonal to a disordered state. The values for the symmetric stretching in the spectra of the PCER:CHOL:FFA mixtures at 0°C indicate a fully extended chain conformation for this mixture (Moore et al., 1997a).

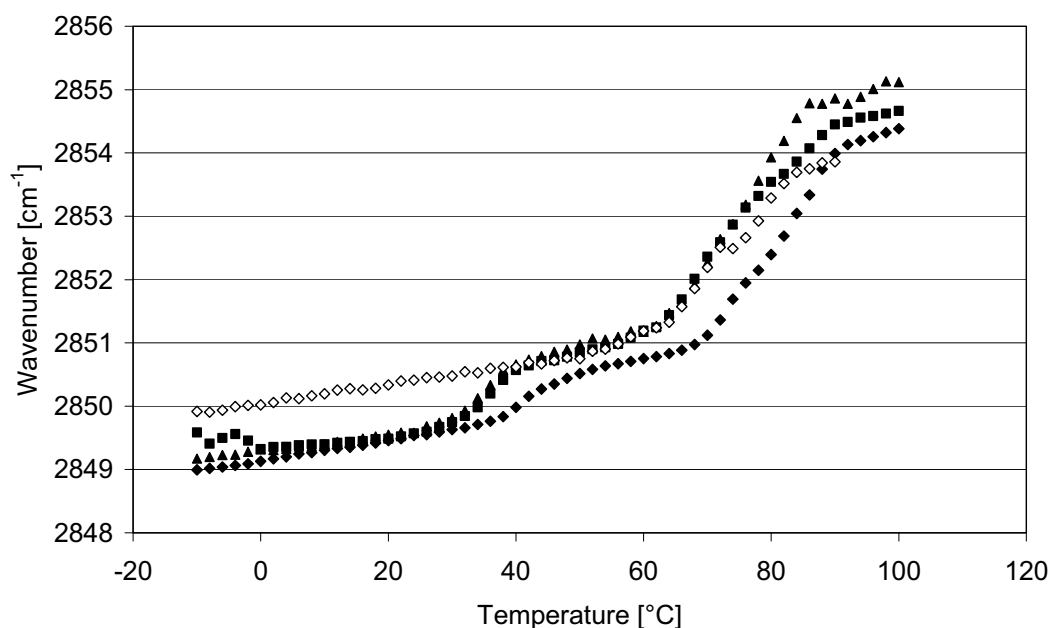


FIGURE 1E. The thermotropic response of the CH₂ stretching frequencies in SC. In dry human SC (diamonds), a shift in CH₂ stretching frequency from 2849.8 to 2850.5 cm⁻¹ indicates the lipid transition from orthorhombic to hexagonal packing between 38 and 50°C. A further shift in CH₂ stretching frequency is followed between 66 (2850.7 cm⁻¹) and 94°C (2854.2 cm⁻¹) representing a transition to a disordered state. After treatment with water (squares) and 35% glycerol (triangles), these transitions occur at slightly lower temperatures, i.e. between 32 and 44°C and 62 and 92°C. Lipids in dry porcine SC (open diamonds) are packed in a hexagonal lattice even at low temperatures. A transition from 50°C (2850.7 cm⁻¹) to 85°C (2853.9 cm⁻¹) reprints the transition of lipids into a disordered state.

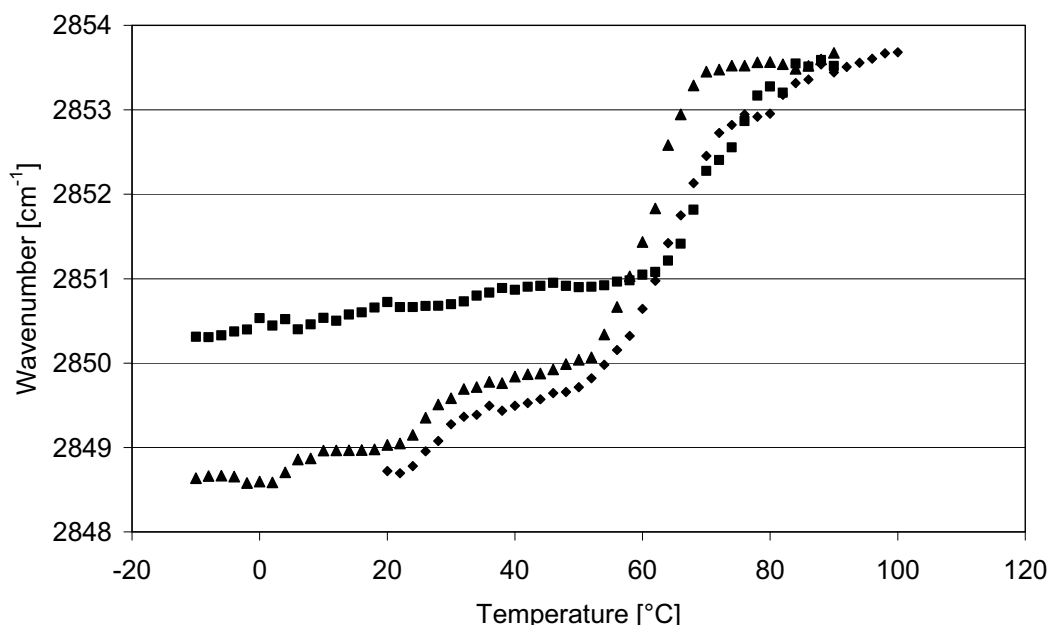


FIGURE 2A. The thermotropic response of the CH₂ stretching frequencies of equimolar HCER:CHOL:FFA (diamonds), PCER:CHOL (squares) and PCER:CHOL:FFA (triangles) mixtures. In the spectrum of PCER:CHOL mixture the CH₂ stretching frequency at 0°C is at 2850.5 cm⁻¹. A shift is observed in the temperature range between 60°C (2851.0 cm⁻¹) and 80°C (2853.3 cm⁻¹) representing a hexagonal to disordered state transition. In the spectrum of the PCER:CHOL:FFA mixture the CH₂ stretching frequency at 0°C is at 2850.5 cm⁻¹. Between 22°C and 32°C an orthorhombic to hexagonal transition is indicated by a shift in CH₂ symmetric frequency from 2849.0 cm⁻¹ and 2849.7 cm⁻¹. Between 50 (2849.7 cm⁻¹) and 70°C (2853.5 cm⁻¹), the transition to a disordered state occurs. In the spectrum of the HCER:CHOL:FFA mixture the CH₂ stretching frequency at 20°C is 2848.7 cm⁻¹ and shift to 2849.4 at 34°C. A second transition occurs between 52 (2849.7 cm⁻¹) and 78°C (2852.9 cm⁻¹).

The transition from an orthorhombic to a hexagonal organization is better visible in figure 2c, in which the CH₂ scissoring bands of the equimolar PCER:CHOL:FFA mixture are shown. At 20°C, a doublet with peaks at 1473.2 and 1463.7 cm⁻¹ indicates an orthorhombic organization. The high degree of splitting (10.5 cm⁻¹) is evidence of large, orthorhombic domains. A weak singlet is observed in between the doublet at 1467 cm⁻¹. This peak is more clearly visible at 24°C and is representative for hexagonal packing. The intensity of this singlet peak continues to increase, while the doublet disappears at approx. 30°C, indicating that the lipids are organized entirely in a hexagonal lattice.

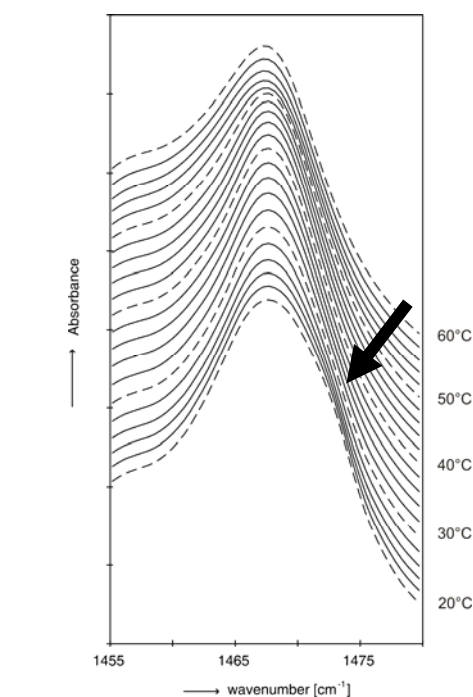
CONFORMATIONAL DISORDERING AND LATERAL PACKING OF EQUIMOLAR HCER:CHOL:FFA MIXTURES

In figure 2a, the wavenumbers of the CH₂ symmetric stretching vibrations of an equimolar HCER:CHOL:FFA mixture are plotted against temperature. At 20°C, the stretching vibration of the equimolar mixture containing HCER has a frequency of 2848.7 cm⁻¹ and remains at this

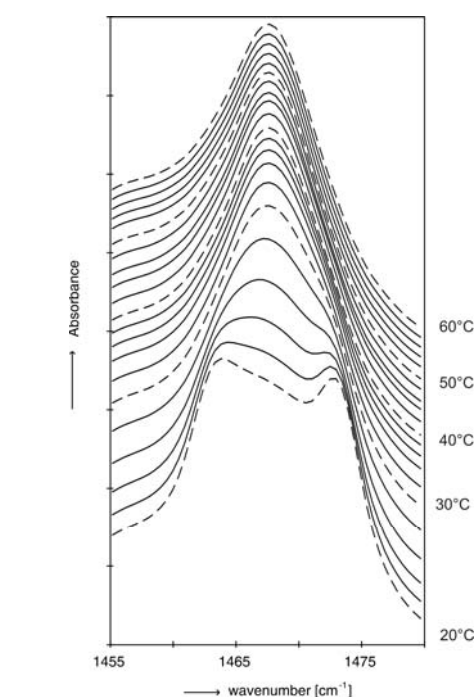
frequency until 24°C. A further increase in temperature results in a frequency increase to 2849.4 cm⁻¹ at 34°C. This shift indicates a transition from an orthorhombic to a hexagonal organization. Increasing the temperature results in a slight increase in frequency until a temperature of around 52°C. Between 52 and 78°C a steep increase in frequency from 2849.7 to 2852.9 cm⁻¹ indicates a second transition, from a hexagonal organization to a disordered state. Figure 2d shows spectra of the CH₂ scissoring bands of the HCER:CHOL:FFA mixture. At 20°C, a doublet contour of the CH₂ scissoring frequency is observed at 1463.1 and 1473.2 cm⁻¹, respectively, indicating the orthorhombic organization. A singlet at approx. 1466 cm⁻¹ indicating the presence of hexagonal packing is first observed at 24°C. However, the doublet is visible until 38°C in shape of a slight shoulder, indicating an orthorhombic to hexagonal phase transition between 24 and 38°C.

CONFORMATIONAL DISORDERING AND LATERAL PACKING IN ISOLATED HUMAN SC SHEETS TREATED WITH GLYCEROL

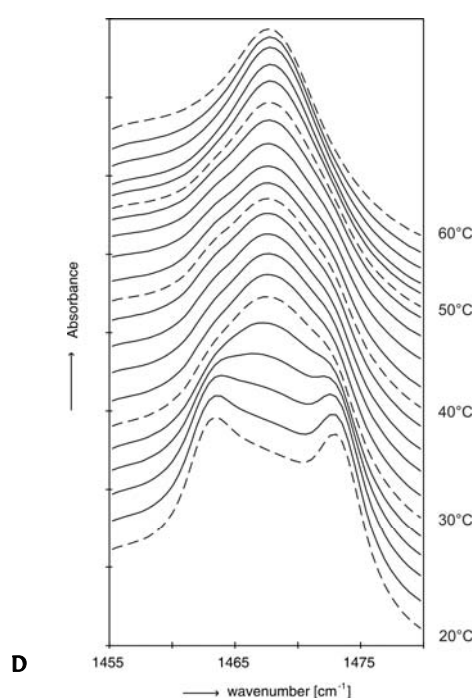
To investigate the effect of glycerol on the lateral packing of isolated SC, SC sheets treated with water and 35% glycerol in water were studied using FTIR spectroscopy. The relationship between wavenumber of the symmetric stretching band of the intercellular SC lipids and temperature is shown in figure 1e. The curves SC treated with water or 35% glycerol in water are very similar to those of dry SC, but a decrease in transition temperatures of the two phase transitions are observed. Treatment with water appears to decrease the onset temperatures of the transition from orthorhombic to hexagonal compared to dry skin, which takes place between 32 and 44°C in hydrated SC sheets compared to 38 and 50°C in dry SC sheets. The wavenumber increase during this transition is, however, similar as in dry SC sheets, namely from 2849.8 to 2850.7 cm⁻¹. The second transition, from hexagonal to a liquid disordered state is commenced at 62°C at a frequency of 2851.2 cm⁻¹ and ends at 92°C at a frequency of 2854.5 cm⁻¹, while in dry human SC sheets this transition starts at 66°C. The curve describing the relationship between temperature and the symmetric stretching frequency of lipids in SC treated with 35% (w/v) glycerol in water closely follows the curve of treatment with water, except that in case of glycerol treatment, the disordered state appears to be reached at a lower temperature of 86°C, with a slightly increased frequency of 2854.8 cm⁻¹.



B



C



D

FIGURE 2BCD. Spectra of the CH₂ scissoring band of lipid mixtures in a temperature range of 20 and 60°C. Each successive spectrum represents a temperature shift of 2°C. (b) PCER:CHOL mixture: A singlet is observed at 1467.7 cm⁻¹ with only very slight broadening of the peak at the lowest temperatures shown (arrow). (c) PCER:CHOL:FFA mixture: Below 24°C a doublet is observed at 1463.7 and 1473.2 cm⁻¹ and a weak peak at 1467 cm⁻¹. Increase in temperature increases the intensity of the singlet and reduces the doublet contour. At 32°C only the singlet remains. (d) HCER:CHOL:FFA mixture: Until 32°C, a sharp doublet contour (1463.1 cm⁻¹ and 1473.2 cm⁻¹) represents an orthorhombic organization. A singlet indicating the presence of hexagonal packing is first observed at 26°C. The double disappears at 38°C.

Figures 1c and 1d illustrate the transition from orthorhombic to hexagonal organization with selected spectra of the CH₂ scissoring bands of human SC treated with water and 35% (w/v) glycerol, respectively. After water treatment, the CH₂ scissoring band is seen at 20°C as a doublet with peaks at 1463.9 cm⁻¹ and 1472.9 cm⁻¹, indicating that the lipid domains in this

sample is predominantly organized in an orthorhombic lattice. The intensity of the peaks of the observed doublet is, however, lower than in dry SC (figure 1a). In between the bands of the doublet, another peak is observed at a frequency of approx. 1467 cm^{-1} , representing a hexagonal or liquid packing. This peak increases in size, while the doublet decreases in size until it is no longer visible at 32°C . After treatment with 35% (w/v) glycerol, the CH_2 scissoring band is also visible as a doublet, with peaks at 1463.1 and 1472.6 cm^{-1} , indicating the presence of orthorhombic packing. A very weak broad peak at approx. 1467 cm^{-1} suggests the presence of very small lipid domains with a hexagonal or liquid lateral packing. The doublet is clearly visible until 32°C , after which temperature only the 1472 cm^{-1} peak is observed. The disappearance of this peak at 40°C indicates that at this temperature, the lipids are present in mainly a hexagonal phase.

LAMELLAR ORGANIZATION IN ISOLATED SC SHEETS TREATED WITH GLYCEROL

To determine the lamellar organization of the intercellular lipids in SC, SAXD measurements were performed. Figure 3 shows SAXD curves of SC treated with water or 35% w/v glycerol. In hydrated SC, 3 orders of the LPP (referred to as 1, 2 and 3 in the figure) with q-values of 0.5, 1.0 and 1.36 nm^{-1} , respectively, corresponding to a repeat distance of 12.4 nm can clearly be discerned, as well as the diffraction peak of separated crystalline CHOL. Most probably the peak at around $q=1.0\text{ nm}^{-1}$ is also due to the 1st order peak of the SPP. After treatment with glycerol, again three orders of the LPP corresponding to the same repeat distance, as well as a peak for phase separated crystalline CHOL are observed in the diffraction profile.

CONFORMATIONAL ORDERING AND LATERAL PACKING OF EQUIMOLAR PCER:CHOL:FFA MIXTURES TREATED WITH GLYCEROL

To determine the effect of glycerol on the lateral organization of equimolar PCER:CHOL:FFA mixtures, the mixtures were treated with glycerol during the equilibration step or glycerol was added to the lipids during preparation of the sample. To increase the exposure of the lipid mixtures to glycerol, the duration of the equilibration was extended from 10 to 60 minutes. The increased equilibration period only slightly changed the transition temperatures of the equimolar PCER:CHOL:FFA sample (not shown). In figure 4a the frequencies of the CH_2 symmetric stretching peaks of an equimolar PCER:CHOL:FFA mixture equilibrated for 60 minutes in buffer with 35% (w/v) glycerol are plotted as function of temperature. The symmetric stretching frequencies of both lipid mixtures are very similar to the control

mixtures, which were equilibrated in perdeuterated acetate buffer (no glycerol). In equimolar PCER:CHOL:FFA samples equilibrated for 60 minutes in 35% glycerol the symmetric stretching band appears at a frequency of 2849.4 cm^{-1} . Increasing the temperature revealed a phase transition from orthorhombic to hexagonal between 23°C and 33°C , with a frequency increase from 2849.5 to 2849.9 cm^{-1} . At 48°C , a second transition takes place: a frequency increase from 2850.0 to 2853.3 cm^{-1} indicates a transition to a liquid, disordered phase, which is completed at 70°C . The contours of the CH_2 scissoring bands of the equimolar PCER:CHOL:FFA mixtures equilibrated in 35% glycerol are shown in figure 4b. At 20°C the doublet indicating orthorhombic organization appears at 1463.6 cm^{-1} and 1473.2 cm^{-1} . Similar as in control equimolar mixtures (fig. 2c) and 60 minutes equilibrated samples (not shown) it disappears in favour of a singlet at 30°C . The very similar conformational disordering and the contours of the CH_2 scissoring bands of the equimolar mixture with 20% glycerol (PCER:CHOL:FFA; glycerol=1:1:1:0.75) are depicted in figures 4a and 4c, respectively.

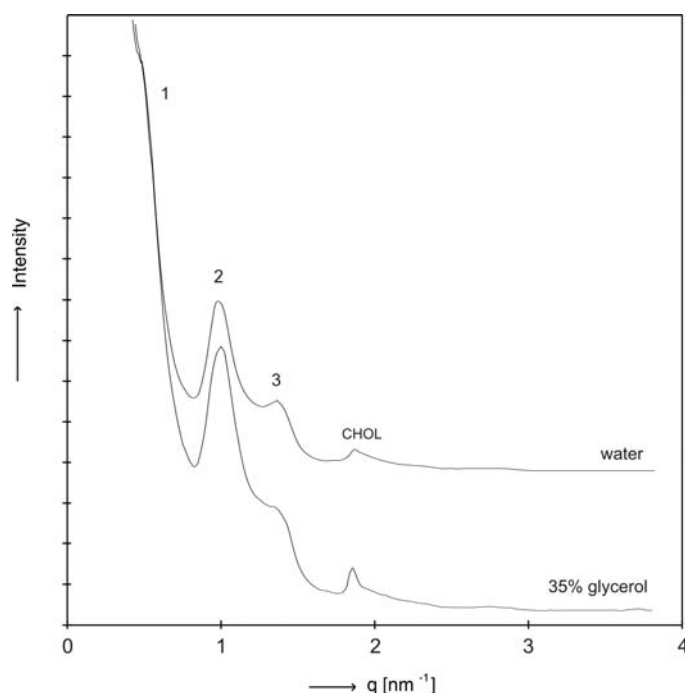


FIGURE 3. SAXD profiles of human SC sheets. Curves of SC treated with water or 35% glycerol are very similar. Three peaks that can be attributed to the LPP can be observed at approximately $q = 0.5, 1$ and 1.36 nm^{-1} , corresponding to a repeat distance of 12.4 nm , as well as the diffraction peak of separated crystalline cholesterol at 1.8 nm^{-1} . Most probably the peak at around $q=1\text{ nm}^{-1}$ is also due to the 1st order peak of the SPP.

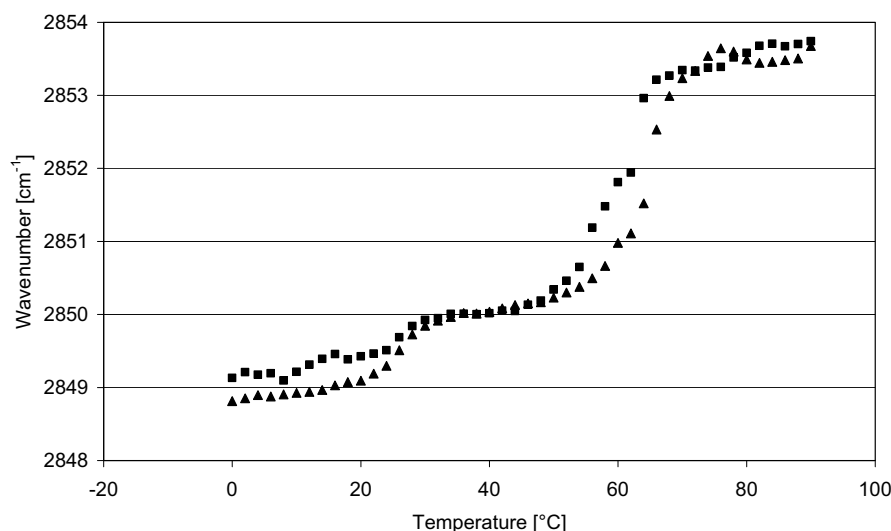


FIGURE 4A. The thermotropic response of the CH_2 stretching frequencies of equimolar PCER:CHOL:FFA mixtures treated with glycerol. Equilibration in 35% glycerol (squares) reveals a CH_2 symmetric stretching frequency of 2849.4 cm^{-1} at 0°C . Increasing the temperature reveals a shift in peak position from 2849.5 to 2849.9 cm^{-1} between 23°C and 33°C . A second shift in CH_2 symmetric frequency is observed between 48°C (2850.0 cm^{-1}) and 70°C (2853.3 cm^{-1}). The thermotropic response of the equimolar PCER:CHOL:FFA mixtures equilibrated with 20% glycerol (triangles) is very similar to that of mixtures those have been equilibrated in 35% glycerol.

Substitution of the FFA by perdeuterated FFA (DFFA) resulting in the equimolar CER:CHOL:DFFA mixtures equilibrated for 60 minutes (control), equilibrated for 60 minutes in 35% glycerol or with 20% glycerol resulted in the contours of the CD_2 scissoring band appearing as singlets, as can be seen in figure 4d, which depicts the CD_2 scissoring band of an equimolar PCER:CHOL:DFFA mixture to which 20% glycerol has been added. No additional splitting of the CD_2 scissoring band was observed, suggesting that the short-range interaction of the carbon tails in the sample was decoupled, indicating that DFFA and CER participate in one lattice.

LAMELLAR ORGANIZATION OF EQUIMOLAR PCER:CHOL:FFA MIXTURES TREATED WITH GLYCEROL

To determine the effect of glycerol on the lamellar organization of equimolar PCER:CHOL:FFA mixtures, similar samples to those described above were also studied using SAXD. The resulting SAXD curves are shown in figure 5. In the curve of a 60 minute equilibrated equimolar PCER:CHOL:FFA mixture seven orders of the LPP with q-values of 0.53, 1.03, 1.48, 1.94, 2.27, 2.61 and 2.87, corresponding to a repeat distance of 12.7 nm are observed (figure 5). Furthermore, two additional peaks are present, the 1st order of the SPP is visible as a small shoulder on the right side of the 2nd order peak of the LPP and a small peak indicating the presence of phase separated, crystalline CHOL. After addition of 35% glycerol to the

equilibration buffer or 20% glycerol to the lipid mixtures prior to spraying, at least three orders of the LPP are still present and a slight shift in peak position is observed compared to the control mixture.

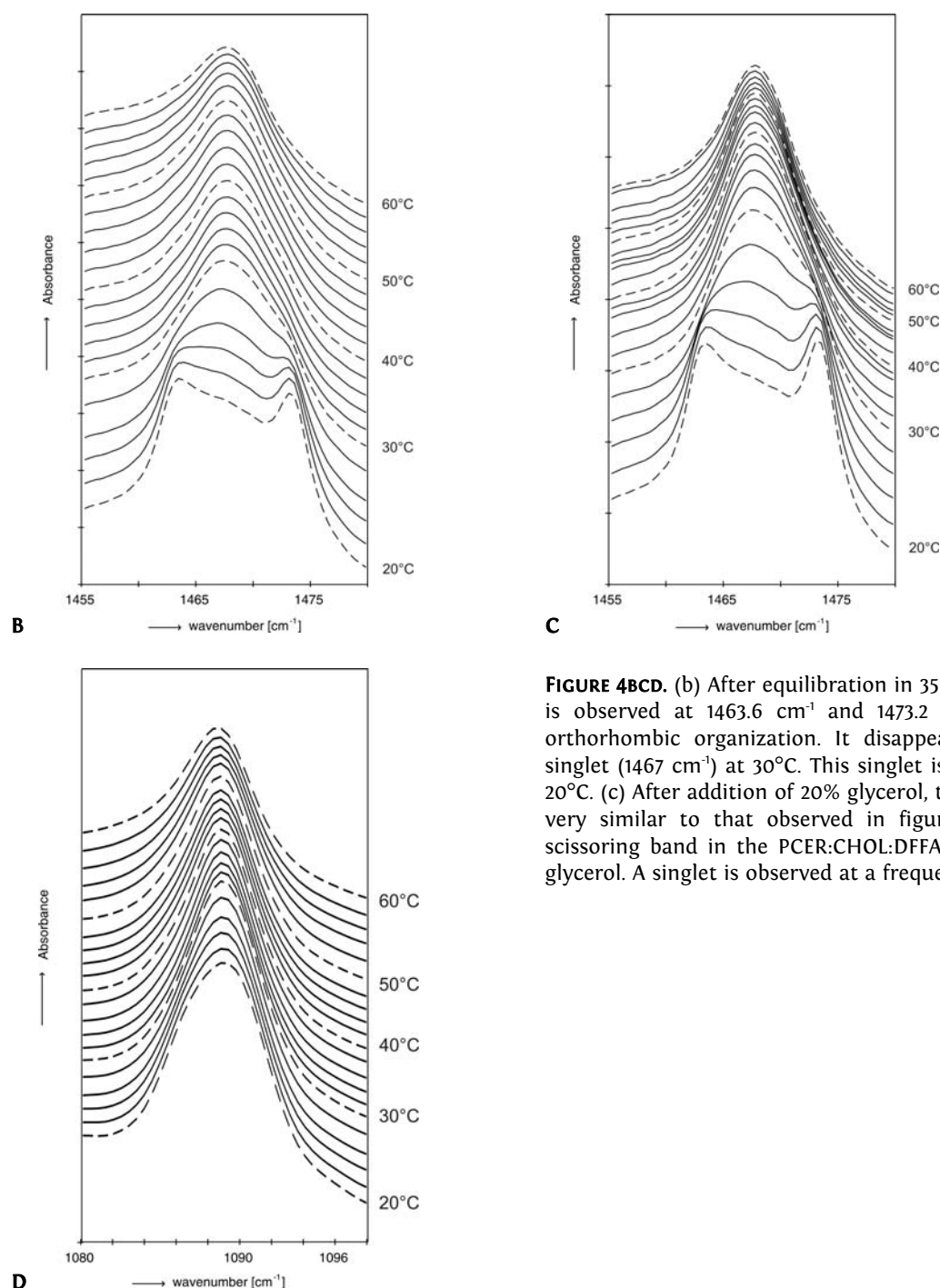


FIGURE 4BCD. (b) After equilibration in 35% at 20°C a doublet is observed at 1463.6 cm^{-1} and 1473.2 cm^{-1} , indicating an orthorhombic organization. It disappears in favour of a singlet (1467 cm^{-1}) at 30°C. This singlet is already present at 20°C. (c) After addition of 20% glycerol, the CH_2 scissoring is very similar to that observed in figure 4b. (d) The CD_2 scissoring band in the PCER:CHOL:DFFA mixture with 20% glycerol. A singlet is observed at a frequency of 1088.0 cm^{-1} .

This indicates a slight increase in repeat distance to 13.2 nm. The 1st order peak of the SPP has increased in intensity compared to the 2nd order peak of the LPP, indicating that glycerol

promotes the formation of the SPP at the expensive of the LPP. The corresponding spacing of the LPP 1st order peak is approx. 5.6 nm. Additionally, a high intensity is observed at the left hand side of the 1st order LPP peak (asterisk). Such a high intensity at low q-value might be due to small domains (low angle scattering). As it is only observed in the glycerol containing samples, this steep decreasing curve might be due to small lipid domains surrounded by glycerol (phase separation).

5. DISCUSSION

SC lipid mixtures have widely been used to gain more insight into the organization of the intercellular lipids of the SC. For this purpose, even mixtures prepared with a single CER or single FFA have been used and provided very useful information on fundamental issues concerning molecular interactions (Bouwstra et al., 1997; Kitson et al., 1994; Lafleur, 1998; Percot, 2001; Velkova, 2002). If however the goal is to determine the effect of substances on the SC lipid phase behaviour, the model composition should match the composition and organization of human SC closely. In this study, first, the lateral organization of human and porcine SC has been studied by FTIR. In subsequent studies lipid mixtures based on PCER have been examined aiming to provide insight in the differences in lateral packing between porcine and human SC.

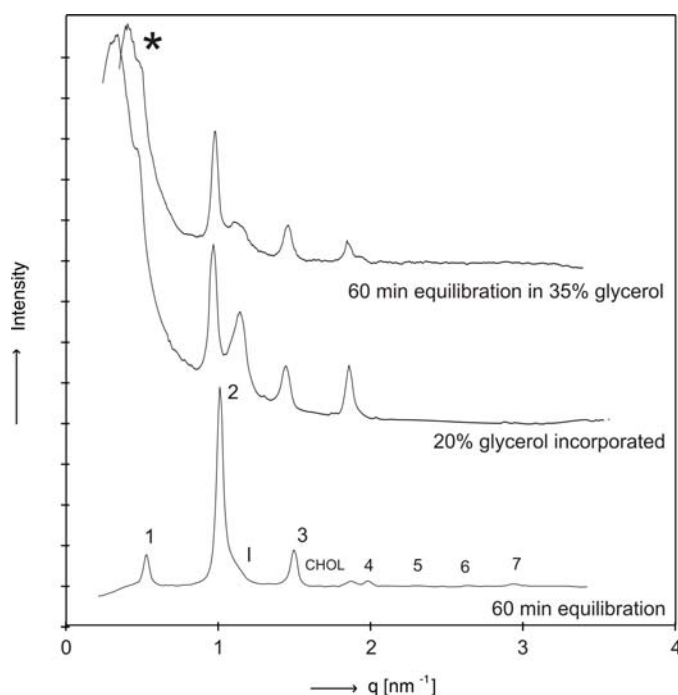


FIGURE 5. SAXD profiles of equimolar PCER:CHOL:FFA mixtures. In the curve of a 60 minute equilibrated equimolar PCER:CHOL:FFA mixture seven orders of the LPP are observed at $q = 0.53, 1.03, 1.48, 1.94, 2.27, 2.61$ and 2.87 ,

corresponding to a repeat distance of 12.7 nm⁻¹. The 1st order of the SPP is observed as a shoulder to the right of the 2nd order LPP peak. A small peak at 1.48 nm⁻¹ indicates the presence of phase separated, crystalline CHOL. After addition of 35% glycerol to the equilibration buffer, four orders of the LPP are still present but at a slightly lower q-value, which is 0.97 and 1.42 nm⁻¹ for the 2nd and 3rd order, respectively, corresponding to a repeat distance of 13.2 nm⁻¹. The 1st order peak of the SPP has increased in intensity and is now better visible at 1.12 nm⁻¹. Additionally, a high intensity is observed at the left hand side of the 1st order LPP peak (asterisk). This high intensity is also visible in the diffraction curve of PCER:CHOL:FFA mixtures to which 20% glycerol has been added prior to spraying of the sample. This curve is very similar to the curve of the mixture equilibrated in 35% glycerol, except that the 4th order of the LPP is not detected. Also in this sample, the 1st order peak of the SPP appears to have increased in intensity.

HUMAN AND PORCINE SC MARKEDLY DIFFER IN LATERAL ORGANIZATION

Previous studies reporting the organization of lipids in porcine SC (Bouwstra et al., 1995; Ongpipattanakul et al., 1994) provided conflicting data concerning the lateral packing of the lipids. While wide angle x-ray diffraction (WAXD) studies reported no evidence of an orthorhombic packing (Bouwstra et al., 1995), other studies using FTIR (Ongpipattanakul et al., 1994) described the presence of the orthorhombic lateral packing up to temperatures of 50-60°C. Furthermore, while FTIR describes the presence of an orthorhombic phase in human SC until temperatures approaching 60°C (Gay et al., 1994), WAXD reveals the disappearance of the orthorhombic phase between 32 and 34°C (Bouwstra, J.A., unpublished data). Therefore, the first aim of the present study was to examine the lateral organization and conformational disordering of isolated human and porcine SC. The CH₂ scissoring bands in spectra of human SC appear as a doublet at room temperature, but most importantly, also at skin temperature (32°C). This indicates that the majority of the lipids is organized in an orthorhombic lattice as has been reported previously (Bouwstra et al., 1992; Gay et al., 1994; Pilgram et al., 1999). The orthorhombic to hexagonal phase transition takes place between 38 and 46°C in dry human SC, but shifts to lower temperatures in hydrated SC in agreement with previous studies (Bouwstra et al., 2003; Gay et al., 1994). However, in the present study the orthorhombic to hexagonal phase transition ends at much lower temperature than observed in one of these FTIR studies (Gay et al., 1994) and is in agreement with results obtained with WAXD studies (Bouwstra, J.A., unpublished data). A weak peak within the doublet indicates that a small portion of the lipids is organized in a hexagonal lattice or in a liquid phase at 20°C, suggesting the coexistence of different domains. A hexagonal lateral packing might be induced by sebum lipids located at the skin surface as has been reported before (Pilgram et al., 1999). However the presence of a liquid phase cannot be excluded and has been observed in lipid mixtures prepared from human CER, CHOL and FFA previously (Bouwstra et al., 2002a).

The scissoring bands of porcine SC appear quite differently. No doublet is observed, the location of the singlet indicating that in porcine SC, the majority of the lipids are organized in a hexagonal packing. Only a barely discernable shoulder at approximately 1473 cm^{-1} in the contour of the singlet suggests that a very small portion of lipids is organized in an orthorhombic packing. At first sight, our study appears to contradict studies of Ongpipattanakul et al (Ongpipattanakul et al., 1994), who reported orthorhombic packing in porcine SC. The group observed a weak peak at -10°C at approx. 1473.3 cm^{-1} . After deconvolution of their spectrum, the second peak at around 1464.5 cm^{-1} was also visible, accompanied by the observation of a singlet indicating a hexagonal phase. However, the weakness of the doublet found by Ongpipattanakul et al (Ongpipattanakul et al., 1994), its small interpeak distance (6.5 cm^{-1}) and the strong presence of the singlet indicates also in their studies a predominant hexagonal lateral packing, while only a small population of lipids form small orthorhombic domains. Summarizing, while the lamellar organization has been shown to be very similar (Bouwstra et al., 1995) to human SC, the lateral organization of porcine and human SC is fundamentally different.

WHICH FACTORS ARE RESPONSIBLE FOR THE DIFFERENCE IN LATERAL ORGANIZATION IN HUMAN AND PORCINE SC?

In human as well as porcine SC, the main lipid classes are CER, CHOL and FFA and these lipid classes are present in an approximately equimolar ratio (Wertz, 1991). However, the composition of FFA and CER are different. In previous studies it has been demonstrated that FFA play an important role in the formation of the orthorhombic lateral packing, while the presence of CHOL has only a minor influence on the lateral organization (Bouwstra et al., 1996a). In the present study, it was decided to determine the contribution of the different CER composition in porcine and human SC on the lipid packing and conformational disordering using mixtures prepared with isolated HCER and PCER. Therefore, the FFA composition and the CHOL:CER:FFA molar ratio was kept the same in all lipid mixtures. We chose an equimolar ratio as has been reported to be present in human (Weerheim and Ponec, 2001) and porcine SC (Wertz, 2000).

THE CONFORMATIONAL ORDERING AND LATERAL ORGANIZATION OF EQUIMOLAR PCER:CHOL:FFA AND HCER:CHOL:FFA ARE VERY SIMILAR

It has previously been shown that equimolar mixtures of PCER, CHOL and FFA can be used to mimic the lamellar ordering of human SC lipids and that these lipids form an orthorhombic packing at room temperature (Bouwstra et al., 1996a) and a hexagonal packing at elevated temperatures. However, using WAXD it is difficult to determine whether besides an orthorhombic phase a hexagonal lateral packing is also present, as the orthorhombic reflections obscure the single strong reflection attributed to the hexagonal lateral packing. In the present study, lipid mixtures containing PCER were prepared and their organization and phase behaviour examined using FTIR as function of temperature. An equimolar PCER:CHOL mixture is organized in a hexagonal lattice as reported previously (Bouwstra et al., 1996b). Addition of an equal amount of FFA, resulting in equimolar PCER:CHOL:FFA mixtures, created large, orthorhombic domains, demonstrating the important role of FFA for the formation of orthorhombic phases. As the interpeak distance in the CH₂ scissoring doublet (approx. 10 cm⁻¹) is similar to that observed in mixtures prepared with HCER, the number of lipids in the domains exceed 100 in both mixtures prepared with HCER and PCER (Mendelsohn et al., 1995; Moore et al., 1997c). When focussing on the transition from orthorhombic to hexagonal in the equimolar mixtures with HCER, as detected by the scissoring vibrations, this transition starts at approximately 24°C, which is only approx. 2°C higher than in the equimolar mixtures prepared with PCER. Furthermore, when focusing on the CH₂ symmetric stretching vibrations, the frequency in the HCER mixtures is slightly lower than in the PCER mixtures, indicating a slightly higher conformational ordering in the former. The thermotropic ordered-disordered transition in the equimolar HCER:CHOL:FFA and PCER:CHOL:FFA mixtures starts at approx. the same temperature (around 52°C) (Gooris and Bouwstra, 2007). From these studies we can conclude that only minor differences in conformational ordering and lateral packing are observed between HCER and PCER mixtures. These differences are much less pronounced at around room temperature and 32°C than the differences observed between the lipid organization in human SC and porcine SC.

The small differences in thermotropic lateral packing and conformational ordering between equimolar HCER:CHOL:FFA and PCER:CHOL:FFA mixtures most likely originate from the difference in CER composition as the fatty acid composition in both mixtures are similar. The differences in CER composition are:

- i) The presence of acylCER that are thought to be of great influence on the formation of the LPP by providing a 'rivet' that links the trilayer structure together and by providing fluidity to the central layer of the trilayer with their unsaturated ester linked linoleic acid (Bouwstra et al., 2000a). In human SC, three acylCER (EOS, EOH and EOP) have been identified, while in PCER, only one acylCER (EOS) is present. The influence of EOS on the lateral organization of equimolar CER:CHOL:FFA mixtures has been studied and only little effect on the formation of the orthorhombic lateral packing has been observed (de Jager et al., 2004a). Furthermore, in the PCER and HCER mixtures in the present study approx. equal percentages of acylCER are present.
- ii) The difference in head group architecture between PCER and HCER, as well as the contribution of each of the head groups to the total CER mixture. In HCER phytosphingosine (40%), sphingosine (42%) and 6-hydroxysphingosine (18%) based CER are present, while in PCER only phytosphingosine (15%) and sphingosine (85%) based CER are present. Sphingosine based CER have the smallest number of hydroxyl groups in the head group region. The difference in head group architecture might affect the lateral packing as well as the formation of hydrogen bonds in the head group region. Moore et al showed that sphingosine based CER promote the formation of an orthorhombic packing, while phytosphingosine based CER promote the hexagonal packing (Rerek et al., 2001). However, these studies were performed using single CER without addition of CHOL or FFA and it is not clear whether the results can be extrapolated to more complex mixtures. The influence of CER head group architecture on the lipid organization is therefore an interesting subject for further investigation.
- iii) The fatty acid chain length distribution in the CER. Fatty acids of HCER on average are longer than those in porcine SC (Wertz, 1991). It has been shown that the ability to pack in an orthorhombic lattice is dependent on chain length. Longer chains have stronger Van der Waals interactions promoting the orthorhombic lateral packing, whereas shorter chains promote the hexagonal lateral packing and thus these mixtures are thermotropically less stable (Snyder et al., 1996). Therefore, the difference in fatty acid chain length of the CER may contribute to the small thermotropic differences between equimolar CER:CHOL:FFA mixtures prepared with PCER or HCER.

THE LATERAL ORGANIZATION OF INTERCELLULAR LIPIDS IN HUMAN SC IN THE LOW TEMPERATURE RANGE CAN BE MIMICKED USING HCER AS WELL AS PCER

The phase behaviour of the equimolar CER:CHOL:FFA mixtures prepared with PCER or HCER is slightly different from that of hydrated human SC. Firstly, the transition from orthorhombic to hexagonal organization occurs at a slightly lower temperature in the equimolar PCER:CHOL:FFA and HCER:CHOL:FFA mixtures (22-32°C or 24-34°C opposed to 30-40°C for hydrated human SC). Secondly, also the transition from hexagonal organization to a liquid, disordered state commences at a lower temperature (52°C for the PCER containing mixtures as opposed to 62°C for the HCER containing mixtures). This does not answer the question why there is a difference in lateral packing between the equimolar PCER:CHOL:FFA mixtures and the lipids in porcine SC. The difference may be caused by small changes in FFA composition between the FFA mixture used in this study and those observed in pig SC. Another reason might be the presence of additional lipid classes in pig SC that are not included in our mixtures, such as cholesterol esters, cholesterol sulphate or small fractions of sphingosines or phytosphingosines. In fact, in previous studies in which the total extracted porcine lipid mixture has been examined, mostly a hexagonal lateral packing was observed (Ongpipattanakul et al., 1994). However, mixtures prepared from the total lipid extracts do not form the LPP (Bouwstra, J.A., unpublished data).

GLYCEROL IN WATER HAS LITTLE EFFECT ON THE LATERAL PACKING AND LAMELLAR ORGANIZATION IN ISOLATED HYDRATED HUMAN SC

The effect of glycerol and water on the lateral packing and conformational disordering of the SC lipid matrix was studied using FTIR. Similarly as in dry human SC, the lipids in human SC treated with 35% glycerol in water have two transitions, although these transitions take place at lower temperatures. The reduction in transition temperatures is also observed after treatment of dry human SC with water and is therefore mainly due to water.

Curves of hydrated human SC display the 1st, 2nd and 3rd order peaks of the LPP with a repeat distance of 12.4 nm. The SPP is not visible, but it is most likely obscured by the 2nd order peak of the LPP. Phase separated CHOL is also detected as a separate peak. After a 24 hour treatment with 35% glycerol, all 3 peaks corresponding to the LPP are still visible, as well as the CHOL peak, while the positions of the peaks did not change compared to the peaks in the

profile of human SC treated with water. Therefore, glycerol does not affect the lamellar organization in human SC.

ORTHORHOMBIC DOMAINS ARE FORMED EVEN IN THE PRESENCE OF HIGH CONCENTRATIONS OF GLYCEROL

The lateral packing of equimolar PCER:CHOL:FFA mixtures equilibrated with 35% glycerol or sprayed in the presence of 20% glycerol was examined using FTIR and similar results were obtained irrespective of the treatment, namely addition of glycerol into the lipid matrix appears to have no dramatic effect on the formation of orthorhombic domains or the thermotropic stability of these domains: until a temperature of approx. 28°C mainly an orthorhombic packing is present, similarly to equimolar PCER:CHOL:FFA mixtures. As the interpeak distance of the CH₂ scissoring doublet is approximately 10 cm⁻¹, the size of the domains exceeds 100 lipids, very similar to the domains in the lipid mixtures in the absence of glycerol.

These findings contradict those of Froebe et al (Froebe, 1990), who found that the presence of glycerol in lipid samples facilitated the formation of liquid crystalline domains. However, Froebe's lipid sample differed from the equimolar mixtures used in this study and e.g. contained large amounts of the triglyceride triolein (22 wt. %) next to cholesterol and a variety of FFA. Additionally, no details on CER composition was provided. In short, Froebe's lipid mixtures did not resemble the lipid composition that is now known to be present in SC. This is most likely the reason that not only their control samples, but also the samples with glycerol showed a liquid crystalline organization.

PRESENCE OF GLYCEROL TO SOME EXTENT PROMOTES THE FORMATION OF THE SPP AT THE EXPENSIVE OF THE LPP

Examination of the lamellar ordering in the lipid PCER:CHOL:FFA mixtures revealed first of all that also after prolonged equilibration the LPP and SPP are formed. These lamellar phases are also present in human SC. When glycerol is added to the equilibration buffer or directly to the lipids, the formation of the SPP is promoted, as can be inferred from the intensity increase of the 1st order diffraction peak of the SPP. The LPP is, however, still prominently present.

DOES GLYCEROL AFFECT THE SKIN BARRIER FUNCTION?

Glycerol has been shown to have no effect on the organization of the intercellular lipid matrix of isolated SC, other than the effects that are caused by water. Studies with SC lipid mixtures suggest that the formation of the lipid lamellae with a LPP may be inhibited by the presence of glycerol and that the LPP swells slightly, whereas FTIR results suggest that the domains that are formed are substantial in size and as stable as their counterparts that have been formed in the absence of glycerol. However, to maximize the possible changes in the lipid organization induced by glycerol, the chosen glycerol concentrations were very high. The experimental conditions in these studies are therefore quite extreme compared to the in vivo situation, wherein glycerol concentrations as high as 20 or 35% are very unlikely to be achieved. Therefore, it is unlikely that treatment with glycerol, even at high concentrations, could result in a decreased orthorhombic organization or a reduced barrier function.

6. CONCLUSION

Easily available PCER can be used to prepare orthorhombically packed equimolar CER:CHOL:FFA mixtures similar to the extracellular lipid matrix in human SC. These mixtures can be used as a model to determine the effect of foreign substances on the lipid organization in SC.

Using this SC lipid model, it has been shown that although glycerol inhibits the formation of the LPP in lipid mixtures, its effect on the formation of the orthorhombic organization is very limited and unlikely to affect the skin barrier in vivo by changing the SC lipid organization

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