1. Introduction

The skin protects the body from the invasion of pathogens and other harmful substances in the external environment, as well as preventing desiccation from within. This skin’s barrier function is ascribed to its thin outermost layer known as the stratum corneum (SC) [1–3]. The SC comprises of keratin filled, flattened dead cells referred to as corneocytes, embedded in a lipid matrix. The corneocytes show a reduced permeability by the presence of a densely cross-linked protein envelope thereby redirecting the permeation of substances along the intercellular lipid matrix [4–6]. Consequently, the composition and organization of the SC lipids are essential to the barrier function.

The SC lipids are arranged into intercellular lamellae aligned approximately parallel to the cell surface [7]. Two distinct lamellar phases, the long periodicity phase (LPP) and the short periodicity phase (SPP) are detected by X-ray diffraction studies [8,9]. The LPP is exclusively present in the SC and is considered to be important for the skin barrier function [10,11]. X-ray and neutron diffraction revealed that the LPP is a trilayer structure [12,13]. Perpendicular to the basal layer of the lamellae, the lipids adopt predominantly the dense orthorhombic lateral packing. This packing plays a role in the low permeability of the skin barrier [14].

The SC intercellular lipid domains are primarily composed of approximately an equimolar ratio of cholesterol (CHOL), free fatty acids (FFAs), and ceramides (CERs) [15,16]. The FFAs are predominantly saturated and display a chain length distribution between 12 and 30 carbon atoms [17,18]. CERs are a structurally heterogeneous group of sphingolipids, comprising of a sphingoid base linked to an acyl chain via an amide bond. Currently, 18 CER subclasses have been identified in human SC [19–23]. Four of the subclasses often referred to as acylCERs, constitute of an ultra-long ω-hydroxy acyl chain, which contains up to 30–34 carbon atoms, ester-linked to an unsaturated fatty acid (usually linoleic acid) and amide-linked to one of the various sphingoid bases. These acyl CERs are only present in the SC and their concentration is reduced in skin diseases such as atopic dermatitis, psoriasis, Netherton syndrome, and autosomal recessive congenital ichthyosis, but also in dry skin [24–30]. Consequently, their role in barrier function has attracted attention [10,31,32]. The relative abundance of the acylCERs is ~8–13

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the effect of CER EOS on barrier function, SC model membranes pre-
located in the middle and end segment of the chain [40]. With regards to
showed that concerning the linoleate chain, the fluidity is primarily
Raman spectroscopy, and NMR techniques [42]. Another NMR study
transform infrared spectroscopy (FTIR) were used to study the lipid
deuterated CERs, which provide more detailed insight into the lipid
NS in the models since SC models prepared with CER NS easily formed
the LPP [44–46]. Furthermore, this composition allowed us to use
deuterated CERS, which provide more detailed insight into the lipid
phase behavior. Small angle X-ray diffraction (SAXD) and Fourier
transform infrared spectroscopy (FTIR) were used to study the lipid
arrangement and phase behavior of the models. The barrier function of
the models was assessed by carrying out permeation studies and trans-
epidermal water loss (TEWL) measurements.

2. Materials and method
2.1. Materials

The synthetic CERs used in the study: i) N-(tetrascanoyl)-sphingo-
sine (CER NS C24), ii) CER NS C24 with a deuterated fatty acid chain
denoted as D-NS, iii) N-(30-Linoleoyloxy-triacontanoyl)-sphingosine
(CER EOS (C30)-L), and iv) CER EOS (C30)-O with deuterated oleate
chain denoted as D-EOS, were generously donated by Evonik (Essen,
Germany). The CERs had a purity of ≥90% (as determined by mass
spectrometry). Using mass spectrometry method for CER analysis [47],
the CERS had a purity of ≥98%. CHOL, ethyl-p-aminobenzoate (E-
PABA), acetate buffer salts, and the FFAs: palmitic acid (C16), stearic
acid (C18), arachidic acid (C20), behenic acid (C22), and lignoceric acid
(C24), were purchased from Sigma-Aldrich Chemie GmbH (Schnelldorf,
Germany). The perdeuterated FFAs (DFFAs) with chain lengths of C18
and C20 were obtained from Cambridge Isotope Laboratories (Andover,
Massachusetts). The DFFAs with chain lengths of C16 and C22 were
purchased from Larodan (Malmö, Sweden). The DFFA with a chain
length of C24 was obtained from Arc Laboratories B.V. (Apeldoorn,
The Netherlands). All organic solvents were of analytical grade. The water
was Millipore quality, obtained through a Milli-Q integral water puri-
fication system with a resistivity of 18 MΩ cm at 25 °C (Millipore,
Bedford, MA). Nucleopore polycarbonate filter disks (pore size 0.05 μm)
were purchased from Whatman (Kent, UK).

2.2. Composition of the model lipid mixtures

SC lipid models were prepared as equimolar mixtures of CER, CHOL,
and FFAs. The FFA component comprised of C16, C18, C20, C22, and
C24 at relative molar percentages of 1.8, 4.0, 7.6, 47.8, and 38.8
respectively, a composition adapted from the FFA composition in the
native SC [17]. The CER fraction of the models differed by the CER EOS
concentration (10/30/50/70/90 mol%) and was counterbalanced with
CER NS. The resulting lipid models are named EOS-10, EOS-30, EOS-50,
EOS-70, and EOS-90. Secondly, similar models were prepared but with
the FFAs replaced by their deuterated counterparts (CER EOS/CER NS/
CHOL/DFFAs) denoted with -DFA as a suffix to the model name. In the
third set of models, the fatty acid acyl chain of CER NS was replaced with
the deuterated chain (CER EOS/CER D-NS/CHOL/FFAs), resulting in the
models identified by the suffix -D-NS. Finally, since CER EOS with
deuterated linoleate chain, is not available, the oleate counterpart was
used in the preparation of the fourth set of models (CER D-EOS/CER NS/
CHOL/FFAs). Therein, the oleate moiety of CER EOS oleate was
substituted with the deuterated counterpart and the resulting models are
denoted by D- as a prefix to the model name. CER EOS linoleate and
oleate are structural analogues (Fig. 1) and have a similar influence on
lipid phase behavior in SC models both resulting in similar phase tran-
sition temperatures and leading to the formation of LPP in SC models
[37,48]. The composition of the models are shown in Table 1.

2.3. Preparation of samples for permeability studies, X-ray diffraction,
and FTIR spectroscopy

For the permeability and X-ray diffraction studies, 0.9 mg of the
appropriate lipid composition was dissolved in 200 μl hexane:ethanol
(2:1) solution, to a final concentration of 4.5 mg/ml. For FTIR spec-
troscopy, 1.5 mg of the applicable lipid composition was dissolved in
chloroform:methanol (2:1) to a concentration of 5 mg/ml. Using a
Linomat IV device (Camag, Muttenz, Switzerland) the solution was

Fig. 1. Molecular structure of the acylCERS used in the study.
The CERS consist of an unsaturated fatty acid esterified to an α-hydroxy fatty acid acyl chain (black), which is linked to a sphingoid base (grey) via an amide bond. The unsaturated fatty acid is either A) linoleic acid (CER EOS (C30)-L) or B) oleic acid (CER EOS (C30)-O).
sprayed on a suitable substrate (nucleopore polycarbonate filter disk with 0.05 μm pore size for the permeability and X-ray studies, and AgBr window for FTIR spectroscopy). It was necessary to use hexane:ethanol as solvent for spraying on the polycarbonate membrane since this membrane is not resistant to chloroform [49]. However, this did not cause any difference in phase behavior. Spraying was performed at the rate of 14 μl/s, over an area of 10 × 10 mm, under a gentle stream of nitrogen. An approximate distance of 1 mm was maintained between the nozzle and the spraying surface. The samples were equilibrated at 85 °C for 30 min, which was sufficient to ensure that the lipid mixtures had fully melted, and then gradually cooled to room temperature. Finally, the samples for X-ray studies were hydrated over 27% NaBr in milli-Q water (water vapour) for at least 15 h. While FTIR samples were hydrated in deuterated acetate buffer (pH 5.0) and incubated at 37 °C for at least 15 h to ensure that the samples were fully hydrated. The membranes used in permeability studies were thus hydrated with phosphate-buffered saline inside the flow-through diffusion cell to mimic the in vivo situation as much as possible [49].

### Table 1
Composition of the various models used in this study. CER:CHOL:FFA mixture is prepared as equimolar. For clarification, the CER EOS concentration in this study refers to the percentage of CER EOS relative to the CER fraction, not the total lipid content.

| Lipid model name | Composition and molar ratio (1:1:1) |
|------------------|-----------------------------------|
| EOS-10           | [EOS(C30)-L 10% + NS(C24) 90%] : CHOL: FFAs |
| EOS-30           | [EOS(C30)-L 30% + NS(C24) 70%] : CHOL: FFAs |
| EOS-50           | [EOS(C30)-L 50% + NS(C24) 50%] : CHOL: FFAs |
| EOS-70           | [EOS(C30)-L 70% + NS(C24) 30%] : CHOL: FFAs |
| EOS-90           | [EOS(C30)-L 90% + NS(C24) 60%] : CHOL: FFAs |
| EOS-10-DFFA      | [EOS(C30)-L 10% + NS(C24) 90%] : CHOL: DFFAs |
| EOS-30-DFFA      | [EOS(C30)-L 30% + NS(C24) 70%] : CHOL: DFFAs |
| EOS-50-DFFA      | [EOS(C30)-L 50% + NS(C24) 50%] : CHOL: DFFAs |
| EOS-70-DFFA      | [EOS(C30)-L 70% + NS(C24) 30%] : CHOL: DFFAs |
| EOS-90-DFFA      | [EOS(C30)-L 90% + NS(C24) 60%] : CHOL: DFFAs |
| EOS-10-D-NS      | [EOS(C30)-L 10% + D-NS(C24) 90%] : CHOL: FFAs |
| EOS-30-D-NS      | [EOS(C30)-L 30% + D-NS(C24) 70%] : CHOL: FFAs |
| EOS-70-D-NS      | [EOS(C30)-L 70% + D-NS(C24) 30%] : CHOL: FFAs |
| D-EOS-10         | [D-EOS(C30)-O 10% + NS(C24) 90%] : CHOL: FFAs |
| D-EOS-30         | [D-EOS(C30)-O 30% + NS(C24) 70%] : CHOL: FFAs |
| D-EOS-70         | [D-EOS(C30)-O 70% + NS(C24) 30%] : CHOL: FFAs |

### 2.4. Permeability studies

Permegear in-line diffusion cells (Bethlehem PA, USA) with a diffusion area of 0.28 cm² were used for the in-vitro permeation studies. The model membranes were mounted in the diffusion cells and hydrated for an hour with the acceptor phase consisting of phosphate-buffered saline (PBS 0.1 M solution: NaCl, Na₂HPO₄, KH₂PO₄, and KCl in milli-Q water with a concentration of 8.13, 1.14, 0.20, and 0.19 g/l respectively) at pH 7.4 prior to the experiment. Before use, the PBS was filtered and degassed. The donor compartment consisted of 1400 μl of a saturated E-PABA (0.65 mg/ml) in acetate buffer solution (pH 5) and was sealed with an adhesive tape to prevent solvent evaporation. The acceptor phase was constantly stirred and perfused at a flow rate of 2 ml/h through an in-line degasser to remove air bubbles that may form during the experiment. The temperature of the membranes was maintained at 32 °C. The acceptor fluid was collected over 15 h at 1-hour intervals. At the end of the diffusion study, the volume per collected fraction of PBS was determined by weight and the concentration of E-PABA was determined by ultra-high performance liquid chromatography (UPLC). The flux of E-PABA was calculated using Fick’s first law of diffusion [30]. Permeation of multiple samples of each composition was analyzed, n ≥ 4. The steady-state flux values were calculated using a time interval between the 5th and 15th hour.

### 2.5. UPLC analysis

UPLC was run with Acquity UPLC systems (Waters Co., Milford, MA, USA) for the analysis of E-PABA. The UPLC systems consisted of a quaternary solvent manager (a high-pressure pump), a tunable ultraviolet/visible absorbance detector, and a sample manager. The stationary phase consisted of a UPLC special analytical column packed with 1.7 μm, bridged, ethyl siloxane, hybrid particles. The column temperature was set at 40 °C. The mobile phase was composed of a mixture of 0.1% trifluoroacetic acid in acetonitrile: milli-Q water at 40:60 (v/v) ratio. The flow rate of the mobile phase was 1 ml/min. 10 μl of the sample was injected on the column. The detector wavelength was set at 286 nm. Data were collected and processed by MassLynx and TargetLynx software V4.1 SCN951 (Waters Co., Milford, USA).

The standard stock solution of E-PABA, 0.5 mg/ml was prepared in a 1:1 solution of methanol and milli-Q. Ten different concentrations were prepared by serial dilutions from the stock, with milli-Q water, to plot the standard curve. The linearity of the relationship was evaluated in a concentration range between 0.1 and 10 μg/ml covering the range of concentrations obtained when analyzing the concentration of E-PABA permeating the model membranes. The calibration curves were obtained using least square linear regression fitting and the linearity was confirmed with the R² values. R² value very close to 1 indicates excellent linearity.

The UPLC method was previously validated for E-PABA analysis as per ICH (International conference on harmonization) guidelines concerning linearity, precision, limit of detection (LOD), and limit of quantification (LOQ) [46,51,52].

### 2.6. TEWL measurements

An AquaFlux (AF200, Biox Systems Ltd., London, UK) was used to measure the water loss from the lipid models. The measurement has been previously described elsewhere [50]. Briefly, the sprayed membranes were mounted in flow-through diffusion cells, which were subsequently filled with milli-Q water. The cells were left to hydrate for at least 30 min before measuring. The TEWL device was then coupled vertically with the donor compartment of the diffusion cell using a special measurement cap (Biox Systems Limited, UK) in order to seal the compartment and ensure vapour tight connectivity. The TEWL values for the models studied were recorded for 30 min. The final 10 min of the measurement was used to calculate the TEWL value, n = 7.

### 2.7. Data analysis for the permeability and TEWL measurements

One-way ANOVA with Bonferroni’s multiple comparison test was performed to analyze the permeability and TEWL data. Differences in mean are considered statistically significant when P < 0.05.

### 2.8. FTIR measurements

FTIR spectra were acquired on a Varian 670-IR spectrometer (Agilent Technologies, Inc., Santa Clara, CA) equipped with a broad-band mercury cadmium telluride detector, cooled by liquid nitrogen. The sample was kept under a continuous dry air purge, starting 30 min before data acquisition. The spectra were generated in the transmission mode by the coaddition of 256 interferograms, at a resolution of 1 cm⁻¹, collected over 4 min. To determine the phase transition in relation to the temperature, the spectra were collected between 0 and 100 °C at a heating rate of 0.25 °C/min, resulting in a 1 °C temperature rise per recorded spectrum. Samples were measured over a range of 600–4000 cm⁻¹, and an enhancement factor of 1.6. The software used was Agilent resolution pro (Agilent Technologies, Palo Alto CA, USA). The conformational ordering and phase transition of the lipid chains were obtained by examination of the protiated methylene symmetric stretching modes observed at
~2850 cm\(^{-1}\) and the deuterated symmetric stretching modes at ~2090 cm\(^{-1}\), referred to as \(\nu_2\text{CH}_2\) and \(\nu_2\text{CD}_2\) modes respectively. The linear regression curve fitting method was used to determine the mid-transition temperature as described previously [53]. The \(\text{CH}_2\) scissoring mode (\(\delta\text{CH}_2\)), 1462–1473 cm\(^{-1}\) and \(\text{CD}_2\) scissoring mode (\(\delta\text{CD}_2\)), 1085–1095 cm\(^{-1}\) were analyzed to evaluate the lateral packing and mixing properties of the lipid chains respectively. Multiple samples of each composition were measured, \(n \geq 2\).

2.9. SAXD studies

To determine the long-range ordering, SAXD experiments were conducted at the European Synchrotron Radiation Facility (ESRF, Grenoble) at station BM26B. The X-ray wavelength (\(\lambda\)) was 0.1033 nm and the sample-to-detector distance was 2.1 m. Diffraction patterns were recorded using a Pilatus 1 M detector. The spatial calibration of the detector was performed using silver behenate. Samples were measured for 90 s. The scattering intensity (\(I\)) was measured as a function of the scattering vector (\(q\)) which is proportional to the scattering angle (2\(\theta\)) according to the equation, defined as \(q = (4\pi \sin \theta)/\lambda\). From the positions of a series of equidistant peaks (\(q_n\)), the periodicity of a lamellar phase was calculated using the equation \(d = 2h\pi/q_0\) in which \(h\) is the order of the diffraction peak. Samples were prepared and measured in triplicate. The peak intensities attributed to the LPP were determined from the diffraction peak. Samples were prepared and measured in triplicate. The peak intensities attributed to the LPP were determined from the diffraction peak. Multiple samples of each composition were measured, \(n \geq 2\).

3. Results

3.1. A high concentration of CER EOS increases the permeability of the SC model membranes

To evaluate the effect of CER EOS concentration on the permeability of the model membranes, we compared their permeability to E-PABA. The average fluxes of E-PABA across the models with a gradual increase in CER EOS concentration are displayed in Fig. 2A. The E-PABA average steady-state flux values are plotted in Fig. 2B. Increasing the CER EOS concentration from 10 to 30% of the total CER fraction in the model membrane resulted in no significant difference in permeability (7.8 ± 2.9 to 8.2 ± 4.8 \(\mu\text{g/cm}^2/\text{h}\)). On further increase in CER EOS concentration to 50, 70, and 90%, the permeability increased (14.5 ± 8.6, 22.2 ± 5.0 and 25.2 ± 3.3 \(\mu\text{g/cm}^2/\text{h}\) respectively), with EOS-70 and EOS-90 being significantly more permeable than EOS-10 and EOS-30.

Water transport was also monitored by performing TEWL measurements. The water loss across EOS-90 and EOS-70 was 3.5 ± 0.2 and 2.9 ± 0.5 \(\mu\text{g/cm}^2/\text{h}\) respectively, both significantly higher than that across EOS-30 (1.8 ± 0.2 \(\mu\text{g/cm}^2/\text{h}\)). See Fig. 2C.

3.2. The phase transition temperature and packing density of the lipid chains are higher with increasing CER EOS concentration

The packing density of the lipids is important for understanding the changes in permeability at increasing CER EOS content. In the FTIR spectrum, \(\nu_2\text{CH}_2\) and \(\delta\text{CH}_2\) frequencies provide information about lipid chain conformational ordering and packing density [55–57]. The thermotropic response of the \(\nu_2\text{CH}_2\) modes of EOS-10 and EOS-90 are plotted in Fig. 3A. The initial wavenumber of the fully protiated models appeared below 2850 cm\(^{-1}\) indicating highly ordered hydrocarbon chains [56,58]. EOS-90 \(\nu_2\text{CH}_2\) wavenumber was higher than that of EOS-10 suggesting lower conformational ordering of the lipid chains, but the difference was not significant. The increase in temperature between 20 and 40 °C resulted in an approximate 1 cm\(^{-1}\) rise in the wavenumber, representing the orthorhombic-hexagonal phase transition. Further increase in temperature resulted in a larger wavenumber shift of 3–4 cm\(^{-1}\) between 60 and 80 °C. This indicates the transition from a hexagonal to

- **Fig. 2.** Permeability of the model membranes.
- A) Average flux of E-PABA across the model membranes over 15 h. B) The average steady-state flux of E-PABA across the model membranes (5–15 h). Data presented as the mean ± SD, \(n \geq 4\). E-PABA steady-state flux was significantly higher in EOS-70 and EOS-90. C) TEWL across the model membranes, EOS-70 and EOS-90 showing significantly higher permeability compared to EOS-30, \(n \geq 3\), \(^*P < 0.05\), \(^{**}P < 0.01\), \(^{***}P < 0.001\).
the disordered liquid phase \cite{59,60}. The midpoint temperatures of the orthorhombic-hexagonal phase transition \(T_{\text{M,OR-HEX}}\) in the various fully protiated models are presented in Fig. 3 B. The \(T_{\text{M,OR-HEX}}\) increased gradually with increasing CER EOS concentration.

Concerning the lateral packing of the lipid chains, the \(\delta\)CH\(_2\) mode was split into two peaks, centered at \(\sim1462\) cm\(^{-1}\) and \(1473\) cm\(^{-1}\) signifying an orthorhombic packing \cite{59,61}. This doublet is a direct result of short-range coupling between adjacent hydrocarbon chains of the same isotope \cite{61}. While the \(\delta\)CH\(_2\) mode of the less dense hexagonal packing is characterized by a singlet positioned at \(\sim1467\) cm\(^{-1}\) in the spectrum. The \(\delta\)CH\(_2\) modes of the various models at \(10^\circ\)C and \(32^\circ\)C are presented in Fig. 4 A and B respectively. At \(10^\circ\)C, the \(\delta\)CH\(_2\) modes of all protiated models displayed two strong peaks typical of orthorhombic packing. At \(32^\circ\)C, the \(\delta\)CH\(_2\) modes in the spectrum of EOS-10, EOS-30, and EOS-50 exhibited strong central asymmetric peaks positioned at \(\sim1467\) cm\(^{-1}\) indicating an increased fraction of lipids adopting a hexagonal lateral packing at the expense of the fraction of lipids forming an orthorhombic packing (Fig. 4B). In contrast, EOS-70 and EOS-90 \(\delta\)CH\(_2\) modes retained the two characteristic peaks attributed to orthorhombic packing.

3.3. The proportion of the liquid phase increases with CER EOS concentration

In a lipid mixture, selective deuteration of the various components enables the simultaneous monitoring of the conformational ordering and phase behavior of individual species \cite{56}. This is because the vibrational energy of protiated and deuterated chains is sufficiently different when detected in the infrared spectrum. We deuterated the FFA chains in the various models. The compositions of the resulting samples are shown in Table 1. The \(\nu\)CD\(_2\) and \(\nu\)CH\(_2\) peak positions attributed to DFFA and CER chains respectively were analyzed in the temperature range between 0 and \(90^\circ\)C (Fig. 5 A). At the initial temperature, \(\nu\)CD\(_2\) peak positions were consistent with previous reports of high
conformational ordering of the methylene chains [46] indicating that the DFFA chains were highly crystalline, even at high CER EOS level. The $\nu_{\text{CH}_2}$ mode of EOS-10-DFFA and EOS-30-DFFA revealed rearrangement of the protiated chain just before the hexagonal-liquid phase transition (between 60 and 80 °C), attributed to a high concentration of CER NS, as reported previously [46]. As the continued presence of orthorhombic packing in EOS-70 and EOS-90 at 32 °C in the fully protiated mixtures (Fig. 4 B) could result from phase-separated FFA, we analyzed the $\delta_{\text{CH}_2}$ and $\delta_{\text{DH}_2}$ modes of the CER EOS/CER NS/CHOL/DFFAs mixtures. When deuterated and protiated chains are mixed in an orthorhombic lattice, the short-range coupling and peak splitting described in Section 3.4 are eliminated as adjacent $\delta_{\text{CH}_2}$ and $\delta_{\text{DH}_2}$ modes will not interact due to differences in vibrational energy. Analysis of the $\nu_{\text{CD}_2}$ modes of EOS-70-DFFA and EOS-90-DFFA at 10 °C (Fig. 4 C) showed significantly reduced peak splitting width compared with the maximum peak splitting in a fully deuterated environment that has been previously determined [46], indicating extensive mixing of the CERs and DFFA chains. At 32 °C, the CD$_2$ modes of EOS-70-DFFA and EOS-90-DFFA transformed to single peaks. This indicates that the orthorhombic packing at elevated temperatures cannot be due to phase-separated FFA. Lipid mixtures including CER NS with a deuterated C24 acyl chain were also examined. The $\nu_{\text{CD}_2}$ mode of EOS-10-D-NS, EOS-30-D-NS, and EOS-70-D-NS were located initially at ~2088 cm$^{-1}$ (Fig. 5 B) indicating that CER NS chains are highly conformationally ordered in the lipid models, even at high CER EOS concentration. As the temperature increased, the order-disorder transition of the protiated chains and the deuterated CER NS chains occurred in the same temperature range. Finally, we analyzed the spectra of the lipid mixtures in which the linoleate moiety of CER EOS was replaced by deuterated oleate. The thermotropic response of the $\nu_{\text{CH}_2}$ and $\nu_{\text{CD}_2}$ modes of D-EOS-10, D-EOS-30, and D-EOS70 are displayed in Fig. 5 C.

3.4. CER EOS concentration affects the LPP repeat distance

The influence of CER EOS concentration on the lamellar phase behavior of SC lipid models was studied. The SAXD profiles of the models with a gradual increase in CER EOS content are displayed in Fig. 7 A–E. In CER EOS-10, the lipids form both the SPP and LPP with repeat distances of 5.4 and 12.2 nm respectively. Increasing the concentration of CER EOS from 10 to 30% resulted in the disappearance of the SPP. No significant difference in LPP repeat distance was observed between EOS-10 and EOS-30. Further increase in CER EOS concentration to 50, 70, and 90%, resulted in a gradual increase in LPP repeat distance (Fig. 7 F) and the intensity ratio between the 2nd and 1st order of the LPP decreased concurrently. The diffraction curve of CER EOS-90, showed the 6th order to be absent, while the 1st order intensity surpassed the 2nd order, thus deviating from the characteristic LPP.
are not entirely comprehended. To understand the skin barrier thoroughly, knowledge of the molecular arrangement of the lamellar unit cell is vital. Previous experiments have identified that the linoleate chain of CER EOS is located in the inner head group regions and in the central region of the trilayer LPP, while the C(30) acyl chain extends from the boarders of the unit cell towards the central layer. It has been suggested that the linoleate/oleate moiety in the matrix could be a key element contributing to the SC impermeability by acting as an obstacle for the diffusion of hydrophilic compounds through the SC and traps for the apolar species. The results from the current study have shown that this hypothesis does not hold when CER EOS concentration is greater than 70%. The SC lipid matrix is endowed with a characteristic composition and organization required for the barrier function. The physiological concentration of CER EOS in the SC is associated with low permeability indicating good barrier function. Data from our study shows that the barrier capability of the SC model is maintained somewhat up to a 50% increase in CER EOS concentration. Such robustness is an advantage to withstand the numerous challenges faced by the body due to the external environment.

4. Discussion

For the first time, the influence of a gradual increase of CER EOS over wide range concentrations, namely from 10% to 90% of the CER fraction of SC models was investigated. Despite the crucial role of CER EOS in the LPP formation, its influence on lipid phase behavior and barrier function are not entirely comprehended. To understand the skin barrier thoroughly, knowledge of the molecular arrangement of the lamellar unit cell is vital. Previous experiments have identified that the linoleate chain of CER EOS is located in the inner head group regions and in the central region of the trilayer LPP, while the C(30) acyl chain extends from the boarders of the unit cell towards the central layer. It has been suggested that the linoleate/oleate moiety in the matrix could be a key element contributing to the SC impermeability by acting as an obstacle for the diffusion of hydrophilic compounds through the SC and traps for the apolar species. The results from the current study have shown that this hypothesis does not hold when CER EOS concentration is greater than 70%

4.1. Effect of CER EOS concentration on barrier function

In SC lipid model systems, CER EOS induces a co-existence of crystalline and disordered liquid domains. We examined the impact of increasing CER EOS concentration on the permeability of the SC lipid models. The results show that the permeability of the membrane to E-PABA did not differ when CER EOS concentration was raised from 10 to 30% of the total CER content. Similarly, it was reported that permeability of indomethacin and theophylline through a complex lipid model was not improved when the concentration of either individual acylCER or an acylCER mixture was raised above the physiological concentration to 30% [43]. In contrast, a previous study demonstrated that the permeability of a SC model prepared with pig CERs with CER EOS concentration of 14% reduced when an additional 20% mol CER EOS was incorporated [64]. However, in this case, the difference is not solely due to the difference in the acylCER content as isolated pig CERs were used, which contain a wide distribution in chain lengths, while the additional synthetic CER EOS had only a single-chain length. Thus a change in chain length distribution might also have affected the permeability as demonstrated previously. In their study, there was also a reduction in the permeability of a simple SC model membrane when the concentration of CER EOS was increased from 10 to 30% [31]. In their study also differed from ours, signifying a different structure. In the present study, E-PABA steady-state flux only increased when CER EOS concentration was raised to 50%, becoming significantly higher when raised to ≥70%. This finding was corroborated by the TEWL values, which were also significantly higher for EOS-70 and EOS-90.

4.2. Effect of CER EOS concentration on lateral lipid organization

Several studies have associated a higher lipid chain packing density with reduced permeability of SC model membranes [32,64,65]. This relationship was not observed in the current study. The remarkably high permeability of EOS-70 and EOS-90 was irrespective of the presence of a high concentration of lipids forming an orthorhombic phase at 32 °C, the physiological temperature at which the permeation studies were carried out. Analysis of the FTIR data showed that the increased permeability was not due to phase separation as the hexagonal-liquid phase transition of FFA and CERs occurred in the same temperature range and the scissoring vibrations demonstrated mixing of the CERS and FFA chains in the EOS-70 and EOS-90 models. Thus, the delayed orthorhombic-hexagonal phase transition observed in the 6CD2 modes of EOS-70 and EOS-90 may be attributed to the increased level of the exceptionally long acyl chain of CER EOS.

To study the behavior of the unsaturated C18 acyl chain moiety of CER EOS, we used mixtures in which the oleate moiety of CER EOS-O was deuterated (Table 1). Several studies have reported a similar phase behavior for CER EOS-L and CER EOS-O containing mixtures. de Sousa et al. [48] analyzed equimolar mixtures of CERS, CHOL, and FFAs. The CER fraction contained either 30 mol% CER EOS-L or 30 mol% CER EOS-O. The results show that the permeability of the SC model to E-PABA steady-state flux only increased when CER EOS concentration was raised to 50%, becoming significantly higher when raised to ≥70%. This finding was corroborated by the TEWL values, which were also significantly higher for EOS-70 and EOS-90.

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The results showed that the model prepared with CER EOS-L and that prepared with the oleate analogue have a similar lipid phase behavior, both resulting in similar phase transition temperatures and leading to exclusive formation of the LPP with similar repeat distance. Bouwstra et al. [37] reported similar phase behavior in model mixtures with human CERs containing either synthetic CER EOS-L or synthetic CER EOS-O. In both mixtures, the LPP, as well as the SPP with very similar repeat distances, were formed. In addition using CER EOS-L, with the linoleate chain deuterated, Janssens et al. [41] determined that the linoleate moiety of CER EOS-L was already disordered at 20 °C indicated by the high δCD2 wavenumber of 2099 cm⁻¹, which were very similar values as that of the deuterated oleate chain of CER EOS in this present study. Based on these studies we conclude that CER EOS linoleate and CER EOS oleate are very similar as far as the conformational ordering and the phase behavior is concerned, which are the properties we focused on in the present study.

In the spectrum of CER D-EOS-O/CER NS/CHOL/FFAs mixtures, the deuterated oleate chains were in the liquid phase in the entire temperature range studied (0–100 °C). Despite increasing CER EOS concentration, there was no increasing shift in the νsCD2 frequencies of the indicated by the high δCD2 wavenumber of 2099 cm⁻¹, which were very similar values as that of the deuterated oleate chain of CER EOS in this present study. Based on these studies we conclude that CER EOS linoleate and CER EOS oleate are very similar as far as the conformational ordering and the phase behavior is concerned, which are the properties we focused on in the present study.

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deuterated oleate chains to indicate any further degree of disordering. In mixtures with the deuterated oleate the CD2: CH2 peak area ratio is an indication of the proportion of the liquid phase; an increase in this ratio with CER EOS concentration is observed.

Based on the carbon number in the model mixtures, the total fraction of CER moiety that is liquid in EOS-10 is 2.7% of the total CER volume and 1% of the total lipid volume given the equimolar ratio of CERS, FFAs, and CHOL. Likewise, the liquid fraction of the total lipids in EOS-30 and EOS-70 are 2.7% and 6.4% respectively. At very high CER EOS concentration (>70%), the disordered linoleate located in the inner head group regions and the central layer of the LPP probably can no longer act as isolated fluid droplets that trap materials. Rather, there is a sufficient fraction of the disordered phase to increase the transport of substances along the inner headgroup region through the SC model membrane. Thus, the increased permeability of the SC models when CER EOS concentration is increased may be attributed to the increased proportion of the liquid phase. Our data demonstrate that the contribution of the increased fraction of lipids forming a disordered phase impacted more the barrier than the higher fraction of lipids forming the orthorhombic phase.

5. Conclusions

In the present paper, we have advanced the previously reported studies of disordered lipid domains in the crystalline SC model, attributed to the unsaturated fatty acid moiety of CER EOS. Increasing CER EOS concentration induced a higher fraction of lipids forming orthorhombic and liquid phases at the expense of the hexagonal phase. Only when CER EOS concentration was raised to >70%, did the permeability of the SC model increase significantly. This could be attributed to the increased fraction of lipids forming a liquid phase. Such sturdiness contributes to the skin’s primary function which is to protect the body from the invasion of pathogens and other harmful substances in the external environment as well as preventing uncontrolled loss of fluid from the body.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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