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ORIGINAL PAPER

Arrangement of ceramide [EOS] in a stratum corneum lipid model matrix: new aspects revealed by neutron diffraction studies

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Abstract The lipid matrix in stratum corneum (SC) plays a key role in the barrier function of the mammalian skin. The major lipids are ceramides (CER), cholesterol (CHOL) and free fatty acids (FFA). Especially the unique-structured ω-acylceramide CER[EOS] is regarded to be essential for skin barrier properties by inducing the formation of a long-periodicity phase of 130 Å (LPP). In the present study, the arrangement of CER[EOS], either mixed with CER[AP] and CHOL or with CER[AP], CHOL and palmitic acid (PA), inside a SC lipid model membrane has been studied for the first time by neutron diffraction. For a mixed CER[EOS]/CER[AP]/CHOL membrane in a partly dehydrated state, the internal membrane nanostructure, i.e. the neutron scattering length density profile in the direction normal to the surface, was obtained by Fourier synthesis from the experimental diffraction patterns. The membrane repeat distance is equal to that of the formerly used SC lipid model system composed of CER[AP]/CHOL/PA/ChS. By comparing both the neutron scattering length density profiles, a possible arrangement of synthetic long-chain CER[EOS] molecules inside a SC lipid model matrix is suggested. The analysis of the internal membrane nanostructure implies that one CER[EOS] molecule penetrates from one membrane layer into an adjacent layer. A 130 Å periodicity phase could not be observed under experimental conditions, either in CER/CHOL mixtures or in CER/CHOL/FFA mixture. CER[EOS] can be arranged inside a phase with a repeat unit of 45.2 Å which is predominately formed by short-chain CER[AP] with distinct polarity.

Keywords Stratum corneum lipids · Ceramide · Neutron diffraction · Internal membrane structure

Abbreviations

CER[AP]	*N*-(α-hydroxyoctadecanoyl)-phytosphingosine
CER[EOS]	30-Linoyloxy-triacontanoic acid-[(2S,3R)-1, 3-dihydroxyocta-dec-4-en-zyl]-amide
PA	Palmitic acid
CHOL	Cholesterol
ChS	Cholesterol sulphate

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Introduction

The outermost layer of the mammalian skin, the stratum corneum (SC), forms the main barrier for drug penetration

across the skin. The SC consists of corneocytes embedded in a multilamellar lipid matrix containing mainly ceramides, cholesterol and free fatty acids (Elias 1981; Downing et al. 1987; Grubauer et al. 1989). Ceramides (CER), which represent the main component of the SC lipids, play a key role in structuring and maintaining the epidermal barrier function. At least nine different classes of CER have been identified in human SC (Wertz et al. 1985; Robson et al. 1994; Stewart and Downing 1999; Ponec et al. 2003). They can be subdivided into three groups based on the nature of their base [sphingosine (S), phytosphingosine (P) and 6-hydroxysphingosine (H)]. Through amide bonding, long-chain nonhydroxy (N) or α-hydroxy (A) fatty acids with varying acyl chain lengths are chemically linked to the sphingosine bases. The acylceramides CER [EOS], CER [EOP] and CER[EOH] have a unique structure as they contain linoleic acid linked to a ω-hydroxy fatty acid (EO) with a chain length of 30–32 carbon atoms (Raith et al. 2004). At the moment, the function of each ceramide class is not known, however, the acylceramides CER[EOS], CER[EOP] and CER[EOH] were reported to be of considerable importance for skin barrier function due to their extremely long fatty acid chain (Bouwstra et al. 1998; Jager et al. 2004a).

Several studies have been performed to obtain insights into the complex lipid organisation, therefore, confirming the skin barrier properties. Conventional electron microscopy studies, in which the lipid samples are dehydrated, chemically fixed by ruthenium tetroxide and stained, have demonstrated that the intercellular lipids in the SC have a lamellar organisation with a repeating pattern of approximately 130 Å, consisting of a broad–narrow–broad sequence of electron lucent bands (Madison et al. 1987; Swartzendruber et al. 1989), namely trilamellar organisation or long periodicity phase (LPP). Ohta et al. (2003) suggested the coexistence of 50 and 130 Å phases on the basis of X-ray diffraction studies on hairless mouse SC. In contrast, other studies relying on cryo-electron microscopy of vitreous human skin reported that the trilamellar conformation could not be observed (Al-Amoudi et al. 2005). This discrepancy could be caused by morphological changes due to ruthenium tetroxide-fixation or dehydration in conventional sample preparation for electron microscopy. In line with this, several studies have reported that chemical fixation by ruthenium tetroxide, applied by Madison et al. (1987) in order to visualise lipid structures in transmission electron microscopy, led to severe changes in the skin's ultra structure (Pfeiffer et al. 2000).

Small-angle X-ray diffraction (SAXD) studies on isolated SC lipids have shown reflections of two lamellar phases with periodicities of 64 and 130 Å, which led to the suggestion of the "sandwich model" of the SC lipid matrix (Bouwstra et al. 2000). In this model, the SC membrane consists of 130 Å trilamellar repeat units with internal layers of 50, 30 and 50 Å.

According to McIntosh et al. (1996), X-ray studies on a hydrated mixture of pig CER:cholesterol:palmitic acid (molar ratio 2:1:1) revealed a single repeat unit of 130 Å, whose existence appears to be linked with the presence of the acylceramide CER[EOS]. In a continuation of this study (McIntosh 2003), the calculation of the electron density profile was performed after inducing swelling of the 130 Å repeating unit. The major finding was a repeating unit containing two asymmetric bilayers in which CHOL is asymmetrically distributed.

Summarising, the existence of the 130 Å lamellar repeat pattern in SC in vivo is currently a matter of debate, comprising many pros and cons about the organisation of the LPP and the inducing or preventing conditions for the formation. Apart from some conventional electron micrographs (Swartzendruber et al. 1989), the 130 Å repeat unit has only been observed in some SAXD studies (White et al. 1988; Bouwstra et al. 1991), while it has not been confirmed in other SAXD studies (Garson et al. 1991) or in cryo-transmission electron microscopy studies on native hydrated epidermis samples (Al-Amoudi et al. 2005) or in neutron diffraction studies on hydrated SC (Charalambopoulous et al. 2004). From those various experimental results it can be concluded that the detection of the 130 Å repeating pattern cannot be regarded as an evidence for the biological relevance and correct preparation of the model systems applied.

It is difficult to obtain information about the internal structure of the SC using either native SC or isolated SC lipid mixtures due to the variability of the native tissue and the extensive isolation and separation of the SC lipids as reviewed in Kessner et al. (2008a). The use of synthetic ceramides with well-defined acyl chain length and head group architecture can overcome these problems (Jager et al. 2004b). Recent studies have mainly concentrated on the proof of the LPP phase in synthetic ceramides based SC lipid membranes by applying small-angle X-ray diffraction (Jager et al. 2004a, b).

The calculation of the scattering length density profile based on X-ray or neutron diffraction experiments is a suitable tool to determine the internal structure of the SC lipid matrix. The crucial limitation for the application of X-ray diffraction is to find the sign of the structure factor (McIntosh 2003). By applying neutron diffraction, the sign of the structure factor can be decided by substituting D_2O with H_2O (Worcester 1976). It is also common to achieve a reasonable structural resolution in a neutron diffraction experiment using an oriented multilamellar stack of lipids on a quartz slide (Worcester 1976; King and White 1986; Wiener and White 1991; Kiselev et al. 2005).

The development of a SC lipid model system composed of defined synthetic lipids, which allows for the determination of the influence of a single lipid species on the bilayer structure. It was found that mixtures prepared with cholesterol, free fatty acids and ceramides mimic the SC lipid organisation (Bouwstra et al. 1996, 2001; McIntosh et al. 1996). The presence of CER[EOS] appears to be a prerequisite for the formation of the LPP phase of 130 Å (Bouwstra et al. 2002). Not only the presence of CER [EOS] but also the proper composition of the other CER is regarded as being important for the LPP formation (Jager et al. 2003). Recent works present an SC lipid composition that mimics SC lipid behaviour to a high extent and can substitute for SC lipid matrix in drug diffusion experiments (Jager et al. 2005, 2006). The related results add information on the influence of the entire SC lipid system on diffusion processes and, therefore, this can be important in questions related to the design of drug delivery systems. Additionally, the same emphasis has to be placed on the creation of a simplified model matrix composed of defined ceramide types and/or FFA types. It is known that ceramides are important in structuring and maintaining SC barrier function, but little is known about the role of each ceramide class concerning this matter. The development of a simplified model of an oriented SC lipid matrix containing ceramides in a mixture with other prominent SC components for investigation by neutron diffraction offers the possibility to identify the internal nanostructure of the membrane. This opportunity allows one to study the influence of each single lipid species on the structure of the model matrix and to determine their major properties in the constitution of the lipid matrix.

Mostly, neutron diffraction was used for the characterisation of phospholipids bilayer in a partly dehydrated state (Worcester 1976; Wiener and White 1991; Nagle and Tristram-Nagle 2000). It has been demonstrated that neutron diffraction studies allow new insights into the nanostructure of the lipid bilayer (Kiselev et al. 2005; Kessner et al. 2008b; Ruettinger et al. 2008a). The internal structure and hydration of a SC lipid model membrane composed of CER[AP]/CHOL/PA/ChS were characterised by neutron diffraction (Kiselev et al. 2005). Three main features could be revealed: (1) neutron diffraction was shown to be an appropriate tool for the investigation of the internal nanostructure of SC lipid model membranes. (2) A low hydration of approx. 1 Å of the inter-membrane space at 60% r.H. was observed. This finding was related to the certain amount of applied CER[AP] assumed to exist at full extended conformation, which existence was already described (Raudenkolb et al. 2005). CER[AP] at full extended conformation appears to create an extremely strong inter-membrane attraction, which tighten neighbouring bilayers to a dense contact and decrease the water diffusion in the lateral direction (Kiselev et al. 2005). (3) From the calculated neutron scattering length density profile, the localisation of CHOL inside the model matrix was proposed. In order to use the benefit of enhancing the local contrast, specific deuterated CHOL-derivates were applied and the previously assumed localisation of CHOL inside the SC lipid model membrane was proofed and specified (Kessner et al. 2008b).

The present work is a continuation of the work of Kiselev et al. (2005) on a SC lipid model matrix composed of CER[AP]/CHOL/palmitic acid (PA)/cholesterol sulphate (ChS). The chosen lipid mixtures are not suited to monitor the in vivo SC situation as the SC lipid matrix contains complex lipid classes as ceramides (40% of total weight), FFA and cholesterol. Inside the CER fraction, nine subclasses have been identified so far. Among them, the ω-acylceramide CER[EOS] (8.3% of total CER mass) and CER[AP] are minor structurally components (Ponec at al. 2003).

Inside the FFA fraction, the 22- and 24 carbon entities are the most abundant ones. Palmitic acid is only present in a very small quantity (Wertz and van den Bergh 1998).

In the present study, the internal membrane nanostructure of the CER[AP]/CHOL/PA/ChS membrane with a weight ratio of 55/25/15/5%, taken from the neutron diffraction patterns, is used as a reference system. Therefore, the relation to the original lipid composition was kept.

The main objective of the present study is to answer the question, whether CER[EOS] is the major and essential component for the formation of a LPP by preparing simplified ternary and quaternary SC lipid model multilamellar membranes based on CER[EOS] whose internal nanostructures are then characterised by neutron diffraction.

Materials and methods

Materials

The CER[EOS] was generously provided by Evonik Goldschmidt GmbH (Essen, Germany). In order to increase chemical purity above 96%, the substance was treated using a MPLC technique on a silica gel column with a chloroform/methanol gradient. The CER[AP] (Fig. 1) with a purity above 96% was also a gift from Evonik Goldschmidt GmbH and used as received. The identity of both ceramides indicated by the parameter of molecular mass (CER[EOS] = 1,012 g mol^{-1}; CER[AP] = 600 g mol^{-1}) was proved by mass spectrometry. Cholesterol, cholesterol sulphate and palmitic acid were purchased from Sigma–Aldrich (Taufkirchen, Germany). Quartz slides (Spectrosil 2000) were received from Saint–Gobain (Germany).

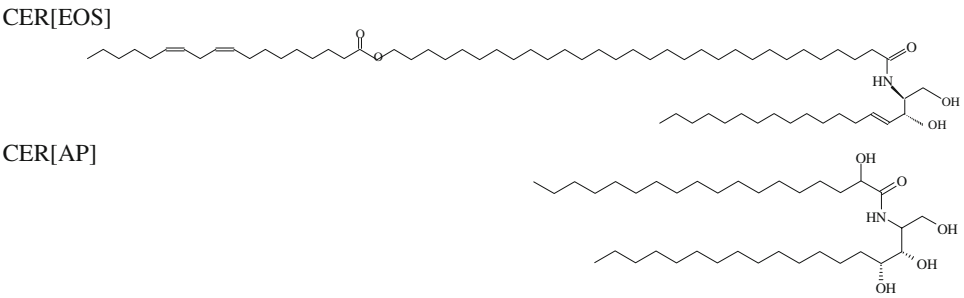

Fig. 1 Chemical structures of 30-linoyloxytriacontanoic acid-[(*2S, 3R*)-1,3-dihydroxyoctadec-4-en-zyl]-amide (*CER[EOS]*) and *N*-(α-hydroxyoctadecanoyl)-phytosphingosine (*CER[AP]*)

Sample preparation

Four compositions of SC lipid model systems were studied (see Table 1). The appropriate mixture of lipids was dissolved in chloroform/methanol (1/1 w/w) at a concentration of 10 mg ml^{-1}. A volume of 1,200 µl of the solution was spread over a 6.4 × 2.5 cm quartz surface and was first dried at room temperature and afterwards under vacuum. After the removal of the organic solvent, a subsequent heating (above 60°C) and cooling (room temperature) cycle was applied, whereby the sample was kept in horizontal position and at 100% relative humidity to decrease the mosaicity of the sample. This annealing procedure was necessary in order to obtain a reasonable orientation of the membranes. The averaged thickness of the lipid film on the quartz slide was 7.5 µm. The pH value was measured by absorption spectroscopy and equaled to 5.5, which is near to the physiological value of the skin. Such an oriented multilamellar stack of lipids on a quartz slide [preparation according to the procedure of Seul and Sammon (1990)] is commonly used in neutron diffraction experiments (Worcester 1976; Wiener and White 1991; Katsaras et al. 1992; Kiselev et al. 2005; Kessner et al. 2008b; Ruettinger et al. 2008a).

Neutron diffraction experiment

Neutron diffraction patterns from the sample were collected by the V1 diffractometer of the Hahn–Meitner Institute, Berlin, located at a cold neutron source ($\lambda = 4.517$ Å) with a sample-to-detector distance of 101.8 cm. The neutron diffraction in the reflection set-up was used to collect the data of the one-dimension diffraction experiment. The single peak position could be determined with a precision of 0.1° (in 2θ). The experimental design in detail is described in the work of Ruettinger et al. (2008a).

Diffraction patterns were recorded as $\theta-2\theta$ scans from 0° to 30°. The two-dimensional position sensitive ^{3}He detector (20 × 20 cm area, 1.5 × 1.5 mm spatial resolution) was used.

In order to vary the difference in the scattering length density between the lipid membrane and water (neutron contrast), the atmosphere of the sample chamber was adjusted up to two types of contrast [H_2O/D_2O 50:50 and H_2O/D_2O 0:100 (w/w)]. The application of high D_2O content allows for detection of higher diffraction orders which is extremely important in Fourier analysis (for calculation of the scattering length density profile). An equilibration time of 12 h was allowed after each change of aqueous solution.

Table 1 Composition of SC lipid model membranes used in this study in % (w/w)

Mixture	Composition	Component ratio % (w/w)
I	CER[AP]/CHOL	50/50
II	CER[EOS]/CHOL/PA	55/25/20
III	CER[EOS]/CER[AP]/CHOL	33/22/45
IV	CER[EOS]/CER[AP]CHOL/PA	33/22/25/20

The samples were equilibrated for 12 h in a thermostat in portable and lockable aluminium cans at fixed humidity of 60%, stored at a saturated salt solution of sodium bromide and a temperature of 32°C prior to the measurements as depicted in detail in Hauss et al. (2002). After introducing the chamber into the neutron beam, the measurements were running for approximately 12 h prior to the experimental set-up.

In order to verify the collected data, each measurement was repeated under the same experimental conditions. For the case of the CER[EOS]/CER[AP]/CHOL model membrane, two samples received from independent preparation cycles were applied and delivered identical neutron diffraction patterns.

In the neutron diffraction patterns, the scattering intensity I (in arbitrary units *a.u.*) was measured as a function of the scattering vector Q (in reciprocal Å). The latter is defined as $Q = (4\pi\sin\theta)/\lambda$, in which θ is the incident angle and λ is the wavelength. From the positions of the series of equidistant peaks (Q_n), the lamellar repeat unit d, or d-spacing, of a lamellar phase was calculated using the equation $Q_n = 2n\pi/d$, n being the order number of the diffraction peak. When the neutron diffraction pattern from an oriented lipid bilayer system shows at least three

diffraction orders, the neutron scattering length density profile across the bilayer $\rho_s(x)$ can be reconstructed by Fourier synthesis as shown in Eq. 1:

$$\rho_s(x) = \frac{2}{d}\sum_{h=1}^{h_{max}} F_h \cos\left(\frac{2\pi h x}{d}\right) \quad (1)$$

where F_h is the structure factor of the diffraction peak of the order h; d is the lamellar repeat distance calculated from the peak position. The absolute value of the structure factors $|F_h| = \sqrt{h \cdot I_h}$ is given by the integrated intensity of the hth diffraction peak I_h. The sign of the structure factor (phase angle) having only the values +1 or −1 for centrosymmetric bilayers, can be determined by H_2O/D_2O exchange (Worcester 1976; Frank and Lieb 1979). The procedure for the evaluation of the neutron diffraction data is described elsewhere (Worcester 1976; Wiener and White 1991; Kiselev et al. 2005; Kessner et al. 2008b; Ruettinger et al. 2008a).

Results

Lamellar ordering of the SC lipid model systems

No multilamellar arrangement was observed for the mixtures CER[AP]/CHOL and CER[EOS]/CHOL/PA. On the contrary, multilamellar arrangement was observed for the mixture CER[EOS]/CER[AP]/CHOL. The neutron diffraction pattern for the membrane CER[EOS]/CER[AP]/CHOL at a composition ratio of 33/22/45% (w/w) is depicted in Fig. 2 for the case of 60% humidity, H_2O/D_2O 50:50 and 32°C. Each diffraction peak has been fitted by a Gaussian function after background subtraction. The centre of the Gaussian function was used to characterise the peak position. The lamellar repeat distance d then was calculated by a linear fit procedure of the order number n of the diffraction peaks versus the value of $2\sin\theta/\lambda$ (θ incident angle; λ wavelength), according to the Bragg equation, whereby the errors given correspond to the errors derived from the fit procedure (Table 2). The membrane repeat distance d equals 45.2 Å and averaged over two contrast values. Additionally, phase-separated cholesterol crystals were present in the model membrane which can be deduced from the [010] reflection located at $Q = 0.18$ Å^{-1} and [020] reflection at $Q = 0.37$ Å^{-1}, representing diffraction from triclinic crystal with lattice parameters a = 14.172 Å, b = 34.209 Å, c = 10.481 Å and α = 96.64°, β = 90.67°, γ = 96.32°.

For the membrane CER[EOS]/CER[AP]/CHOL/PA at the composition ratio of 33/22/25/20% (w/w), the periodicity d slightly shifted to 42.2 Å (data not shown). Crystalline CHOL was again detected, this is similar to the CER[EOS]/CER[AP]/CHOL membrane.

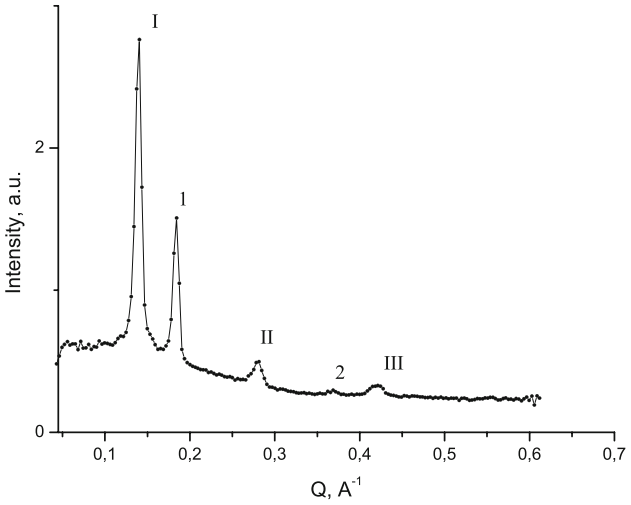

Fig. 2 Neutron diffraction pattern measured as $\theta-2\theta$ scan from 0 to 30° for the CER[EOS]/CER[AP]/CHOL membrane with the composition of 33/22/45% (w/w) at 60% humidity, H_2O/D_2O 50:50 and 32°C. *Roman numbers* indicate the first to third order diffraction peaks for the model membrane and *arabic numbers* indicate the [010] and [020] diffraction peaks from pure CHOL crystals

Table 2 Parameters of the internal membrane structure

	CER[EOS]/ CER[AP]/CHOL (33/22/45% w/w)	CER[AP]/ CHOL/PA/CHS (55/25/15/5% w/w)
Repeat distance d (Å)	45.2 ± 0.4	45.63 ± 0.04
Membrane thickness d_m (Å)	45.2 ± 0.4	45.63 ± 0.04

Calculation of the neutron scattering length density profile of the CER[EOS]/CER[AP] CHOL model matrix

The absolute values of the structure factors $|F_h| = \sqrt{h \cdot I_h}$ (h is the diffraction order) were calculated from the integrated peak intensity I_h. The influence of the absorption on the structure factor values is negligible for the thickness of a lipid film of 7.5 μm, as described by Kiselev et al. (2005). In a statistical mixed composition of lipids forming a membrane with zero curvature, there is no reason for the molecules to arrange themselves asymmetrically in both leaflets of the membrane. Therefore, such a membrane has to be regarded as symmetrical and the signs of the structure factor can be determined by H_2O/D_2O exchange in the case of centrosymmetric bilayers, which only have the values +1 or −1. For the CER[EOS]/CER[AP]/CHOL membrane with 33/22/45% (w/w) at H_2O/D_2O 50:50, the signs of the structure factors were determined by 6 possible combinations as −, +, − for the diffraction orders h = 1, 2, 3, respectively. The neutron scattering length density (in

arbitrary units) across the bilayer ρ_s (x) was restored by Fourier synthesis as:

$$\rho_s(x) = a + b\frac{2}{d}\sum_{h=1}^{h_{max}} F_h \cos\left(\frac{2\pi hx}{d}\right) \quad (2)$$

where a and b are coefficients for the relative normalisation of $\rho_s(x)$ (Nagle and Tristram-Nagle 2000). Usually, neutron scattering length density profiles are presented independently in arbitrary units. In the present study, the identity of the membrane repeat units for the CER[EOS]/CER[AP]/CHOL membrane as well as for the CER[AP]/CHOL/PA/ChS membrane allows one to compare both profiles in a relative scale. The calculated neutron scattering length density profile for the CER[EOS]/CER[AP]/CHOL model matrix studied at 60% humidity and at H_2O/D_2O 50:50 and the neutron scattering length density profile of the reference system CER[AP]/CHOL/PA/ChS [ratio of 55/25/15/5% (w/w)] determined under the same conditions, are shown in arbitrary units (Fig. 3) and therefore, need to be arranged in a relative scale to each other. For applying this procedure, the values of the scattering length densities of the methyl (-0.087×10^{11} cm^{-2}) and methylene groups (-0.030×10^{11} cm^{-2}) are important. In the case of the CER[AP]/CHOL/PA/ChS multilamellar membrane, the centre of the membrane $x = 0$ is only formed by CH_3 groups, whose scattering length density equals -0.087×10^{11} cm^{-2} (Kiselev et al. 2005). For simple comparison, the membrane centre $x = 0$ was arranged at $\rho(x) = -1$ in arbitrary units. As seen from Fig. 3, differences occur at the centre of the membrane for the ternary system CER[EOS]/CER[AP]/CHOL. For this reason, the CH_2 region was used for relative normalisation because this region is never formed by any other molecular groups than methylene groups, consequently this can be regarded as a constant. Hence, the CH_2 region was arranged at -0.3 in our arbitrary scale, which corresponds to the scattering length of the methylene groups (-0.030×10^{11} cm^{-2}).

The main results taken from the neutron scattering length density profiles are:

(1) Similar to the reference system described in Kiselev et al. (2005), the calculated Fourier profile for the CER[EOS]/CER[AP]/CHOL membrane demonstrates a zero value for the intermembrane space. Thus, the membrane thickness is equal to the membrane repeat distance as described in Kiselev et al. (2005). The calculated nanostructure values for both membranes are presented in Table 2. As summarised in Table 2, the membrane thicknesses of the reference system and CER[EOS]/CER[AP]/CHOL membrane are equal within the experimental error. The two maxima of the neutron scattering length density profile correspond to the position of the polar head groups of the lipid bilayer. The polar head group region of CER[EOS]/CER[AP]/CHOL multilamellar membrane is at the same position as the polar head group region of the reference system.

(2) The intensities at the polar head group regions differ. For the CER[EOS]/CER[AP]/CHOL multilamellar membrane, the intensity at this region reveals a smaller value than in the reference system.

(3) The minimum of $\rho_s(x)$ in the centre of the bilayer corresponds to the position of CH_3 for the reference membrane. As depicted in Fig. 3, the intensity in the centre of the bilayer of the CER[EOS]/CER[AP]/

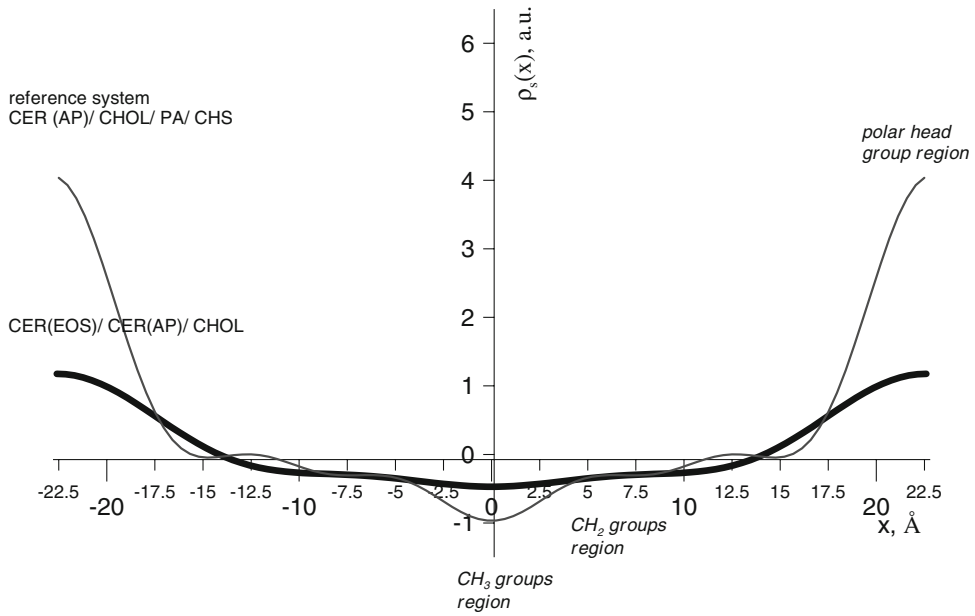

Fig. 3 Neutron scattering length density profile $\rho_s(x)$ across the CER[EOS]/CER[AP]/CHOL membrane [33/22/45 % (w/w)] at 60% humidity for the case of H_2O/D_2O 50:50 and for the CER[AP]/CHOL/PA/ChS (55/25/15/5 % w/w) membrane measured under same conditions

CHOL multilamellar membrane is not equal to that of the reference system. It is diminished when compared to the reference system. This experimental fact is very important because it shows that the composition of the CER[EOS]/CER[AP]/CHOL membrane in the centre of bilayer is not only formed by CH_3 groups.

Discussion

The aim of the present work is to find a lipid mixture prepared with commercially available CER [EOS], which can be used to investigate the internal nanostructure of a model SC lipid matrix by neutron diffraction. Thereby, the focus is put on studying the properties as well as the influence of CER[EOS] on the self-assembly of the lamellar membrane structure. It is important to investigate the role of CER[EOS] as being regarded as a prerequisite for the formation of the long-periodicity phase (LPP) due to its unique molecular structure (Bouwstra et al. 1998; McIntosh 2003). Thus, the extremly lipophilic character of the long hydrocarbon chains as well as the low polar properties of the sphingosine-based headgroup has to be taken into account.

The present study is one of the first attempts to use neutron diffraction in SC lipid research. Therefore, the chosen experimental conditions followed the one for well-studied phospholipids membranes (Worcester 1976; Frank and Lieb 1979; Gordeliy and Kiselev 1995; Nagle and Tristram-Nagle 2000). In the work of Kiselev et al. (2005), the calculation of the neutron scattering length density profile for a SC lipid model membrane was employed for the first time. Thereby, an extensive comparison to the knowledge about the internal membrane structure of phospholipid-based membranes was given. The low hydration of the intermembrane space of the SC lipid model matrix is a major distinction from a phospholipid membrane. Further, a constant membrane thickness under hydration of the SC lipid model membrane from 60 or 30–40% humidity (room humidity) to the fully hydrated system was assumed. For a suitable correlation of that study to the present one, the same experimental conditions were applied. In future studies, the behaviour of SC lipid model membranes under water excess may be investigated in order to determine the influence of water excess on the thickness of the water layer. For the present task, it was required to find a suitable composition of the lipids, which exhibits multilamellar ordering with low mosaicity as a prerequisite for Fourier synthesis. As shown, no lamellar arrangement was observed for the mixture CER[AP]/CHOL. This finding is in accordance with Jager et al. (2003), describing that a varying distribution of acyl chain length (from either the FFA or the CER moiety) promotes a better mixing of ceramides, FFA and the solubilisation of CHOL.

No multilamellar ordering was obtained for the membrane CER[EOS]/CHOL/PA. This finding underlines that not only the presence of CER[EOS] but also an appropriate composition of the other ceramides is important for the lipid organisation (Jager et al. 2003).

In contrast, the mixture of CER[EOS]/CER[AP]/CHOL at a weight ratio of 33/22/45% showed one phase with a periodicity of 45.2 Å. The diffraction patterns reveal phase-separated cholesterol. There is much published data (Huang et al. 1999; Brzustowicz et al. 2002; Jager et al. 2005; Ali et al. 2006), which reveal that the presence of crystalline cholesterol does not affect proper multilamellar arrangement of the lipid system. For a better identification of the reflections due to cholesterol crystals we introduced calculations done for the determination of the lattice parameters of a triclinic crystal. The diffraction pattern enables the restoration of the neutron scattering length density profile by Fourier synthesis. For further characterisation, the profile was then compared to a formerly used model of an SC lipid matrix as a reference system (Kiselev et al. 2005). The identity of the membrane repeat unit of the reference system to that composed of CER[EOS]/CER[AP]/CHOL allows one to compare both neutron scattering length density profiles in arbitrary scale. To visualise results derived from the neutron scattering length density profiles, a sketch of a reasonable model of the CER[EOS]/CER[AP]/CHOL membrane based on the structure of lipid components is presented in Fig. 4. This model was checked by a comparison of the Fourier profile of the reference system to that of the CER[EOS]/CER[AP]/CHOL membrane. For the sake of interpretation, both neutron scattering length density profiles are presented in a relative scale to each other. The values of the scattering length densities of the methyl (-0.087×10^{11} cm^{-2}) and methylene groups (-0.030×10^{11} cm^{-2}) are important in this purpose.

(1) The smaller intensity at the position of polar head groups of the CER[EOS]/CER[AP]/CHOL membrane is caused by additional CH_2 groups of the overlapping chain of CER[EOS]. The negative scattering length density of CH_2 groups lead to a reduction in the maximum related to the polar head group region of the reference system.

(2) The minimum in the centre of the bilayer representing the CH_3 groups is less pronounced. This finding evidences that the same amount of CH_3 groups ($\rho_s = -0.087 \times 10^{11}$ cm^{-2}) are replaced by CH_2 with $\rho_s = -0.030 \times 10^{11}$ cm^{-2}. Therefore, methylene groups from the overlapping CER[EOS] chain

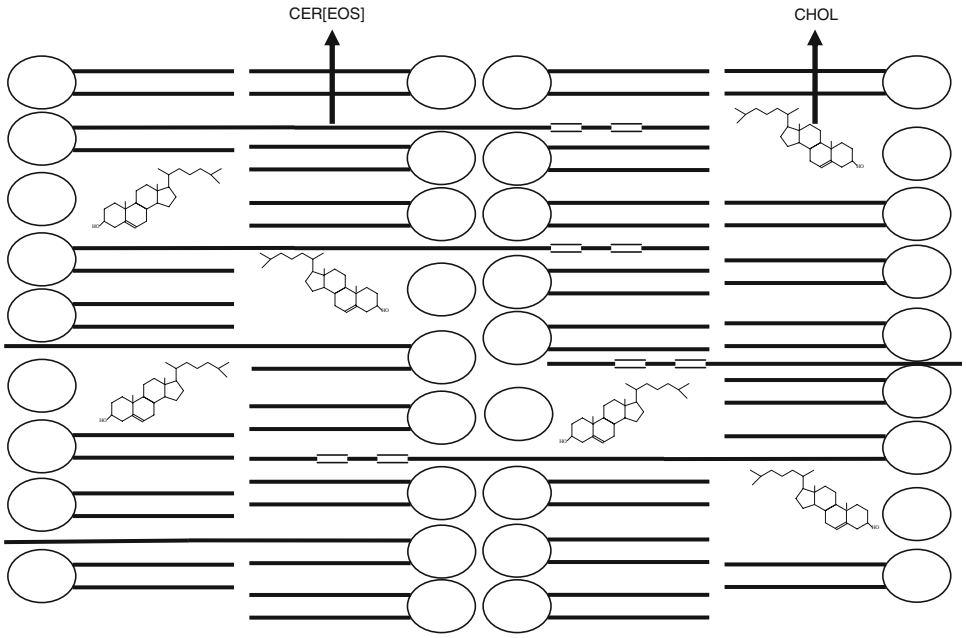

Fig. 4 Schematic representation of the CER[EOS]/CER[AP]/CHOL model membrane

substitute a certain amount of CH_3 groups. The decreased number of CH_3 groups at this position causes the lower minimum in the centre.

The results obtained from the neutron scattering length density profile of the CER[EOS]/CER[AP]/CHOL membrane are consistent with the model shown in Fig. 4. CER[EOS] can be placed inside a phase with a repeat distance of 45.2 Å. One CER[EOS] molecule can span a layer and extends into adjacent layer, which is similar to the short-periodicity phase (SPP). Our calculated Fourier profile is the first experimental proof of such a location.

This assumption may be underlined by the amphiphilic character of ceramides with the lipophilic alkyl chains anchor the molecules in the lipophilic center of the membrane and the hydrophilic head groups containing free hydroxyl groups and amide group which enables ceramides to act both as hydrogen bond donors and acceptors. If these groups participate in lateral hydrogen bonds within the lipid matrix, the stability and impermeability of the membrane will considerably increase (Pascher 1976).

In the present work, the low intermembrane hydration has additionally to be minded, which excluded intermembrane water as a partner for the formation of hydrogen bonds. In fact, latter are formed between OH-groups of adjacent membrane layers. This behaviour induces an internal neutralisation of these polar forces and lead to a decrease of the polarity of the hydrophilic region in the bilayer. Thus, it is possible to propose such an arrangement of long-chain ceramides in a short-periodicty phase by extending the alkyl chain into neighbouring layer in spite of passing the hydrophilic membrane region.

Many studies focused on the characterisation of native SC or on SC lipid matrix substitutes that give diffraction patterns with a periodicity of 130 Å. The ω-acylceramide CER[EOS] is regarded to be a necessity for the formation of the LPP. There are reports which describe that an appropriate mixture of CER[EOS] with a suitable composition of other CERs, CHOL and FFA is essential for the formation of the LPP (Jager et al. 2003). Our studies confirm that a proper mixture of CERs with a suitable fatty acid chain length distribution is a necessity for the solubilisation of CHOL in the matrix. An oriented multilamellar sample with low mosaicity as a prerequisite for data evaluation was observed in the mixture of CER[EOS]/CER[AP]/CHOL. The completion of the SC lipid matrix by FFA is regarded to be crucial for the formation of the LPP, in addition to the presence of CER[EOS] (Jager et al. 2003). However, investigations of the model system composed of CER [EOS]/CER[AP]/CHOL/PA by neutron diffraction did not reveal the formation of the LPP. The membrane repeat unit was slightly shifted to a lower value of $d = 42.2$ Å. Obviously, the observed phase-separation occurs due to different chain lengths of short-chain PA and long-chain CER[EOS] preventing proper lipid mixing. Former studies (Kiselev et al. 2005) on the reference system CER[AP]/CHOL/PA/ChS with a similar chain length of CER[AP] and PA did not reveal a phase separation, indicating that matching chain length is necessary for a good lipid mixing and a reasonable lamellar orientation of the model system. Additionally, the minor role of PA inside the lipid matrix has to be mentioned. In preceding neutron diffraction studies, the influence of FFA with biological relevance as behenic acid

was studied (Ruettinger et al. 2008b). But as a first step, it was necessary to stuck on the original lipid composition of Kiselev et al. (2005) for a substantiated analysis and comparison.

The major result of the present work is the absence of the LPP under the experimental conditions used, indicating that the presence of CER[EOS] appears not to be the only prerequisite for the formation of the 130 Å periodicity. We conclude that additional parameters have to induce the LPP. The existence of the LPP and its inducing or preventing conditions (Pfeiffer et al. 2000; Al-Amoudi et al. 2005) as well as the organisation inside this phase (Bouwstra et al. 2000; McIntosh 2003) is still under discussion. The detection of crystalline cholesterol should not be added to this discussion. According to Jager et al. (2005), cholesterol crystals were detected in the presence of the LPP, therefore, indicating that phase-separated cholesterol does not prevent the formation of the long repeat distance.

Further, the influence of short-chain CER[AP] on the membrane assembly process can be studied. Initially, the application of CER[AP] as a minor representative of the SC ceramide fraction is chosen because of keeping the comparability to the reference system. Moreover, an interesting influence of CER[AP] on the membrane nanostructure was monitored. The detected lamellar repeat unit of 45.2 Å is in the range of the molecular size of two opposite CER[AP] molecules. The polar head group architecture of CER[AP] with four neighbouring OH-groups is known to induce strong lateral hydrogen bonds (Rerek et al. 2001). This property appears to create a superstable membrane nanostructure with a short-periodicity phase and forces long-chain but less polar CER[EOS] to arrange inside this phase. A similar influence of CER[AP] on the nanostructure of a SC lipid model matrix was observed in the work of Ruettinger et al. (2008a). Polar CER[AP] dictates a main phase of approx. 45 Å and determines the bilayer stability. Therefore, CER[AP] obligates various long-chain FFA to arrange inside this phase by interdigitating of the chains in the center of the membrane or by separating as a FFA rich phase. More in-depth studies of the influence of CER[AP] on the nanostructure of the SC lipid model membrane are in progress.

The investigation of multilamellar oriented SC lipid model systems by neutron diffraction offers a promising possibility to characterise the internal membrane nanostructure. Both, the sample preparation method (Seul and Sammon 1990; Katsaras et al. 1992; Kiselev et al. 2005) and the data evaluation procedures (Katsaras et al. 1992; Worcester 1976; Wiener and White 1991) are well established in neutron science. There are other research groups who tried to carry out neutron diffraction experiments on isolated as well as on synthetic SC based samples (Steriotis 2002; Bouwstra et al. 2004). The impact of our work is that for the first time an oriented, multilamellar SC lipid model system containing synthetic CER[EOS] could be characterised by neutron diffraction. The main results are the non-detection of a LPP under the experimental environment chosen and a superstable membrane nanostructure based on CER[AP]. We are aware that the applied SC lipid model membrane cannot simulate the structure of the complex lipid matrix of the SC, particularly due to the use of monodistributed lipid mixtures which cannot mimic the phase behaviour of the SC lipid matrix (Norlén et al. 2007). But neutron diffraction studies on a multilamellar oriented model system with a defined composition offer the possibility to characterise the influence of single lipids on the membrane assembling process. It is not the intention to create a model system that mimic the in vivo situation which further can be applied as a substitute in drug diffusion experiments. We rather try to achieve a SC lipid model membrane containing a selection of relevant synthetic lipids in a reasonable ratio. The characterisation of those simplified models by neutron diffractions appears to be a suitable tool to investigate basic model membrane qualities. From the corresponding neutron scattering length density profiles we expect significant insights in the membrane nanostructure that allows analysing the function of each of the lipids applied on the membrane assembling process.

Therefore, the collected experimental data, presented in this work and used for the determination of the internal membrane structure, assume the proposed arrangement of a long-chain ceramide inside a short-periodicty phase.

In conclusion, the results of the presented neutron diffraction study demonstrate for the first time the arrangement of synthetic CER[EOS] inside a SC lipid model matrix by analysing the internal membrane nanostructure. A 130 Å periodicity phase could not be observed under experimental conditions, either in CER/CHOL mixtures without FFA or in mixtures with FFA. CER[EOS] can be arranged inside a phase with a repeat unit of 45.2 Å. Our data show that CER[EOS] spans a layer and extends into another layer.

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