Experimental report

03/11/2016

Proposal: 9-13-677 **Council:** 4/2016

Title: Investigation of the stratum corneum multilamellar lipid membrane structure in dependence of asymmetric CER[NP]-C24 and CER[AP]-C24

Research area: Soft condensed matter

This proposal is a continuation of 9-13-566

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Samples: ceramides, fatty acids, cholesterol

Instrument	Requested days	Allocated days	From	To
D16	13	8	03/10/2016	11/10/2016

Abstract:

The stratum corneum (SC) as the main penetration barrier of the mammalian skin is very important for ter¬rest¬ri¬al life[1]. The barrier properties are dependent on the SC lipid matrix, which is com¬po¬sed of ceramides (CER), cholesterol and free fatty acids. Un¬fort¬unately their mol¬ec¬u¬lar arrangement is still not completely understood. This led to the development of sev-er¬al different theoretical models, trying to explain SC properties, which are still under de¬ba-te[2]. The aim of this study is to yield more information that will help to fur¬ther sup¬port one of these mo¬dels. Special focus shall be set to the two most recently pro¬po¬sed models, the ar¬ma-tu¬re reinforcement model[3, 4] and the asymmetry model[5]. For both models the full extended conformation of CER is indispensable[3, 4, 5]. That for the asym¬me¬tric CER[NP]-C24 and CER[AP]-C24 shall be in¬ves¬tigated for their influence on the lipid ma¬trix ar¬chi¬tec¬ture, using neutron diffraction and for the first time defined deuterations within these CER molecules.

Investigation of the stratum corneum multilamellar lipid membrane structure in dependence of asymmetric CER[NP]-C24 and CER[AP]-C24

Introduction and state of science

The Stratum corneum (SC) is the outermost layer of the mammalian skin and thus represents the main barrier against exogenous noxae and the penetration of foreign substances. The SC consists of dead corneocytes, enwrapped within a complex mulilamellar organized lipid matrix. This matrix mainly contains ceramides (CER) about 50 %, cholesterol (CHOL) and free fatty acids (FFA)[1]. In the native SC, the two most abundant CER are the CER[NP] and [AP]. It was furthermore shown, that most CER within the SC are of an asymmetric type with mainly a C18 sphingoid base and ≥C20 acyl chain, very often C24[2,3]. It was determined, that the CER are of utmost importance for a functional skin barrier[4,5]. This remarkable purpose makes a more substantiated knowledge of the role of individual species, of the very variable CER necessary. This is especially the case since there is not yet a consensus on the overall organization of the lipid matrix, in fact several different models were proposed and are still controversially discussed[6]. The most recent additions are the *armature reinforcement* (ARM)[7,8] and the *asymmetry model* (ASM)[9]. The ARM proposes an array of multiple stacked bilayers arranged from hairpin folded CER assuming a small amount of CER in a full extended conformation, linking two lamellae together[7,8]. The ASM proposes an array of multiple stacked monolayers with solely fully extended CER and asymmetrically arranged CHOL and FFA[9]. Especially controversial is the long periodicity phase (LPP) with a 12-13 nm lamellar repeat distance, in contrast to the above described short periodicity phase (SPP) with only 5-6 nm, that was observed in the native SC[10]. This second phase was also observed in other experiments and its appearance is thought to be dependent on the presence and ratio of the very long chain ω-acyl-CER[EOS] within the lipid mixture[11]. The CER[EOS] is furthermore supposed to stabilize the lipid interactions and lamellar structure of the SPP. It was shown before, that well-defined mixtures of synthetic lipids with predefined acyl chain and head group can be used to generate simple model membranes. This way, the highly variable CER can be brought to a level where the influence of the individual species is much easier accessible. It could furthermore be shown, that specific deuteration of lipid components can be used to obtain even more information about the system and identify the position and orientation of the lipids within a lamellar layer. This is possible due to the difference in neutron scattering length for the Hydrogen (^{1}H) and Deuterium (^{2}H/D) and provides essential information on the organization of the lipid matrix[6].

Aim of this work

The primary aim of this study is to determine the typical arrangement of the two asymmetrical CER[NP]-C24 and CER[AP]-C24 within the lipid model membrane. These results will then be compared to the ones for the symmetric C18 variants which were used in most older studies. Furthermore shall the influence of the CER[EOS] be accessed first of all on the SPP arrangement and secondly in terms of a possible LPP formation. These results can than give a profound insight on the influence of the two most abundant CER on the lamellar structure as well as that of the very long CER[EOS] with its proposed outstanding function both for the SPP and LPP.

Method

Model membrane lipid mixtures were applied as shown in (Table 1) below.

Table 1: Sample composition

SC lipid model system	Molar ratio	H_2O/ D_2O
CER[NP]-C24_CER[AP]-C24_CER[EOS]_CHOL_LA	0.6/0.3/0,1/0,7/1	0/100; 50/50, 92/8
CER[NP]-C24-**D3**_CER[AP]-C24_CER[EOS]_CHOL_LA	0.6/0.3/0,1/0,7/1	0/100; 50/50, 92/8
CER[NP]-C24_CER[AP]-C24-**D3**_CER[EOS]_CHOL_LA	0.6/0.3/0,1/0,7/1	0/100; 50/50, 92/8
CER[NP]-C24_CER[AP]-C24_CER[EOS]-**D3**_CHOL_LA	0.6/0.3/0,1/0,7/1	0/100; 50/50, 92/8

Terminally deuterated CER[NP]/[AP] and [EOS] (Fig. 1) were especially synthesized for our experiments and were used to further investigate the exact positioning and arrangement of these lipids within the lamellae. As FFA lignoceric acid (LA) was used, corresponding to the CER acyl chain length of 24 carbon atoms[9]. It is also known to be the most abundant of the fatty acids in the native SC[2]. For the CER[EOS] an analogue with a 10-methyl-branched C16 ω-acyl-chain that is

more stable than the linoleic acid, that is the most abundant in the native [EOS][12]. This analogue was however shown, to properly partake in the formation of a lamellar lipid matrix[13].

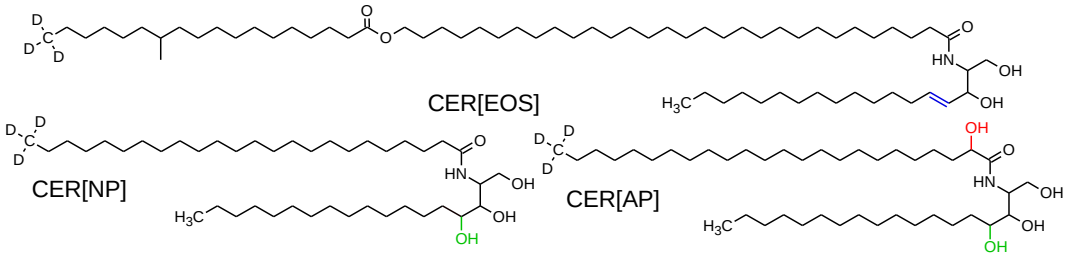

Fig. 1: Terminally deuterated CER for this experiment.

The samples were measured at the average natural skin temperature of 32 °C. To detect possible swelling, two different relative humidity (RH) values, 57 % which is about the humidity of the native skin and 98 % were applied. Three different H_2O/D_2O contrasts were applied to determine the water distribution as well as the sign of the structure factors. The obtained reflexes were fitted using gauss functions. The resulting intensities and 2θ-positions were then used to determine the structure factors $|F_n| = \sqrt{hA_nI_n}$. These were than transform into wave functions that were summated to generate a complete scattering length density profile according to the following equation:

$$p_s(x) = a + b\frac{2}{d}\sum_{n=1}^{n_{max}} F_n\left(\frac{2\pi nx}{d}\right)$$

Results

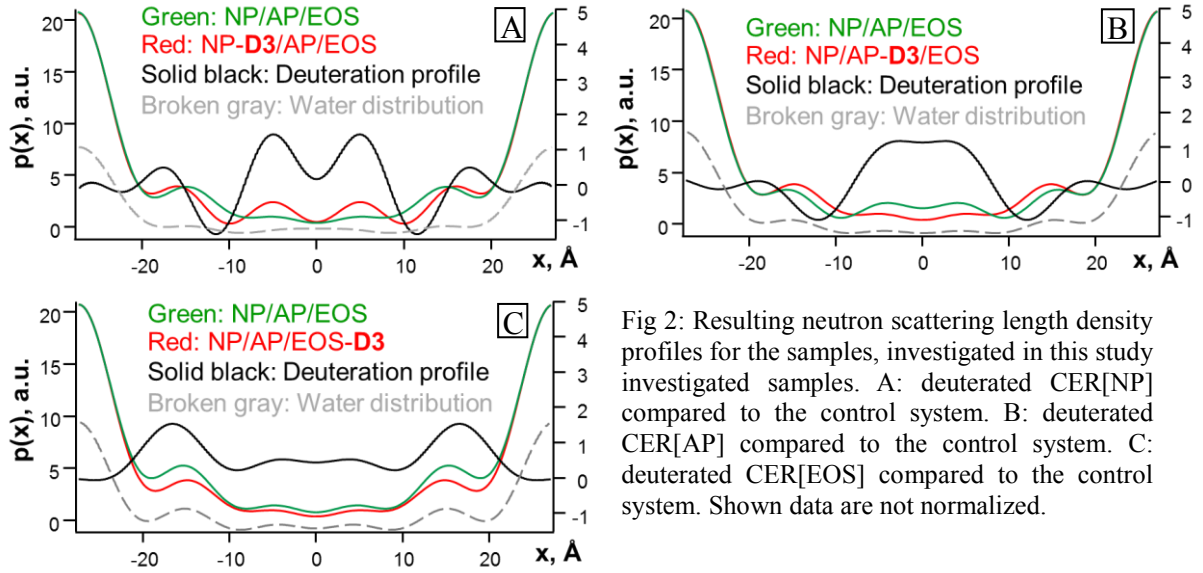

Fig 2: Resulting neutron scattering length density profiles for the samples, investigated in this study investigated samples. A: deuterated CER[NP] compared to the control system. B: deuterated CER[AP] compared to the control system. C: deuterated CER[EOS] compared to the control system. Shown data are not normalized.

What could be observed for the investigated systems is that the asymmetric long chain C24 CER form a SPP with a lamellar repeat distance of 5,465±0,018 nm. Within this phase, the deuteration at the end of the long acyl-chain was detected slightly shifted outward from the lamellar mid-plane. At two positions these could be well resolved for the CER[NP] and a bit less for the [AP]. These CER are thus arranged with their long tails slightly interdigating into the opposing lamellar leaflet. A separation into a CER and a FFA rich phase like described in some older studies was not observed. The water distribution could only show water arranged within the head group region. Also the CER[EOS] was detected within the SPP. The deuteration at the end of ω-acyl-chain was detected slightly inward from the head group region. The CER[EOS] thus almost completely spans both lamellar leaflets with the end of its ω-acyl-chain slightly inward from the head group region of the opposing lamellar leaflet. Using the about native ratio of the CER[EOS] together with both of the most common SC CER the often discussed LPP was not detected in a measurable amount. Only for the sample containing the deuterated CER[EOS] where the signal for the[EOS] should be the strongest, small traces of a second phase with an estimated repeat distance of 12,5-13,0 nm that would be in accordance to an LPP was detected. A swelling and thus increased repeat distance of the lamellae due to increased humidity was not observed.

Discussion

The results discussed above yield much important information on the properties of the most abundant SC CER, phytosphingosine type CER in general and the system with a native amount of CER[EOS] especially. The observed lamellar repeat distance within the SPP is consistent with two opposing long chain CER, with their C24 acyl chains slightly overlapping in the lamellar mid-plane. This is in accordance with the spacing of a comparable system without the [EOS] as measured before, that had a spacing of about 5.46 nm (data not jet published). The very long CER[EOS] could thus be shown, to be incorporated into the SPP, not influencing the lamellar spacing at all. In both systems two distinct positions for the deuteration shifted slightly outward from the lamellar mid-plane were observed Fig. 3.

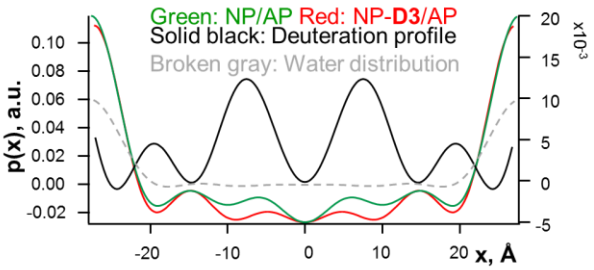

Fig 3: Resulting neutron scattering length density profiles for the CER[NP]/[AP]/CHOL/LA system with a 0.66:0.33:0.7:1 molar ratio measured before at the V1 in berlin. Shown data are not normalized.

The slight inward shift of the deuteration position which also leads to a partial overlap of both peaks for the system additionally containing the CER[EOS] can most likely be attributed to a stabilizing effect of the very long acyl chain, that crosses both lamellar leaflets. This could be reducing the mobility of the long overhangs straightening them out, locating the terminal end slightly inward. Furthermore, despite the addition of the CER[EOS] in a native like ratio of 10 % no distinct LPP was observed. This confirms the observation that was made using CER[AP]-C18 as a racemic mixture of both the D- and L-form together with [EOS] with an even higher ratio of 23 % (w/w) within the whole mixture, which would be equal to about 57 mol-% of the CER portion. In this experiment also no LPP could was observed, despite the much higher [EOS] content[14]. The small amount of a 12,5-13 nm phase observed within the sample containing the deuterated [EOS] that should give the strongest signal for a possible CER[EOS] based LPP however hints to the fact that the formation of such a phase is possible. That the LPP was not observed in this experiments however suggests, that the presence of CER[EOS] alone is not enough to form this phase but other conditions have to be met. Furthermore no change in lamellar spacing was observed, neither for the system with nor for the one without the CER[EOS], upon hydration of about 100 %. A swelling of membranes, based on the CER[NP] and [AP] thus does not take place. This speaks against the ARM, that is suggesting a slight swelling due to a chain flip mechanism transforming full extended CER to hairpin folded CER.

Refrences

[1] P. W. Wertz,B. van den Bergh, *Chem. Phys. Lipids*, 1998**;** *Vol. 91* **2**, 85–96.
[2] M. Ponec,A. M. Weerheim, et al; *J. Invest. Dermatol.*, 2003**;** *Vol. 120* **4**, 581–588.
[3] Y. Masukawa,H. Narita, et al; *J. Lipid. Res.*, 2008**;** *Vol. 49* **7**, 1466–1476.
[4] W. M. Holleran,M.-Q. Man, et al; *J. Clin. Invest.*, 1991**;** *Vol. 88* **4**, 1338–1345.
[5] L. Coderch,O. López, et al; *Am. J. Clin. Dermatol.*, 2003**;** *Vol. 4* **2**, 107–129.
[6] D. Kessner,A. Ruettinger, et al; *Skin. Pharmacol. Physiol.*, 2008**;** *Vol. 21* **2**, 58–74.
[7] M. A. Kiselev,N. Y. Ryabova, et al; *Eur. Biophys. J.*, 2005**;** *Vol. 34* **8**, 1030–1040.
[8] M. A. Kiselev, *Cryst. Rep.*, 2007**;** *Vol. 52* **3**, 525–528.
[9] I. Iwai,H. Han, et al; *J. Invest. Dermatol.*, 2012**;** *Vol. 132* **9**, 2215–2225.
[10] K. C. Madison,D. C. Swartzendruber, et al; *J. Invest. Dermatol.*, 1987**;** *Vol. 88* **6**, 714–718.
[11] M. de Jager,G. S. Gooris, et al; *J. Lipid. Res.*, 2005**;** *Vol. 46* **12**, 2649–2656.
[12] A. Hinder,C. E. Schmelzer, et al; *Skin. Pharmacol. Physiol.*, 2011**;** *Vol. 24* **3**, 127–135.
[13] T. N. Engelbrecht,T. Hauss, et al; *Soft Matter*, 2011**;** *Vol. 7* **19**, 8998.
[14] A. Schroeter,D. Kessner, et al; *Biophys. J.*, 2009**;** *Vol. 97* **4**, 1104–1114.