Experimental report

16/03/2017

Proposal: 9-13-700 **Council:** 10/2016

Title: Investigation of the different head group H-bonding of D- and L-CER[AP] and its influence on stratum corneum model membrane architecture

Research area: Soft condensed matter

This proposal is a new proposal

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Samples: ceramides, fatty acids, cholesterol

Instrument	Requested days	Allocated days	From	To
D16	16	8	26/01/2017	03/02/2017

Abstract:

The stratum corneum, as the main penetration barrier of the mammalian skin, is very important for all terrestrial life[1]. Its barrier properties are dependent on the lipid matrix (LM), composed of ceramides (CER), cholesterol and free fatty acids[1]. Its arrangement however is still not completely understood. The influence of the individual CER head groups on the overall LM structure for example was not yet investigated thoroughly. For the long chain C24 CER so far no investigations were performed at all, even though they are the most common type within the SC[13, 14]. Especially the omega-hydroxy-CER have a very special influence on the overall lamellar architecture an phase behaviour[8, 9, 17, 18]. This study is aiming to gain substantiated knowledge on the influence of the most common long chain omega-hydroxy-CER, the CER[A24P18] on the LM architecture. The omega-hydroxy-CER furthermore exist as two enantiomers, the D- and the L- form which greatly differ in their behaviour, so both forms will be investigated.

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), the outermost layer of the mammalian skin, represents the main barrier against exogenous noxae and foreign substances. It consists of dead corneocytes, within a complex multilamellar lipid matrix. This matrix consists mainly of ceramides (CER), cholesterol (CHOL) and free fatty acids (FFA)[1]. It could be shown, that the CER are the most important factor for the sustaining of the SC barrier function[2]. They furthermore determine the overall phase behaviour of the lipid matrix[3,4]. This essential function makes it necessary, to learn more about the influence of each individual CER species. In comparison to many other lipids like phospholipids, the CER head groups (HG) are smaller and less polar. Nonetheless they show strong and stable interactions through a complex H-bonding network (HbN)[5]. It could further be shown, that the HbN in the HG of the CER influences their chain packing and phase behaviour. Generally it was observed, that the more OH-groups, the stronger the HbN and the stronger the HbN, the weaker the chain ordering[6–9]. The α-OH-group however seems to be the exception to this rule[7,9,10]. Naturally the α-OH-CER are solely found with a D-(2R)-conformation, synthetically however also the L(2S)-entantiomer could be obtained. H-bonds are strictly directional so even such a slight conformational change for a single OH can influence the HbN arrangement, especially, since polar CER interactions are exclusively based on H-bonds[5]. Well-defined mixtures of synthetic lipids with predefined acyl chains and HG can serve to generate more simple model membranes, which keep the influence of the individual CER species at an observable level[11]. The model membranes for this experiment were prepared using a well-established method[12]. The CER[AP] C24 as the most common α-OH-CER in the SC was used in this study. As FFA lignoceric acid (LA) was used, corresponding to the CER acyl chain length and because it is one of the most abundant FFA within the native SC[13]. For these kinds of experiments, neutron diffraction can serve as a very valuable tool. A partial deuteration of the CER acyl chain(Fig. 1) enables the determination of the CER position within the model membrane, due to the different neutron scattering length of 1H and 2H=Deuterium (D). This will allow an assessment of the overall lamellar structure of the model membrane[11].

Aim of this work

Synthetically it is easier and cheaper to generate a mixture of both α-OH-CER isomers. For scientific and especially pharmaceutical applications it is important to know, how the different head groups influence the formation and nanomolecular arrangement of the lipid matrix. The aim of this work was to clarify the influence of the conformation of the α-OH-group on the formation and nanostructure of a multilamellar lipid matrix. This can serve to see, if the L-enantiomer can also be used to generate SC-substitutes and models or if it may have other interesting applications.

Method

Table 1: Sample composition

SC lipid model system	Molar ratio	RH [%]	H_2O/ D_2O [w/w]
CER[N24P18] / D-CER[A24P18] / CHOL / LA	0.66/0.34/0,7/1	57/98	0/100; 50/50, 92/8
CER[N24P18] / L-CER[A24P18] / CHOL / LA	0.66/0.34/0,7/1	57/98	0/100; 50/50, 92/8
CER[N24P18] / D-CER[A24P18]-**D_3** / CHOL / LA	0.66/0.34/0,7/1	57/98	0/100; 50/50, 92/8
CER[N24P18] / L-CER[A24P18]-**D_3** / CHOL / LA	0.66/0.34/0,7/1	57/98	0/100; 50/50, 92/8
D-CER[A24P18] / CHOL / LA	1/0,7/1	57/98	0/100; 50/50, 92/8
L-CER[A24P18] / CHOL / LA	1/0,7/1	57/98	0/100; 50/50, 92/8
D-CER[A24P18]-**D_3** / CHOL / LA	1/0,7/1	57/98	0/100; 50/50, 92/8
L-CER[A24P18]-**D_3** / CHOL / LA	1/0,7/1	57/98	0/100; 50/50, 92/8

The deuterated D- and L-CER[AP] (Fig. 1) were especially synthesized for our experiments. They were used to observe the formation of a multilamellar lipid matrix within the applied mixtures. Furthermore, to investigate the exact position and arrangement of these lipids within this lamellar structure. The CER[AP] as most abundant of the α-OH-CER in the SC was used[14]. As FFA lignoceric acid (LA) was used, corresponding to the CER C24 acyl chain length [15]. It is also known to be the most abundant of the fatty acids in the native SC[14]. 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 diffraction signals 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 transformed into wave functions that were summated to generate a complete scattering length density profile according to the following equation: $p_s(x) = a + b\frac{2}{d}\sum_{n=1}^{n_{max}} F_n\left(\frac{2\pi nx}{d}\right)$

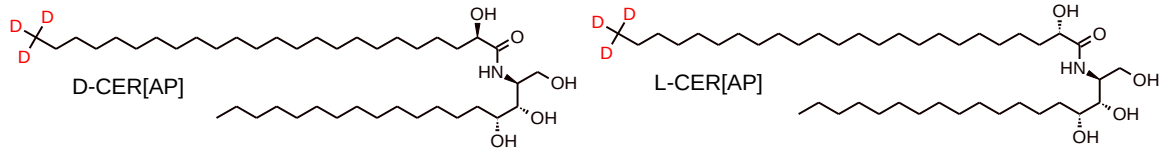

Fig. 1: D and L-form of the terminally deuterated CER[A24S18], as will be used for these experiments

Results

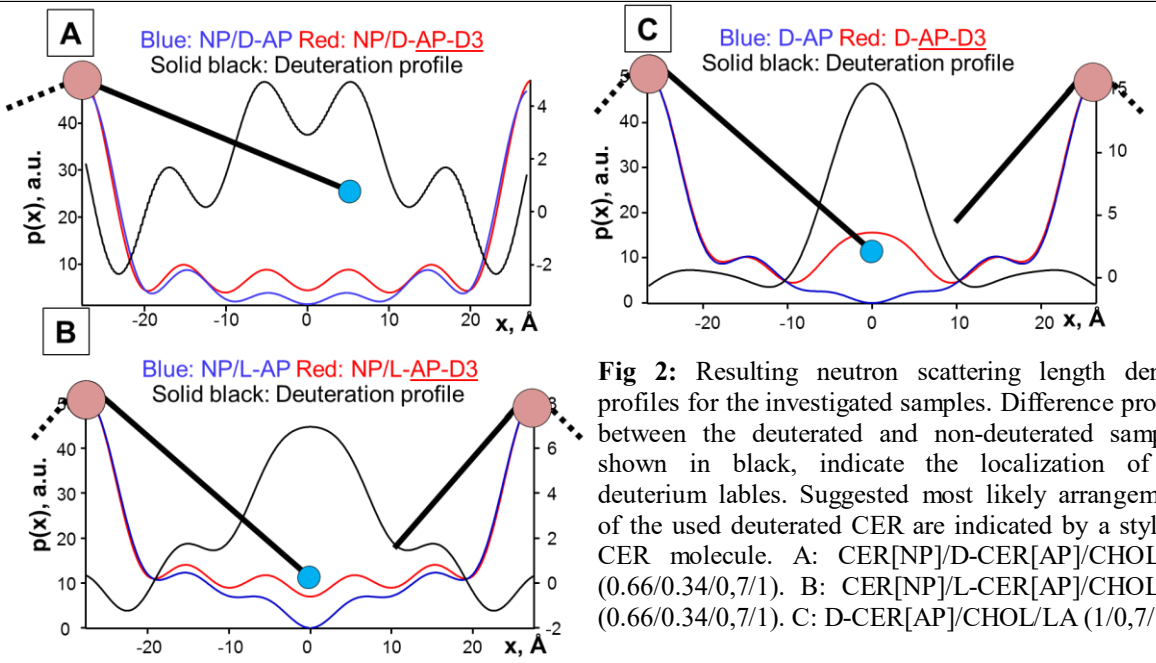

Fig 2: Resulting neutron scattering length density profiles for the investigated samples. Difference profiles between the deuterated and non-deuterated samples, shown in black, indicate the localization of the deuterium lables. Suggested most likely arrangements of the used deuterated CER are indicated by a stylized CER molecule. A: CER[NP]/D-CER[AP]/CHOL/LA (0.66/0.34/0,7/1). B: CER[NP]/L-CER[AP]/CHOL/LA (0.66/0.34/0,7/1). C: D-CER[AP]/CHOL/LA (1/0,7/1).

In this study, it could be observed that the D- and the L-CER[AP] have a distinctly different influence on the lamellar structure, both together with other CER and as only CER in a lipid mixture. Despite the small difference between the two, they promote a different nanostructur. For the CER[NP]/D-[AP] system, the overall scattering profile is flat, hinting to a mostly straight carbon chain arrangement. No distinct minimum in the lamellar middle was observed. This together with the two observed deuteration positions indicates the C24 chains overlapping in the lamellar mid-plane. The system shows a repeat distance of 5.48±0.09 nm, which also conclusive with straight overlapping lipid chains. For the CER[NP]/L- [AP] system the profile is instead V-shaped with a distinct scattering minimum in the lamellar middle. This indicates a tilted arrangement, with both CH_3 located in the lamellar-centre, leading to the minimum. The single very strong deuteration positon in the lamellar centre is also conclusive with this arrangement and results from both CD_3 being in direct proximity. The repeat distance of 5.49±0.04 nm is equal to the first system. This and the single deuteration position is conclusive with a chain tilt. Otherwise without eth overlapping long chains, the repeat distance would increase.The D-CER[AP] as only CER led to a again led to V-shaped scattering profile with a distinct minimum in the lamellar centre, again indicating a tilted chain arrangement. The very strong single deuteration signal also supports such an arrangement. Also the very strong deuteration signal furthermore hints to a very rigid chain arrangement, not dispersing it due to movement of the terminal groups. This could be hinting to a more crystalline-like arrangement compared to other CER mixtures. The thickness of 5.28±0.03 nm is shorter than fort he mixed systems. This together with the single deuteration position suggests a strong chain tilt (Fig. 2). For the system with the L-CER[AP] as only CER, no lamellar structure could be observed. Different annealing conditions with higher temperatures were used to promote lamellae formation. However no change could be observed up to 85 °C were all signal was lost, indicating disordering. For none of the systems, which formed a lamellar phase swelling could be observed upon raising the humidity from 57 to 98 %. Also water could only be located within the lamellar HG region.

Discussion

The observed results are conclusive with, prior observations. During the synthesis and purification, the CER[AP] expressed crystalline-like characteristics, the L- even more, than the D-enantiomer. Crystalline CER[NP] C18 and C24 were shown to be polymorphic, yielding multiple different crystal modifications, depending on the crystallization conditions[16,17]. Either a V-shaped or extended conformation were concluded for most modifications. For the C24 variant partial interdigitation of the long chains could be observed for some modifications[17,18]. In a monolayer, the D-CER[AS] C18 could be shown, to assumes a tighter chain packing than the L-enantiomer, hinting to a disturbance of the chain packing for the D-enantiomer, making it less crystalline[3]. Crystalline D-CER[AP] C18 showed two and the L-enantiomer three crystalline modifications. A fully extended conformation was suggested for the D- and a V-shaped one for the L-enantiomer[10]. Overall it can be concluded, that crystalline CER mainly prefer V-shaped conformations. For lipid mixtures however this was not observed. For the mixture of CER[NP]and L-[AP] and the one with only D-CER[AP], the arrangement rather hints to such a crystalline-like behaviour. However no Phase separation was observed, meaning the CHOL and FFA partake in the formation of this phase. An increased repeat distance upon hydration assumingly caused by the V-shaped CER was not observed. It is possible, that the sample was already fully hydrated due to humidity in the air or the CHOL and FFA prevent this change in repeat distance. The mixture of CER[NP] and D- [AP] on the other hand seemed to be arranged with straight chains and overlapping long chains in the lamellar mid-plane. This is in good conclusion with observations made for the native SC as well as native-like model mixtures[19,20]. The Lamellar thickness for all three systems is near the native SC short periodicity phase with 5-6 nm[21]. For the SPP of a native-like model a value of 5.45 nm was observed. The two CER[NP]/[AP] mixtures fit this thickness while the D-CER[AP] mixture is slightly shorter. Overall, the CER[NP]/D-[AP] mixture closely resembles the native lipid matrix, while the other systems had a more crystalline-like behaviour. This should be enough to showcase the strong influence of even minor differences within the CER HG. This demonstrates that even tough differences between most CER species are rather minute, no high redundancy in their function is to be expected. The differing characteristics of the D- and L-CER[AP] furthermore is of much importance for future investigations. No isomer mixture can be used, since the unnatural L- has a different influence than the D-enantiomer. This is even more important for pharmaceutical applications. The L-CER[AP] is an unnatural substance and not contained in the native SC. Since it shows a different behaviour than the natural D-enantiomer, it cannot yet be considered safe. It is possible, that the more crystalline character would lead to a disturbance within the lamellar structure rather than supporting or repairing it which could lead to negative side effects. On the other hand, if this tendency to form a more crystalline like arrangement has a positive influence on the lipid matrix, the L-CER[AP] could be an attractive substance for pharmaceutical applications.

Refrences

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