Experimental report

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Proposal: DIR-153 **Council:** 4/2017

Title: Comparative Investigation of the Lamellar Phase Behaviour of Plant and Mammalian Ceramides

Research area: Biology

This proposal is a new proposal

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Samples: ceramides, Cholesterol, fatty acid

Instrument	Requested days	Allocated days	From	To
D16	5	5	08/03/2018	13/03/2018

Abstract:

Comparative Investigation of the Lamellar Phase Behaviour of Plant and Mammalian Ceramides

Introduction and state of science

The skin, one of the main barrier organs of the human body, protects against the invasion of pathogens, and physical as well as chemical noxae. It also limits the epidermal water loss, keeping the viable cells underneath moisturized[2]. Especially its outermost layer, the *stratum corneum* (SC), is of utmost importance for this function. This layer consists of rigid corneocytes which are enclosed in a complex multilamellar lipid matrix (LM), responsible for the skin barrier function. It is comprised of about equimolar ratios of ceramides (CER), cholesterol (CHOL) and free fatty acids (FFAs)[1], with the CER as main contributor to the barrier function[2,3]. A reduced CER content impairs this function[4], increasing the risk of infection and skin damage and leads to itching and inflammation[5]. Skin diseases such as psoriasis but also aged skin are associated with reduced CER levels and the corresponding symptoms[6]. Treating damaged skin with CER[7] and CER-analogues can partially restore the barrier[8]. Synthesis or isolation of skin CERs is expensive, time consuming and isolation furthermore ethically questionable. Plant-derived CERs are an alternative for the replenishment of depleted CERs if delivered to the skin for example in a cream. However, plant and mammalian CERs have some different hydroxylations, chain length and double bonds. Possibly yielding some different properties.

Aim of this work

This investigation aims towards understanding the influence of OatCERs on the lamellar structure of the skin and compare them to mammalian CERs. These results can hopefully provide evidence for the usefulness of them for repairing damaged skin and strengthening the skin barrier. The data can be used for further comparative studies planned for other plant-derived CERs in the future.

Method

In this study, CERs from oat were used. The two predominant CERs contain hydroxypalmitic acids (h16:0/h20:0, 1.25:1, OatCER I/ II) amide-linked to sphingenine (d18:1$^{\Delta 8}$). And are most similar to CER[AP] which was used for comparison, together with the most common skin CER[NP][9]. An intermediate chain length of C18 was chosen, also for the fatty acid component stearic acid (SA), as intermediate between C16 and C20. CHOL was added in a slightly reduced ratio of 0.7 instead of the natural equimolar ratio, to minimize the formation of a separate crystalline CHOL phase.

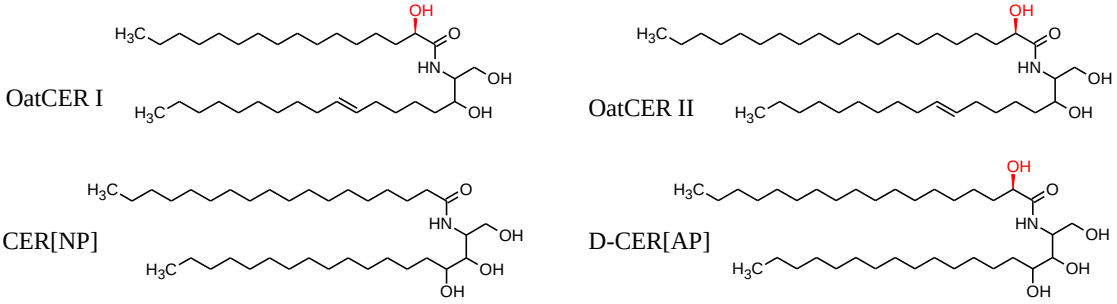

Figure 1: Oat-derived CERs and CER [NP] and CER [AP]

The model systems for these experiment were prepared, using a well-established method[10]. The lipids for the model systems were solved in chloroform/methanol (2:1) and sprayed at 75-80 °C, followed by annealing (repeated heating+hydration/cooling) 5 °C below spraying temperature.

SC lipid model system	Molar ratio	H_2O/ D_2O contrast; w/w
OatCER/CHOL/SA	1 / 0,7 /1	0/100; 50/50, 92/8
CER[NP]/[AP]C18/CHOL/SA	0.67 / 0.33 / 0.1 / 0,7 / 1	0/100; 50/50, 92/8
CER[NP]/[AP]C18/CHOL/SA	0.33 / 0.67 / 0.1 / 0,7 / 1	0/100; 50/50, 92/8

The samples were measured at 32 °C which is about the average skin temperature. Two different relative humidity (RH) values, 57 % and 98 % were applied. Furthermore, each sample was measured at three different H_2O/D_2O contrast, to determine the structure factor sign. After a condition change (R.H., H_2O/D_2O) the samples were equilibrated for at least 6 h.

Results

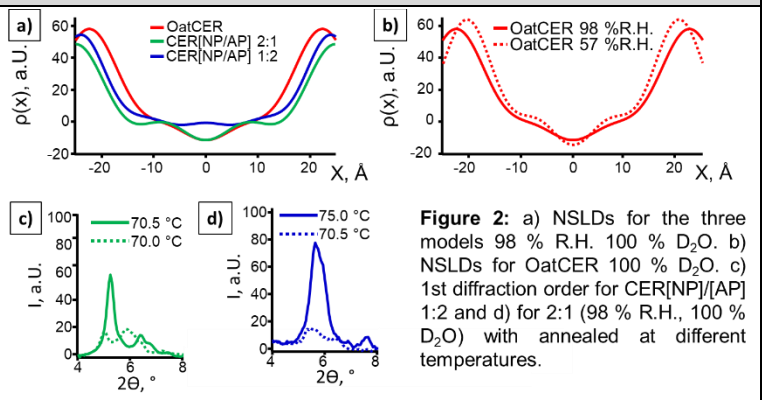

Figure 2: a) NSLDs for the three models 98 % R.H. 100 % D_2O. b) NSLDs for OatCER 100 % D_2O. c) 1st diffraction order for CER[NP]/[AP] 1:2 and d) for 2:1 (98 % R.H., 100 % D_2O) with annealed at different temperatures.

The diffraction experiments were able, to show several important properties of the OatCERs, but also the skin CERs, which they are compared to. For all three models, a neutron scattering length density profile (NSLD) could be calculated (Figure 2 a). All three diffraction profiles looked relatively similar, with the head groups and water located only at the outer boards of the inner tail region, with small differences in lamellar repeat distance: CER[NP]/[AP] 2:1; 4.99±<0.01 nm, [NP]/[AP] 1:2; 4.79±0.04 nm and OatCER with a slightly shorter repeat distance of 4.48±0.01 nm. For the 2:1 and OatCER system. A minimum -indicating the localisation of all CH_3-groups- is observed in the lamellar middle. This minimum is not observed for the 1:2 system. Changing the relative humidity from 98 % to a lower value of 57 % did not induce any changes in the skin CER. For the OatCER, the repeat distance decreased to 4.08±0.02 nm demonstrating a hydration dependent swelling of the lipid lamellae (Figure 2 b). However, the observed NSLDs have a relatively low level of detail, because for all 3 systems, the intensity of the diffraction signals was relatively low, with only 3 (OatCER, 2:1)-4 (1:2) diffraction orders observable (data not shown). For the skin CER systems, this was mainly due to a strong phase separation into at least two lamellar phases with different intensity and repeat distance, dispersing the total diffraction signal between them (data not shown). For the OatCERs, there was only a less phase separation with smaller proportions of a second phase (4.06±0.07 nm) was also only present at 98 % R.H.. Nonetheless, only 3 lamellar orders with a relatively strong first order could be observed. This shows, that the low number of diffraction orders in this case mainly indicates a lower order of the acyl chains. Using different spraying and annealing temperatures did furthermore demonstrate, that the behaviour within the skin CER is strongly dependent on the preparation conditions, with even 0.5 °C already completely changing the phase behaviour (Figure 2 c,d). It was furthermore observed that the 1:2 mixture, which's phase behaviour is mainly determined by the CER[AP] had a 5 °C higher optimal spraying/annealing temperature and a higher diffraction peak intensity and number (higher order). Its two main phases had a more similar repeat distance (I/II: 4.79±0.04/4.56±0.06 nm) than for the 2:1 system in which the second predominant phase was much shorter than the main phase (I/II: 4.99±<0.01/4.07±<0.01 nm) (data not shown).

Discussion

Even though, the NSLDs are low detail, they are suitable to demonstrate, that the lamellar phase structure under at 98 % R.H. is similar for all three systems. The slightly shorter repeat distance for the OatCER is a result of the slightly higher content of C16 CER. Due to the mixture of C16 and C20 however it is as expected very similar to the repeat distance with C18 skin CER. The additional double bond of the OatCER's chains most likely also contributes to this shorter repeat distance, by decreasing the lateral chain order and thus the perpendicular space requirement due to the slightly less extended chains. The lack of a minimum in the lamellar centre for the 1:2 system is a result of the corresponding error wave for the 4th diffraction order which would usually be compensated by the 5th and possibly 6th order. The NSLD without the 4th order looks similar to the two other profiles with only 3 orders. The similarities between the structure of the skin CER and the OatCER models clearly demonstrate that they are in principle suitable to be used for substitution or replenishment of reduced skin CER. A quantification of the proportion of each phase is unfortunately not possible, since the integral peak intensity is not directly comparable, because it is also dependent on the order, head group composition, water content and other factors. Even though it did become clear that the OatCER have a slight advantage in this aspect. The OatCER did show less phase separation in the sense that only two phases instead of up to four plus separated CHOL were observed. The second phase also had a relatively weak signal and thus most likely small proportion. This decreased phase separation is most likely a result of the additional C8,9 double bonds, reducing the

lateral order and thus miscibility within the system and with the CHOL. This phase behaviour is comparable to the C24 CER, which are by far more abundant in the skin than the C18 variants[9,11]. For these, no phase separation is observed, in comparably simple mixtures of CER[NP]/[AP]CHOL and lignoceric acid[12]. However, the main difference between the OatCER and the skin CER is the sensitivity to hydration demonstrated by the swelling upon extreme hydration 98 % R.H.. Since this is higher than the natural hydration of the skin would be, the phase with the repeat distance of 4.08±0.02 nm observed at 57 % R.H. would actually have to be considered the more natural phase. Which is much shorter than the native like 5.45 nm phase observed for the C24 CER[12]. This shorter repeat distance is undoubtedly a result of the high proportion of the short C16 CER. The order of all three systems showing only 3-4 lamellar orders is generally lower than for the comparable C24 CER systems, showing 5-6 lamellar orders[12,13]. This shows, that longer plant CER or at least a higher proportion of the longer variants would be more desirable, to better suit the properties of the natural LM. The observed sensitivity to hydration could have two possible causes. It could be an intrinsic property of the investigated OatCER, possibly caused by the decreased lateral order due to the C8,9 double bonds, also allowing for water to be incorporated into the resulting free space between the head groups. The other possibility is that, even though the CER are about 95 % pure, this effect is caused by an impurity, still remaining in the sample. This could possibly be a sugar-moiety containing glycol-CER or other glycosylated lipids which have a much higher water binding capacity, allowing for more water to be absorbed and possibly change the structure, since the bigger sugar containing head groups take up more space, especially, if they are hydrated. The pure OatCER were obtained from hydrolysis of glycosylated species, making this a reasonably likely possibility. The compared to the skin CER slightly brown-yellow discoloration and sticky, slightly viscous consistency of the original sample could also hint towards this. An increase in repeat distance, upon integration of molecules with a larger more hydrophilic head group like phospholipids in a CER-based system, was already shown to lead to such an increase in spacing[14]. The observed sensitivity to hydration is also not a natural and possibly unfavourable feature for the lipids, since it could possibly reduce the barrier function of the skin by disturbing the head group interaction. However at this point, it is unclear, if the increased hydration would necessarily be negative. It could possibly also be utilized, to treat dry skin, if it is a result of the CERs themselves and not a possible impurity.

Conclusion and Outlook

In general, it can be concluded, that plant derived CER could be suitable for applications in skin care and protection, since they have similar properties as the skin CER. They did even show less phase separation, similar to the more abundant C24 skin CER. The biggest uncertainty at this point is their sensitivity to hydration. It has to be investigated further, if this is caused by an impurity or by the CERs themselves and if it could possibly be utilized in a positive way. It would furthermore be favourable, to have longer chain plant CERs, which are more similar to skin CER.

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