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Experimental report

The role of EO-class ceramide in the arrangement of the stratum corneum model membranes

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Introduction

The stratum corneum (SC) extracellular lipid matrix comprises ceramides (Cer), free fatty acids (FFA) and cholesterol (Chol) – in approximately equimolar fractions, with a minor amount of cholesteryl sulfate (CholS)[1]. These skin lipids create a highly organized lamellar structure with a repeat distance ~ 13 nm (so-called long periodicity phase)[2] and provide a barrier against water loss and the entering of exogenous substances through the skin. The role of individual ceramide classes in the SC structure is still not clear. The ultra-long ω-acylCer (EO-class Cer), which contain 30-34C acyls with linoleic acid ester-linked to ω-hydroxyl, are indispensable in the homeostasis of the skin permeability barrier[3]. They are supposed to be essential for the formation of the long periodicity phase. With the aim to shed more light on the ceramide structure - SC lipid model arrangement relations, we prepared samples with Cer having long (C24) or ultra-long (EO-class Cer) acyl chains for the experiment on DN-2 neutron diffractometer. The samples were prepared in a form of aligned oriented multilayers.

Experiment

Ultra long-chain ceramide CerEOS (from the EO-class ceramides), which is not commercially available, and $_{d\text{-}47}$CerNS24 with deuterated acyl chain of 24 carbons were synthesized at the Faculty of Pharmacy of Charles University in Hradec Králové. Very long-chain CerNS24 was purchased from Avanti Polar Lipids (Alabaster, USA). Other components of SC model lipid membrane such as cholesterol and free fatty acids with different lengths of hydrocarbon chains were purchased from Sigma-Aldrich Chemie Gmbh (Schnelldorf, Germany). SC matrix was recreated in two compositions with very long-chain CerNS24 (CerNS24/FFA/Chol), and both ultra long-chain CerEOS and very long-chain CerNS24 (CerEOS/CerNS24/FFA/Chol). Samples were prepared as oriented multilayers on the 25x65 mm^2 rectangular single crystal silicon wafers. In parallel, two samples of the same composition, but with deuterated labels in CerNS24 were prepared as well. Each sample was duplicated. Thus, a total of 8 samples were prepared. The cholesterol content in each sample was 45 mol%. During the preliminary testing on the X-ray diffractometer, the best-oriented sample of each species was chosen. X-ray measurements were also performed after the neutron experiment.

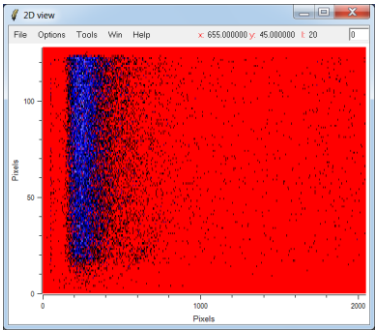
The neutron diffraction experiment was carried out at maximum contrast variation condition (100% D_2O) on hydrated samples (relative humidity 100%) at room temperature (20 ^oC). The diffraction data were collected at the DN-2 real time diffractometer in the time-of-flight (TOF) mode while utilizing 2D position sensitive detector. The collection time spanned several hours (up to 15 hours in some cases).

Results and discussion

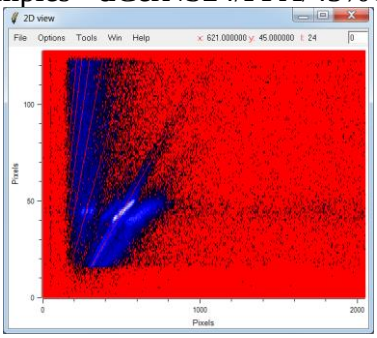
Our diffraction data analysis shows that periodic lamellar structures are formed in hydrated samples of the SC model membrane with deuterated ceramides both with an ultra long-chain CerEOS and with a long-chain CerNS. Figure 1 shows the improvement in the quality of the sample after hydration

and annealing observed especially in the samples with CerEOS. We can see up to 4 reflection orders in these measurements.

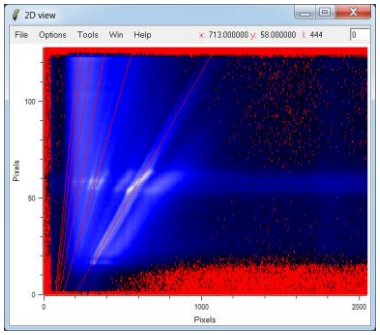
Samples - **d**CerNS24/FFA/45%Chol



before hydration (dry sample)

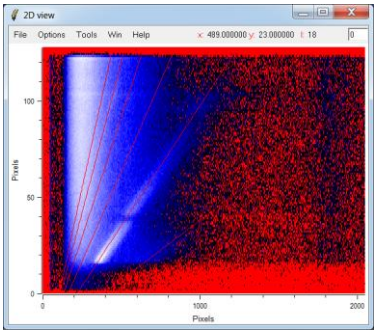


after hydration;
time of measurement 2x5min

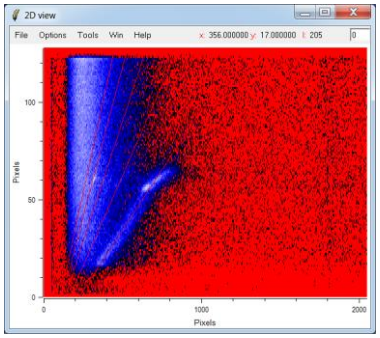


after annealing;
time of measurement 5x3h

Samples - CerEOS/**d**CerNS24/FFA/45%Chol



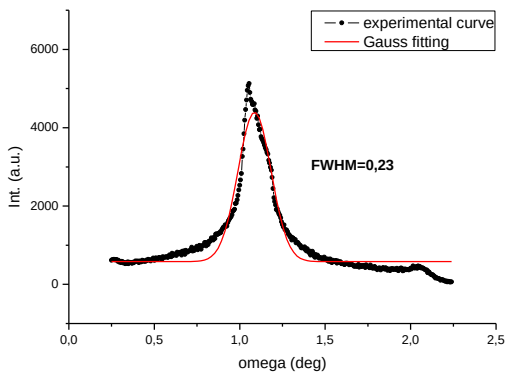
after hydration
time of measurement 6x1h



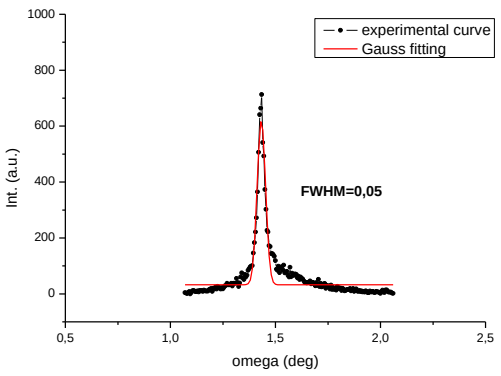
after annealing
time of measurement 1x1,5h

Figure 1. 2D TOF diffraction patterns of samples with deuterated ceramides hydrated with 100% D_2O.

It is clear from our measurements that annealing process affects the ordering of lipid molecules forming the model membrane. It improves the orientation of multilayers in sample and reduces its mosaicity. This is confirmed by the measurements of so-called "rocking curves" (RC). The curves are well described by a Gaussian function with the peak's full-width at half-maximum (FWHM) characterizing the sample mosaicity. FWHM values for the **d**CerNS24/FFA/Chol are 0.21º and 0.04º, and for the CerEOS/**d**CerNS24/FFA/Chol they are 0.23º and 0.05º before and after annealing, respectively (Figure 2).



RC before annealing measured at the first Bragg peak (2θ=2.49º).



RC after annealing measured at the first Bragg peak (2θ=3.13º).

Figure 2. Rocking curves for CerEOS/**d**CerNS24/FFA/Chol before and after sample annealing.

The long periodicity phase is observed in the sample with the ultra long-chain ceramide CerEOS/**d**CerNS24/FFA/Chol. The repeat distance is 122.6 Å. This value is in a good agreement with our X-ray measurements before and after neutron experiment (120.8 Å and 123.6 Å). As can be seen from

the repeated X-ray measurements, the microstructure of the SC model lipid membrane does not change during annealing. The long periodicity phase in the sample is conserved.

On the other hand, the sample with long chain ceramide (**d**CerNS24/FFA/45%Chol) having the insignificant long periodicity and strong short-period phase (d = 105.5 Å and d = 53.0 Å, X-ray data) prior to hydration, undergoes conformational changes in the structure of the lipid matrix during hydration and annealing. The long periodicity phase completely disappears. In this case, four reflection orders corresponding to the short periodicity structure are well determined by the neutron diffraction (Figure 3). Moreover, the new structure is characterized by triplets with the most intense peak in the middle. Such a structure is even better seen in the X-ray spectra measured after neutron diffraction experiment. The repeat distance of this structure is 52.8 Å and 53.9 Å (neutron and X-ray data).

Apparently, changes in the structure of SC model lipid matrix occurred due to the transition of ceramide molecules (partly at least) from one conformation to another. Initially, the molecules of d-CerNP were supposedly in a full-extended conformation. However, after hydration and annealing of the sample, d-CerNP molecules may have taken a hairpin-shape form. The question of the reversibility of such a transformation remains yet to be understood. Most likely in our case, there may not have been enough time allowed after annealing and hydration as the repeated X-ray measurements were performed only 4 days apart.

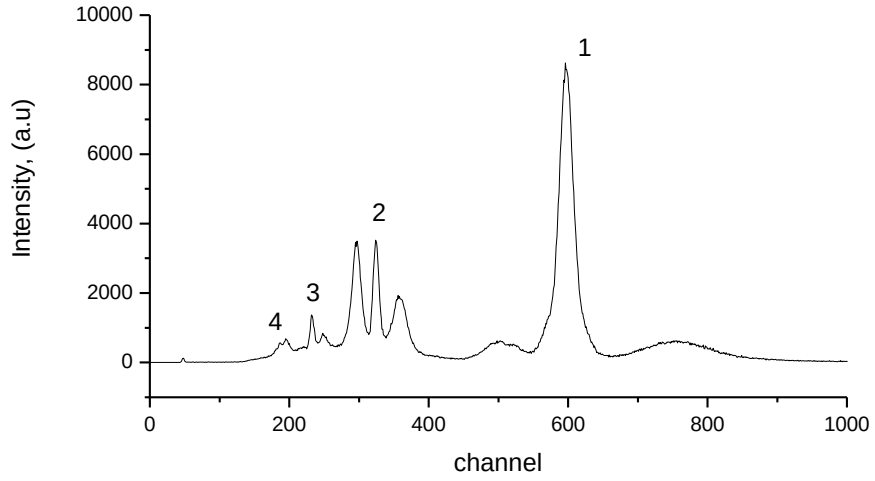


Figure 3. Neutron diffraction spectra collected after sample annealing (**d**CerNS24/FFA/45%Chol) at 100% D_2O, room temperature during 15 hours. Numbers indicate peaks corresponding to the reflection orders from the lamellar structure.

Conclusions

Unfortunately, the results obtained in this experiment do not allow us to reconstruct neutron scattering density profiles for the determination of ceramide spatial arrangement in the SC model lipid matrix. It is necessary to make measurements with different contrast variations D_2O. In the meantime, at a lower D_2O/H_2O ratio, as well as in samples with protonated CerNS24, it was not possible to obtain a sufficient number of diffraction peaks (4 or more).

References

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(2) White, S. H.; Mirejovsky, D.; King, G. I. Structure of Lamellar Lipid Domains and Corneocyte Envelopes of Murine Stratum Corneum. An X-Ray Diffraction Study. *Biochemistry (Mosc.)* **1988**, *27* (10), 3725–3732.

(3) Uchida, Y.; Holleran, W. M. Omega-O-Acylceramide, a Lipid Essential for Mammalian Survival. *J. Dermatol. Sci.* **2008**, *51* (2), 77–87.