Experimental report

14/02/2024

Proposal: **9-10-1738** **Council:** 10/2022

Title: Ceramide Crystals in Phospholipid Liposomes

Research area: Soft condensed matter

This proposal is a new proposal

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Samples: D2O
Ethanol
Lipids (lecithin, DOPC, ceramides, lyso-lipids) ~C44H77D9NO7P

Instrument	Requested days	Allocated days	From	To
D16	2	2	12/04/2023	14/04/2023
IN15	4	0		
D22	1	1	09/04/2023	10/04/2023

Abstract:

Ceramides (CERs) are one of the main lipids in the Stratum Corneum (SC), and play an essential role on the skin¿s barrier function. Damage to the SC either by internal or external stimulus results in lower CER levels and com- promises its barrier properties. A promising therapy is to restore CERs by delivering them into the skin, but its widespread use is limited as current formulations use stabilisers that may cause adverse skin effects. A promising alternative are phospholipid (PL) liposomes as they are established delivery systems in pharmaceutic and cosmetic applications. However, limited colloidal stability caused by CER crystallization is one of the main drawbacks for PL:CER systems. We intend to apply S/WANS and NSE to elucidate the mechanism for the failure of stability of these formulations.

Ceramide crystals in Phospholipid Liposomes

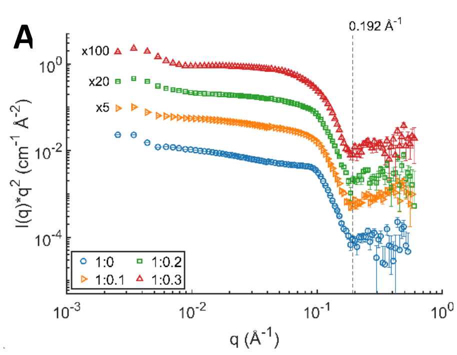
Experimental report for experiment 09-10-1738 (April 2022)

Ceramides (CERs) are one of the main lipids in the Stratum Corneum (SC), and play an essential role on the skin's barrier function. Damage to the SC either by internal or external stimulus results in lower CER levels and compromises its barrier properties[1]. A promising therapy is to restore CERs by delivering them into the skin, being phospholipid (PL) liposomes a promising delivery system. However, limited colloidal stability caused by CER crystallization is one of the main drawbacks for PL:CER assemblies[2]. The aim of our study was firstly, to elucidate the impact of ceramide addition in liposome and membrane structural architecture and secondly, to follow the evolution of particle morphology up until crystal formation to understand the destabilization process of the PL:CER vesicles. Evolution of liposome architecture was studied by means of Small-Angle Neutron Scattering (SANS) and was complemented with neutron diffraction experiments to access higher wavenumber values.

Experimental setup and samples

For this experiment, we prepared liposomes from DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine) and plant-derived phospholipids with a content of >94% phosphatidylcholine (HPC) with and without ceramide NP (CER-NP) in different manners: For DOPC based liposomes, the method employed was the following: DOPC and CER were dispersed in glycerin under heating and thereafter, heavy water was added to the dispersion. The liposomes were extruded with an Avanti mini extruder for 21 times using a 200 nm pore size membrane to reduce particle size and increase colloidal stability. For HPC based systems, samples were prepared as follows: HPC and CER were dispersed in glycerol under heating followed by addition of heavy water. Homogenization was done using a high-performance dispersing instrument (Ultra-Turrax IKA) and at this stage, one aliquot was set apart for characterization. Another aliquot was taken from the homogenized dispersion and sheared by extrusion, following the parameters described above. A series of both DOPC and HPC based liposomes were prepared up to 6 weeks in advance of the experiment and in the consecutive weeks leading to the week of the experiment to study the evolution of particle morphology.

Samples were measured with their respective background at D22 at sample-to-detector distances of 17.6 m and 1.4 m at a neutron wavelength of 6 Å to access information in q between 0.002 1/ Å and 0.64 1/ Å. For all samples, measurements were taken for empty cuvettes, backgrounds, and transmissions. Data reduction was done with the GRASP program. Aged samples were also measured in D16. Measurements were carried out at a neutron wavelength of λ = 4.5 Å and at two detector positions, namely 43.5° and 90° at room temperature.



Results and discussion

Analyzing the SANS curves of DOPC-based 1%w liposomes, it is evident that vesicles composed solely of DOPC are predominantly multilamellar. This is supported by the presence of a correlation peak around q = 0.09 1/Å in Figure 1A. Moreover, the scattering at mid-q conforms to a power law of -2.4, hinting towards the presence of polydisperse multilamellar vesicles.

[1] M. Shao, Z. Hussain, H. E. Thu, S. Khan, H. Katas, T. A. Ahmed, M. Tripathy, J. Leng, H.-L. Qin and S. N. A. Bukhari, Coll. Surf. B, 2016, 147, 475–491.

[2] E. Khazanov, A. Priev, J. P. Shillemans and Y. Barenholz, Langmuir, 2008, 24, 6965–6980.

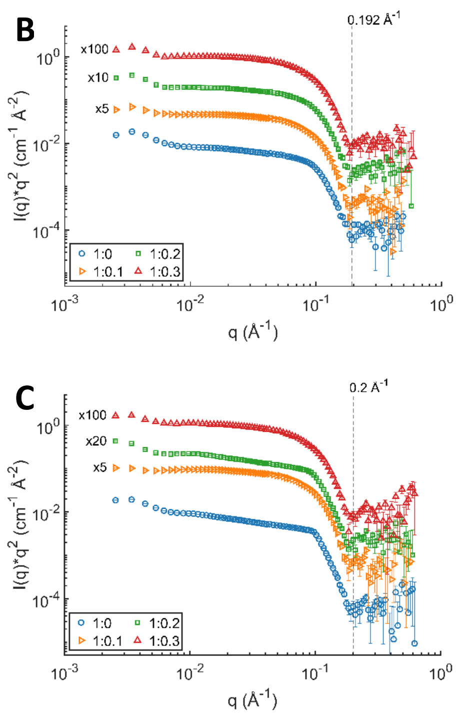


Figure 1. Experimental SANS curves of different phospholipid-ceramide liposome systems. (A) DOPC 1% - CER extruded liposomes, (B) HPC 1% - CER liposomes homogenized with the UltraTurrax, (C) HPC 1% - CER extruded liposomes. The legends indicate the PL:CER ratios

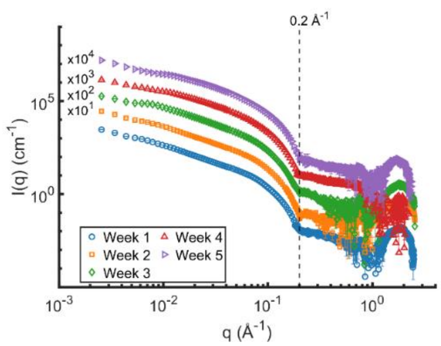


Figure 2.. Results of neutron scattering experiments on extruded and aged DOPC-CER (11 %w) liposomes

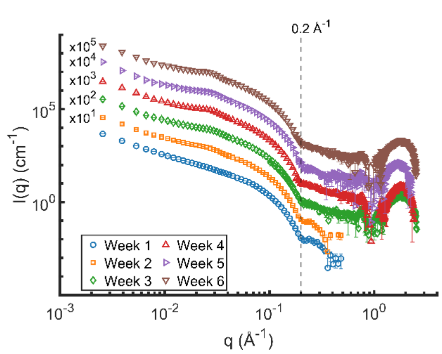


Figure 3. Results of neutron scattering experiments on homogenized (UltraTurrax) and aged HPC-CER (11 %w) liposomes

However, as ceramide (CER) is introduced, liposomes tend to transition towards a more unilamellar structure. This is observed by the smearing out of the correlation peak and a gradual contraction of the power law to 2.2 at the highest ceramide concentration. The same behavior is observed for other DOPC concentrations. In the case of HPC-CER liposomes homogenized using the UltraTurrax, the change in lamellarity is more noticeable when comparing pure HPC vesicles to the 1:0.1 and 1:0.3 HPC:CER mixtures (Figure 1B). As the amount of CER in the sample increases, the vesicles exhibit reduced multilamellarity. Finally, there are no significant structural differences amongst the extruded HPC:CER samples other than increasing polydispersity at higher ceramide content (Figure 1C). The same behavior is observed for samples with 2%w HPC.

Aged PL-CER liposomes

Samples at 10% phospholipid mass ratios were prepared up to 6 weeks prior to the experiment day in order to analyze their structural evolution over time. Joint SANS and neutron diffraction (where available) curves are presented in Figure 2, 3 and 4.

Looking at the scattering curves of DOPC-based vesicles (Figure 2), it is evident that the liposomes exhibit polydispersity as indicated by the smearing of the form factor oscillation near 0.004 1/Å. Additionally, the Guinier plateau is inaccessible in the employed SANS configuration, suggesting the presence of assemblies with an average radius of at least 300 nm. Analyzing the scattering curves in relation to the sample aged for 1 week facilitates the observation of structural changes over time. The increase in scattering intensity near 0.025 1/Å appears as a delicate bump in the SANS curves starting at week 2 and intensifying towards week 5. The q position of this signal suggests a characteristic length of approximately 251 Å, unrelated to vesicle-vesicle correlations. It is possible that over time, assemblies with structures distinct from the core-shell formation develop, potentially contributing to the loss in intensity near 0.08 1/Å. Examining the HPC-CER assemblies reveals a behavior similar to that observed in aged DOPC-CER liposomes, as depicted in Figure 3. In general, the HPC-CER liposomes exhibit high polydispersity, indicated by the absence of form-factor oscillation, and particle sizes larger than those accessed with the SANS configuration (i.e. >300 nm). Interestingly, a discernible bump at q ~ 0.03 1/Å emerges in the sample aged for two weeks and becomes more prominent in older samples. The increase in scattering intensity appears as a peak in the normalized curves, corresponding to characteristic lengths of 209 Å, similar to those observed in DOPC-CER liposomes.

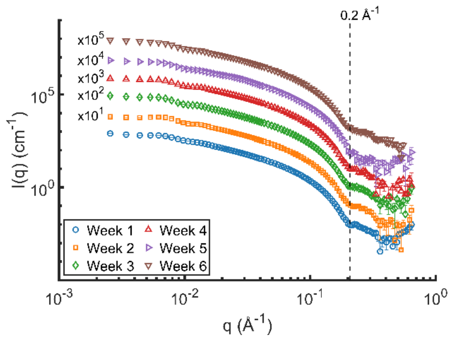


Figure 4. Results of neutron scattering experiments on extruded and aged HPC-CER (11 %w) liposomes

The samples that were also extruded, differ in behavior. Notably, there is an absence of the bump formation (near q ~ 0.03 1/Å) over time, as depicted in Figure 4. This absence indicates that the vesicles maintain higher colloidal stability, as there are no discernible structural changes within the observed 6-week period. These samples were peculiar in that they showed gel-like flow behavior, which could indicate a rearrangement of the liposomes caused by the extrusion. Shearing could have created a system with closed-packed vesicles of higher colloidal stability than the other two formulations discussed so far. This phenomena gives reason to believe that the samples could have experienced the formation of a lamellar phase derived from the colloidal instability of these systems.

Unfortunately, no distinct peak associated with ceramide crystallization is observed in the high-q region of the curves, i.e., above 0.1 1/Å, possibly due to insufficient contrast between the phospholipids and the ceramide crystals.

Addition of a surfactant

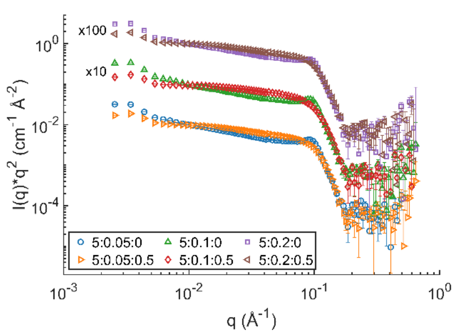


Figure 5. Kratky plots of DOPC:LPC and DOPC:LPC:CER (1%) extruded liposomes. The legend refers to the DOPC:LPC:CER ratios

The introduction of a surfactant with a smaller packing parameter is of interest as it can enhance the colloidal stability of PL-CER fluid bilayers. Here, we present the effect of lysophosphatidylcholines (LPC) on DOPC-LPC extruded liposomes, with or without CER. SANS curves are reported in Figure 5, where it can be seen that DOPC-LPC liposomes are multilamellar in the absence of ceramide. This situation changes by CER addition, as the disappearance of the correlation peaks near 0.1 1/Å would suggest.

Conclusion

The SANS and neutron diffraction experiments provided insights into the structural changes and colloidal stability of phospholipid-ceramide (PL-CER) liposomes. The incorporation of ceramides into DOPC or HPC liposomes results in a reduction in lamellarity, as evidenced by the disappearance of correlation peaks at 0.1 1/Å in the SANS curves. Regarding the aged samples, it is believed that the changes in scattering curves are due to the formation of a lamellar phase with time. This phase change is prevented in HPC liposomes upon extrusion. Finally, there is an evident reduction in lamellarity upon the addition of ceramide in DOPC-LPC liposomes. Such structural changes raise questions about the nature of multilamellarity, since it is possible that other morphologies (lipid bilayer fragments, stacked lamellar phase) are present in the dispersion. To corroborate the nature of the observed lamellarity, additional characterization such as cryo-TEM or SAXS experiments are needed.