Experimental report

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Proposal: 8-02-866 **Council:** 4/2019

Title: Structural investigation of natural deuterated phospholipid multilayers

Research area: Soft condensed matter

This proposal is a continuation of 8-02-811

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Samples: deuterated phospholipids
hydrogenous phospholipids

Instrument	Requested days	Allocated days	From	To
D16	7	7	30/01/2020	06/02/2020

Abstract:

Selective deuteration is a useful strategy to highlight specific sample components in different techniques such as NMR, infrared spectroscopy and neutron scattering techniques. Recently, we optimized a protocol for purifying the different phospholipid classes from the deuterated lipids extracted from yeast cells grown in deuterated culture media. In particular phosphatidycholine (dPC), phosphoethanolamine (dPE), phosphatidylserine (dPS) and Cardiolipin (dCL) lipids were successfully isolated in high yields. During our last experiment on D16, we prepared a lipid multilayer with the extracted dPC lipids and the collected experimental data showed the formation of a highly ordered multilayer. Here we propose to continue the characterization of the purified phospholipids and compare it with the results we previously reported for the total lipid and phospholipid extracts.

Structural investigation of natural deuterated phospholipid multilayers

Introduction. Selective deuteration is a useful strategy to highlight specific sample components in different techniques such as NMR, infrared spectroscopy and neutron scattering techniques. Nevertheless, the production of deuterated phospholipids in high amounts and as different molecular species (i.e. different headgroup and acyl chain compositions) is currently the limiting factor in the full exploitation of these experiments. While the chemical synthesis of deuterated phospholipids is currently limited to fully saturated lipids (with rare exceptions), the extraction of lipids from bacteria or yeast cells grown in deuterated media is a high throughput approach to produce several different deuterated phospholipids. In this context, we previously reported the extraction of deuterated lipids from *P. Pastoris* cells grown in deuterated culture media [1]. In particular, both the total lipid extract, including several phospholipid species, sterols, free fatty acids and triglycerides [2] and the phospholipid fraction [3] were separately characterized by means of Neutron Diffraction (ND) measurements. More recently, we optimized a protocol for purifying the different phospholipid classes from the total deuterated lipid extract and in particular phosphatidycholine (dPC), phosphatidylglycerol (dPG), and Cardiolipin (dCL) lipids. While dPC lipids are efficiently extracted from P.Pastoris cells, dPG and dCL were extracted from E. Coli. During this D16, we prepared lipid multilayers with hPC, dPC, hPG and dPG lipids and characterized their structure as reference. Subsequently we characterized multilayers prepared with mixtures of PC and either PG and CL in both cases with 70/30 mol/mol.

Experimental section. Lipid films corresponding to 2 mg of hydrogenous or deuterated lipids were prepared by drying proper amount of lipid solution in 1:4 chloroform: methanol on the bottom of a glass vial under nitrogen. The film was re-dissolved with 600 μl of ultrapure water and sonicated for ~5min with a tip sonicator to obtain a homogenous sample. The vesicle suspension was deposited on freshly cleaned quartz slides and subsequently dried, and treated with 3 cycles of 2h in vacuum at 45°C and 2h at 98% RH and 45°C. The multilayers were equilibrated at 97%RH for 24h and then placed in the humidity chambers, aligned, and equilibrated at 57% Relative Humidity (RH) for 12h. After the measurements in this condition, the samples were further equilibrated at 98% (12h) and the corresponding diffraction data were collected. The reservoir of the humidity chamber was filled with H_2O for the deuterated multilayers and D_2O for the hydrogenous multilayers to guarantee the best contrast between the lipids and the hydration water. Diffraction data were collected at detector angle (γ) 11 deg by scanning the sample angle (ω) in the range -1:8 deg, with a step of 0.05 deg. Data reduction was carried out with the ILL software Lamp. The efficiency of the detector was considered during data treatment by loading the proper calibration file in Lamp. The reduced detector images were integrated in the ω range corresponding to the observed diffraction peaks in order to obtain intensity vs 2θ plot. The positions of the Bragg peaks in the plots were determined by fitting the peaks with a Gaussian function. Figure 1 shows the data collected for the hPG multilayer at the two explored RH values. Similar results were also obtained for the dPG multilayer. Co-existence of two different lamellar phases at 57% RH, while a single lipid phase dominated the diffraction profile at 98% RH. This observation is in agreement with previously observed results for PC multilayers (8-02-844).

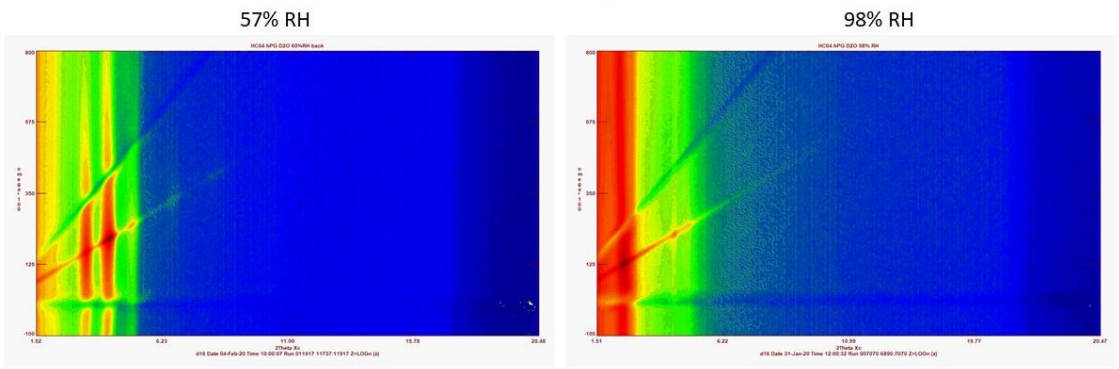

Figure 1: 2D detector images collected for the hPG multilayer at 57% RH and 98 % RH.

Interestingly the d-spacing calculated for both humidity conditions resulted to be substantially larger than the one calculated in the case of the dPC multilayer. In particular, at 98% RH the d-spacing for dPG and hPG resulted to be respectively 80Å and 100Å while 61Å and 64Å were observed for hPC and dPC respectively. Difference in the d-spacing between dPC and dPC, as well as dPG and hPG, are due to a difference in acyl chain composition of the deuterated lipids compared to the corresponding hydrogenous version. Indeed, deuterated lipids both in the case of PC and PG headgroups are characterized by a higher content of C18 acyl chain, which induce thicker lamellae in the multilayer. Interestingly the difference between the d-spacing of PC and PG multilayer suggests that the PG multilayer might be characterized by a thicker water layer between the lamellae compared to PC and most likely due to the electrostatic repulsion between the headgroups of difference lamellae. We also attempted to prepare multilayers with PC and either PG or CL (70/30 mol/mol) in their hydrogenous and deuterated version. Unfortunately all the prepared samples, showed a very low order and indeed in most of the cases only one diffraction peak was observed. The very low ordering of these multilayers cannot be ascribed to the combination of two different headgroups, since ordered multilayers were previously produced with the entire *Pichia Pastoris* phospholipid extract, where several different phospholipid species were simultaneously present. More reasonably we believe that the PG and CL lipids, which mainly contain C14 and C16 acyl chains, add further acyl chain species compared to the PC lipids, which mainly contain C16 and C18 acyl chains. The increased heterogeneity of the acyl chain composition in the PC/PG and PC/CL mixtures might be the cause for the low ordering of these multilayers.

Conclusions. In this experiment, we performed the characterization of new natural phospholipid multilayers, hPG and dPG extracted from E. Coli, and compared their structure with hPC and dPC multilayers extracted from Pichia Pastoris, and used in this study as a reference. The PG multilayers exhibited a structural organization in different lipid phases as also observed for the PC multilayers. However, the PG multilayers are characterized by a much larger d-spacing compared to the PC multilayers and most likely associated to the increased repulsion among the lamellae, which favors the formation of thicker water layer. Further investigation is required to characterized the PC/PG and PC/CL mixtures.

References: [1] A. De Ghellinck et al., PloS One, 2014; [2] A. Luchini et al., Colloids and Surfaces B, 2018. [3] A. Luchini et al., Chemistry and Physics of Lipids, 2020.