Experimental report

21/05/2017

Proposal: 8-02-796 **Council:** 10/2016

Title: Deuterated cholesterol and final contrasts for completeness of the investigation of AmB mechanism withinnatural yeast biomembranes.

Research area: Biology

This proposal is a continuation of 8-02-746

Main proposer: **Robin DELHOM**

Experimental team: Robin DELHOM
Giovanna FRAGNETO

Local contacts: Giovanna FRAGNETO

Samples: Silicon Blocks
Natural Pichia pastoris lipids (Deuterated)
Natural Pichia pastoris lipids (hydrogenous)
Deuterated cholesterol

Instrument	Requested days	Allocated days	From	To
D17	3	3	30/01/2017	02/02/2017
FIGARO	3	0		

Abstract:

It is widely accepted that the activity of Amphotericin B (AmB) results from its specific interaction with ergosterol. We have carried out a detailed characterization of the lipid composition as well as the structural consequences of AmB over native fungal extracts from Pichia pastoris. The structure of yeast membranes differs considerably from typical model lipid bilayers composed of synthetic lipids. AmB inserts in yeast membranes both in the absence and presence of ergosterol, and our results confirmed directly that AmB forms a thick extramembranous aggregate, proposed recently to act as an ergosterol-extracting sponge, only in presence of the complex natural lipid mixture.
We propose to make advantage of the new fully deuterated cholesterol available from D-Lab in order to complete our measurements and quantify the differences of AmB action between ergosterol and cholesterol-containing reconstituted biomembranes, proposed to be the origin of AmB toxic side effects in mammalian cells. We will also investigate the impact of the surrounding apolar lipids that are present in addition to the phospholipids in biological membranes, by performing two yet untried contrasts.

Experimental Report
D17 - 8-02-796

Background:

The activity of Amphotericin B against systemic fungal infections is widely accepted to result from its specific interaction with ergosterol. We have recently carried out a detailed characterization of the lipid composition and structure of native fungal membrane extracts from *Pichia pastoris*, as well as the consequences of AmB action. The structure of yeast membranes differs considerably from typical model lipid bilayers composed of synthetic lipids and depends on the degree of lipid polyunsaturation. AmB inserts in yeast membranes both in the absence and presence of ergosterol, and our results confirmed directly that AmB forms a thick extramembranous aggregate, proposed recently to act as an ergosterol-extracting sponge. We proposed to complete our investigation of P. pastoris membranes by quantifying the effect of the different lipid fractions (polar/non-polar lipids, sterols) on the ergosterol and cholesterol extraction and AmB insertion by using membranes where the lipid fractions and sterols are selectively labelled.

Physiologically, natural yeast membranes are functional at the growth temperature (in our case 30°C) and are composed of around 15-20mol% of ergosterol. The viability of the cells depends on its membrane composition, and the strong antifungal agent amphotericin B (AmB) is known to interact with the ergosterol and induce fungal or protozoal cell death. Moreover, due to its preference to ergosterol, mammalian cells containing cholesterol as main sterol, are less impacted, explaining the use of AmB as antifungal treatment. In our experiments and studies, we perform deposition of bilayers of yeast lipids by vesicle fusion, allowing us to use different compositions in order to investigate the influence of sterol content in the mechanism of AmB. Most of the studies are performed with simple model membranes, composed of one phospholipid molecular species and sterol content, when we use a natural mixture of phospholipids, hydrogenated and deuterated, to which we add sterol content. This experiment aimed at investigating the extraction of ergosterol by AmB and at the same time, the mechanism inducing toxic side effect, by interaction with cholesterol.

Results:

We measured 4 different samples both before and after AmB addition during the three days allocated on D17, as well as two other bilayers for structural characterization only – no AmB involved. The mixtures were sonicated in the corresponding water solution (D_2O or H_2O) containing 100mM of NaCl and 20mM of $CaCl_2$, before being injected in the sample cells at a controlled temperature of 52°C. The nature of the water, light or heavy, was selected in order to match the overall density of the lipid mixture. Thus, a fully deuterated sample was prepared in a D_2O solution, when a partially hydrogenous sample (ex h-Polar d-Erg*) was prepared in H_2O. Note that the deposition was performed in a horizontal geometry, offline, the supporting block being place below the lipid-containing solution. For the last two samples – missing from proposal 8-02-775 and performed in the spare time of this experiment – the deposition has been ‘forced’ as previous configuration did not lead to any deposition. As already mentioned in precedent reports, the mismatch of the nature of the water solution can be used in order to ‘force’ the lipids to interact with the surface of the block used. For deuterated lipids, the combination of the use of a light water solution and a configuration where the block seats below the solution gives better deposition (even multilayers if timing is not controlled). For hydrogenous sample, the reverse combination; heavy water and block above the solution gives similar results.

The listed samples in Table 1. were performed. Four contrasts could be achieved either before and after AmB injection. This assure good reliability of the model obtained from fitting the data. Unless difficulty of deposition of the last two samples, resolved as explained above, the instrument and the experiment was successful and use of neutron has been optimized at best.

Table 1: Samples performed during the 3 days on D17

Samples (30mol% sterol)		Reason
h-Polar d-Cholesterol		Impact of deuterated cholesterol – Improved contrast
d-Polar d-Cholesterol		
d-Polar h-Ergosterol		Repeat previous measurements
d-Polar h-Cholesterol		
h3Q10		Complete 8-02-787
d3Q10		

*Polar stands for Polar fraction (phospholipids), h3 correspond to the hydrogenated total C. *glabrata* extract when d3 correspond to the deuterated version. Q10 stands for co-enzyme Q10. h & d are the indication of hydrogen or deuterium-labelled molecules.

The collected data have to be analysed together with the complementary data obtained over the past two years in the different experiments performed at ILL. Nevertheless, early fitting showed good depositions (water in hydrophobic tails less than 4% for all samples) as well as good adequacy with previous model established with corresponding, i.e. lipids and a given sterol, independently of the labelling of each of the parts. The use of deuterated cholesterol allows us to affine the quantification involved and could lead us to understand better the selectivity of AmB with ergosterol more than for cholesterol.

The data obtained (see Figure 1.) show good statistics and support the idea that the headgroup region, mainly the one facing the solution is impacted by AmB. Indeed, the cholesterol seem to be present in the headgoup region as the deuterated cholesterol gives better contrast. A fact not observe, or to very limited proportions, with ergosterol. Completeness of fittings will allow us to demonstrate if the presence of cholesterol at the interface with solvent can explain the relative protection towards AmB.

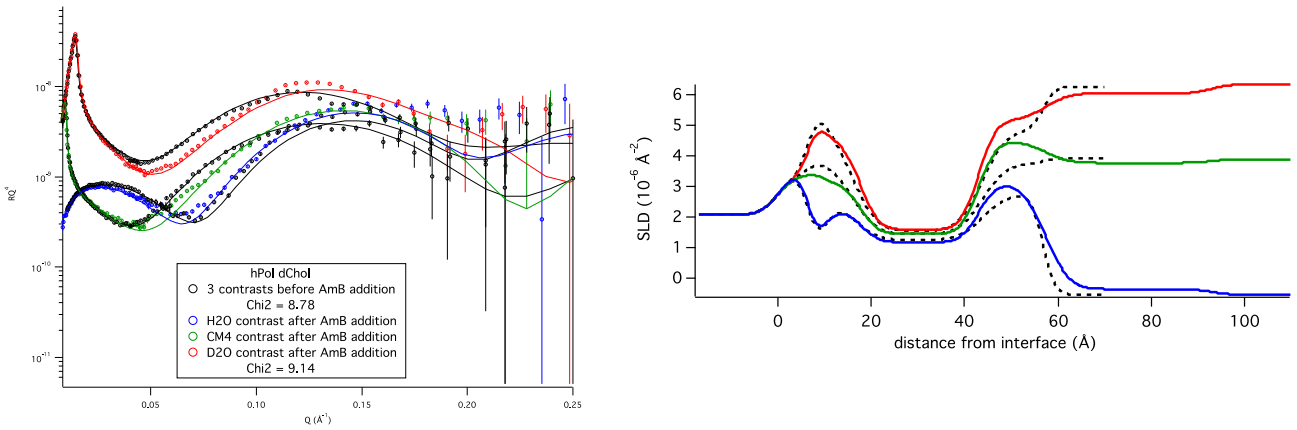

Figure 1. h-Polar d-Cholesterol; A) black curves correspond to contrasts performed before AmB addition and coloured ones to contrasts measured after AmB addition. B) Scattering length density profiles obtained from fittings.

References:

1. Production and analysis of perdeuterated lipids from *Pichia pastoris* cells, de Ghellinck A., Schaller H., Laux V., Haertlein M., Sferrazza M., Marechal E., Wacklin H., Jouhet J., Fragneto G., PloS One, (2014), 9, e92999-1-e92999-9
2. Lipid polyunsaturation determines the extent of membrane structural changes induced by Amphotericin B in *Pichia pastoris* yeast, de Ghellinck A, Fragneto G, Laux V, Haertlein M, Jouhet J, Sferrazza M, Wacklin H. Biochim Biophys Acta. 9, 2317-2325 (2015)
3. Anderson, T.M. et al. Amphotericin forms an extramembranous and fungicidal sterol sponge. Nat Chem Biol 10, 400-406 (2014).