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The impact of deuteration on natural and synthetic lipids: A neutron diffraction study

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ABSTRACT

The structural investigation of cellular membranes requires access to model systems where the molecular complexity is representative of the cellular environment and that allow for the exploitation of structural techniques. Neutron scattering, and in particular neutron diffraction can provide unique and detailed information on the structure of lipid membranes. However, deuterated samples are desirable to fully exploit this powerful method. Recently, the extraction of lipids from microorganisms grown in deuterated media was demonstrated to be both an attracting route to obtain complex lipid mixtures resembling the composition of natural membranes, and to producing deuterated molecules in a very convenient way. A full characterization of these deuterated extracts is hence pivotal for their use in building up model membrane systems. Here we report the structural characterization of lipid extracts obtained from *Pichia pastoris* by means of neutron diffraction measurements. In particular, we compare the structure of membranes extracted from yeast cells grown in a standard culture medium and in a corresponding deuterated culture medium. The results show that the different molecular composition of the deuterated and protiated lipid extracts induce different structural organization of the lipid membranes. In addition, we compare these membranes composed of extracted yeast lipids with stacked bilayers prepared from synthetic lipid mixtures.

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1. Introduction

Cellular membranes are complex systems involving different lipid species as well as proteins. Lipids contribute to the membrane structural scaffold, however, recent studies have clearly shown that their role is not limited to only providing a fluid matrix in which membrane proteins are embedded [1]. Indeed, specific lipid-lipid interactions as well as lipid-protein or protein-protein interactions play a pivotal role in the biological function of cellular membranes [2,3]. With the aim of understanding the complex mechanisms occurring at the edges of every living cell, physico-chemical properties of complex lipid membranes are nowadays increasingly investigated [4,5]. The fluid-like nature of these systems prevents the characterization of their atomic-level structure by means of conventional crystallography techniques. However, other techniques such as X-ray and neutron reflectometry have provided detailed structural information on lipid membrane structure along the bilayer surface normal [6–9].

Since the early history of membrane models, x-ray and neutron diffraction from the nanometer-scale structure of lipid multilayers has been intensively exploited [10–17]. Indeed, detection of Bragg peaks arising from the multilayer architecture allows direct determination of the membrane repeat distance. In addition, in the case of neutrons, the H/D contrast variation approach [6] can be used to extract the nuclear density distribution across the membrane from the diffraction data. The great success of neutrons to probe biological samples relies on their non-destructive and highly-penetrating nature, and their ability to distinguish hydrogen isotopes, e.g.

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protium (H) and deuterium (D). The highly different scattering cross-section of these two isotopes allows for the powerful isotopic labelling method used for most of the neutron scattering experiments. Besides neutron scattering techniques, deuterium-labelling is also widely used in NMR experiments, and thus represents an attractive approach for different scientific communities [18].

Mixtures of one to three predominant lipid molecular species, mainly synthetic lipids, are widely used as a preferred tool in neutron diffraction studies of model membranes [19–25]. Such systems have enabled many detailed investigations of the role and impact of phospholipid classes and/or the unsaturation of lipids on membrane structure and properties [26–28]. In addition, sterols – in particular cholesterol – have been targeted due to their impact on membrane fluidity and their importance in many biological processes related to health [29,30]. Among all PC and PC/Chol bilayers represent commonly recognized references to reproduce the main features of cellular lipid membranes [29–31].

More recently, complex mixtures of lipids extracted from natural sources have been successfully characterized by means of neutron diffraction. In particular, it is worth mentioning the characterization of stratum corneum (SC) lipid matrices as well as plant lipids [32–36]. These highly organized systems provide useful information on the phase behavior of relevant biological interfaces.

However, the need for deuteration of phospholipids, sterols and other lipid constituents of biological membranes, with the aim to take full advantage of neutron contrast variation, has been the limiting step for further complex investigations [37]. The growth of high-density cell cultures in deuterated media as natural lipid sources has resulted in a powerful route to overcome this limit and allows the production of complex membrane models without the need of sophisticated chemical synthesis of the individual labelled compounds [38,39].

Direct extraction of lipids from cells results in complex mixtures which can be used to build membrane models closer to real systems [40–43]. Indeed, lamellar lipid bilayers can be prepared by using lipids extracted from microorganisms [44]. These systems not only provide relevant information on the physicochemical properties of the real cellular lipid mixtures but also represent a more realistic platform for the characterization of the interaction between membranes and proteins or biomedical systems, i.e. nanoparticles and drugs [45,46].

In this work, we used neutron diffraction to investigate and compare the multilamellar organization of protiated (h-) and deuterated (d-) phospholipid mixtures extracted from *Pichia pastoris* according to the protocol reported elsewhere [39]. This microorganism was already successfully used in a previous neutron diffraction study, where the structure of the overall lipid extract was analyzed [40]. Here we focus our attention particularly on the phospholipid portion of the lipid extract. Results collected on the phospholipids extracted from *Pichia pastoris* are compared to those obtained from mixtures of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and cholesterol, the sterol being either fully protiated or fully deuterated. POPC and POPC/Chol are commonly selected to mimic the lipid portion of cellular membranes; the present experiments aimed at highlighting overall differences and similarities with respect to more complex lipid mixtures, which are closer to the composition of real membranes.

2. Methods

2.1. Chemicals

Protiated and deuterated total lipid extracts (h or d-total extract) were obtained from *Pichia pastoris* yeast cells grown in H_2O or D_2O media as described below. Further purification allowed isolation of the respective protiated and deuterated natural phospholipids (h or d-phospholipids). 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and cholesterol (h-Chol) were purchased from Avanti Polar Lipids (US), the lipid standard 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) and all the organic solvents were purchased from Sigma Aldrich (France) and used without further purification. 1,2-Diacyl-*sn*-glycero-3-phospho-L-serine sodium salt from bovine brain (PS) and cardiolipin sodium salt from bovine heart (CL) were purchased from Sigma Aldrich (France), dissolved in H_2O and extracted in chloroform before being used as TLC lipid standards. Analytical grade (≥99% purity) protiated methyl pentadecanoate was purchased from Larodan (Sweden) and deuterated methyl pentadecanoate was synthesized by the STFC Deuteration Facility at the ISIS Neutron and Muon Facility in Didcot (UK). H_2O was produced from a purifying system (MilliPore; resistivity >18 MΩcm) and D_2O (>99% purity) was purchased from Sigma Aldrich (France). Deuterated cholesterol (d_{46}Chol,>98% purity) was produced at the Deuteration Laboratory of the Institut Laue-Langevin (D-Lab) and used as received.

2.2. Sample production

Cell cultures were grown and lipid were extracted as described in de Ghellinck et al. [39]. Briefly, harvested freeze-dried yeast cells were poured in boiling ethanol in order to prevent lipase activation. A classical Folch extraction was then executed [47] via addition of methanol and chloroform. The remaining cell pellets were filtered through glass wool and rinsed with chloroform/methanol solution 2:1, v/v. After addition of NaCl 1% and decantation, the recovered organic phase containing the lipid extract was dried under argon and kept at −20 °C during storage. The obtained samples correspond to the total extracts, containing mainly phospholipids but also sterols, steryl-esters, free fatty acids and triglycerides. In order to isolate the phospholipid fraction, part of the total extracts obtained underwent a flash column chromatography containing silica (Roth, mesh size 0.04-0.063) equilibrated with a chloroform/acetic acid solution, 100:1, v/v. This solution was used in order to elute the nonpolar lipids listed above. Once all apolar fractions eluted, methanol was used as eluent in order to recover the phospholipid fraction. Thin Layer Chromatography (TLC, Merck) with chloroform/methanol/water (65:25:4, v/v) as solvent system, and I_2 as the detecting agent, was used to check the quality of separation.

2.3. Lipid analysis

The phospholipids were dissolved in the minimum amount of chloroform and the equivalent of 200 μg of lipids was deposited on a 20 × 20 cm glass plate coated with silica (silica gel 60, Merck) using a Hamilton syringe. Two-dimensional thin layer chromatography (2D-TLC) was performed with chloroform/methanol/water (65:25:4) as the first solvent system. The plate was then dried under a stream of argon, and rotated of 90° before the second elution was performed with chloroform/acetone/methanol/glacial acetic acid/water (50:20:10:10:5). Lipids were visualized by using I_2. The marked spots, assigned with respect to standard lipids (POPC, POPE, PS and CL), were scraped off the TLC plates. The recovered silica, corresponding to each phospholipid class, was placed in capped glass tubes to which a precise amount of internal standard, protiated methyl pentadecanoate was added. Hydrolysis of the phospholipids and methylation of the fatty acids was then performed by the addition of 3 mL of H_2SO_4 in methanol (2.5% in volume), and incubation at 100 °C for one hour. The reaction was quenched by addition of 3 mL of water, and 3 mL of *n*-hexane was finally added in order to extract the fatty acid methyl esters (FAMEs) formed. The extraction

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