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Solid-State NMR of highly ^{13}C-enriched cholesterol in lipid bilayers

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ABSTRACT

Cholesterol (Chol) is vital for cell function as it is essential to a myriad of biochemical and biophysical processes. The atomistic details of Chol's interactions with phospholipids and proteins is therefore of fundamental interest, and NMR offers unique opportunities to interrogate these properties at high resolution. Towards this end, here we describe approaches for examining the structure and dynamics of Chol in lipid bilayers using high levels of ^{13}C enrichment in combination with magic-angle spinning (MAS) methods. We quantify the incorporation levels and demonstrate high sensitivity and resolution in 2D ^{13}C-^{13}C and ^{1}H-^{13}C spectra, enabling *de novo* assignments and site-resolved order parameter measurements obtained in a fraction of the time required for experiments with natural abundance sterols. We envision many potential future applications of these methods to study sterol interactions with drugs, lipids and proteins.

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1. Introduction

Sterols are essential in numerous biochemical and biophysical processes inside the cell. Cholesterol (Chol), the major sterol present in mammalian cells, is a key regulator of membrane order, permeability, thickness and lateral organization [1–3] and ultimately membrane protein function [1,4,5]. The regulatory roles of Chol depend directly upon its atomistic interactions with other sterols and phospholipid molecules [6–8]. In fact, Chol has also recently been implicated to have an essential role in HIV-mediated viral fusion [9] and ligand binding leading to apoptosis [10]. The orientation and dynamics of Chol were demonstrated to be essential for its regulatory roles [11,12], although molecular details of the mechanism are still lacking. Additionally, the atomistic specificity of sterol interactions with a variety of proteins is of increased interest, especially because Chol is a major component of mammalian membranes (typically 50 mol%) [13]. For example, Chol interacts with the amyloid precursor protein and may play a key role in amyloidogenesis related to Alzheimer's disease [14]. These many applications underscore the broad significance of impactful methods to study this ubiquitous molecule in its native bilayer environment.

Much of what is known about the detailed structure and dynamics of Chol in lipid bilayers comes from experimental NMR studies, combined with molecular dynamics (MD). Extensive ^{2}H and ^{13}C studies of Chol have reported order parameters and restraints on the orientation in the bilayer from ^{2}H NMR [15–27] and ^{13}C NMR [28–30]. However, previous NMR dynamics investigations focused on a limited number of labeled sites [15–25] and/or used bulky analogs of Chol [31,32], which are known to behave differently in membranes than the native sterol [2,33]. Additionally, MD simulations of Chol in bilayers have been utilized to ascertain the orientation and fast timescale dynamics (usually <100 ns) at some sites [11,34]. Unfortunately, the rigorous comparison of MD with NMR data has often been limited to the A ring and tail of Chol, due to the relative ease of labeling these portions of the molecule [29]. For example, the most readily available commercial

Abbreviations: Chol, cholesterol; MAS, magic angle spinning; MD, molecular dynamics; SSNMR, solid-state nuclear magnetic resonance; CTUC COSY, constant-time uniform-sign cross-peak correlation spectroscopy; ALT, average Liouvillian theory; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; NAV, N-acetyl valine.

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versions of ^{2}H and ^{13}C-Chol are (3,4-$^{13}C_2$), (2,3,4-$^{13}C_3$), (23,24,25,26,27-$^{13}C_5$), (3-D_1), (6-D1), (7-D1), (2,2,3,4,4,6-D_6) or (25,26,26,26,27,27,27-D_7) (from Cambridge Isotope Laboratories, Sigma Aldrich, and Avanti Polar Lipids). Highly enriched Chol would potentially enable more complete global analyses of structure and dynamics, as has been demonstrated to be critical for the high-resolution determination of membrane protein structures.

Here we provide methodological contributions into this active research field including: (1) a cost-effective production of highly ^{13}C-enriched Chol on the >10 mg scale; (2) quantitative analysis of labeling patterns for uniform and 2-^{13}C-acetate forms; (3) collection and assignment of multidimensional scalar and dipolar magic-angle spinning (MAS) solid-state NMR (SSNMR) spectra; and (4) determination of ^{1}H-^{13}C dipolar order parameters.

2. Materials and methods

2.1. Cholesterol biosynthesis

2.1.1. Growth

Several yeast extract, peptone and dextrose (YPD) plates were prepared using 10 g/L of yeast extract, 20 g/L of peptone, and 20 g/L of agar. Water was then added to this mixture and autoclaved for 30 min. The mixture was cooled for about 20 min and 50 mL of 40% dextrose solution was added. The plates were then poured and left to cool and solidify. Once the plates solidified, the Rh6829 strain [35] was streaked onto several plates and left to incubate for 24–48 h until single colonies were visible. Approximately 1 L of media was prepared for the inoculation of the cell colonies. The media consisted of 950 mL milliQ water, 40 mg uracil, 40 mg leucine, 0.90 g ^{13}C-sodium acetate, 7 g yeast nitrogen base with amino acids, and 5 g yeast extract. The mixture was autoclaved for 30 min and 50 mL of 40% sterile-filtered dextrose solution was subsequently added to the mixture. 5 colonies of the Rh6829 strain were added to 1 mL of media. 1 mL of the inoculated media was poured back into the 1 L cultures. The 1 L cultures were then incubated at 30 °C for 48–72 h until the mixture was confluent.

2.1.2. Harvesting

Once confluent, the 1 L cell cultures were spun down at 3000*g* for 30 min, 950 mL of the media were poured off and the cells were re-suspended in approximately 50 mL of the remaining media. The re-suspended cell mixtures were transferred to 50 mL conical vials and were then spun down in a centrifuge at 3000*g* for 5 min. The supernatant was discarded and the cells were re-suspended in 50 mL milliQ water and were then spun down at 3000*g* for 5 min, (repeated 3 times). After pouring off the water from the final wash, 15 mL of 0.1 M HCl was added to each vial. The mixtures were vortexed and added to a 1 L round bottom flask. The cells were heated at 90 °C and stirred at 700 rpm for 1 h. The mixture was then transferred to a 5 L 3-neck round bottom flask. The 1 L round bottom flask was rinsed with 300 mL of 200 proof ethanol and the solution was transferred to the 5 L 3-neck flask. Additionally, the 1 L round bottom flask was rinsed with 1 L of 50% aqueous KOH and transferred to the 5 L flask. The mixture was stirred at 150 rpm and refluxed for 1 h. After allowing the flask to cool to ambient temperatures, the mixture was transferred to a 4 L separatory funnel. The 3-neck flask was rinsed with 400 mL petroleum ether and poured into the separatory funnel. The organic layer was collected and aqueous layer was rinsed with petroleum ether (4 × 400 mL). The organic layers were combined, dried with sodium sulfate and filtered into a round bottom flask. The solvent was removed under reduced pressure to *ca.* 5 mL and transferred to a 40 mL IChem vial and the volatiles were removed *in vacuo* and the sample was dried overnight.

2.1.3. Purification

The crude material after harvesting is light tan (~70% pure). Purification was carried out by preparative HPLC using an Agilent Prep-C18 10 μm, 30 × 150 mm column at 4 mL/min, with detection at 282 nm. The labeled cholesterol was dissolved in ethyl acetate and filtered into an HPLC vial. A mixture of isocratic acetonitrile: ethanol (70:30) was used as the mobile phase. The retention time was about 20 min. Once the sample has been retained after purification, the solvent was removed under reduced pressure yielding a colorless solid, >95% pure. The sample vial was stored at −20 °C.

2.2. SSNMR data collection & analysis

2.2.1. ^{13}C-^{13}C Constant-time uniform-sign cross-peak (CTUC COSY) [36] NMR spectra

750 MHz (^{1}H frequency) spectra were acquired at 20 °C. Spectra were processed using NMRPipe [37] and analyzed using the Sparky program (3.115) for peak-picking [38].

2.2.2. $R48_3^{18}$–Symmetry NMR experiments

600 MHz (^{1}H frequency) $R48_3^{18}$ 2D ^{1}H-^{13}C dipolar recoupling spectra were acquired at 20 °C, and a MAS rate of 13.051 kHz; 36 points were collected in the dephasing dimension, for a maximal dephasing period of 1.38 ms. Spectra were processed using NMRPipe [37] and analyzed in the Sparky program for peak picking. NMRPipe was used to extract out the time-domain trajectories.

2.2.3. $R48_3^{18}$–Symmetry data analysis

The time-domain dipolar coupling dephasing trajectories were analyzed using an in-house model-free fitting program, Iota_MF (written in Python), which Fourier transforms the data to the frequency domain prior to fitting. The Iota_MF program analyzes the data by Average Liouvillian Theory (ALT). The resulting simulated fits yield a scaling factor and angle for each coupling as well as an overall relaxation value. Line shapes were first fitted using only directly attached protons.

2.3. Lipid bilayer reconstitution for solid-state NMR

SSNMR samples were prepared in approximately 20 mg batches of either 10:3 or 40:1 (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC): U-^{13}C Chol. ^{13}C-Chol was added to the lipid (Avanti, 850457C, 10 mg/mL in chloroform), swirled until dissolved, and dried under a flow of nitrogen or argon gas. The lipid-sterol film was then placed under high vacuum overnight. Freshly prepared HEPES buffer of pH 7.0 and water were added to the dry film, which was subsequently vortexed (1 min) and sonicated (3 min). The solution then underwent 5 freeze-thaw steps that included freezing the sample vial in liquid nitrogen and thawing under running water. The solution was frozen a sixth time and immediately placed in the lyophilizer overnight to yield a white powder. After lyophilization, the sample container was flushed with argon before removing the powder and recording its final mass, and the powders were stored under argon. The powders were packed into 3.2 mm standard rotors and hydrated to ~33% by mass with deionized water.

2.4. Determining ^{13}C labeling efficiency using quantitative solution NMR

To ascertain the labeling percentage, quantitative proton and carbon spectra were collected. The quantitative ^{1}H NMR spectrum was collected without ^{13}C decoupling, in order to observe the

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