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^{17}O NMR spectroscopy as a tool to study hydrogen bonding of cholesterol in lipid bilayers†

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Cholesterol is a crucial component of biological membranes and can interact with other membrane components through hydrogen bonding. NMR spectroscopy has been used previously to investigate this bonding, however this study represents the first ^{17}O NMR spectroscopy study of isotopically enriched cholesterol. We demonstrate the ^{17}O chemical shift is dependent on hydrogen bonding, providing a novel method for the study of cholesterol in bilayers.

Cholesterol is an important constituent of lipid bilayers both in biological and synthetic membranes. It modifies bilayer fluidity and rigidity and can lead to the formation of phase separated or micro domains.[1–3] Additionally, it is a key component of liposomal formulations for drug delivery, as it reduces drug leakage.[4] Cholesterol is capable of forming hydrogen bonds with other lipids and water through the hydroxyl (3β-OH) group. Understanding the nature of these bonds is crucial to understanding membrane structure and bilayer physical properties.[5,6]

In a phospholipid bilayer cholesterol is orientated perpendicular to the plane of the bilayer, with the hydroxyl group aligned with the carbonyl of the ester linkage in phospholipids.[7,8] There have been multiple modes of hydrogen bonding proposed, some of which can be seen in Fig. 1. The two main proposed modes of cholesterol to lipid hydrogen bonding: a direct lipid-cholesterol hydrogen bond, and hydrogen bonding through a bridging water molecule.[6,9,10]

Hydrogen bonding of cholesterol in membranes has been investigated using several techniques including: infrared and Raman spectroscopy;[11–14] molecular dynamics;[9,10,15,16] and NMR.[16–20] NMR spectroscopy studies are particularly useful due to the detailed molecular picture they can provide. These usually rely on nuclei not directly involved, such as ^{13}C or on atoms that are exchangeable such as in the case of proton NMR.[16–18] ^{17}O NMR spectroscopy has been used to great effect in investigations of hydrogen bonds in both organic small molecule and biological systems.[21–24] Observing ^{17}O rather than the ^{1}H nucleus can lead to more accurate results due to the non-exchangeable nature of the oxygen site, and wide chemical shift range.[25] However, there have been no reports of cholesterol hydrogen bonding using ^{17}O NMR spectroscopy, mainly owing to the difficulties of observing unenriched

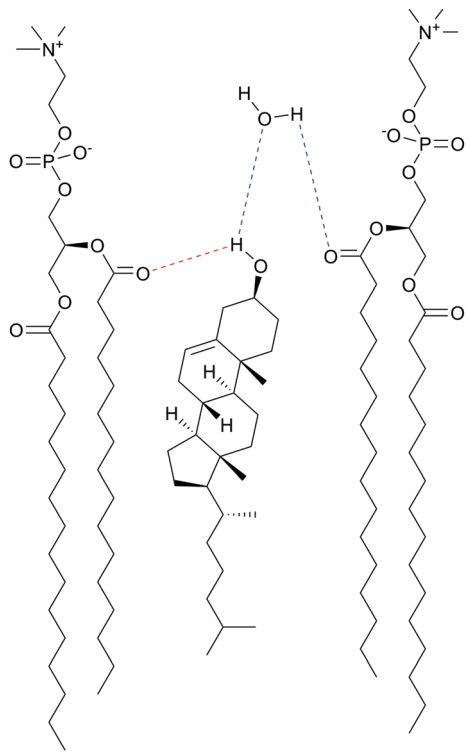

Fig. 1 Diagram of previously proposed models of cholesterol hydrogen bonding, blue lines indicate hypothetical water–bridge bonds and red indicates direct lipid–cholesterol hydrogen bonds.[6,9,10]

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Table 1 Solution state ^{17}O NMR chemical shift and FWHM values

Solvent	^{17}O NMR chemical shift (ppm)	Full width half maximum (kHz)
Toluene	31.2	2.37
Acetone	33.3	1.30
Chloroform	35.3	2.48

cholesterol (^{17}O natural abundance = 0.0373%), and the practicalities of measuring quadrupolar NMR. In this work ^{17}O enriched cholesterol was synthesised using previously published conditions,[26] and utilised in solid state ^{17}O NMR spectroscopy studies of hydrogen bonding. This exploits the single oxygen site on the cholesterol to provide information about hydrogen bonding. The chemical shift of ^{17}O cholesterol in three different lipid bilayers was investigated. Under MAS regimes, the chemical shift can be observed to change depending on the other lipid moiety in the bilayer, which indicated the extent of hydrogen bonding within the bilayer.

The isotropic ^{17}O chemical shift of the enriched cholesterol in toluene, acetone, and chloroform are shown in Table 1. Previously, it has been reported that the ^{17}O NMR chemical shift of cholesterol in acetonitrile at 75 °C was 38.8 ppm with a full width half maximum of 0.7 kHz.[27] However, this temperature is not physiologically relevant and is often incompatible with some phospholipids. Therefore, the chemical shifts in this instance were measured at room temperature. The chemical shift is consistent with previous reports and shows the ^{17}O NMR chemical shift of cholesterol is between 30–40 ppm with a small solvent dependence. The spectrum of cholesterol in acetone exhibits a narrower line width than in chloroform and toluene. This may be related to cholesterol self-association. Previous reports have indicated that cholesterol can form dimers in chloroform and toluene.[28,29] However, acetone is a proton acceptor, and can interrupt self-association by forming hydrogen bonds with cholesterol, leading to smaller line widths.

The solid state NMR spectrum of the anhydrous cholesterol is shown in Fig. 2. The anhydrous nature of the cholesterol was determined from microscope images (S1, ESI†), as anhydrous crystalline cholesterol forms needle shaped crystals.[30] In the NMR spectrum of the anhydrous cholesterol, the characteristic quadrupolar lineshape can be seen. Fitting of this peak reveals the quadrupolar coupling constant (C_q) is 9.1 MHz, the asymmetry constant (η) is 0.76 and the isotropic chemical shift is 34.6 ppm. As this is the only known example of a ^{17}O NMR spectrum of a steroid, there are limited options for comparison spectrum available. Values for the hydroxyl peak on carbohydrate rings found by Sefzig *et al.* range from 8.76–9.51 MHz for C_q and 0.83–1.00 for η which are similar to the values found in this study.[31]

In order to use the enriched cholesterol to understand hydrogen bonding of cholesterol in bilayers, three 50 mol% cholesterol bilayers were made with DPPC, DOPC, and eSM. The static and MAS spectra can be seen in Fig. 3. The static ^{17}O NMR spectra (Fig. 3A) of cholesterol in a bilayer possess an asymmetric line shape overlain with a sharper peak from

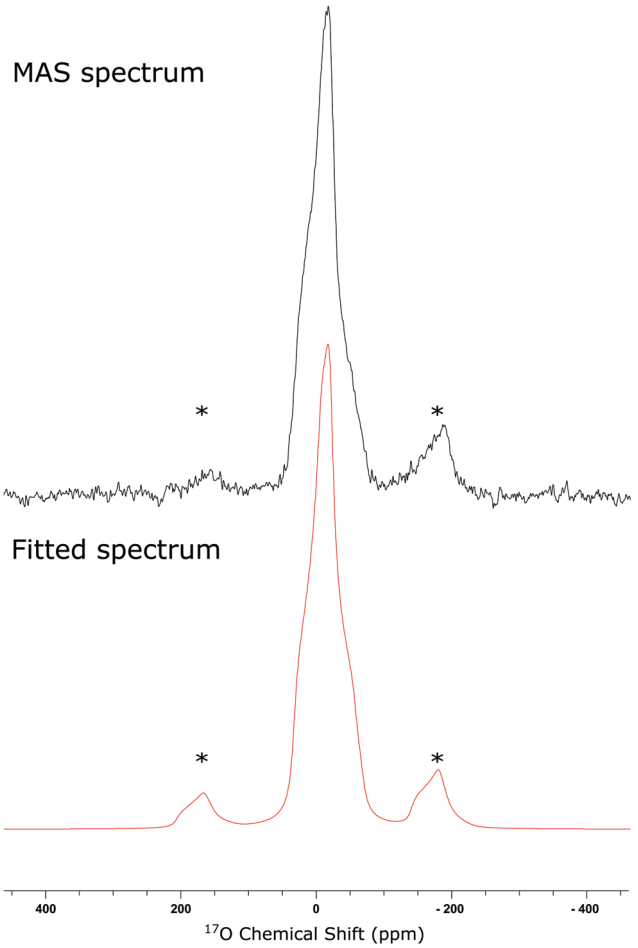

Fig. 2 Real (top) and simulated fitted (bottom) ^{17}O NMR spectrum of anhydrous enriched cholesterol spun at 20 kHz. Spinning sidebands are indicated by *.

residual ^{17}O NMR spectrum of water, whereas, the MAS spectra (Fig. 3B) appear as two Lorentzian peaks from water and cholesterol. Cholesterol in a bilayer is a viscous gel, and all lipid bilayer systems used in this work are in the liquid ordered phase.[32,33] Within this phase there is lateral motion and axial rotation in the bilayer, meaning that for this specific system, the second order quadrupolar and CSA contributions are minimal. Fitting the static ^{17}O NMR line shapes produces small values of C_q (<1 MHz), and η values of 0 (Table 2). This indicates that the major influence on the line shape is due to a distribution of isotropic chemical shifts because of the different chemical environments of the hydroxyl group. Therefore, upon spinning the loss of the first order quadrupolar and CSA effects leaves a Lorentzian peak.

The fitting of the static peaks produces values shown in Table 2. Values of isotropic chemical shift vary according to the other lipid components in the bilayer and are further upfield than the values found from the cholesterol in solvent and the MAS spectrum of the anhydrous crystalline cholesterol alone. Previously, it has been found that the CSA and quadrupolar values of some functional groups vary depending on hydrogen bond length.[24,34] However, this has not been characterised for

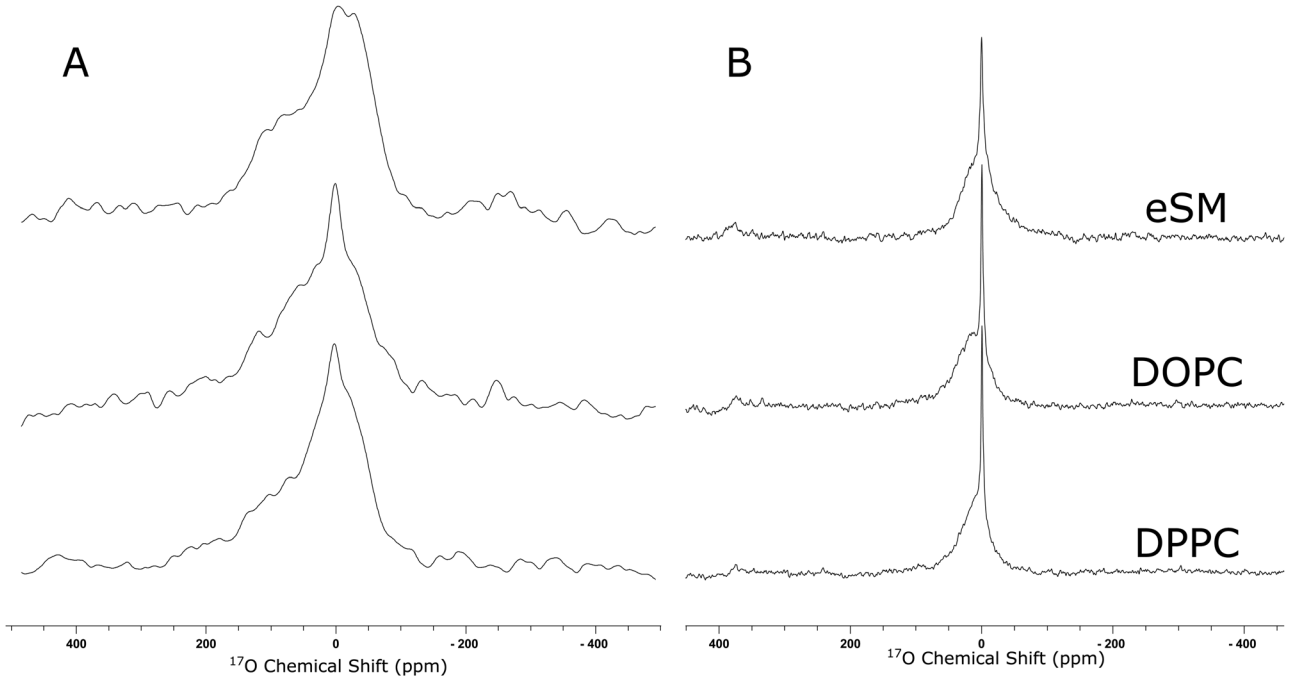

Fig. 3 Static (A) and MAS (B) spectra of cholesterol in bilayers at 50 mol%. Lipids which form the bilayer are indicated in the labels. Sharp peak indicates water in hydrated sample. MAS spectra were acquired using spinning speeds of 20 kHz.

Table 2 Values for the CSA and quadrupolar components obtained from fitting the static data of cholesterol in bilayers

	δ(iso)/ppm	δ(11)/ppm	δ(22)/ppm	δ(33)/ppm	C_q/MHz	η
eSM	15.9	140.6	140.7	−65.5	0.40	0
DOPC	26.8	140.3	0.8	−60.7	0.93	0
DPPC	25.4	152.0	−21.7	−54.0	0.00	0

this functional group, and there does not appear to be a trend in this data set.

Fitting the two Lorentzian peaks in the MAS spectra (Fig. 3B) enables discernment of the cholesterol peak and Table 3 shows the chemical shift and full width half maximum of both the cholesterol and water peaks. To indicate a fully hydrated sample, crystalline cholesterol monohydrate was used as a control. The X-ray crystal structure of cholesterol monohydrate has been reported previously as possessing three hydrogen bonds per oxygen atom making it ideal to represent an extensively hydrogen bonded cholesterol molecule.[35] The unusually narrow line width of crystalline monohydrate may derive from the crystal structure. In the unit cell of cholesterol monohydrate, the ^{17}O of the hydroxyl has eight inequivalent crystallographic sites.[35] This sometimes appears as multiple sites in the MAS NMR. We can only hypothesise that this was the case here, and has manifested as a broad distribution of sites, of which only a few are readily observed.

Table 3 Values of chemical shift and full width half maximum of the cholesterol and water peaks from the MAS spectra shown in Fig. 3B

	Cholesterol		Water	
	Chemical shift/ppm	FWHM/ kHZ	Chemical shift/ppm	FWHM/ kHz
Cholesterol monohydrate	2.74	3.09	−3.30	0.23
eSM	3.82	6.54	0.12	0.54
DOPC	18.13	6.54	−0.37	0.50
DPPC	11.42	5.59	−0.43	0.40

All chemical shift values of cholesterol in bilayers (static and MAS) are further upfield than that of cholesterol in solution and the isotropic chemical shift found from the solid anhydrous cholesterol, closer to the chemical shift of crystalline cholesterol monohydrate. However, there is a difference in chemical shift values found between the static and MAS samples. This is likely due to the poor signal-noise ratio in the static samples causing a lower quality fit and less reliable values. Errors for MAS values can be found in S7 (ESI†). The upfield shift of the NMR signal of the enriched cholesterol in bilayers is attributed to the cholesterol engaging in extensive hydrogen bonding. Whilst cholesterol can form a variety of different types of hydrogen bond when in a phospholipid bilayer, they are predominantly through the proton on the hydroxyl group.[10] The hydrogen bond causes an increase in electron density around the oxygen, and an upfield shift closer to zero ppm. This is consistent with previous reports indicating that hydrogen bonding *via* the proton causes an upfield shift in the ^{17}O NMR signal of the attached oxygen.[25] If cholesterol engages in hydrogen bonding it shifts upfield, providing a simple method of measuring the hydrogen bonding in bilayers.

Whilst the cholesterol in all three lipid systems has a more upfield chemical shift than that of the cholesterol in the solution state, there also appears to be differences between the cholesterol in the egg sphingomyelin and the phosphocholine membranes.

The NMR chemical shift of cholesterol in the sphingomyelin bilayer is further upfield than that of the phosphocholines. This is likely due to stronger cholesterol–lipid interactions. In some cases, cholesterol has been shown to preferentially interact with sphingomyelin over phosphocholines.[6,36,37] This is due to the extra hydrogen bonding site, originating from the amide on sphingomyelin.

The NMR chemical shift of the water peak also moves depending on bilayer composition as can be seen from Table 3. Pure water has an ^{17}O NMR chemical shift of 0.0 ppm, however this signal depends on the environment that the water is in, and the presence of dissolved salts can cause this to change.[38] The chemical shift of water in the monohydrate sample shows a value of −3.30 ppm, which is primarily from the bulk water surrounding the hydrated crystal. The chemical shift of water in the samples containing a bilayer has a more mixed character because these samples are hydrated at the minimum required to achieve excess hydration (65 wt%). The chemical shift of water in the sphingomyelin sample is 0.12 ppm which is higher than the phosphocholine samples at −0.37 ppm and −0.43 ppm for DOPC and DPPC respectively. This could indicate the extent that water is involved in the hydrogen bonding.

Previous work has already demonstrated the power of ^{17}O NMR spectroscopy to elucidate hydrogen bonding in biological systems.[21–24] This work has shown that it can also be used to clearly demonstrate this interaction in lipid bilayers. After enrichment of cholesterol, the 1D ^{17}O NMR spectra provide a measure of hydrogen bonding from chemical shift alone, where an upfield shift indicates more hydrogen bonding. This allowed for simple comparison of three lipid systems, which supported previous ascertains that cholesterol binds more strongly to sphingolipids. A limitation of this, however, is that this method alone does not indicate of the mode of hydrogen bonding, and future studies incorporating 2D-NMR spectroscopy or isotopically enriched phospholipids would provide further detail and may be able to elucidate the nature of the hydrogen bonds.

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Conflicts of interest

There are no conflicts to declare.

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