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# **Sterols associated with Small Unilamellar Vesicles (SUV): intrinsic mobility role for $^1\text{H}$ NMR detection**

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## **Abstract**

Small unilamellar vesicles (SUV) of phospholipids are often used as a membrane model system for studying the interaction of molecules. When using NMR under the standard liquid state conditions, SUV phospholipid proton spectra can be recorded, exhibiting sharp signals. This is not only due to the fast vesicular tumbling, but rather to the combination of this tumbling with the individual motion of the lipids inside the bilayer. This appears evident because addition of cholesterol is responsible of broader resonances due to the slowing down of the lipid motion. On the other hand, no  $^1\text{H}$  signal is detected for cholesterol in the bilayer. This lack of detection of the inserted molecules explains why generally SUV are not considered as a good model for NMR studies under the standard liquid state conditions. Here we use two other sterols in order to demonstrate that an increase of the molecular mobility inside the bilayer could allow the detection of their proton resonances. For desmosterol and lanosterol, which show higher mobility inside the bilayer, with increasing lateral diffusion rates,  $^1\text{H}$  sterol signals are detected in contrast to cholesterol. For the fast diffusing lanosterol, no significant improvement in detection is observed using deuterated lipids, demonstrating that homonuclear dipolar coupling is fully averaged out. Furthermore, in the case of low mobility such as for cholesterol, the use of a fast magic angle spinning probe is shown to be efficient to recover the full proton spectrum.

## **Introduction**

Interaction of molecules with the membrane is the first step in a wide range of cellular processes. Therefore, the characterization of these interactions represents an important approach for understanding various biological phenomena. Because of the complexity of native membranes, artificial models are often used for studying these interactions, with each model having its own advantages and limitations and being more or less convenient for each biophysical technique of analysis.

Here, we focus on small unilamellar vesicles (SUV) as a membrane model for studying these interactions by nuclear magnetic resonance experiments. This model has been widely used in several biophysical studies based on various techniques such as fluorescence, circular dichroism or dynamic light scattering, and currently forms a standard model for analysing processes involving interactions with the membrane. NMR has been only sparingly used for the analysis of such interactions. Cholesterol was one of the first molecules studied in phospholipid SUV<sup>1-5</sup> and the complete absence of inserted cholesterol  $^1\text{H}$  signals led many authors to conclude that SUV are inappropriate for  $^1\text{H}$  NMR experiments.<sup>6</sup> Consequently, it was only seldom used in NMR studies. Other models such as multilamellar vesicles (MLV) or oriented samples were favoured due to the wide development of solid-state NMR methods over the last 20 years.<sup>7-16</sup>

However, the small diameter of SUV – between 30 nm and 50 nm – ensures a fast tumbling motion, with a correlation time in the microsecond range, which, combined with the individual movements of phospholipids, leads to an efficient averaging out of dipolar couplings and chemical shift anisotropy. This gives rise to sharp NMR resonances for the lipids under standard liquid state conditions, even in proton NMR. This is not true with larger vesicles (LUV) which display proton broad resonances for the lipids, highlighting the role of the vesicular tumbling. This strong sensitivity of the lipid signals linewidths has been used in the case of SUV to characterize the decrease of the individual motion of the phospholipids, induced for example by naphthalene derivative<sup>17</sup> or to obtain evidence of vesicles fusion induced by a peptide.<sup>18</sup>

Studying molecules interacting with SUV is more complex since their signals are not always detected. However, few NMR studies have been reported using SUV, as already pointed out in a review<sup>19</sup> where we described these studies and, more specifically, discussed the conditions for good <sup>1</sup>H NMR detection of molecules interacting with lipids in SUV. For efficient averaging out of anisotropic interactions – essentially dipolar couplings for the <sup>1</sup>H nuclei – it is important to take into account not only the vesicle tumbling, but also the association-dissociation dynamics of the interacting molecules. In the review, as well as in a previous study on porphyrins and cytochrome *c* interactions with SUV, we discussed the role of association-dissociation dynamics in the <sup>1</sup>H detection of associated molecules, through the dissociation kinetic rate constant ( $k_{\text{off}}$ ).<sup>19, 20</sup> In fact, a high  $k_{\text{off}}$  is required to ensure a good averaging out of dipolar couplings, thus allowing the detection of sharp resonances. As an example, the neurotensin structure in presence of SUV was recently solved by NMR under the standard liquid state conditions.<sup>21</sup>

In this study, we focus in more detail on the role of the mobility of interacting molecules inside the bilayer for ensuring good <sup>1</sup>H NMR detection. For this purpose, we investigate different sterols incorporated in phospholipid SUV: cholesterol and two of its precursors, desmosterol and lanosterol. About sterol interactions with SUV, the impact of the kinetic rate constant can be ruled out because of its high value; it has been reported as less than 1 h<sup>-1</sup> for cholesterol.<sup>22-24</sup> Although we failed to find similar data for the two other sterols, desmosterol and lanosterol, strong dynamic behaviour are not expected. The behaviour of these compounds inside a lipidic bilayer have already been studied using multilamellar vesicles (MLV)<sup>25, 26</sup> and the main purpose of our study was to determine the condition of good proton NMR detection when using SUV.

Cholesterol is a major constituent of eukaryotic cell membranes, especially plasma membranes, where it can account for up to 50% of the lipid fraction. It is absolutely essential for normal cell functioning, viability and proliferation. Despite extensive studies on cholesterol over the last 40 years, its biological role is still not fully understood. Its most important function seems to be its ability to modulate the physicochemical properties of the membrane.<sup>27</sup> It influences the lateral organization of the membrane constituents, contributing to the formation of caveolae and, along with sphingomyelin, the development of membrane domains called lipid rafts.<sup>28-31</sup>

These modifications of membrane properties result from the ability of cholesterol to promote strong variations of the lipid phase. These variations brought about by cholesterol are complex and have been widely studied by several techniques.<sup>25, 32-43</sup> They still represent an important research area because they are at the origin of the various cholesterol properties in the cellular membrane. This aspect is not discussed here in more detail, since, for our purpose, we only consider cholesterol properties related to its slow motion, compared to other sterols, when incorporated into a bilayer as well as its ability to slow down lipid motion by stiffening the membrane and ordering the lipid acyl chains.<sup>26, 34, 36, 44-47</sup>

The aim of our study is to show that, in addition to the fast tumbling of the small vesicles, fast internal motion inside the SUV is sufficient to sharpen enough the  $^1\text{H}$  signals of interacting molecules for NMR experiments under standard liquid state conditions. With this in mind, we studied desmosterol and lanosterol incorporated in SUV. These two cholesterol precursors in the biosynthetic pathway have been studied in lipid membranes by Pulsed Field Gradient Magic Angle Spinning NMR, showing increased diffusion rates inside the bilayer.<sup>48</sup>

Desmosterol differs from cholesterol by having only one double bond between  $\text{C}_{24}$  and  $\text{C}_{25}$  (Figure 1). Again, the modifications of the phase induced by this sterol are complex and dependent on lipid composition. Desmosterol seems to have only a weak effect on stabilizing raft formation<sup>49</sup> and altering the membrane lateral pressure profile<sup>50</sup>. The small structural difference with cholesterol induces an increasing mobility inside the bilayer, with higher diffusion rates not only for desmosterol but also for the membrane lipids.<sup>48</sup>

Lanosterol differs more significantly from cholesterol than desmosterol. It has the same double bond between  $\text{C}_{24}$  and  $\text{C}_{25}$  as desmosterol. Otherwise, there is a double bond between  $\text{C}_8$ - $\text{C}_9$  instead of  $\text{C}_5$ - $\text{C}_6$ , and three additional methyl groups, two at the  $\text{C}_4$  and one at the  $\text{C}_{14}$  positions, which lead to a less planar structure of the molecule (Figure 1). This less flat structure seems to give rise to weaker interactions with the lipids, and produces a higher mobility for the molecule inside the bilayer<sup>51-53</sup> along with a lower membrane rigidity.<sup>54</sup> Lanosterol undergoes a higher lateral diffusion rate in the membrane than desmosterol<sup>48</sup> as well as a higher flip-flop rate than cholesterol.<sup>55</sup> It induces lower order parameters of the acyl lipid chains, whereas the order parameters are of the same magnitude for cholesterol and desmosterol inserted in the bilayer.<sup>8, 48, 56-58</sup>

Here, we focus on the increasing mobility of the three sterols inside the bilayer. By studying these sterols in SUV, we show that, when motion inside the bilayer is sufficiently high, the interacting molecule can be detected with good precision by  $^1\text{H}$  NMR under standard liquid state conditions.

As in many artificial membrane studies, DMPC (1,2-Dimyristoyl-*sn*-Glycero-3-Phosphocholine) was chosen since it mimics the saturated natural membrane lipids that are preferentially associated with cholesterol in lipid microdomains. The transition temperature between the gel and liquid crystalline phase is  $23^\circ\text{C}$ , which facilitates the preparation of multilamellar vesicles (MLV) at convenient working temperatures. We chose a molar ratio of 70:30 for the preparation of DMPC:Sterol vesicles since 30% corresponds to a typical cholesterol content in mammalian membrane cells.

## Materials and methods

### *Materials*

Cholesterol, desmosterol and lanosterol were purchased from Sigma-Aldrich Co. (St. Louis, MO). The phospholipids 1,2-Dimyristoyl-*sn*-Glycero-3-Phosphocholine (DMPC), 1,2-Dimyristoyl-*d*<sub>54</sub>-*sn*-Glycero-3-Phosphocholine (DMPC-*d*<sub>54</sub>) and 1,2-Dioleoyl-*sn*-Glycero-3-Phosphocholine (DOPC) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). Sterols and lipids were used without further purification.

### *Preparation of vesicles*

Lipids and sterols in appropriate amounts were mixed in chloroform and dried under vacuum to obtain a thin film. For desmosterol-lipid and lanosterol-lipid mixtures, the film was sometimes quite inhomogeneous, so further solubilisation in cyclohexane was required followed by a second and eventually a third evaporation under vacuum.<sup>8</sup>

Multilamellar vesicles were then obtained by rehydrating the film, at concentrations between 10 mM and 125 mM of total lipids depending on the mixtures.  $\text{D}_2\text{O}$  was used for rehydration,

and MLV preparations then were placed without further transformation in the HR-MAS rotor for HR-MAS NMR experiments.

SUV were obtained as previously described<sup>59</sup> by sonication of the MLV preparation at the chosen concentration for a few minutes at room temperature, using the micro-tip of a sonicator (U200S, UKA Labortechnik). Centrifugation was applied to eliminate titanium debris from the sonicator probe. SUV samples were always freshly prepared. Vesicles formation and stability during NMR acquisition at the working temperature were then directly checked on <sup>1</sup>H NMR spectra, which display sharp resonances with a choline headgroup methyl <sup>1</sup>H splitting due to the curvature differences between intra and extra layers of the vesicles<sup>60</sup>. Further control of the absence of aggregation was performed as previously by dynamic light scattering (4700/PCS100 Malvern) and <sup>31</sup>P NMR experiments, exhibiting resonance linewidths below 150 Hz as expected for SUV.<sup>59</sup>

### ***NMR measurements***

<sup>1</sup>H NMR measurements for SUV and MLV samples were carried out on two Bruker AVANCE 500 spectrometers operating at 500.12 MHz and 500.15 MHz and equipped with a 5 mm cryo-probe TXI and a 5 mm BBO probe, respectively. <sup>31</sup>P spectra were obtained with the 5 mm BBO probe. <sup>1</sup>H HR-MAS NMR measurements for MLV samples were performed on the second spectrometer with a triple-inverse HR-MAS probe, using 50  $\mu$ l rotors and at a spinning frequency up to 12000 Hz. Different working temperatures were used, and the spectra are presented at 323K. Spectra using very high spinning rate at the magic angle were acquired on a Bruker Avance 750 spectrometer operating at 750.12 MHz with a MAS 1.3 mm triple resonance probe. In all experiments, the residual water HDO peak was suppressed by using presaturation.

### **Results and discussion**

When cholesterol is incorporated into SUV made up of phospholipids, we firstly observe that the lipid signals are broadened with increasing amounts of cholesterol in the SUV. This is because cholesterol stiffens the membrane and reduces the phospholipid motion. As already described in many papers (see for example reference 40) several lipid movements, on different time scales, take place in a bilayer : rotation around chemical bonds (picoseconds), *trans-gauche* isomerisation (picoseconds), axial diffusion (rotation) around the lipid molecule axis (nanoseconds), wobbling of this axis (nanoseconds), lateral diffusion inside the bilayer (microseconds), flip-flop (from milliseconds to seconds) and undulatory membrane motion (from milliseconds to seconds). As already mentioned it has been largely described in the literature that incorporating cholesterol in the membrane has the effect of increasing the acyl-chain phospholipid order parameters. The axial diffusion and wobbling seems to remain unperturbed<sup>33, 61</sup> whereas cholesterol drastically limits the *trans-gauche* isomerisation favouring the *trans* conformation<sup>46</sup> as well as it reduces the lateral diffusion of the lipids.<sup>48</sup>

The resulting effect on SUV spectra has been previously discussed in several papers<sup>62-64</sup> and is illustrated in Figure 2 which compares the spectra of <sup>1</sup>H pure DMPC SUV with DMPC:Cholesterol SUV at molar ratios of 95:5 and 70:30. The acyl chain signals clearly broaden with increasing cholesterol content. The intensity ratio between the signals of methylene acyl chains and the terminal acyl chain methyl groups shows a strong reduction as the membrane stiffens owing to increased amounts of cholesterol.

On the other hand, as mentioned in the introduction, no cholesterol signal is detected on these spectra. When using protonated lipids, a large number of cholesterol resonances are masked by the lipid signal themselves, as can be seen by comparing the spectra of protonated and deuterated DMPC:Cholesterol SUV <sup>1</sup>H at a ratio of 70:30 (see Figure 3; A.a and B.a). How-

ever, even with protonated lipids, some cholesterol resonances should be detected outside the lipid signal range (Figure 4; A.a). The invisibility of the sterol signal in SUV has thus been generally attributed to the tumbling motion of the vesicles, which is not fast enough to average out dipolar couplings for the inserted molecule. However, averaging out of these couplings can be obtained by using MAS techniques with MLV,<sup>3, 4, 32</sup> as illustrated by the HR-MAS MLV spectrum showing cholesterol signals (see Figure 4; A.a).

Nevertheless, when comparing SUV <sup>1</sup>H NMR spectra of DMPC: Sterol and DMPC-*d*<sub>54</sub>:sterol at 70:30 molar ratios (Figure 3), we can firstly see that, with protonated lipids, the broadening of phospholipid acyl chain signals progressively decreases when changing from cholesterol to desmosterol and lanosterol. For the methylene acyl chains signals, full width at half height decreases from 100 Hz for SUV with cholesterol to 70 Hz and 60 Hz for SUV with desmosterol and lanosterol, respectively. This should be compared to a width of 50 Hz for sterol-free vesicles. At the same time, the intensity ratio between the <sup>1</sup>H signals of methylene acyl chains and the terminal acyl chain methyl groups increases from about 1 for SUV with cholesterol, to 1.6 with desmosterol and 1.7 with lanosterol, compared to 1.8 for sterol-free SUV. While the CH<sub>2</sub>-O-P protons of the glycerol backbone are detected near 4.2 ppm in DMPC: Lanosterol, they mostly disappear in the DMPC: Cholesterol SUV spectrum. These results suggest that the phospholipids recover higher mobility with changing sterol type from cholesterol to desmosterol and lanosterol, in agreement with several previous studies showing a progressive lower ordering effect as well as an increase of the lipid lateral diffusion.<sup>48, 53, 56</sup>

It can also be seen that desmosterol and lanosterol signals are detected, such as the olefinic proton of C<sub>24</sub>, which appears near 5.3 ppm. Proton signals of the C<sub>26</sub> and C<sub>27</sub> methyl groups are detected near 1.8 ppm, both in desmosterol and in lanosterol. The corresponding cholesterol signal is more shielded due to the absence of the double C<sub>24</sub>-C<sub>25</sub> bond; while this signal is masked by the lipid resonances in protonated SUV, it is visible at 1 ppm in deuterated SUV (Fig. 3, B.a). For lanosterol, the signals near 2.2 ppm, 1.2 ppm and 1 ppm corresponds to C<sub>11</sub> and C<sub>12</sub> protons, C<sub>29</sub> and C<sub>6</sub> protons and C<sub>18</sub> protons, respectively. These attributions are based on previous studies obtained by solid-state NMR, for <sup>1</sup>H and <sup>13</sup>C cholesterol signals<sup>65</sup> and for <sup>13</sup>C desmosterol and lanosterol signals in DMPC bilayers,<sup>4</sup> and comparison between the three sterol spectra (<sup>1</sup>H and <sup>1</sup>H-<sup>13</sup>C HSQC) in SUV and CDCl<sub>3</sub> (not shown here).

As pointed out in the introduction, these two sterols have been shown to have a faster dynamic within the bilayer, with higher lateral diffusion and flip-flop rates. Once again several movements take place for the sterol molecules inside the bilayer. Cholesterol has been reported to exhibit fast axial diffusion and axis wobbling with correlation times in the nanosecond range.<sup>66-68</sup> An axial diffusion increase is unexpected for desmosterol, only slightly different from cholesterol by one double bond in the hydrophobic tail. The less flat structure of lanosterol, exhibiting several additional methyl groups, might result in different axial diffusion behaviour but no axial diffusion data were found in the literature neither wobbling ones. Measurements of the lateral diffusion coefficients have been carried out by Scheidt and co-workers using <sup>1</sup>H PFG MAS NMR experiments in DPPC (1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphocholine) membranes.<sup>48</sup> They showed that DPPC and cholesterol diffusion rates are of about the same order (only slightly higher values for cholesterol) and decrease with the amount of cholesterol inserted. DPPC diffusion is faster with desmosterol or lanosterol in the membrane. For desmosterol, the diffusion rates are only slightly higher than for the phospholipid, while they become much faster for lanosterol, indicating weaker interaction with the phospholipids. The apparent diffusion coefficients has been reported to vary, at 320 K, from 8 to 16 × 10<sup>-12</sup> m<sup>2</sup>.s<sup>-1</sup> for cholesterol and lanosterol respectively, with a median value at 12 × 10<sup>-12</sup> m<sup>2</sup>.s<sup>-1</sup> for desmosterol.<sup>48</sup> The flip-flop rate constants of cholesterol and lanosterol in DPPC bilayer at low sterol concentration and 323 K, where recently theoretically calculated by Parisio and co-workers,<sup>55</sup> quite in agreement with other molecular dynamic studies of cholesterol

flip-flop,<sup>69-71</sup> showing an increase of the rate constants from  $2 \times 10^4 \text{ s}^{-1}$  for cholesterol to  $6.6 \times 10^4 \text{ s}^{-1}$  for lanosterol. Our results show that as motion increases for the inserted molecule, it becomes possible to detect its  $^1\text{H}$  resonances in SUV. Considering that quite small differences in the diffusion coefficient and flip-flop rates, only a factor of two or three between cholesterol and lanosterol, highlights the fact that the fast vesicular tumbling is probably playing a major role for an efficient averaging out of dipolar couplings. But the several kinds of motion are important and should be high enough: the vesicular tumbling, the lipid motion and the inserted molecule motion.

Figure 3 also presents a comparison of the protonated and deuterated SUV spectra, showing that deuteration yields sharper signals for desmosterol in the case of signals that are not overlapping with the lipid ( $\text{C}_{24}$  and  $\text{C}_{26,27}$  protons). On the other hand, deuteration only slightly reduces the signal linewidths in the case of lanosterol.  $^1\text{H}$  homonuclear dipolar couplings depend on two components: intra-molecular couplings and inter-molecular couplings including sterol-lipid and sterol-sterol couplings. Using deuterated phospholipids prevents inter-molecular homonuclear dipolar coupling between the sterol and the phospholipid acyl chain protons. Thus, with protonated lipids, residual  $^1\text{H}$  dipolar couplings remain between lipid and sterol molecules for desmosterol in DMPC SUV, but not for the more mobile lanosterol. Hence, for molecules with sufficiently fast dynamic, we can clearly detect  $^1\text{H}$  resonances in SUV even without using deuterated lipids.

Experiments with DOPC SUV were also carried out with the intermediate desmosterol molecule to see if it is possible to obtain sharper signals using unsaturated phospholipids. Although these latter increase the membrane disorder and exhibit lower-order acyl chain parameters,<sup>46, 72</sup> no major improvement was observed (not shown).

In Figure 4, we compare the spectra of DMPC: Sterol MLV with HR-MAS and SUV in the standard liquid conditions without rotation. Clearly, whatever the nature of the sterol, good detection is observed in the case of MLV with magic angle spinning. Such a good detection in SUV is only observed with lanosterol, dipolar couplings being efficiently averaged out by the combination of SUV tumbling as well as lipid and molecular motion inside the bilayer.

As previously described,<sup>73, 74</sup> no improvement is obtained on the DMPC:Cholesterol SUV spectra when using HR-MAS with a spinning rate up to 12 kHz, the vesicular tumbling interfering with the MAS (not shown). We then checked the efficiency of higher MAS rate on SUV composed of DMPC:Cholesterol with molar ratio of 70:30. This Lipid:Cholesterol ratio corresponds to the worse spectrum in the standard liquid state conditions. The  $^1\text{H}$  NMR spectrum, recorded at 750 MHz and 40 kHz spinning rate is shown in the Figure 5 and the corresponding MLV spectrum recorded at 500 MHz and 10 kHz is also displayed for comparison. Clearly, both spectra are pretty similar in term of linewidths. The increase of the spinning rate up to 40 kHz enables the detection of signals of inserted cholesterol in SUV. This opens the possibility of working with SUV as a model membrane even for molecules exhibiting very slow dynamics inside the bilayer. This should constitute an interesting approach when SUV is the most relevant membrane model, for example when studying molecules interacting with highly curved membranes.

## Conclusions

SUV are generally believed as an unsuitable model membrane for NMR. We show in the present study that as long as the inserted molecules display sufficiently high mobility inside the bilayer sharp  $^1\text{H}$  signal are obtained. By studying three sterols inserted in phospholipid SUV, we show that the  $^1\text{H}$  signals sharpness is related to the molecule motion inside the bilayer. Cholesterol is invisible in SUV and broadens the lipid signals due to its slow motion inside

the membrane and its stiffening and ordering effects on the bilayer, which also slows down the lipid motion. By contrast, while desmosterol and lanosterol display increasing mobility inside the membrane, they do not have such a broadening effect on the lipid signals and can be detected under NMR standard liquid state conditions. For molecules such as cholesterol with very low mobility inside the bilayer, we demonstrate that spinning at the magic angle with a very high spinning rate permits to recover the molecule proton resonances. Finally, SUV constitute an interesting model for studying molecule-membrane interactions provided that the interacting molecule has sufficiently fast dynamics. In the case of molecules with low dynamics inside the bilayer the use of fast magic angle spinning could be an efficient approach to work with SUV.

### **Acknowledgments**

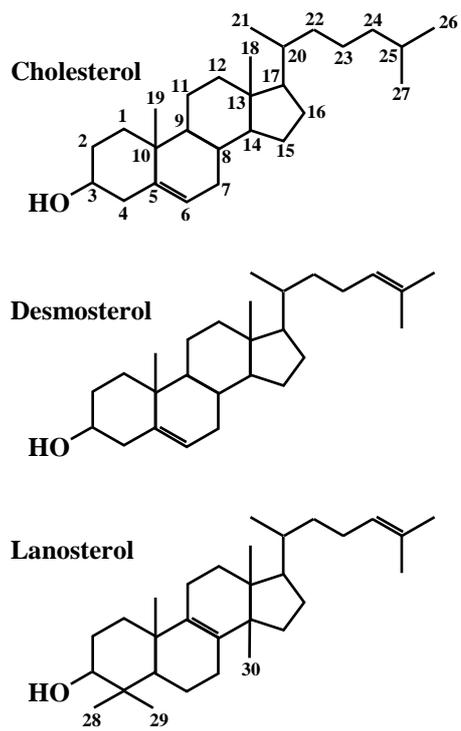
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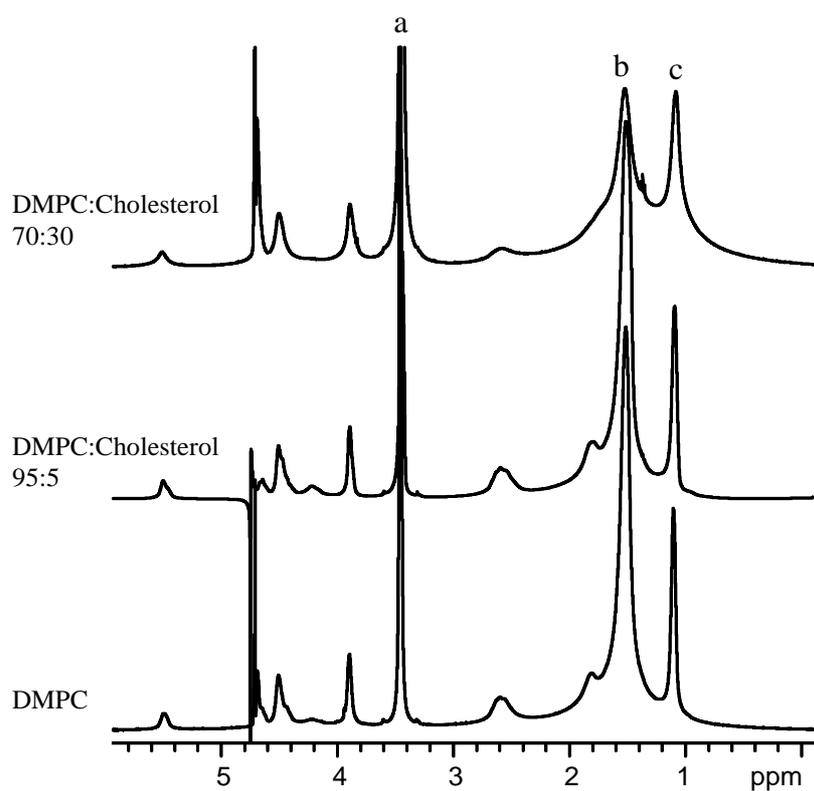
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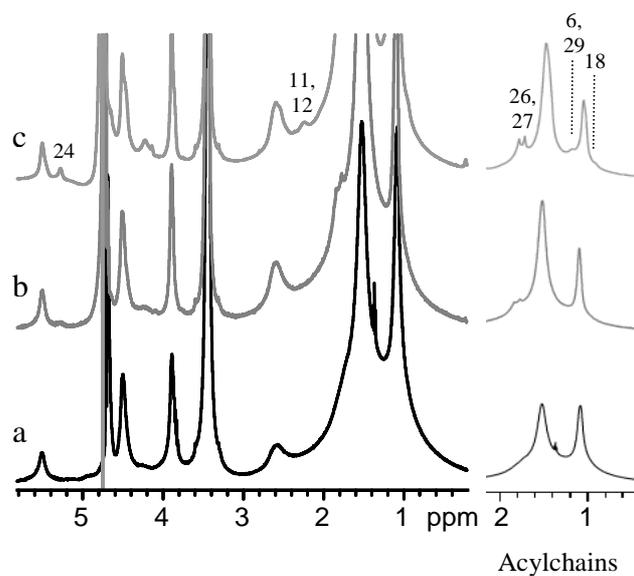


**Figure 1:** Structures of the three sterols and their classical nomenclature

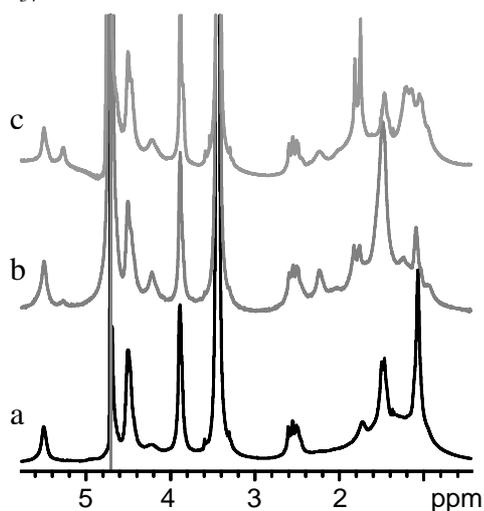


**Figure 2 :**  $^1\text{H}$  NMR spectra of SUV composed of pure DMPC and DMPC:Cholesterol with molar ratios of 95:5 and 70:30, recorded at 323K. Lipid signals are labelled as follows: (a) choline methyl headgroups, (b) methylene acyl chains, (c) terminal acyl chain methyl groups. Intensities are arbitrary. Intensities are normalized using the resonance of the choline methyl headgroup while ignoring peak broadening.

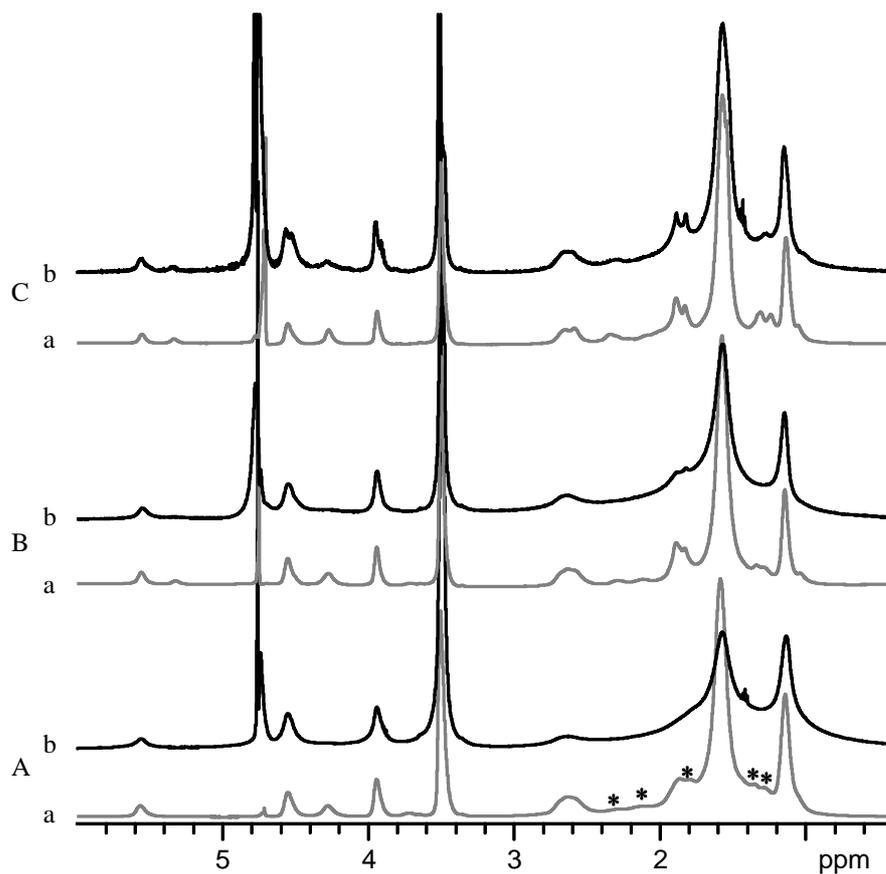
A: DMPC:Steryl



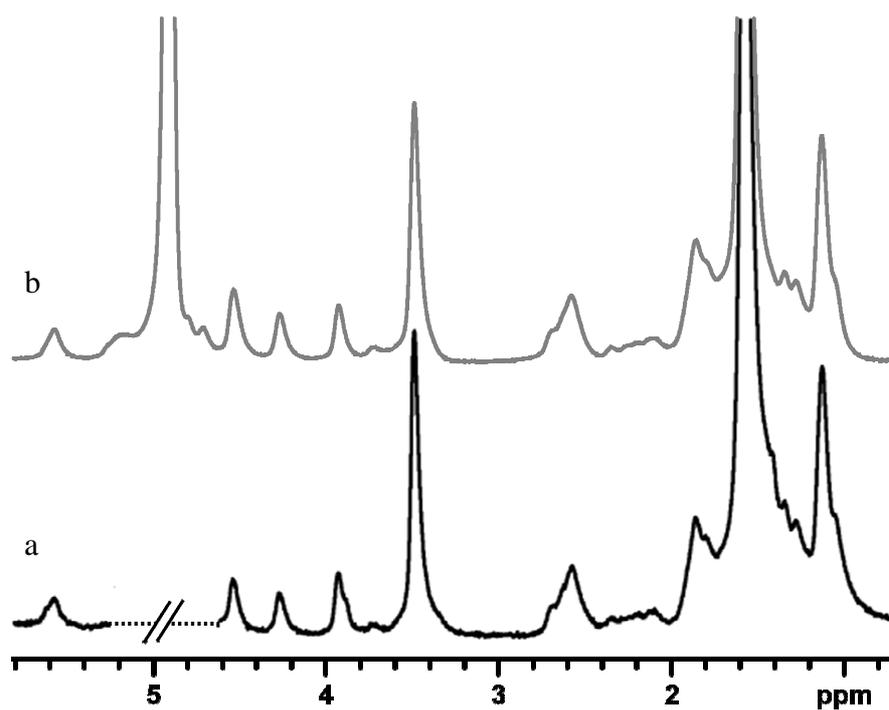
B: DMPC-*d*<sub>54</sub>:Steryl



**Figure 3** :  $^1\text{H}$  NMR spectra of SUV composed of DMPC:Steryl (A) and DMPC- $d_{54}$ :Steryl (B) with molar ratio of 70:30, recorded at 323K, for (a) cholesterol, (b) desmosterol and (c) lanosterol. Detail of lipid acyl chain signals is given on right of (A). *i* numbers over DMPC:lanosterol signals refer to lanosterol  $C_i$ -protons following nomenclature of Figure 1. Intensities are arbitrary. Intensities are normalized using the resonance of the choline methyl headgroup while ignoring peak broadening.



**Figure 4** :  $^1\text{H}$  NMR spectra of DMPC:Cholesterol (A), DMPC:Desmosterol (B), DMPC:Lanosterol (C), with molar ratio of 70:30, recorded at 323K, for (a) MLV with HR-MAS NMR at 12000Hz spin rate and (b) SUV under the standard liquid state NMR conditions. Intensity units are arbitrary. \* in A.a. are cholesterol peaks standing outside the lipid signal range, detected in MLV but not in SUV.



**Figure 5** :  $^1\text{H}$  NMR spectra of DMPC:Cholesterol with molar ratio of 70:30, recorded at 323K for (a) SUV with MAS at 40 kHz spin rate, at 750 MHz, for clarity water resonance was removed, and (b) MLV with MAS at 10 kHz spin rate, at 500 MHz. Intensity units are arbitrary.