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The thickness of cholesterol sulfate-containing membranes depends upon hydration

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Abstract

The ordering of 30 mol % cholesterol (CH) or cholesterol sulfate (CS) on chain deuterated dimyristoylphosphatidylcholine (DMPC) was investigated by ^{2}H-NMR for different hydrations. It is found that: (i) hydration has merely no influence on chain order (chain length) for DMPC-cholesterol systems, (ii) in CS-containing mixtures chain order (length) is greater at low hydration (DMPC-to-water molar ratio, R_i, of 11.3) than in excess water ($R_i \approx 500$) and (iii) at low hydration the ordering is about the same for CS or CH-containing systems whereas it is not at high hydration. DMPC-CS bilayer thickness is therefore very sensitive to hydration. © 1997 Elsevier Science B.V.

Keywords: ^{2}H-NMR; Membrane thickness; Cholesterol; Cholesterol sulfate; Hydration; Chain order

1. Introduction

In biological systems, cholesterol (CH) is known to play a fundamental role in regulating membrane fluidity [1] whereas cholesterol sulfate (CS) is often referred to as a membrane stabilizer [2,3]. They are both present in many natural lamellar systems such as spermatozoon plasma membrane [4], stratum corneum, the outermost layer of skin [5] or erythrocyte membranes [6]. Alteration of their population ratio may lead to dysfunction as serious as reduction of the fertilization efficiency of spermatozoa [7–9] or skin scaling [10,11]. These dramatic outcomes reflect how different their behavior is in biological systems although their chemical structure is merely identical. In model systems, such differences are found again: comparative studies showed that CS ordering effect on fluid phase phospholipid chains is much lower than CH when lamellae are hydrated in excess water [12,13]. Because it has been demonstrated that a measure of C–C bond order parameters lead to an accurate estimate of average lipid chain length and hence to bilayer thickness [15,16], such an observation implies that bilayer hydrophobic thickness is much lower in the presence of CS than with CH. It has been also found that CS can bind 12 more water molecules [12] than CH underlining the pronounced hydrophilic character of cholesterol sulfate. Such a disparity led us to wonder whether hydration might play a role in the ordering effect of both molecules,

Abbreviations: DMPC, dimyristoylphosphatidylcholine; [*sn*-1-$^2H_{27}$]DMPC, 1-[$^2H_{27}$]myristoyl-2-myristoyl-*sn*-glycero-3-phosphocholine; CH, cholesterol; CS, cholesterol sulfate; R_i, water-to-DMPC molar ratio

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i.e. in modulating membrane thickness. In the following we will study by ^{2}H-NMR the order along the *sn*-1 $^2H_{27}$ myristoyl chain of DMPC in the presence of CH or CS for different hydration conditions. Namely, steroid-containing DMPC lamellae will be hydrated in excess water or for a H_2O-to-DMPC molar ratio of 11.3 where it has been shown that hydration of the interface has just been completed [12].

2. Material and methods

[*sn*-1-$^2H_{27}$]DMPC was purchased from Avanti Polar Lipids (Alabaster, USA). Cholesterol was obtained from Sigma (St. Louis, USA) and cholesterol sulfate from Genzyme (Sulfolk, England). Deuterium depleted water was from Aldrich (Milwaukee, USA). The purity of [*sn*-1-$^2H_{27}$]DMPC, CH and CS was checked by thin layer chromatography prior and after completion of experiments, no degradation was detected.

50 mg of [*sn*-1-$^2H_{27}$]DMPC was used for sample preparation. Mixtures of [*sn*-1-$^2H_{27}$]DMPC with 30 mol % CH or CS were prepared by cosolubilization in chloroform/methanol (2/1 v/v) or methanol, respectively. To achieve complete dissolution, the cholesterol sulfate methanolic solution was heated to 60°C. The solvent was removed by blowing a stream of nitrogen over the sample for two hours and pumping the residue under high vacuum for one hour. Excess water was then added and samples heated to 45°C for ca. 30 min, shaken in a vortex mixer and cooled down into liquid nitrogen. This cycle was repeated several times and the samples were lyophilized overnight. A white dry fluffy powder was thus obtained and transferred into 10 mm diameter glass tubes. Eventually, the appropriate amount of deuterium depleted water was added and the tubes were sealed. Homogenization of samples was performed by successive heating, shaking and cooling cycles as described before and then transferred into a 8 mm diameter NMR glass tube. For samples with low hydration (R_i = 11.3), all the procedure was realized in a glove bag under dry nitrogen atmosphere to control hydration.

Solid state ^{2}H-NMR experiments were performed at 30.7 MHz using a Bruker MSL 200 spectrometer. ^{2}H-NMR spectra were obtained on resonance using the quadrupolar echo pulse sequence with a 8-pulse cyclops sequence [14,17]. Acquisition parameters for [*sn*-1-$^2H_{27}$]DMPC were 250 kHz spectral window, 90° pulse duration of 8 μs, interpulse delay of 30 μs, and recycle time of 2 s. Spectral "de-Paking" was performed as described by [18,19] and "oriented-like" spectra calculated for bilayer normals at 90° with respect to the magnetic field direction. Quadrature detection was utilized. Samples were allowed to equilibrate at least 30 min at a given temperature before signal acquisition. Temperature was regulated to ±1°C. Data treatment was performed on Bruker Aspect 3000 and VAX/VMS 4000 computers. A Lorentzian broadening of 100 Hz was applied to the free induction decays before Fourier transformation.

3. Results and discussion

^{2}H-NMR experiments were performed as a function of temperature for all systems investigated in 5°C steps from 10 to 50°C. Fig. 1 shows selected spectra obtained at 45°C on CH- and CS-containing systems for both studied hydrations, i.e. R_i = 11.3 and $R_i \approx 500$ (excess water). They all present axially symmetric powder line shapes indicative of a random bilayer distribution with respect to the magnetic field [14]. Spectra at R_i = 11.3 exhibit comparable width whereas the system at $R_i \approx 500$ is markedly narrower than that containing CH at the same hydration. Increasing hydration slightly increases the width of the CH-containing spectrum and markedly decreases that of the CS-containing system. All spectra consist of overlapping powder patterns arising from the nonequivalent CD_2 along the *sn*-1 acyl chain [14,20], each one being characterized by a quadrupolar splitting, $\Delta\nu_Q$. In the whole range of temperatures, spectra show similar features as those of Fig. 1. This indicates that the DMPC gel-to-fluid phase transition is no longer detected by ^{2}H-NMR of fatty acyl chains in the presence of CH or CS. This has already been shown in samples hydrated in excess water [12] but our results demonstrate that this is also true for very low hydration. All spectra were de-Paked to afford separation of most of the doublets corresponding to each C–D bond of the acyl chain. Assignment of labeled positions was made in the

light of previous works on selectively deuterated systems [21] and calculated order parameter profiles [15,16,22,23]. Each deuterated methylene is thus characterized by a quadrupolar splitting whose value is directly proportional to the order parameter S_{CD} ($|S_{CD}| = (4/3)(\Delta\nu_Q/A_Q)$, A_Q = 167 kHz [24]). The resulting values at 45°C are plotted in Fig. 2 as a function of labeled carbon position for both [*sn*-1-$^2H_{27}$]DMPC-CH (30 mol %) and [*sn*-1-$^2H_{27}$]DMPC-CS (30 mol %) systems and for two hydrations, R_i = 11.3 and $R_i \approx 500$. All the plots reflect the well-known order gradient along the DMPC acyl chains [15,16,20,25] independently of the steroid nature and hydration, i.e. a comparable order for CD_2 segments near the glycerol backbone (positions 2 to 7/8) and a smooth decrease when going to the hydrophobic core.

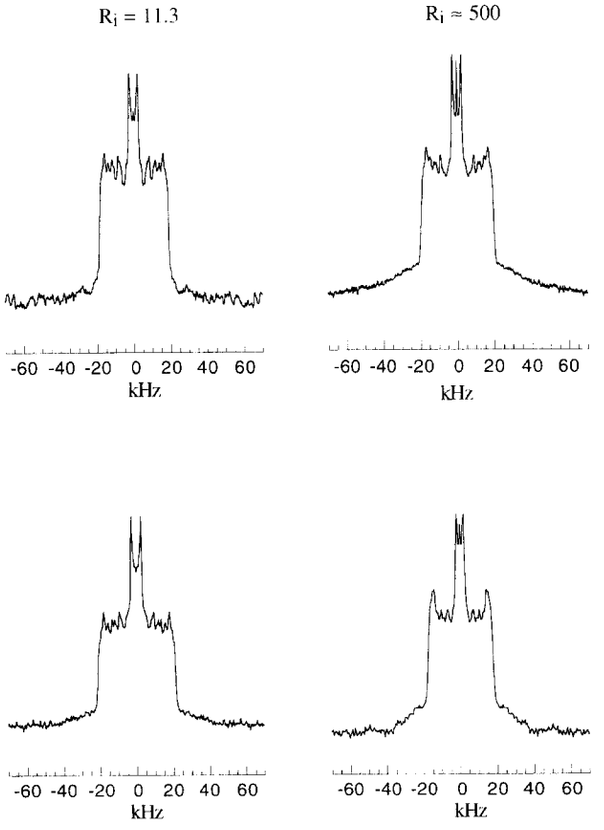

Fig. 1. ^2H NMR powder spectra of [*sn*-1-$^2H_{27}$]DMPC-CH (30 mol %)-H_2O (top) and [*sn*-1-$^2H_{27}$]DMPC-CS (30 mol %)-H_2O (bottom) systems at 45°C. Systems are either weakly hydrated (left column, R_i = 11.3) or in excess water (right column, $R_i \approx 500$).

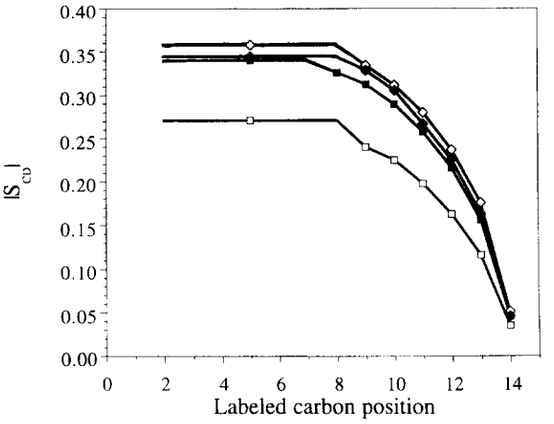

Fig. 2. Acyl chain S_{CD} order parameter as a function of labeled carbon position for [*sn*-1-$^2H_{27}$]DMPC-steroid (30 mol %)-H_2O systems, at 45°C.(– ■ –, R_i = 11.3, and – □ –, $R_i \approx 500$) CS-containing samples, (– ◆ –, R_i = 11.3, and – ◇ –, $R_i \approx 500$) CH-containing samples. Data points are connected to help in reading the figure. Experimental error in S_{CD} is ±0.001, i.e. within symbol size.

S_{CD} values for CH-containing system hydrated in excess water are slightly greater than those for the low-hydrated sample showing that hydration has only a weak influence on DMPC order in the presence of CH. Both curves corresponding to R_i = 11.3 are almost superimposed indicating that the effect of CH and CS on chain order is similar when sample hydration is low. This is no longer valid in excess water: chain order along the DMPC *sn*-1 chain is much greater in the presence of cholesterol than in the presence of cholesterol sulfate. The same results are observed for all temperatures investigated, the S_{CD} values concomitantly decreasing when increasing temperature (not shown). As early shown by Seelig and coworkers [21], such a behavior may be used to calculate the temperature variation of the hydrophobic chain length, $\langle L \rangle$. According to Douliez et al. [15,16], calculation of $\langle L \rangle$ requires the knowledge of S_{CD} and S_{mol}, the molecular order parameter. Unfortunately, no S_{mol} value is known for the CS-containing system. For DMPC-CH and by making use of ^2H-NMR and X-rays data it has been found to be 0.8 at 30°C [26]. Recently, independent calculations reported a value of 0.95 [15]. As a consequence, S_{mol} = 1 was taken for both systems, as this approximation was shown to have little effect on $\langle L \rangle$ [15,27].

For all systems, the hydrophobic length of DMPC *sn*-1 chain is thus plotted versus temperature in Fig. 3. $\langle L \rangle$ regularly decreases with increasing temperature. The lowering in $\langle L \rangle$ value from 15 to 50°C, $\langle \Delta L \rangle$, is equal to 1.6 Å for both steroid-containing mixtures for $R_i = 11.3$ whereas $\langle \Delta L \rangle$ is respectively equal to 1.7 and 2.4 Å for the CH- and CS-containing systems in excess water. This progressive decrease of $\langle L \rangle$ with temperature, as observed for all systems, is easily understandable by thermally driven order decrease which counterbalances the steroid ordering effect. Curves corresponding to CH-containing mixtures are close to each other: their separation never exceeds 0.3 Å at a given temperature, which is very close to the experimental error (0.2 Å) [27]. On the opposite, curves describing CS-containing mixtures are well separated: their separation ranges from 0.7 to 1.3 Å on the whole thermal range. To summarize, DMPC chains in contact with CS are much shorter in excess water than in weakly hydrated samples. In these conditions they are also much shorter that in the DMPC-CH system.

The previous paragraph allows to conclude that: (i) hydration has merely no influence on chain order (chain length) in mixtures containing cholesterol, (ii) the effect of hydration on mixtures containing cholesterol sulfate is clearly demonstrated: chain order

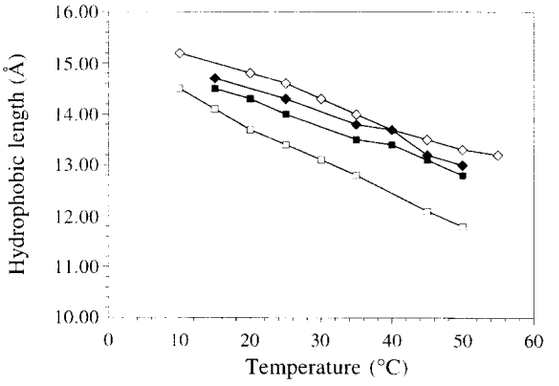

Fig. 3. Hydrophobic length of the *sn*-1 DMPC chain as a function of temperature for [*sn*-1-$^2H_{27}$]DMPC-steroid (30 mol%)-H_2O systems calculated according to Douliez et al. [16]. (– ■ –, $R_i = 11.3$, and – □ –, $R_i \approx 500$) CS-containing samples, (– ◆ –, $R_i = 11.3$, and – ◇ –, $R_i \approx 500$) CH-containing samples. Data points are connected to help in reading the figure. Error on the hydrophobic length is ±0.1Å and is included in symbol size.

(length) is greater at low hydration than in excess water and (iii) at low hydration the ordering is about the same for CS or CH-containing systems whereas the converse is observed at high hydration. A decrease in order parameters or hydrophobic length can be accounted for by a decrease in van der Waals attraction between steroid cycles and phospholipid chains [28–30]. This can be produced either by an increase in lipids headgroup volume (a) or by a change in their relative vertical position within lamellae (b). Our findings will now be discussed according to these two hypotheses. Increasing lipid headgroup volume by an increase in intrinsic hydration would lead to further separate phospholipids from steroids: DMPC acyl chains would then be more apart from steroid cycles and experience more conformational freedom [28–30]. Chain order would then decrease together with their average length. However, in previous works, we showed that the lipidic interface of DMPC-CH (30 mol%) and DMPC-CS (30 mol%) lamellae are water-saturated for respectively $R_i = 3.3$ and $R_i = 8.7$ [12]. In the experiments we carried out, low hydration was fixed to $R_i = 11.3$. Thus, both types of interfaces are completely hydrated and further addition of water does not modify lipid hydration so that this first hypothesis (a) must be dismissed. Alternatively, CS vertical location in the bilayer could be altered by lamellae swelling (b). Indeed, if one supposes that CS is localized upper than CH in the monolayer, then the number of DMPC methylene carbons in contact with the CS fused ring system will decrease, so the attractive van der Waals forces will also decrease. As a result, the ordering effect of CS on DMPC chains would then be weakened. At this level of the study, it is good to remind the behavior of the interbilayer distance, d_w with hydration. For $R_i = 11.3$, d_w is equal to ca. 10 Å for both systems at 25°C as established by small angle X-ray diffraction experiments [12]. This closeness could force CH and especially CS to be buried in the membranes. Several studies demonstrated that CH is indeed embedded in lamellae such that its hydroxyl moiety is hydrogen bonded to one of the carbonyl groups of phospholipid chains [31–34]. In the case of CS, it is reasonable to think that electrostatic repulsion between CS molecules from two opposite bilayers distant by 10 Å and steric reasons could favor a location well inside the bilayer. It is then understandable that

when both steroids are well embedded in the lamellae they will exert comparable effects on chain order as observed in Fig. 2. The addition of water in excess results in a small increase in d_w (15 Å) for CH-containing membranes and a tremendous swelling for the CS-containing system (d_w = 93 Å) [12]. Such a swelling of the lamellar system will of course decrease the electrostatic and steric repulsion between CS molecules from two opposite bilayers. In addition, and as already mentioned in the introduction, CS is much more hydrophilic than CH because of its sulfate moiety rich in oxygen atoms. These can form hydrogen bonds with water molecules and thus would rather be in contact with the aqueous phase than with the hydrophobic chains area. Therefore, a position of CS nearer to the aqueous phase could be induced by water addition. On the opposite, CH would remain deep in the membrane because of the low hydrophilic character of its hydroxyl moiety and also to the poor swelling observed. As a consequence, no effect is expected on chain order (length) upon hydration of CH-containing membranes whereas a protrusion of the CS molecules by several angstroms above the bilayer plane would lead to the observed decrease in chain order upon swelling. The existence of such a protrusion for the CS molecules is clearly hypothetical at this stage of the study. However, preliminary neutron diffraction experiments recently performed on aqueous dispersions of DMPC containing either 30 mol % of deuterated CS or protonated CS reveal large differences in molecule vertical position (Faure and Dufourc, in preparation) and hence support hypothesis (b). This would then imply that the bilayer thickness which is closely related to the average acyl chain length [15] is thinner when containing CS as compared to CH.

In conclusion, it appears that DMPC-CS lamellae thickness is much more sensitive to hydration than that of DMPC-CH. This finding emphasizes the importance of steroid headgroup hydrophilicity and suggests that differences observed between CH vs. CS in biological systems are likely to be related to this property. On the other hand, and though made on model membranes, this finding is of fundamental importance in its biological implications: CS-containing membranes could "adapt" their thickness to hydration. The consequences can be dramatic in systems such as the skin where CS is present and whose hydration decreases from the deeper layers to the upper.

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