Cyclodextrin-Induced Lipid Lateral Separation in DMPC Membranes: ^{2}H Nuclear Magnetic Resonance Study

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ABSTRACT Cholesteryl cyclodextrins, obtained by grafting a cholesterol moiety on the oligosaccharide core, combine the size selectivity of the cyclodextrin cavity with the carrier properties of model membrane systems such as micelles or liposomes. The cholesteryl cyclodextrins were incorporated as guests in chain perdeuterated dimyristoyl phosphatidylcholine (DMPC-d54) membranes. The deuterium nuclear magnetic resonance (NMR) spectra obtained with the A form of cholesteryl-β-cyclodextrin (βCC_A), with a succinyl spacer inserted between the cholesterol moiety and the cyclodextrin headgroup, indicated that this compound induces a lateral phase separation of DMPC-d54, into a pure lipid phase and a cholesteryl cyclodextrin-rich phase. The lipid exchange rate between the two phases was slow on the NMR timescale ($>10^{-5}$ s), and two well-resolved spectral components could be detected. The laterally segregated mixed phase was observed at various membrane concentrations of cholesteryl cyclodextrin, even with dispersions containing only 5% of the derivative. The dePaked spectra allowed the determination of the relative amount of DMPC-d54 molecules contained in each phase, giving $\sim$1 to 1.5 DMPC molecules per unit of βCC_A. This ratio was found to be independent of the total membrane concentration of βCC_A. The cholesteryl cylodextrin-rich phase was detected on a large range of temperature from -12°C to 25°C and exhibits a smooth transition from a fluid environment to a more ordered state, occurring $\sim$0°C. A boundary phase between the pure lipid and cyclodextrin-rich phase was detected at 19°C just below the fluid-to-gel transition. The average orientational order was reduced in the cholesteryl cyclodextrin-rich phase, and quasi-independent of temperature, as opposed to the order parameters measured for the NMR signals of the pure lipid phase. However, the NMR data obtained with βCC_A deuterated on the cyclodextrin headgroup indicated that the latter was quasistatic, with very large order parameters ($\sim$120 kHz) at all temperatures, suggesting strong interactions between neighboring cyclodextrin headgroups. The interactions of DMPC-d54 membranes with the B form of cholesteryl-β-cyclodextrin, lacking the succinyl spacer, was also investigated in a parallel study. No lateral phase separation was found with this compound, indicating that the spatial location and a precise positioning (allowed by the spacer) of the cyclodextrin headgroup at the membrane interface was crucial for the stability of the cholesteryl cyclodextrin lamellar phase.

INTRODUCTION

Cyclodextrins (CD) are water-soluble natural cyclic oligosaccharides composed of 6, 7, or 8 glucose units (α, β, and γ cyclodextrins, respectively). Owing to their torus-shaped structure, they delimit an internal rather hydrophobic cavity although the external part of the molecule is hydrophilic. They are therefore able to include hydrophobic compounds leading to water-soluble inclusion complexes. This property has been used extensively in a wide number of applications in fields such as pharmaceutical, cosmetics, or food industries (Duchêne, 1990). Chemical modifications of the natural oligosaccharidic core allow orientation and modulation of the properties of these cage-molecules toward specific applications. A promising field concerns the addition of one or several highly hydrophobic moieties on the hydrophilic carbohydrate, giving amphiphilic compounds prone to self-organization in aqueous media or to insertion in supramolecular assemblies such as liposomes or micelles. It was observed that the grafting of one cholesteryl unit on natural CD (the coupling can be direct or through a spacer) leads to amphiphilic molecules that can be efficiently inserted as guests in phospholipid liposomes. Using small-angle x-ray scattering, we have shown that the insertion of cholesteryl-β-cyclodextrin in multibilayers of dimyristoyl phosphatidylcholine (DMPC) results in the formation of two different lamellar phases, one is made of pure DMPC, the other one being strongly loaded by the cholesteryl cyclodextrin guest (Auzély-Velty et al., 1999). This would indicate that the polar CD moieties experience strong lateral interactions, sequestrating lipid molecules in a two-dimensional network. Thus, it appears that besides its biological interest, the cholesteryl cyclodextrin/DMPC system offers also an interesting approach of the molecular-induced lateral segregation of phospholipids within bilayers membranes.

To gain detailed molecular information on the cyclodextrin-induced lateral segregation process, we have carried out

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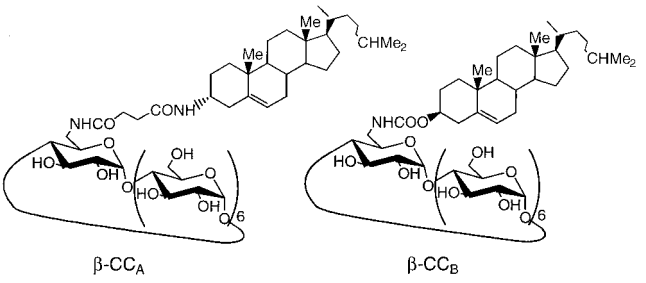

Scheme 1.

a comprehensive deuterium magnetic nuclear resonance (^{2}H NMR) study with DMPC membranes containing derivatives of cholesteryl-β-cyclodextrin, differentiated by the presence (A) or the absence (B) of a succinyl spacer linking the cholesteryl moiety and the cyclodextrin headgroup. Chain-deuterated DMPC probing the cholesteryl cyclodextrin-induced perturbations, allowed the various phases to be monitored separately on a large range of temperatures (−12°C–37°C) and at various cholesteryl cyclodextrin concentrations, providing detailed information on their nature and the order of the lipids.

MATERIALS AND METHODS

Synthesis of cyclodextrin derivatives

6^I-(cholest-5-en-3α-ylamido)succinyl-amido-6^I-deoxy-cyclomaltoheptaose (βCC_A) and 6^I-(cholest-5-en-3β-yloxycarbonyl)amino-6^I-deoxy-cyclomaltoheptaose (βCC_B) (Scheme 1) were synthesized as described previously (Auzély-Velty et al., 1999). The deuterated analogues of β-cyclodextrin and of βCC_A, regio-specifically labeled on the C-2 carbon of all glucose units, were obtained according to a published procedure (Djedaïni et al., 1990). All compounds were fully characterized by high field proton NMR and high resolution mass spectrometry.

Sample preparations

DMPC and chain deuterated DMPC-d54 were purchased from Avanti Polar Lipids (Alabaster, AL) and the cholesterol from Sigma (St. Louis, MO). Multilayered liposomes were prepared by mixing chloroform lipid solutions and methanol solutions of the appropriate cyclodextrin derivative. The solvent was then removed by evaporation under N_2. The solid residues were dried under vacuum (10^{-2} mm Hg) for 12 h and dispersed by continuous vortexing at 20°C in 100 to 300 μl of deuterium depleted water (Eurisotop, France) equilibrated at pH 8.0 giving ~200 mM lipid dispersions.

^{2}H NMR experiments

^{2}H NMR spectra were recorded at 46 MHz on a Bruker DMX 300 spectrometer equipped with a probe specifically designed for solid state deuterium NMR experiments (Morris Instruments Inc., Gloucester, ON, Canada). Spectra were acquired from −12°C to 37°C with a dwell time of 2 μs, 4-K data points, and a recycling time of 200 ms. A quadrupolar echo pulse sequence (Davis et al., 1976) was used with pulse length of 3 μs and pulse separation, τ, of 40 μs. The phase was adjusted to obtain no signal in the imaginary channel, which was then discarded before the Fourier transform of the echo. When necessary, the free induction decay was shifted by a fraction of the dwell time using an orthogonal polynomial interpolation routine so that the Fourier transform could start at the top of the echo (Davis, 1983). Oriented ^{2}H NMR spectra (0°) were obtained by the numerical dePake-ing procedure described by Sternin et al. (1983). The method of moments (Davis, 1979; Davis et al., 1979) was also applied to the chain deuteron spectra. The narrow (<3 Hz) dePaked methyl resonances found in the −10 to 10 kHz range were simulated with a Gaussian line shape after baseline correction of the data. Each resonance was fitted with three independent parameters namely the frequency, the line width, and the intensity.

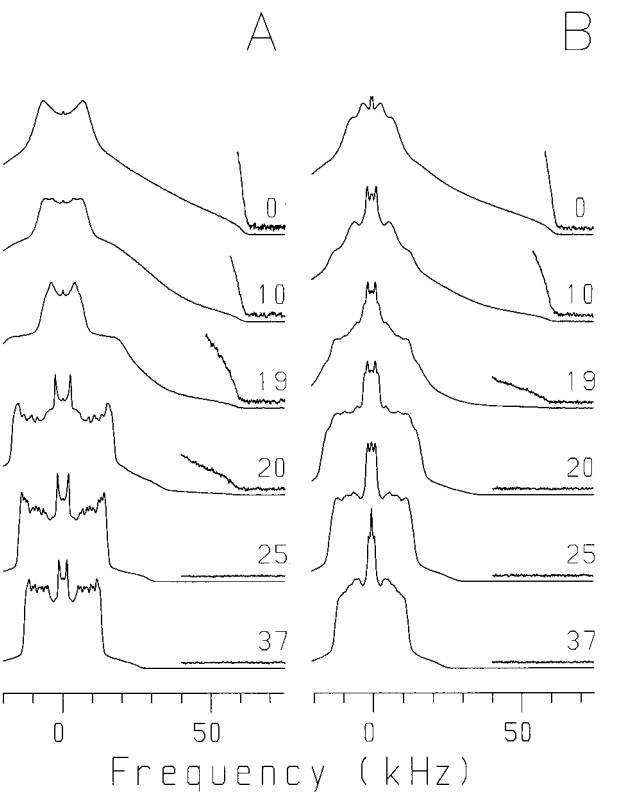

FIGURE 1 Temperature dependence of the ^{2}H NMR spectra of DMPC-d54 membranes. Pure (*A*) and with 20% (*B*) βCC_A in molar %. The spectra were recorded at 37, 25, 20, 19, 10, and 0°C. The thin line in the 40- to 75-kHz region shows the spectra scaled by a factor of 10.

RESULTS

^{2}H NMR of deuterated phospholipids provides a suitable tool for studying the lipid membrane organization through the direct measurement of the local orientational order parameters of the C—D bonds of the lipid acyl chains and polar headgroups (Davis, 1983). In particular gel-to-liquid crystalline phase transitions of phospholipid membranes can be precisely monitored by following the line shape changes of ^{2}H NMR spectra of the chain-deuterated lipid derivatives (Davis, 1979; 1983). In Fig. 1 *A*, the line shapes of ^{2}H NMR spectra of saturated DMPC-d54 change dramatically between 20°C and 19°C from a well-resolved, axially sym-

metric distribution of quadrupolar splittings typical of the fluid L_α phase above 20°C, to a much broader distribution of lipid molecules in the gel $P_{\beta'}$ phase below 20°C. At higher temperatures, the quadrupolar splitting distribution is typical of phospholipid bilayers in the fluid state with a large quadrupolar splitting, or plateau, containing the methylene groups near the membrane interface, smaller resolved quadrupolar splittings for the methylene closer to the bilayer center, and a narrow doublet due to the methyl group at the end of the chain. The gel-to-liquid crystalline transition of perdeuterated DMPC occurs at lower temperature (20°C) than the value of ~23°C, measured by various techniques with pure unlabeled DMPC membranes (Marsh, 1990; Koynova and Caffrey, 1998). This effect is due to the chain-chain deuteron interactions that lower the phase transition temperatures of deuterated phospholipids (Peterson et al., 1975).

DMPC-d54 bilayers with cholesteryl-β-cyclodextrin A

The ^{2}H NMR spectra recorded in the presence of 20% molar βCC_A (Fig. 1 *B*) significantly differ from those obtained with pure DMPC. Above 20°C, the lipids are all in the fluid state as probed by the liquid crystalline line shape of the ^{2}H NMR powder patterns, and apparently there is only one component at 37°C. A shoulder is detected at 25°C, on each side of the large quadrupolar splitting of the plateau methylene groups, suggesting that at least two components coexist. This shoulder appears more clearly when the temperature is decreased. At 20°C, the resonances of the methyl terminal deuterons are also split in two signals (±1.5 and ± 2.0 kHz), indicating the coexistence of two distinct types of DMPC molecules in the fluid state, in slow exchange on the ^{2}H NMR timescale. At 19°C, a gel phase component is detected with signal intensity out to ≈±63 kHz, and also ≈±6 kHz, where the signal of methyl groups in the gel phase appears on pure DMPC-d54 spectra at the same temperature. Now we can detect three components at 19°C, the DMPC molecules in the gel phase coexisting with at least two species of DMPC in the fluid-like state monitored by the outer and inner splittings, which will be referred as component (I) and (II) in the following, of the methylene deuterons of the plateau region and of the terminal methyl groups. On cooling the sample further, the amount of the gel phase spectrum increases while the maxima of the fluid component (I) detected on both methyl and plateau resonances collapse. In contrast, the intensities of the other fluid component (II) remain constant, although a progressive broadening of the NMR lines occurs below 10°C.

The effect induced by βCC_A on the DMPC-d54 acyl chains can be analyzed in more detail after dePake-ing of the ^{2}H NMR data (Fig. 2, bottom spectra). As shown on the spectrum recorded with pure DMPC (Fig. 2, trace *a*), this procedure allows clear monitoring of the individual quadrupolar splittings of the myristoyl acyl chains, i.e., the terminal methyl groups (1), the methylene groups localized at the end of the acyl chains and those contributing to the plateau region (2). The two components (I) and (II) observed in the presence of βCC_A appear clearly on the dePaked spectrum recorded with 20% (Fig. 2 *b*) or 30% (Fig. 2 *c*) of the cyclodextrin derivative. An estimation of the relative intensity of the methyl resonances of component (I) and (II) gives ~30% and 50% of (II) at, respectively, 20% or 30% molar βCC_A. The ratio of the two methyl components was found to be independent of the pulse separation used in the quadrupolar echo sequence (for values of τ between 40 and 200 μs; see Materials and Methods). This indicates that the measurement is not distorted by differences in echo decay times for the methyl groups in the two lipid phases.

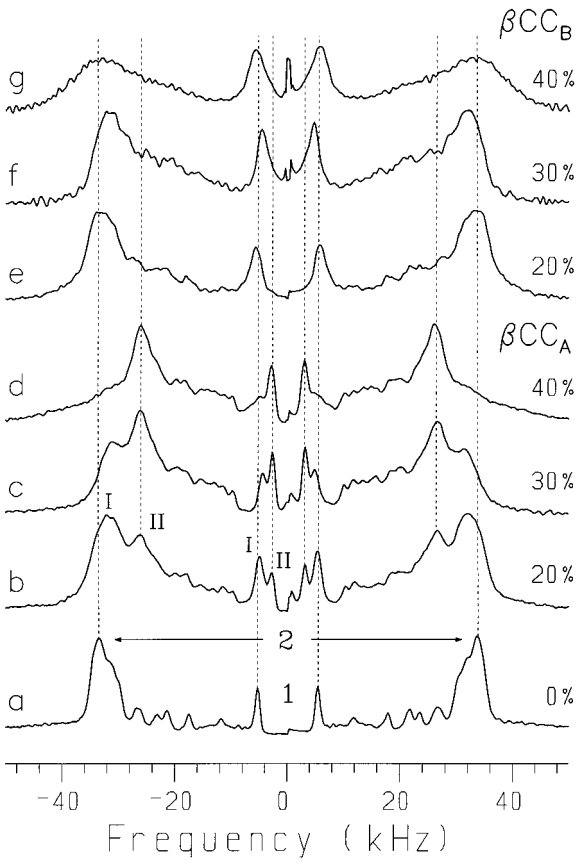

FIGURE 2 ^{2}H NMR dePaked spectra of DMPC-d54 membranes recorded at 20°C with 20%, 30%, and 40% βCC_A (*b*, *c*, *d*) or βCC_B (*e*, *f*, *g*) expressed in molar %. The bold letters on the pure DMPC spectrum *a* point to the quadrupolar splittings attributed to the methyl deuterons (1) and to the methylene deuterons of the plateau region (2). The components (I) and (II) described in the text are indicated on spectrum *b*. The dePaked spectra were scaled with normalization factors obtained by area-normalization of their related Fourier transform spectra shown in Fig. 1. The intensity of spectrum *a* obtained without cholesteryl cyclodextrin (0%) is rescaled by a factor of 0.7.

Thus, the signal ratio of the two components is not equal to 1 and changes with the guest/lipid ratio. This indicates that the two lipid forms characterized by the splitting of the methyl resonances do not result from the magnetic inequivalence of the *sn*-1 and *sn*-2 chains, as observed in the presence of cholesterol (Sankaram and Thomson, 1990). When 40% of the guest is added to the bilayers (Fig. 2 *d*), we observe that component (I) has almost disappeared, so that most of the signal of DMPC deuterons are now under component (II). The dePaked deuterium NMR spectra displayed in Fig. 2 show that for all membranes containing βCC_A, the quadrupolar splittings of the methyl and plateau methylene groups of component (II) do not depend on the βCC_A-to-DMPC molar ratio, i.e., the order parameters of the DMPC acyl chains in the new phase are approximately invariant whatever the guest concentration in the membrane.

DMPC-d54 with cholesteryl-β-cyclodextrin B

In the B form of cholesteryl-β-cyclodextrin (βCC_B) the cholesteryl moiety is directly linked to the amino group of cyclodextrin without the succinyl spacer found in the A form. As seen in Fig. 2 (*e–g*), the dePaked ^2H NMR spectra of DMPC membranes obtained at 20°C with the two derivatives are quite different. Apparently, there is only one fluid component with βCC_B, when the membrane is in the liquid crystalline state. The quadrupolar splittings of the plateau and methyl deuterons have approximately the same values than those measured with pure DMPC-d54, and are not decreased as observed with βCC_A.

The dePaked lines are broadened in the presence of βCC_B, while they are still narrow and resolved with βCC_A (Fig. 2, *b–d*). This line broadening is quite pronounced when the membrane βCC_B concentration reaches 40% (Fig. 2 *g*).

Temperature dependence of DMPC-d54 bilayers with cholesteryl cyclodextrins

The temperature dependence of the DMPC/cholesteryl cyclodextrin interaction was probed on large range of temperature from 37°C to −12°C. The dePaked spectra obtained with 20% βCC_A or βCC_B are respectively shown in the stacked plots of Fig. 3, *A* and *B*. The line widths and quadrupolar splittings measured for the methyl deuterons from these spectra are plotted in Fig. 4.

Temperature above 0° C

The first occurrence of the component (II) induced by the membrane incorporation of βCC_A, is detected ~25°C at the level of the plateau methylene signals, followed by a splitting of the methyl resonances at 23°C.

It appears clearly that one component (I) is temperature dependent, whereas the other (II) is barely affected. As shown in Fig. 4, there is a clear line broadening (Fig. 4 *B*) and a sharp increase of the quadrupolar splitting (from 5–13 kHz, Fig. 4 *A*) of component (I) below 20°C, at the $L_\alpha \rightarrow P_{\beta'}$ transition temperature of pure DMPC-d54. In fact, it can be shown that the temperature dependence of the plateau and the methyl quadrupolar splittings measured with the signals of component (I) are similar to that obtained with pure DMPC-d54. In contrast, the quadrupolar splittings of component (II) of both methylene and methyl deuterons remain quasiconstant below 20°C, without significant line broadening. As seen on the dePaked spectra displayed in Fig. 3 *A*, the signal intensity of the plateau methylene deuterons starts to decrease below 15°C, and becomes barely detectable at 5°C. However, there is no loss of intensity of the methyl signal, and no important quadrupolar splitting changes or line broadening occur until the temperature reaches 5°C, well below the fluid-to-gel phase transition. Cooling the sample below 5°C leads to further broadening of the methyl resonances of component (II), with a simultaneous increase of the quadrupolar splitting, suggesting the formation at these temperatures of a more ordered phase (Fig. 4, *A* and *B*).

As mentioned above, there is only one component on the ^2H NMR spectra of DMPC-d54/βCC_B membranes, although the dePaked resonances of the fluid methyl group measured above Tc are slightly broadened (Fig. 3 *B*). The chain quadrupolar splittings are temperature dependent in the presence of the B form of cholesteryl-β-cyclodextrin, as observed with pure DMPC, and no temperature-invariant component can be detected on the whole temperature range (Fig. 3 *B* and Fig. 4, *C* and *D*), as opposed to the results obtained with βCC_A-containing membranes. Intensities of gel phase lipids at ±63 kHz are detected at 19°C on the powder spectra, indicating that a fluid to gel phase transition occurs (spectra not shown). The chain methyl quadrupolar splitting is still detected below this temperature and continues to increase regularly with a marked inhomogeneous line broadening of the dePaked NMR lines ~15°C (Fig. 3 *B*). The distribution of the methyl deuteron intensities is asymmetric, retaining a Gaussian line shape on the inner half width, i.e., on the side of the center frequency of the spectrum, similar to that measured in the fluid phase above 19°C, and a broader shoulder on the outer width of the signal, which spreads over the frequency range of the methyl signal of the pure lipid in the gel phase. The intensity of the outer shoulder increases when the temperature decreases, and at 0°C the methyl NMR lines are almost superimposable with the corresponding resonances of the pure lipid spectrum at the same temperature.

The gel-to-fluid transition appears clearly from the temperature dependence shown in Fig. 4 of the methyl

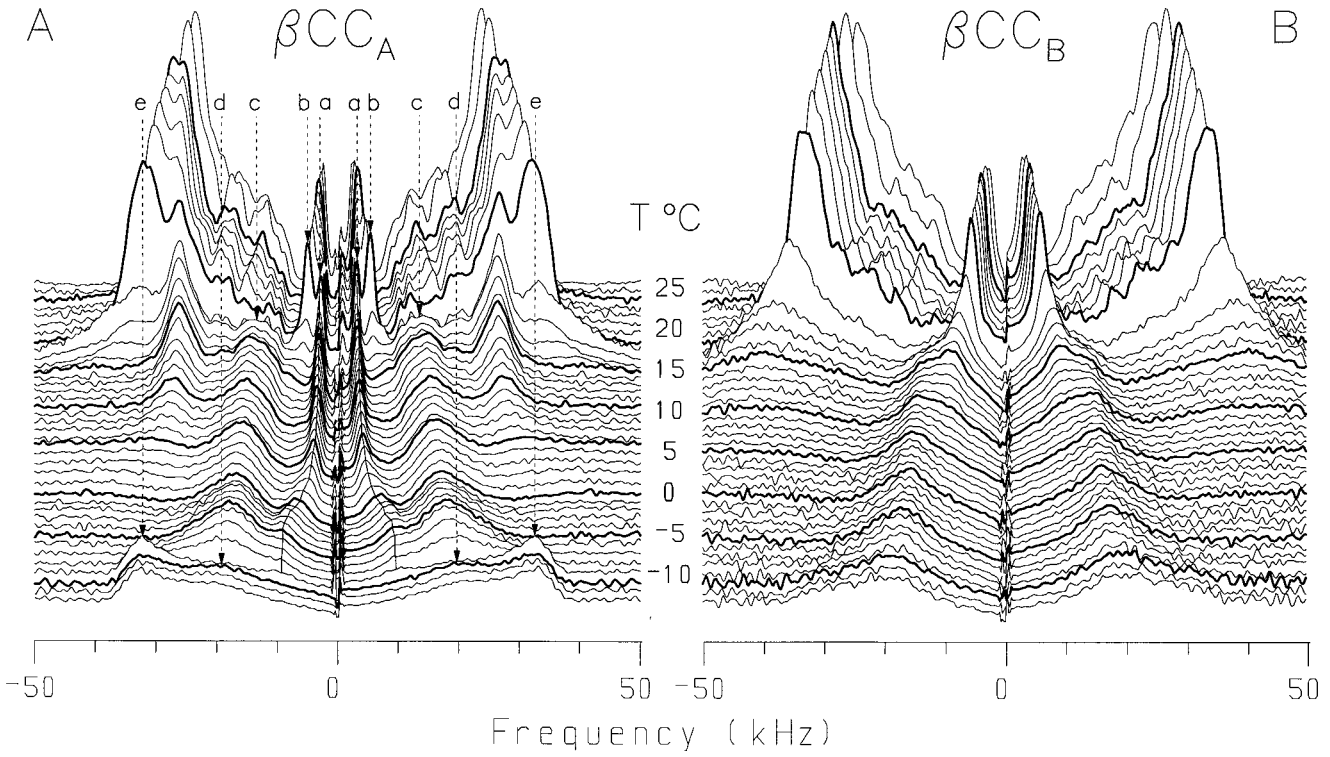

FIGURE 3 ^{2}H NMR dePaked spectra of DMPC-d54 membranes recorded with 20% molar of βCC_A (*A*) or βCC_B (*B*). The stacked plots show the spectra recorded at 37 and 30°C, and from 25 to −12°C (1°C step, going from the upper traces to the lower traces; selected temperatures are indicated by the bold traces). The dePaked spectra were scaled with normalization factors obtained by area normalization of their related Fourier transform spectra shown in Fig. 1. (*A*) The letters indicate the quadrupolar splittings of component (I) in the fluid state at 20°C (*b*), in the gel phase at 18°C (*c*), in the lamellar gel phase at −9°C (*e*), and the quadrupolar splittings of component (II) in the fluid L_{CD} phase at 20°C (*a*) and in a gel-like state at −10°C (*d*) (see Discussion). The methyl resonances of component II are also indicated by a thin solid line connecting their maxima, showing the transition occurring between 5 and −5°C, from the fluid L_{CD} phase to an ordered L_{CD} phase (see Discussion).

line widths (Fig. 4 *D*) and quadrupolar splittings (Fig. 4 *C*), although it is significantly smoothed, ranging between 20°C and 15°C. For the A form of cholesteryl-β-cyclodextrin, the transition probed by the signal of component (I) is steeper and is already achieved at 18°C (Fig. 4, *A* and *B*).

The chain methyl line widths and quadrupolar splittings were also measured at high concentrations (40%) of cholesteryl-β-cyclodextrin. The temperature dependence obtained for these two parameters with the component (II) of DMPC membranes containing either 20% or 40% of βCC_A are similar, with the same slope increase below 5°C. Likewise, the related data obtained with the B form of cholesteryl-β-cyclodextrin show that the temperature-induced variations of the methyl line widths and quadrupolar splittings measured at 20% and 40% of βCC_B are similar.

The lipid phase changes were also monitored by the first moment M_1 analysis of the powder pattern spectra shown in Fig. 1, and is displayed in Fig. 5 for various concentrations (0, 5%, 20%, 30%, and 40%) of the A and B form of cholesteryl-β-cyclodextrin. For all samples, the first moment M_1 is increased when the temperature is decreased due to the thermally induced increase of the average orientational order of the lipid acyl chains. In the case of pure DMPC, there is a sharp change ~20°C associated with the gel-to-liquid crystalline transition, as discussed above. The incorporation of either A or B form of cholesteryl-β-cyclodextrin leads to a decrease of M_1 values, indicating a decrease of the average orientational order of the hydrophobic chains in the presence of the cyclodextrin derivatives. There is a large decrease of M_1 value between 10°C and 20°C in the presence of the βCC_A, leading to a progressive broadening of the fluid-to-gel transition, due to the growing fraction of the fluid lipids found under component (II) on the deuterium spectra. At 40% βCC_A the first moment variations are almost linear without sigmoid transition but with a sixfold increase of the slope occurring ~18°C. The decrease of M_1 values measured below the transition for a given amount of cholesteryl-β-cyclodextrin is less important with the B form than with the A form, and the fluid-to-gel transition is less affected with the former derivative. In particular, there is still a sigmoid transition in the presence of 40% of βCC_B, although shifted at lower temperature by ~3°C.

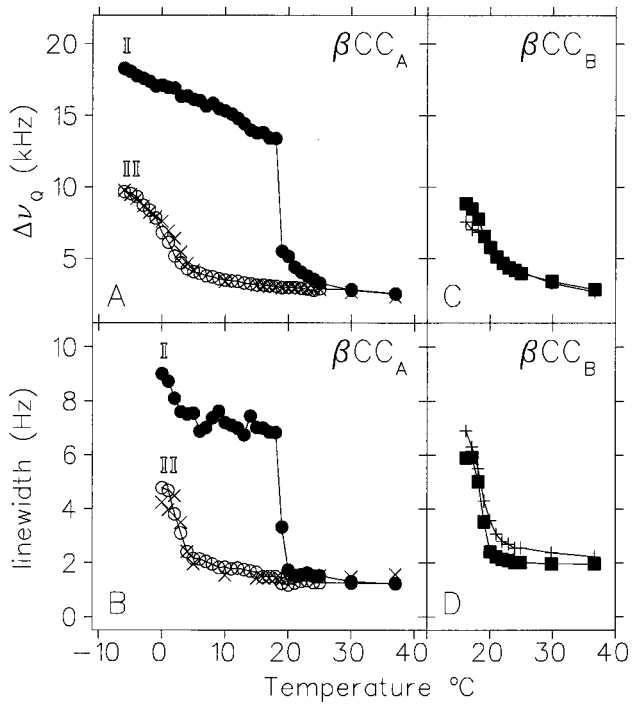

FIGURE 4 Quadrupolar splittings (*A* and *C*) and line widths (*B* and *D*) of the DMPC-d54 methyl resonances, measured from the dePaked spectra shown in Fig. 3, obtained in the presence of βCC_A (*A* and *B*) or βCC_B (*C* and *D*) as follow: (●,○) 20% βCC_A; (×) 40% βCC_A; (■) 20% βCC_B; and (+) 40% βCC_B. In the presence of the inhomogeneous line broadening occurring below 20°C with βCC_B-containing membranes (*C* and *D*), line widths, and quadrupolar splitting were determined from the inner shoulder of the methyl resonances of the dePaked spectra of Fig. 3 *B*. The measurements below 15°C were found to be unreliable.

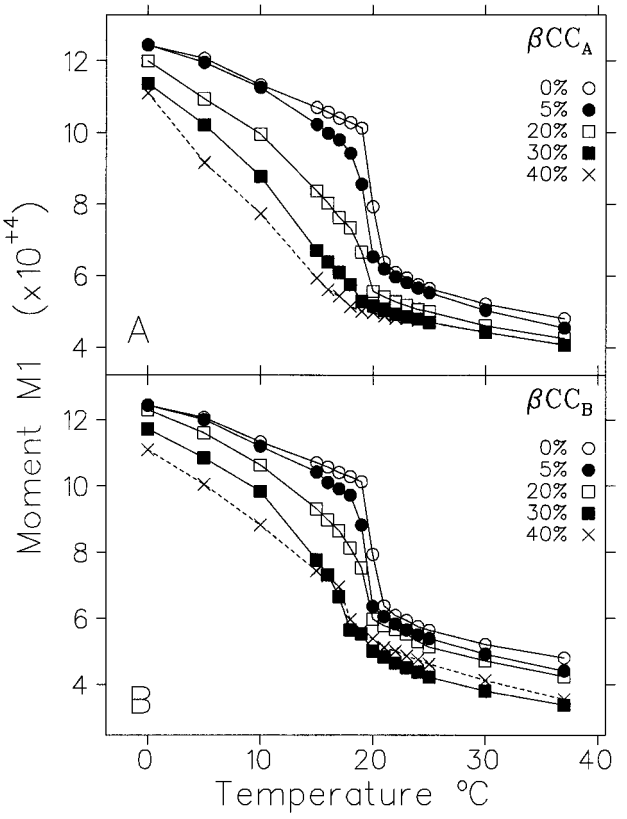

FIGURE 5 Moment M1 of the ^2H NMR spectra of DMPC d54 membranes pure (○) and with 5% (●), 20% (□), 30% (■), and 40% (×) (molar %) of βCC_A or βCC_B.

Temperatures below 0°C

As observed for the $L_\alpha \rightarrow P_{\beta'}$ transition at 19°C, there is another sudden increase (20–32 kHz) of the methyl quadrupolar splitting of the component (I) observed with the A form of cholesteryl-β-cyclodextrin at approximately −8°C (Fig. 3 *A*). This transition is also detected with the corresponding pure DMPC-d54 sample (Roux et al., unpublished results). A similar transition, occurring at approximately −4°C, has been observed previously by ^2H NMR with DMPC specifically deuterated on the methyl group of the *sn*-2 chain (Westerman et al., 1982). It is probable that these spectral changes observed upon cooling the DMPC membranes below 0°C, reflect the $P_{\beta'} \rightarrow L_{\beta'}$ transition of the phospholipid, from the gel ($P_{\beta'}$) phase to a pure lamellar gel ($L_{\beta'}$) phase (Trahms et al., 1983; Koynova and Caffrey, 1998). A close inspection of the dePaked spectra obtained below 0°C with 20% βCC_A displayed in Fig. 3 *A* shows that the methyl quadrupolar splitting of component (II) (±10 kHz) is still detected at these temperatures even at −9°C below the second transition observed with the pure lipid component (I). When the temperature reaches −10°C, the dePaked methyl resonances found at ±10 kHz disappear and are replaced by another signal appearing approximately ±20 kHz, not detected at this temperature on the corresponding ^2H NMR of pure DMPC-d54. This new quadrupolar splitting appears to be similar to that measured at −7°C for the pure laterally segregated lipids (I) in the gel state, i.e., before the putative $P_{\beta'} \rightarrow L_\beta$ transition. Interestingly, this latter perturbation is correlated with the freezing of the bulk water, as probed on the corresponding deuterium powder spectrum by the complete and sudden loss of the narrow isotropic signal attributed to residual deuterated water.

As opposed to βCC_A membrane data, there is no NMR evidence of a sharp transition below 0°C with βCC_B, although the NMR lines show a marked asymmetry below −8°C with intensities spreading over ±30 kHz.

DMPC-d54 bilayers with low concentrations of cholesteryl-β-cyclodextrins

To test further the ability of βCC_A to induce the formation of a second lipid environment (II), we have also obtained data at low concentration (5%) of βCC_A. It appears that, despite this membrane dilution, a second component is still detected, as shown in Fig. 6. Due to the low amount of

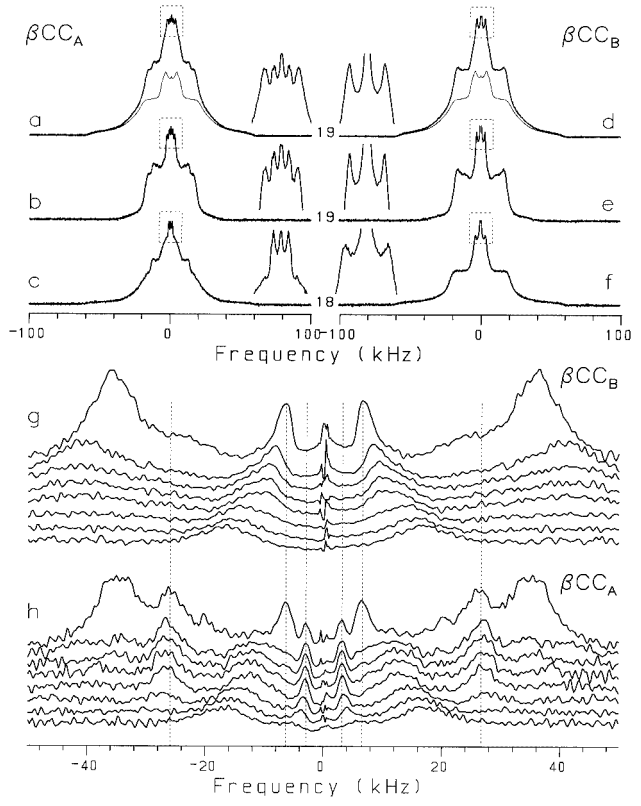

FIGURE 6 ^{2}H NMR difference spectra of DMPC-d54 membranes with 5% of βCC_A (*a*, *b*, *c*, and *g*) or βCC_B (*d*, *e*, *f*, and *h*). Original (*a* and *d*) and difference (*b*, *e*, *c*, and *f*) spectra at 19°C (*b* and *e*) and 18°C (*c* and *f*). The expanded methyl region is shown for each spectrum (*a–f*). The stacked plot (*g* and *h*) show the dePaked spectra obtained from the difference spectra at 19, 18, 17, 16, 15, 5, and 0°C.

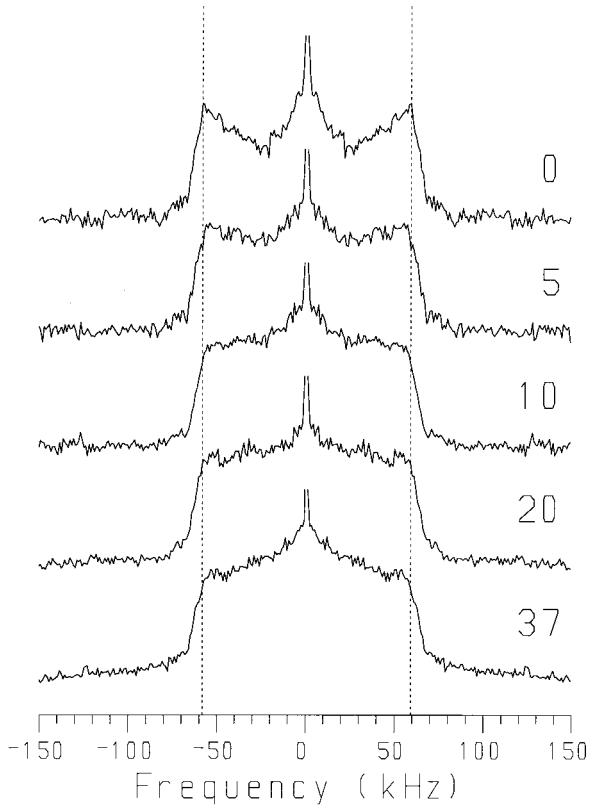

FIGURE 7 Temperature dependence of the ^{2}H NMR spectra of 20% molar of βCC_A-d7, deuterated on the C2 carbon of the seven glucose units, incorporated in DMPC membranes.

βCC_A, this component is not resolved on the DMPC-d54 spectra recorded in the fluid phase (data not shown), but it appears clearly on the ^{2}H NMR spectrum recorded at 19°C, just below the fluid-to-gel phase transition (Fig. 6 *a*). The analysis of the methyl region indicates very clearly the coexistence of two fluid lipid environments with gel phase lipids, as observed on the corresponding NMR spectra recorded with 20% of βCC_A. To isolate these fluid components, we have removed the gel signal by subtracting a fraction ($\sim$ 62%) of the pure DMPC-d54 gel phase spectrum recorded at the same temperature. We were able to obtain a difference spectrum (Fig. 6 *b*) with no signal left beyond $\pm$40 kHz, containing only the signals of the fluid lipids. The same procedure was applied to the NMR data recorded at lower temperature, i.e., the same fraction (62%) of the pure lipid gel phase spectrum at the corresponding temperature was subtracted. The difference spectrum derived from the data measured after lowering the temperature of one degree from 19°C to 18°C indicates clearly that the fraction of the pure lipid remaining in the fluid state has now moved into the gel state (Fig. 6 *c*). After dePake-ing of the obtained difference spectra (Fig. 6 *h*), it can be shown that the temperature dependencies of the line widths and quadrupolar splittings of the methyl signal follow closely those obtained at 20% and 40% βCC_A. Similar difference spectra were obtained at 10% of βCC_A (data not shown), and lead to the same conclusions.

^{2}H NMR spectra were also obtained with 5% (Fig. 6, *d–g*) and 10% (data not shown) of the B form of cholesteryl-β-cyclodextrin. The analysis of the difference spectra obtained with the same procedure, as described above, confirms the results obtained at higher concentrations, showing that there is no additional component in the presence of this cholesteryl-β-cyclodextrin derivative.

Deuterated βCC_A in DMPC bilayers

The interaction of cholesterol cyclodextrin with zwitterionic DMPC bilayers was probed with βCC_A-d7 deuterated on the oligosaccharide headgroup, at the level of the C_2 carbon of the seven glucose units. The ^{2}H NMR spectra obtained with this derivative, shown in Fig. 7, contain a very large quadrupolar splitting ($\sim$120 kHz) with a broad featureless signal spread over the whole frequency range. The larger splitting has no thermal dependence, retaining the same value at all temperatures. On the other hand, the intensity of

TABLE 1 Lipid distribution at different temperatures and for various molar % of βCC_A, among the gel phase, the fluid phase, and the L_{CD} phase

$\%\beta CC_A$	T°C	F_{gel}*	F_{fluid}†	F_{Lcd}†	N_{gel}‡	N_{fluid}‡	N_{Lcd}‡	$W_{fluid\ Hz}$†	$W_{Lcd\ Hz}$†
5	19	0.62	0.16	0.22	118	57	16	2.5	1.4
10	19	0.39	0.31	0.30	35	38	17	2.1	0.9
20	19	0.18	0.47	0.35	7	21	11	2.3	1.1
30	19	0	0.49	0.51	0	11	12	2.2	1.1
20	20	0	0.68	0.32	0	27	13	1.8	1.2
20	21	0	0.67	0.33	0	27	13	1.6	1.1
20	22	0	0.70	0.30	0	28	12	1.6	1.0
30	20	0	0.50	0.50	0	12	12	1.7	1.1
30	21	0	0.54	0.46	0	13	11	1.7	1.0
30	22	0	0.59	0.41	0	14	10	1.7	1.0

F_{gel}, F_{fluid}, and F_{Lcd} are the fraction of the gel, fluid, and L_{CD} phase with $F_{gel} + F_{fluid} + F_{Lcd} = 1$.
N_{gel}, N_{fluid}, and N_{Led} are the number of lipid molecules found respectively, in the gel phase, the fluid phase and the L_{CD} phase for 10 molecules of βCC_A.
W_{fluid} and W_{Lcd} are the computed methyl line widths of the lipids found in the fluid phase (I) and the L_{CD} phase (II).
*The amount of pure lipid spectrum subtracted to the corresponding spectrum of cholesteryl-β-cyclodextrin-containing membranes.
†Obtained by fitting the methyl resonances with a Gaussian lineshape.
‡Derived from F_{gel}, F_{fluid}, and F_{Lcd}.

the central signals decreases progressively when the temperature is lowered, revealing a well-defined quadrupolar splitting at 0°C. The broad signal distribution observed at high temperatures could either reflect differences in the order parameters of the glucose units or different environments of the βCC_A molecules. We have also recorded ^2H NMR spectra with native deuterated β-cyclodextrin-d7 (without the cholesteryl moiety) and obtained a single narrow isotropic resonance, which indeed confirms that the hydrophilic guest does not interact spontaneously with phosphatidylcholine liposomes in the absence of a hydrophobic anchor (data not shown). There is a 200-Hz difference between the chemical shifts of the isotropic signal of free β-cyclodextrin-d7 and the central narrow resonance found on the spectra of membrane-bound βCC_A shown in Fig. 7. In the latter case the isotropic signal is due to the deuterons of residual heavy water.

DISCUSSION

Our study shows that the A form of cholesteryl-β-cyclodextrin, which contains a spacer between the sterol and the cyclodextrin moiety, induces a lateral separation of lipid molecules in two distinct phases containing pure DMPC and DMPC sequestrated between βCC_A molecules. We report two components of the DMPC-d54 spectra, with exchange rates that are slow on the ^2H NMR timescale ($>10^{-5}$ s^{-1}), probing the occurrence of two different lipid environments. Component (I) has the same thermal behavior as pure DMPC membranes, namely 1) a line broadening at the gel-to-fluid transition temperature of pure DMPC-d54 with the appearance of spectral intensities characteristic of lipids in the gel state and 2) the same quadrupolar splitting temperature dependence. Thus, this component is associated with a pure DMPC phase, co-existing with a cyclodextrin-rich phase appearing as component (II) on the NMR traces. These ^2H NMR results are in good agreement with our previous data obtained for the DMPC/βCC_A system using small angle x-ray scattering and differential scanning calorimetry, which indicate also the coexistence of two lamellar phases at 30°C (Auzély-Velty et al., 1999). In the present study, we have obtained NMR data providing detailed molecular information on the DMPC/cholesteryl-β-cyclodextrin system on a large range of concentrations and temperatures. A phase separation was found to occur with only 5% of βCC_A. The overall perturbation of the bilayer is weak at such concentration (almost probe level) so the membrane order is close to that of pure DMPC, as shown in the corresponding moment M1 curve (Fig. 5). Under these conditions, we can partially dissociate discrete effects from global membrane perturbations induced by larger concentrations of the guest. We have found that there are at least three lipid environments at 19°C just below the fluid-to-gel transition of DMPC-d54. A first one, with the smaller splitting, is associated with lipids interacting with βCC_A, in a cholesteryl cyclodextrin-rich phase, which we will refer to as the L_{CD} phase in the following, and appearing as component (II) on the ^2H NMR spectra. A second one is associated to the fluid signal with the larger quadrupolar splitting and should correspond to lipids located at the boundary of the L_{CD} phase, remaining in the fluid state at 19°C and a third one is that of pure lipids in the gel phase, not interacting with the L_{CD} phase. These three lipid environments are clearly distinguished on the difference spectrum obtained with 5% βCC_A at 19°C (Fig. 6). A quantitative analysis of the dePaked NMR spectra has been attempted by simulating the fluid dePaked methyl resonances of component (I) and (II) with Gaussian line shapes, and the results are shown in Table 1. For the sample containing 5% of βCC_A at 19°C, we have estimated that for 10 molecules of βCC_A, the amount of DMPC in the L_{CD} phase, at the boundary of the L_{CD} phase and in the gel state are (within

the experimental error, respectively), 16, 57, and 118. As shown in Table 1, the number of lipids in the gel phase is quickly reduced when the concentration of βCC_A is increased, and for 30% βCC_A there is no more lipids in the gel phase. Indeed, the total number of fluid lipid per molecule of βCC_A is also reduced, but it is mostly at the expense of the fluid boundary lipids, whereas the number of lipid molecules found in the L_{CD} phase remains approximately constant in the range of 1 to 1.5 DMPC per βCC_A molecule. A similar ratio is also found at temperatures above the fluid-to-gel transition at either 20% or 30% of βCC_A. At 40% βCC_A, which corresponds already to a global ratio of 1.5 molecules of DMPC per cholesteryl-β-cyclodextrin, almost all the lipids should be in the L_{CD} phase. The calculated line width values are indeed consistent with the data plotted in Fig. 4, showing that the pure lipid resonances start to broaden at 19°C at the onset of the fluid-to-gel transition, whereas those of the lipids in the L_{CD} phase are not affected by this transition. Thus, the DMPC-to-βCC_A ratio in the L_{CD} phase appears to be remarkably invariant, i.e., independent on both the temperature variations and the total amount of cholesteryl-β-cyclodextrin. This strongly suggests that the two phases are fully separated with DMPC either pure or mixed with βCC_A. A possible explanation could be that these two phases are in fact macroscopically distinct. In such case, the two ^2H NMR components would just reflect the heterogeneity of the sample with membrane aggregates enriched in cholesteryl-β-cyclodextrin and pure lipid particles. This assumption can be ruled out by the observation that, at all βCC_A concentrations, the pure lipid phase does interact with the L_{CD} phase through the boundary fluid lipids detected at the onset of the fluid-to-gel transition.

It appears that the lateral separation of the L_{CD} phase, detected at membrane concentrations as low as 5% of βCC_A, has to be associated with strong interactions between the cyclodextrin headgroups. The β form of cyclodextrin alone, without cholesterol, is actually well known to form aggregates in water and is 10-fold less soluble than the α and γ derivatives. Interestingly, the chemical modifications of the cyclodextrin hydroxyls, for instance by methylation, disrupt these interactions and lead to completely different behaviors (Auzély-Velty et al., 2000). In our case, the βCC_A cyclodextrin headgroups incorporated in DMPC bilayers appear to be very rigid, because the deuterons of the cyclodextrin headgroup exhibit a very large quadrupolar splitting, independent of the temperature variations, giving a high order parameter, corresponding to an almost complete absence of motion.

Thus, the emerging picture is that the stability of the L_{CD} phase should be governed primarily by the hydrophilic interaction between cyclodextrin headgroups. In this case hydrophobic interaction between the sterol and the phospholipid acyl chains could be of secondary importance in maintaining the cohesion of the L_{CD} phase. The L_{CD} phase seems to contain a maximum of 1.5 DMPC per βCC_A. Considering the ratio of the specific area of the cyclodextrin headgroup and of the cholesteryl hydrophobic anchor, it can be roughly estimated that, in the case of a packed network of cyclodextrin headgroups, the volume left in between two adjacent cholesteryl cyclodextrin molecules should effectively accommodate for no more than two to three phospholipid molecules. The acyl chains of these sequestrated lipids are more disordered than in the pure lipid phase. This is shown by the decrease of the first moment M_1 and the reduction of the quadrupolar splittings of the methyl and plateau deuterons observed in the presence of βCC_A. This effect is thus completely opposed to the ordering of the phosphatidylcholine fluid phase, or "condensing" effect, induced by cholesterol alone, which leads in similar experimental conditions, and with the same cholesterol to phospholipid ratio, to an average ~70% increase of the DMPC-d54 quadrupolar splittings (data not shown; Vist and Davis, 1990). The acyl chains quadrupolar splittings are also remarkably insensitive to temperature variations in the L_{CD} phase, as opposed to what is observed in the pure lipid phase, and remain fluid well below Tc. The above remarks support the model in which the packing order of the L_{CD} phase is not primarily determined by chain-chain or cholesterol-chain interactions, as it occurs in DMPC bilayers, but is rather dominated by the interactions between the large cyclodextrin moities. However, the chain deuterons become temperature sensitive between 5°C and −5°C, showing that the DMPC molecules do undergo a phase transition from a fluid phase to more a ordered environment, as shown in Fig. 3 *A* and Fig. 4. Yet, the methyl quadrupolar splittings are still smaller than those measured in DMPC gel phase, indicating that the orientational order of this new L_{CD} state is smaller than in the $P_{\beta'}$ gel phase of the pure lipid. This ordered L_{CD} phase seems to be quite stable and appears to be preserved at low temperatures, even at −9°C when the pure lipids appear to be in the rigid $L_{\beta'}$ lamellar gel phase. However, it apparently breaks down at −10°C, where the lipids associated to component (II) seem to undergo another transition. The lipids should have evolved into a gel-like state, considering that the quadrupolar splittings of their acyl chain methyl groups are now comparable with those measured for the component (I) of the pure lipid in the gel phase (Fig. 3 *A*). This transition could be due to the freezing of the bulk water molecules observed simultaneously at −10°C, which probably lead to disorganization or even a disruption of the cholesteryl-β-cyclodextrin headgroup network and a rearrangement or a suppression of the L_{CD} phase.

The role of the spacer introduced between the sterol and the cyclodextrin is certainly decisive by increasing the conformational space offered to the βCC_A headgroups anchored at the membrane interface. Such a conformational flexibility is probably required to achieve head to head interactions efficient enough to induce the clustering effect leading to the sequestration of the DMPC molecules in the

L_{CD} phase. The results obtained with the B form of cholesteryl β-cyclodextrin, lacking the succinyl spacer, support this model. In the presence of this derivative, only one phase is detected with spectrum line shapes indicating that the bilayers are still stable. In the fluid phase, there is a decrease of the average orientational order as probed by the first moment M1 values, which are approximately similar to those obtained with bilayers containing βCC_A. In fact, the global disordering effects induced by these two cyclodextrin derivatives on the DMPC acyl chains in the fluid phase are quantitatively similar, although the molecular mechanisms involved in the interaction of βCC_A and βCC_B with the lipid membrane appear to be qualitatively different. In the absence of succinyl spacer, the B form of cholesteryl-β-cyclodextrin must be distributed in the whole bilayers, perturbing almost each phospholipid molecule, whereas with the A form, two laterally segregated distinct phases are observed. This appears quite clearly on the ^2H NMR dePaked spectra obtained with 20% of the cholesteryl-β-cyclodextrin derivatives displayed in Fig. 3. With βCC_B there is a smoothed gel-to-fluid phase transition involving the ensemble of the lipids, whereas sharp phase transitions concerning only a fraction of lipids separating from the βCC_A-induced L_{CD} phase are observed at 19°C and also at −8°C. At high concentrations of βCC_A, there is formation of an almost pure L_{CD} phase without cooperative fluid-to-gel transition, whereas a sigmoid transition is still detected with βCC_B, indicating that the DMPC molecules are still able to undergo a cooperative, although smoothed, transition to the gel state (Fig. 5).

The ensemble of our NMR data provide conclusive evidences that there is globally one lipid phase with βCC_B, whereas a pure lipid phase and a laterally segregated cholesteryl cyclodextrin-rich phase are detected with βCC_A. Without the succinyl link to the cholesterol moiety, the cyclodextrin headgroup of βCC_B must be constrained at the membrane surface with a reduced conformational space, hindering probably the adequate positioning of adjacent headgroups and the formation of the L_{CD} phase observed with the βCC_A. Thus, the cholesteryl cyclodextrin/DMPC system gives a straightforward example on how an amphiphilic molecule can affect lipid membranes with the local formation of a two-dimensional molecular network within the bilayer, through finely-tuned intermolecular headgroup interactions at the membrane interface.

The ability of bilayer membranes to incorporate the cholesteryl-β-cyclodextrin derivatives should permit the liposome transportation of hydrophobic cyclodextrin-bound drugs, standing out at the bilayer surface toward the external medium, facilitating their interaction with circulating molecules or macromolecular assemblies. Indeed, the primary pharmacological interest of the cholesteryl-β-cyclodextrin lies in the inclusion property of the cyclodextrin cavity. Further efforts will thus be dedicated to investigate the effect of the inclusion of a guest compound on the cholesteryl-β-cyclodextrin liposome insertion and on the formation of the L_{CD} phase. Special attention will be given to pharmacologically active compounds to design new formulations for the administration of drugs combining the size-specificity of the cyclodextrin moiety and the carrier properties of phospholipid liposomes. It will be in particular interesting to evaluate the relative potencies, if any, of the βCC_A and βCC_B derivatives and determine whether the formation of the laterally segregated L_{CD} phase is pharmacologically relevant.

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