Lipid Lateral Segregation Driven by Diacyl Cyclodextrin Interactions at the Membrane Surface

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ABSTRACT Cyclodextrins are hydrophilic molecular cages with a hydrophobic interior allowing the inclusion of water-insoluble drugs. Amphiphilic cyclodextrins obtained by appending a hydrophobic anchor were designed to improve the cell targeting of the drug-containing cavities through their liposome transportation in the organism. After insertion in model membranes, they were found to induce a lateral phase separation into a pure lipid phase and a fluid cyclodextrin-rich phase (L_{CD}) with reduced acyl chain order parameters, as observed with a derivative containing a cholesterol anchor (M. Roux, R. Auzely-Velty, F. Djedaïni-Pilard, and B. Perly. 2002. *Biophysical Journal*, 8:813–822). We present another class of amphiphilic cyclodextrins obtained by grafting aspartic acid esterified by two lauryl chains on the oligosaccharide core via a succinyl spacer. The obtained dilauryl-β-cyclodextrin (βDLC) was inserted in chain perdeuterated dimyristoylphosphatidylcholine (DMPC-d54) membranes and studied by deuterium NMR (^{2}H-NMR). A laterally segregated mixed phase was found to sequester three times more lipids than the cholesteryl derivative (~4–5 lipids per monomer of βDLC), and a quasipure L_{CD} phase could be obtained with a 20% molar concentration of βDLC. When cooled below the main fluid-to-gel transition of DMPC-d54 the βDLC-rich phase stays fluid, coexisting with pure lipid in the gel state, and exhibits a sharp transition to a gel phase with frozen DMPC acyl chains at 12.5°C. No lateral phase separation was observed with partially or fully methylated βDLC, confirming that the stability of the segregated L_{CD} phase was governed through hydrogen-bond-mediated intermolecular interactions between cyclodextrin headgroups at the membrane surface. As opposed to native βDLC, the methylated derivatives were found to strongly increase the orientational order of DMPC acyl chains as the temperature reaches the membrane fluid-to-gel transition. The results are discussed in relation to the "anomalous swelling" of saturated phosphatidylcholine multilamellar membranes known to occur in the vicinity of the main fluid-to-gel transition.

INTRODUCTION

Biomembranes can be viewed as a mosaic of lipid domains with unique biochemical compositions, controlled by a variety of lateral segregation processes occurring within the lipid matrix. Besides its fundamental importance in membrane biophysics, phase segregation in phospholipid model membranes has regained much interest in the past few years, with the emerging concept of functional lipid rafts, primarily related to the noncovalent clustering of cholesterol and sphingolipids in the presence of other phospholipids (1–4). Numerous earlier studies report on lipid lateral segregation in model membranes containing binary or ternary mixtures of phopholipids differing by their headgroups or by their acyl chain length and unsaturation (see Veatch and Keller (5) and references therein). Electrostatic-driven interactions of negatively charged phospholipid-containing membranes with cations, charged peptides, or proteins can also lead to lipid lateral segregation (6–11). In this study, we describe phospholipid lateral segregation induced by intermolecular headgroup interactions of an amphiphilic polysaccharide at the membrane surface.

Amphiphilic cyclodextrins, designed to combine the inclusion ability of the cyclodextrin cavity (12,13) with the carrier properties of model membrane systems such as micelles or liposomes, were found to induce lateral segregation of a cyclodextrin-enriched lipid phase and a pure lipid phase (14). For instance cholesteryl-β-cyclodextrin, obtained by grafting a cholesterol anchor onto the oligosaccharide core, is able to induce the formation of laterally segregated fluid microdomains (L_{CD}) containing ~1–1.5 lipid per cyclodextrin within dimyristoylphosphatidylcholine (DMPC) multilamellar membranes. The segregated L_{CD} phase is stable and remains in the fluid state below the main transition of DMPC, coexisting with pure lipids in the gel state. β cyclodextrin monomers are able to aggregate in aqueous solution (15,16), and the formation of the L_{CD} phase is believed to be mediated through intermolecular interactions of cyclodextrin headgroups at the membrane surface. Accordingly, the flexibility and size of the cyclodextrin headgroups were found to be crucial for the thermodynamic stability of the cholesteryl cyclodextrin-rich lamellar phase. Restraining the cyclodextrin molecular space by removing the flexible spacer inserted between the cholesterol anchor and the cyclodextrin

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headgroup prevents L_{CD} phase lateral separation. Likewise, increasing the cyclodextrin headgroup size by substituting the β-cyclodextrin by the γ form while retaining the spacer leads also to the L_{CD} phase suppression (M. Roux unpublished results). Amphiphilic cyclodextrins appear to provide a straightforward case of microdomain formation within a lipid bilayer through finely tuned intermolecular interactions at the membrane surface.

In this study, we investigate the dependence of the L_{CD} phase stability on the nature of the hydrophobic anchor. We have substituted the bulky sterol nucleus by two short C_{12} acyl chains and inserted various concentrations of the obtained dilauryl-β-cyclodextrin (βDLC) in membranes of DMPC with perdeuterated acyl chains. The formation of a βDLC-induced L_{CD} phase as monitored by deuterium NMR is detailed in this report. Related NMR spectra were also recorded from membranes containing βDLC with a partially or fully methylated headgroup, and no lateral segregation could be detected. The phase properties of the βDLC-containing membranes are also discussed in relation to molecular events known as ''anomalous swelling'' occurring in the fluid phase at temperatures near the fluid-to-gel transition of saturated phosphatidylcholine membranes (17,18).

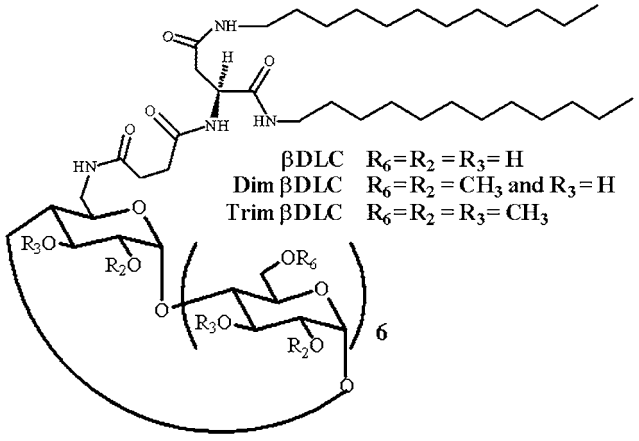
MATERIALS AND METHODS

Synthesis of cyclodextrin derivatives

Synthesis of βDLC and methylated derivatives has been achieved as already described elsewhere (19). This new class of amphiphilic cyclodextrins is obtained by grafting aspartic acid bearing acyl chains on succinylamido β-cyclodextrin, succinylamido DimβDLC, or succinylamido TrimβDLC. In this study, the amino acid and the acyl chain are aspartic acid and lauryl amine, respectively. The chemical structures of the final compounds, βDLC, dilauryl-di-2,6-*O*-methyl β-cyclodextrin (DimβDLC), and dilauryl-tri-2,3,6-*O*-methyl β-cyclodextrin (TrimβDLC), are displayed in Scheme 1.

Sample preparations

DMPC and chain deuterated DMPC-d27 and DMPC-d54 were purchased from Avanti Polar Lipids (Alabaster, AL) and the cholesterol from Sigma



SCHEME 1 Chemical structures of the βDLC derivatives.

(St. Louis, MO). Multilamellar liposomes were prepared by mixing chloroform solutions of the lipid and appropriate cyclodextrin derivative. The solvent was then removed by evaporation under reduced pressure and the solid residues suspended in 1–2 ml of water equilibrated at pH 7 and lyophilized. The lyophilizated powder was dispersed by continuous vortexing at 20°C in 100–300 μl of buffer (50 mM Tris, 40 mM NaCl) in deuterium-depleted water (Euriso-Top, Saint-Aubin, France) equilibrated at pH 7.5 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, Gloucester, Ontario, Canada). Membrane samples were cooled from 37°C to −12°C, and NMR spectra were acquired with a dwell time of 2 μs, 4 K data points, and a recycling time of 200 ms. A quadrupolar echo pulse sequence (20) was employed with pulse length of 4 μs and pulse separation, τ, of 40 μs. The phase was adjusted to obtain no signal in the imaginary channel. 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 (21). Oriented ^{2}H-NMR spectra (0°) were obtained by the numerical de-Pake-ing procedure (22). Order parameters S_{CD} of the methyl and methylene groups of fluid acyl chains were obtained from their de-Pake-ed quadrupolar splittings $\Delta\nu_Q$ according to

$$S_{CD} = (4/3)(h/e^2qQ)\Delta\nu_Q, \quad (1)$$

where (e^2qQ/h) is the deuteron quadrupolar coupling constant, which is taken as 167 kHz for a C-D bond (21,23). The first moments M1 of the deuterium powder spectra were determined, and the average order parameters $\langle S_{CD}\rangle$ of the DMPC-d54 acyl chains calculated according to Davis (21,23):

$$\langle S_{CD}\rangle = (4/3)\,(h/e^2qQ)\left(\frac{3\sqrt{3}}{4\pi}\right)M1. \quad (2)$$

Quantification and removal of the gel component from composite gel/fluid powder pattern spectra recorded in the presence of the cyclodextrin derivatives were done by subtraction of the area-normalized spectra of the pure lipid in the gel state recorded at the same temperature. This subtraction was done until complete extinction of spectral wings typical of gel phase lipids, found in the ±60 kHz region of the composite spectra. To test for coexistence of fluid phases, the de-Pake-ed methyl resonances found in the −10–10 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. When split into two components, the relative intensities of the individual resonances were found to be independent of the pulse separation used in the quadrupolar echo sequence, indicating that they were not distorted by differences in echo decay times of the two observed species.

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 lipid acyl chain and polar headgroup C-D bonds (21). Deuterium NMR spectra of membranes recorded above the gel-to-fluid transition are characterized by a well-resolved distribution of quadrupolar splittings typical of the liquid crystalline L_α phase (Fig. 1 *a*). This quadrupolar splitting distribution reflects the order profile of phospholipid bilayers in the fluid state, seen more clearly after de-Pake-ing

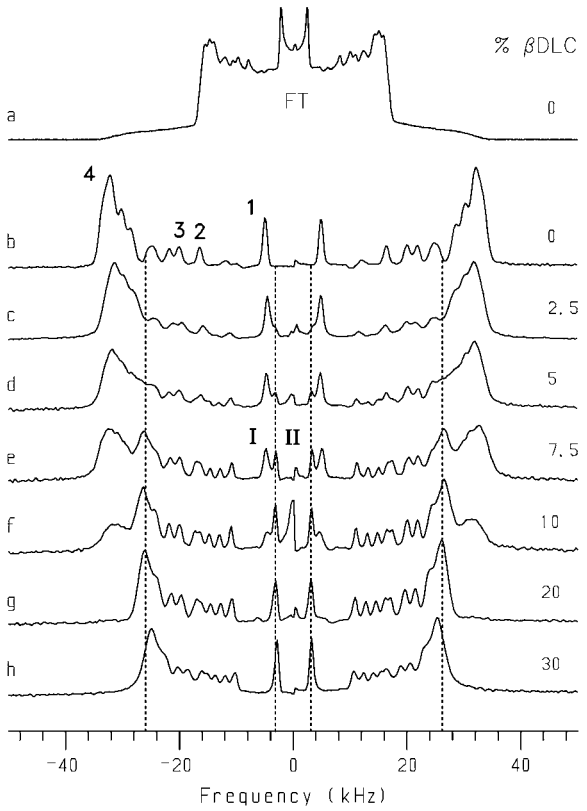


FIGURE 1 ^{2}H-NMR powder (*a*) and de-Pake-ed spectra (*b–h*) of DMPC-d54 membranes recorded at 21°C, without (*a*, *b*) and with (*c–h*) 2.5%, 5%, 7.5%, 10%, 20%, and 30% βDLC expressed in molar %. The bold digits on the pure DMPC spectrum (*b*) point to the quadrupolar splittings attributed to the methyl deuterons (**1**), the vicinal C13 methylene deuterons of the *sn*-1 (**2**), and *sn*-2 (**3**) chains and those of the plateau region (**4**). The components (**I**) and (**II**) described in the text are indicated on spectrum (*e*). The de-Pake-ed spectra were scaled with normalization factors obtained by area normalization of their related FT spectra (*a*).

of the ^{2}H-NMR data, which allows monitoring of the individual quadrupolar splittings of the myristoyl acyl chains (Fig. 1 *b*). The large composite quadrupolar splitting, or plateau (**4**), is for the ordered methylene groups located near the membrane interface. The smaller resolved quadrupolar splittings are for the less ordered methylenes found near the bilayer center, and the narrow doublet of ~4 kHz (**1**) is attributed to the methyl group of the disordered end of the acyl chain (C14). The full assignment of the resolved quadrupolar splittings can be deduced from comparison with data obtained i) in this work with DMPC-d27 perdeuterated on the *sn*-2 chain, and ii) in previous studies with DMPC (24) or dipalmitoylphosphatidylcholine (DPPC) (25,26) selectively labeled at each acyl chain position.

Dilauryl-β-cyclodextrin

The bilayer NMR signature is retained after incorporation of βDLC for molar concentrations up to 30%. The membrane insertion of increasing amounts of βDLC is associated with a progressive appearance of a second spectrum component (**II**), correlated with a decrease of the intensity of the pure lipid signal (**I**). The two components in slow exchange on the ^{2}H-NMR timescale (10 μs), are clearly distinguished for the methyl resonances but not the methylene groups, which are overlapping, giving crowded regions on the NMR spectra. At 20% of βDLC, the component associated with the pure lipid has disappeared and all lipids are found in a single component spectrum displaying well-resolved quadrupolar splittings even in the previous crowded regions. The quadrupolar splittings measured on this spectrum are ~10%–15% smaller than those of the pure lipid NMR spectrum and do not depend on the βDLC/DMPC molar ratio, i.e., the acyl chain order parameters are approximately invariant whatever the cyclodextrin concentration in the membrane. The relative intensities of the methyl resonances of components (**I**) and (**II**) change with the cyclodextrin/lipid ratio. The overall results are similar to others obtained with the related cholesteryl-β-cyclodextrin derivative for which lateral segregation of a cholesteryl cyclodextrin-rich phase was seen (14).

The temperature dependence of the DMPC/βDLC interaction was probed by cooling the membrane samples on a large range of temperatures from 37°C to −12°C at all concentrations investigated. The de-Pake-ed spectra obtained with 7.5% and 20% βDLC are shown in the stacked plots of Fig. 2. The component (**II**) induced by the membrane incorporation of 7.5% βDLC can be already distinguished at 37°C in the feature having the largest quadrupolar splitting associated with the plateau region (see Fig. 2 *a*). It separates more clearly from that of the pure lipid around 25°C and below where the methyl resonances are split in two signals (Fig. 2 *b*). As observed with the cholesteryl derivative (14), this new component (**II**) is barely affected by temperature as opposed to the larger increase of the quadrupolar splittings of the pure lipid (**I**). At the fluid-to-gel transition temperature of pure DMPC-d54 (19.5°C), the signal of the pure lipid in the gel phase disappears in the noise of the spectrum, whereas the individual quadrupolar splittings of the second spectrum induced by the βDLC are still well resolved and characteristic of lipids remaining in the fluid state. Below 13°C the whole spectrum is considerably broadened, indicating a transition to a gel state of the lipids interacting with the βDLC. A similar transition is also observed below 13°C with the DMPC-d54 sample containing 20% of βDLC (Fig. 2, *c* and *d*). In the latter case, no significant signal associated with pure lipid is detected. There is only a single fluid spectrum corresponding to component (**II**), without detectable change at the DMPC-d54 main transition temperature. The spectra recorded in the fluid state at 15°C with these membranes (spectrum *e*) and with pure DMPC liposomes at 30°C (spectrum *f*) are similar, indicating that the acyl chain quadrupolar splitting distribution, the so-called order profile, of the lipid associated with βDLC is similar to that of the pure lipids.

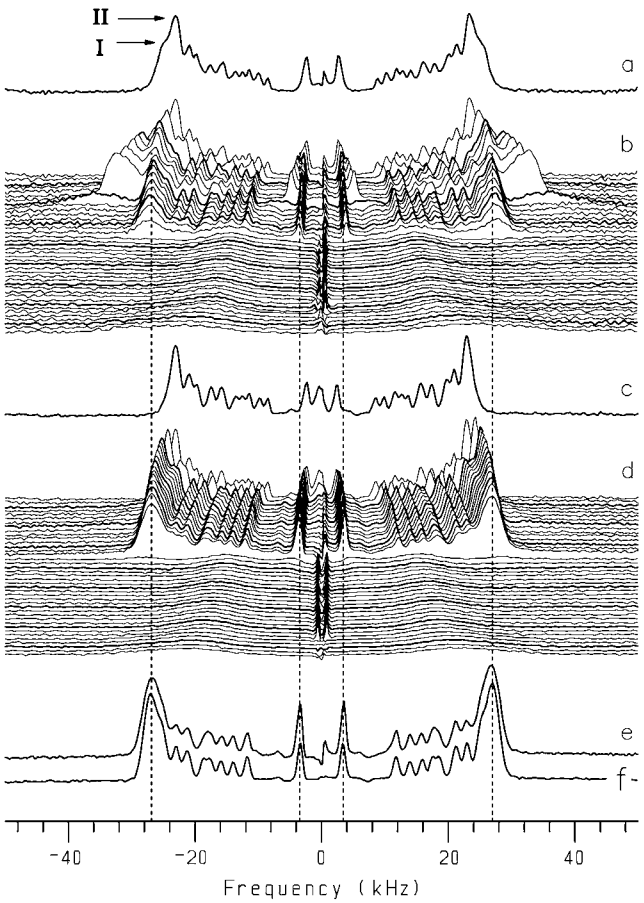


FIGURE 2 ^{2}H-NMR de-Pake-ed spectra obtained from DMPC-d54 membranes containing 7.5% (*a*, *b*) and 20% (*c*, *d*) molar of βDLC. The stacked plots (*b*, *d*) show the spectra recorded at 37°C and 30°C and from 25 to −12°C (1°C step, going from the *upper traces* to the *lower traces*). The traces (*a*) and (*c*) show the spectra obtained at 37°C. The components (**I**) and (**II**) described in the text are indicated on spectrum (*a*). The de-Pake-ed spectra were scaled with normalization factors obtained by area normalization of their related FT spectra. Spectrum *e*: DMPC-d54 membranes containing 20% molar of βDLC at 15°C. Spectrum *f*: pure DMPC-d54 membranes at 30°C.

Below 13°C, the quadrupolar splitting of the acyl chain methyl groups, which are still experiencing axial symmetry due to the fast rotation of the trimethyl rotor, can be measured (~20 kHz) and is found to be similar to that obtained for the pure lipids at these temperatures (Fig. 3, *a* and *b*). Deuterium NMR spectra recorded at lower temperatures displayed another transition of the lipid acyl chains occurring around −7°C with DMPC-d54, characterized by a large increase of the methyl quadrupolar splitting to 32 kHz (Fig. 3 *c*). This spectral change is associated with the transition of the phospholipids from the gel $L_{\beta'}$ phase to the lamellar crystalline gel L_c phase (27–29). The latter phase is still detected at low concentrations (2.5%, 5%, and 7.5%) of βDLC (Fig. 3, *d–f*) but progressively disappears when the concentration is raised up to 30% of βDLC (Fig. 3, *g* and *h*).

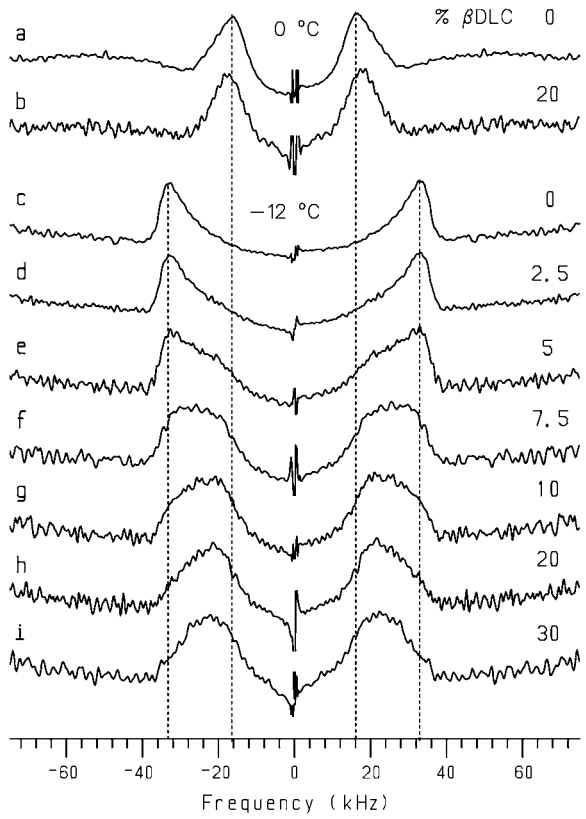


FIGURE 3 ^{2}H-NMR de-Pake-ed methyl resonances of DMPC-d54 membranes recorded at 0°C (*a*, *b*) and −12°C (*c–i*), without (*a*, *c*) and with (*b*, *d–i*) 2.5%, 5%, 7.5%, 10%, 20%, and 30% βDLC expressed in molar %.

The spectral changes observed with the DMPC-d54 membranes can be analyzed in more detail with the temperature dependence of the first moment M_1 measured from the powder pattern deuterium spectra. As seen on Fig. 4 the first moment M_1 increases when the temperature is decreased due

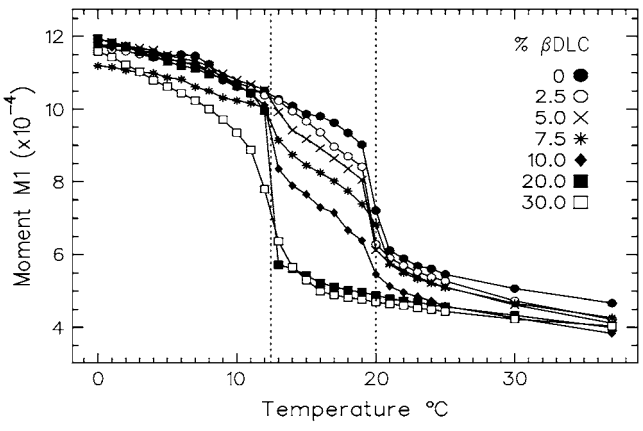


FIGURE 4 First moment M1 of the ^{2}H-NMR powder spectra of DMPC-d54 membranes as a function of temperature (°C): (●) pure and with (○) 2.5%, (×) 5%, (*) 7.5%, (♦) 10%, (■) 20%, and (□) 30% (molar %) of βDLC.

to the thermally induced increase of the average orientational order of the lipid acyl chains. In the absence of βDLC, there is a sharp change of M_1 values around 20°C, associated with the main gel-to-liquid crystalline transition of the pure lipids, as discussed above. Another sharp transition is found at 12.5°C on the moment curve of the sample prepared with 20% of βDLC. For intermediate concentrations of βDLC, the moment curves appear as a combination of the two limiting ones obtained at 0% and 20% of βDLC. The main transition at 20°C can be detected at all concentrations below 20%, whereas the other sharp transition at 12.5°C appears at βDLC concentrations above 2.5%. Below 12°C, the moment curves are roughly superimposable up to 20% of βDLC, indicating that the average order in the lipid bilayer is globally the same at these concentrations. With 20% and 30% of βDLC the M_1 traces are not superimposable below 12°C. The transition detected at this temperature is smoothed with 30% of βDLC, indicating that the DMPC acyl chains are more disordered in large excess of βDLC.

Methylated dilauryl-β-cyclodextrins

Data were also obtained with methylated βDLCs. The β-cyclodextrin exhibits a wide number of hydroxyl groups. They can be separated into two classes, namely primary hydroxyls (on carbons C6) and secondary hydroxyls (on carbons C2 and C3) located on the narrower and the wider rims of the cyclodextrin, respectively. Since primary and secondary hydroxyl groups exhibit different chemical reactivities, regiospecific methylation can be performed (30,31). So, the synthesis of DimβDLC and TrimβDLC has been achieved.

The NMR spectra recorded after incorporation of these compounds in deuterated DMPC membranes contained only one component, as opposed to those obtained with nonmethylated βDLC (Fig. 5). This indicates that the free lipids and those interacting with the methylated βDLC exchange rapidly on the NMR timescale, leading to a time-averaged spectrum. At 20°C, the quadrupolar splittings are significantly larger in the presence of 10% of either DimβDLC or TrimβDLC, with a 25% and 29% increase for the methylenes of the plateau and the terminal methyl groups, respectively. This effect is opposed to that observed with the nonmethylated βDLC, which was found to decrease the DMPC chain quadrupolar splittings (Fig. 5 *b*). Besides this increased lipid chain order, there is also a splitting of the chain methyl signal into two NMR lines of equal intensities with both methylated cyclodextrin derivatives. If the experiment is conducted with DMPC-d27 deuterated on the *sn*-2 acyl chain, only one methyl signal is detected, corresponding to the larger methyl quadrupolar splitting of DMPC-d54 (spectra not shown). This indicates clearly that each quadrupolar splitting observed with the DMPC-d54 membranes is attributed to a methyl group of a given acyl chain. The distinction of the methyl group quadrupolar splittings of the *sn*-1 and *sn*-2 acyl

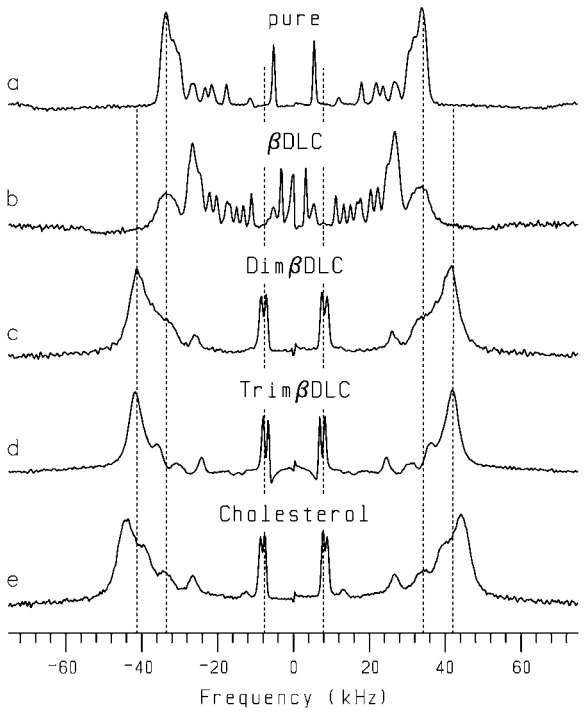


FIGURE 5 ^{2}H-NMR de-Pake-ed spectra of DMPC-d54 membranes recorded at 20°C, either pure (*a*) or with a 10% molar concentration of βDLC (*b*), DimβDLC (*c*), TrimβDLC (*d*), or cholesterol (e).

chains starts around 22°C, just above the main transition of DMPC-d54. A resolution of the methyl signals has also been observed in the presence of cholesterol, near the fluid-to-gel transition of similar membranes of deuterated DMPC or DPPC (32,33). We found the same result under our experimental conditions with DMPC-d27 and DMPC-d54 membranes containing 10% of cholesterol, with an increase of the *sn*-2 methyl quadrupolar splitting (Fig. 5 *e*). This effect is also correlated with an increase of the other quadrupolar splittings, as observed in the presence of the methylated βDLCs. The de-Pake-ed spectra obtained with the methylated βDLC- and cholesterol-containing membranes are in fact very similar, indicating that they have approximately the same order profile.

The acyl chain order parameters have been measured in the fluid phase for all membranes. The temperature dependence of the methylene deuterons located at each extremity of the chains, namely those of the plateau region, and of the carbon (C13) next to the methyl group of the *sn*-1 acyl chain is shown in Fig. 6. For all membranes the order parameters increase upon cooling, with more pronounced variations just below the main transition. At 30°C, the C13 deuteron order parameter is the same with or without TrimβDLC. Below 30°C, this order parameter increases sharply for the TrimβDLC-containing membranes, so that the curve finally diverges from that of the pure lipids. Above 30°C, the order parameter is slightly decreased, and the TrimβDLC curve goes under that of the pure lipids. Similar results are obtained with

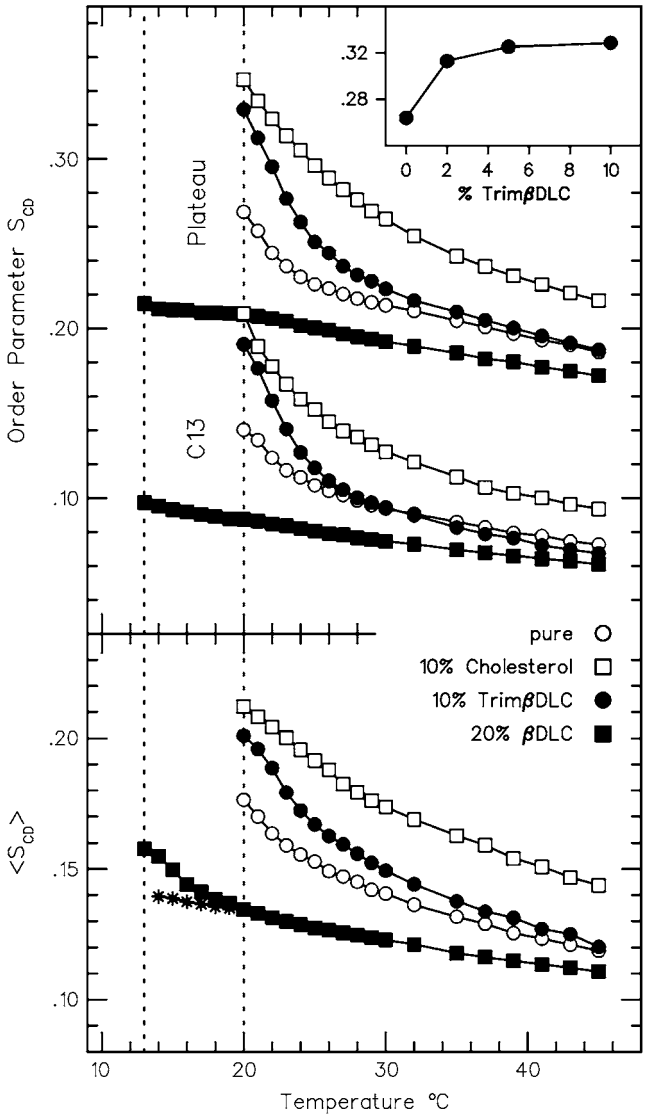


FIGURE 6 Temperature dependence of DMPC-d54 acyl chain order parameters S_{CD} of the plateau region and *sn*-1 C13 methylene resonances measured from the deuterium de-Pake-ed spectra (*top*) and of the average order parameters $\langle S_{CD} \rangle$ of the myristoyl chains calculated from the first moment M1 of the powder spectra (*bottom*). The membranes were either pure (○) or contained 10% molar concentration of βDLC (■), TrimβDLC (●), or cholesterol (□). With the exception of the βDLC data (■), all spectral moments M1 were measured from deuterium NMR spectra of DMPC-d54 membranes in the fluid state, containing no component which could be attributed to gel state lipids, i.e., no signal around ±61 kHz. Average order parameters $\langle S_{CD} \rangle$ of βDLC-containing membranes below *T*c, calculated from a fluid powder pattern obtained after subtraction of a small gel component with intensities around ±61 kHz (<10% of the whole signal) is also shown (*). The inset in the top panel shows the TrimβDLC concentration dependence of the methylene plateau deuteron order parameters at 20°C (●).

the deuteron order parameters of the *sn*-2 C13 carbon, the terminal methyl groups (C14) (data not shown), and the plateau region, except that for the latter the curve never goes below that of pure lipids. All order parameters measured with the cholesterol-containing membranes display increased values, even at high temperatures. Order parameters of the plateau and C13 methylene groups are also plotted for the βDLC-containing membranes (Fig. 2 *d*). There is a weak monotonic increase with a single slope on the whole temperature range, even at 13°C, just above the fluid-to-gel transition of these membranes. The value of this slope is similar to those measured with the other membranes at high temperatures. Identical results were obtained with the data of the DMPC membranes containing 7.5% (Fig. 2 *b*) and 10% (spectra not shown) of βDLC. The curves obtained with these membranes are all found to be superimposable. Additionally, order parameters of the plateau methylene groups derived from the spectral component (**I**) of the pure lipids remaining in the latter membranes were found to be identical with those of the pure DMPC membranes plotted on Fig. 6, with the same slope increase near the main transition.

The average order parameter $\langle S_{CD} \rangle$ of each membrane has also been calculated from the moment M1 of the deuterium NMR powder spectra recorded in the fluid state. The $\langle S_{CD} \rangle$ temperature dependences plotted on Fig. 6, are qualitatively similar to the curves displaying the variations of the individual order parameters of the plateau and C13 methylene groups detailed above. In particular, a steeper slope is also observed just above the transition temperature *T*c with all membranes, except those containing βDLC. The larger slope was also measured for the TrimβDLC-containing membrane. For the membranes containing nonmethylated βDLC, an almost linear variation of the average order parameter with temperature is observed until 17°C, followed by a slope increase just below the transition observed at 12.5°C with these membranes. The latter slope increase is due to the occurrence in the powder spectra of weak but significant intensities around ±61 kHz, due to residual free lipids in the gel state, increasing the average order of the overall lipid acyl chains. If the spectral moment is derived only from the fluid powder pattern obtained after subtraction of this gel component, which accounts for <10% of the whole signal, the $\langle S_{CD} \rangle$ curve retains a constant slope over the whole temperature range as found with the plateau and C13 methylene group order parameters. Indeed, the deuterium NMR powder pattern of the cholesterol- or TrimβDLC-containing membranes used for the calculation of the average order parameter $\langle S_{CD} \rangle$ plotted in Fig. 6 did not contain any gel component, i.e., there was no signal around ±61 kHz.

NMR data were also obtained at lower TrimβDLC concentrations. The results obtained above the fluid-to-gel transition of DMPC-d54 are plotted in the top inset of Fig. 6, which displays the TrimβDLC concentration dependence of the plateau methylene order parameters at 20°C. The TrimβDLC-induced order parameter increase reached a plateau at ~5%–10% of the cyclodextrin derivative. Increasing the amphiphilic methylated cyclodextrin concentration to 20% does not lead to an additional increase of the chain quadrupolar splittings, which were found to be similar to

those measured at 10% of these derivatives, confirming that a plateau is effectively reached at this concentration. About 60% of this maximum value is already achieved in the presence of 2.5% βDLC. NMR spectra were also obtained below the DMPC-d54 fluid-to-gel transition temperature, showing well-resolved methyl quadrupolar splittings indicating the occurrence of fluid lipids below *T*c. The discussion of these data is beyond the scope of these article and will be detailed elsewhere.

DISCUSSION

We have shown previously that cyclodextrins inserted in DMPC-d54 membranes via a hydrophobic cholesteryl anchor were prone to self-organize at the membrane surface, sequestering lipids and inducing a laterally segregated mixed L_{CD} phase (14). Similar results were obtained in this work, with βDLCs prepared by replacing the cholesterol moiety by an aspartic acid esterified by two lauryl chains. This compound is also found to promote a phase separation in similar DMPC membranes. The lateral segregation of a cyclodextrin-enriched phase is clearly evidenced by the appearance of a second component (**II**) on the fluid state ^{2}H-NMR spectra of DMPC-d54, coexisting with the pure lipid signal (**I**). A quantitative analysis of the de-Pake-ed NMR spectra has been attempted by simulating the fluid methyl resonances of component (**I**) and (**II**) with Gaussian line shapes. The results shown in Table 1 indicate that this new L_{CD} phase does accommodate 4–5 lipids per cyclodextrin molecule. This composition is found at all βDLC concentrations investigated and is thus characteristic of the βDLC-induced L_{CD} phase. Such an amount is approximately three times that estimated for the L_{CD} phase observed with the cholesteryl derivative (14). The larger number of lipids trapped in the βDLC-induced L_{CD} phase must be associated with differences in the average area per cyclodextrin headgroup, relative to the lateral area of its dilauryl anchoring unit. The orientation and packing of the cyclodextrin headgroups in the L_{CD} phase might also be sensitive to the way they are anchored in the bilayer, with a possible increase of the cyclodextrin effective area upon bilayer insertion with lauryl acyl chains.

TABLE 1 Lipid distribution between the pure lipid and L_{CD} phases for various amounts of βDLC at 21°C

% βDLC	F_{fluid}	F_{Lcd}	N_{fluid}	N_{Lcd}	W_{fluid} Hz	W_{Lcd} Hz
2.5	0.86	0.14	335	55	1.0	0.8
5.0	0.76	0.24	145	45	1.1	0.8
7.5	0.56	0.44	69	54	1.4	0.7
10.0	0.49	0.51	44	46	1.4	0.7
20.0	0.00	1.00	0	40	–	0.8

The fractions of the pure lipid and L_{CD} phase, F_{pure} and $F_{Lcd,}$ were determined by a simulation of the methyl resonances (see Materials and Methods). N_{pure} and N_{Lcd} are the number of lipid molecules found, respectively, in the pure lipid and L_{CD} phase for 10 βDLC molecules. W_{pure} and W_{Lcd} are the computed methyl line widths (kHz) of the lipids found in the fluid phase (I) and the L_{CD} phase (II).

There is a marked line broadening of the pure lipid signal (**I**) at the main gel-to-fluid transition temperature of DMPC-d54 (19.5°C), characteristic of lipids going through the gel state as in pure DMPC-d54 membranes. As observed with the cholesteryl-β-cyclodextrin derivative (14), the second component (**II**) associated with the βDLC-induced L_{CD} phase is not affected, indicating that the trapped lipids remain in the fluid state below the transition, coexisting with pure lipids in the gel state. Below 13°C, the fluid L_{CD} spectral component disappears completely, leading to a gel phase NMR spectrum, showing that the lipids sequestered in the L_{CD} phase have now turned to a gel-like state. Even if they are not distinguished on the NMR spectra, it is very likely that there is still a lateral separation of an L_{CD} phase within pure lipids, as evidenced by the NMR data obtained at low temperatures (Fig. 3). At −12°C, some lipids are in the lamellar crystalline L_c gel phase, whereas others, probably associated to lipids trapped in a βDLC-rich ordered phase, appear to remain in an L'_β gel-like state. Accordingly, the signal of the pure lipids in the L_c gel phase disappears progressively upon incorporation of increasing βDLC concentrations, whereas the L'_β gel-like signal becomes predominant (see Fig. 3). As shown previously, the L_{CD} phase induced by the cholesteryl derivative had a more complex phase behavior (14). Well-resolved fluid-like quadrupolar splittings of the acyl chain methyl groups could be observed until 5°C with this derivative, indicating that the segregated L_{CD} phase retained a certain degree of fluidity, at least in the bilayer center, at such low temperature. Cooling the sample below 5°C led to a broadening of the methyl resonances and a quadrupolar splitting increase, suggesting the formation at these temperatures of a more ordered L_{CD} phase.

The different thermal behavior observed between the two segregated L_{CD} phases induced by the cholesteryl- and dilauryl-β-cyclodextrin, respectively, should be indeed related to the different nature of their hydrophobic anchors. With their almost identical chemical structure, it is quite probable that the myristoyl acyl chains of DMPC adjust more closely with the flexible lauryl chains than with the bulky and rigid sterol nucleus. It is actually well known that cholesterol itself induces a ''fluidifying'' effect of the lipid acyl chains below the main transition, inhibiting the formation of the gel phase, the lipid phase staying fluid well below the transition (see Vist and Davis (33) and references therein). This cholesterol ''fluidifying'' effect could indeed explain the partial fluidity remaining at low temperatures of the L_{CD} phase, whose cohesion in the bilayer hydrophobic region is controlled by interactions between the lipid acyl chains and the sterol anchor of the cholesteryl derivative. Conversely, the almost ''perfect'' match occurring between the saturated acyl chains of the dilauryl derivative and DMPC allows a more efficient packing and cooperative ''freezing'', leading to the transition toward an ordered gel phase observed at 12.5°C. It is useful to consider what would be expected if βDLC was replaced by

dilaurylphosphatidylcholine (DLPC) to obtain a binary mixture with dimyristoyl phosphatidylcholine. In the case of an ideal mixing of the two saturated chain phosphatidylcholines and by assuming the DLPC main transition is around −2°C, we would expect that DMPC-d54 membranes containing ~18% DLPC should also display a fluid-to-gel transition around 12.5°C. Indeed packing constraints imposed by interactions between the cyclodextrin moieties must also modulate the L_{CD} phase transitional behavior. To differentiate effects due to chain length or headgroup packing, it would be interesting to reproduce our experiments with the dimyristoyl-β-cyclodextrin derivative.

At a concentration approximately equal to the stoichiometry found for the L_{CD} phase (~20%), βDLCs are able to sequester all the lipid molecules, leaving a single stable mixed lipid/βDLC phase, with only traces of free lipids. The well-defined NMR spectra obtained denote a homogeneous L_{CD} phase, with a remarkably sharp fluid-to-gel transition as shown on the moment M1 curves (Fig. 4). This sharp fluid-to-gel transition is retained at low concentrations of βDLC, highlighting the cohesion and stability of the L_{CD} phase in the presence of a large excess of lipids even in the gel state. Now, if we increase the βDLC concentration above the L_{CD} phase stoichiometry determined experimentally, there should be a shortage of lipids, with two limiting cases. In the first case, the composition of the L_{CD} phase is preserved, and the ''free'' exceeding βDLC rejected outside of the mixed lipid phase in some kind of free βDLC clusters. A second alternative would be an L_{CD} phase containing ''holes'' partially filled with lipids exchanging rapidly within a cyclodextrin cluster. For instance, with 30% βDLC there are only 2.3 lipids available per molecule of βDLC, leaving approximately two vacant lipid sites, and the interactions between lipid and βDLC acyl chains are expected to be loosened. Indeed the fact that the moment curve obtained at this concentration shows a much less cooperative fluid-to-gel transition favors this second hypothesis.

The results obtained with the βDLC support and refine the model developed previously (14) of a segregated lipid phase primarily stabilized by hydrophilic interactions of cyclodextrin headgroups at the membrane surface. Monomers of β cyclodextrin can form aggregates in solution through hydrogen bonds between the free hydroxyl groups of their glucose units (15,16). It is very likely that the same driving forces can control the formation of a membrane-bound cyclodextrin network at the membrane surface, as suggested by our data. This model is indeed strongly supported by the results obtained here with the di- and trimethylated βDLC. No lateral segregation could be detected with these methylated derivatives. The NMR spectra contained only one component, showing that methylation of the cyclodextrin headgroups inhibits the formation of the laterally segregated lipid phases observed with amphiphilic cyclodextrin with free hydroxyl groups. These compounds are then distributed evenly in the membrane, interacting with all the lipids on the NMR time-scale, leading to the observed well-resolved averaged single component NMR spectra.

The methylated βDLC have a clear ordering effect on the acyl chains just above the main transition, evidenced by the increase of their quadrupolar splittings and the resolution of the *sn*-1 and *sn*-2 methyl groups. In this respect, the Trimβ DLC-induced membrane perturbation can be related to the cholesterol action on DMPC membranes in the fluid L_α phase, which is also characterized by an increase of the acyl chain order parameters and a resolution of the two methyl signals of DMPC (32,33). These effects observed in the fluid phase with cholesterol are a consequence of the well-known straightening or ''condensing'' effect on the fluid lipid acyl chains induced by the bulky rigid ring system of this compound. The resolution of the *sn*-1 and *sn*-2 acyl chain methyl groups has been tentatively interpreted as a change in average orientation or fluctuation of the last *sn*-2 chain segment, which extends farther in the bilayer center than the *sn*-1 chain, relieving possible packing problems occurring when the chains are highly ordered (33). This model is consistent with our observation that the larger order parameter measured in the presence of cholesterol and Trimβ DLC is precisely that of the *sn*-2 methyl group. The methylated βDLC has to be deeply inserted into the bilayer to increase the chain order and perturb the reorientation of the last segment of the DMPC-d54 acyl chains. To achieve this and to compensate for its short lauryl acyl chain anchor, the cyclodextrin headgroup has to penetrate into the bilayer. Indeed the methylation of the hydroxyl groups increases the cyclodextrin hydrophobicity, favoring a deeper bilayer insertion of the polysaccharide headgroup below 25°C. The absence of chain ordering at higher temperatures could be explained by a temperature dependence of the average bilayer transverse location of the methylated cyclodextrin moiety. Increasing the temperature would bring the methylated cyclodextrins toward the bilayer surface to finally reach a location perhaps similar to that of the nonmethylated cyclodextrin in the L_{CD} phase. This would leave more disordered acyl chain segments beyond the plateau region, near the end of the myristoyl chains as observed experimentally above 30°C (see Fig. 6).

The Trimβ DLC-induced chain ordering becomes very important a few degrees above the main transition, suggesting it might be related to molecular events occurring near this transition. In this temperature range there is actually a clear slope increase of the order parameter temperature dependence for all membranes investigated, including that of pure lipids. It is well known that due to enhanced density fluctuations in the vicinity of the transition, this temperature region is associated with various abnormal behaviors of saturated phosphatidylcholine membranes, such as bilayer permeability (34), heat capacity (35), fluorescence lifetime (36), or ultrasound velocity (37), the most studied phenomenon being a nonlinear increase of the lamellar repeat distance D, known as ''anomalous swelling'' of the multilamellar

phosphatidylcholine liposomes. For example, neutron scattering studies of DMPC multilamellar membranes in the fluid phase have reported an anomalous increase of the interbilayer lamellar spacing as temperature approaches the main transition (17,38). This effect is not observed with the ethanolamine derivative DMPE (39) and is attenuated with long chain phosphatidylcholine (40). It is also progressively inhibited when the hydrostatic pressure is increased (41). It has been shown that this interbilayer increase is due to both i) an increase in the water layer, and ii) a thickening of the lipid hydrophobic region of ~0.5 Å due to a straightening of the lipid acyl chains (18,40). The water layer increase is believed to arise from a decrease of the bilayer bending rigidity, resulting in enhanced bilayer undulations and increased bilayer repulsions (18). The thickening of the hydrophobic region is not necessarily coupled with the water layer increase and can occur alone, as observed with long chain phosphatidylcholine such as the distearoyl derivative (40). It can be monitored by ^{2}H-NMR (42) via the increase of the acyl chain deuteron order parameters near the main transition of saturated lipids such as DMPC, as observed in this study (Fig. 6). Thus, it is possible that the enhanced density fluctuations associated with the anomalous pretransitional behavior of DMPC-d54 multibilayers could favor a deeper membrane penetration of the methylated cyclodextrin headgroups, leading, as discussed above, to a cholesterol-like bilayer perturbation with the observed increase of the acyl chain order parameters and concomitant resolution of the *sn*-1 and *sn*-2 methyl NMR signals. Interestingly, a similar perturbation has also been reported near the transition of gramicidin D-containing DMPC membranes (43).

There is no such pretransitional ordering of the DMPC acyl chains in the vicinity of the sharp fluid-to-gel transition occurring at 12.5°C within the composite L_{CD} phase found in βDLC-containing membranes. There is only a monotonous increase of the acyl chain quadrupolar splittings when the sample is cooled, without any slope change, followed by a sudden transition as monitored by the line shape change of the deuterium NMR spectra (Fig. 6). However, the temperature dependence of the NMR spectral component (**I**) of the pure lipids coexisting with the L_{CD} phase continue to display nonlinear order parameter increases near the main transition. This shows that the pure lipids are still undergoing some anomalous pretransitional behavior despite the presence of the L_{CD} clusters and suggests that the two segregated phases are well separated. In this respect, monitoring the cyclodextrin-induced L_{CD} domains at the macroscopic level with techniques such as fluorescence microscopy should provide meaningful data.

CONCLUSION

Cyclodextrins anchored at the surface of DMPC membranes with covalently bound dilauryl chains were found to induce a laterally segregated phase (L_{CD}) containing ~4–5 lipids per monomer of βDLC. The L_{CD} phase exhibits physical properties different from those of pure DMPC bilayers. It displays a sharp first order fluid-to-gel transition at 12.5°C, ~7° below that of pure DMPC-d54. There is no evidence for a nonlinear increase of the DMPC acyl chain order parameters in the fluid phase near the transition, as observed during the pretransitional anomalous swelling of pure DMPC membranes. However, the pure lipid phase which coexists with the L_{CD} phase is not perturbed and keeps the physical properties of pure DMPC membranes, with a fluid-to-gel transition at 19.5°C and a nonlinear increase of acyl chain order parameters in the vicinity of the transition. Thus, DMPC membranes in the fluid state were shown to accommodate laterally segregated fluid and long-lived (>10 μs) microdomains exhibiting different physical and mechanical properties from the pure lipid phase. The segregation process is believed to occur through intermolecular hydrogen bonds between adjacent polysaccharide headgroups at the membrane surface and lipid sequestration in the obtained cyclodextrin network. Accordingly, methylation of the βDLC hydroxyl groups was found to inhibit the formation of the L_{CD} phase. The bilayer insertion of trimethylated βDLC was found to considerably amplify the nonlinear increase of the DMPC acyl chain order parameters in the vicinity of the main DMPC transition. Whether the particular properties of the membrane-bound acylated cyclodextrin derivatives detailed in this article can be pharmacologically relevant regarding the potency of a drug trapped in the cyclodextrin cavities will be investigated in forthcoming studies.

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