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Temperature-Dependence of Cationic Lipid Bilayer Intermixing: Possible Role of Interdigitation

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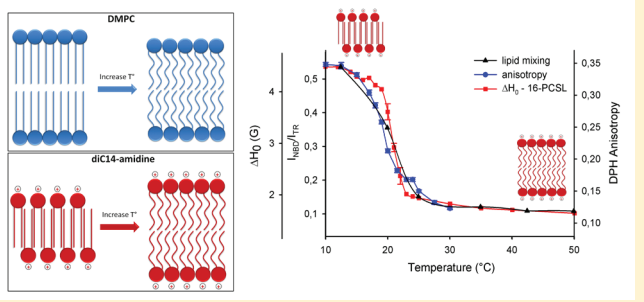
ABSTRACT: In this work, we investigated the properties of a fusogenic cationic lipid, diC14-amidine, and show that this lipid possesses *per se* the capacity to adopt either an interdigitated structure (below and around its transition temperature) or a lamellar structure (above the transition temperature). To provide experimental evidence of this lipid bilayer organization, phospholipids spin-labeled at different positions of the hydrocarbon chain were incorporated into the membrane and their electron spin resonance (ESR) spectra were recorded at different temperatures. For comparison, similar experiments were performed with dimyristoyl phosphatidylcholine, a zwitterionic lipid (DMPC) which adopts a bilayer organization over a broad temperature range. Lipid mixing between diC14-amidine and asolectin liposomes was more efficient below (10−15 °C) than above the transition temperature (above 25 °C). This temperature-dependent "fusogenic" activity of diC14-amidine liposomes is opposite to what has been observed so far for peptides or virus-induced fusion. Altogether, our data suggest that interdigitation is a highly fusogenic state and that interdigitation-mediated fusion occurs via an unusual temperature-dependent mechanism that remains to be deciphered.



INTRODUCTION

Fusion of biological membranes is involved in several biological processes including virus infection, fertilization, and trafficking.[1] Lipid bilayer intermixing is one of the most intriguing phenomena associated with biomembrane fusion. Its mechanism has been extensively investigated over the last 30 years. It has been proposed that the mechanism implicates the formation of a stalk where the two leaflets are fused together, followed by the formation of a fusion pore, but other studies have identified hemifusion structures as intermediates involved in the fusion of the two outer leaflets, leaving the inner leaflets separated.[1−4]

Protein-free fusion could be achieved by modifying lipid composition or by forcing contacts between lipid bilayers. For example, adding lipids having a negative spontaneous curvature (with unsaturated acyl chains or small uncharged headgroups) facilitates the formation of fusion intermediates.[5−7] For charged lipid species, liposomal fusion can be induced by pH changes or addition of multivalent ions[8,9] suggesting that charge neutralization, causing dehydration of bilayer surfaces, could promote fusion.[10−13] Another mechanism of membrane fusion, although less documented, relies on lipid tail interdigitation: correlation between interdigitated membranes and fusion has been suggested for ethanol-induced interdigitated phospholipid vesicles.[14−16] Interdigitation could be involved in many different biological processes relying on architecture and stability of biomembranes, which is a reason we think this question merits further investigation.

DiC14-amidine is a cationic lipid that efficiently transports nucleic acids in mammalian cells[17−23] but also displays unexpected cellular activities like TLR4-agonist activity,[24−26] anti-inflammatory,[25−28] or immunomodulatory properties.[29] We have recently demonstrated that diC14-amidine cationic liposomes fuse very efficiently and rapidly with cells in the absence of a fusion-enhancing colipid.[30] Molecular dynamics simulations suggested the formation of a rather unstable and partly interdigitated organization of diC14-amidine bilayer[31] that might influence its fusogenic property.[30,31]

In this paper, we observe that interbilayer lipid mixing of liposomes is enhanced at low temperature in contrast to usual fusogenic phospholipids which need increasing disorder of their hydrocarbon tails for fusion. In order to better understand this behavior and gain access to the mobility parameters of diC14-amidine hydrocarbon chains, we have used the electron spin resonance technique (ESR). ESR was previously used successfully to monitor structural alterations due to diC14-amidine/DNA association or changes in pH.[32,33] In the present work, in order to monitor the degree of diC14-amidine membrane interdigitation, phospholipids spin-labeled at different positions of the hydrocarbon chain were incorporated into the membrane, and their electron spin resonance spectra recorded at different temperatures. We compared the spectra

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obtained with those of dimyristoyl phosphatidylcholine, a zwitterionic lipid (DMPC), which adopts a bilayer organization over a broad temperature range. We observed that the fluidity gradient is drastically different for each lipid below the transition temperature but shows a similar profile above the transition temperature. These results are consistent with the formation of the nonbilayer interdigitated organization as already suggested by molecular dynamics simulations.[31]

MATERIALS AND METHODS

Reagents. Texas Red-DHPE (Texas Red 1,2-dihexadecanoyl-*sn*-glycero-3-phospho-ethanolamine, triethylammonium salt) and NBD-PE (*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt) are Invitrogen (Molecular Probes) products. DMPC (dimyristoyl phosphatidylcholine), Asolectin (L-a-phosphatidylcholine (Soy-20%)) and spin labels 1-palmitoyl-2-(*n*-doxylstearoyl)-*sn*-glycero-3-phosphocholine (*n*-PCSL, *n* = 5, 7, 10, 12, or 16) were purchased from Avanti Polar Lipids (Birmingham, AL, USA). DiC14-amidine (3-tetradecylamino-*N*-*tert*-butyl-*N*′-tetradecyl-propionamidine) was synthesized as described[19] and stored as a powder at −20 °C.

Liposome Preparation. A lipid film was formed from a chloroform solution, dried under a stream of N_2, and left under reduced pressure for a minimum of 2 h, to remove all traces of organic solvent. Dispersions were prepared by the addition of Hepes buffer (10 mM, pH 7.4) followed by vortexing for about 2 min above T_m (~40 °C). The samples were used right after preparation. For ESR measurements, spin labels were added to the lipid chloroform solution at 0.2 mol % relative to the lipid concentration; hence, no spin−spin interaction was observed. For fluorescence measurements, NBD-PE and Texas Red-DHPE (at 0.8 mol % each) were dissolved with diC14-amidine in chloroform before lipid film formation. Liposomes were then stored at 4 °C and heated at 55 °C for 10 min just before the experiment.

ESR Spectroscopy. ESR measurements at X-band were performed with a Bruker EMX spectrometer. Field-modulation amplitude of 1G and microwave power of 5 mW were used. The temperature was controlled to about 0.2 °C with a Bruker BVT-2000 variable temperature device, and monitored with a Fluke 51 K/J thermometer with a probe placed just above the cavity. The magnetic field was measured with a Bruker ER 035 NMR Gausmeter. All ESR data shown are means of the results of at least three experiments, and the uncertainties are the standard deviations. When not shown, the uncertainty was found to be smaller than the symbol in the graph.

The effective order parameter, S_{eff}, was calculated from the expression[34]

$$S_{\mathrm{eff}} = \frac{A_{\parallel} - A_{\perp}}{A_{ZZ} - (1/2)(A_{xx} + A_{yy})} \frac{a_o'}{a_o}$$

where $a_o' = (1/3)(A_{xx} + A_{YY} + A_{ZZ})$, $a_o = (1/3)(A_{\parallel} + 2A_{\perp})$, $A_{\parallel}$ (= A_{max}) is the maximum hyperfine splitting directly measured in the spectrum (see Figure 5), $A_{\perp} = A_{min} + 1.4[1 - ((A_{\parallel} - A_{min})/(A_{zz} - (1/2)(A_{xx} + A_{yy})))]$, A_{min} is the measured inner hyperfine splitting (see Figure 5) and A_{xx}, A_{yy}, and A_{zz} are the principal values of the hyperfine tensor for doxylpropane.[35]

Temperature Dependence of Fluorescence Anisotropy of DPH Inserted into diC14-Amidine Liposomes. The fluorescence probe 1,6-diphenylhexa-1,2,5-triene (DPH)(Sigma Aldrich) was introduced into the amidine liposomes and incubated 30 min at 50 °C (DPH/lipid molar ratio 1:450). Fluorescence anisotropy measurements were performed in a SLM 8000C spectrofluorometer with Glan-Thompson polarizers placed in T-geometry. Excitation was performed at 358 nm and emission was recorded at 429 nm. For each experiment, the sample was loaded in a quartz cuvette, and the mixture was stirred continuously (small magnetic bar) in a thermostatic chamber. Anisotropy measurements were carried out by simultaneously measuring the vertical and horizontal components of the polarized emission. Fluorescence anisotropy (r) is defined by the equation

$$r = \frac{I_{vv} - (G_f \times I_{vh})}{I_{vv} + (2G_f \times I_{vh})}$$

where I_{vv} and I_{vh} are the vertically and horizontally polarized emission intensities, respectively, with the direction of the excitation light beam being vertically polarized. Correction factor G_f is given by the equation

$$G_f = \frac{I_{hv}}{I_{hh}}$$

where I_{hv} and I_{hh} are the vertically and horizontally polarized emission intensities, respectively, with excitation beam horizontally polarized. Fluorescence intensities I_{vv}, I_{vh}, I_{hv}, *and* I_{hh} of blanks (liposomes in the absence of DPH) were subtracted before calculating anisotropy of DPH samples.

Lipid Mixing Assay. Lipid mixing between cationic liposomes and asolectin liposomes was monitored using fluorescence resonance energy transfer assay (FRET) as described.[30] Asolectin liposomes were added at a mass ratio of 10:1 to diC14-amidine liposomes (labeled with NBD-PE and Texas Red-PE at 0.8 mol % each) and loaded in a quartz thermostated fluorescence cell. The samples were gently stirred throughout the experiment. The fluorescence was monitored using an SLM-8000 spectrofluorometer with excitation and emission bandwidths of 4 nm. Generally, samples were excited at 470 nm and emission spectra were recorded between 500 and 625 nm. Control emission spectra were performed in parallel before and after lipid mixing.

For each temperature, the ratio between donor (NBD) and acceptor (Texas Red) fluorescence intensities was calculated as

$$\frac{I_{\mathrm{NBD}}}{I_{\mathrm{TR}}} = \frac{I^s_{\mathrm{NBD}}}{I^s_{\mathrm{TR}}} - \frac{I^i_{\mathrm{NBD}}}{I^i_{\mathrm{TR}}}$$

where $(I^s_{NBD})/(I^s_{TR})$ is the ratio between fluorescence intensity at 535 nm and fluorescence intensity at 605 nm for the sample, and $(I^i_{NBD})/(I^i_{TR})$ the ratio between initial respective fluorescence intensities of the labeled liposome suspension before addition of asolectin liposomes.

RESULTS AND DISCUSSION

Transition Temperature of DMPC and diC14-Amidine. DiC14-amidine and DMPC share the same hydrophobic domain (C14 atoms chains) with a large zwitterionic headgroup for DMPC, and a smaller positively charged group for diC14-amidine (Figure 1) (+1 at pH 7.4 used here). The calculated enthalpy of the gel−fluid transition is found similar for diC14-amidine and DMPC, $\Delta H \sim 6.5$ kcal/mol. However, while a sharp gel−fluid transition is observed for DMPC, diC14-amidine displays a broader transition at a slightly lower temperature (Figure 2), suggesting that the electrostatic repulsion among headgroups destabilize the gel phase at low ionic strength. Such a broad gel−fluid transition was also reported for the anionic phospholipid DMPG and was attributed to repulsion between charged phosphate groups at the bilayer surface (ref 36 and references therein). It is also interesting to point out that diC14-amidine bilayers do not display a pretransition like DMPC (T_p around 13 °C, Figure 2), or other cationic or anionic amphiphilic membranes.[36,37] Such a disappearance of the pretransition has been previously reported for several interdigitated phases.[38−41]

Existence of an Interdigitated Phase below the Gel−Fluid Transition of diC14-Amidine Liposomes. Phospholipids spin-labeled at different positions along the hydrocarbon chain were incorporated into bilayers of diC14-amidine and DMPC. Figure 3 shows the ESR spectra of 5-, 7-, 10-, 12-, and

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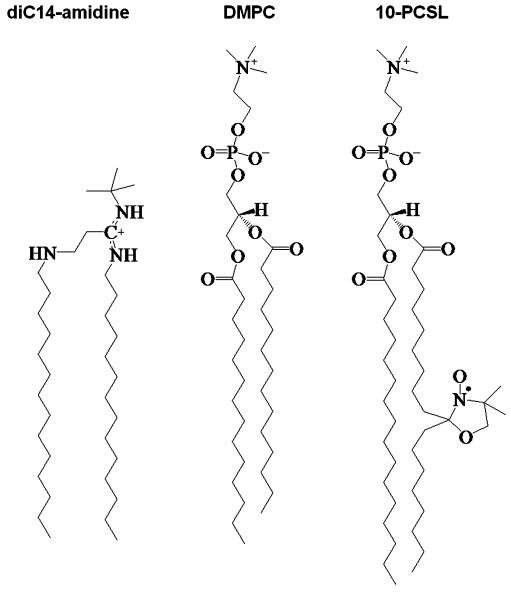

Figure 1. Chemical structures of diC14-amidine, DMPC, and the spin label 10-PCSL.

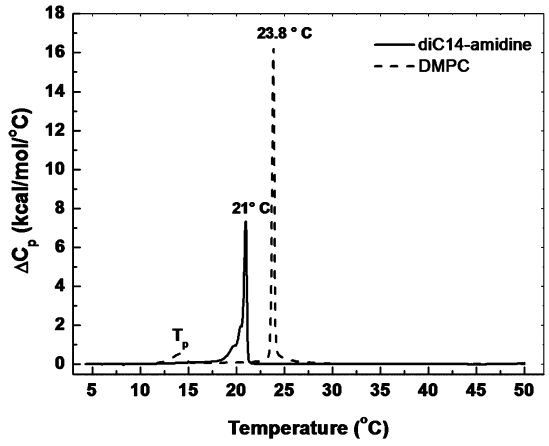

Figure 2. Excess heat capacity (ΔC_p) of diC14-amidine and DMPC (10 mM). Phase transition temperatures are indicated.

16-PCSL inserted into gel phase membranes of diC14-amidine and DMPC (T = 5 °C). The maximum hyperfine splitting, A_{max}, directly measured from the ESR spectra, is sensitive to the label microenvironment viscosity or packing[42] (Figure 3). As chain order and mobility parameters are both evaluated, it is a measure of fluidity of the membrane, and consequently, the word fluidity will be used to refer to these two parameters. DMPC exhibits a typical bilayer pattern: A_{max} decreases when the paramagnetic moiety goes deeper into the bilayer (Figure 4a), reflecting the known bilayer flexibility gradient.[35,43] Hence, even in the gel phase, the fatty acyl chains are not all extended in the all-trans conformation. The A_{max} parameter is clearly temperature dependent; the 16-PCSL probe indicating a considerable increase in chain flexing motions at higher temperatures (Figure 4a). On the other hand, diC14-amidine spectra (Figure 4b) shows that A_{max} values obtained with paramagnetic centers located at different depths of the lipid chains are rather similar and only slightly dependent on temperature, which is representative of interdigitated lipid bilayers.[34,44] In interdigitated lipid membranes, the fluidity gradient is nearly abolished, as the microenvironment

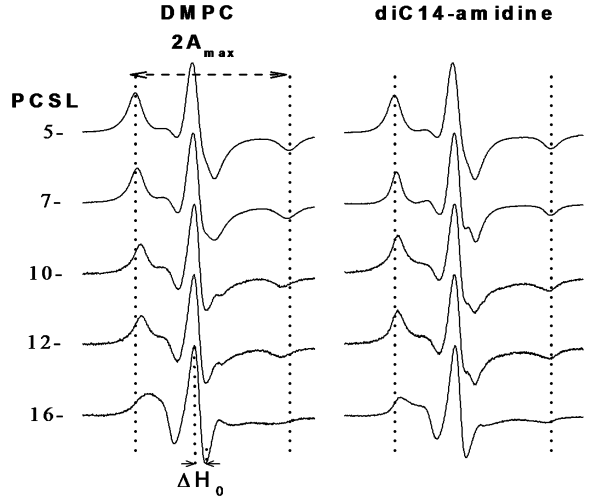

Figure 3. ESR spectra of 5-, 7-, 10-, 12-, and 16-PCSL incorporated in DMPC and diC14-amidine, at 5 °C. Dotted lines indicate the positions of the outer features of the 5-PCSL spectra (outer hyperfine splitting, $2A_{max}$). Total spectra width 100 G.

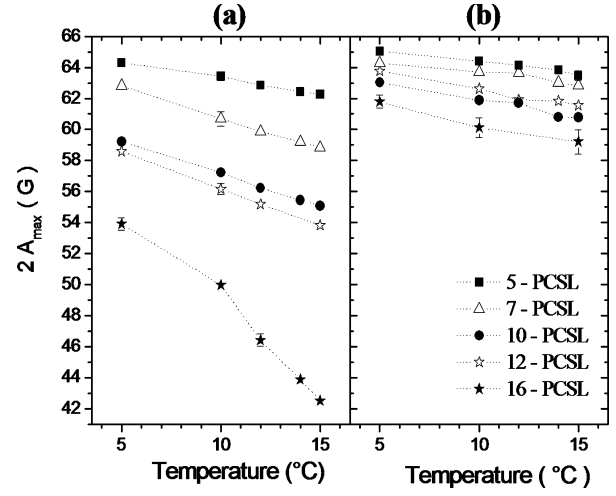

Figure 4. Outer hyperfine splitting ($2A_{max}$) measured on ESR spectra of different spin labels incorporated in the gel phase of (a) DMPC and (b) diC14-amidine.

monitored by a nitroxide at the fifth C-atom position is similar to that monitored by the probe located close to the acyl chain end and A_{max} values measured by 5- and 12- or 16-PCSL are similar (Figure 4b). Furthermore, due to interdigitation, the diC14-amidine gel phase is expected to be more packed than in DMPC bilayer, and the measured A_{max} values are larger for the former, for all the positions along the hydrocarbon chain (Figure 4b).

Existence of a Lamellar-Phase above the Gel–Fluid Transition of diC14-Amidine Liposomes. Above the transition temperature ($T > T_m$), distinct parameters have to be used to analyze the ESR spectra of the different spin labels. The effective order parameter (S_{eff}) is the most adequate parameter to evaluate the bilayer fluidity of diC14-amidine and DMPC bilayers with the probes 5- and 7-PCSL. Indeed, due to the position of the nitroxide group closer to the bilayer surface, in a quite ordered microenvironment, their ESR spectra are rather anisotropic, and A_{max} and A_{min} values can be directly measured (the maximum and minimum hyperfine splittings), as

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shown in Figure 5, and S_{eff} values can be calculated (see Materials and Methods and ref 45). S_{eff} includes contribution

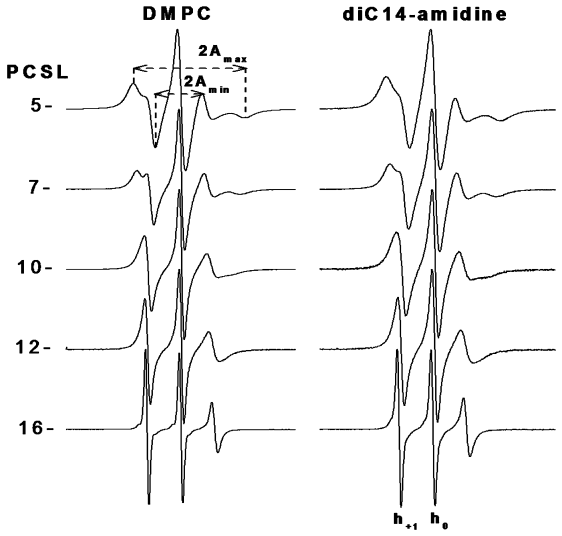

Figure 5. ESR spectra of 5-, 7-, 10-, 12-, and 16-PCSL incorporated in DMPC and diC14-amidine, at 40 °C. Total spectra width 100 G. Outer and inner hyperfine splittings (A_{max} and A_{min}) are indicated, as well as the position of the h_{+1} and h_0 lines.

from both chain order and rate of motion, but the principal contribution is the amplitude of segmental motion of the acyl chains.[45] Spin labels, namely, 10-, 12-, and 16-PCSL, located deeper in the bilayer, in a less ordered environment, yield isotropic ESR signals, and A_{max} and A_{min} values cannot be measured accurately. However, the ratio of the amplitudes of the central and low field lines (h_{+1} and h_0 lines are indicated in Figure 5), which tends to unity as the spin label mobility increases, can be evaluated accurately. Figure 6 compares these parameters of spin labels incorporated in diC14-amidine and DMPC bilayers above T_m. Spin labels incorporated into diC14-amidine and DMPC fluid bilayers display similar ESR spectra, and for both, the bilayer packing decreases toward the bilayer core, as the temperature increases, suggesting that, above T_m, diC14-amidine adopts a bilayer organization instead of an interdigitated state.

Bilayer Organization of diC14-Amidine Liposomes around the Gel–Fluid Transition. To further decipher the diC14-amidine and DMPC bilayers organizations, we analyzed ESR spectra of 16-PCSL inserted into both diC14-amidine and DMPC membranes at temperatures around the gel–fluid transition (Figure 7). The 16-PCSL is the most sensitive probe to detect bilayer fluidity changes (and coexistence of different phases). As expected, 16-PCSL spectra in DMPC indicate a rigid bilayer up to 23 °C, and a fluid one at and above 24 °C (Figure 7), consistent with the sharp gel-fluid transition at 23.8 °C (Figure 2). However, the same spin label in diC14-amidine clearly shows the coexistence of gel and fluid phases between 19 and 21 °C (the low field feature of the gel component is shown in Figure 7) and a fluid bilayer at and above 22 °C. These ESR data are in agreement with diC14-amidine DSC profile (Figure 2), which indicates a broader gel–fluid transition (therefore with possible coexistence of gel and fluid phases), and the end of the transition before 22 °C.

Temperature-Dependence of diC14-Amidine Liposome—Asolectin Lipid Mixing. We monitored the lipid

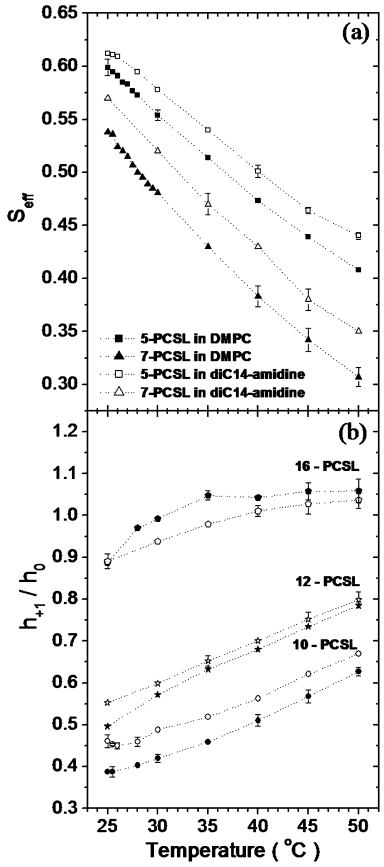

Figure 6. (a) Effective order parameter (S_{eff}) measured on ESR spectra of 5- and 7-PCSL incorporated in the fluid phase of DMPC (full symbols) and diC14-amidine (open symbols). (b) Ratio between the amplitudes of the central and low field lines (h_{+1}/h_0) measured on ESR spectra of 10-, 12-, and 16-PCSL incorporated in the fluid phase of DMPC (full symbols) and diC14-amidine (open symbols).

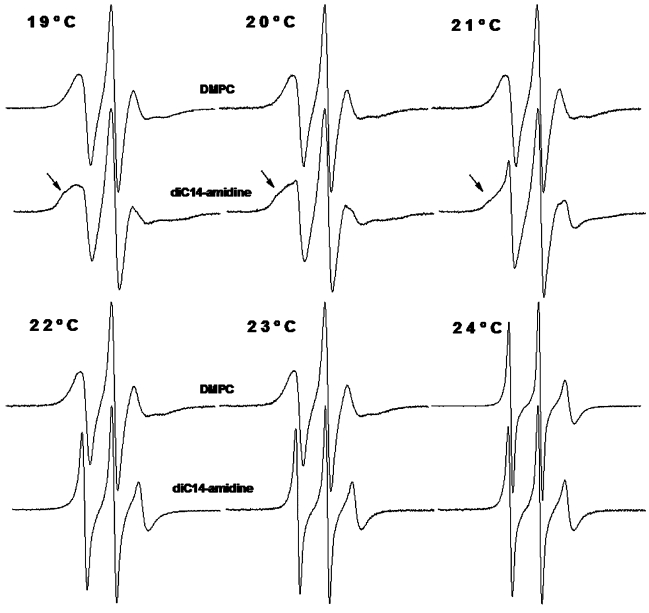

Figure 7. ESR spectra of 16-PCSL incorporated in DMPC (1st and 3rd rows) and in diC14-amidine (2nd and 4th rows). Arrows indicate the position of the low field feature of the gel component in the composite spectra. Total spectra width 100 G.

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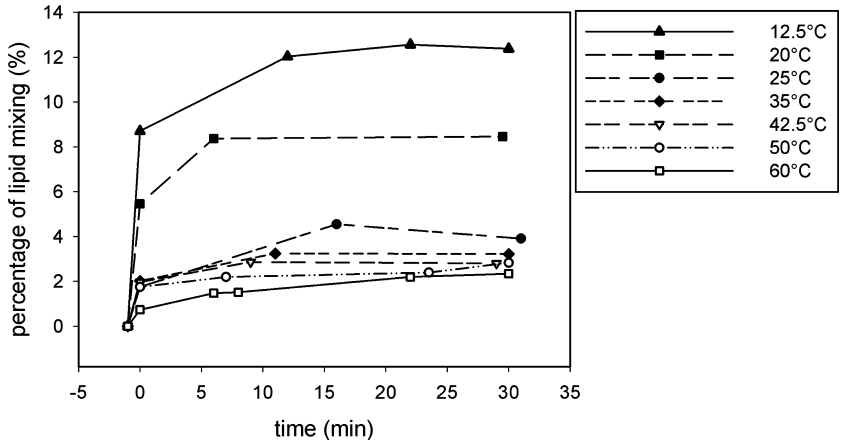

Figure 8. Lipid mixing between diC14-amidine liposomes and asolectin liposomes as a function of temperature. Asolectin liposomes were added to diC14-amidine liposomes (labeled with 0.8 mol % of NBD-PE and Texas Red-PE) at a mass ratio of 10:1 and loaded in a quartz fluorescence cell thermostated at different temperatures. The fluorescence was monitored using an SLM-8000 spectrofluometer with excitation and emission bandwidths of 4 nm. The ratio between fluorescence of NBD and fluorescence of Texas Red was calculated as described in Materials and Methods.

mixing between diC14-amidine liposomes and asolectin liposomes at different temperatures using fluorescence resonance energy transfer assay (FRET). The ratio between the fluorescence intensity of the donor (NBD) and fluorescence intensity of the acceptor (Texas Red) after addition of asolectin liposomes for each temperature is plotted in Figure 8 (see Materials and Methods). Surprisingly, lipid mixing between diC14-amidine and asolectin liposomes is more efficient at low temperatures (10−15 °C) than at high temperatures (above 25 °C). This temperature-dependent fusogenic property has not been observed previously since, for peptides and virus-induced fusions, fusion is generally enhanced at higher temperatures.[46−49]

The bilayer gel−fluid transition can be monitored by the central field line-width, ΔH_0, directly measured in the ESR spectra of 16-PCSL intercalated into the membrane (see Figure 3). ΔH_0 gets smaller as the microenvironment monitored by the spin label is less packed,[35] and is measured over the whole range of temperatures, from 5 to 50 °C. Likewise, the bilayer gel−fluid transition can be monitored by the decrease in the fluorescence anisotropy of the probe DPH inserted into the diC14-amidine bilayer. Figure 9 shows the overlay of the diC14-amidine transition monitored either by a fluorescence probe (DPH) or spin labeled probe (16-PCSL) and the lipid mixing efficiency as a function of temperature expressed as ratio between donor and acceptor fluorescence intensities 30 min after addition of asolectin liposomes. The observed transition between the "high fusogenic state" and the "low fusogenic state" fits pretty well the bilayer gel−fluid transition measured with the two probes. This strongly suggests that the fusion is maximal at temperatures where the diC14-amidine bilayer is organized as an interdigitated phase, but decreases drastically when a more classical bilayer organization predominates.

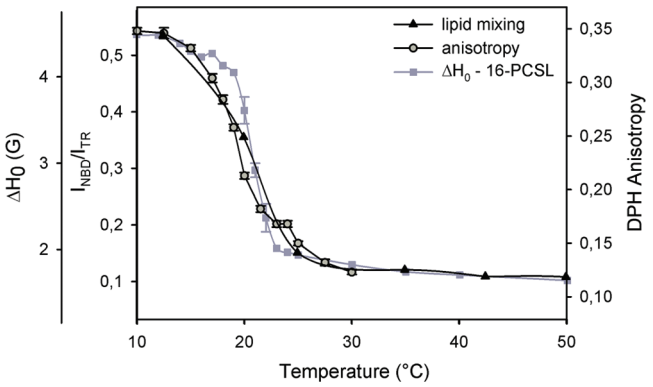

Figure 9. Temperature dependence of the central field line-width, ΔH_0, of 16-PCSL, and the fluorescence anisotropy of DPH, both probes intercalated into di-C14-amidine bilayers, and the lipid mixing efficiency of the liposomes. *Triangles*: Lipid mixing efficiency after 30 min of diC14-amidine liposomes with asolectin liposomes (as explained in Figure 8) was reported as a function of temperature. *Circles:* Temperature dependence of fluorescence anisotropy of DPH into diC14-amidine liposomes. *Squares*: Temperature dependence of ΔH_0 of 16-PCSL in diC14-amidine liposomes. Values are means of two experiments, and the uncertainties are standard deviations. When not shown, the uncertainty was found to be smaller than the symbol in the graph.

CONCLUSIONS

In the literature, interdigitated phases of symmetric lipids have been mostly induced by perturbations at the interfacial region of the bilayer (e.g., addition of alcohol,[50−53] glycerol,[34] acetonitrile,[54] polymyxin B,[34] ions like thiocyanate,[55] or pressure modifications[38,56]), resulting in modifications of the polar headgroup area or dehydration of the bilayer.[54] Interdigitation has also been described for asymmetric lipids or mixed-chains lipids of different chain length.[39,57−59] In this work, we demonstrated, using electron spin resonance, that a cationic lipid with two C14 alkyl chains, diC14-amidine does adopt, *per se,* a nonlamellar interdigitated organization below and around its transition temperature T_m (23 °C) and a lamellar bilayer organization above T_m (Figures 2−5). Evaluation of bilayer thicknesses by atomic force microscopy (C. Yip, unpublished data) or by X-ray diffraction[60] are in agreement with these data.

Such behavior had already been observed for other cationic lipids presenting a big headgroup with short acyl chains[61,62] or for cationic lipids with a high surface charge density.[63,64] In those examples, interdigitation was attributed to the net charge density of the polar heads, resulting in repulsion between headgroups, and could be modified by neutralization of this charge, by adding neutral lipids, DNA or NaCl. Furthermore, Ryhanen et al.[64] suggested that DHAB (dihexadecyldimethy-

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lammoniumbromide) vesicles also present an interdigitated phase in the fluid state.

In the case of diC14-amidine liposomes, without changing the surface charge density of the lipid, the lipid organization changes from a preferentially interdigitated state to a lamellar phase by increasing the temperature. Therefore, at low temperature, since close contacts between the charged diC14-amidine headgroups are unfavorable (due to repulsive electrostatic forces), the lipids are forced to interdigitate in order to condense the tail groups while keeping the neighboring headgroups apart. At higher temperature, thermal motion enables the lipid tails to occupy a larger lateral space, which satisfies the spacing constraints between the repulsive headgroups, and the more common nonoverlapped bilayer motif is adopted.

Furthermore, our results (Figures 8 and 9) suggest that the interdigitated phase of diC14-amidine liposomes correlates with the more potent fusogenic property of diC14-amidine vesicles at low temperature. Actually, previous studies on liposomes treated with small concentrations of ethanol in water already suggested the role of interdigitation in fusion processes, but destabilization of the membranes was not dependent on the intrinsic properties of the lipids since addition of ethanol was required to observe this effect.[14−16,53] Another study of fusogenic cationic liposomes made of asymmetric chain length showed an unusual nonlamellar phase in these liposomes.[65] Our data show here that transition to an interdigitated phase of a pure cationic lipid can be induced by a decrease in temperature, allowing the fusogenicity of the lipid to be modulated. This temperature-dependence behavior is in contrast with well-known fusogenic phospholipids such as the hexagonal phase-promoting phospholipids which become more fusogenic when their hydrocarbon tails occupy a larger dynamical volume as thermal disorder increases.

In conclusion, we think that interdigitation is an alternative membrane fusion mechanism that could be involved in biological processes and could be used for applied purposes such as cargo delivery to cells. In this regard, it would be important to synthesize new amidine derivatives with transition temperatures above 37 °C, to take advantage of the interdigitated organization to improve intracellular delivery. We already have demonstrated the fusogenic properties of an amidine derivative with a longer alkyl chain like diC16-amidine (T_m = 41 °C); however, more studies are required to demonstrated its interdigitated organization at 37 °C.[30]

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Notes

The authors declare no competing financial interest.

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