



DNA alters the bilayer structure of cationic lipid diC14-amidine: A spin label study

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ABSTRACT

Cationic lipids–DNA complexes (lipoplexes) have been used for delivery of nucleic acids into cells *in vitro* and *in vivo*. Despite the fact that, over the last decade, significant progress in the understanding of the cellular pathways and mechanisms involved in lipoplexes-mediated gene transfection have been achieved, a convincing relationship between the structure of lipoplexes and their *in vivo* and *in vitro* transfection activity is still missing. How does DNA affect the lipid packing and what are the consequences for transfection efficiency is the point we want to address here. We investigated the bilayer organization in cationic liposomes by electron spin resonance (ESR). Phospholipids spin labeled at the 5th and 16th carbon atoms were incorporated into the DNA/diC14-amidine complex. Our data demonstrate that electrostatic interactions involved in the formation of DNA–cationic lipid complex modify the packing of the cationic lipid membrane. DNA rigidifies the amidine fluid bilayer and fluidizes the amidine rigid bilayer just below the gel–fluid transition temperature. These effects were not observed with single nucleotides and are clearly related to the repetitive charged motif present in the DNA chain and not to a charge–charge interaction. These modifications of the initial lipid packing of the cationic lipid may reorient its cellular pathway towards different routes. A better knowledge of the cationic lipid packing before and after interaction with DNA may therefore contribute to the design of lipoplexes capable to reach specific cellular targets.

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1. Introduction

Cationic lipids are positively charged amphiphilic molecules made of a cationic polar head group (usually amino groups), a hydrophobic domain (comprising alkyl chains or cholesterol), and a linker connecting the polar head group with the non-polar tail. Most of them form positively charged liposomes, sometimes in combination with a neutral helper lipid [1–3]. Felgner and colleagues designed and synthesized the first cationic lipid used as a transfecting agent, and provided evidence that this positively charged lipid formed with DNA a stable complex allowing delivery and expression of the gene material into the cell [4]. This seminal work has opened the way to the cationic lipid-mediated intracellular delivery of hydrophilic molecules (nucleic acids, messenger RNA, peptides and proteins) [4–13].

In contrast to neutral or negatively charged liposomes, cationic liposomes efficiently interact with negatively charged molecules (such as nucleic acids, including plasmids, messengers RNAs and synthetic oligonucleotides, or proteins and peptides), mainly through electrostatic interactions [4–13]. Despite the fact that, over the last decade, significant progress in the understanding of the cellular pathways and mechanisms involved in lipoplexes-mediated gene transfection [14] have been achieved, a convincing relationship

between the structure of lipoplexes and their *in vivo* and *in vitro* transfection activity is still missing.

Spectroscopic methods (Raman, FTIR, UV), small-angle-X-rays scattering (SAXS) cryo-electron microscopy and isothermal titration calorimetry [15–24] have been used to evaluate the structure of lipid and DNA components in lipoplexes.

How does DNA affect cationic lipid packing and what are the consequences for transfection activity is the point we want to address.

We investigated the bilayer organization in cationic liposomes by electron spin resonance (ESR). The technique monitors the hydrocarbon chain microenvironment in the proximity of phospholipids labeled with paramagnetic probes localized along the acyl chains [25].

Phospholipids spin labeled at the 5th and 16th carbon atoms were incorporated into the DNA/diC14-amidine complex. The ESR spectra of the labeled samples, in the presence and absence of DNA, are interpreted in terms of changes in lipid packing and mobility caused by DNA–liposome interaction. Data were compared with the perturbation induced by a single nucleotide in order to evaluate the role of a repetitive charged motif present in the DNA chain.

2. Materials and methods

DiC14-amidine (3-tetradecylamino-N-tert-butyl-N-tetradecylpropionamide) was synthesized as described [26]. Spin labels 1-palmitoyl-2-(*n*-doxylstearoyl)-*sn*-glycero-3-phosphocholine (*n*-PCSL, *n* = 5 or 16) were purchased from Avanti Polar Lipids (Birmingham,

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AL, USA). Deoxyribonucleic acid (DNA), sodium salt from Salmon Testes (D-1626, 033K7012), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), sodium chloride (NaCl) and deoxyadenosine-5'-monophosphate (dAMP) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.1. DiC14-amidine liposomes preparation

A film was formed from a chloroform:methanol (2:1) solution of diC14-amidine and spin label (0.3 mol% relative to the lipid for 16-PCSL, and 0.6 mol% for 5-PCSL were found to be the maximum spin label concentrations required to avoid spin–spin interaction). The film was dried under a stream of N₂ and left under reduced pressure for a minimum of 2 h, to remove all traces of organic solvent. It was then hydrated with 10 mM Hepes at pH 7.4, to a final lipid concentration of 4 mM, heated above the phase transition for 5 min, and vortexed.

2.2. DiC14-amidine DNA lipoplexes preparation

About 12 h before the experiment, DNA was hydrated in 10 mM Hepes and kept overnight at 8 °C, at a nucleotide concentration of

22 mM. A volume of DNA solution was added into an equal volume of the DiC14-amidine liposome while gently shaking the tube, to a final lipid concentration of 4 mM, and the desired DNA concentration (0.0, 0.36, 0.70, 1.00, 1.20, 1.70 and 2.00, nucleotide/lipid molar ratios). The liposome/DNA mixture was allowed to stand for 30 min at room temperature. The same protocol was used to prepare the di-C14-amidine/dAMP complexes.

2.3. ESR spectroscopy

ESR measurements at X band were performed with a Bruker EMX spectrometer. The sample temperature was controlled within 0.2 °C by a Bruker BVT-2000 variable temperature device. The temperature was checked with a Fluke 51 K/J thermometer with the probe placed just above the cavity. The sample temperature was decreased in the interval 50–5 °C. To ensure thermal equilibrium, before each scan, the sample was left at the desired temperature for at least 10 min. The ESR data were acquired immediately after sample preparation. Magnetic field-modulation amplitude of 1 G and microwave power of 10 mW were used. The magnetic field was measured with a Bruker ER 035 NMR gaussmeter.

All data shown are means of the results of at least two experiments, and the uncertainties are the standard deviations. For the 16-PCSL, the isotropic hyperfine splitting, a_o , was taken to be one-half of the distance between the low and high field lines. For the highly anisotropic spectra of 5-PCSL, the isotropic hyperfine splitting was calculated from the expression [27,28],

$$a_o = (1/3)(A_{//} + 2A_{\perp})$$

where $A_{//}$ ($=A_{\max}$) is the maximum hyperfine splitting directly measured in the spectrum (see Fig. 1), and

$$A_{\perp} = A_{\min} + 1.4 \left[1 - \frac{A_{//} - A_{\min}}{A_{zz} - (1/2)(A_{xx} + A_{yy})} \right]$$

where A_{\min} is the measured inner hyperfine splitting (see Fig. 1) and A_{xx} , A_{yy} and A_{zz} are the principal values of the hyperfine tensor for doxylpropane [29].

Effective order parameters, S_{eff} , were calculated from the expression [30]

$$S_{\text{eff}} = \frac{A_{//} - A_{\perp}}{A_{zz} - (1/2)(A_{xx} + A_{yy})} \frac{a_o'}{a_o}, \quad \text{where } a_o' = (1/3)(A_{xx} + A_{yy} + A_{zz}).$$

Rotational correlation times for isotropic motion, according to the motional narrowing theory, can be calculated from the peak-to-peak width of the ESR Lorentzian lines [31,32]:

$$\Delta H_L(m) = A + B_m + C_m^2.$$

Where m is the m -th component of the nitrogen nuclear spin, A is the Lorentzian linewidth of the central line, $\Delta H_L(0)$, and B and C are

$$B = \frac{1}{2} \Delta H_L(0) \left(\frac{\Delta H_L(+1)}{\Delta H_L(0)} - \frac{\Delta H_L(-1)}{\Delta H_L(0)} \right)$$

$$C = \frac{1}{2} \Delta H_L(0) \left(\frac{\Delta H_L(+1)}{\Delta H_L(0)} + \frac{\Delta H_L(-1)}{\Delta H_L(0)} - 2 \right).$$

The correlation time for doxyl labels is either $\tau_B = -1,22$ B or $\tau_C = 1,19$ C, ($\tau_B = \tau_C$ for isotropic movement). Lorentzian linewidths are calculated using a computer program, which performs nonlinear least-square fitting of the experimental ESR spectrum using a model of a Lorentzian–Gaussian functional for corrections for non-resolved hyperfine splitting [33,34].

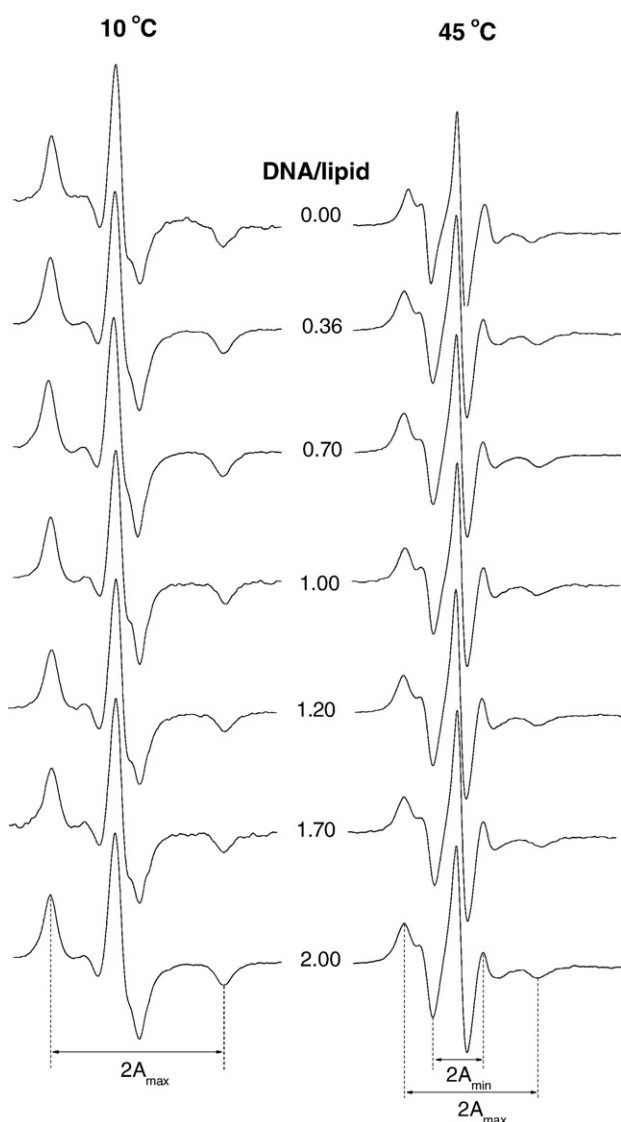


Fig. 1. ESR spectra of 5-PCSL in diC₁₄-amidine liposomes at 10 and 45 °C, at different DNA/lipid molar ratios (nucleotide/lipid molar ratios). The hyperfine splitting A_{\max} and A_{\min} are indicated. Total spectra width 100 G.

3. Results and discussions

The mobility and/or order of the hydrocarbon chains at different C-atom positions along the lipid acyl chain are monitored by spin-labeled phospholipids.

3.1. DNA–liposome interaction monitored by a spin probe located close to the bilayer surface

The hydrocarbon chain microenvironment in the proximity of the membrane surface is monitored by the ESR signal of a phospholipid labeled at the 5-C atom (5-PCSL). Fig. 1 shows the ESR spectra of 5-PCSL incorporated in pure diC14-amidine liposomes, and in DNA–diC14-amidine complexes. The spectra were recorded at two different temperatures, below (10 °C) and above (45 °C) the diC14-amidine gel–fluid transition ($T_m \approx 23$ °C) [35]. The DNA/lipid ratios are nucleotide/lipid molar ratios (0.36, 0.70, 1.00, 1.20, 1.70 and 2.00).

No major difference caused by the presence of DNA is detectable by visual observation of the spectra. The spectra shown in Fig. 1 are all typical bilayer spectra. Due to the flexibility gradient towards the bilayer core [29], the spectra of 5-PCSL are much more anisotropic (larger A_{\max}) than those of 16-PCSL (see Fig. 4), which monitors the center of the membrane. At 10 °C, the spectra are characteristic of nitroxides in a tightly packed environment, consistent with a bilayer in the gel phase. At 45 °C, the spectra of 5-PCSL are typical of fluid bilayers, in which the label moves fastly around its long axis [29]. Hence, 5-PCSL ESR spectra reveal that the diC14-amidine bilayer is intact in the presence of DNA up to ratio of 2 nucleotides per lipid.

Even though visual observation does not detect significant changes, DNA induced packing differences in the bilayer around the 5-C atom position can be detected via parameters that can be directly measured in the spectra. As discussed [36], whenever the ESR spectra are relatively anisotropic (out of the motional narrowing region, as

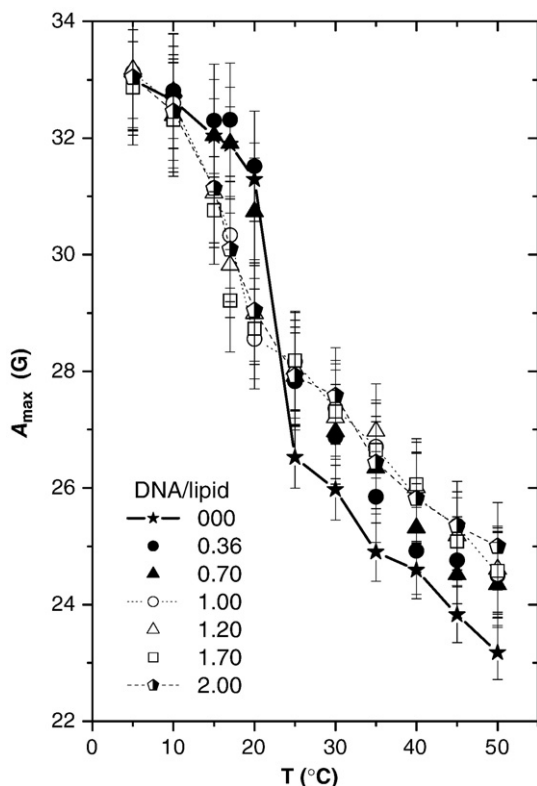


Fig. 2. Temperature dependence of the outer hyperfine splitting (A_{\max}) of 5-PCSL in diC14-amidine at 0.00, 0.36, 0.70, 1.00, 1.20, 1.70 and 2.00 DNA/lipid molar ratios. Data from spectra in Fig. 1.

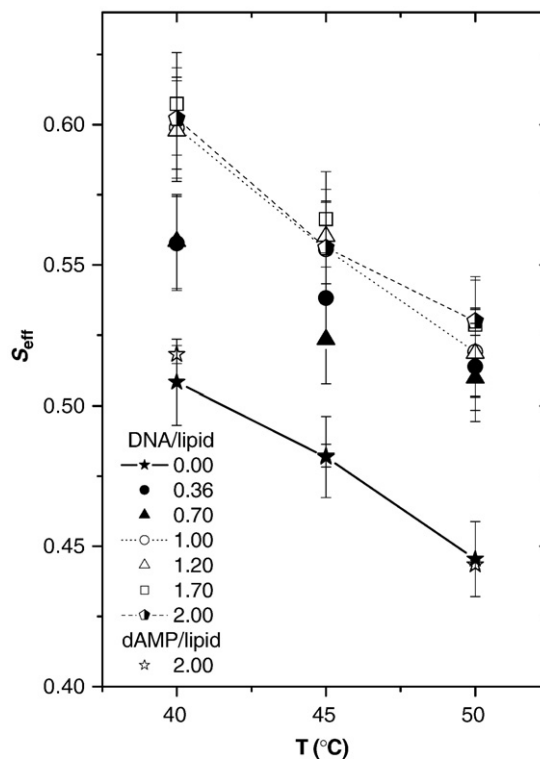


Fig. 3. Order parameter (S_{eff}) of 5-PCSL in diC14-amidine fluid phase, at 0.00, 0.36, 0.70, 1.00, 1.20, 1.70 and 2.00 DNA/lipid molar ratios, and at 2.00 dAMP/lipid molar ratio. Data from spectra in Fig. 1.

those shown in Fig. 1), indicating a spin label slow movement, the analysis of the signal is not straightforward. However, the lipid bilayer structure can be satisfactorily described by empirical parameters, directly measured from the ESR spectra, which depend on the spin label micro-environment viscosity (including both order and mobility). Parameters sensitive to bilayer packing/organization (see, for instance, [37,38]), successfully used in the literature as comparative parameters, were chosen. Distinct parameters are used for different spin labels and for the different ranges of temperature, depending on the accuracy of their direct measurement on the spectra and their sensitivity to the bilayer structure.

A_{\max} , the outer hyperfine splitting of 5-PCSL (shown in Fig. 1), is used over the whole range of temperature. Its value increases with the label microenvironment viscosity or packing [36], and the bilayer phase transition can be monitored (Fig. 2) through the variation of this parameter (see a mild decrease of A_{\max} from 5 to 20 °C, and a sharp drop around 23 °C, for pure diC14-amidine bilayer). At 5 and 10 °C, around the 5th C-atom position, the bilayer structure is not much affected by DNA. However, DNA significantly reduces the bilayer phase transition cooperativity, fluidizing the gel phase at and above 15 °C (lower A_{\max} values), and rigidifying the fluid phase (increase in A_{\max} for temperatures above 25 °C). The almost complete disappearance of the sharp diC14-amidine phase transition in the presence of DNA confirms previous differential scanning calorimetry results [35]. This effect could be attributed either to partial penetration of DNA into the bilayer or to electrostatic interactions affecting the hydrocarbon chains interactions.

To check the role of a repetitive charged motif in the DNA chain, experiments were repeated with a single nucleotide, dAMP. Interestingly, no 5-PCSL A_{\max} variation was detected in diC14-amidine bilayers in the presence of dAMP, both in the lipid gel or fluid phases, up to 2.0 nucleotide/lipid molar ratio (not shown).

With 5-PCSL inserted into fluid membranes, the bilayer organization can be better analyzed by the effective order parameter, S_{eff} ,

defined in Materials and methods (Fig. 3). The main contribution to S_{eff} is the amplitude of movement of the hydrocarbon chain moiety [30]. As indicated by the increase in A_{max} , DNA significantly increases the packing/order of the diC14-amidine bilayer in the fluid phase (increases S_{eff} in Fig. 3). It seems that the DNA effect is maximal around a DNA/lipid ratio of 1.0, hence equal molar concentrations of DNA nucleotide and lipid. The fact that no change of S_{eff} was observed with dAMP demonstrates that the ordering effect of DNA on diC14-amidine fluid phase is related to the repetition of a charged motif rather than to a charge-charge interaction (Fig. 3).

The presence of water in the bilayer can be estimated from the magnitude of the isotropic nitrogen hyperfine splitting a_o , which can only be well measured on the ESR spectra of fluid membranes, where the label movement around the nitroxide z axis is fast in the ESR time-scale [39]. In the proximity of the bilayer surface, monitored by 5-PCSL, a_o was found to be 15.03 ± 0.08 G for all systems studied, namely pure diC14-amidine and/or incubated with DNA or dAMP. This value, typically reported for 5-PCSL inserted into lipid bilayers (see, for instance, [40]), provides evidence that the bilayer hydration around the 5th C atom position is not altered by the presence of DNA or dAMP.

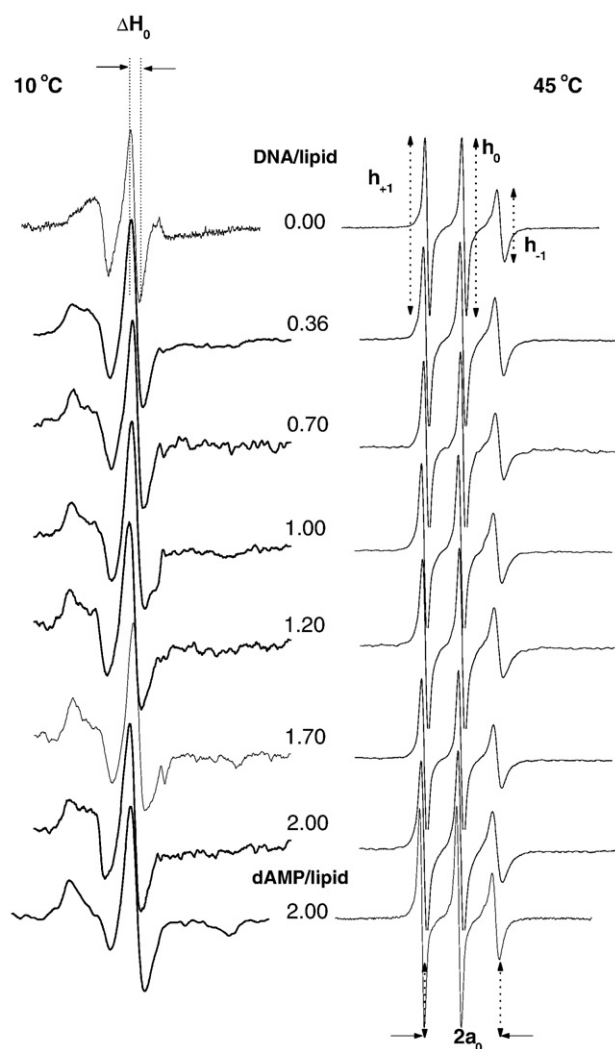


Fig. 4. ESR spectra of 16-PCSL in diC₁₄-amidine at 10 and 45 °C, with different DNA/lipid molar ratios (nucleotide/lipid molar ratios). The three nitrogen hyperfine line amplitudes (h_{+1} , h_0 and h_{-1}) corresponding to $m_l = +1, 0$ and -1 , respectively, the central field linewidth (ΔH_0), and the isotropic hyperfine splitting (a_o) are indicated. Total spectra width 100 G.

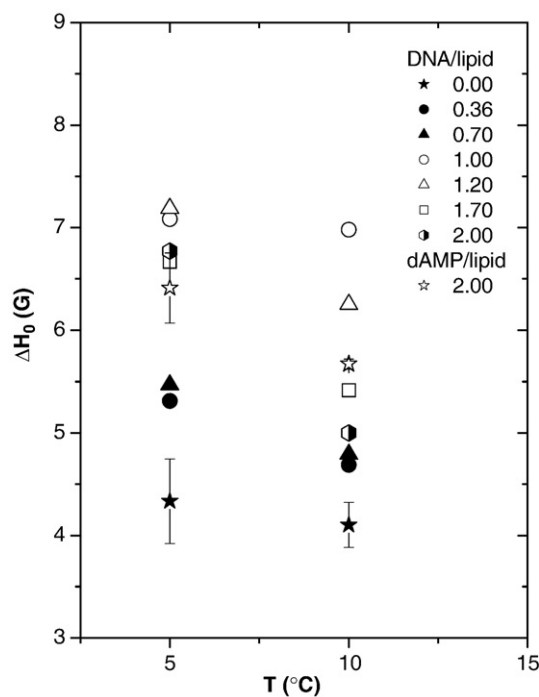


Fig. 5. The central field linewidth (ΔH_0) of 16-PCSL in diC₁₄-amidine gel phase, at 0.00, 0.36, 0.70, 1.00, 1.20, 1.70 and 2.00 DNA/lipid molar ratios and at 2.00 dAMP/lipid molar ratio. Data from spectra in Fig. 4.

3.2. DNA–liposome interaction monitored by a spin located in the bilayer core

A phospholipid labeled close to the end of the acyl chain, like 16-PCSL, is sensitive to the order/mobility at the bilayer center, where the nitroxide moiety is located. Fig. 4 shows the ESR spectra of 16-PCSL incorporated in diC14-amidine liposomes, in the absence and in the presence of DNA and dAMP. The spectra are shown at 10 and 45 °C, corresponding to the lipid gel and fluid lipid phases, respectively. As mentioned before, the bilayer, at the gel and fluid phases, is less packed in the core than to the surface, as attested by the more isotropic spectra obtained for 16-PCSL as compared to those of 5-PCSL.

ESR spectra of the probe incorporated in the gel (10 °C) and fluid (45 °C) phases of diC14-amidine, with and without DNA, need to be analyzed separately (Fig. 4). There is indeed no single parameter that can be used over the whole range of temperatures and which is sensitive to the packing in both the gel and the fluid phases. At 10 °C, the decrease on the spin probe movement, observed in the presence of both DNA and dAMP, is evidenced by the increase of the ESR signal anisotropy (broader signals), indicating a better bilayer packing. The central field linewidth, ΔH_0 , becomes larger when the probe movement gets slower [29]. Fig. 5 shows that both DNA and dAMP lead to a better bilayer packing at 5 and 10 °C (increase ΔH_0). (Between 15 and 20 °C, no reliable parameter could be measured in the 16-PCSL spectra of diC14-amidine bilayer, both with and without DNA). The rigidifying effect of DNA and dAMP, observed in the diC14-amidine bilayer core (with 16-PCSL) at low temperatures, and not observed with 5-PCSL, is not unusual (Fig. 2). In the gel phase, considering the high packing in the region of the bilayer located around the 5th C-atom, it is known that the ESR of spin probes can detect modifications in the bilayer core, caused by interacting molecules, but not at the bilayer surface (for instance, see [38]). The rigidifying effect of both DNA and dAMP, at 5 and 10 °C, can possibly be attributed to a non specific shielding of the electrostatic repulsion between diC14-amidine head groups, allowing the lipids to

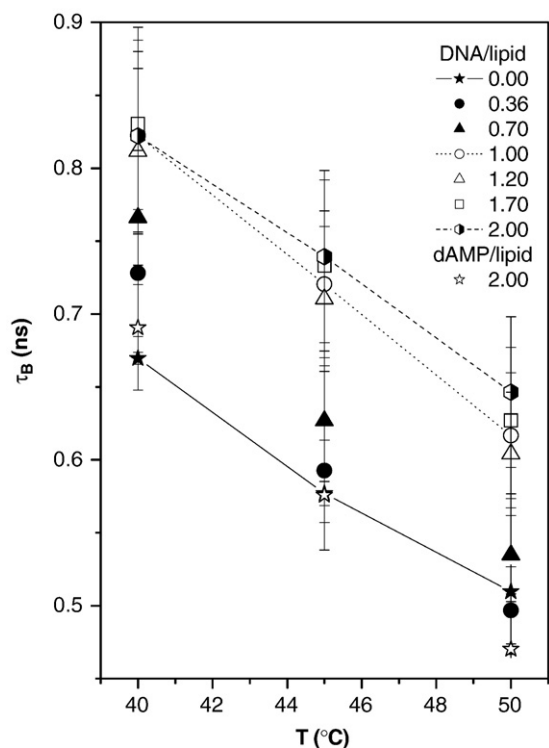


Fig. 6. Rotational correlation times (τ_B) of 16-PCSL in diC₁₄-amidine fluid phase, at 0.00, 0.36, 0.70, 1.00, 1.20, 1.70 and 2.00 DNA/lipid molar ratios and at 2.00 dAMP/lipid molar ratio. Data from spectra in Fig. 4.

get close together in the gel phase. It is most likely that head group electrostatic repulsion is the determinant factor keeping diC₁₄-amidine molecules apart at low temperatures.

Calculation of rotational correlation times from ESR spectra of 16-PCSL, inserted into a fluid phase, provides direct information on the fluid phase packing (see Materials and methods). The rotational correlation times τ_B and τ_C were found to be similar, indicating that the nitroxide moiety of 16-PCSL is located in a quite isotropic environment when incorporated into the fluid phase of diC₁₄-amidine bilayers [32]. Values of τ_B for diC₁₄-amidine alone or mixed with dAMP and DNA at different concentrations are shown in Fig. 6. As observed with 5-PCSL (Fig. 3), the nucleotide dAMP does not alter the diC₁₄-amidine fluid phase packing (similar τ_B values obtained without and with [dAMP]/[lipid] = 2.0, Fig. 6). However, in agreement with the results obtained with the nitroxide at the 5th C-atom position (Fig. 3), DNA significantly increases the packing in the bilayer core (Fig. 6). Also as observed with 5-PCSL (Fig. 3), DNA alters the lipid packing up to around a [nucleotide]/[lipid] ratio equal to 1.0.

Considering the bilayer polarity at the 16-C atom position for fluid membranes, a_o was found to be 14.28 ± 0.04 G for all systems studied, namely pure diC₁₄-amidine and the lipid complexed with DNA or dAMP. This is a typical value obtained with 16-PCSL inserted in lipid bilayers [40], lower than that obtained at the 5th C-atom position (15.03 ± 0.08 G) as a consequence of the bilayer polarity gradient [39].

Hence, though DNA significantly increases the bilayer order at the 5th C-atom position (Fig. 3) and leads to a better packing at the bilayer core (Fig. 6), it does not alter the hydration of the membrane hydrocarbon region. It is important to have in mind that alterations of bilayer hydration can be detected by spin labels. For instance, cationic peptides, which bind at anionic bilayer surfaces, enhance the bilayer packing and the bilayer hydration [37]. In contrast, cholesterol, which completely penetrates the bilayer, increases the bilayer order but decreases the bilayer polarity [37,41].

4. Concluding remarks

At and below 10 °C, DNA-induced changes in lipid organization on the gel phase of diC₁₄-amidine are similar to those observed with single nucleotide, suggesting a non specific electrostatic shielding effect, bringing the amidine head groups close together. Between 15 and 20 °C, DNA, and not dAMP, fluidizes the bilayer of pure diC₁₄-amidine, still in the gel phase. The interaction between the phosphate moieties of the free nucleotide and the charge associated to the amidine moiety does not modify significantly the lipid packing in the fluid phase, showing that free nucleotide electrostatic shielding effect is not relevant for lipids in fluid diC₁₄-amidine. On the opposite, DNA rigidifies the fluid diC₁₄-amidine phase (Figs. 2 and 6). In summary, a main conclusion of our study is that DNA modifies the packing of the amidine bilayer and that such changes depend on the initial membrane packing. Globally, DNA stabilizes the amidine fluid bilayer and fluidizes the amidine rigid bilayer.

It is largely accepted that fluid lipoplexes penetrate into cells rather by fusion with the plasma membrane whereas more rigid lipoplexes do prefer the endocytic pathway [14]. Fluidity and rigidity were referring in that case to the state of the bilayer before interacting with DNA. The present work provides evidence that interaction with DNA does modify the initial packing and fluidity of the cationic lipid in a way that could reorient the cellular pathway towards the endocytic route (rigidifying effect) or the fusion route (fluidizing effect). A better knowledge of the cationic lipid packing, before and after interaction with DNA, can therefore contribute to the design of lipoplexes capable to reach specific cellular targets.

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