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Chemistry and Physics of Lipids 131 (2004) 197–204
CPL CHEMISTRY AND PHYSICS OF LIPIDS
www.elsevier.com/locate/chemphyslip

Structural characterization of diC$_{14}$-amidine, a pH-sensitive cationic lipid used for transfection

Carlos R. Benatti [a], Jean-Marie Ruysschaert [b], M. Teresa Lamy [a,*]

[a] *Instituto de Física, Universidade de S. Paulo, CP 66318, CEP 05315-970, S. Paulo, SP, Brazil*
[b] *Laboratoire de Structure et Fonction des Membranes Biologiques, Université Libre de Bruxelles, Campus Plaine CP 206/2, B-1050 Brussels, Belgium*

Received 8 March 2004; received in revised form 19 May 2004; accepted 19 May 2004

Available online 14 July 2004

Abstract

The structure of *N*-*t*-butyl-*N*′-tetradecyl-3-tetradecylaminopropionamidine (diC$_{14}$-amidine) cationic vesicles, used for transfection, was investigated at different pH values and ionic strengths, through the analysis of the electron spin resonance (ESR) spectra of spin labels. Phospholipid derivatives, spin labeled at the 5th and 16th C-atoms along the hydrocarbon chain, incorporated in diC$_{14}$-amidine bilayers, show that the bilayer structure is highly sensitive to the pH value of the medium, due to the two titratable groups present in the amphiphile. Compared with samples at higher pH values, the double charged diC$_{14}$-amidine at pH 3 presents a rather non-organized bilayer gel phase, and a much lower gel-fluid temperature transition, in accord with a strong headgroup electrostatic repulsion. In addition, the structure was found to be highly dependent on the ionic strength of the medium. However, pH 3 diC$_{14}$-amidine bilayer, in the fluid phase, was found to be slightly more closely packed than those at pH 7.4 or 9.0, which are less charged. Parallel to that, the larger isotropic hyperfine splitting measured for nitroxides in the center of the pH 3 diC$_{14}$-amidine bilayer suggests a higher membrane polarity for the highly charged low pH sample.

Keywords: diC$_{14}$-amidine; Spin label; Cationic liposomes; Structural and thermal properties; pH-sensitive

1. Introduction

With the advance in the knowledge of the genetic origins of many diseases, several research groups have concentrated on the study of vehicles for gene transfer to mammalian cells (Gao and Huang, 1991; Paukku et al., 1997; Curiel and Douglas, 2002). Less immunogenic non-viral systems, cationic amphiphiles have shown great potential for gene delivery (El Ouahabi et al., 1996; Bragonzi et al., 2000; Ramesh et al., 2001). The apparent simplicity of the interaction between cationic lipids and DNA can be one of the attractive features in the use of this system for transfection (Felgner and Ringold, 1989). However, the efficiency of the cationic lipid/DNA complex (lipoplexes) is dependent on many properties, such as surface charge density, particle size, lipid composition, etc. The efficiency of a good DNA carrier is related to both the binding and the release of the genetic material.

The exact mechanism responsible for DNA release remains speculative, but may include a destabilizing environment provided by low pH at regions of molecu-

* Corresponding author. Tel.: +55-11-3091-6829; fax: +55-11-3813-4334.
E-mail address: mtlamy@if.usp.br (M.T. Lamy).

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doi:10.1016/j.chemphyslip.2004.05.002

lar interactions. Several lipoplexes have been prepared with a "helper" lipid (Noguchi et al., 1998; Smisterová et al., 2001), so called because of their ability to improve transfectivity. Considering the possible important role played by the endosomes in transfection, pH sensitive liposomes have been designed to exploit their acidic environment, which can reach values below 5.0 (Ohkuma and Poole, 1978; Daleke et al., 1990). The stability of lipoplexes at acidic endosomal pHs will depend either upon the intrinsic acidity constants of the cationic amphiphiles or on titratable non-cationic amphiphiles used for making the liposomes pH-sensitive.

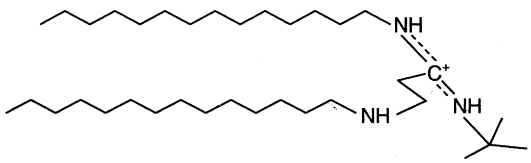
The present work focuses on the cationic amphiphile *N*-*t*-butyl-*N*′-tetradecyl-3-tetradecylaminopropionamidine (called diC_{14}-amidine or Vectamidine). This lipid was found to be capable of transfecting DNA and mRNA into mammalian cells with high efficiency (Ruysschaert et al., 1994; El Ouahabi et al., 1996). Compared with many other cationic lipids, diC_{14}-amidine has the advantage that it does not need a helper lipid, significantly simplifying lipoplex formulation (Ruysschaert et al., 1994; El Ouahabi et al., 1997). Still different from other lipids used for transfection, diC_{14}-amidine is a cationic amphiphile with two titratable groups, an amine and an amidine, showing two pK_a values (Pector et al., 1998) that are significantly affected by the ionic strength of the medium; the amine and the amidine pK_a values vary from 4.6 and 8.8 (30 mM NaCl) to 5.2 and 9.4 (130 mM NaCl), respectively.

In view of the possible relationship between the structural properties of diC_{14}-amidine liposomes at different acidic environments and its high efficiency as a transfecting agent, the present work studies those liposomes at different pH values, 3.0, 7.4 and 9.0, where the amphiphile will be positively double-charged, mono-charged, and less than mono-charged, respectively. The structure of the diC_{14}-amidine liposomes are investigated through the analysis of the electron spin resonance (ESR) spectra of spin labels incorporated in them. This technique has been extensively used to monitor the viscosity and polarity of the microenvironment where the probes are localized (see, for instance, Schreier et al., 1978; Marsh, 1981; Benatti et al., 2001, and references therein). Phospholipids, spin labeled at different acyl chain positions, were used to monitor the diC_{14}-amidine bilayer at different depths.

2. Materials and methods

2.1. Reagents

DiC_{14}-amidine, synthesized as described (Ruysschaert et al., 1994), is available from Biotech Tools (Brussels, Belgium). 4-(2-Hydroxyethyl)-1-piperizineethanesulfonic acid (Hepes) and NaCl were purchased from Sigma Chemical Co. (St. Louis, MO, USA), and were used as furnished. The spin labels 1-palmitoyl-2-(*n*-doxylstearoyl)-*sn*-glycero-3-phosphocholine (*n*-PCSL, $n = 5$ or 16) were purchased from Avanti Polar Lipids (Birmingham, AL, USA).



Molecular structure of diC_{14}-amidine

2.2. Lipid dispersion preparation

A film was formed from a chloroform solution of diC_{14}-amidine and spin label (0.2 mol%, relative to the lipid, for 16-PCSL, and 0.6 mol% for 5-PCSL, were found to be the maximum spin label concentrations to display no spin–spin interaction), dried under a stream of N_2 and left under reduced pressure for a minimum of 2 h, to remove all traces of the organic solvent. Dispersions were prepared with film hydration by the addition of the desired buffer solution with or without salt, heated above the phase transition at 58 °C for ∼2 min and vortexed. The buffer systems used were phosphate 20 mM (low ionic strength) and phosphate 20 mM + NaCl 150 mM (high ionic strength), both at pH 3.0, 7.4 and 9.0. DiC_{14}-amidine concentration was 4 mM, and 60 μL were loaded in a capillary for ESR measurements.

2.3. ESR spectroscopy

ESR measurements 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 varied from 50 to 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. 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 Gausmeter. All data shown are means of the results from at least three experiments, and the uncertainties are the standard deviations. When not shown, the uncertainties are smaller than the size of the symbols.

The parameters of the 16-PCSL ESR spectra were found by fitting each line to a Gaussian–Lorentzian sum function (Halpern et al., 1993), taking advantage of the fact that the sum function is an accurate representation of a Gaussian–Lorentzian convolution, the Voigt function (Bales, 1989). The isotropic hyperfine splitting, a_0, was taken to be one-half the difference in the resonance fields of the high- and low-field lines.

For the highly anisotropic spectra of 5-PCSL, the isotropic hyperfine splitting was calculated from the expression (Griffith and Jost, 1976; Gaffney, 1976),

$$a_0 = \tfrac{1}{3}(A_{//} + 2A_{\perp}), \tag{1}$$

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

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

where $2A_{\min}$ 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 (Hubbell and McConnell, 1971).

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

$$S_{\text{eff}} = \frac{A_{//} - A_{\perp}}{A_{zz} - (1/2)(A_{xx} + A_{yy})}\frac{a_0'}{a_0}$$
$$\text{where} \quad a_0' = \tfrac{1}{3}(A_{xx} + A_{yy} + A_{zz}) \tag{3}$$

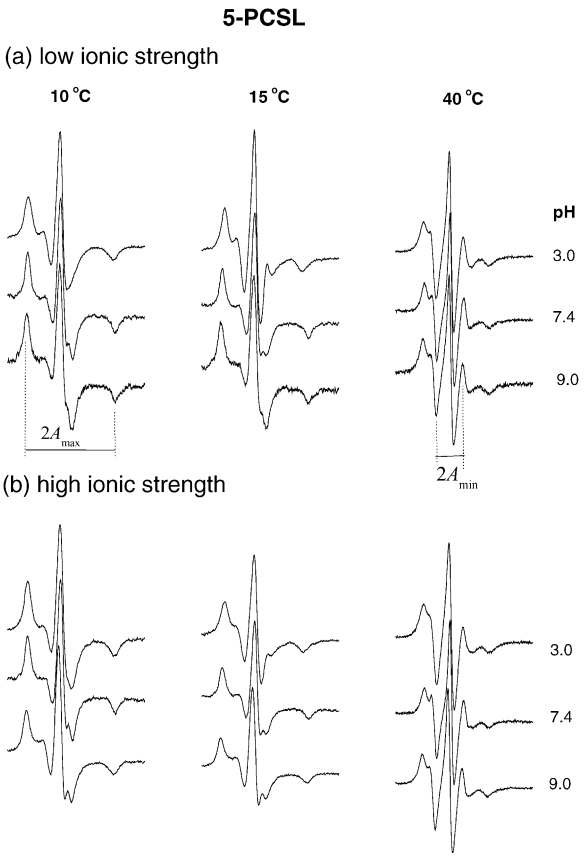


Fig. 1. ESR spectra of 5-PCSL in diC$_{14}$-amidine liposomes at (a) low and (b) high ionic strength, at pH 3.0 (first), 7.4 (second) and 9.0 (third row), at 10, 15 and 40 °C, as shown in the figure. The hyperfine splittings $2A_{\max}$ and $2A_{\min}$ are indicated. Total spectra width 100 G.

3. Results and discussions

3.1. Liposome structure monitored by a spin label close to the bilayer surface

ESR spectra of 5-PCSL incorporated in diC$_{14}$-amidine liposomes are presented in Fig. 1. The spectra are due to dispersions prepared at three different medium pH values, 3.0, 7.4 and 9.0, each at low and high ionic strength (see Section 2). The ESR spectra shown in Fig. 1 were obtained at 10, 15 and 40 °C. As discussed further, the 10 and 40 °C bilayers are in the gel and fluid lipid phases, respectively, whereas the lipid phase of the 15 °C samples is highly depend on the medium pH. The ESR spectra indicate a

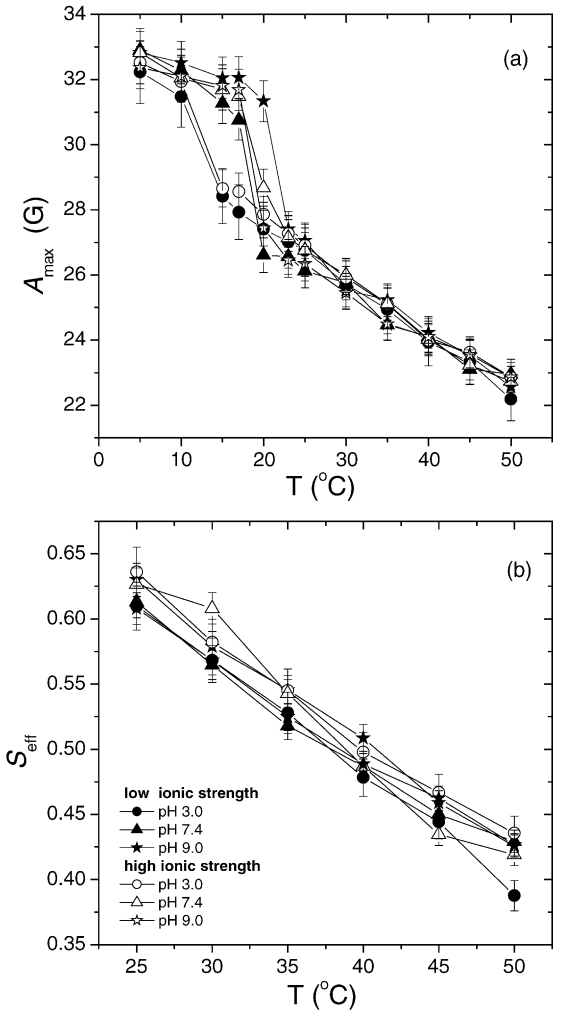


Fig. 2. Temperature dependence of (a) the outer hyperfine splitting (A_{max}) and (b) effective order parameter (S_{eff}) measured on the ESR spectra of 5-PCSL in diC_{14}-amidine at low (solid symbols) and high (open symbols) ionic strength, at pH 3.0 (circle), 7.4 (triangle) and 9.0 (star). The data were acquired from 50 to 5 °C.

structural difference for diC_{14}-amidine liposomes at pH 3.0, as compared to the higher pH values, 7.4 and 9.0, (Fig. 1). The observed spectral differences are evident in the empirical parameter A_{max} (Fig. 2a), the outer hyperfine splitting (shown in Fig. 1a), which can be used as an empirical parameter that increases with the label microenvironment viscosity or packing (Freed, 1976). All samples studied presented clear gel-fluid transition temperatures, well distinguished by relatively sharp A_{max} variations at the 5-PCSL position, between 10 and 20 °C. It is also important to note that the 5-PCSL spectra of the label incorporated in fluid diC_{14}-amidine, at the three pH values studied, are typical of bilayer structure (Seelig, 1976).

Fig. 2a indicates that at low pH values, with the diC_{14}-amidine double charged, the gel phase is less tightly packed and less stable, leading to a much lower gel-fluid phase transition temperature (T_m). Almost no difference among the packing of the samples can be observed at the lipid fluid phase, monitored either by the A_{max} value or by the effective order parameter, S_{eff}. The latter parameter (see Section 2) contains contributions from both order and rate of motion, although the principal contribution to S_{eff} is the amplitude of segmental motion of the acyl chains (Schindler and Seelig, 1973).

It is interesting to note that the presence of salt does not significantly alter the behavior of the low pH diC_{14}-amidine samples at the 5-PCSL nitroxide position. At pH 9, a considerable difference in T_m is observed between the samples at low and high ionic strength, with the low salt sample presenting a higher gel-fluid lipid phase transition. That can certainly be attributed to the expected dependence of the diC_{14}-amidine apparent pK value on the medium ionic strength. As mentioned before, it was observed that diC_{14}-amidine at low ionic strength (20 mM Hepes buffer, pH 7.3, plus 30 mM NaCl) titrates at pK_a 8.8, whereas the lipid at higher ionic strength (20 mM Hepes buffer, pH 7.3, plus 130 mM NaCl) titrates at pK_a 9.4 (Pector et al., 1998). Considering that, at pH 9, the low ionic strength diC_{14}-amidine bilayer studied here is less charged than the high ionic strength sample, hence presenting a more stable gel phase, with a higher transition temperature value (Fig. 2a).

3.2. Liposomes structure monitored by a spin label at the membrane core

The diC_{14}-amidine liposomes were also monitored by 16-PCSL, a phospholipid that bears a nitroxide group positioned at the bilayer core. Fig. 3 shows the ESR spectra at three temperatures of 16-PCSL incorporated in diC_{14}-amidine bilayers at pH values 3.0, 7.4 and 9.0, at low and high ionic strength. It is not clear whether the ESR signal from 16-PCSL in diC_{14}-amidine liposomes at pH 3, at low temperature, is a one-site signal, however, it will be analyzed as such

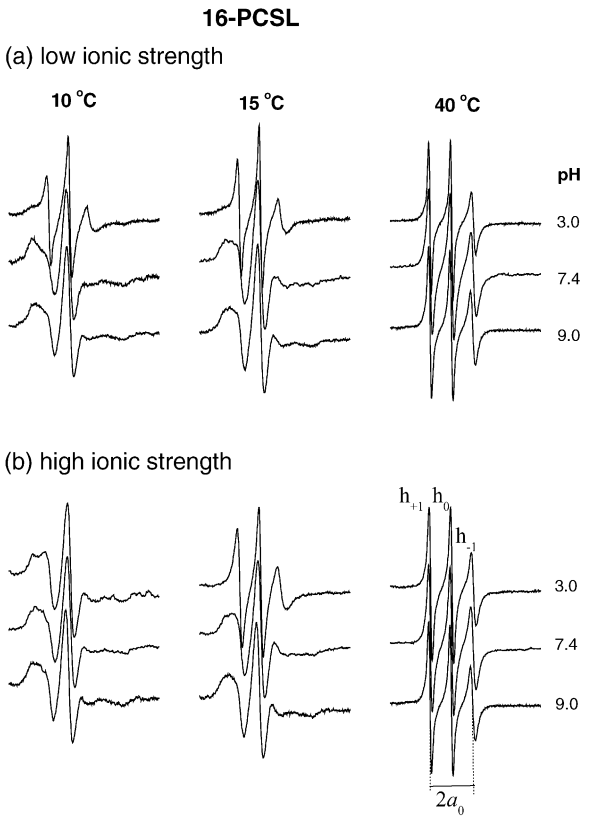


Fig. 3. ESR spectra of 16-PCSL in diC$_{14}$-amidine liposomes at (a) low and (b) high ionic strength, at pH 3.0 (first), 7.4 (second) and 9.0 (third row), at 10, 15 and 40 °C, as shown in the figure. The three nitrogen hyperfine lines (h_{+1}, h_0 and h_{-1}, corresponding to $m_I = +1$, 0 and -1, respectively) and the isotropic hyperfine splitting (a_0) are indicated. Total spectra width 100 G.

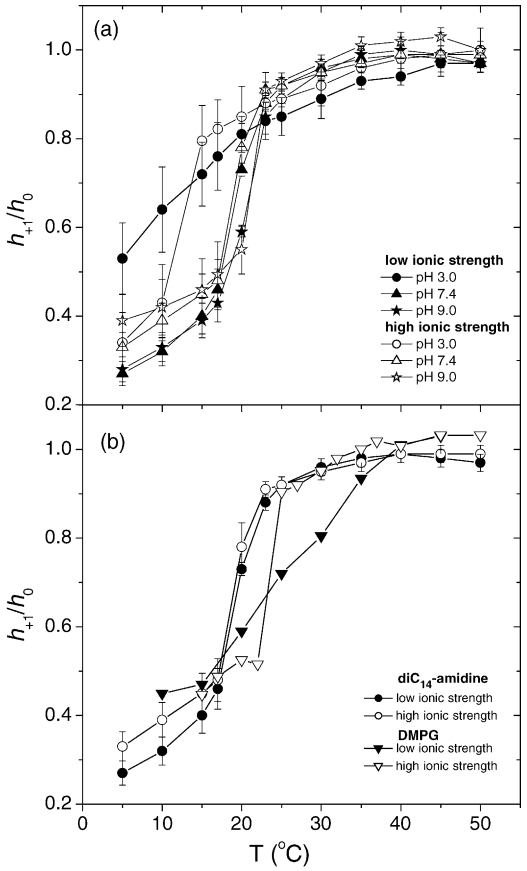


Fig. 4. (a) Temperature dependence of the ratio between the amplitudes of the low and the central field lines (h_{+1}/h_0) measured on the ESR spectra of 16-PCSL in diC$_{14}$-amidine at low (solid symbols) and high (open symbols) ionic strength, at pH 3.0 (circle), 7.4 (triangle) and 9.0 (star). The data were acquired from 50 to 5 °C. (b) Comparison of the diC$_{14}$-amidine data at pH 7.4 with those of DMPG, at low and high ionic strength.

(see, for instance, Freed, 1976). In contrast to 5-PCSL, 16-PCSL, which monitors the membrane at the bilayer center, provides evidence of much greater fluidity of the gel phase (at 10 °C) of the highly charged pH 3 diC$_{14}$-amidine bilayer at low ionic strength (Fig. 3). However, the 16-PCSL data corroborate the results obtained with 5-PCSL, indicating a much lower phase transition temperature for the low pH samples; pH 3 diC$_{14}$-amidine bilayers at 15 °C, at both low and high ionic strength, yield an ESR spectra typical of a fluid lipid phase (first spectra in center columns in Fig. 3), distinctly different from the spectra obtained for the other samples at that temperature.

For the 16-PCSL, the best experimental parameter to be used over the whole range of temperature is the ratio between the amplitudes of the low and central field lines (h_{+1}/h_0, see Fig. 3). This parameter is highly sensitive to chain order/mobility and can be measured from 50 to 5 °C, therefore allowing one to monitor the lipid fluid-gel transition. With respect to the lipid phase transition, it is interesting to note that, in contrast to the results obtained with 5-PCSL, the pH 9 sample at the bilayer core is not sensitive to salt concentration, yielding similar T_m values at low and high ionic strength (Fig. 4a). The samples at pH 9 and 7 yield rather sharp gel-fluid temperature transitions, with the pH 9 samples presenting a slightly higher T_m value (~20 °C) as compared to the pH 7

samples (∼18 °C), probably due to the lower charge in the bilayer surface of the former.

The behavior of the highly charged pH three samples is rather peculiar in that the high ionic strength sample displayed a sharp temperature transition, but at a quite low temperature, around 12 °C. The low salt sample presents a very loosely packed gel phase at the 16-PCSL-monitored microenvironment, with high h_{+1}/h_0 values, and a smooth phase transition (Fig. 4a). It is interesting to note that the gel-phase packing of the high salt samples, at pH values 3, 7.4 and 9, is rather similar (see experimental data at 5 and 10 °C in Fig. 4a), although the phase transition temperatures differ considerably. Also interesting is the result that mono-charged diC_{14}-amidine bilayers at pH 7.4 and 9, in low ionic strength, present a rather tightly packed gel phase, indicated by the low h_{+1}/h_0 values. Fig. 4b reproduces the results shown in Fig. 4a, for diC_{14}-amidine bilayers, at pH 7.4, and compares them with the results yielded by the diC_{14} anionic phospholipid 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol (DMPG), evincing that cationic diC_{14}-amidine membranes are much less sensitive to the presence of salt than those of DMPG (Lamy-Freund and Riske, 2003), despite the similarity between the gel and fluid phase packing.

Surprisingly, in the fluid phase, the highly charged pH 3 diC_{14}-amidine membrane seems to be more closely packed at the bilayer core (as monitored by 16-PCSL) than the higher pH samples, displaying lower h_{+1}/h_0 values (Fig. 4a). This is clearly indicated by the 16-PCSL rotational correlation times, with the low pH samples presenting rather higher values for both τ_B and τ_C (Fig. 5a and b). Due to the difficulty in establishing a nitroxide preferential rotational axis, the τ_B and τ_C values were calculated according to Schreier et al. (1978), using the corrections for non-resolved hyperfine splitting proposed by Bales (1989). The similarity between the τ_B and τ_C values indicates that the nitroxide of 16-PCSL that is incorporated in the fluid phase of diC_{14}-amidine bilayers is in a quite isotropic environment (Schreier et al., 1978).

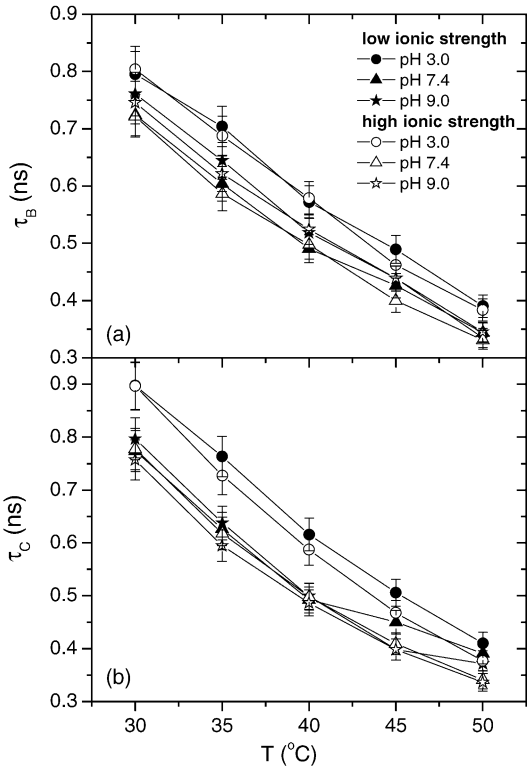


Fig. 5. Temperature dependence of the rotational correlation times (a) τ_B and (b) τ_C calculated (see text) from the ESR spectra of 16-PCSL in diC_{14}-amidine at low (solid symbols) and high (open symbols) ionic strength, at pH 3.0 (circle), 7.4 (triangle) and 9.0 (star).

3.3. Membrane polarity

It has been shown that the magnitude of the nitrogen isotropic hyperfine splitting (a_0, one-third of the trace of the hyperfine tensor) depends on several factors which increase the unpaired electron spin density at the nitrogen nucleus, such as solvent polarity, the presence of electric fields or the existence of electron transfer complex (Griffith et al., 1974; Schwartz et al., 1997). For labels inside a lipid bilayer, there are strong indications that an increase in a_0 is mainly related to the increase in the amount of nitroxide-water hydrogen bonding (Griffith et al., 1974). Therefore, the presence of water in the bilayer can be estimated from the magnitude of the isotropic nitrogen hyperfine splitting.

The nitrogen isotropic hyperfine splitting a_0 can be relatively well determined in ESR spectra typical of probes displaying high order and fast movement, where the A_{max} and A_{min} values can be precisely measured (see Section 2) like those of 5-PCSL at high

temperature (Fig. 1). Otherwise, for relatively isotropic ESR signal, in the motional narrowing region (Hudson and Luckhurst, 1969), like that of 16-PCSL, a_0 can be directly measured in the ESR spectrum, as indicated in Fig. 3, or more accurately obtained by fitting the three hyperfine lines to Voigh functions (see Section 2). Properly measured a_0 values are expected to be rather independent of temperature. The variation of the isotropic hyperfine splitting of spin labels in aqueous media, where a_0 can be very accurately evaluated, is less than 0.3% between 20 and 50 °C (data not shown). However, inside the lipid bilayer, a small increase of a_0 with temperature could possibly indicate an actual increase in the number of water molecules with temperature.

Fig. 6 shows the calculated a_0 values for 5- and 16-PCSL in the different samples studied. Considering the strong dependence of a_0 with the microenvironment polarity (Griffith et al., 1974), the data presented here strongly suggest that the nitroxide in 5-PCSL ($a_0 \approx 15.0$ G) is, in average, positioned in a shallower position in the bilayer than that attached to 16-PCSL ($a_0 \approx 14.3$ G). Moreover, they are both inside the bilayer, as in aqueous medium spin labels present a much higher a_0 value, 15.78 ± 0.01 G (Fernandez and e Lamy-Freund, 2000).

The a_0 values for 5-PCSL in the different samples studied (Fig. 6a) indicate that the diC_{14}-amidine bilayer polarity close to the 5th carbon atom is independent of the medium pH value or salt concentration. However, at the bilayer core, which is monitored by 16-PCSL, the higher values of the isotropic hyperfine splitting suggest that the low ionic strength sample at low pH is significantly more hydrated. Hence, it is interesting that the substantial increase in packing measured at the bilayer core for the highly charged low pH diC_{14}-amidine bilayers (higher rotational correlation times, Fig. 5) seems to parallel the increase in membrane polarity.

Comparison of several cationic lipids has suggested that disruption of endosomes was more frequently observed with DNA-diC14-amidines complexes than with other cationic lipids (El Ouahabi et al., 1997) and that efficient transfection mediated by cationic lipids is not only correlated to their percentages of cellular uptake but also to their ability to escape and destabilize endosomes. Since a major characteristic of di-C14 amidine, as compared to other cationic lipids, is that it bears two positive charges at low pH value, it was proposed that this high charge density could be responsible for a strong destabilization of the endosomal membrane.

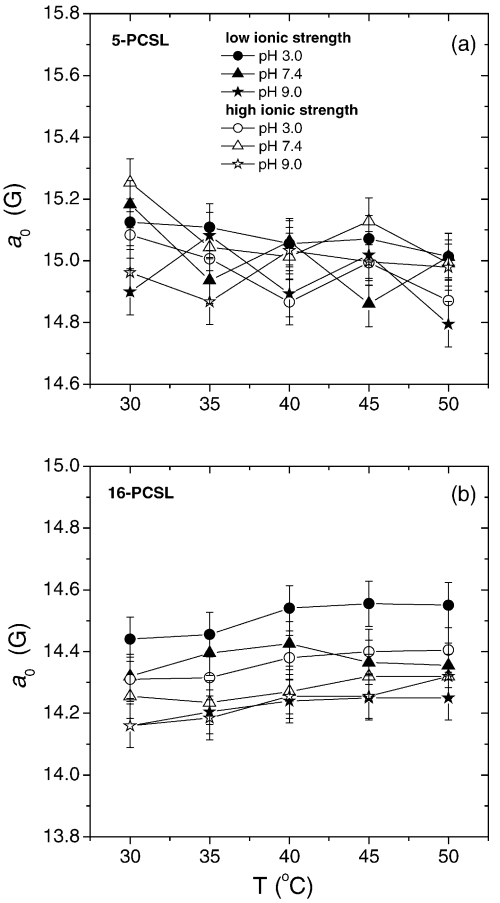


Fig. 6. Temperature dependence of the isotropic hyperfine splitting (a_0) calculated from the ESR spectra of (a) 5-PCSL and (b) 16-PCSL in diC_{14}-amidine at low (solid symbols) and high (open symbols) ionic strength, at pH 3.0 (circle), 7.4 (triangle) and 9.0 (star).

Acknowledgements

This work was supported by USP (Universidade de São Paulo), FAPESP (Fundação de Apoio à Pesquisa do Estado de São Paulo) and a joint project of CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) and FNRS (Fonds

National de la Recherche Scientifique). Fellowships for C.R.B. (FAPESP, 01/06925-9) and M.T.L. (CNPq, 522536/95) are acknowledged.

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