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Low cholesterol solubility in DODAB liposomes

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Abstract

Through the analysis of the ESR spectra of spin labels, we investigated the thermotropic properties of dioctadecyl dimethylammonium bromide (DODAB) liposomes, in low and high ionic strength, with different cholesterol contents. The cationic lipid gel phase is stabilized by the presence of ions, the bilayer having a higher gel/fluid transition temperature (T_m) in high ionic strength. As found for low ionic strength [Benatti, C.R., Feitosa, E., Fernandez, R.M., Lamy-Freund, M.T., 2001. Structural and thermal characterization of dioctadecyldimethylammonium bromide dispersions by spin labels. Chem. Phys. Lipids, 111, 93–104], high salt DODAB membranes also present a clear coexistence of the two phases around T_m. Cholesterol solubility in DODAB bilayers seems to be rather low, as the coexistence of DODAB and cholesterol-rich domains can be clearly detected by spin labels, for cholesterol concentration as low as 15 mol% of the total lipid. For lower cholesterol concentrations, the effect of cholesterol in DODAB bilayers is similar to that in phospholipids. For concentrations at or above 45 mol% of cholesterol, spin labels do not detect the coexistence of structurally different domains.

Keywords: DODAB; Cationic amphiphile; Cholesterol solubility; ESR; Ionic strength

1. Introduction

The possibility of using cationic amphiphiles as drug or gene delivery systems has motivated many studies with cationic amphiphile/gene complexes (lipoplex) in recent years. Dimethyldioctadecylammonium bromide (DODAB) was one of the first cationic amphiphiles synthesized (Kunitake et al., 1977), with two long hydrocarbon saturated chains. When hydrated, DODAB aggregates form closed bilayers or liposomes (Carmona-Ribeiro, 1992). Recently, the thermotropic behavior of DODAB in pure water was investigated by electron spin resonance (ESR), with two different preparation protocols: bath-sonication and simple mechanical agitation (non-sonicated) (Benatti et al., 2001). It was shown that the non-sonicated dispersions are formed mainly by one population of DODAB bilayers, either in the gel or in the fluid phase, depending on the temperature (gel–fluid transition temperature T_m around 43 °C).

Considering that most eukaryotic cell membranes contain sterols, and that mammalian cells contain around 25–50 mol% of cholesterol (Yeagle, 1985), this lipid could play a role in the interaction between cells and lipoplexes. Hence, it is important to investigate the structural alterations caused by the presence of cholesterol in the cationic vesicles. For instance, Bhattacharya and Haldar (2000) observed that the addition of cholesterol

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into cationic lipid vesicles reduces the leakage rates, irrespective of lipid molecular structure, and induces changes in membrane properties.

Cholesterol plays important roles in eukaryotic cells, modulating the physical properties of membranes (Yeagle, 1985). Several studies have shown that cholesterol fluidizes and/or induces disorder in the gel amphiphile organization, below T_m, while rigidifying the membrane above T_m (Finean, 1990; Huang et al., 1993; Silvius et al., 1996; Bhattacharya and Haldar, 1996, 2000). Usually, there is an upper limit to cholesterol incorporation in lipid bilayers, above which cholesterol seems to precipitate as crystals of pure cholesterol either in the monohydrate or in the anhydrous form (Loomis et al., 1979). This cholesterol incorporation limit was observed by Huang et al. (1999) for phosphatidylcholine (PC) (66 mol%) and phosphatidylethanolamine (PE) bilayers (51 mol%) or in the range 35–40 mol%, and by Bach (1984) for phosphatidylserine (PS) bilayers (33 mol%, depending on the acyl chain composition). As the concentration of cholesterol increases, the system undergoes a process of phase separation to form cholesterol-rich domains (Pata and Dan, 2005). It is known that in the presence of cholesterol the number of bound water molecules increases in the region where phase separation takes place (Bach and Miller, 1997). The formation and composition of cholesterol-rich domains vary with lipid type (Crane and Tamm, 2004; Veatch and Keller, 2002).

In the present work, we analyze DODAB dispersions at different ionic strengths, in the presence of increasing amounts of cholesterol. They are investigated through the analysis of the electron spin resonance (ESR) spectra of hydrophobic spin labels incorporated in the aggregates. This technique has been extensively used to monitor the viscosity and polarity of the microenvironment where the probes are localized (see, for example, Schreier et al., 1978; Marsh, 1989; Benatti et al., 2001, and references therein). Phospholipids, spin labeled at different acyl chain positions, were used to monitor the bilayer, and/or cholesterol aggregates, at different depths.

2. Materials and methods

2.1. Materials

Dioctadecyl dimethylammonium bromide (DODAB), cholesterol and spin labels 1-palmitoyl-2-(*n*-doxylstearoyl)-*sn*-glycero-3-phosphocholine (*n*-PCSL, $n = 5$, 12, 14 or 16) were purchased from Avanti Polar Lipids (Birmingham, AL, USA). 4-(2-Hydroxyethyl)-1-piperizineethanesulfonic acid (Hepes) and NaCl were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Lipid dispersion preparation

A film was formed from a chloroform solution of DODAB or DODAB/cholesterol at the desired ratio 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 concentration to display no spin–spin interaction). The film was 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 with film hydration by the addition of the desired buffer solution, with or without salt, and samples were three times heated at 58 °C for ~2 min, above DODAB phase transition, and each time vortexed for about 3 min. That was found to be enough for fully hydrate the bilayers as ESR spectra taken immediately after this procedure was found to be identical to those taken after the sample had been a few hours standing at room temperature. The buffer systems used were 20 mM (Hepes) (low ionic strength) and 20 mM Hepes + 150 mM NaCl (high ionic strength), at pH 7.4. The total lipid concentration was 4 mM.

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 varied in the interval 5–50 °C, either by heating or cooling. 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 gaussmeter.

All data shown are means of the results of at least three experiments, and the uncertainties are the standard deviations. When not shown, the uncertainties are smaller than the size of the symbols. For the 16-PCSL, the isotropic hyperfine splitting, a_0, 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 (Griffith and

Jost, 1976; Gaffney, 1976):

$$a_0 = \left(\frac{1}{3}\right)(A_{\parallel} + 2A_{\perp})$$

where $A_{\parallel}$ (=A_{max}) is the maximum hyperfine splitting directly measured in the spectrum (see Fig. 1), and $A_{\perp} = A_{min} + 1.4\left[1 - \frac{A_{\parallel} - A_{min}}{A_{zz} - (1/2)(A_{xx} + A_{yy})}\right]$ 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 McConnel, 1971).

Effective order parameters, S_{eff}, were calculated from the expression (Schindler and Seelig, 1973):

$$S_{eff} = \frac{A_{\parallel} - A_{\perp}}{A_{zz} - (1/2)(A_{xx} + A_{yy})}\frac{a_0'}{a_0},$$

$$\text{where } a_0' = \left(\frac{1}{3}\right)(A_{xx} + A_{yy} + A_{zz})$$

3. Results and discussion

3.1. DODAB liposomes in different ionic strengths

Fig. 1 shows the ESR spectra of two phospholipid spin labels incorporated in DODAB vesicles, at different temperatures, in high and low ionic strength media. They are typical bilayer spectra, at the 5th and 16th C-atom position, similar to those obtained with unsonicated DODAB dispersion in pure water (Benatti et al., 2001). Due to the flexibility gradient towards the bilayer core (Hubbell and McConnel, 1971), the spectra of 5-PCSL are much more anisotropic (larger A_{max}) than those of 16-PCSL, which monitor the center of the membrane. The spectra shown in Fig. 1 were obtained by cooling the samples, from the fluid to the gel phase. At 20 °C, the spectra of both labels are characteristic of nitroxides in a tightly packed environment, consistent with a bilayer in the gel phase. It is important to note that even in the gel phase the membrane is less packed at the core than close to the surface, as evident by the rather more anisotropic signal of 5-PCSL as compared to that of 16-PCSL, both at 20 °C. At 44 and 50 °C, the spectra of 5-PCSL are typical of fluid bilayers, yielded by the label in a rather fluid and ordered location, close to the bilayer interface, where the label has fast movement around its long axis (Hubbell and McConnel, 1971). At those temperatures, the core of the bilayer is rather fluid, so the 16-PCSL spectra indicate fast and nearly isotropic movement for the nitroxide moiety, typical of the motional narrowing region (Hubbell and McConnel, 1971). As discussed before (Benatti et al., 2001), between 38 and 42 °C, the presence of two signals can be detected in the ESR spectra, corresponding to the coexistence of the gel and fluid phases. For the low ionic strength samples, the coexistence of the two phases is evident at the 38 °C spectra, with the two labels, whereas in high salt DODAB the labels indicate the presence of two signals at higher temperatures, 40 and 42 °C.[1]

The DODAB phase transition can be monitored through the variation of parameter A_{max}, the outer hyperfine splitting (shown in Fig. 1), measured on the 5-PCSL spectra, which can be used as an empirical parameter that increases with the label microenvironment viscosity or packing (Freed, 1976). As expected for ionic lipids, salt decreases the head-group interactions, stabilizing the gel phase, therefore increasing the gel–fluid transition temperature (Fig. 2). As discussed above, there is a temperature range of coexistence of phases in DODAB bilayers, clearly seen in the ESR spectra. However, A_{max} values monitor sharp transitions, as the values are measured as the distance between the outer extreme features, therefore, when there is coexistence of signals, this parameter monitors the more rigid signal only. In low and high ionic strength, DODAB phase transition shows a hysteresis effect (Fig. 2), as observed with the amphiphile in unsonicated water dispersion (Benatti et al., 2001).

Though the DODAB gel–fluid transition temperature is dependent on the ionic strength, with 5-PCSL monitoring the micro-region close to the bilayer interface, no difference can be observed in the DODAB liposomes packing at the gel phase, for low and high ionic strength. A similar result was obtained with 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol (DMPG), an anionic phospholipid (Fernandez and Lamy-Freund, 2000). Above T_m, the bilayer can be better analyzed by the parameter S_{eff} (Fig. 3). The main contribution to the effective order parameter, S_{eff}, is the amplitude of movement of the hydrocarbon chain moiety (Schindler and Seelig, 1973). In the DODAB fluid phase, the presence of ions at the bilayer surface slightly decreases the packing of the bilayer (decreases S_{eff}).

For the 16-PCSL, which monitors the lipid bilayer packing at the membrane core, 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. 1) (Marsh, 1989). This parameter approaches unity as the movement of the

[1] It is interesting to point out that the fact that two well-separated spectra are obtained shows that the exchange rate between the labels in the two different phase domains is slow on the ESR time scale (lower than about 10^8 s^{-1}).

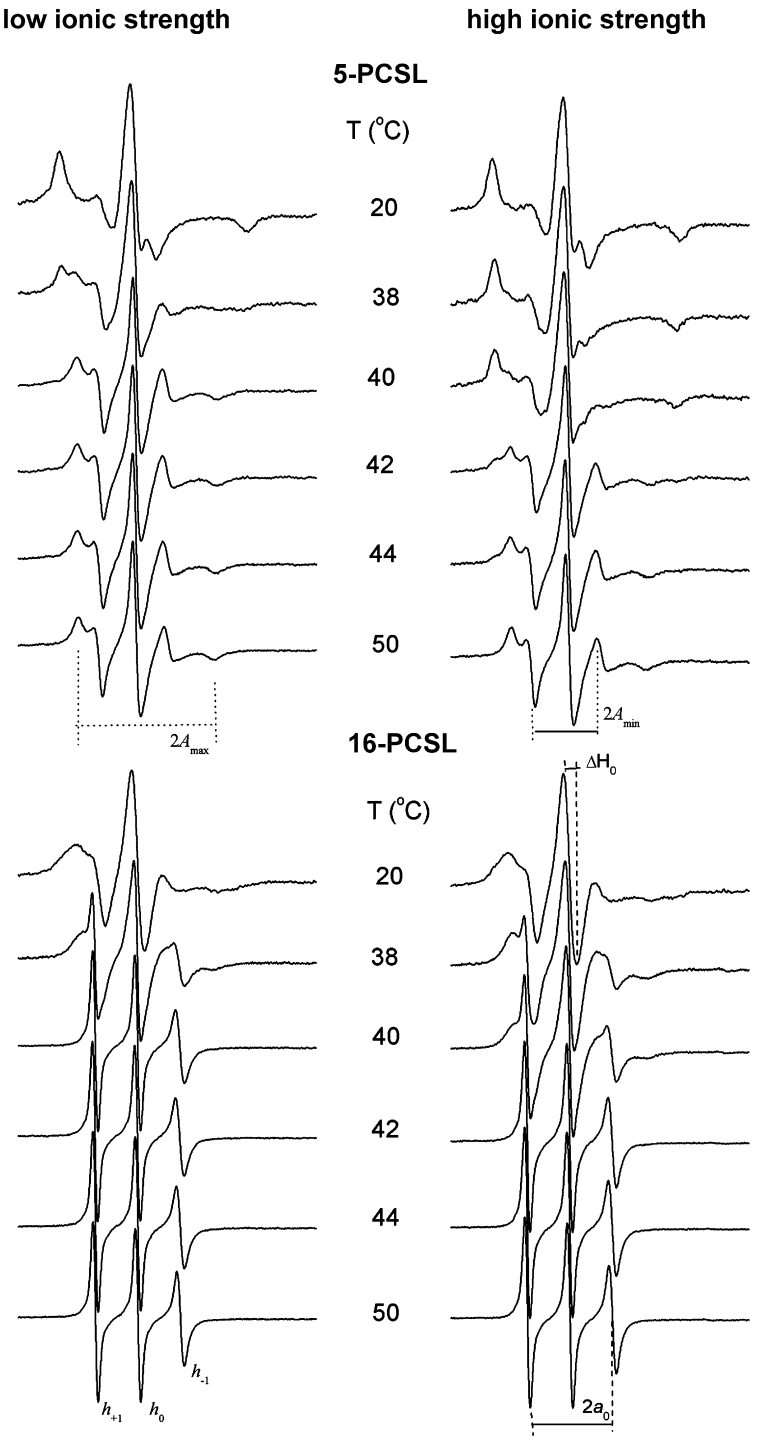

Fig. 1. ESR spectra of 5-PCSL and 16-PCSL in DODAB liposomes at low (left column) and high (right column) ionic strength, at different temperatures (cooling procedure). The outer hyperfine splitting (A_{max}), the three nitrogen hyperfine line amplitudes (h_{+1}, h_0 and h_{-1}, corresponding to $m_I = +1$, 0 and -1, respectively) and the isotropic hyperfine splitting (a_0) are indicated. Total spectra width 100 G.

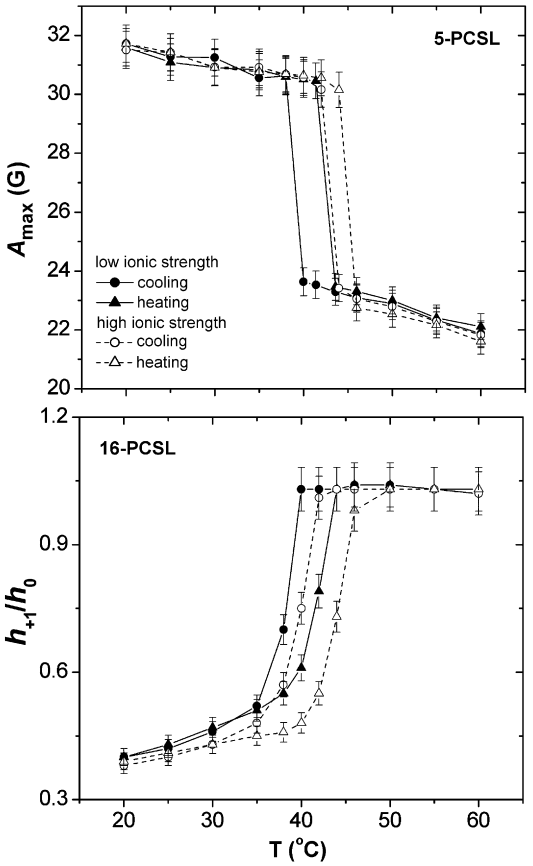

Fig. 2. Temperature dependence of the outer hyperfine splitting (A_{max}) measured on the 5-PCSL ESR spectra and the ratio of the low and the central field line amplitudes (h_{+1}/h_0) measured on the 16-PCSL ESR spectra, in DODAB liposomes at low (full symbols) and high (open symbols) ionic strength, at cooling (●, ○) and heating (▲, △) procedures.

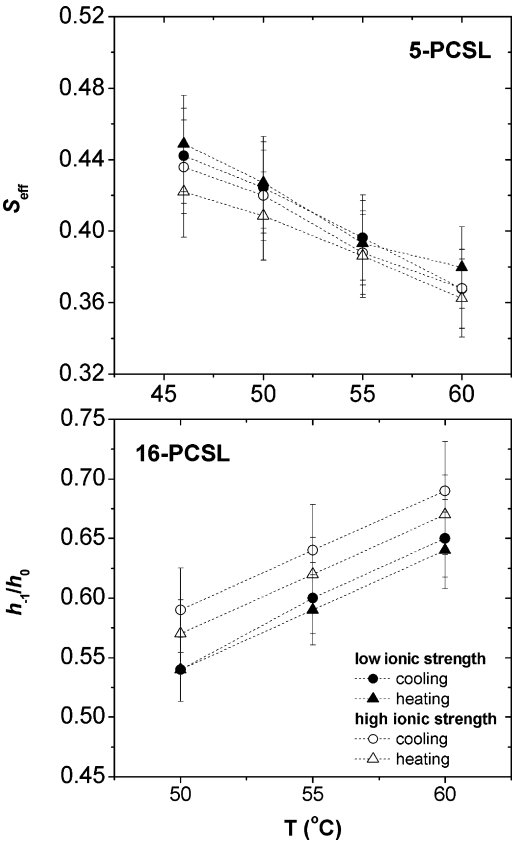

Fig. 3. Temperature dependence of the order parameter (S_{eff}) of 5-PCSL and the ratio between the high and the central field line amplitudes (h_{-1}/h_0) of 16-PCSL in DODAB liposomes at low (full symbols) and high (open symbols) ionic strength, at cooling (●, ○) and heating (▲, △) procedures.

label gets faster and more isotropic, being sensitive to chain order/mobility over the range of temperature used, hence allowing one to monitor the lipid gel–fluid transition (Fig. 2). In the gel phase, the decrease in head-group repulsion caused by the presence of salt significantly increases the hydrocarbon packing at the bilayer core (16-PCSL), decreasing h_{+1}/h_0. Above T_m, the bilayer can be better analyzed by the parameters h_{-1}/h_0 (Fig. 3), which also monitors the bilayer fluidity, getting close to unity when the viscosity at the microenvironment monitored by the spin label decreases (Marsh, 1989). Opposite to the effect in the gel phase, in the DODAB fluid phase the presence of ions at the bilayer surface slightly decreases the packing of the bilayer, both at the 5th and at the 16th carbon atom positions (decreases S_{eff} and increases h_{-1}/h_0) (Fig. 3). It seems that, in the fluid phase, the decrease in the head-groups electrostatic repulsion by the presence of ions is less important than the ions adsorption at the bilayer surface, possibly forcing the head groups to get further apart.

For spin labels inside a lipid bilayer, there are strong indications that the presence of water in the bilayer can be estimated from the magnitude of the nitrogen isotropic hyperfine splitting a_0, which can only be well measured for fluid membranes (Griffith et al., 1974). The bilayer polarity was found to be independent of the medium ionic strength or measurement procedure (heating or cooling). Close to the bilayer surface, monitored by 5-PCSL, a_0 was found to be around 15.00 G, and 14.35 G at the bilayer core, monitored by 16-PCSL. Similar results were obtained with the anionic lipid DMPG (Fernandez and Lamy-Freund, 2000).

3.2. DODAB/cholesterol liposomes

Fig. 4 shows the ESR spectra of 5-PCSL in DODAB bilayers in the gel (20 °C, low salt) and fluid (50 °C, low and high salt) phases, with different cholesterol con-

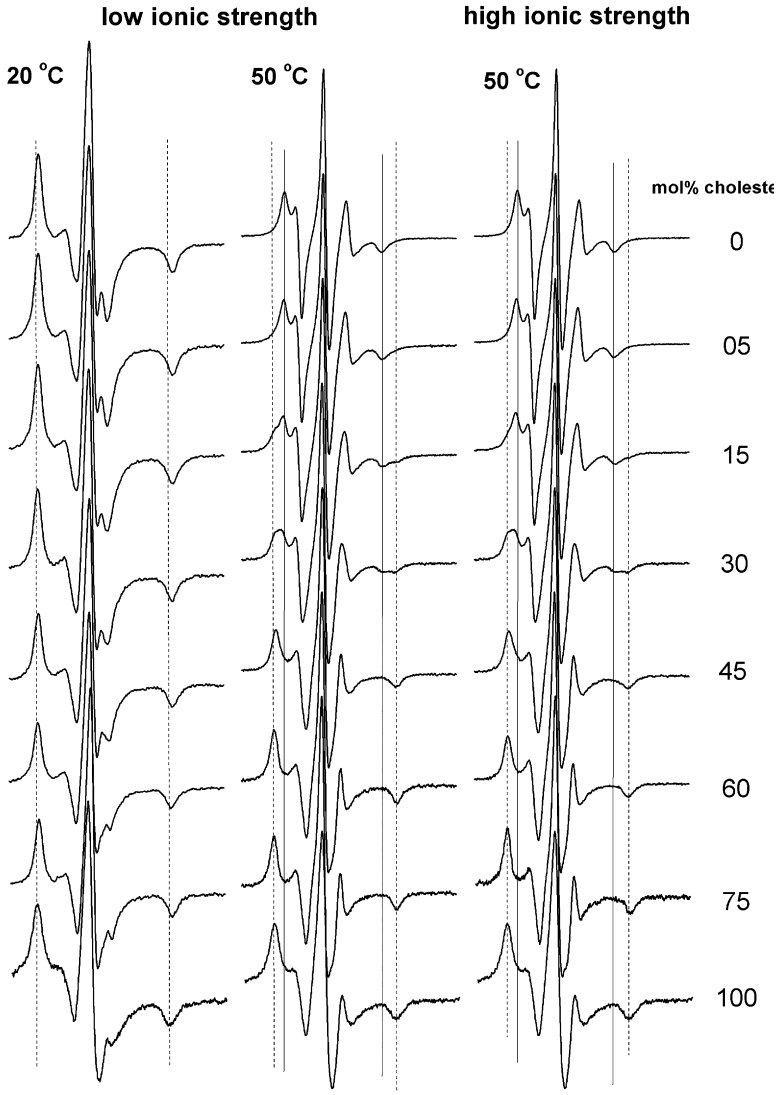

Fig. 4. ESR spectra of 5-PCSL in DODAB dispersions with different cholesterol concentrations, at 20 °C (left) and 50 °C (right), the latter at low and high ionic strength. The vertical full and dash lines indicate the features of the ESR signal of the label in DODAB and pure cholesterol aggregates, respectively. Total spectra width 100 G.

centrations. As 5-PCSL incorporates in pure cholesterol crystals, for comparison, the spectra of 5-PCSL in pure cholesterol are also shown (bottom spectra in Fig. 5).

In DODAB fluid phase (50 °C), with 15 and 30 mol% cholesterol, at both high and low ionic strength, the coexistence of two signals is evident, indicating the coexistence of DODAB and cholesterol-rich domains at concentrations as low as 15 mol%, the latter either inside or outside the bilayer. The coexistence of ESR signals could not be detected for cholesterol concentrations lower than 15 mol% (13 mol%, results not shown). Throughout this work it is important to have in mind that phase separation can happen without being monitored by spin labels, for at least three reasons: the label does not significantly partition in one of the phases; the ESR signals yielded by the label in the two distinct phases are similar; the labels exchange quickly between the different environments, in the ESR time scale.

At 45 mol% cholesterol, or more, the ESR spectra of 5-PCSL is similar to the spectra of 5-PCSL incorporated in pure cholesterol aggregates, but not identical. Hence, as discussed above, for those conditions, 5-PCSL ESR would not be sensitive to the possible coexistence of DODAB/cholesterol domains and cholesterol crystallites. The continuous and dotted vertical lines in Fig. 4 help the visualization of the features of 5-PCSL in a pure

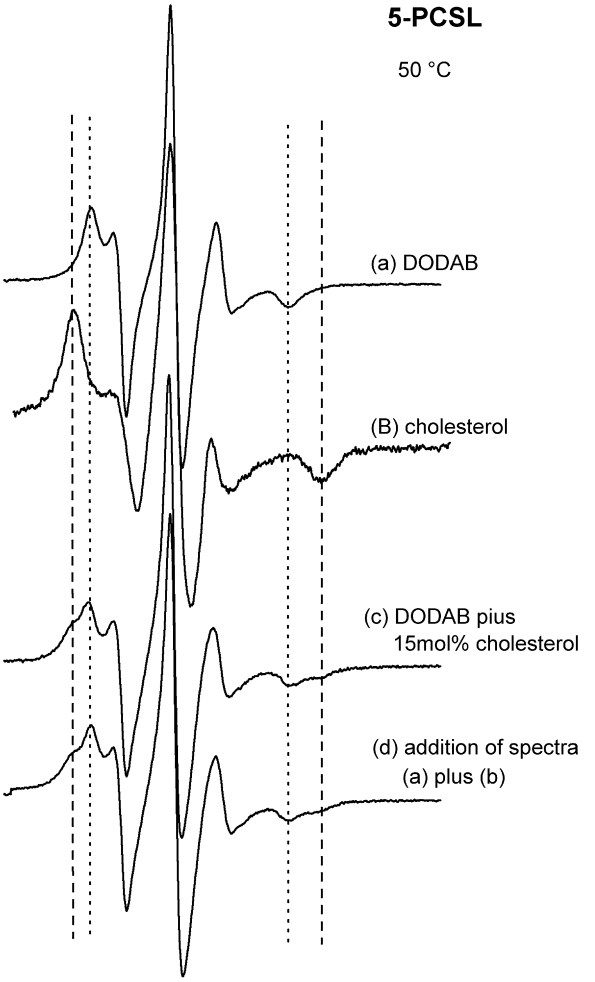

Fig. 5. (a)–(c) reproduce spectra at 0, 100, and 15 mol% cholesterol from Fig. 5, respectively; (d) was obtained from the addition of fractions of spectra (a) and (b).

DODAB bilayer, and in cholesterol aggregate, respectively. At 20 °C, the spectra of 5-PCSL incorporated in DODAB bilayer or cholesterol aggregate are similar, indicating a rather packed label micro-environment. Here again the coexistence of phases could exist, but would not be detectable by the 5-PCSL ESR signal.

Fig. 5 reproduces the ESR spectra of 5-PCSL incorporated in pure DODAB bilayers and in cholesterol crystals, at 50 °C (a and b). Bellow them we show the ESR spectra yielded by 5-PCSL in DODAB dispersion with 15 mol% of cholesterol (c) (these three spectra are reproduced from Fig. 4). The bottom spectrum (d) was created by the addition of fractions of signals (a) and (b), showing that the ESR spectra of 5-PCSL in DODAB dispersion with 15 mol% cholesterol (c) can be somehow reproduced as a sum of 5-PCSL in pure DODAB bilayer (a) and in cholesterol aggregate (b) (Fig. 5). That

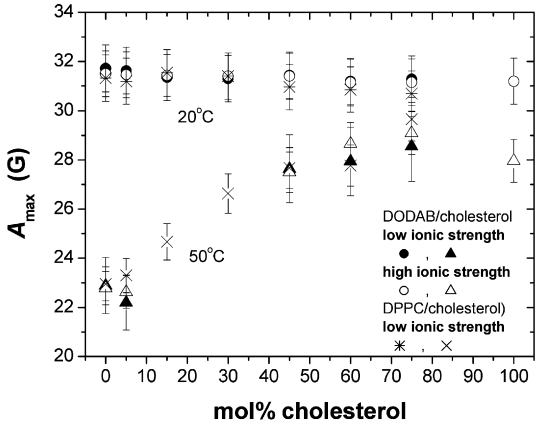

Fig. 6. Cholesterol content dependence of the outer hyperfine splitting (A_{max}) measured on the ESR spectra of 5-PCSL in DODAB liposomes in low (●, ▲) and high (○, △) ionic strength and in DPPC in low ionic strength (*, ×), at 20 and 50 °C.

strongly indicates the coexistence of cholesterol-rich domains with DODAB/cholesterol bilayers, at concentrations as low as 15 mol% of cholesterol. The DODAB small head-group and the strong interaction between the long hydrocarbon chains are possibly responsible for the low solubility of cholesterol in DODAB bilayers (Huang, 2002).

For 5-PCSL incorporated in DPPC bilayers, with different cholesterol concentrations (results not shown), there is no clear indication of cholesterol-rich domains, showing the high solubility of cholesterol in PC bilayers with saturated acyl chains, as reported before (Huang et al., 1999). However, like DODAB, at and above 45 mol% cholesterol, the ESR spectra of 5-PCSL in DPPC/cholesterol dispersions are similar to the spectra of 5-PCSL incorporated in pure cholesterol aggregates, but not identical. It is important to have in mind that the presence of cholesterol crystals was detected in PC dispersions at 25 °C, by differential scanning calorimetry (DSC), at 50 mol% of cholesterol (Mabrey et al., 1978).

Fig. 6 shows the A_{max} values for the different DODAB/cholesterol and DPPC/cholesterol dispersions. It clearly indicates that the effect of cholesterol in DODAB dispersions at low and high ionic strength is rather similar. In DODAB fluid phase, at 50 °C, DODAB/cholesterol A_{max} values are shown for cholesterol concentrations below 15 mol%, and above 45 mol%, where the ESR signals indicate the presence of one population only. At 50 °C, in DPPC/cholesterol dispersions, A_{max} progressively increases with cholesterol addition, indicating an increasing incorporation of cholesterol into the DPPC bilayer. In DODAB/cholesterol, at 5 mol%, no signif-

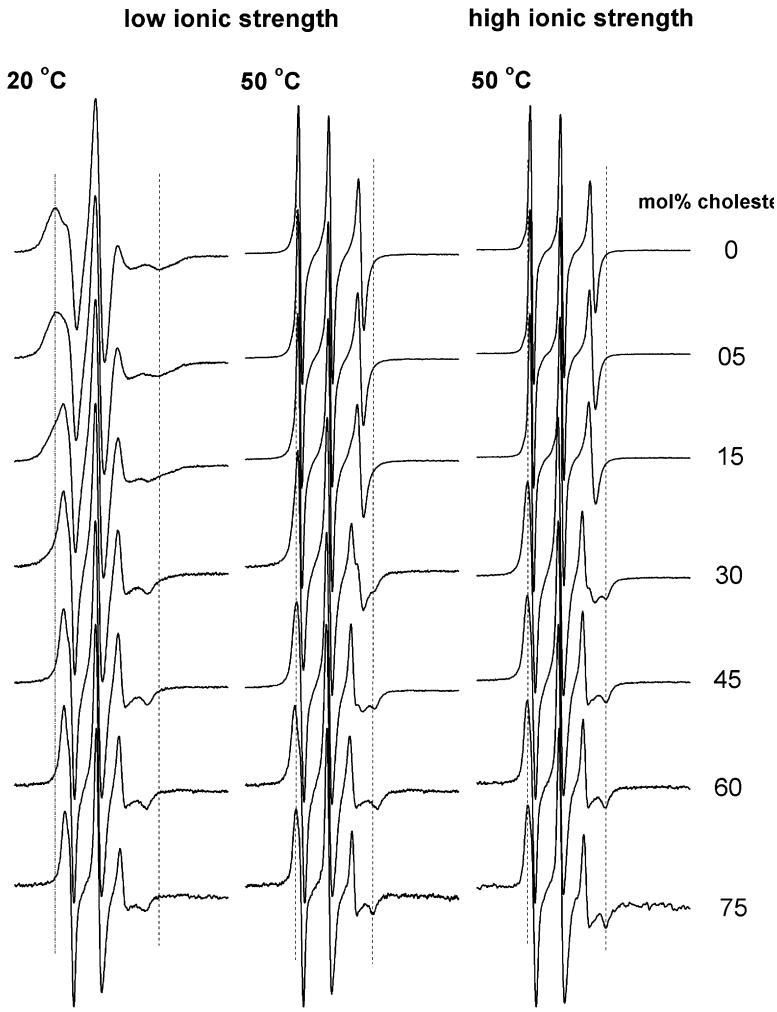

Fig. 7. ESR spectra of 16-PCSL in DODAB dispersions with different cholesterol contents, at 20 and 50 °C, the latter at low and high ionic strength. The vertical dash lines indicate the features of the ESR signal of the label in DODAB and high cholesterol amount aggregates. Total spectra width 100 G.

icant change in A_{max} is observed. As discussed above, for cholesterol concentrations above 45 mol%, A_{max} values are rather similar to the value yielded by 5-PCSL in a pure cholesterol aggregate.

As mentioned, with DODAB and DPPC in the gel phase, at 20 °C, 5-PCSL ESR spectra are not very sensitive to possible alterations caused by the presence of cholesterol. However, there is a slight decrease in the A_{max} value with cholesterol.

It is interesting to note that, as expected, no bilayer significant structural change with temperature (phase transition) is observed for DODAB with high cholesterol concentrations (above ~60 mol%), as the A_{max} parameters from the ESR spectra at 20 and 60 °C are rather similar (Fig. 6). This result is confirmed by DSC (data not shown).

Fig. 7 shows the ESR spectra of 16-PCSL in DODAB dispersions, with increasing amounts of cholesterol. Whereas 5-PCSL incorporates in cholesterol aggregates, the same phospholipid spin labeled at the 16th carbon atom, 16-PCSL, does not partition in pure cholesterol crystals. Hence, 16-PCSL either monitors pure DODAB bilayer or DODAB/cholesterol domains. Their different partition characteristics are also evident in Figs. 4 and 7, for instance, at low ionic strength DODAB bilayers at 50 °C: cholesterol-rich domains are monitored by 5-PCSL with 15 mol% cholesterol, whereas for them to be seen by 16-PCSL more cholesterol is necessary (30 mol%). For higher cholesterol concentrations, the spectra yielded by 16-PCSL at 20 and 50 °C are rather similar. In the gel phase, we can observe the rather anisotropic spectrum of 16-PCSL in pure

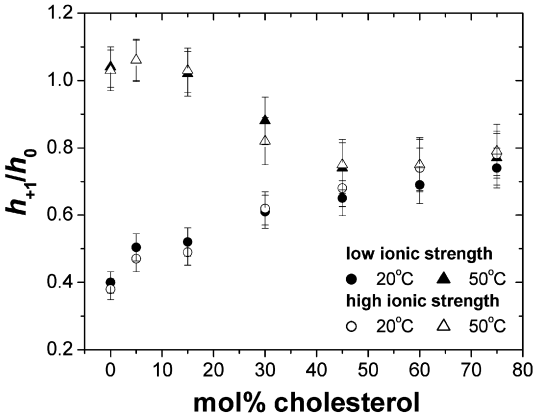

Fig. 8. Cholesterol content dependence of the ratio between the amplitudes of the low and central field lines (h_{+1}/h_0) measured on the ESR spectra of 16-PCSL in DODAB dispersions at low (full symbol) and high (open symbol) ionic strength, at 20 °C (●, ○) and 50 °C (▲, △).

DODAB bilayer, turning to a much less anisotropic signal with cholesterol addition, while the opposite behavior is observed with DODAB in the fluid phase.

Considering that the ratio between the ESR line amplitudes (h_{+1}/h_0 or h_{-1}/h_0) decreases as the signal gets more anisotropic (more rigid environment), for DODAB gel phase (20 °C) cholesterol seems to be progressively incorporated, fluidizing the bilayer, up to around 45 mol%, for both ionic strengths (Fig. 8). For DODAB fluid phase, h_{-1}/h_0, which can be measured at low cholesterol concentrations, is more sensitive than h_{+1}/h_0 to bilayer packing. This parameter also indicates that cholesterol rigidifies fluid DODAB bilayers, as expected (results not shown). As mentioned before, 30 and 45 mol% cholesterol spectra are due to a mixture of two populations. For higher cholesterol concentrations, no additional change on the bilayer packing is observed, and the 16-PCSL ESR spectra at 20 and 50 °C are rather similar (see parameters in Fig. 8), indicating the absence of a temperature phase transition for those samples containing high percentage of cholesterol. These results are similar to those obtained with 5-PCSL.

4. Conclusions

The comparison of the results shown here, which investigate the thermal behavior of DODAB dispersions in buffer systems, with those published before, with DODAB in water (Benatti et al., 2001), leads to the conclusion that there is no significant structural difference in DODAB bilayers prepared in media of different ionic strengths. As found for water dispersions, high salt DODAB membranes also present a clear coexistence of two phases around T_m, and a thermal hysteresis of about 3 °C. Salt was found to significantly increase T_m, stabilizing the gel phase, possibly by screening the head groups electrostatic interaction and increasing the lipid packing at the bilayer core. In the DODAB fluid phase, the presence of ions at the bilayer surface slightly decreases the membrane packing, both at the 5th and 16th carbon atom positions, possibly by physically increasing the distance between the polar head groups.

Cholesterol solubility in fluid DODAB bilayers seems to be rather low. 5-PCSL monitors the coexistence of DODAB and cholesterol-rich domains for concentrations as low as 15 mol% cholesterol, both in low and high ionic strength. ESR spectroscopy cannot inform if the different lipid domains are inside the bilayer or not. Differential scanning calorimetry could possibly be useful in the detection of the coexistence of pure cholesterol crystals with DODAB/cholesterol bilayers. The low solubility of cholesterol in DODAB bilayers should be related to the small head group of this amphiphile.

In DODAB gel phase, due to the highly anisotropic spectra of the labels, it is not possible to detect cholesterol-rich domains, if present.

For cholesterol concentrations lower than 15 mol% of the total lipid, cholesterol effect is similar to that in phospholipid membranes: cholesterol is incorporated in DODAB bilayers, loosening the gel phase bilayer packing, and turning more rigid the fluid membrane.

It is known that cholesterol has lower solubility in membranes with polyunsaturated acyl chains (Brzustowicz et al., 2002; Brzustowicz et al., 1999; Fanani et al., 2004). This observation can be rationalized by the fact that the polyunsaturated chains have an irregular contour that does not pack well with the smooth surface of cholesterol. However, in addition, anionic lipids, particularly phosphatidylserine (Bach and Wachtel, 2003) mix poorly with cholesterol. To our knowledge, this is the first work to study the relative solubility of cholesterol in cationic membranes. We find that the solubility is also low, suggesting that it is low in charged membranes independent of the nature of the charge. This may be a consequence of the contrast between the more hydrophilic charged group of the amphiphile and the hydrophobic nature of cholesterol.

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References

Bach, D., 1984. Differential scanning calorimetric study of mixtures of cholesterol with phosphatidylserine or galactocerebroside. Chem. Phys. Lipids 35, 385–392.

Bach, D., Miller, I.R., 1997. Hydration of phospholipid bilayers in the presence and absence of cholesterol. Biochim. Biophys. Acta 1368, 216–224.

Bach, D., Wachtel, E., 2003. Phospholipid/cholesterol model membranes: formation of cholesterol crystallites. Biochim. Biophys. Acta 1610, 187–197.

Benatti, C.R., Feitosa, E., Fernandez, R.M., Lamy-Freund, M.T., 2001. Structural and thermal characterization of dioctadecyldimethylammonium bromide dispersions by spin labels. Chem. Phys. Lipids 111, 93–104.

Bhattacharya, S., Haldar, S., 1996. The effects of cholesterol inclusion on the vesicular membranes of cationic lipids. Biochim. Biophys. Acta 1996, 21–30.

Bhattacharya, S., Haldar, S., 2000. Interactions between cholesterol and lipids in bilayers membranes. Role of lipid headgroup and hydrocarbon chain-backbone linkage. Biochim. Biophys. Acta 1467, 39–53.

Brzustowicz, M.R., Stillwell, W., Wassall, S.R., 1999. Molecular organization of cholesterol in polyunsaturated phospholipid membranes: a solid state ^{2}H NMR investigation. FEBS Lett. 451, 197–202.

Brzustowicz, M.R., Cherezo, V., Zerouga, M., Caffrey, M., Stillwell, W., Wassall, S.R., 2002. Controlling membrane cholesterol content. A role for polyunsaturated (docosahexaenoate) phospholipids. Biochemistry 41, 12509–12519.

Carmona-Ribeiro, A.M., 1992. Synthetic amphiphile vesicles. Chem. Rev. 21, 209–214.

Crane, J.M., Tamm, L.K., 2004. Role of cholesterol in the formation and nature of lipid rafts in planar and spherical model membranes. Biophys. J. 86, 2965–2979.

Fanani, M.L., Topham, M.K., Walsh, J.P., Epand, R.M., 2004. Lipid modulation of the activity of diacylglycerol kinase α- and ζ-isoforms: activation by phosphatidylethanolamine and cholesterol. Biochemistry 43, 14767–14777.

Fernandez, R.M., Lamy-Freund, M.T., 2000. Correlation between the effects of a cationic peptide on the hydration and fluidity of anionic lipid bilayers: a comparative study with sodium inos and cholesterol. Biophys. Chem. 87, 87–102.

Finean, J.B., 1990. Interaction between cholesterol and phospholipid in hydrated bilayers. Chem. Phys. Lipids 54, 147–156.

Freed, J.H., 1976. Theory of slow tumbling ESR spectra for nitroxides. In: Berliner, L.J. (Ed.), Spin Labeling: Theory and Applications, vol. 1. Academic Press, New York, pp. 53–132.

Gaffney, B.J., 1976. Practical considerations for the calculation of order parameters for fatty acid or phospholipid spin labels in membranes. In: Berliner, L.J. (Ed.), Spin Labelling. Theory and Applications. Academic Press, New York, p. 567.

Griffith, O.H., Dehlinger, P.J., Van, S.P., 1974. Shape of hydrophobic barrier of phospholipid bilayers (evidence for water penetration in biological membranes). J. Membr. Biol. 15, 159–192.

Griffith, O.H., Jost, P.C., 1976. Lipid spin label in biological membranes. In: Berliner, L.J. (Ed.), Spin Labelling. Theory and Applications. Academic Press, New York, p. 567.

Huang, J., 2002. Exploration of molecular interactions in cholesterol superlattices: effect of multibody interactions. Biophys. J. 83, 1014–1025.

Huang, J., Buboltz, J.T., Feigenson, G.W., 1999. Maximum solubility of cholesterol in phosphatidylcholine and the phosphatidylethanolamine bilayers. Biochim. Biophys. Acta 1417, 89–100.

Huang, T.H., Lee, C.W., Das Gupta, S.K., Blume, A., Griffin, R.G., 1993. A ^{13}C and ^{2}H nuclear magnetic resonance study of phosphatidylcholine/cholesterol interactions: characterization on liquid–gel phases. Biochemistry 32, 13277–13287.

Hubbell, W.L., McConnel, H.M., 1971. Molecular motion in spin-labeled phospholipids and membranes. J. Am. Chem. Soc. 93, 314–326.

Kunitake, T., Okahata, Y., Tamaki, K., Kumamaru, F., Takayanagi, M., 1977. Formation of the bilayer membrane from a series of quaternary ammonium salts. Chem. Lett., 387–390.

Loomis, C.R., Shipley, G.G., Small, D.M., 1979. The phase behavior of hydrated cholesterol. J. Lipid Res. 20, 525–535.

Mabrey, S., Mateo, P.L., Sturtevant, J.M., 1978. High-sensitivity scanning calorimetric study of mixtures of cholesterol with dimyristoyl- and dipalmitoylphosphatidylcholines. Biochemistry 17, 2464–2468.

Marsh, D., 1989. Experimental methods in spin-label spectral analysis. In: Berliner, L.J., Reuben, J. (Eds.), Spin Labeling. Theory and Applications, vol. 8. Plenum Press, New York, pp. 255–303.

Pata, V., Dan, N., 2005. Effect of membrane characteristics on phase separation and domain formation in cholesterol–lipid mixtures. Biophys. J. 88, 916–924.

Schindler, H., Seelig, J., 1973. EPR spectra of spin labels in lipid bilayers. J. Chem. Phys. 59, 1841–1850.

Schreier, S., Polnaszek, C.F., Smith, I.C.P., 1978. Spin labels in membranes. Problems in practice. Biochim. Biophys. Acta 515, 375–436.

Silvius, J.R., del Giudice, D., Lafleur, M., 1996. Cholesterol at different bilayer concentration can promote or antagonize lateral segregation of phospholipids of differing acyl chain length. Biochemistry 35, 15198–15208.

Veatch, S.L., Keller, S.L., 2002. Organization of lipid membranes containing cholesterol. Phys. Rev. Lett. 89, 2681011–2681014.

Yeagle, P.L., 1985. Cholesterol and the cell-membrane. Biochim. Biophys. Acta 822, 267–287.