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Cationic amphiphiles and the solubilization of cholesterol crystallites in membrane bilayers

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Abstract

Cationic amphiphiles used for transfection can be incorporated into biological membranes. By differential scanning calorimetry (DSC), cholesterol solubilization in phospholipid membranes, in the absence and presence of cationic amphiphiles, was determined. Two different systems were studied: 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC)+cholesterol (1:3, POPC:Chol, molar ratio) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-L-serine] (POPS)+cholesterol (3:2, POPS:Chol, molar ratio), which contain cholesterol in crystallite form. For the zwitterionic lipid POPC, cationic amphiphiles were tested, up to 7 mol%, while for anionic POPS bilayers, which possibly incorporate more positive amphiphiles, the fractions used were higher, up to 23 mol%. 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and DOTAP in methyl sulfate salt form ($DOTAP^{mss}$) were found to cause a small decrease on the enthalpy of the cholesterol transition of pure cholesterol aggregates, possibly indicating a slight increase on the cholesterol solubilization in POPC vesicles. With the anionic system POPS:Chol, the cationic amphiphiles dramatically change the cholesterol crystal thermal transition, indicating significant changes in the cholesterol aggregates. For structural studies, phospholipids spin labeled at the 5th or 16th carbon atoms were incorporated. In POPC, at the bilayer core, the cationic amphiphiles significantly increase the bilayer packing, decreasing the membrane polarity, with the cholesterol derivative 3β-[*N*-(*N*′,*N*′-dimethylaminoethane)-carbamoyl]-cholesterol (DC-chol) displaying a stronger effect. In POPS and POPS:Chol, DC-chol was also found to considerably increase the bilayer packing. Hence, exogenous cationic amphiphiles used to deliver nucleic acids to cells can change the bilayer packing of biological membranes and alter the structure of cholesterol crystals, which are believed to be the precursors to atherosclerotic lesions.

Keywords: Cationic amphiphiles; Cholesterol; Spin labels; DSC; DOTAP; DC-chol

1. Introduction

Since the first report by Felgner and Ringold [1] cationic amphiphiles have been largely used as vectors for gene delivery. Presently, some effects caused by those exogenous lipids in the organisms have been reported [2,3]. However, the toxicity of the cationic amphiphiles is still an obstacle to their large use in gene therapy [4]. Considering that cationic amphiphiles used for transfection can incorporate into biological membranes, changing their structure and composition, the present work focuses on the possible alterations those lipids can cause in model membranes, with and without cholesterol.

Cholesterol is a major constituent of mammalian plasma membranes, playing important roles in eukaryotic cells, modulating the physical properties of bilayers [5]. In an effort to understand the interactions that can occur between cholesterol and phospholipids, numerous studies have been made focusing on the interaction between cholesterol and single lipid bilayers. Several studies have shown that cholesterol induces disorder (fluidizes) to the gel amphiphile organization, below the gel-fluid transition of bilayers, T_m, while rigidifying the membrane above T_m [6–10]. 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 [11]. Huang et al. [12] observed that for a membrane composed by phosphatidylcholine (PC) the

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maximum cholesterol solubility is about 66 mol% and by phosphatidylethanolamine (PE) the limit is about 51 mol%, and Bach et al. [13] observed that the membrane of phosphatidylserine (PS) has a limit of cholesterol solubilization at about 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 [14].

The thermotropic phase behavior of cholesterol crystals has been investigated, and three transitions have been observed. One, around 36 °C, involves the conversion of one crystalline form of anhydrous cholesterol [15] to another anhydrous form. A second one above 70 °C, has been identified as the conversion of cholesterol monohydrated [16] to anhydrous cholesterol, and a last one about 151 °C, corresponding to crystal–liquid transition [11,17]. The thermal manipulation of the sample can have profound effects on the appearance of the polymorphic phase transition of cholesterol. Differential scanning calorimetry (DSC) and X-ray have been much applied to investigate the cholesterol in crystallite form.

In the present work, by DSC, cholesterol solubilization in phospholipid membranes, in the absence and presence of cationic amphiphiles, was followed through the crystalline cholesterol thermal transition at around 36 °C. Two different systems were studied: POPC (1-pamitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine) with cholesterol (1:3, POPC:Chol, molar ratio), and POPS (1-pamitoyl-2-oleoyl-*sn*-glycero-3-[phospho-L-serine]) with cholesterol (3:2, POPS:Chol, molar ratio). As mentioned above, those lipid systems were shown to present excess of cholesterol, not solubilized in the phospholipid bilayers, forming cholesterol aggregates. We determined the consequences of adding low percentages of cationic amphiphiles, from 2 to 7 mol % to the zwitterionic lipid POPC. For anionic POPS bilayers, which possibly incorporate more positive amphiphiles, due to electrostatic attraction, the fractions of cationic amphiphile were higher, from 9 to 23 mol%. The cationic amphiphiles used were DOTAP (1,2-dioleoyl-3-trimethylammonium-propane), DOTAP^{mss} (methyl sulfate salt of DOTAP) and the cholesterol derivative DC-chol (3β-[*N*-(*N*′,*N*′-dimethylaminoethane)-carbamoyl]-cholesterol).

For the structural studies, phospholipids spin labeled at the 5th and 16th carbon atoms were incorporated into the lipid systems. The ESR spectra of the labeled samples in the presence and absence of the cationic amphiphiles provide information about structural and polar alterations caused by these molecules on the model membranes.

2. Materials and methods

Cholesterol (99% pure) was purchased from the Norther Lipids Inc. (Vancouver, CA). The phospholipids 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-L-serine] (POPS), the cationic amphiphiles 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and the methyl sulfate salt form (DOTAP^{mss}), and the spin labels 1-palmitoyl-2-(*n*-doxylstearoyl)-*sn*-glycero-3-phosphocholine (*n*-PCSL, *n* = 5 and 16) were purchased from Avanti Polar Lipids (Birmingham, AL, USA). 3β-[*N*-(*N*′,*N*′-dimethylaminoethane)-carbamoyl]-cholesterol (DC-chol) was purchased from Sigma Chemical Company (St. Louis, MO). All products were used as received. Their chemical structures are illustrated in Chart 1.

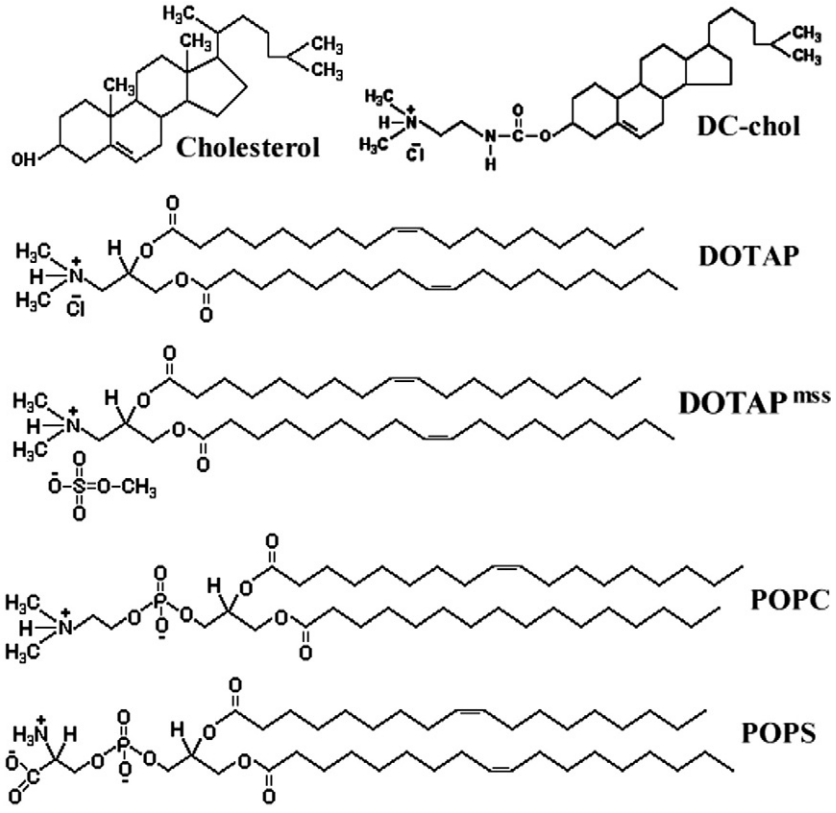

Chart 1. The molecular structures of cholesterol and the used amphiphiles DC-chol, DOTAP, DOTAP^{mss}, POPC and POPS.

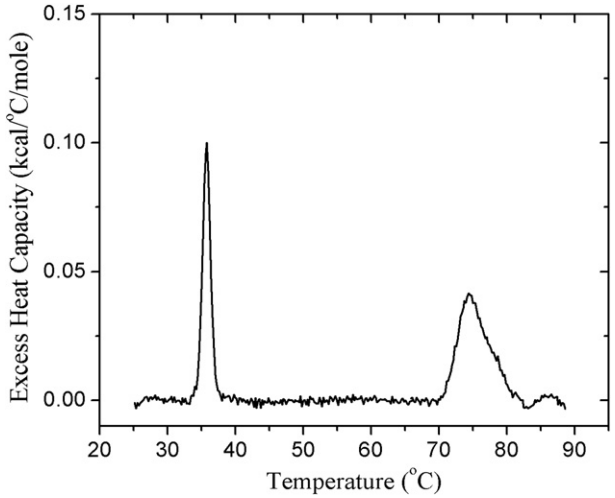

Fig. 1. First DSC heating scan of POPC:Chol dispersion (1:3, molar ratio). Scan rate 0.75 °C/min.

2.1. Preparation of multilamellar vesicles

Phospholipid and cholesterol, when desired, were dissolved in chloroform/methanol (2:1, v/v), and the solvent evaporated under a stream of nitrogen with constant rotation of the test tube so as to deposit a uniform film of lipid over the bottom of the tube. The tube was placed in a vacuum chamber for at least 2 h, to remove the last traces of solvent. The lipid film was hydrated with 20 mM Pipes, 1 mM EDTA, 150 mM NaCl, 0.002% NaN_3, and pH 7.4 buffer [17], quickly heated to 90 °C, and kept in an ice bucket for 5 min. The heating can convert most of the cholesterol monohydrate to the anhydrous form, though some cholesterol monohydrate forms can usually be found in the preparation. The rate of rehydration of cholesterol, even in the presence of excess water, is very slow, requiring several hours. All samples contain the same total lipid concentration (16.4 mg/ml). When cholesterol was incorporated, the phospholipid:cholesterol molar ratio was kept constant (1:3 and 3:2 in POPC:Chol and POPS:Chol, respectively). The cationic lipids were added so that their molar concentrations, relative to total lipids (phospholipid+cholesterol+cationic lipid) were those specified (2, 5 or 7 mol% for POPC samples, and 9, 17 or 23 mol% for POPS samples).

2.2. Dsc

Measurements of DSC were made using a MicroCal calorimeter model MC-2, with two cells containing 1.42 ml each, and data acquired with MicroCal Origin software. A constant scan rate of 0.75 °C/min was used, from 25 to 90 °C.

2.3. Esr

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 from 15 to 50 °C. To ensure the sample's thermal equilibrium, before each scan the sample was left at the desired temperature for 5 min. 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.

The ESR data were acquired about 30 min after sample preparation. The sample was inserted in the ESR cavity already equilibrated at 15 °C, and ESR spectra were acquired while heating the sample from 15 to 50 °C. Different samples were prepared for each procedure, and the results were in good agreement.

For ESR spectra corresponding to spin labels in the motional narrowing regime [18], the isotropic hyperfine splitting, a_o, 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, and 16-PCSL in POPC:Chol samples, the isotropic hyperfine splitting was calculated from the expression [19,20],

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

where $A_{//}$ ($=A_{max}$) is the maximum hyperfine splitting directly measured in the spectrum (see Fig. 5), and

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

where $2A_{min}$ is the measured inner hyperfine splitting (see Fig. 5) and A_{xx}, A_{yy} and A_{zz} are the principal values of the hyperfine tensor for doxylpropane [21].

Effective order parameters, S_{eff}, were calculated from the expression [22]

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

3. Results and discussions

3.1. Dsc

Using a sample prepared with the zwitterionic phospholipid POPC with excess of cholesterol, 75 mol%, we investigated the thermal transition of cholesterol crystalline by DSC, at a heating scan rate of 0.75 °C/min. Fig. 1 illustrates the first heating DSC scan of cholesterol crystallite in POPC:Chol mixture, from 25 to 90 °C. The two peaks indicate two conversions of cholesterol, at about 36 and 75 °C, related to the conversion of one crystalline form of anhydrous cholesterol to another and the conversion of cholesterol monohydrate to anhydrous cholesterol, respectively [11]. So, some monohydrate cholesterol could still be found after the heating protocol used. The present work focuses on the first peak at ca. 36 °C.

The enthalpy values (ΔH) obtained from the area under the DSC peak at 36 °C, in POPC/cholesterol mixtures at 50, 66, 75 and 80 mol% cholesterol, are shown in Fig. 2. For lower percentages of cholesterol, no transition could be detected by DSC, indicating that all cholesterol is solubilized in the POPC

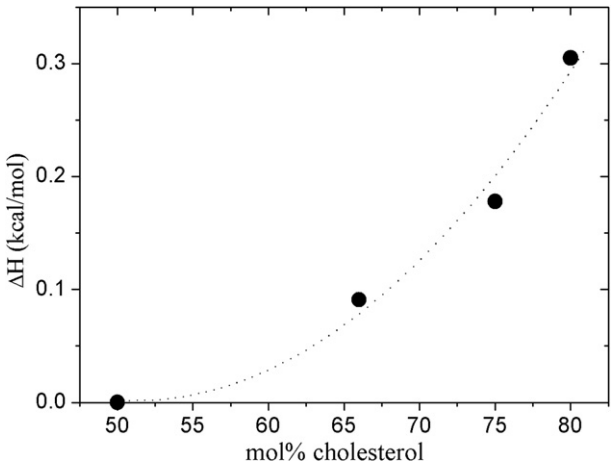

Fig. 2. Enthalpy per mole cholesterol (area under the peak at ~35 °C) as a function of cholesterol content (mol%), in POPC dispersions.

Table 1
Cholesterol transition enthalpy in POPC:Chol[a] dispersions in the presence of cationic amphiphiles

Systems[a]	Cationic amphiphile (mol%)	ΔH (kcal/mol)[b]
POPC:Chol	0	0.18±0.02
POPC:Chol+DOTAP	2	0.13±0.02
	5	0.12±0.02
	7	0.11±0.02
POPC:Chol+DOTAP^{mss}	2	0.16±0.02
	5	0.15±0.02
	7	0.12±0.02
POPC:Chol+DC-chol	2	0.19±0.03
	5	0.26±0.04
	7	0.20±0.03
POPC:Chol+POPS	2	0.15±0.02
	5	0.13±0.02
	7	0.18±0.03

[a] POPC:Chol means 1:3 (POPC:Chol), molar ratio.
[b] Mol of cholesterol. Enthalpy obtained from the area under the first thermal peak at about 36 °C, with scan rate 0.75 °C/min.

membrane. Accordingly, the enthalpy of the cholesterol crystal transition rises with increasing amounts of added cholesterol, above 50 mol% of cholesterol. So, to investigate the effect of cationic amphiphiles on the solubility of cholesterol in POPC membranes, a fixed amount of 75 mol% of cholesterol was used (3:1, POPC:Chol, molar ratio). As expected, cholesterol solubility in POPS membranes is lower than in POPC, hence, from a similar argument [23], we fixed the amount at 40 mol% cholesterol for POPS membranes (3:2, POPS:Chol, molar ratio).

In POPC:Chol systems, DOTAP and DOTAP^{mss} were found to somewhat decrease the enthalpy of the cholesterol thermal transition in cholesterol crystals (Table 1), hence slightly increasing the cholesterol solubility in POPC vesicles. However, considering the small alterations, and that a small percentage of DOTAP was added to the bilayer and the POPC:Chol molar ratio kept constant, a dilution effect cannot be ruled out. Up to the concentrations used (2 to 7 mol%), these cationic amphiphiles do not seem to incorporate into cholesterol aggregates, as the shape and the position of the cholesterol thermal transition peak do not change significantly (Fig. 3a and b). Opposite to that, the cationic cholesterol derivative DC-chol seems to incorporate into cholesterol aggregates, changing the peak position (Fig. 3c), and raising the cholesterol transition enthalpy (Table 1). Hence, as DC-chol probably mixes with cholesterol in cholesterol crystals within POPC bilayers, it is impossible to estimate the alterations in cholesterol solubility in POPC caused by DC-chol. For comparison, the presence of the anionic lipid POPS was also tested for cholesterol solubilization in POPC bilayers. The alterations caused by POPS on the enthalpy of the cholesterol thermal transition are not very significant (Table 1), but POPS somehow alters the cholesterol aggregate structure, making its thermal transition sharper, hence more cooperative (Fig. 3d).

For the anionic POPS bilayer, where the incorporation of positive lipids should be higher, larger concentrations of cationic amphiphiles were tested. Interestingly, with the anionic system POPS:Chol, those higher percentages of cationic amphiphiles (9 to 23 mol%) dramatically change the cholesterol crystal

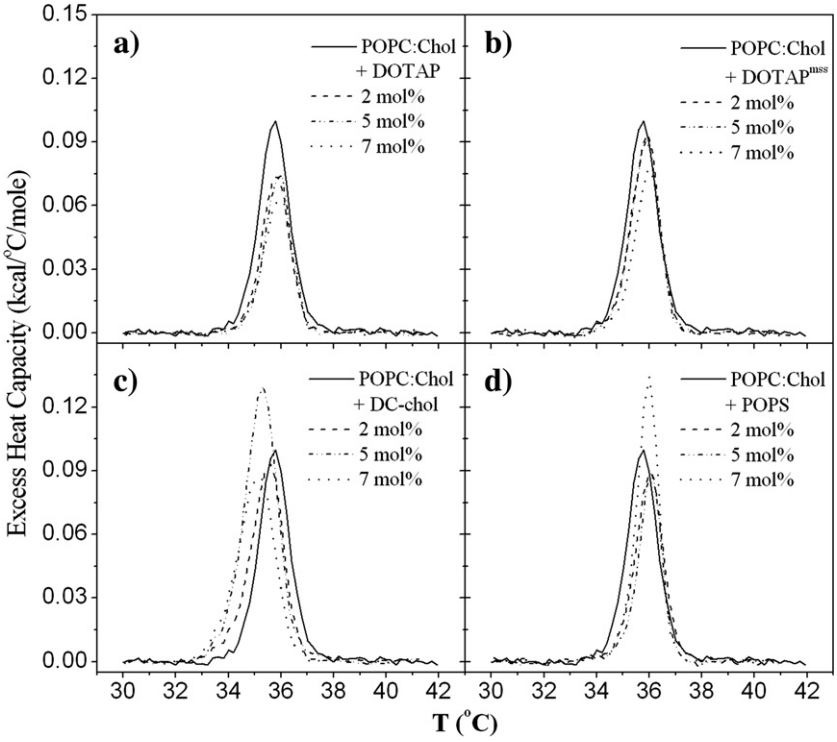

Fig. 3. First DSC heating scans of POPC:Chol (1:3, molar ratio) dispersions, with a) DOTAP, b) DOTAP^{mss}, c) DC-chol, and d) POPS, at (—) 0, (---) 2, (-···-) 5 and (····) 7 mol%.

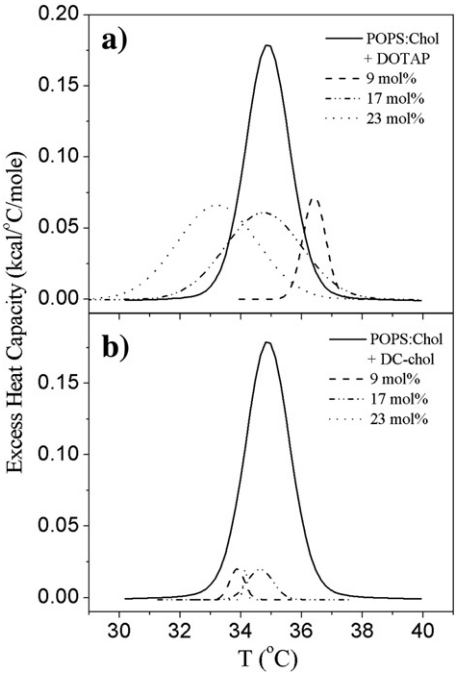

Fig. 4. First DSC heating scans of POPS:Chol (3:2, molar ratio) dispersions, with a) DOTAP, and b) DC-chol, at (—) 0, (---) 9, (-···-) 17, and (····) 23 mol%.

thermal transition (Fig. 4), indicating significant changes on the cholesterol aggregates caused by the presence of the cationic amphiphiles. Hence, that makes impossible the analysis of the cholesterol solubilization in POPS bilayers through the evaluation of the cholesterol thermal transition enthalpy (Table 2).

Considering that the DSC data can only monitor the alterations cationic amphiphiles cause on the cholesterol crystals, we found it important to monitor the lipid bilayer structure via the ESR signal of spin labels incorporated into the membranes. The phospholipid spin labels used, 5- and 16-PCSL, monitor the bilayer close to the aqueous surface and at the bilayer hydrophobic core, respectively.

3.2. Esr

To complement the DSC data, spin labels were incorporated in four different lipid systems, with and without the cationic amphiphiles: POPC, POPC:Chol (1:3 molar ratio), POPS and POPS:Chol (3:2 molar ratio). As discussed before, in the two phospholipid/cholesterol dispersions, phospholipid/cholesterol bilayers and cholesterol aggregates coexist. The four lipid systems were structurally analyzed in the presence and in the absence of DOTAP and DC-chol, and POPC dispersions were also studied with the addition of DOTAP^{mss} and the anionic phospholipid POPS, for comparison.

With spin labels in POPC systems, similar to the DSC experiments, cationic amphiphiles were used in three different concentrations, 2, 5 and 7 mol%, and at higher concentrations with negative POPS systems, namely 9, 18 and 23 mol%.

Table 2
Cholesterol transition enthalpy in POPS:Chol[a] dispersions in the presence of cationic amphiphiles

Systems[a]	Cationic amphiphile (mol%)	ΔH (kcal/mol)[b]
POPS:Chol	0	0.35±0.02
POPS:Chol+DOTAP	9	0.08±0.01
	17	0.27±0.04
	23	0.19±0.03
POPS:Chol+DC-chol	9	0.03±0.01
	17	0.01±0.01
	23	0.01±0.01

[a] POPS:Chol means 3:2 (POPS:Chol), molar ratio.
[b] Mol of cholesterol. Enthalpy obtained from the area under the first thermal peak at about 36 °C, with scan rate 0.75 °C/min.

3.3. POPC dispersions

Fig. 5 shows the ESR spectra of 5-PCSL incorporated in aggregates present in POPC and POPC:Chol dispersions, at 45 °C. They are spectra of highly mobile and ordered spin labels, typical of the label at the 5th carbon atom position in a fluid lipid bilayer, where the labeled phospholipid has fast movement along its long axis [21]. Up to 7 mol%, DOTAP, DOTAP^{mss}, or POPS do not affect POPC bilayer order at the 5th C-atom position, as shown by the rather similar calculated

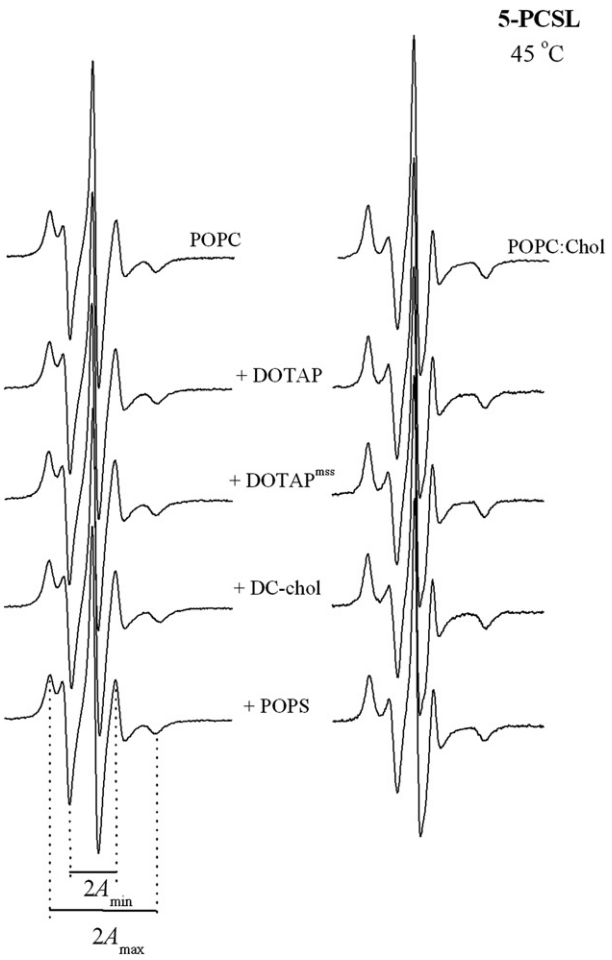

Fig. 5. ESR spectra of 5-PCSL in POPC (left side) and POPC:Chol (1:3, molar ratio; right side) liposomes, at 45 °C, without and with 7 mol% of DOTAP, DOTAP^{mss}, DC-chol and POPS. The hyperfine splittings A_{max} and A_{min} are indicated. Total spectra width 100 G.

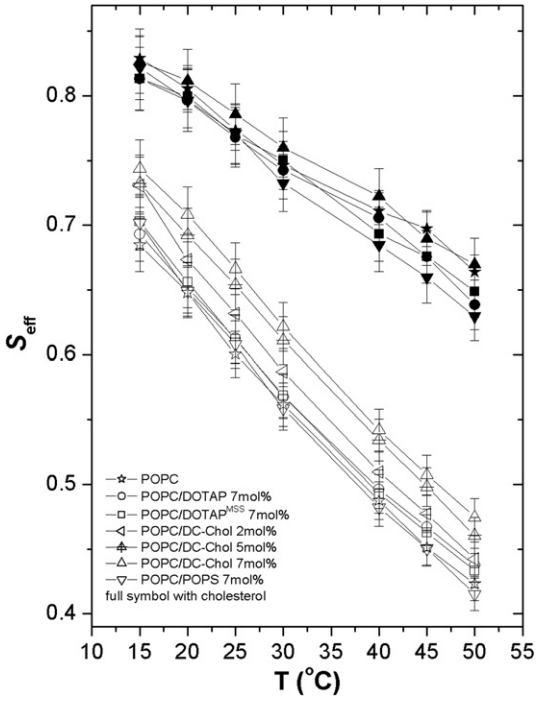

Fig. 6. Temperature dependence of the order parameter (S_{eff}) of 5-PCSL in (open star) POPC membranes, and POPC with (○) 7 mol% DOTAP, (□) 7 mol% DOTAP^{mss}, (▽) 7 mol% POPS, (◁) 2 mol% DC-chol, (△ hachured) 5 mol% DC-chol, (△) 7 mol% DC-chol, and in (★) POPC:Chol membranes, and POPC:Chol with (●) 7 mol% DOTAP, (■) 7 mol% DOTAP^{mss}, (▲) 7 mol% DC-chol and (▼) 7 mol% POPS. Results obtained by heating the samples.

effective order parameter values[1], S_{eff}, shown in Fig. 6, at temperatures varying from 15 to 50 °C. However, even low concentrations of DC-chol significantly increase POPC bilayer order (Fig. 6). This is an effect similar to that observed with cholesterol in fluid membranes [5,24]. Accordingly, cholesterol significantly increases the bilayer order, as monitored by S_{eff} (Fig. 6). In those highly ordered membranes, no structural alteration could be observed, at the 5th carbon atom position, in the presence of the concentrations of the cationic amphiphiles or POPS used (Fig. 6). It is interesting to note that, though 5-PCSL incorporates in cholesterol aggregates [25], in these aggregates its ESR spectrum is not similar to those obtained with the lipid dispersions studied here, the calculated effective order parameter being significantly higher (S_{eff}=0.72 at 50 °C). Hence, in the dispersions studied here, 5-PCSL is mostly (or totally) monitoring the POPC:Chol bilayers.

The presence of nitroxide hydrogen bonds inside the bilayer, probably related to the presence of water molecules around the polar group, can be estimated from the magnitude of the nitrogen isotropic hyperfine splitting (a_o), which can be well measured for fluid membranes [26]. As the a_o parameter of 5-PCSL, in either POPC or POPC:Chol bilayers, is not affected by the presence of the cationic amphiphiles, or POPS, we can say that the membrane polarity at the 5th C-atom position remains unchanged upon the incorporation of those lipids. For POPC, a_o=15.03±0.03 G for all samples studied, and, surprisingly, with cholesterol there is a slight increase in the isotropic hyperfine splitting, a_o=15.14±0.03 G, for all samples studied (a_o values were measured at 45 and 50 °C). Hence, somehow cholesterol seems to increase water molecules penetration close to the bilayer surface. Alternatively, this small increase in a_o could be related to a small probability of cholesterol making a hydrogen bond with the nitroxide around the 5th C-atom position.

In order to study the structural alterations caused by the cationic amphiphiles at the phospholipid bilayer core, ESR spectra yielded by 16-PCSL in POPC and POPC:Chol bilayers were analyzed (Fig. 7). As mentioned before, cholesterol significantly increases the bilayer order, turning the ESR signal much more anisotropic. As expected, this effect is much more evident at the bilayer core. The POPC samples, without cholesterol, can be well studied by the ratio h_{-1}/h_0, between the amplitudes of the high and the central field lines (see Fig. 7). This parameter is sensitive to chain order/mobility [27], getting close to unity as the viscosity at the microenvironment monitored by the spin label decreases, and the movement of the label gets faster and more isotropic. Interestingly, DOTAP, DOTAP^{mss}, DC-chol and the anionic lipid POPS increase the

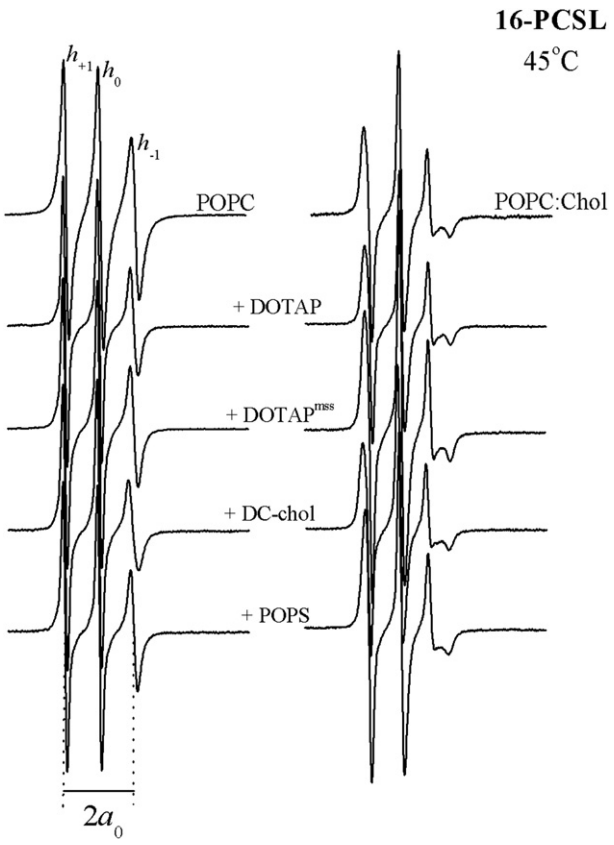

Fig. 7. ESR spectra of 16-PCSL in POPC (left side) and POPC:Chol (1:3, molar ratio; right side) liposomes, at 45 °C, without and with 7 mol% of DOTAP, DOTAP^{mss}, DC-chol and POPS. The three nitrogen hyperfine line amplitudes (h_{+1}, h_0 and h_{-1}) corresponding to m_I=+1, 0 and −1, respectively, and the isotropic hyperfine splitting (a_o) are indicated. Total spectra width 100 G.

[1] The main contribution to the effective order parameter is the amplitude of movement of the hydrocarbon chain moiety [22].

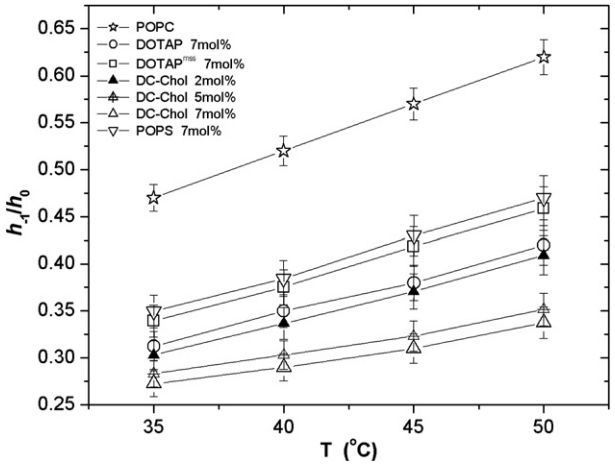

Fig. 8. Temperature dependence of the ratio between the amplitudes of the high and the central field lines (h_{-1}/h_0) measured on the ESR spectra of 16-PCSL in (open star) POPC and POPC with (○) 7 mol% DOTAP, (□) 7 mol% DOTAP^{mss}, (▽) 7 mol% POPS, (▲) 2 mol% DC-chol, (△ hachured) 5 mol% DC-chol, and (△) 7 mol% DC-chol. Results obtained by heating the samples.

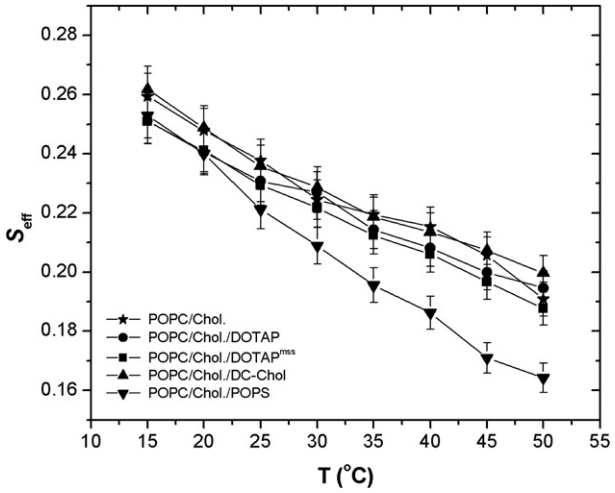

Fig. 9. Temperature dependence of the order parameter (S_{eff}) of 16-PCSL in (★) POPC:Chol membranes (1:3, molar ratio), and POPC:Chol with (●) 7 mol% DOTAP, (■) 7 mol% DOTAP^{mss}, (▲) 7 mol% DC-chol and (▼) 7 mol% POPS. Results obtained by heating the samples.

POPC bilayer packing, as their presence in the bilayer significantly decreases the 16-PCSL parameter h_{-1}/h_0 (Fig. 8). As expected, DC-chol shows a stronger effect on the POPC bilayer. Moreover, the cationic amphiphiles decrease the bilayer polarity at the bilayer core, again DC-chol displaying a stronger effect (Table 3). Hence, even small percentages (up to 7 mol%) of cationic amphiphiles make the fluid POPC bilayer slightly more packed and less hydrated at the bilayer core. That could possibly be related to the small headgroup of those lipids. It is important to note that DC-chol causes a very significant effect.

With POPC:Chol bilayers, due to their high organization, the best parameter to be used in the analysis of possible structural alterations is the effective order parameter, S_{eff}, already discussed for the analysis of the 5-PCSL spectra. Fig. 9 shows that POPC:Chol bilayer structure is not much affected by the charged lipids, apart from the negative POPS, which significantly fluidizes the bilayer, decreasing S_{eff}. However, all charged lipids slightly decrease the bilayer polarity, somehow decreasing the POPC:Chol a_o values (Table 3). 16-PCSL a_o value in POPC:Chol bilayer, as expected, is slightly lower than that in pure POPC (values in Table 3). It is interesting to note that, due to the bilayer polarity gradient [26–28], all 16-PCSL a_o values are lower than those obtained with 5-PCSL, mentioned above.

Table 3
16-PCSL isotropic hyperfine parameter (a_o in Gauss)

	Pure bilayer	+DOTAP	+DOTAP^{mss}	+DC-chol	+POPS
POPC				14.27±0.02	
	14.41±0.02	14.25±0.03	14.30±0.05	14.15±0.02	14.33±0.02
				14.04±0.02	
POPC:Chol	14.39±0.01	14.38±0.01	14.35±0.02	14.34±0.02	14.34±0.01

a_o values, measured between 45 and 50 °C, were found to be rather similar at 2, 5 and 7 mol% of the ionic lipid, so average values considering the three concentrations are shown, apart from DC-chol, where the three values correspond to the three cationic amphiphile concentrations. a_o values for the POPC samples were directly measured on the spectra, and those for POPC:Chol samples were calculated from the A_{max} and A_{min} parameters, as discussed in Materials and methods.

3.4. POPS dispersions

POPS dispersions, with and without cholesterol, were studied in the presence of 9, 17 and 23 mol% of DOTAP and DC-chol. As the features of the 5-PCSL spectra are not very much different from those in Fig. 5, the ESR spectra are not shown, and S_{eff} parameters are directly presented in Fig. 10. Similar to the effect in POPC membranes, cholesterol organizes POPS bilayers, increasing the 5-PCSL effective order parameter (Fig. 10). According to the lower incorporation of cholesterol into anionic POPS membranes, as compared with POPC, cholesterol saturated POPS bilayers are less organized than those of the zwitterionic lipid POPC (lower S_{eff} for POPS:Chol, Fig. 10, as compared with POPC:Chol, Fig. 6). Interestingly, the higher concentrations of DC-chol used with POPS:Chol bilayers strongly organize the membrane at the 5th C-atom position (significantly increase S_{eff} in Fig. 10). Hence, in POPS:Chol dispersion, the cationic derivative of cholesterol seems to both penetrate and disturb the cholesterol crystallites (Fig. 4), and penetrate into POPS:Chol bilayers, increasing the membrane order. Alternatively, DC-chol could also lead to an increase of the percentage of cholesterol incorporation into POPS membranes. DOTAP slightly decreases the POPS:Chol bilayer order parameter (Fig. 10).

In pure POPS bilayers, the higher concentrations of DOTAP and DC-chol used (as compared with those used with POPC) significantly increase the bilayer order at the 5th C-atom position, with DC-chol displaying a much stronger effect. However, similar to the effect obtained with POPC, the cationic amphiphiles do not alter the bilayer polarity, a_o values being 15.08±0.05 G for POPS, and 15.05±0.05 G for POPS:Chol, with or without the cationic amphiphiles.

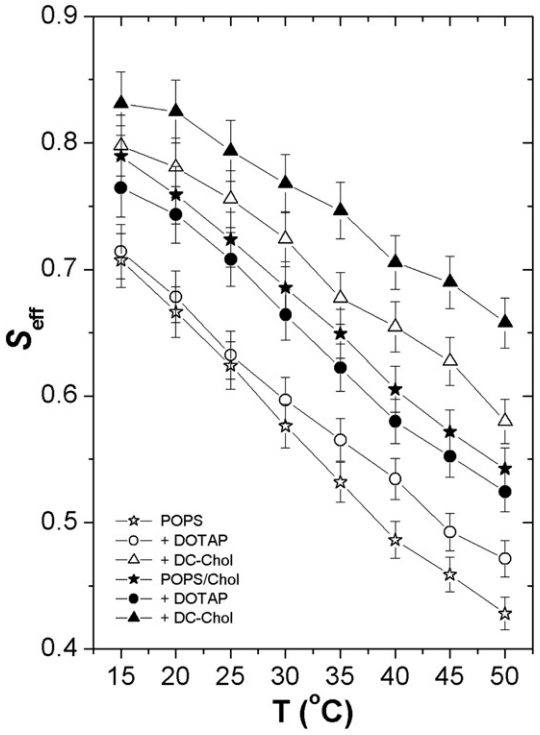

Fig. 10. Temperature dependence of the order parameter (S_{eff}) of 5-PCSL in (open star) POPS membranes, and POPS with (○) 23 mol% DOTAP, (△) 23 mol% DC-chol, and in (★) POPS:Chol membranes (3:2, molar ratio), and POPS:Chol (●) 27 mol% DOTAP, (▲) 23 mol% DC-chol. Results obtained by heating the samples.

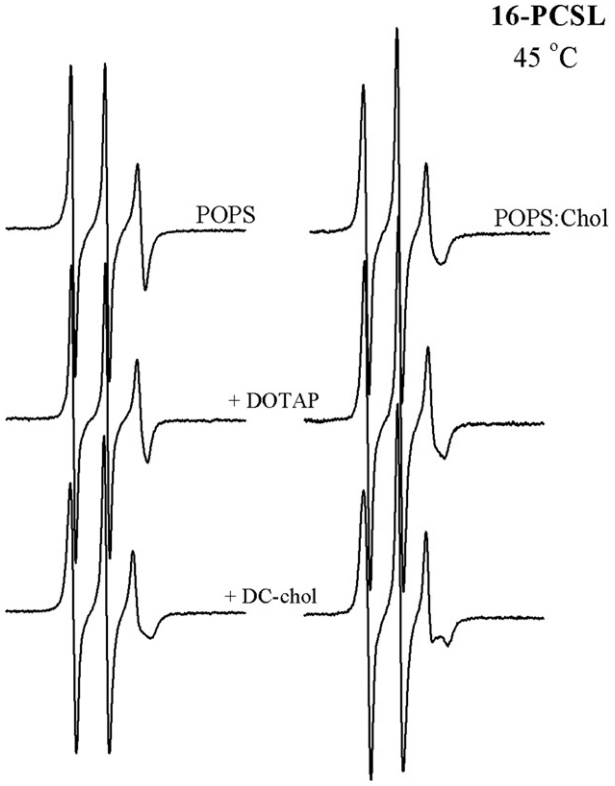

Fig. 11. ESR spectra of 16-PCSL in POPS (left side) and POPS:Chol (3:2, molar ratio; right side), at 45 °C, without and with 23 mol% of DOTAP, and DC-chol. Total spectra width 100 G.

Fig. 11 shows the ESR spectra of 16-PCSL incorporated into POPS and POPS:Chol dispersions, with 23 mol% of DOTAP and DC-chol. Similar to the effect observed with 5-PCSL, at the bilayer core, monitored by 16-PCSL, POPS-cholesterol saturated bilayers (POPS:Chol) are much less organized than cholesterol saturated POPC membranes (POPC:Chol) (top spectra on the right in Fig. 7 and 11). Hence, due to the lower order presented by 16-PCSL in POPS:Chol membranes, S_{eff} values cannot be correctly measured, and the best parameter to be used for the structural analysis of all samples is the ratio between the low and the central field lines, h_{+1}/h_0, shown in Fig. 12 for temperatures between 15 and 50 °C. The higher organization of POPS:Chol bilayers, as compared with POPS, is evinced by the lower h_{+1}/h_0 ratio values yielded by 16-PCSL incorporated in the cholesterol saturated bilayer.

At the bilayer core, DOTAP does not alter much the structure of pure POPS bilayers (Fig. 12), and slightly decreases the hyperfine splitting parameter ($a_o = 14.26 \pm 0.03$ G for POPS, and 14.16 ± 0.04 G for POPS + 23 mol% DOTAP). The spectra of 16-PCSL in POPS:Chol (or in POPS + DC-chol) do not allow a direct measurement of reliable isotropic hyperfine splittings.

The effect of DC-chol at the bilayer core of POPS:Chol membranes is not easy to be interpreted: the cholesterol derivative fluidizes the membrane for temperatures below 30 °C (increases h_{+1}/h_o), but rigidifies the bilayer above 40 °C (decreases h_{+1}/h_o). Though the data presented here do not allow much speculation, it is interesting to note that the cholesterol crystallite thermal transition is between 35 and 40 °C, and it is profoundly affected by the presence of DC-chol (Fig. 4). As also indicated by 5-PCSL, it can be concluded that DC-chol can still be incorporated in cholesterol saturated POPS bilayers, causing significant effects on the bilayer packing. In pure POPS, DC-chol strongly rigidifies the bilayer, significantly decreasing the h_{+1}/h_0 ratio (Fig. 12). Similar

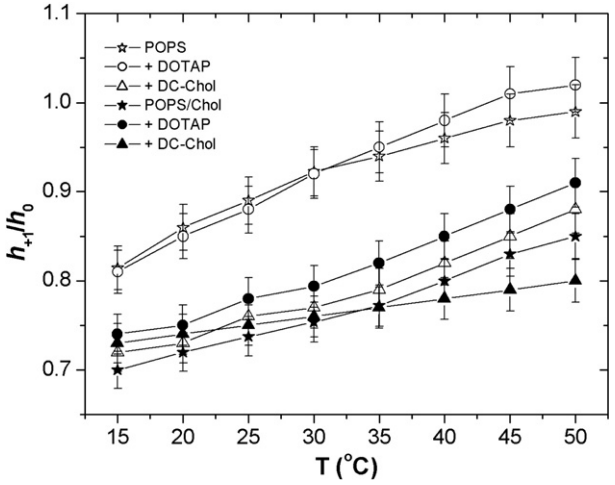

Fig. 12. Temperature dependence of ratio between the amplitudes of the low and central field lines (h_{+1}/h_0) measured on the ESR spectra of 16-PCSL in (open star) POPS membranes, and POPS with (○) 23 mol% DOTAP, (△) 23 mol% DC-chol, and in (★) POPS:Chol membranes (3:2, molar ratio), and POPS:Chol (●) 23 mol% DOTAP and (▲) 23 mol% DC-chol, results obtained by heating the samples.

effect was observed at the 5th C-atom position (Fig. 6), as discussed above.

4. Conclusions

When inserted into a pure zwitterionic lipid, such as POPC, the three cationic amphiphiles tested, DOTAP, DOTAP^{mss} and DC-chol, significantly increase the bilayer packing and decrease the polarity at the center of the membrane. As expected, the cholesterol derivative, DC-chol, produces a stronger effect. However, in highly organized cholesterol saturated POPC bilayers, the data from spin label studies indicate that the effect of these cationic amphiphiles (up to 7 mol%) is rather small. This is in agreement with the DSC results, which indicate that DOTAP and DOTAP^{mss} cause only a small increase in the solubilization of cholesterol in POPC:Chol membranes.

In contrast to the relatively weak effect of the cationic amphiphiles with mixtures of cholesterol with zwitterionic lipids, the results are very different when an anionic phospholipid is substituted. With POPS saturated with cholesterol, the presence of DOTAP strongly disturbs the cholesterol crystal thermal transition around 36 °C. The mixture of DC-chol in cholesterol crystallites is even greater, nearly completely eliminating the thermal transition of anhydrous cholesterol. In addition to the increase of cholesterol solubility in POPS-cholesterol mixtures, DC-chol in particular, significantly increases the bilayer packing. DOTAP is also incorporated into POPS-cholesterol mixtures, but causes only a small decrease in the packing.

The above observations are relevant to the manner in which lipofection agents interact with cell membranes. All lipofection analogs exhibit favorable partitioning from aqueous solution to a membrane. This is in agreement with our results. The initial interaction of these cationic amphiphiles will be with the extracellular monolayer of the plasma membrane of mammalian cells that is composed largely of zwitterionic lipids and cholesterol. The outer monolayer is important for internalization. No specific interactions are required and endocytosis appears to be a major route of entry [4]. Once inside the cell the cationic amphiphile may transfer from binding to nucleic acid to associating with the anionic lipids of the cytoplasmic face of the plasma membrane. These amphiphiles and particularly DC-chol, would not be excluded from cholesterol-rich domains in the membrane and may therefore more efficiently liberate the associated nucleic acid.

DC-chol has the strongest effect in ordering the membrane. Not surprisingly, it acts in a manner analogous to cholesterol. However, because DC-chol is charged, it is less likely to form domains in membranes. It may thus provide a useful tool to distinguish between effects of cholesterol on the “fluidity” of biological membranes and its ability to promote the formation of cholesterol-rich domains. DC-chol will mimic cholesterol with regard to “fluidity” effects but not with regard to increased domain formation.

DC-chol and other cationic amphiphiles have been used to deliver nucleic acids to cells. However, one could also consider using non-coding nucleic acid to deliver cationic amphiphiles to cells. This could prove a particularly useful strategy in the case of DC-chol, where the amphiphile would transfer from its complex with nucleic acid and bind to the cell membrane or other locations of deposits of cholesterol crystals. There is evidence that cholesterol crystals form in foam cells and are a precursor to atherosclerotic lesions [29–33]. Anionic lipids on the cytoplasmic face of the membrane would promote formation of these crystallites [34], while cationic amphiphiles, such as DC-chol would reduce the amount of cholesterol crystals that may have a beneficial effect in protecting from atherosclerosis.

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References

[1] P.L. Felgner, G.M. Ringold, Cationic liposome-mediated transfection, Nature 337 (1989) 387–388.
[2] A. Elouahabi, V. Flamand, S. Ozkan, F. Paulart, M. Vandenbranden, M. Goldman, J.M. Ruysschaert, Free cationic liposomes inhibit the inflammatory response to cationic lipid–DNA complex injected intravenously and enhance its transfection efficiency, Molecular Therapy 7 (2003) 81–88.
[3] H. Lv, S. Zhang, B. Wang, S. Cui, J. Yan, Toxicity of cationic lipids and cationic polymers in gene delivery, J. Contr. Release 114 (2006) 100–109.
[4] J. Zabner, Al.J. Fasbender, T. Moninger, K.A. Poellinger, M.J. Welsh, Cellular and molecular barriers to gene transfer by a cationic lipid, J. Biol. Chem. 270 (1995) 18997–19007.
[5] P.L. Yagle, Cholesterol and the cell-membrane, Biochim. Biophys. Acta 822 (1985) 267–287.
[6] J.B. Finean, Interaction between cholesterol and phospholipid in hydrated bilayers, Chem. Phys. Lipids 54 (1990) 147–156.
[7] T.H. Huang, C.W. Lee, S.K. Das Gupta, A. Blume, R.G. Griffin, A ^{13}C and ^{2}H nuclear magnetic resonance study of phosphatidylcholine/cholesterol interactions: characterization on liquid–gel phases, Biochemistry 32 (1993) 13277–13287.
[8] J.R. Silvius, D. del Giudice, M. Lafleur, Cholesterol at different bilayer concentration can promote or antagonize lateral segregation of phospholipids of differing acyl chain length, Biochemistry 35 (1996) 15198–15208.
[9] S. Bhattacharya, S. Haldar, The effects of cholesterol inclusion on the vesicular membranes of cationic lipids, Biochim. Biophys. Acta 1283 (1996) 21–30.
[10] S. Bhattacharya, S. Haldar, Interactions between cholesterol and lipids in bilayer membranes. Role of lipid headgroup and hydrocarbon chain–backbone linkage, Biochim. Biophys. Acta 1467 (2000) 39–53.
[11] C.R. Loomis, G.G. Shipley, D.M. Small, The phase behavior of hydrated cholesterol, J. Lipid Res. 20 (1979) 525–535.
[12] J. Huang, J.T. Buboltz, G.W. Feigenson, Maximum solubility of cholesterol in phosphatidylcholine and phosphatidylethanolamine bilayers, Biochim. Biophys. Acta 1417 (1999) 89–100.
[13] D. Bach, Differential scanning calorimetric study of mixtures of cholesterol with phosphatidylserine or galactocerebroside, Chem. Phys. Lipids 35 (1984) 385–392.
[14] V. Pata, N. Dan, Effect of membrane characteristics on phase separation and domain formation in cholesterol–lipid mixtures, Biophys. J. 88 (2005) 916–924.
[15] H.S. Shieh, L.G. Hoard, C.E. Nordman, Crystal structure of anhydrous cholesterol, Nature 267 (1977) 287–289.

[16] M.C. Bryan, Crystal structure of cholesterol monohydrate, Nature 260 (1976) 727–729.
[17] R.M. Epand, D. Bach, N. Borochov, E. Watchtel, Cholesterol crystalline polymorphism and the solubility of cholesterol in phosphatidylserine, Biophys. J. 78 (2000) 866–873.
[18] M. Moser, D. Marsh, P. Meier, K.H. Wassmer, G. Kothe, Chain configuration and flexibility gradient in phospholipid membranes, Biophys. J. 55 (1989) 111–123.
[19] O.H. Griffith, P.C. Jost, Lipid spin label in biological membranes, in: L.J. Berliner (Ed.), Spin Labeling. Theory and Applications, Academic Press, New York, 1976, p. 453.
[20] B.J. Gaffney, Practical considerations for the calculation of order parameters for fatty acid or phospholipid spin labels in membranes, in: L.J. Berliner (Ed.), Spin Labeling. Theory and Applications, Academic Press, New York, 1976, p. 567.
[21] W.L. Hubbell, H.M. McConnel, Molecular motion in spin-labeled phospholipids and membranes, J. Am. Chem. Soc. 93 (1971) 314–326.
[22] H. Schindler, J. Seelig, EPR spectra of spin labels in lipid bilayers, J. Chem. Phys. 59 (1973) 1841–1850.
[23] D.E. Bach, E. Wachtel, N. Borochov, G. Senisterra, R.M. Epand, Phase behavior of heteroacid phosphatidylserines and cholesterol, Chem. Phys. Lipids 63 (1992) 105–113.
[24] J.A. Urbina, S. Pekerar, H.B. Le, J. Patterson, B. Montez, E. Oldfield, Molecular order and dynamics of phosphatidylcholine bilayer membranes in the presence of cholesterol, ergosterol and lanosterol: a comparative study using ^{2}H-, ^{13}C- and ^{31}P-NMR spectroscopy, Biochim. Biophys. Acta 1238 (1995) 163–176.
[25] C.R. Benatti, R.M. Epand, M.T. Lamy, Low cholesterol solubility in DODAB liposomes, J. Phys. Chem. 145 (2007) 27–36.
[26] O.H. Griffith, P.J. Dehlinger, S.P. Van, Shape of hydrophobic barrier of phospholipid bilayers (evidence for water penetration in biological membranes), J. Membrane Biol. 15 (1974) 159–192.
[27] D. Marsh, Experimental methods in spin-label spectral analysis, in: L.J. Berliner, J. Reuben (Eds.), Spin Labeling. Theory and Applications, vol. 8, Plenum Press, New York, 1989, pp. 255–303.
[28] D. Kurad, G. Jeschke, D. Marsh, Lipid membrane polarity profiles by high-field EPR, Biophys. J. 85 (2001) 1025–1033.
[29] Y.J. Geng, J.E. Phillips, R.P. Mason, S.W. Casscells, Cholesterol crystallization and macrophage apoptosis: implication for atherosclerotic plaque instability and rupture, Biochem. Pharmacol. 66 (2003) 1485–1492.
[30] P.M. Yao, I. Tabas, Free cholesterol loading of macrophages induces apoptosis involving the fas pathway, J. Biol. Chem. 275 (2000) 23807–23813.
[31] G. Kellner-Weibel, P.G. Yancey, W.G. Jerome, T. Walser, R.P. Mason, M.C. Phillips, G.H. Rothblat, Crystallization of free cholesterol in model macrophage foam cells, Arterioscler. Thromb. Vasc. Biol. 19 (1999) 1891–1898.
[32] A.M. Klinkner, C.R. Waites, W.D. Kerns, P.J. Bugelski, Evidence of foam cell and cholesterol crystal formation in macrophages incubated with oxidized LDL by fluorescence and electron microscopy, J. Histochem. Cytochem. 43 (1995) 1071–1078.
[33] D.M. Small, George Lyman Duff memorial lecture. Progression and regression of atherosclerotic lesions. Insights from lipid physical biochemistry, Arteriosclerosis 8 (1988) 103–129.
[34] D. Bach, E. Wachtel, Phospholipid/cholesterol model membranes: formation of cholesterol crystallites, Biochim. Biophys. Acta 1610 (2003) 187–197.