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The effects of cholesterol inclusion on the vesicular membranes of cationic lipids

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Abstract

Small unilamellar vesicles formed from four cationic lipids in the absence and the presence of varying amounts of cholesterol were studied using fluorescence polarization and ^{1}H-NMR techniques. The fluorescence polarization data clearly indicate that the packing order in the cationic lipid bilayers are affected by inclusion of cholesterol. Importantly, this effect exists also with a cationic lipid that is devoid of any formal linkage region where the interaction of the lipid with cholesterol through hydrogen bonding is not feasible. The interactions of cholesterol with different types of cationic lipids in excess water have also been examined in multilamellar dispersions using proton magnetic resonance spectroscopy. In all the cases, the methylene proton linewidths in the NMR spectra respond to the addition of cholesterol to vesicles. Hydrophobic association of the lipid and cholesterol imposes restriction on the chain $(CH_2)_n$ motions, leaving the terminal CH_3 groups relatively mobile. On the basis of energy-minimized structural models, a rationale of the cholesterol-cationic lipid assembly has also been presented.

Keywords: Membrane vesicle; Cationic lipid; Headgroup-chain linkage; Cholesterol; Fluorescence anisotropy; Phase transition

1. Introduction

We have been interested in developing different *cationic* lipids [1] for the purposes of introducing DNA into eukaryotic cells. These lipids are biologically interesting as they are effective in-vitro, non-viral transfection agents for mammalian cells [2]. Furthermore, they modulate reactivities of phospholipases through *isothermal* bilayer → hexagonal aggregate transitions [3]. Cationic lipids when mixed with DOPE, in-vivo transfection can also be effected [4]. However, several such cationic amphiphiles often show toxic effects to the treated cells [5,6]. Whatever may be the case it appears that there is a consensus that lipid with cationic headgroup is required for gene delivery although specific structural details of the hydrophobic segment may also be important.

When administered for transfection, cationic lipids interact with other membrane subunits, including cholesterol, protein and even other cellular lipids of the eukaryotic cell. Therefore, it is important to examine the nature of mutual interactions that may be present between a cationic lipid and each cellular components under well defined conditions. Thus before undertaking an elaborate program on the design and synthesis of different cationic lipids with predictable affinity towards DNA [7], we thought that it might be useful to explore the molecular basis of the mutual interactions that exist between the cholesterol and different types of cationic lipids. This is particularly relevant because cholesterol appears to be ubiquitously present in all mammalian cell membranes [8]. Moreover, cholesterol remarkably increases the orientational order and consequently the packing of the lipid hydrophobic chains [9–12]. It is now universally accepted that cholesterol supramolecularly associates with different kinds of lipids within the membranous portions of the cell [13–15]. One possible role of cholesterol molecule is to modulate the hydrocarbon chain fluidity of the lipid components of the membranes [16]. This in turn alters the thermotropic phase transition temperature in the lipid bilayer region of cell membrane and also influences their permeability behavior [17]. However, there is a lack of consensus about the specific nature of interactions that exist between cholesterol and a lipid of given structure.

In this work, we have chosen four different cationic lipids (Fig. 1) that vary around the *connector* between the

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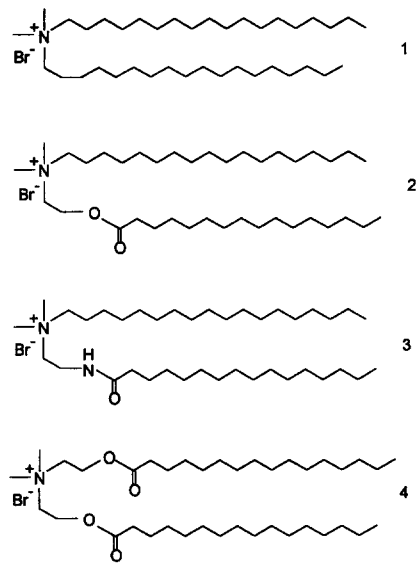

Fig. 1. The structures of the cationic lipids used in this study: **1**, DH_{18}; **2**, $E_{15}H_{16}$; **3**, $A_{15}H_{16}$; **4**, DE_{15}.

hydrophobic and polar segments. Earlier, we have shown how vesicular properties are influenced by variation of the nature of the linkage between the cationic Me_2N^+ headgroup and the hydrophobic segments [1]. The cationic lipids used in this study differ in terms of their molecular features specifically at the chain-headgroup linkage region. In the dialkyldimethylammonium lipid **1**, DH_{18}, the chain-polar headgroup linkage consists of CH_2 groups that are *inert* to hydrogen bonding interactions. No analogous lipid exists in nature that is devoid of any formal linkage function such as amide, ester or ether. Therefore, studying the interaction between the cholesterol and dialkyldimethylammonium lipid, **1** offers scope to explore the lipid-cholesterol association which originate only through hydrophobic contacts. The lipids, **2** and **3** contain dissimilar chain-connector region within the same lipid molecule. **2** contains one alkyl chain and one ($CH_2CH_2OCOC_{15}H_{31}$) acyl chain both connected directly to the Me_2N^+ headgroup. Similarly **3** contains one n-$C_{16}H_{33}$ (alkyl) chain directly attached to the Me_2N^+ headgroup and the other one is linked via a $CH_2CH_2NHCOC_{15}H_{31}$ chain (*amide* linkage). Although cationic in character, **3** resembles naturally occurring lipid sphingomyelin, in that it contains one hydrophobic chain connection via an amide (NH-C = O) type linkage. **4** contains symmetrical diacyl chain linkages and thus represents a cationic lipid analogous to diester lipids that occur naturally. Earlier studies using differential scanning calorimetry indicated that cationic lipids with diester linkages closely resemble phosphatidylcholine (diester) lipids of identical chain lengths in terms of their melting temperature (T_m) [1].

From a biophysical standpoint, these studies are of interest as they depict the interaction of positively charged lipids with the neutral membrane lipid cholesterol. In addition the synthetic availability allows investigation of the role of connector functional groups in this interaction. In this paper, we provide some quantitative estimates on the fluidity of the interior of various cationic lipid vesicles and lipid-cholesterol coaggregates by measuring fluorescence depolarization of vesicle doped 1,6-diphenyl-1,3,5-hexatriene (DPH). The NMR studies indicate that cholesterol inclusion influences the CH_2 proton linewidths of hydrophobic chains of different cationic lipid above their phase transition temperature. Finally, specific interactions that exist between cholesterol and lipid were examined by molecular modelling via energy minimization methods.

2. Materials and methods

All the reagents and chemicals used in this study were of the highest purity available. Except for the cationic lipid with an amide linkage **3**, $A_{15}H_{16}$, the other lipids were prepared according to the procedure described [1]. Cholesterol (C 8667, 99 + %) and 1,6-diphenyl-1,3,5-hexatriene (DPH) (D 4380) were purchased from Sigma (St. Louis, MO, USA) and used as received. The purity of all the lipids was checked by thin-layer chromatography (TLC) on silica gel G-60 plates (Merck) prior vesicle preparation. TLC employed chloroform/methanol mixture as eluant as indicated in the synthesis of $A_{15}H_{16}$ and spots were visualized by staining the plates with iodine vapor and all the lipids in this study exhibited single spots on TLC plates.

2.1. Synthesis

The lipid $A_{15}H_{16}$, **3** was synthesized as shown in the following.

$$Me_2NCH_2CH_2NH_2 + C_{15}H_{31}COCl \xrightarrow[\text{(ii) Work up 83\%}]{\text{(i) DMF}}$$

$$\underset{\mathbf{5}}{C_{15}H_{31}CONHCH_2CH_2NMe_2} \xrightarrow[\text{EtOH, Reflux, 45\%}]{n-C_{16}H_{33}Br} \mathbf{3}$$

2.1.1. N-Hexadecanoylaminoethyl-N,N-dimethylamine (5)

In 10 ml dry DMF 1.0 ml (9.1 mmol) of *N,N*-dimethylethylenediamine was taken in an Erlenmeyer flask and cooled in an ice-salt bath. To this 2.9 g (10.5 mmol) of palmitoyl chloride was added with constant stirring. Just after the addition, white solids were seen. After waiting for 0.5 h, 10 ml EtOAc was added and the solid was filtered from the reaction mixture. The residue was recrystallized twice from EtOAc. The solid so obtained after filtration and recrystallization and filtration was taken up in $CHCl_3$ and washed with 1 N NaOH and passed through a short column of anhydrous Na_2SO_4. On evaporation of the solvent from this solution 2.43 g (7.46 mmol) of compound, **5** (83% yield) was produced. ^{1}H-NMR ($CDCl_3$, 200 MHz): δ 0.87 (*t*, 6H, -CH_3), 1.25–1.37 (*br m*, 26H, -CH_2-), 1.52–1.62 (*m*, 2H, $C\underline{H}_2CH_2C=O$), 2.17 (*t*, 2H,

-CH_2C = O), 2.22 (*s*, 6H, CH_3-N), 2.39 (*t*, 2H, NCH_2CH_2NHC = O), 3.33 (*q*, 2H, CH_2NH-C = O), 5.97 (*br m*, 1H, NH-C = O), IR, 1670 cm^{-1} (-C = O str.), 3225 cm^{-1} (N-H str.), m.p.: 65°C.

2.1.2. *N,N-Dimethyl-N-hexadecyl-N-hexadecanoylaminoethylammonium bromide (3)*

In 25 ml of dry EtOH, 0.40 g (1.23 mmol) of **5** and 0.5 ml (1.63 mmol) of n-hexadecyl bromide were taken. The mixture was kept under reflux for 24 h. The solvent was evaporated and the residue was recrystallized several times from acetone until a single spot was observed on TLC plate. The yield after purification was 45% (0.35 g, 0.56 mmol). ^{1}H-NMR ($CDCl_3$, 200 MHz): δ 0.87 (*t*, 6H, -CH_3), 1.25–1.40 (*br m*, 56H, -CH_2-), 1.52–1.62 (*m*, 2H, CH_2CH_2C = O), 2.27 (*t*, 2H, -CH_2C = O), 3.45 (*s*, 6H, CH_3-N), 3.54 (*m*, 2H, $N^+CH_2(CH_2)_nCH_3$), 3.78 (*m*, 4H, $N^+CH_2CH_2$NHC = O), 8.25 (*br m*, 1H, NH-C = O), IR: 1670 cm^{-1}(C = O, str.), 3220 cm^{-1} (N-H, str.), m.p.: 105°C (soften) and 152°C (clear melt). Anal.: Calcd. for $C_{36}H_{75}ONBr \cdot 1.5\ H_2O$: C, 65.6; H, 11.9; N, 4.2; Found: C, 65.6; H, 11.9; N, 3.9.

2.2. *Unilamellar vesicle preparation*

Separate solutions of cationic lipid in the presence or absence of cholesterol in chloroform were evaporated to dryness in glass vials under stream of dry nitrogen gas. This allowed the formation of thin lipid films on the walls of the vials. To ensure complete removal of the last traces of the organic solvent, the vials containing lipid was left under high vacuum overnight. Either the dried lipid or the lipid/cholesterol mixture was dispersed in water (Millipore) and the resulting mixture was then allowed to get hydrated for ca. 1 h at 4°C. Then, the suspension was thawed to 65°C for 10 min and was then frozen to 4°C for 15 min. The freeze-thaw cycles were repeated two or three times to ensure optimal hydration. The lipid or lipid-cholesterol dispersion thus obtained was sonicated individually above their thermotropic phase transition temperatures using a probe type sonicator at 20 W (Heat Systems Ultrasonic Processor model XL-2020) for 5 min to give an optically translucent suspension. The dispersions were carefully separated from titanium debris from the probe and from the poorly dispersed solid lipid particles.

2.3. *Fluorescence polarization measurements*

Fluorescence anisotropies of the vesicle doped DPH were measured by excitation of DPH at 360 nm and emission at 430 nm. The slit-widths were 5 nm for both the excitation and emission. The fluorescence intensities of the emitted light polarized parallel ($I_\parallel$) and perpendicular ($I_\perp$) to the excited light were recorded at different temperatures. These fluorescence intensities were corrected for the scattered light intensity. Fluorescence polarization, P values at each temperature were calculated according to the equation, $P = (I_\parallel - G \cdot I_\perp) / (I_\parallel + G \cdot I_\perp)$, where G is the instrumental grating factor. The corresponding anisotropies were obtained by employing the Perrin's equation.

The gel to liquid-crystalline phase transition temperatures were calculated from the mid-points of the breaks related to the temperature-dependent anisotropy values [18–20]. The temperature range for the phase transition was calculated from the two temperature points for each experiment which marked the beginning and the end of the phase transition process.

2.4. *^{1}H-NMR experiment*

^{1}H-NMR spectra were recorded at 400 MHz on a Bruker AMX-400 Fourier-transform spectrometer equipped with ASPECT X32 computer and the chemical shift due to the HDO peak was taken as internal standard for each samples prepared in D_2O. The typical spectral conditions were as follows: pulse width 13.0 μs (flip angle 90°), sweep width 5555.56 Hz, data points 16K, acquisition time 1.474 s, FID resolution of 0.33908 Hz/point, the number of scans 600.

^{1}H-NMR experiments were carried out using cationic lipid-cholesterol vesicular mixtures (10, 20 and 30 mol% cholesterol). For comparison, the ^{1}H-NMR spectra were also taken in the absence of cholesterol for each of the lipid vesicles. For each experiment the lipid concentration was maintained constant at 17.8 mM. Cast lipid films (with or without cholesterol) were first made in flasks which were then hydrated in D_2O for 1h at room temperature. The resulting mixtures were then vortexed thoroughly and were then subjected to several freeze-thaw cycles (above T_m of the corresponding lipid) keeping the flask protected during all the manipulations. The stable multilamellar lipid suspensions thus produced were rapidly transferred into separate NMR tubes and immediately closed under dry N_2. The NMR spectra were recorded at 60°C (i.e., above the phase transition temperature of all the vesicular systems) to examine the effect of cholesterol above the phase transition temperatures of the respective vesicular assemblies.

2.5. *Molecular modeling studies*

The molecular modeling studies were conducted with BIOSYM software in a Silicon Graphics Indigo work station. The individual molecules were constructed using standard bond lengths, angles and dihedral angles. The atoms within each molecule were assigned their appropriate hybridization, charge and bond order utilizing the 'Builder module' of INSIGHT (version 2.3.5). The CVFF forcefield provided by DISCOVER module was chosen for the minimization constraints. This forcefield was applied to draw the lipid or steroid molecules and evaluated using

conjugate gradient method. The conformational preference for each system was determined by dynamic simulation at 300 K. The resulting lowest energy conformations for the 2:1 cationic lipid-cholesterol complexes were selected as the minima for the parameter sets and are given in Fig. 6.

3. Results and discussion

Close look at the specific molecular features of various naturally occurring lipids reveals that there exist remarkable differences at various levels of lipid architectures. Thus, one finds significant differences in their polar headgroup structure, hydration and rigidity [21]. The fatty acyl chains also differ in terms of the length, in the degree of unsaturation and in the nature of branching etc. Moreover, the variations around the connector region between the hydrophobic and polar portions within lipid monomer structures are also important. While both the *ester* or the *ether* linkages between polar headgroup and non-polar chains occur in glycerol based phosphatidylcholines, the polar and apolar segments are attached through an *amide* linkage in sphingomyelin [22]. Alkyl and alk-1-enyl ether phospholipids also occur in eukaryotic cell membranes of normal healthy cells and at higher levels in malignant cancer cells [23]. Earlier studies with model and reconstituted ether and ester derived phospholipid vesicles showed significant differences in their diffusion properties and in their intermolecular interactions with other membrane constituents [10]. Such differences in the connector region suggest a role of the linkage functional units in modulating mutual intra- and inter-monomer interactions within the membranous assemblies and their association with other molecules when present in the membranous matrix.

Earlier Yeagle et al. examined the headgroup conformation and egg phosphatidylcholine lipid/cholesterol assemblies in vesicles using ^{31}P-^{1}H nuclear Overhauser effects [24]. Based on their findings, it was proposed that 3β-OH group of cholesterol is hydrogen bonded to the ester carbonyl region of the phospholipid molecules. Grönberg et al. examined the interaction of cholesterol with sphingomyelin analogous in mixed monolayers [25]. Lateral organizations of liquid-crystalline cholesterol-dimyristoyl phosphatidylcholine mixed bilayer have been recently examined and revealed the existence of domain with hexagonal and centered rectangular cholesterol superlattices [26].

With this background on natural lipid-cholesterol association, in the present study we attempted to gain information about the interaction of non-biological, synthetic, bilayer forming cationic lipids with neutral membrane lipid cholesterol. As an obvious approach to understand the nature of such cationic lipid-cholesterol association, we first examined the fluorescence polarization due to DPH in different cholesterol laced and cholesterol free vesicular assemblies.

3.1. Fluorescence anisotropy

Different cationic lipids were separately hydrated and sonicated in the absence as well as in the presence of varying concentration of cholesterol. The alkyl or acyl chain disorders in the cationic lipid bilayers were measured by determining the fluorescence polarization of DPH as a function of temperature [19]. As shown in Fig. 2, the anisotropy values for all the lipids decreased with temperature.

Fig. 2 further shows the dependence of anisotropy values due to DPH doped in different pure and cholesterol laced cationic lipid vesicles as a function of temperature. All the profiles indicated inflections related to the thermotropic phase transition processes. Fig. 2(a–d) further shows that the incorporation of cholesterol in all the cationic lipid types affected the thermal behavior of the mixtures profoundly. Distinct thermal phase transitions could only be seen up to an inclusion of 10 mol% of cholesterol into individual lipid system. But even addition of 10 mol% cholesterol generally causes broadening of the phase transition process. Further increase in cholesterol content abolished the sharp individual phase transition profiles.

For the dialkyl lipid, DH_{18}, **1**, a phase transition temperature of ≈ 42°C (width 9 K) was obtained from the midpoint of the breaks related to the anisotropy–temperature plot (Fig. 2a). This value for T_m corresponds well with the value obtained by differential scanning

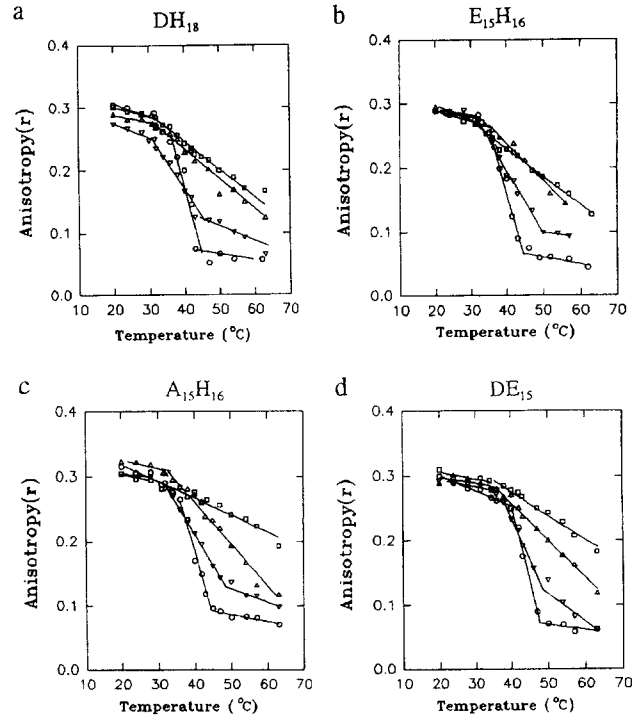

Fig. 2. Fluorescence anisotropy (r) vs. temperature plots for cationic lipid vesicles: (a) DH_{18}, (b) $E_{15}H_{16}$, (c) $A_{15}H_{16}$, (d) DE_{15} with 0 mol% (○), 10 mol% (▽), 20 mol% (△) and 30 mol% (□) of cholesterol.

calorimetry as reported earlier [1]. Incorporation of 10 mol% cholesterol into DH_{18} resulted in an apparent depression in T_m to ≈ 38°C and the width of the anisotropy–temperature profile became broad (≈ 14.5 K) (Fig. 2a). Further increase in the cholesterol (20 or 30 mol%), abolished the phase transition related breaks in the anisotropy (r) vs. temperature plot.

For the mixed chain lipid with one acyl and one alkyl chain, $E_{15}H_{16}$, **2**, a phase transition temperature of ≈ 39.5°C (width 5.3 K) was obtained (Fig. 2b). Unlike with dialkyl lipid, DH_{18}, the incorporation of 10 mol% cholesterol into $E_{15}H_{16}$ did not depress the melting temperature although there was a broadening effect on the phase transition process (T_m ≈ 40.5°C, width ≈ 18.8 K) (Fig. 2b). Further addition of cholesterol again vanished the phase transition profile totally. Increase in temperature only resulted in practically monotonous decrease in anisotropy values.

We also examined another mixed chain lipid $A_{15}H_{16}$, **3**, composed of palmitoyl and one n-$C_{16}H_{33}$ chain. The acyl chains connected to Me_2N^+ group via an amide linkage and thus could be considered as putative mimics of sphingomyelin. The phase transition temperature for this type of cationic lipid was not significantly different from its mixed chain alkyl-acyl (ester) counterpart, **2**, except that with the former, the width of the phase transition profile was broader (9.5 K) (Fig. 2c). Addition of cholesterol resulted in the broadening of the melting profile and as was observed with DH_{18} and $E_{15}H_{16}$, clear phase transition disappeared.

When diester lipid, DE_{15}, **4**, was examined, a phase transition temperature of 43.5°C was found (Fig. 2d). In this case, even addition of 10 mol% cholesterol practically abolished the clear phase transition profile although a broad T_m of 39.8°C could still be detected (Fig. 2d). With further increase in cholesterol, melting profile became extremely broad and practically monotonous.

The anisotropies of different lipid vesicular system at their respective T_m values were also plotted as a function of cholesterol as given in Fig. 3. With all the lipid types, cholesterol addition enhanced anisotropies progressively. The pattern of anisotropy variation with cholesterol addition appears to be along identical lines with lipids that contain similar chain-polar headgroup connectors (DH_{18} or DE_{15}). The increments in anisotropies for DPH doped in vesicular mixed chain lipids (EH or AH types) follow different trends. While with $E_{15}H_{16}$, the anisotropy approximately rises with increase in cholesterol, the $A_{15}H_{16}$ system shows a more complex behavior.

Overall the fluorescence anisotropy measurements as a function of temperature indicate that the effect of cholesterol is less pronounced in the solid gel-like phases below phase transition temperature for all the four types of cationic lipids examined herein (Table 1). But although the effects are similar, closer examinations reveal some differences with individual lipids carrying various linker groups.

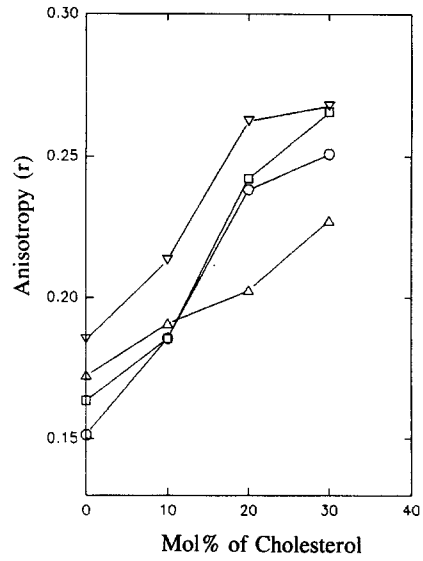

Fig. 3. The plots of the fluorescence anisotropies of different lipid vesicular systems at their T_m as a function of cholesterol concentration. (○) DH_{18}, (△) $E_{15}H_{16}$, (▽) $A_{15}H_{16}$, (□) DE_{15}.

The results with the parent lipids (i.e., without cholesterol) indicate that the anisotropy is comparatively less in case of the lipid that contain amide group. It indicates that there could be greater tightness in the intermolecular packing. The intermolecular hydrogen-bonded interaction present between the amide bonds of (-NH · · · O = C-) the neighboring monomers could provide greater tightness in the chain packing. Thus the present findings clearly show that the nature of the linkage of the lipid backbone does have a bearing on the fluidity of the system.

Table 1
Phase transition parameters of cationic lipids and their cholesterol doped membranes as determined by fluorescence spectroscopy [a]

Lipid [b]	%Cholesterol	T_m [c] (°C)	ΔT_m (K)	$r(T_m)$
DH_{18}	0	42.0	9.00	0.1513
	10.0	38.0	14.54	0.1854
	20.0			0.2381
	30.0			0.2508
$E_{15}H_{16}$	0	39.5	5.25	0.1723
	10.0	40.5	18.75	0.1908
	20.0			0.2026
	30.0			0.2271
$A_{15}H_{16}$	0	40.0	9.50	0.1852
	10.0	39.75	17.75	0.2131
	20.0			0.2626
	30.0			0.2679
DE_{15}	0	43.7	9.50	0.1634
	10.0	42.7	11.36	0.1855
	20.0			0.2421
	30.0			0.2658

[a] See text for notations. [b] See Fig. 1 for abbreviated notations for individual lipids. [c] The melting transition temperatures are accurate to ±2°C.

Despite 'loose' association relative to that in PC/cholesterol mixture (see below), the incorporation of cholesterol in the cationic lipid vesicles profoundly affects, the bilayer thermotropic transition properties. The thermal phase transitions was observable only up to 10 mol% incorporation of cholesterol into the membrane. After that the clear phase transition process disappears. In our previous studies [1], we have seen that diester containing lipids are more thermotropically stable than their dialkyl or alkyl-acyl counterparts. Since the disposition of the linker functionality in the lipid backbone has a major role in the lipid packing [27], one might anticipate that the interaction of cholesterol with such systems should depend on the specific details of chain-headgroup connector function.

From the anisotropy measurements with different cholesterol containing cationic lipid vesicular systems, we find that the anisotropic perturbation by cholesterol is the least in the gel state whereas the effect is more pronounced only after the beginning of the process of phase-transition. The anisotropy data further show that the amide containing lipids are more 'tightly' packed in the gel state than its counterparts with ester type of connector. As order parameter is directly related to the lipid packing, the available data on order parameter also support the results in the similar direction. At 25°C (below the phase transition temperature for all the lipid vesicles), the relative change in the order parameter from one system to another is not significant. The effect of cholesterol addition is more pronounced in the solid gel-liquid crystalline coexistence region for all the cationic lipid types. Thus, at this stage of transition, the order parameter increases steadily with the increase in cholesterol content in the vesicular lipid/cholesterol mixture.

Upon addition of the cholesterol in the lipid matrix, the cholesterol molecules are incorporated into the bilayer resulting in a greater headgroup separation between the lipid monomers. In our previous studies [1] we have observed that in the process of vesicular phase transition involving cationic lipids, the cooperativity is significantly lower than that of the lecithins of comparable chain length. This suggests that both the role of the repulsive interaction between the pendant Me_2N^+ units at the level of headgroup and the attractive forces between the hydrophobic components of these lipid bilayers are important. 'Looser' association in the vesicles of cationic lipids could be envisaged on the basis of repulsive interactions at the level of headgroups. Phosphatidylcholine lipids are zwitterionic and hence do not experience such electrostatic repulsions. Instead, dipolar interactions between phosphatidylcholine headgroup stabilize the bilayer assembly [24]. Consequently, addition of cholesterol into cationic lipid vesicles should lead to greater stabilization of assembly as a result of the mitigation of headgroup-headgroup repulsions relative to that present in pure cationic lipid assemblies. Such stabilization should be augmented further with the increase in the associative capacities at the linker region.

3.2. NMR studies

To further understand the nature of cationic lipid–cholesterol interactions in membranes, various cholesterol-free and cholesterol-laced vesicular prepara-

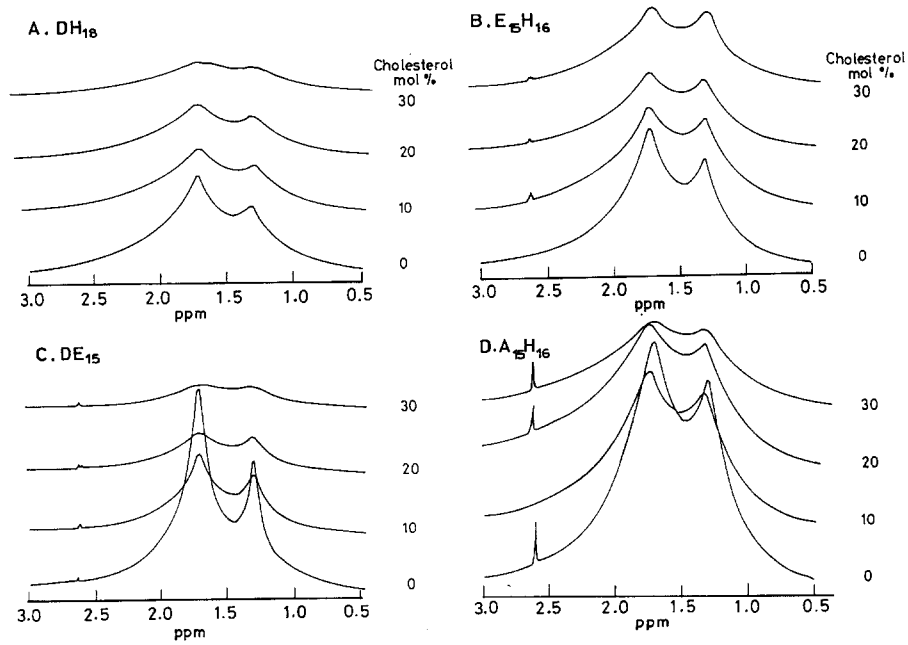

Fig. 4. The proton magnetic resonance spectra of chain polymethylene $(CH_2)_n$ and terminal methyl (CH_3) groups for different lipid systems in the absence and in the presence of different mol% of cholesterol at 60°C. (A) DH_{18}, (B) $E_{15}H_{16}$, (C) DE_{15}, (D) $A_{15}H_{16}$.

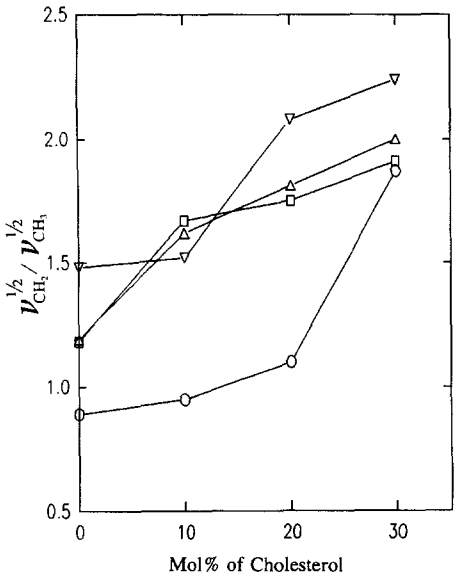

Fig. 5. The plots of the ratio of the widths at half-height of polymethylene $(CH_2)_n$ to terminal methyl (CH_3) proton signals against cholesterol concentration. (○) DH_{18}, (△) $E_{15}H_{16}$, (▽) $A_{15}H_{16}$, (□) DE_{15}.

tions were analyzed by ^{1}H-NMR spectroscopy. From the anisotropy measurements at various temperatures on different cholesterol doped lipid assemblies, it was found that the perturbation induced by cholesterol in individual preparation was minimum in their solid gel-like states (below phase transition temperature). The effect of cholesterol was more pronounced above their phase transition temperatures. Based on such considerations, we examined ^{1}H-NMR spectra of different vesicular preparation at 60°C which most likely represented 'liquid-crystalline' states for all the samples. Fig. 4 shows the chain $(CH_2)_n$ and terminal CH_3 resonances for different lipid systems in the absence and in the presence of cholesterol. For all the lipid systems, addition of cholesterol in gradually greater amounts (0–30 mol%) led to progressive broadening of the downfield methylene proton signals indicating that cholesterol significantly restricted the mobility of the $(CH_2)_n$ units of the acyl or alkyl chain, but had less effect on the terminal CH_3 groups. In other words, cholesterol appears to have resisted the flexing of the methylene units from the headgroup-chain connector region spanning the entire length of cholesterol (in the liquid-crystalline state). Similar results were obtained when cholesterol was added to phosphatidylcholine vesicles [28,29].

Fig. 5 shows the plots of the ratio of the widths at half-height of methylene to terminal methyl proton signals as a function of cholesterol concentration. In all the cases, increase in cholesterol resulted in the increase in $\nu_{CH_2}^{1/2}/\nu_{CH_3}^{1/2}$ ratio for the mixed membranes. Although, upon addition of cholesterol, each cationic lipid behaved nearly similarly, the plot shown in Fig. 5 brings out finer differences that exist among cholesterol and different cationic lipid complexes. The lipid containing ester linkages ($E_{15}H_{16}$ or DE_{15}) showed rather steep increase in the rigidity upon increase in the cholesterol content up to 10 mol%. Then further increase in cholesterol up to 30 mol% appears to increase $\nu_{CH_2}^{1/2}/\nu_{CH_3}^{1/2}$ only moderately. In contrast, DH_{18}, which does not contain any formal connector group, resisted an abrupt increase in rigidity even after inclusion of 20 mol% of cholesterol. However, there was a steep rise in $\nu_{CH_2}^{1/2}/\nu_{CH_3}^{1/2}$ value at 30 mol% cholesterol. $A_{15}H_{16}$, which contains an amide linker on the other hand shows a more complex behavior in its interaction with cholesterol. The observed, minor but detectable differences in the interactions of the cholesterol with different type of cationic lipids as observed in the NMR experiment could be explained on the basis of variations in the molecular structures of these lipids in the vesicular matrix. The interaction between DH_{18} and cholesterol should originate exclusively from the hydrophobic contacts where cholesterol could act as a filler. In contrast with lipids containing ester or amide connectors, the lipid monomer could participate through hydrogen bonding interaction with 3β-OH of cholesterol in vesicular membranes in addition to sterol/lipid hydrophobic contacts. Since lipids that contain ester or amide connector, possess hydrogen bonding potential, if bound through such association, the cholesterol molecule could be sort of 'immobilized' within vesicles as long as the cholesterol content is less than lipid. But in the case of DH_{18}, such interaction is not feasible and hydrophobic association should be more 'flexible' and should be able to accommodate cholesterol with the membrane depending on the miscibility of the latter.

It would be instructive to compare the nature of cholesterol contacts with 1-palmitoyl-2-oleoyl-*sn*-glycerophosphocholine (POPC) above their phase transition temperature (−5°C) which is known to have pronounced interactive capacity with cholesterol [30]. Cholesterol had been shown to exert a greater ordering effect on phosphatidylcholine which contain an oleolyl chain in position-2 and a saturated palmitoyl chain in position-1 of their glyceryl diester backbone [31]. Such preference of cholesterol for 1-acyl-2-oleyl PCs has been attributed to the presence of *sn*-2 acyl chain 9,10-*cis* double bond, which allows accommodation of the sterol C-18 and C-19 angular methyl groups [32]. Since the cationic lipids examined herein lacked any such double bonds at the level of the steroid angular methyl groups, the cationic lipid/sterol contacts in the middle of the bilayer are 'looser' than the same in POPC/cholesterol assemblies.

3.3. Optimized conformations of cationic lipids

As is known with fully saturated diacyl phosphatidylcholines, the differences in the depths of chain penetration play a crucial role in the thermotropic phase transition of the lipid membrane assemblies [33]. Consideration of the conformation of the dioctadecyldimethylammonium lipids as available from the crystal structure of dioctadecyldimethylammonium bromide monohydrate, **1** [34], pro-

vides a basis for explaining the effect of the modification of the connector regions that link the hydrophobic segments with the polar dimethylammonium headgroup. Assuming that the lipid monomer structure in vesicles resembles the structural features of the lipid in their crystalline states. We minimize the lipid structures. In the crystal structure of **1**, the n-octadecyl chain attached to Me_2N^+ center have been shown to be *inequivalent* to one another. One of the alkyl chain assumes the *s-trans* conformation and penetrates farther into the bilayer. The other chain of identical length however takes up a folded conformation (ggtgg) near the Me_2N^+ group and the *s-trans* conformation towards the chain terminus. This results in an inequivalence in chain penetration which has also been observed with phospholipids [33]. The conformations of other lipids **2**–**4** respectively, which were derived from the structure of **1** by extending and modifying the chain/Me_2N^+ linkage region to incorporate ester or amide group wherever necessary. We further assume herein that the lipid molecule architecture of **2**, **3** and **4** may be derived by modification and extension of structure of **1**. Examination of structure generated after modification followed by energy minimization reveals, that in each model, the ester on the acyl chain points toward a neighboring lipid molecule. In the case of mixed chain lipid **2** or **3**, the n-hexadecyl chain prefers to assume the *s-trans* conformation and the acyl chain ($-CH_2CH_2OC(O)C_{15}H_{31}$) assumes a *folded* conformation near the Me_2N^+ headgroup and a straight (zigzag) chain conformation toward the chain terminus. This plan assists in minimization of the mismatch in the depths of chain penetration. Cationic diester lipids **4**, also under condition of similar modeling studies gave indications of inequivalence in chain penetration into the bilayer midplane.

3.4. Cholesterol interaction

In case of DH_{18} system, where there is no group capable of hydrogen bonding, only the alignment of the cholesterol changes with respect to the neighboring lipid molecules. The headgroups of the individual lipid molecules were also affected in the presence of water molecules to accommodate them in the inter amphiphilic cavity. The distance between the two Me_2N^+ headgroups increase from ≈ 5.4 to ≈ 11.4 Å upon inclusion of cholesterol. The structural perturbations on the rigid cholesterol backbone do not seem to be significant in the assembly. The 3β-OH group in cholesterol appears to get hydrated under these conditions (Fig. 6).

In case of $E_{15}H_{16}$, there exists an ester-carbonyl group which can form hydrogen bond with 3β-OH from a donor molecule such as cholesterol. The above conclusion however, did not indicate existence of any significant extent of hydrogen bonding between the cholesterol 3β-OH and the ester-carbonyl of the lipid backbone. It appears that cholesterol prefers to lie deeper toward the bilayer midplane during hydrophobic stabilization. Under such situation, the

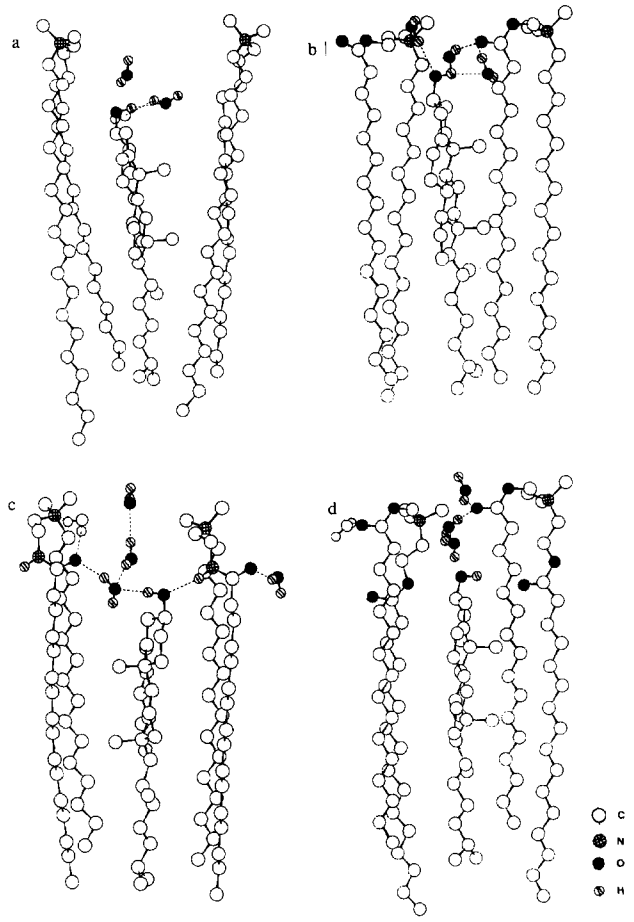

Fig. 6. Energy minimized structural models (INSIGHT) of 2:1 cationic lipid/cholesterol complexes. (a) DH_{18}, (b) $E_{15}H_{16}$, (c) $A_{15}H_{16}$, (d) DE_{15}.

ester-carbonyl group (bent chain) tends to stay more to the hydrophilic side of the bilayer. Under these circumstances the distance between the 3β-OH of cholesterol and the C = O of the bent chain lipid increases rending the formation of hydrogen bond between them not feasible.

In case of DE_{15}, there exists two ester groups in either of the bent as well as in the straight chain. The position of the *bent* chain ester remains nearer to the hydrophilic headgroup region whereas the *straight* chain ester extends more toward the hydrophobic side of the bilayer. In this situation, the straight chain ester group stays nearer (3.2 Å) to the 3β-OH group of cholesterol than that of the bent chain ester (≈ 6 Å). It was found that direct cholesterol-lipid (DE_{15}) hydrogen bonding could not be ensured.

In case of $A_{15}H_{16}$, **2**, there exists an amide group in the chain-polar group linker region. The molecular mechanics calculation shows that the amide group resides in the bent chain. The mismatch between the straight and the bent chain was found to be similar to that found in $E_{15}H_{16}$ amphiphile. In this case, an interesting phenomena was however, observed. It is the N-H hydrogen of the amide group that takes part in the hydrogen bonding with 3β-OH of cholesterol and the OH hydrogen forms hydrogen bond

with the C = O of the neighboring lipid molecule through bridges from intervening water molecules near the surfaces. This results in the formation of a complex hydrogen bonding network through the participation of interfacially adhering water molecules (Fig. 6).

In order to have a feasible hydrogen bonding between 3β-OH group and the carbonyl of the linker region from the lipids, the cholesterol and the interacting lipid molecule have to come within ≈ 2.5 Å at the level of the hydrogen bonding interaction. This also results into bringing the cationic lipids containing saturated hydrophobic chain and steroid molecule closer at the deeper interior of the membrane. However, such proximity creates unfavorable contacts between the axial methyls of the steroid backbone with the polymethylene chains of the lipids. When energy minimization was done, this scenario does not appear to be preferred. Instead in order to avoid such unfavorable interactions, the steroid molecule rather repositions itself in such a way that the axial methyls of the 'rigid' steroid backbone are accommodated within the spaces between the two lipid chains. This plan of steroid–lipid interaction (Fig. 6) indicates that the hydrophobic association is the more important factor which controls the lipid-cholesterol association. Thus the lipid molecules with ester or even amide linkages containing C = O position themselves at such distances that *direct* hydrogen bonding with the 3β-OH of cholesterol are not feasible. Although lipid C = O · · · H-O-H · · · O-H (3β-cholesterol) interactions appear to be present through intervention of water molecules that adhere along the membrane interfaces. This conclusion is further supported from the fact that similar type of steroid-lipid assembly is also preferred when a lipid (such as DH_{18}) is devoid of any linkage function that could participate in hydrogen bonding with the 3β-hydroxyl group.

3.5. Summary

The lipids studied herein are rather 'loosely' held because of repulsive interactions among the pendant cationic Me_2N^+ groups. One would expect therefore that the addition of cholesterol as a filler molecule would lead to reduction in the headgroup repulsion. The headgroup sizes of the cationic lipids used herein are quite different than that of the diacylphosphatidylcholine lipids. These cationic lipids are inert to hydrogen bonding interaction at the headgroup level Me_2N^+ group). But the phospholipid molecules could participate in monomer to monomer contacts at the headgroup level both through dipolar and hydrogen bonding interactions. Despite these fundamental differences in their specific structural features, polar character and mutual lipid monomer–monomer interactions within vesicles, the effects of inclusion of cholesterol in cationic lipid vesicles seem to be remarkably similar to that observed with cholesterol-phospholipid covesicles. The mutual hydrophobic association between cationic lipid and cholesterol appears to be more important than any possible hydrogen bonding interaction between lipid and cholesterol. Examination of the effects of cholesterol inclusion into vesicles of other cationic lipids with defined structural features [35] is underway to explore the generality of the present findings.

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