The Influence of Cholesterol on the Interaction between *N*-Dodecyl-*N*,*N*-dimethyl-*N*-benzylammonium Halides and Phosphatidylcholine Bilayers

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Effects of *N*-dodecyl-*N*,*N*-dimethyl-*N*-benzylammonium halides (DBeAX) on thermotropic phase behavior of phosphatidylcholine/cholesterol bilayers as well as on ^{1}H NMR spectra were studied. The surfactants were added either to the water phase or directly to the lipid phase (a mixed film was formed). The benzyl group, opposite to liposomes without cholesterol, is not incorporated into the bilayer in the gel state but only in the liquid state. All the halides DBeAX (particularly the chloride DBeAC) showed greater ability to destabilize the membrane structure in the presence than in the absence of cholesterol. The interaction of DBeAX with DPPC/cholesterol bilayers and subsequent changes in the phospholipid bilayer organization depended on the kind of counterion. The strongest effects were observed for chloride (most electronegative ion) and for iodide (largest ion). The effects of chloride and bromide on phase transition and ^{1}H NMR spectra in the presence and absence of cholesterol were opposite. This is discussed in terms of the influence of counterions on the pair cholesterol-DPPC interactions.

Key words: DPPC, Cholesterol, *N*-Dodecyl-*N*,*N*-dimethyl-*N*-benzylammonium Halides

Introduction

For a long time quaternary ammonium surfactants (QAS) have been widely applied in technology and medicine, as well as in membrane biology and in everyday life. As a consequence, numerous papers have been devoted to studies of the QAS interaction with model membranes (Eliasz *et al.*, 1976; Lichtenberg *et al.*, 1983; Lichtenberg, 1985; Balgavy *et al.*, 1984; Lasch, 1995; Koynowa and Tenchov, 2001). Recently, the interest in QAS and their interaction with model membranes, and particularly interaction with DNA, has grown up because liposomes containing cationic surfactants proved to be able to transfer DNA into cells (Dias *et al.*, 2001, 2002; Woude *et al.*, 1997; Clamme *et al.*, 2000; Fisicaro *et al.*, 2005).

In our previous papers (Różycka-Roszak and Pruchnik, 2000a, b; Różycka-Roszak and Przyczyna, 2003; Różycka-Roszak *et al.*, 2004), we have studied the interaction of dodecyltrimethylammonium halides (DTAX), *N*-dodecyl-*N*,*N*-dimethyl-*N*-benzylammonium halides (DBeAX) and 1-decyloxymethyl-3-carbamoylpyridinium salts (PS-X) with DPPC liposomes and also the interaction of PS-X with DPPE and DTAX with DPPC/cholesterol liposomes. We have shown that the interaction and subsequent changes in the phospholipid bilayer organisation depend on the kind of counterion.

The objective of the present paper was to study the interaction between DBeAX and phosphatidylcholine bilayers containing cholesterol (DPPC/cholesterol). The long chain *N*-dodecyl-*N*,*N*-dimethyl-*N*-benzylammonium halides, like other QAS, show strong biological activity, and one of them, *N*-dodecyl-*N*,*N*-dimethyl-*N*-benzylammonium chloride, is applied as a major component of "benzalkonium chloride (BkCl)" which is widely used as an antiseptic in pharmaceutical preparations (Possidonio *et al.*, 1999). Besides, carbopol/BkCl aggregates are studied as potential carriers of hydrophobic drugs (Barreiro-Iglesias *et al.*, 2003).

The study should contribute to a deeper understanding of the role of counterions in the biological activity of surfactants. DPPC/cholesterol systems resemble the biological environment more than DPPC liposomes without cholesterol. Cholesterol is a major component of the plasma membrane in many cells of higher organisms (McMullen *et al.*, 2004). Besides, cholesterol-rich domains in DPPC/cholesterol systems can be treated as a

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model for rafts in natural membranes (Loura *et al.*, 2001). The present studies should also contribute to understand the role of surfactant (especially counterion) in cholesterol-DPPC interactions. This seems to be important because transfection efficiency is increased when cholesterol is included into cationic liposomes (Zhang and Anchordoquy, 2004). We studied DPPC/cholesterol bilayers having 2, 5 and 10 mol% cholesterol. This content range seems to be especially interesting. At the molar ratio 2 mol% cholesterol starts to induce phase separation and cholesterol-rich and cholesterol-poor domains are formed (McMullen and McElhaney, 1995). Besides, in the range 0–10 mol% the conformational order parameter (the ratio of the number of ordered lipid chains to the total number of lipid chains) decreases reaching a minimum at 10 mol% cholesterol (Polson *et al.*, 2001). Also at 10 mol% cholesterol the total area of a bilayer dived by the number of DPPC molecules reaches a minimum (Edholm and Nagle, 2005).

As in previous studies (Różycka-Roszak and Przyczyna, 2003; Różycka-Roszak *et al.*, 2004), the compounds were added to liposomes either through the water phase (methods 1a and 1b) or through the lipid phase (method 2). Thanks to that we were able to study the interaction between DBeAX and liposomes containing cholesterol-rich and -poor domains (method 1) as well as the influence of a surfactant on the formation of cholesterol domains (if possible in such a system; method 2). Also as before, we have investigated the influence of the studied compounds on both the thermotropic phase behaviour (using DSC) and ^{1}H NMR spectra of DPPC/cholesterol bilayers.

Materials and Methods

Chemicals

DPPC and cholesterol were purchased from Avanti Polar Lipids, Birmingham, Alabama. *N*-Dodecyl-*N*,*N*-dimethyl-*N*-benzylammonium chloride (DBeAC) and *N*-dodecyl-*N*,*N*-dimethyl-*N*-benzylammonium bromide (DBeAB) were purchased from Fluka, Switzerland.

N-Dodecyl-*N*,*N*-dimethyl-*N*-benzylammonium iodide (DBeAI) was prepared by mixing a concentrated aqueous NaI solution with an aqueous solution of DBeAC at room temperature. A precipitate was obtained, which was redissolved in warm water and precipitated again after cooling. The solution was filtered and recrystallized from EtOH. Purity was verified by ^{1}H NMR measurements. Additionally, a satisfactory elemental analysis was obtained. 99.98% D_2O was purchased from Dr. Glaser AG, Basel, Switzerland.

Sample preparation for differential scanning calorimetry (DSC)

Samples for DSC were prepared on multilamellar vesicles (MLVs) using two methods.

Method 1a

DPPC with appropriate amounts of cholesterol (2, 5 and 10 mol% of total lipid) were dissolved in chloroform. Traces of chloroform were removed with a stream of dry nitrogen under reduced pressure. The lipid film on the flask wall was dispersed by adding aqueous solutions of DBeAC, DBeAB or DBeAI at appropriate concentrations. It was then intensively shaken at about 60 °C for 15 min to give a milky suspension of liposomes.

Method 2

DPPC, cholesterol (2, 5 and 10 mol% of total lipid) and appropriate amounts of DBeAC, DBeAB or DBeAI were dissolved in chloroform. Chloroform was evaporated to form a thin mixed film on the flask wall. Afterwards, distilled water was added and further processing was analogous to method 1a.

Final DPPC concentrations in liposomes with 2, 5 and 10 mol% of cholesterol were equal to 24.5, 23.74 and 22.5 mg/ml, respectively. The lipid suspensions were loaded into the sample cell of a DSC microcalorimeter (Mettler Toledo Thermal Analysis System D.S.C. 821^e). The scan rate was 2 °C/min, and incubation, performed at 4 °C, lasted 5 d.

Sample preparation for ^{1}H NMR

Measurements were performed on SUVs, also prepared using two different methods:

Method 1b

A chloroform solution of DPPC and cholesterol (5 mol%) was evaporated. Traces of chloroform were removed with a stream of dry nitrogen under reduced pressure for 2 h. The formed lipid film was dispersed by adding heavy water and shaking on a vortex mixer, giving a milky suspension of liposomes. This liposome suspension was soni-

cated for 30 min above main phase transition temperature with a 20 kHz sonicator. Then a solution of either DBeAC or DBeAB at the appropriate concentration in heavy water was added. Samples were enclosed in 5 mm NMR tubes. Unfortunately, due to the low solubility of DBeAI, we were not able to prepare liposomes with this salt.

Method 2

DPPC and 5mol% of cholesterol were dissolved in chloroform. Chloroform solutions of DBeAC and DBeAB were also prepared. Then the appropriate amounts of lipids solutions and salts solutions were mixed, chloroform was evaporated to form a film on the flask wall. Traces of chloroform were removed with a stream of dry nitrogen under reduced pressure. The mixed film (DPPC/cholesterol/DBeAX) was dispersed by adding heavy water and agitating the flask on a vortex mixer, resulting in a milky suspension of liposomes. This liposome suspension was sonicated (as in method 1a) and enclosed in NMR tubes. The final phosphatidylcholine concentration was 21.2 mg/ml.

^{1}H NMR spectra were recorded at 25 °C and 45 °C on an Avance Bruker DRX 300 Spectrometer at 300.13 MHz. Signals were acquired using a 6173 Hz spectral window, 10.7 ms pulse and an acquisition time of 2.65 s. Digital resolution was 40.9268 Hz/cm, which is 0.1364 ppm/cm. The residual heavy water signal was used as a chemical shift reference. Samples were incubated for 5 d at 4 °C, and additionally equilibrated in the NMR spectrometer for at least 30 min when the spectra were recorded at 25 °C and at 45 °C.

Results and Discussion

DSC

The effects of DBeAX on the phase transitions of DPPC/cholesterol containing 2, 5 and 10 mol% of cholesterol are listed in the Tables I and II.

At low cholesterol contents (2 and 5 mol%) all compounds affect the phase transitions in a similar way as it was for bilayers without cholesterol (Różycka-Roszak and Przyczyna, 2003). For method 1 the DBeAC compound lowers the main transition

Table I. Transition temperatures (T_m [°C]) and enthalpies (ΔH_m [kJ/mol]) for the main phase transition of DBeAX/DPPC/2 mol% chol mixtures and of DBeAX/DPPC/5 mol% chol mixtures (in parentheses).

$n_{DBeAX}/n_{DPPC/chol}$		DBeAC		DBeAB		DBeAI	
		1a	2	1a	2	1a	2
0.015	T_m	39.6 (39.0)	40.4 (39.1)	39.8 (39.1)	39.8 (38.9)	40.5 (40.2)	39.3 (38.4)
	ΔH_m	29.7 (23.6)	32.3 (30.4)	29.5 (26.6)	31.9 (23.2)	27.9 (25.3)	30.2 (26.2)
0.03	T_m	38.2 (37.9)	38.7 (38.0)	39.0 (37.7)	38.8 (37.7)	40.2 (39.6)	38.7 (38.2)
	ΔH_m	29.7 (20.4)	31.8 (26.2)	28.7 (26.3)	28.6 (22.0)	27.9 (23.8)	28.4 (24.5)
0.06	T_m	36.6 (35.6)	37.5 (36.7)	37.5 (36.4)	37.2 (36.4)	39.4 (38.9)	37.2 (35.8)
	ΔH_m	28.2 (19.6)	30.7 (22.6)	27.0 (21.9)	24.9 (19.9)	27.1 (21.4)	28.5 (21.7)
0.09	T_m	35.1 (33.4)	36.0 (34.3)	36.7 (35.0)	36.7 (34.8)	38.7 (38.7)	35.0 (33.7)
	ΔH_m	25.4 (19.6)	29.8 (20.7)	26.8 (21.6)	20.3 (17.8)	26.8 (20.7)	27.0 (21.0)
0.15	T_m	30.6 (29.8)	31.2 (30.5)	32.9 (31.7)	32.2 (30.6)	36.4 (35.2)	32.5 (30.1)
	ΔH_m	23.9 (18.3)	29.8 (20.0)	25.4 (19.3)	18.8 (14.0)	25.5 (18.9)	25.7 (19.6)
0.21 (0.22)	T_m	30.0 (29.2)	30.3 (29.5)	31.7 (30.0)	29.8 (29.7)	– (–)	29.8 (29.2)
	ΔH_m	20.3 (16.1)	27.6 (18.7)	25.4 (17.1)	18.4 (13.5)	– (–)	23.6 (18.8)
0.30 (0.31)	T_m	27.6 (25.8)	28.9 (25.9)	28.7 (26.2)	27.3 (–)	– (–)	32.0 (–)
	ΔH_m	18.8 (11.6)	25.1 (–)	23.8 (16.0)	17.6 (–)	– (–)	22.0 (–)

temperature T_m and enthalpy ΔH_m more than other DBeAX. In the case of method 2 all compounds decrease the temperature T_m to approx. the same extent while the transition enthalpy is decreased most by DBeAB. Probably, as it was discussed in previous papers for DPPC liposomes without cholesterol, this is due to strong bromide adsorption and the consequent stronger hydration of the polar head group and resulting enhanced head group repulsion.

At a cholesterol content of 10 mol%, for method 1, DBeAC and DBeAB decrease T_m to approx. the same extent while DBeAI decrease T_m significantly less. Transition enthalpy is most decreased by DBeAC. For method 2, all compounds decrease T_m in a similar way. Both DBeAC and DBeAB also decrease ΔH_m in a similar way while DBeAI a little more. The DSC curve

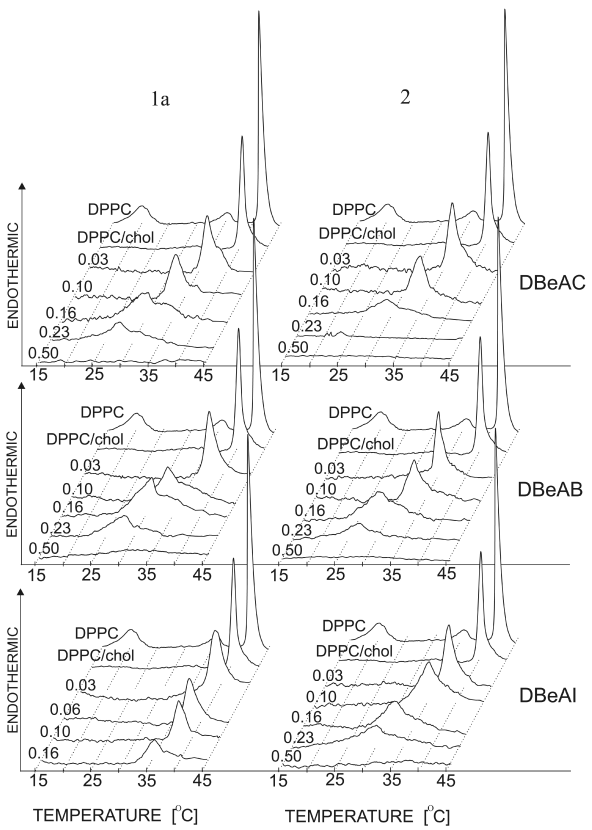

Fig. 1. DSC heating curves of MLVs with increasing molar ratio of DBeAX to DPPC/10 mol% cholesterol. The curves were normalized for the amount of DPPC: (1a), method 1a; (2), method 2.

for gel to liquid-crystalline phase transition of DPPC/cholesterol bilayer progressively broadens and gets asymmetrical with increasing concentration of DBeAX. The effect is more apparent for higher contents of cholesterol as shown on Fig. 1. In the case of DBeAC and DBeAB the peakes are broader and more asymmetrical for method 1 than for method 2 while for DBeAI the peakes are broader for method 2. The phase transition is not detected at the molar ratio DBeAX/DPPC about 0.5, while for DBeAC for method 2 even at the molar ratio 0.23.

At cholesterol contents of approx. 1–2 mol% to 20–25 mol% the main phase transition of DPPC/cholesterol can be considered as consisting of superimposed sharp and broad melting components (McMullen and McElhaney, 1995). The sharp component corresponds to the transition in cholesterol-poor domains and the broad component to the melting of cholesterol-rich domains. Accordingly, we analyzed the heating curves for DBeAX/DPPC/10 mol% cholesterol mixtures in the neighborhood of the main peak by unfolding into the straight baseline and two Gaussian curves fitted to the DSC data using the least-squares method. The resulting parameters, *i.e.* Gaussian's maximum positions T_m^I, T_m^{II} and half-maximum widths (FWHMs) $T_{1/2}^I$, $T_{1/2}^{II}$ were considered as temperatures and widths of the sharp (I) and broad (II) component of the transition, respectively. The obtained values are listed in Table II. For DPPC/cholesterol mixtures without surfactant the transition component I can be assigned to cholesterol-poor and component II to cholesterol-rich domains. In the case of method 1, when a DBeAX molecule enters the phospholipid bilayer in which cholesterol-poor and cholesterol-rich domains already exist, it may locate either in domains of one type or in both. So, the components I and II can be assigned to cholesterol-poor and cholesterol-rich domains. From the changes in widths and in temperatures of both components (Table II) it may be concluded that the compounds seem to locate in both domains but DBeAI enters the cholesterol-rich domain at significantly lower degree. For samples prepared with method 2 uniform location of DPPC, DBeAX and cholesterol molecules in liposomes might be expected. If it is the case, the transitions should be symmetrical as it was observed for DPPC/cholesterol liposomes in the presence of DTAX (Różycka-Roszak and Pruchnik, 2000a). Anyway, in the system studied in the

$n_{DBeAX}/n_{DPPC/chol}$		DBeAC		DBeAB		DBeAI	
		1a	2	1a	2	1a	2
0.03	T_m^I	36.2	36.5	36.5	36.8	39.1	37.4
	T_m^{II}	38.4	38.1	37.5	38.1	40.1	38.8
	$T_{1/2}^I$	1.9	1.6	1.6	1.3	2.2	2.1
	$T_{1/2}^{II}$	2.4	2.8	4.2	2.7	3.7	4.5
0.10	T_m^I	33.1	32.6	32.6	32.7	37.0	31.8
	T_m^{II}	36.3	34.9	36.0	35.5	39.4	33.8
	$T_{1/2}^I$	2.8	2.3	2.8	2.5	2.2	3.9
	$T_{1/2}^{II}$	3.3	4.6	6.5	5.5	4.1	7.1
0.16	T_m^I	28.0	28.7	29.9	30.0	34.7	28.7
	T_m^{II}	33.2	33.8	35.0	29.5	38.3	32.7
	$T_{1/2}^I$	5.5	5.5	5.8	4.8	3.1	4.8
	$T_{1/2}^{II}$	4.6	6.3	9.1	6.7	5.4	8.9

Table II. Temperatures of the maximum (T_m^I and T_m^{II} [°C]) and half-maximum widths ($T_{1/2}^I$ and $T_{1/2}^{II}$ [°C]) of Gaussians after decomposition of the main phase transition DSC peaks for DBeAX/DPPC/10 mol% chol mixtures.

present paper, the transitions are broad and asymmetrical also for method 2 (Fig. 1) and in the case of DBeAI even more than for method 1. For both methods there are no significant differences in the transition components parameters T_m^I and T_m^{II} as well as $T_{1/2}^I$ and $T_{1/2}^I$ (Table II). Therefore it seems to be likely that also for method 2 the domains with different content of cholesterol are formed.

With increasing content all DBeAX decrease the transition temperature of component I more than of component II and they reduce the cooperativity (*i.e.* increase width) of component II more than of component I. DBeAC and DBeAB decrease the transition temperatures of both components for both methods to approx. same extend while DBeAI does it significantly less for method 1. Iodide reduces the cooperativity of both components more for method 2 while bromide for method 1. Chloride affects the cooperativity of component I in a nearly same way for both methods, while lowering of cooperativity for component II is bigger for method 2 than for method 1.

^{1}H NMR

The effects of DBeAC and DBeAB on ^{1}H NMR spectra of DPPC liposomes prepared according to both methods were recorded in the lipid gel state (25 °C) and in the liquid-crystalline state (45 °C). In the lipid gel state the lineshape is broadened compared with the liquid-crystalline state where the lineshape is considerably narrower. Considerable line narrowing results upon addition of DBeAX.

Aromatic protons

In aqueous solution below the critical micelle concentration (CMC) equal to 4.3 mM (Różycka-Roszak, 1990), aromatic protons of DBeAX give only one signal (Fig. 2). At higher concentration (above CMC) the signal separates into two groups of signals (Fig. 2). The effect was in details studied before (Różycka-Roszak and Cierpicki, 1999) and explained as due to changes in the position of the benzyl group during the micellization and its location inside the micelle. On the basis of ^{1}H–^{13}C HMQC and NOESY spectra it was possible to assign a signal at a lower frequency to protons in positions 3, 4, 5 and a signal of higher frequency to protons in positions 2, 6. This indicates that protons 3, 5 and 4 move into a less polar environment rather than protons 2 or 6.

In liposome dispersions the lineshape and linewidth of the aromatic protons resonance originating from the benzyl group of DBeAX are significantly changed compared with an aqueous solution. At 45 °C, like it was in the case DPPC liposome, the signal of aromatic protons in DPPC/cholesterol liposome dispersions separates into two broad signals even at concentrations below CMC (Fig. 2). This suggests that, like in the micellization, the benzyl group also changes its position during the interaction with bilayers and incorporates into them. Likewise, it seems reasonable to

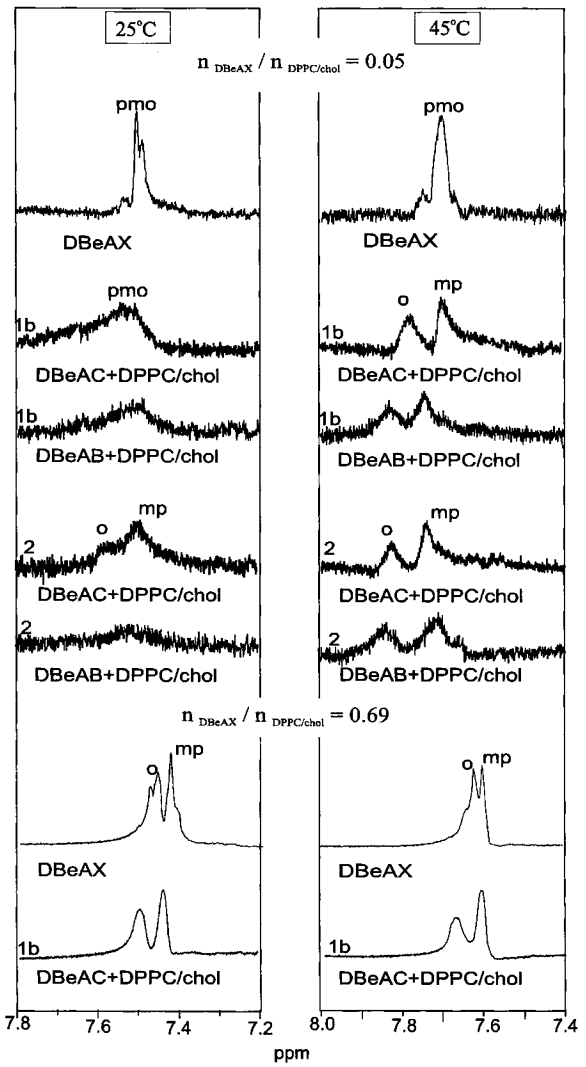

Fig. 2. ^{1}H NMR spectra of DBeAX at 25 °C and 45 °C. The figure shows changes in aromatic signals of DBeAC and DBeAB in the absence (upper curves) and in the presence of DPPC/5 mol% cholesterol liposomes; (1b), method 1b; (2), method 2. The concentrations of DBeAC and DBeAB were 1 mM (molar ratio $n_{DBeAX}/n_{DPPC/chol}$ = 0.05) and 15 mM (molar ratio $n_{DBeAX}/n_{DPPC/chol}$ = 0.69). Small letters denote chemical shifts of protons in positions 2, 6 (*o*, *ortho*), 3, 5 (*m*, *meta*), and 4 (*p*, *para*).

assign a signal at lower frequency to protons in positions 3, 4, 5 and a signal at higher frequency to protons in positions 2, 6 (Różycka-Roszak and Cierpicki, 1999; Różycka-Roszak and Przyczyna, 2003).

At 25 °C (lipid gel state) the NMR lineshape is significantly broadened compared with the liquid-crystalline state. The intensity of the aromatic proton signal is higher for DBeAC than for DBeAB for method 2 while for method 1 it is approx. the same. Differently as in the liquid-crystalline state, the signal of aromatic protons does not separate into two. This suggests that the benzyl group of DBeAX, opposite as it was in the case of DPPC without cholesterol (Różycka-Roszak and Przyczyna, 2003), is not inserted into the gel phase of liposomes for both methods. For steric reasons the benzyl group seems to be more likely located on the surface than inside the phospholipid bilayer. Such location seems to be especially likely in the gel phase, since cholesterol increases the cross section area per head group of DPPC in that phase. In the liquid-crystalline phase cholesterol decreases the cross section area per head group of DPPC (it is known as condensing effect of cholesterol) and probably this is the reason why the benzyl group changes its position and locates inside the lipid bilayer.

Alkyl chain protons

The effects of DBeAC and DBeAB on ^{1}H NMR spectra of DPPC liposomes are in general similar to those without cholesterol (Różycka-Roszak and Przyczyna, 2003). The greatest difference is seen in the effect on $(CH_2)_n$ proton signal in the gel phase at a concentration below CMC. At the concentration 1 mM (below CMC), it seems reasonable to assign the $(CH_2)_n$ signal to protons of lipid alkyl chains. Addition of DBeAX causes narrowing of the $(CH_2)_n$ proton signal, indicating increased mobility of the alkyl chains. For DPPC/cholesterol liposomes the narrowing is more apparent in the case of DBeAC than DBeAB (it was opposite for DPPC liposomes without cholesterol) particularly for method 2. Results presented in this paper show that the interaction of DBeAX with DPPC/cholesterol bilayers and the consequent changes in the phospholipid organization depend on the kind of counterion. The strongest effect was observed for chloride and iodide. This seems to be mainly due to the ability of the counterion to enhance structural nonconformability between cholesterol and DBeAX molecules. DBeAI, with the largest counterion, probably enhances the structural nonconformability mostly for steric reasons while

DBeAC having the smallest but most electronegative counterion causes a similar effect by disturbing cholesterol-lipid interactions.

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