^1H NMR Spectroscopic Studies of Interactions between Dimyristoylphosphatidylcholine (DMPC) and Europium Nitrate in the Presence of Tetracaine

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Abstract— ^1H NMR spectroscopic studies have been carried out for multidispersed vesicles of DMPC and DOPC in the presence of $\text{Eu}(\text{NO}_3)_3$ and anesthetics (tetracaine and lidocaine). ^1H NMR signals of DMPC vesicles disappeared, while those of DOPC did not, in the coexistence of $\text{Eu}(\text{NO}_3)_3$ and the anesthetics. An addition of EDTA-Na_4 to this solution regained the signals of DMPC vesicles, implying cancellation of the effect of $\text{Eu}(\text{III})$ ions by chelating with EDTA anions. Inter-vesicular aggregation was proposed as a molecular mechanism responsible for the signal disappearance.

It is well known that ultrasonication of an aqueous suspension of phospholipids extracted from cell membranes gives the so-called vesicles which are small hollow spheres with a unilamellar wall composed of a bilayer of phospholipid molecules (1) and that this lipid bilayer acts as a barrier for metal ion penetrations (2). This finding permits one to regard the vesicles as a model of the cell membrane and stimulated many investigators to study its properties from various points of view. A number of NMR spectroscopic studies have been carried out since then.

Addition of a small amount of lanthanide ions to egg yolk lecithin dispersions was found to induce a splitting of the choline methyl signals, implying that the two signals correspond to either of the choline methyl protons in the exterior or interior layer (3, 4). This lanthanide-ion-induced splitting was found to be sensitive to some local anes-

thetics. It has been found that tetracaine added to egg lecithin containing $\text{Eu}(\text{III})$ ions reduces the induced splitting, but procaine has very weak power to reduce it (5–8). This suggests that the two local anesthetics might have different molecular mechanisms in interactions with nerve cell membranes. The present study was carried out to examine if such interactions of anesthetics show an acyl chain dependence and to get some information about the molecular mechanism of the interactions, by using synthetic phospholipids, DMPC and DOPC, instead of egg yolk lecithin, a mixture of many kinds of phospholipids.

Materials and Methods

Vesicle preparation: Phospholipids were purchased from Sigma Chemical Company and used without further purification. DMPC powder was suspended in D_2O in 1% w/v, and the resultant mixture was bubbled with N_2 gas for five minutes to purge the dissolved air. After tight sealing, the suspension was sonicated with an ultrasonic cleaner (Sharp Model UT-51N) until it became almost transparent. A chloroform solution of DOPC,

Abbreviation: DMPC, Dimyristoylphosphatidylcholine. DOPC, Dioleoylphosphatidylcholine. DPPC, Dipalmitoylphosphatidylcholine. EDTA-Na_4 , Ethylenediaminetetraacetic acid tetrasodium.

Fig. 1. Molecular structures of tetracaine and lidocaine.

placed in a round bottom flask, was evaporated by flushing N_2 gas to leave a thin film of the phospholipid on the wall. An appropriate amount of D_2O was added to obtain a suspension of 1% w/v concentration. Subsequent sonication was the same as described above. The resultant dispersions of phospholipids were used without any further characterization, probably containing multilamellar vesicles to some extent.

Reagents: Tetracaine hydrochloride, lidocaine hydrochloride (Fig. 1), and europium nitrate hexahydrate were purchased from Sigma Chemical Company.

^1H NMR measurements: ^1H NMR spectra were recorded on a JEOL GX-400 NMR spectrometer at ambient temperature ($24 \pm 0.5^\circ\text{C}$) in the Fourier transform mode. Free induction decays were accumulated under the following conditions: flip angle, 45° ; data points, 16 K; spectral width, 4 kHz; digital resolution, 0.0012 ppm; pulse repetition, 7.05 sec. Chemical shifts were read to adjust the HDO signal at 4.790 ppm. The pH values of these dispersions were between 5 and 6 (± 0.5), as measured with a strip-type pH indicator by Merck.

Results

The ^1H NMR spectra of DOPC and DMPC vesicles are shown in Fig. 2. The two spectra are very similar to each other except for a few signals arising from the protons located around the unsaturated bond in DOPC. Our attention is focussed on the choline methyl

Fig. 2. 400 MHz ^1H -NMR spectrum of DMPC vesicles (1% w/v) (a) and DOPC vesicles (1% w/v) (b).

signals and so they are marked by an asterisk in the figures.

The choline methyl protons of DOPC vesicles give a sharp signal at δ 3.27, whereas those of DMPC give two sharp signals at δ 3.23 and 3.27 with considerably different peak heights. The taller one of the two peaks can be assigned to the choline methyl protons of phospholipid molecules in the exterior layer and the other one to those in the interior layer. The intensity ratio, $I_{\text{out}}/I_{\text{in}}$, obtained for several separate preparations, ranged from 1.2 to 1.4, suggesting that the vesicles' diameters are about 400–600 Å.

In the spectrum of DMPC, some peaks other than the choline methyl signals show unresolved structures, suggesting a different magnetic environment between the exterior

and interior layers. On the other hand, the corresponding signals of DOPC vesicles are more symmetrical and narrower, probably because of a smaller difference in magnetic environment between the two layers.

Almost the same spectra were obtained for these solutions even after being kept at room temperature for a week.

Addition of tetracaine

An aqueous solution of tetracaine was added to a vesicle dispersion of DMPC or DOPC. The ^1H NMR spectrum from tetracaine was slightly broadened, but there was only a single set of signals assignable to tetracaine, indicating that chemical exchange between tetracaine molecules free from and bound to the vesicles can be supposed to be rapid on the NMR time scale. Line broadening is clearly observed on the aromatic proton signals, but is not clear on the *n*-butyl signals because of the partial overlapping with the signals from the acyl side chains of phospholipids (Fig. 3). This suggests involvement of the benzene ring in the interaction and probable involvement of the *n*-butyl. Therefore, it is supposed that the *n*-butyl-aminobenzoyl group is embedded in the lipophilic region of the vesicles.

Addition of tetracaine to DMPC vesicles caused a slight upfield shift of the choline methyl signal from the exterior molecules, while the signal from the interior molecules remains almost unchanged (Table 1). This may be understood as a result of interaction of tetracaine with phospholipid molecules in the exterior layer of vesicles. In the case of DOPC vesicles, addition of tetracaine caused a slight splitting of the choline methyl signal, also suggesting a preferential interaction of tetracaine with phospholipid

molecules in the exterior layer of the vesicles.

Addition of $\text{Eu}(\text{NO}_3)_3$

As the added amount of $\text{Eu}(\text{NO}_3)_3$ was increased, the two choline methyl signals of DMPC vesicles moved oppositely, coalesced at a certain amount of $\text{Eu}(\text{NO}_3)_3$, and separated again at a greater amount of $\text{Eu}(\text{NO}_3)_3$ (Table 2). The movement of the taller signal at lower field toward the upfield direction was greater than the movement of the other signal at higher field toward lower field. The former was broadened while the latter was kept sharp with addition of $\text{Eu}(\text{NO}_3)_3$. Figure 4a shows the ^1H NMR spectrum of the mixture of DMPC (1% w/v, 0.5 ml) and $\text{Eu}(\text{NO}_3)_3$ (40 mM, 0.1 ml). These observations are consistent with the signal assignment given above.

In the case of DOPC, the addition of $\text{Eu}(\text{NO}_3)_3$ induced a splitting of the singlet

Fig. 3. ^1H NMR spectrum of the mixture of DMPC vesicles (1% w/v, 0.5 ml) and tetracaine (20 mM, 0.1 ml). The letter T indicates the signals from tetracaine molecules.

Table 1. Chemical shifts of the choline methyl protons of DMPC vesicles mixed with various amounts of tetracaine

	DMPC (1% w/v)	Tetracaine (x mM)	$\text{N}(\text{CH}_3)_3$	
			In	Out
1	0.5 ml	—	3.226	3.272
2	0.5	0.1 ml (5 mM)	3.224	3.267
3	0.5	0.1 (10 mM)	3.222	3.262
4	0.5	0.1 (20 mM)	3.222	3.258
5	0.5	0.1 (30 mM)	3.223	3.258

signal from the choline methyl protons into two. Figure 4b shows a typical spectrum of such mixtures. This europium-induced splitting clearly relates to the bilayer structure of the vesicles. One of the two signals remains sharp and is located at nearly the

original position, indicating that the signal comes from the molecules in the interior layer, and the other one, broader and shifted upfield, comes from the molecules in the exterior layer.

After 24 hr, almost the same spectra were

Table 2. Chemical shifts of the choline methyl protons of DMPC vesicles in the presence of various amounts of $\text{Eu}(\text{NO}_3)_3$

	DMPC (1% w/v)	$\text{Eu}(\text{NO}_3)_3$ (20 mM)	$\text{N}(\text{CH}_3)_3$	
			In	Out
1	0.7 ml	—	3.226	3.272
2	0.675	0.025 ml	3.240	3.240
3	0.65	0.05	3.246	3.209
4	0.60	0.10	3.260	3.174

Fig. 4. ^1H -NMR spectra of DMPC (a) and DOPC (b) vesicles (1% w/v, 0.5 ml) mixed with $\text{Eu}(\text{NO}_3)_3$ (40 mM, 0.1 ml).

Fig. 5. ^1H -NMR spectra of DOPC vesicles (1% w/v, 0.5 ml) mixed with $\text{Eu}(\text{NO}_3)_3$ (40 mM, 0.1 ml) in the presence of tetracaine (40 mM, 0.1 ml) (a) and lidocaine (40 mM, 0.1 ml) (b).

re-recorded for both vesicles, DMPC and DOPC, in the presence of $\text{Eu}(\text{NO}_3)_3$. This finding is explained in terms of (i) $\text{Eu}(\text{III})$ ions can not penetrate into the phospholipid bilayer membranes, (ii) $\text{Eu}(\text{III})$ ions interact only with the molecule in the exterior layers, and (iii) these dispersions are not unstable. **Co-operative effect of $\text{Eu}(\text{NO}_3)_3$ and tetracaine or lidocaine**

a) DOPC vesicles: Figure 5a shows the ^1H NMR spectrum of a DOPC dispersion containing tetracaine and $\text{Eu}(\text{NO}_3)_3$. As compared with those in Fig. 4b, (i) the distance between the two peaks is smaller, and (ii) the sharpness of the upfield signal is regained. It can be said, therefore, that tetracaine interacts with vesicles to prevent $\text{Eu}(\text{III})$ ions from approaching to the polar head groups.

In Fig. 5b is shown the ^1H NMR spectrum

of a DOPC dispersion containing lidocaine and $\text{Eu}(\text{NO}_3)_3$. It is noticed that the separation between the two choline methyl signals remains almost unchanged compared with that in Fig. 4b and that the sharpness of the upfield signal was not regained. From these observations, it may be said that lidocaine hardly prevents $\text{Eu}(\text{III})$ ions from attacking the choline methyl groups.

Similar results were reported for a system of egg yolk lecithin, EuCl_3 , and tetracaine or procaine (7).

b) DMPC vesicles: The spectra in Fig. 6a and 6b show that the signals from DMPC phospholipid molecules were reduced in intensity for some reason. Especially, in Fig. 6b, the signals from DMPC molecules almost disappear, while those of tetracaine remained apparent, although they are less resolved. Figure 6c and 6d show the ^1H NMR spectra

Fig. 6. ^1H -NMR spectra of DMPC vesicles (1% w/v, 0.5 ml) mixed with $\text{Eu}(\text{NO}_3)_3$ (x mM, 0.1 ml) and tetracaine (y mM, 0.1 ml) or lidocaine (z mM, 0.1 ml): 5a, x=y=20; 5b, x=y=40; 5c, x=z=20; 5d, x=z=40.

for an aqueous dispersions of DMPC accompanied with $\text{Eu}(\text{NO}_3)_3$ and lidocaine instead of tetracaine. Shrinkage of DMPC signals is also observed in Fig. 6d. This observation suggests that $\text{Eu}(\text{III})$ ions play an important role in the signal disappearance.

A close examination of Fig. 6a and 6c indicates that tetracaine can, but lidocaine hardly can, prevent $\text{Eu}(\text{III})$ ions from approaching to the choline methyl groups of the vesicle membranes. Furthermore, a comparison of Fig. 6b and Fig. 4a, in both cases $\text{Eu}(\text{III})$ ions exist at the same concentration, seems to suggest that tetracaine enhances interactions between europium cations and DMPC vesicles through some groups other than the choline methyl group.

The signal disappearance of DMPC molecules could not be prevented by adding CaCl_2 of an amount equimolar to $\text{Eu}(\text{NO}_3)_3$, but could by replacing $\text{Eu}(\text{NO}_3)_3$ by CaCl_2 or $\text{La}(\text{NO}_3)_3$. These results seem to point out an important role of paramagnetic $\text{Eu}(\text{III})$ ions.

Interestingly enough, an addition of EDTA- Na_4 solution to the mixed solution in question (corresponding to Fig. 6b) caused reappearance of the signals arising from DMPC vesicles (Fig. 7). The two peaks of the choline methyl protons seen in the figure might be an indication of the continuance of the bilayer structure of the vesicles.

The signals from the four protons of EDTA may appear around this region. However, no signal other than the HDO signal was observed for a mixture of EDTA-

Na_4 (40 mM, 0.2 ml), $\text{Eu}(\text{NO}_3)_3$ (40 mM, 0.1 ml), and D_2O (0.5 ml). This is evidence for chelation between EDTA anions and $\text{Eu}(\text{III})$ cations.

Discussion

It is of interest that the ^1H NMR spectrum of a system, DMPC vesicles (1% w/v, 0.5 ml)+ $\text{Eu}(\text{NO}_3)_3$ (40 mM, 0.1 ml)+tetracaine (40 mM, 0.1 ml), is not composed of the signals from both components but of only those from tetracaine, whereas in the system where DOPC vesicles were used in place of DMPC vesicles, the ^1H NMR spectrum is composed of the signals from both components. How does the disappearance of proton signals from DMPC occur?

A study of quadrupole splitting of deuterium resonance on selectively deuterated nonsonicated DPPC dispersions (9) revealed that $\text{Eu}(\text{III})$ ions have an effect distinctively different from that caused by any other diamagnetic ions examined. However, no detailed discussion about molecular mechanisms was given.

Replacement of $\text{Eu}(\text{NO}_3)_3$ by $\text{La}(\text{NO}_3)_3$ or CaCl_2 regained the DMPC signals. This result indicates that strong electric fields due to highly charged ions and differences in permeabilities of metal ions with different sizes into phospholipid membranes should not be a main reason for the signal disappearance.

The neutron diffraction studies of several selectively deuterated DPPC revealed that water molecules can penetrate to the depth of the backbone of glycerol moieties (10). This observation suggests a possibility of $\text{Eu}(\text{III})$ ions approaching to the glycerol moiety as well as to the polar head groups. From this simple mechanism, however, it is difficult to expect such a complete suppression, as observed, of signals from the acyl side chain protons, since the pseudocontact effects are proportional to the inverse 3rd power of the distances between $\text{Eu}(\text{III})$ ions and the protons in question (11). Therefore, the paramagnetism of $\text{Eu}(\text{III})$ ions is not a main reason for the signal disappearance.

On the other hand, the signals arising from tetracaine (or lidocaine) are well observed,

Fig. 7. Reappearance of DMPC signals on addition of EDTA- Na_4 (40 mM, 0.2 ml) to the solution of which the spectrum is shown in Fig. 5b.

although they are less resolved, at about the same fields as those of aqueous solutions. The observed signals can be attributed to the molecules of anesthetics freely moving in the aqueous phase. This means that only a small fraction of anesthetics could be directly involved in binding to vesicles and causing the signal disappearance with the cooperation of Eu(III) ions, though Yeagle et al. (12) suggested permeation of tetracaine into the exterior layer of egg-yolk vesicles from their carbon-13 NMR study. With respect to the binding states of tetracaine with DPPC, ^2H NMR studies revealed that there are two kinds of binding sites, strong and weak (13, 14). Taking these results into account, the rather broad spectrum of tetracaine observed in the present study is due to chemical exchange between molecules free in the solvent phase and weakly bound to the vesicles, and the molecules in a small fraction strongly bound to the vesicles are assumed to be undetectable.

NMR signals of vesicles are dependent on fluidity, which is the mobility of phospholipid molecules in the layers, and tumbling rate of vesicles. The same tumbling rates may presumably be assumed for any vesicles examined here when their Dalton numbers are similar. The lateral mobility of phospholipid molecules in the bilayer at room temperature depends upon its phase transition temperature between gel and sol phases. The phase transition temperature was reported as -22 and 23°C for DOPC and DMPC, respectively (15). It is clear that DOPC is fluid but DMPC may not be at $24 \pm 0.5^\circ\text{C}$. Therefore, any factors reducing the fluidity of DMPC vesicles may cause broadening of the signals. In this regard, it has been reported for DPPC vesicles (16) that the vesicle size is an important factor to determine the appearance of the observed signals; the choline methyl signal was easily observed even at room temperature when the vesicles are $300 \text{ \AA}$ in diameter, although the phase transition point of DPPC has been reported as 41°C (15) and that a much broader signal was recorded for the DPPC vesicles with a diameter of $900 \text{ \AA}$. From this, it can be said that much larger DMPC vesicles would not be detected in the ^1H NMR

spectrum even if they exist in the system.

Suppose that the signal disappearance of DMPC vesicles is due to reduction in fluidity. Then, the spectrum in Fig. 6a should be interpreted in terms of two components: one is an invisibly broadened spectrum of DMPC vesicles due to reduction in mobility and the other is that of ones not affected. The observed signals themselves are not so broad. This fact indicates that there are two kinds of vesicles; one lacks fluidity in the bilayer for some reason and the other has normal fluidity. This means an irrational state occurs in which Eu(III) ions and tetracaine molecules interact preferentially with some particular vesicles. It is concluded, therefore, that the signal disappearance of DMPC by addition of $\text{Eu}(\text{NO}_3)_3$ and tetracaine (or lidocaine) could not be ascribed to reduction in molecular mobility in the bilayer vesicles themselves.

Suppose that some vesicles cause fusion or aggregation, as another possible mechanism, to change to a larger size by the cooperation of Eu(III) ions and tetracaine. The larger vesicles should tumble much more slowly than non-fused ones do. The former might give broad signals but the latter sharp ones, as in the case. Since the membrane fusion point (T_f) of DMPC was reported as 59°C (17), it is not easy but imaginable that such fusions occur at about 24°C by cooperation of Eu(III) ions and tetracaine (or lidocaine). However, the formation of aggregates may be more plausible.

The fact that addition of EDTA-Na_4 to the three component mixture regained the signals from DMPC molecules, especially the two split choline methyl signals (Fig. 7), seems to prove that the bilayer structures of the DMPC vesicles were not destroyed even when their signals were not observed. This reappearance of the choline methyl signals by addition of EDTA-Na_4 is difficult to explain in terms of a vesicle fusion mechanism, but is rather easy to explain in terms of a vesicle aggregation mechanism. It is assumed that the vesicle aggregation undergoes through intervesicular bridging by Eu(III) ions through the phosphate groups on the vesicle surfaces. When large vesicles are involved in this aggregation, the signal disappearance

would occur with ease. Addition of a chelating agent with higher affinity to Eu(III) ions may release the vesicles and Eu(III) ions from aggregates. The vesicles thus released naturally show the choline methyl signals characteristic of the bilayer structures. Similar mechanisms were proposed for aggregation of phosphatidylethanolamine and phosphatidylserine vesicles by divalent cations (18).

The neutron diffraction studies (10) on several selectively deuterated DPPC and the X-ray study on DMPC·2H₂O (19) revealed that the acylalkyl chains are extended straightly and the polar head groups are located at almost right angle to them. This leads one to think that the surfaces of phosphatidylcholine vesicles are probably very similar in alignment and distribution of formal charges. However, DOPC and DMPC vesicles respond differently to the cooperation of Eu(NO₃)₃ and anesthetics as described above.

These observations suggest stronger interactions of cations with DMPC vesicles than with DOPC vesicles, probably due to difference of fine structure of the vesicle surfaces. In this regards, it has been reported that permeabilities of cations such as Na⁺ and K⁺ for DMPC vesicles show a peak about the phase transition temperature, i.e., about room temperature (20). The supposed mechanism is that phospholipid molecules exist as an mosaic of solid-phase and liquid-phase, accompanying rather large holes along their boundaries. However, Eu(III) ions also do not seem to penetrate easily as mentioned in the results (Fig. 4a).

The present data suggests to us that Eu(III) ions have a higher affinity to the phosphate groups of the vesicles than La(III) and Ca(II) ions have and that the phosphate groups may be more exposed to the aqueous phase, i.e., more interactive with cations, on the surfaces of DMPC vesicles than of DOPC. This difference may lead to the different response described above of DMPC and DOPC vesicles. As for the affinity, it is known that lanthanide cations can be extracted from an aqueous phase by using kerosene solutions of tri-*n*-butylphosphate (21).

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