NMR Study of Choline Methyl Group of Phospholipids

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Abstract : Dimyristoylphosphatidylcholine (DMPC) and 1,2-dimyristoylamido-1,2-deoxyphosphatidylcholine (D₁₄DPC) were studied with ¹H- and ³¹P- NMR. By using the nuclear Overhauser enhancement difference spectra (NOEDF) and the nuclear Overhauser effect spectroscopy (NOESY) in ¹H NMR, we found that the ¹H NMR chemical shift of N⁺(CH₃)₃ of DMPC liposome is different from that of the D₁₄DPC liposome. It was also found that in D₁₄DPC/DMPC mixed liposome D₁₄DPC might prefer to locate in outer leaflet of the lipid bilayer similarly to the case of sphingomyelin (SPH). ¹H-Detected ¹³C NMR has been successfully used to measure the ¹³C spin-lattice relaxation time (*T*₁) of N⁺(CH₃)₃ of DMPC liposome. The sensitivity with this method was higher by 16 times compared with that of ordinary inversion recovery (IR) method.

Key words : Phosphatidylcholine liposome; artificial boundary lipid; NMR; ¹H-detected ¹³C NMR; spin-lattice relaxation time.

Introduction. We have already synthesized a lipid, D₁₄DPC, which carries two amide bonds instead of ester bonds of regular lecithins.^{1,2)} D₁₄DPC was capable of acting as a boundary lipid enabling a significant increase in the glycoprotein reconstitution in the liposomal bilayers.^{1,2)} Therefore, by using this artificial boundary lipid, we could significantly improve efficiency of membrane protein transfer from erythrocyte,³⁾ B16 melanoma cells,⁴⁾ and human platelets⁵⁾ to the artificial boundary lipid-containing liposome. In addition, Nakamura *et al.* demonstrated that the protein transfer using D₁₄DPC-containing liposome is applicable even to an animal tissue such as epithelium of bullfrog tongue.⁶⁾ Shibata *et al.* found very effective tumor rejection of semi-syngeneic mice when sensitized by the D₁₄DPC-containing egg PC liposome. Also in this case, a tumor surface antigen protein was reconstituted into the liposome by the direct protein transfer method from BALBRVD leukemia cell.^{7,8)}

To understand actual behavior and functions of D₁₄DPC in a liposomal membrane, we further studied

its conformation, location and dynamics by using ¹H-, ²H-, ¹³C-, and ³¹P-NMR techniques. In this work, in addition, we applied the ¹H-detected ¹³C NMR technique, which was first developed by Palmer *et al.*,^{9,10)} for liposomal system to obtain ¹³C-*T*₁ of the choline methyl group of phospholipids in the liposome. We certified that this method can significantly improve the measurement sensitivity compared with the case of ordinary IR (inversion recovery) method.

Experimental. Materials. DMPC (Sigma Chemical Co., St. Louis, U.S.A.), D₁₄DPC (Dojindo Laboratories, Kumamoto, Japan) and all other reagents were commercially available.

Preparation of liposomes. Lipids were dissolved in CHCl₃/CH₃OH (2:1 by volume), and the solvent was removed under reduced pressure. The thin film formed on the wall of a test tube was completely dried overnight under vacuum. The lipid thin film was suspended in deuterium-substituted phosphate buffered saline (PBS) (5 mM sodium phosphate and 150 mM NaCl (pD=7.5)). The lipid suspension was passed through a series of Nucleopore polycarbonate membrane filters with subsequently decreased pore sizes (five times each in the sequence of 1,000, 600, 400, and 200 nm, and then ten times each with 100 and 50 nm) set in an extruder (Lipex Biomembranes, Vancouver, Canada).⁵⁾

NMR experiments. NMR were acquired at 500.00

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Fig. 1. ^1H NMR spectra of 50 nm DMPC liposome at different temperatures.

Fig. 2. ^1H NMR spectra of 50 nm $\text{D}_{14}\text{DPC}/\text{DMPC}$ (1:1, by mol) liposome at different temperatures.

MHz for ^1H , 202.35 MHz for ^{31}P , and 125.65 MHz for ^{13}C spectra on a JEOL α -500 NMR spectrometer with a temperature control.

Results and discussion. With DMPC and $\text{D}_{14}\text{DPC}/\text{DMPC}$ (1:1 by mol) liposomes, no dramatic difference was observed in the chemical shift for $(-\text{CH}_2)_n$ and $\omega\text{-CH}_3$ groups of acyl chains except the intensity. However, the situation was quite different for the choline methyl group. Fig. 1 shows the ^1H NMR signals of pure DMPC liposome measured over a range from 3.215 to 3.355 ppm at various tempera-

tures. Fig. 2 shows the ^1H NMR spectra of $\text{D}_{14}\text{DPC}/\text{DMPC}$ (1:1 by mol) liposome.

In unilamellar liposomal system, the ^1H NMR chemical shift of the choline *methyl* group of D_{14}DPC is different from that of DMPC. Of four broad peaks, two peaks of the lower field are assigned to the choline *methyl* group of DMPC, while another two peaks of the higher field are that of D_{14}DPC . They were of course directly assigned by comparing with the chemical shift of corresponding single lipid liposomes. This is really a new finding in this work, because this is contrary to what was previously reported.¹¹⁾ People has believed that the choline methyl groups of the phosphatidylcholine liposomes are in the same chemical environment and subsequently show the same or almost same chemical shift even in a mixed phospholipid liposome. Therefore, the difference in the chemical shift of the choline *methyl* groups between D_{14}DPC and DMPC (Fig. 2) should come from the different chemical environment between the two in the mixed liposomal membrane. In order to understand this unexpected result, we had to study using (1) a NMR spectrometer with higher magnetic field to distinguish small chemical shift difference, (2) at higher temperature to increase resolution, and (3) with a liposome having a suitable size.

It is well known that the choline *methyl* group of phospholipids in liposome shows NOE with adjacent phospholipids.¹²⁾ Therefore, if they are irradiated with appropriate radio-frequency, we can observe the NOE phenomena from the choline *methylene* protons ($-\text{CH}_2\text{CH}_2\text{-N}^+$). Fig. 3a shows a regular ^1H NMR spectrum of the $\text{D}_{14}\text{DPC}/\text{DMPC}$ liposome over a range of 3.85–3.23 ppm at 60°C. A broad peak at 3.717 ppm (the part of lower magnetic field in Fig. 3a) is the overlapped signal of whole the choline *methylene* protons, while peaks of the right hand side are from the choline *methyl* protons of the lipids as described above (Figs. 1 and 2). Figs. 3b and c are NOEDF of the mixed lipid system. We observed NOE on the choline *methylene* proton signal when we irradiated the choline *methyl* proton signal of DMPC in the outer leaflet at 3.295 ppm (*vide infra*). Similarly, when we irradiated the signal at 3.274 ppm, the same NOE phenomena was again observed on the choline *methylene* proton signal (the part of lower magnetic field of Fig. 3c). We further confirmed this assignment by using NOESY experiment. Fig. 4 shows the NOESY spectrum of the $\text{D}_{14}\text{DPC}/\text{DMPC}$ liposome at 60°C. It is easily understood that there are two cross

Fig. 3. ^1H NMR spectra of 50 nm $\text{D}_{14}\text{DPC}/\text{DMPC}$ (1:1, by mol) liposome at 60°C . (a) Regular ^1H NMR spectrum; (b) NOEDF spectrum irradiated at 3.295 ppm; (c) NOEDF spectrum irradiated at 3.274 ppm.

Fig. 4. NOESY spectrum of $\text{D}_{14}\text{DPC}/\text{DMPC}$ (1:1, by mol) liposome at 60°C .

peaks between the choline *methylene* proton signal and the signals that are expected to be the choline *methyl* protons.

Another interesting finding in this work is the asymmetric location of the two lipids, DMPC and

Fig. 5. ^{31}P NMR spectrum of $\text{D}_{14}\text{DPC}/\text{DMPC}$ (1:1, by mol) liposome at 75°C .

D_{14}DPC , in the lipid bilayer. The choline *methyl* protons of the lipid in the inner leaflet resonance at higher magnetic field, while those in the outer leaflet resonance at lower magnetic field.¹³⁾ As seen in the ^1H NMR of DMPC (Fig. 1), there exist two separated peaks; one of them appeared at the higher field corresponds to the lipid locating in the inner leaflet, and the other intense peak at the lower field comes from the lipid in the outer leaflet. The reason may be the following. The lipids in the inner leaflet are more densely packed than the lipids being in the outer leaflet, leading to a requirement of higher field strength for the resonance. When temperature becomes higher, the difference in the chemical shift between the inner and the outer leaflets becomes smaller. This can be understood as evidence that an increase in the mobility of the lipid headgroup causes a decrease in the effect of packing density. The results of NOEDF with the $\text{D}_{14}\text{DPC}/\text{DMPC}$ liposome (Fig. 3) also supported that the signal at 3.274 ppm is from the choline *methyl* group of D_{14}DPC in the outer leaflet, while the peak at 3.265 ppm is from the lipid in the inner leaflet. This was also true in the ^{31}P NMR for the $\text{D}_{14}\text{DPC}/\text{DMPC}$ (1:1 by mol) liposome (Fig. 5). In the ^{31}P NMR, contrary to what was observed in the ^1H NMR, the phosphorous signal of D_{14}DPC appeared at the lower magnetic field than that of DMPC. The assignment was also made by comparison of the chemical shifts in each single lipid system. In any event, judging from these results, it is clear that

Fig. 6. Schematic drawing of intramolecular hydrogen bonding in the D_{14} DPC head group.

D_{14} DPC prefers to locate more in the outer leaflet compared with DMPC.

What is the reason that D_{14} DPC prefers to be more in the outer leaflet than DMPC in the mixed lipid bilayer? This would be due to formation of an intramolecular hydrogen bonding in D_{14} DPC (Fig. 6).^{14),15)} Such the intramolecular hydrogen bonding was also proposed for SPH by Berden *et al.*¹¹⁾ Through ^{31}P NMR studies of eggPC/SPH liposomal system, they found that SPH prefers to locate more in the outer leaflet of the bilayer upon the formation of intramolecular hydrogen bonding. Curvature of the outer leaflet of liposome is smaller than that of the inner leaflet. These may be reasons that D_{14} DPC also has a preference to be in the outer leaflet of lipid bilayers similarly to SPH. To obtain the exact amount ratio of these lipids in the asymmetric location, either in the inner or in the outer leaflet, we are now in progress by mathematical and computational simulation on data of ^1H and ^{31}P NMR in various mixed lipid systems.¹⁶⁾

Membrane dynamics of the D_{14} DPC-containing liposomes is another important problem to us. For this purpose, ^{13}C - T_1 measurement is promising because the relaxation parameters of ^{13}C are governed by dipolar interaction with protons directly attached. In general, the interpretation of the ^{13}C relaxation data does not require whole chemical structure of the molecule under consideration, unlike ^1H relaxation studies. Studies of the ^{13}C relaxation properties for biomolecules were originally based on direct observation of ^{13}C spectra with 1D NMR technique. However, most unfortunately, either ^{13}C -labelled sample or substantial measuring time is required because of its low sensitivity of ^{13}C .¹⁷⁾ Recently, Palmer^{9),10)} and Nicholson¹⁸⁾ proposed the proton-detected carbon NMR method for measurement of ^{13}C - T_1 for methyl group (IS_3 system). This method provides a significant gain in the sensitivity and is useful to obtain the order parameter and the effective correlation time for

Fig. 7. NMR spectra of 50 nm DMPC liposome. (a) Regular ^1H NMR spectrum; (b) Spectrum measured with ^1H -detected ^{13}C method.⁹⁾

methyl group of proteins.^{10),18)} Hence, we tried to apply this proton-detected carbon NMR method⁹⁾ to liposome system. To certify that this method is correct and applicable even to liposomal system, we of course carefully investigated in advance using a simple model, L-Met-methyl- ^{13}C ($^{13}\text{CH}_3\text{SCH}_2\text{CH}_2\text{CH}(\text{NH}_2)\text{CO}_2\text{H}$), in D_2O . We found a very good agreement within error between the results: The T_1 was 6.0 sec by the regular IR method, while it was 6.3 sec by the present method. This certainly confirmed that the method proposed here is reliable and utilizable.

Fig. 7a shows the regular ^1H NMR spectrum of the DMPC liposome at 37°C . The broad and intense peak at 3.28 ppm comes from $-\text{N}^+(\text{CH}_3)_3$, and a peak at 1.35 ppm is mainly of the methylene protons of acyl chains. Another broad peak at 0.93 ppm is due to $\omega\text{-CH}_3$. Fig. 7b shows the ^1H NMR spectrum of the DMPC liposome measured by the ^1H -detected ^{13}C NMR pulse at 37.0°C .⁹⁾ The peak at 1.35 ppm was completely suppressed, while the peaks at 3.28 and at 0.93 ppm were left. By changing the mixing time of the pulse,⁹⁾ the ^{13}C - T_1 for $\text{N}^+(\text{CH}_3)_3$ of DMPC was obtained to be 0.57 ± 0.01 sec. The T_1 value obtained in this work was in good accordance with that previously reported by One and Prestegard using the regular IR method.¹⁹⁾ It is apparent that this new method enhances the sensitivity higher than the value of $(\gamma_{\text{H}}/\gamma_{\text{C}})^2$,^{9),10)} where γ_{H} and γ_{C} are magnetogyric ratios

Fig. 8. ^{13}C NMR spectra for choline methyl groups. (a) $\text{D}_{14}\text{DPC}/\text{DMPC}$ (1:1, by mol) liposome at 60°C . (b) DMPC/SPH (1:1, by mol) liposome at 75°C .

of ^1H and ^{13}C , respectively.

With $\text{D}_{14}\text{DPC}/\text{DMPC}$ -, DMPC/SPH -, $\text{D}_{14}\text{DPC}/\text{DMPC}/\text{cholesterol}$ - and $\text{D}_{14}\text{DPC}/\text{DMPC}/\text{DMPE}$ -liposomes, it was impossible to obtain the independent information about dynamics of $\text{N}^+(\text{CH}_3)_3$ for each lipid when we employed the regular ^{13}C IR method, because all the ^{13}C signals of the choline methyl groups of lipids were overlapped in liposomal system. Fig. 8 shows ^{13}C NMR spectra for the choline *methyl* groups of the $\text{D}_{14}\text{DPC}/\text{DMPC}$ (1:1 by mol) liposome at 60°C and DMPC/SPH (1:1 by mol) liposome at 75°C . Unfortunately, all the choline *methyl* groups appeared at the same position. Although the resolution was not good enough with this method at present to obtain information about dynamics of the mixed liposome system at physiological temperature (37°C), it seems possible to obtain the information if we used higher magnetic field.

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