NMR Observation on Transbilayer Distribution of *N*-[^{13}C]Methylated Chlorpromazine in Asymmetric Lipid Bilayer of Unilamellar Vesicles

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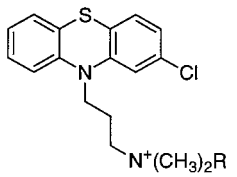
Unilameller vesicles of a suitable size for NMR resolution were successfully prepared with asymmetric bilayer in terms of the phospholipid composition. The preferred distribution of *N*-[^{13}C]methylated chlorpromazine in the inner leaflet of these vesicles were directly observed by liquid NMR using a paramagnetic shift reagent in the external medium, verifying the bilayer couple hypothesis.

Chlorpromazine (**1**; CPZ), an amphiphilic dibenzothiazine being widely used as an antipsychotic medicine, is known to transform erythrocytes from normal discoid to an invaginated "cupped" shape.[1] Such erythrocyte transformation has been generally explained by the bilayer couple hypothesis.[1] According to this hypothesis, cationic molecules such as CPZ tend to reside in the inner monolayer of the erythrocyte membrane due to Coulombic interaction with the acidic polar heads of phospholipids on the inner surface, resulting in asymmetric expansion of the bilayer and thus the invagination of erythrocytes.



1: R = H
2: R = ^{13}CH$_3$

In addition to this hypothesis, there were later found many other factors on the erythrocyte shape change, which are concerned with proteins in erythrocytes.[2] In order to verify contribution of the bilayer couple hypothesis, several microscopic observations have been recently attempted on phospholipid unilamellar vesicles as model membrane systems without proteins.[3] However, there has been no example of the direct observation on the accumulation of small cationic molecules in the inner leaflet enriched with acidic lipids in the unilamellar vesicles.

Under these circumstances, we tried to observe directly an asymmetric distribution of a CPZ derivative in lipid-asymmetric unilamellar vesicles, where acidic lipids are located at the inner leaflet of the bilayer membrane. The fluorescent quenching has been the most common method to investigate behaviors of molecules in vesicle membrane;[4] however, common fluorescent probes are so large (MW > 200) that they may alter the behavior of small molecules such as CPZ (MW = 319) when bound covalently. Thus, we adopted NMR spectroscopy for direct observation of *N*-[^{13}C]methylated CPZ (**2**; [^{13}C]MCPZ, MW = 335), also reported to induce invaginated shape transformation of erythrocytes though not as the isotopically labeled form.[1b,1c] Here the transbilayer distribution of [^{13}C]MCPZ could be estimated by a paramagnetic shift reagent added outside of liposomes, influencing chemical shifts of the molecules located near outer surfaces.[5]

First, preparative conditions of unilamellar vesicles were adjusted for NMR observation. Liquid NMR experiments on liposomes have been carried out in most cases on small unilamellar vesicles, prepared by ultrasonication,[5] of the size under 25 nm diameter[6] in order to minimize the broadening of NMR signals due to dipolar-dipolar interaction between nuclei observed when their isotropic movements are insufficient. These small vesicles, however, are considered not to retain stable asymmetric transbilayer distribution of lipids due to their large curvature at the bilayer. Therefore, we searched for an alternative preparation method of vesicles, which possess stable lipid asymmetry and are yet small enough for NMR resolution (Table 1).

After some experimentation, we found that a method developed by Hope and Cullis[3c,7] is the best among the previously reported procedures. In this method, asymmetric transbilayer distributions of acidic lipids are realized by transbilayer pH gradient. However, the size of asymmetric large unilamellar vesicles (LUVs) prepared according to the reported procedures (Table 1, entry 1) was too large to show sufficient NMR resolution (data not shown). Thus, modified conditions were attempted to obtain smaller asymmetric vesicles. As seen from Table 1, both of the stable lipid asymmetry and relatively small size were realized when asymmetry was introduced to LUVs composed of EggPC-EggPA (10:1) at 70 °C (Table 1, entry 5) after extrusion through two sheets of 50 nm pore filter.[8] This asymmetry was maintained at room temperature at least for 1 day. In the case of DOPC-DOPA (10:1) liposomes of 50 nm diameter, lipid asymmetry introduced at 45 °C was not maintained at room temperature. These results show that EggPA stays more firmly at one side of the bilayer than DOPA, while unsaturated nature of lipids[9] were reported to facilitate their transbilayer movement.[7b]

Table 1. Introduction of lipid asymmetry to unilamellar vesicles. Sizes are based on the pore size of the extrusion filter.

Entry	Composition of Lipids	Size /nm	Temp. /°C	Time /min	PA transported
1	DOPC/DOPA (10:1)	100	45	30	>80%
2	DOPC/DOPA (10:1)	50	45	60	0%
3	EggPC/EggPA (10:1)	100	45	40	0%
4	EggPC/EggPA (10:1)	100	70	90	>80%
5	EggPC/EggPA (10:1)	50	70	60	>80%
6	EggPC/DOPA (10:1)	100	70	80	0%

DO: dioleoyl; Egg: Egg-yolk; PC: phosphatidylcholine; PA: phosphatidic acid.

Next, ^{13}C NMR spectra were recorded for estimating a distribution of [^{13}C]MCPZ in the lipid-asymmetric vesicles as prepared above. The spectra were recorded in time-course from the addition of [^{13}C]MCPZ and paramagnetic Pr^{3+} respectively to the emulsion of EggPC only and lipid-asymmetric vesicles. The results are shown in Figure 1, where the time-course changes in the transbilayer distribution of [^{13}C]MCPZ were shown. The downfield shift of the labeled methyl signal by addition of Pr^{3+} indicates this methyl group of the quaternary ammonium to be located near the surface in the liposomes, as was reported for CPZ.[10] This split became ambiguous when the size of liposomes were larger (data not shown). These split signals should directly reflect the distributions of [^{13}C]MCPZ at the outer and inner surface of the bilayer, since there was a negligible portion of [^{13}C]MCPZ molecules unbound to the vesicles.[11]

Figure 1 revealed that the inner population of [^{13}C]MCPZ molecules was larger in lipid-asymmetric vesicles than that in EggPC vesicles at any measuring time, reflecting accumulation of [^{13}C]MCPZ in the inner leaflet due to its interaction with acidic lipids in lipid-asymmetric unilamellar vesicles, as hypothesized in the bilayer couple hypothesis.

The time-course data also revealed that it took over 10 hours for [^{13}C]MCPZ molecules to be equilibrated between outer and inner leaflets. This is because quaternary ammonium molecules do not diffuse across the lipid membrane so easily as neutral form, as indicated in the previous studies for the behavior of MCPZ in erythrocytes membrane.[1b,1c] According to these studies, CPZ induces invagination of erythrocyte in 10 minutes, whereas MCPZ induces crenation of erythrocytes instead for first 10 minutes and then gradually invaginated the blood cells taking 2 hours.

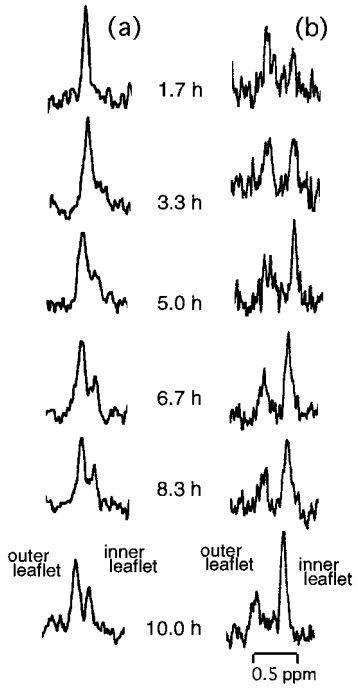


Figure 1. Time-course changes of ^{13}C NMR spectra of the [^{13}C]MCPZ in EggPC/EggPA (10:1) LUVs (size: 50 nm).[12] Signals for the ^{13}C at the outer surface are broadened due to paramagnetic Pr^{3+}, which was present only outside the vesicles.
(a) EggPC. (b) EggPA was rich in inner leaflet.

The present study leads to the first direct observation on accumulation of the cationic compounds in the inner leaflet of the lipid-asymmetric unilamellar vesicles, as the result of successful preparation of unilamellar vesicles with stable lipid asymmetry and the small size enough for NMR resolution. We expect the present study to be widely applicable for extensive NMR investigations on mode of actions for bilayer-targeting smaller molecules.

References and Notes

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8 The typical experimental procedure was as follows in the case of entry 5. Multilamellar vesicles (MLVs) were first prepared by vortexing the lipid film (EggPC, 50 mg; EggPA, 5 mg) in the 2 mL of Tris-HCl buffer [10 mmol dm^{-3} tris(hydroxymethyl)aminomethane, 150 mmol dm^{-3} NaCl, pH 9]. The dispersion of MLVs was then frozen and thawed 15 times and resultant vesicles were extruded through polycarbonate filters of 50 nm 20 times to afford thus sized LUVs. Next in order to induce lipid-asymmetry to LUVs, extravesiculer medium was adjusted to pH 2 by HCl. After LUVs were incubated at 70 °C for 1 hour, the sample was cooled to 25 °C. The extent of asymmetry was estimated with 2-(*N*-*p*-toluidinyl)naphthalene-6-sulfonate, a fluorescent probe sensitive to electric charges of the membrane surface.[7b]

9 The composition of acyl chains of both EggPC and EggPA is: (16:0)/(18:0)/(18:1)/(18:2)/(the others) = 32/17/30/17/4; D. A. White, in "Form and Function of Phospholipids," ed by G. B. Ansell, R. M. C. Dawson, and J. N. Hawthorne, Elsevier, Amsterdam (1973), p 441.

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11 As estimated from the NMR measurement on the supernatant after ultracentrifugation of the corresponding mixture of EggPC MLVs and [^{13}C]MCPZ.

12 In each experiment, LUVs were prepared in Tris-HCl buffer (see Note 8). After the extravesiculer medium was adjusted to pH 2 by HCl, LUVs were incubated at 70 °C for 1 hour, then cooled to 25 °C. To each sample were added $Pr(NO_3)_3$, D_2O, and [^{13}C]MCPZ immediately before NMR measurement; [lipid]/[Pr^{3+}]/[[^{13}C]MCPZ] = 51/9.6/0.034. ^{13}C NMR spectra were recorded on JEOL A-500 (125 MHz, 2000 scans).