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Phosphatidylcholine bearing 6,6-dideuterated oleic acid: A useful solid-state ^{2}H NMR probe for investigating membrane properties

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ABSTRACT

Lipid organization has been at the center of research on lipid rafts. Dioleoylphosphatidylcholine (DOPC) is a typical unsaturated lipid. Very few studies have reported its thermodynamics in raft-like membranes. Herein, we have developed a highly efficient synthetic method for [C6-$^2H_2$] oleic acid, and newly synthesized [C6-$^2H_2$] DOPC. In raft-like oriented bilayers, [C6-$^2H_2$] DOPC shows clear phase separation and characteristic phase behavior at various temperature. It has been successfully utilized for the comparison of membrane properties between sphingomyelin (SM) and dihydrosphingomyelin (DHSM) membranes.

Lipids in cellular membranes heterogeneously distribute and assemble to form microdomains termed lipid rafts, which are usually rich in sphingolipids and cholesterol (chol).[1,2] The rigid chol enhances the ordering of sphingolipids and induces the formation of the liquid ordered (L_o) phase, which has distinct physical properties from that of liquid disordered (L_d) phase.[3,4] Raft formation is thought to be caused by this L_o/L_d phase separation process.[5–7] Uncovering the behavior of individual lipids in these phases is necessary for a better understanding of the molecular mechanisms underlying the biomedical functions of lipid rafts.

Here, we use solid-state ^{2}H NMR to investigate the phase behavior of dioleoylphosphatidylcholine (DOPC), which is a typical L_d component in raft-model membranes, and has often been used to prepare a ternary mixture with sphingomyelin (SM) and chol that forms L_o/L_d-co-existing bilayers. Although incorporation of unsaturated DOPC into L_d phases is energetically favorable,[8] a small amount of DOPC is always found in L_o phases. However, this phase segregation is difficult to detect using a previously reported DOPC probe [C11-$^2H_2$] DOPC.[9,10] In fact, the allylic C11 of oleic acid is not the best position for ^{2}H-labeling since the C9–C10 double bond forces the orientation of the C11–H bonds not to be perpendicular to the membrane normal unlike the orientation of other C–H moieties apart from the C9-C10 double bond. This particular situation of C11 (and C8) results in the smaller magnitude in quadrupolar coupling of the CD_2 moiety, which hampers the observation of the clearly separated ^{2}H signals between L_o and L_d phases. In particular, it becomes more difficult to examine the phase behavior of the L_o domains that contains a small amount of DOPC.

Recent molecular dynamics (MD) simulations suggest that, in DOPC/chol bilayers, chol enhances the order of C6 methylene of an oleoyl group more effectively than that of other carbons in the *sn*-2 acyl chain of the phospholipid.[11] Thus, in ternary SM/chol/DOPC mixtures, [C6-$^2H_2$] DOPC should show clear separated signals of DOPC in the L_o phase. However, the only one synthetic method of [C6-$^2H_2$] oleic acid, the key precursor for the synthesis of [C6-$^2H_2$] DOPC, is a linear 14-step sequence including a classical malonate extension process.[12] Therefore, in this Letter, we first established an efficient synthetic route to this intermediate to prepare [C6-$^2H_2$] DOPC. Then, we compared this new probe with [C11-$^2H_2$] DOPC in the standard wideline ^{2}H NMR recording to confirm the better performance in the L_o/L_d-separated SM/chol/DOPC system. Next, we applied this probe to a more challenging bilayer preparation; SM of the L_o/L_d-co-existing ternary system was replaced by dihydrosphingomyelin (DHSM), which is known to form more ordered domains than SM does.[13] In this highly ordered phase, the content of DOPC probably becomes even smaller, thus making the selective observation of the ^{2}H

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signals of DOPC partitioned in L_o even more difficult. DHSM is known as the major phospholipid of human lens membranes,[14] and also reported to be involved in pathological events such as viral infections.[15,16]

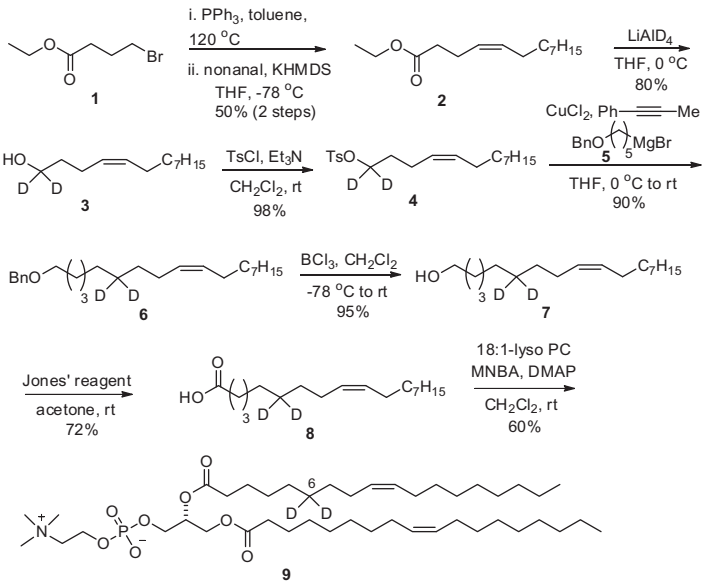
As shown in Scheme 1, the improved synthesis began with the preparation of phosphonium salt from brominated ester **1**, followed by Wittig olefination to give pure *cis*-olefin **2**. Subsequent reduction of the ester with $LiAlD_4$ afforded deuterated alcohol **3**, which was tosylated to give the deuterated intermediate **4**. A recent study suggested that the tosylated precursor is a more suitable coupling partner than the brominated one for C_{sp3}–C_{sp3} Kumada–Corriu coupling.[17] Compound **4** was subsequently coupled with the Grignard reagent **5** prepared from the commercial benzyloxypentyl bromide to afford cross-coupled product **6** in high yield. Benzyl deprotection by boron trichloride followed by Jones' oxidation led to the desired [C6-$^2H_2$] oleic acid **8** in 24% overall yield after 7 steps. Finally, [C6-$^2H_2$] DOPC **9** was prepared using 18:1-lyso PC under typical condensation conditions.

With compound **9** in hand, we first measured the ^2H NMR spectrum for its powder-type hydrated membrane and compared it with the previous probe [C11-$^2H_2$] DOPC in ternary liposomes. The standard wideline spectra were recorded as shown in Figure 1. As expected, **9** generates much more clearly separated peaks than [C11-$^2H_2$] DOPC does. The two doublet peaks in each spectra corresponding to the L_o and L_d phase, respectively, the major fraction of DOPC is in the L_d phase as shown by the larger integrated intensity of the inner doublet. Notably, the peak distance between inner and outer splittings in **9**/SM/chol membranes increased by 1.6 fold compared to [C11-$^2H_2$] DOPC-containing mixtures. The above data confirms that **9** is the better probe appropriate for the L_o/L_d-separated systems.

The macroscopically aligned membranes, in which the bilayer normal is perpendicular to the magnetic field direction, have improved spectral resolution and sensitivity compared to powder pattern samples.[18,19] Thus, we chose to study the thermodynamics of **9** in such an oriented membrane system. In the bottom trace of Figure 2a, the ^2H NMR spectrum of oriented **9**/SM/chol bilayers at 30 °C provided completely separated doublets with even higher resolution than the powder-type ^2H NMR spectra (Fig. 1a). It is clear to see that small fraction of **9** is in the L_o phase, while major fraction pertains to the L_d phase. The integration area ratio of the outer doublet peak to the inner doublet peak is 28/72. As temperature rose, the order of **9** in L_o phase decreased while that in L_d phase increased, and the phase separation was poorly observed at 40 °C. Then, at 45 °C, the spectra gave completely fused signals, and the peak became sharp and less ordered at 50 °C, indicating the membranes were uniformly mixed. The ^2H NMR spectra also shows isotropic components as shown by the central peak. Although the sample contains a small amount of HDO in the water, the isotropic signal is most likely due to the presence of non-oriented structures (such as vesicles) tumbling rapidly in water. As temperature rose, more amounts of isotropic non-oriented phases were formed.

Due to the superior performance of **9** with regard to the clear L_o/L_d phase separation and high sensitivity for phase transitions, we were convinced that this molecule could be further applied to probe the membrane property alteration, which was thought to be closely related to cellular functions. DHSM is the major phospholipid of human lens membranes, whereas being a rather minor constituent in other tissues.[13] However, an enrichment of DHSM was found in the HIV-1 membrane, produced from the host cell through viral release.[20] Recent studies indicated that DHSM is important for HIV-1 gp41-mediated fusion.[15,16] The role of DHSM in regulating membrane properties can be speculated by previous study that DHSM tends to form rigid domains due to the lack of *trans* double bond in the ceramide backbone.[14] To further examine the relevance of DHSM in domain formation, compound **9** was applied to the DHSM-containing ternary system.

As compared with **9**/SM/chol mixtures, less fraction of **9** should be partition to the highly ordered DHSM-enriched phase. Fortunately, small but clearly visible distribution of **9** in L_o phase was detected as shown by the outer doublet in the bottom spectra in Figure 2b. At 30 °C, the integration area ratio of the outer doublet peak to the inner doublet peak drops to 16/84, much lower than that in the SM ternary mixtures, revealing that the greater amount of DOPC is excluded from the DHSM-enriched phase. As temperature rose, the order of **9** in L_o phases decreased slower than that in the SM mixture, and the apparent phase separation was clearly observed even at 45 °C, at which only one doublet appeared in the SM mixtures, implying the thermostability of the L_o domains was obviously enhanced in the DHSM mixture. These results are



Scheme 1. Synthesis of [C6-$^2H_2$] oleic acid **8** and [C6-$^2H_2$] DOPC **9**.

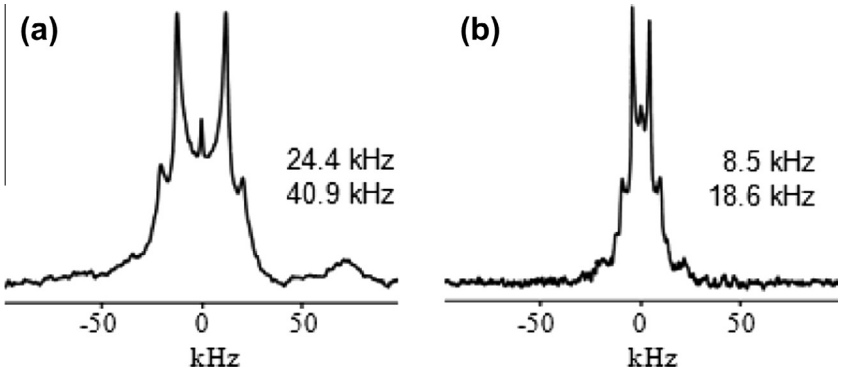


Figure 1. ^{2}H NMR spectra of 50 wt % aqueous multilammellar dispersions at 30 °C: (a) **9**/SM/chol (1/1/1 mol) and (b) [C11-$^{2}H_2$] DOPC/SM/chol (1/1/1 mol).

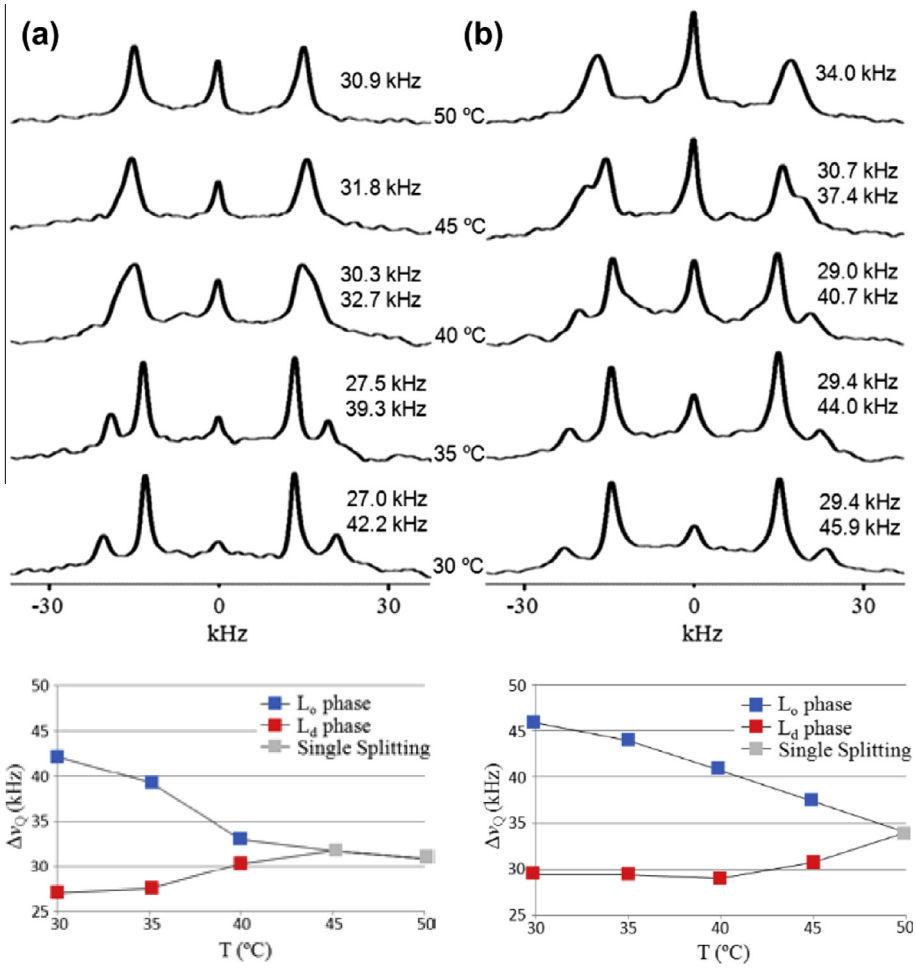


Figure 2. Temperature dependence of ^{2}H NMR spectra and quadrupolar splitting of oriented multi-bilayers formed on glass plates at 96% humidity: (a) **9**/SM/chol (1/1/1 mol) and (b) **9**/DHSM/chol (1/1/1 mol). The angle between the bilayer normal and the magnetic field is set at 90°. The relevant ^{2}H NMR data was compiled in the graphs below for clarity.

in agreement with our recent finding in binary DHSM/DOPC bilayers, in which the DHSM prompts the formation of rigid and stable DHSM-rich domains.[21] Moreover, it is interesting to observe that the order of **9** in both L_o and L_d phases of the DHSM mixture dramatically increased as compared with the SM mixture, indicating that DHSM enhanced the rigidity not only of DHSM-rich L_o phases, but of DHSM-poor L_d phases. It is speculated that DHSM with saturated C_4–C_5 bond increases the accessibility of neighboring lipid molecules, thus leading to an enhanced intermolecular interaction by lipid packing in cellular membranes. The ^{2}H NMR spectra of **9**/DHSM/chol mixtures show larger isotropic components compared to SM mixtures, revealing more amounts of **9**-containing non-oriented structures were formed in DHSM membranes. The distinct differences observed in the ^{2}H NMR spectra of **9** between SM and DHSM ternary mixtures evidently demonstrates the versatility of this DOPC probe for sensing of change in membrane properties.

In conclusion, we established a highly efficient synthetic route to [C6-$^2H_2$] oleic acid **8**, and newly synthesized [C6-$^2H_2$] DOPC **9** to investigate the behavior in raft-like membranes by ^{2}H NMR. Owing to the reasonable deuterated position as well as an appropriate sample preparation of mechanically oriented membranes, compound **9** exhibits completely separated and well-resolved ^{2}H NMR signals stem from the L_o and L_d phases. With these advantages, the DOPC probe was successfully applied to examine difference in membrane properties between SM and DHSM. Their phase behavior between L_o and L_d phases could be easily distinguished. This ^{2}H NMR probe may serve as a useful biochemical tool for investigating various L_o/L_d-co-existing systems.

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Supplementary data

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