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Effect of a Lithocholic Acid Derivative on the Molecular Packing and Stability of Phospholipid Vesicles

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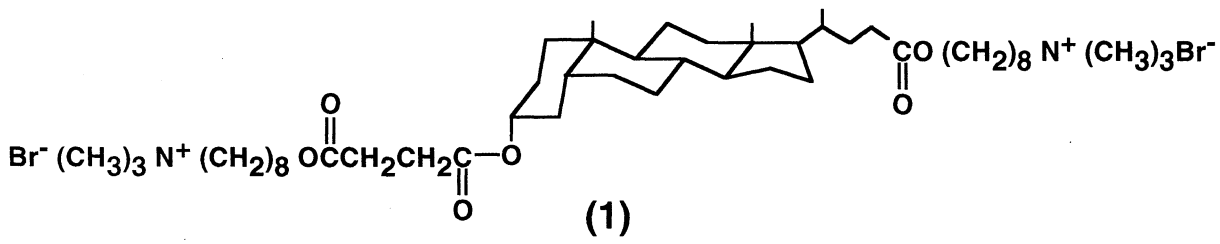
A lithocholic acid derivative having high compatibility with phospholipid was synthesized and incorporated to the phospholipid bilayer membrane. A significant influence of it on the molecular packing and the stability enhancement of the vesicle suspension were clarified by a fluorescence depolarization method and a turbidity measurement, respectively.

The unique properties of bolaamphiphilic molecules in membrane-mimetic chemistry have attracted the curiosities of many scientists in the last decade.[1)] They can self-organize to form monolayer membrane or be incorporated into phospholipid bilayer membrane as membrane-spanning molecules. Based on the extensively studied phenomena of interactions of cholesterol with lipids in a bilayer membrane,[2,3)] some efforts have been made in recent years to introduce a steroid ring as a structural component of synthetic lipids, by incorporation with phospholipids, they can locate their steroid rings near the surface of the membrane.[4,5)] Stimulated by an idea to locate a rigid steroid ring to the center of lipid bilayer membrane and to clarify the different effects of this moiety on the lipid bilayer membrane compared with those of cholesterol, we attempt to use a steroidal moiety as a rigid segment of bolaamphiphile to ensure the entire membrane-spanning packing state in lipid bilayer membrane and thus stabilize the mixed vesicles.[1c)] The first approach we made was the synthesis of a single chain bolaamphiphilic lithocholic acid derivative (**1**), and then the effect of **1** on the molecular packing of phospholipid vesicles was studied by a fluorescence depolarization method and the stability of the mixed vesicles against aggregation was evaluated by measuring the turbidity change.



(1)

Firstly, a diacid compound was synthesized from the reaction of lithocholic acid with succinic anhydride in refluxing dry toluene for 10 h, after recrystallized two times from methanol, it was characterized by IR, NMR spectroscopy (yield 84%). The diacid was then converted to the diacid dichloride by refluxing with

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$SOCl_2$ with a drop of dry DMF as catalyst. After completely dried *in vacuo*, the diacid dichloride was reacted directly with 8-bromo-1-octanol (dry benzene, DMAP, 25 °C, 12h.), affording 45% of the dibromo-intermediate after purification with chromatography (SiO_2, $CHCl_3/CH_3OH$=100/1, v/v). Quaternization of the intermediate with TMA (60 °C, dry DMF, 72 h.) gave 65% of the expected **1**.[6]

Vesicles of dipalmitoylphosphatidylcholine (DPPC) with required molar ratios of **1** for the fluorescence depolarization measurements were prepared by probe-type sonication (60 W, 10 min. 50 °C) of CH_3OH-cast films of DPPC/**1** mixtures in deionized water with a total lipid concentration of 1mM (1M=1mol dm^{-3}). After the vesicle solutions were cooled down to 25 °C, 1,6-diphenyl-1,3,5-hexatriene (DPH) in THF was incorporated into the bilayer of the vesicles at 45 °C ([DPH]/[DPPC]=1/1000). Then the fluorescence spectroscopy was recorded on a spectrofluorometer (JASCO FP-770) equipped with polarizers. The relationship of steady-state fluorescence anisotropy (r) of DPH and temperature was used to evaluate the relative fluidity and phase transition behaviors of the lipid membranes.[7] DPPC/cholesterol vesicles were prepared and studied in the same way as comparison. The mixed vesicles prepared by the same method with a total lipid concentration of 5 mM were used to study the turbidity change of the suspension due to aggregation and fusion by monitoring at 4 °C the change in absorbance at 400 nm (Shimadzu MPS-2000).

When the film of DPPC/**1** mixture was sonicated in an aqueous media, transparent suspensions of mixed vesicles were obtained.[8] The average hydrodynamic diameter of the resulting vesicles was 48±14 nm (Coulter, N4SD), which could be regarded as small unilamellar vesicles. An increase in the ratio of **1** did not change the size of the vesicles obviously. Owing to its high compatibility with DPPC, **1** can be homogeneously incorporated in the phospholipid assembly. Figure 1 shows the temperature-dependent fluorescence anisotropy (r) changes of DPPC vesicles with different amount of cholesterol (A) and **1** (B), respectively. For the DPPC/cholesterol vesicles, at the temperature below the main phase transition of the host DPPC vesicles, incorporation of cholesterol slightly disordered the molecular packing of lipid bilayer. Above the main transition, the effect of cholesterol on the lipid bilayer was much more obvious, r gradually increased with increasing the cholesterol ratio, which suggested the gradual enhancement of the hydrophobic chain packing.[9] These results were coincided very well with those obtained by other methods for DPPC/cholesterol vesicles.[3c] In the case of DPPC/**1** vesicles, however, quite different results were obtained as

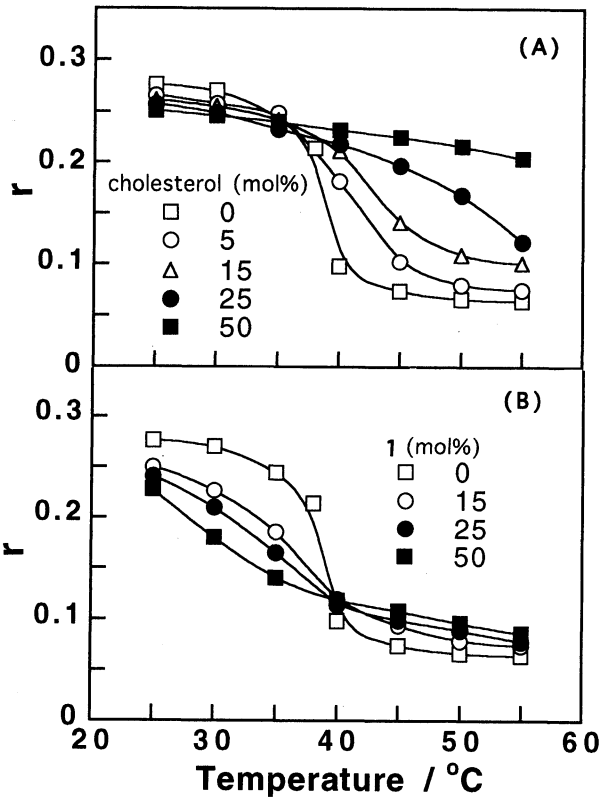

Fig.1. Plots of fluorescence anisotropy (r) vs. temperature for DPPC vesicles of various cholesterol (A) and **1** (B) molar concentrations.

shown in Fig.1(B). Below the main phase transition of DPPC bilayer membrane, the incorporation of **1** decreased the value of r, which meant the increased mobility of DPH in the mixed vesicles. This effect was much clearer when the ratio of **1** was increased in the mixed vesicles. Since DPH locates only in the hydrophobic center of the bilayer membrane, higher movement of DPH also means the decrease of the orientational order of the phospholipid hydrocarbon chains caused by the bulky steroidal moiety at the center of the bilayer membrane. Since the lateral movement of the hydrocarbon chains of **1** might be relatively restricted compared with the lipid hydrocarbon chains, the incorporation of bulky steroidal moiety would create much clefts, and such loose packing state would afford a higher degree of freedom. When results at temperatures above the main phase transition temperature of the host DPPC vesicles were compared, noteworthy phenomenon was observed. Some increase of r with incorporating **1** was clear but not as much as in the case of cholesterol, this might be the weak effect of steroid moiety above T_c similar to the cholesterol effect or it might also be interpreted as that the membrane-spanning packing state of **1** in DPPC vesicles can hinder the movements of DPH and lipid hydrophobic chains as reported earlier that the thermal stability of vesicles was enhanced by membrane-spanning lipids.[1b,c)]

The thermally induced phase transition of the DPPC/**1** mixed vesicles was also studied by differential scanning calorimetric (DSC) measurement (data not shown). It was observed that the endothermal peak of the main phase transition of the pure DPPC vesicles was gradually broadened and moved to lower temperatures with increasing the ratio of **1** ; no obvious phase transition was detected when **1** reached 50 mol%. Since cholesterol incorporation to the phospholipid bilayer membrane broadens the main phase transition peak with slight move to higher temperature, the different effects of steroidal moiety were again clarified. It should be pointed out that head group difference between **1** and DPPC has little effect on the packing state of the hydrophobic region.[10)] As mentioned above, the high compatibility of **1** with DPPC resulted in homogeneous dispersion, no phase separation was observed for the mixed vesicles as studied by DSC.

It is well-known that phospholipid vesicles, especially small unilamellar vesicles, tend to aggregate at temperatures below the gel-to-liquid crystalline phase transition temperature (T_c).[11)] When DPPC vesicles prepared at 50 °C by sonication were incubated at 4 °C, below the T_c of DPPC (41 °C), turbidity inreased rapidly as shown in Fig.2. Incorporation of cholesterol can improve the lipid packing of the bilayer membrane, and thus showed higher stability against aggregation. For the mixed vesicles incorporated with membrane-spanning molecule **1**, good stability against aggregation was also observed even in the incorporation ratio of 15 mol%. The surface charge change of the mixed vesicles might not be the main reason for such an significant effect of **1** on the dispersion state. We thus favored the explanation that the enhancement of molecular motion in the bilayer membrane below the T_c of the mixed vesicles increased

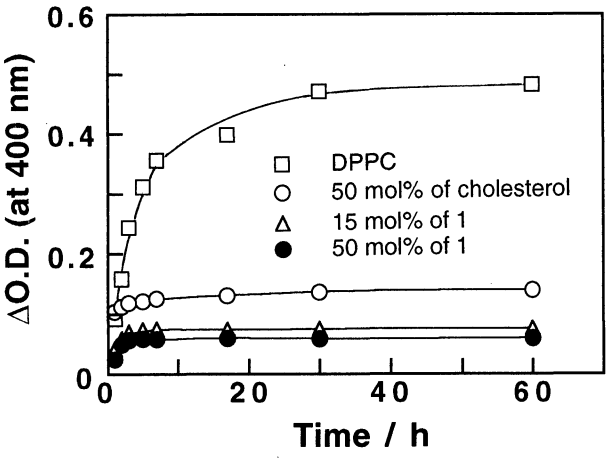

Fig.2. Time-dependent turbidity changes of DPPC vesicles containing cholesterol or **1** at 4 °C.

the disaggregation rate of vesicles.[11]

It is concluded that incorporation of steroidal moiety to the center of DPPC bilayer membrane by using a bolaamphiphilic lithocholic acid derivative **1** can obviously increase the mobility of the membrane below the T_c of DPPC vesicles in comparison with cholesterol and results in higher stability of dispersion state of the vesicles.

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6) Purified by chromatography (SiO_2, 65/30/5, $CHCl_3/CH_3OH/H_2O$); ^1H NMR (270MHz, CD_3OD) δ 4.8ppm (m,1H,C_3 steroid), 4.1 (t,4H,-CH_2OCO-), 3.4(t,4H,-CH_2N-), 3.2(s,18H,-$N(CH_3)_3$), 2.6 (m,4H,-OC(=O)CH_2CH_2C(=O)O-), 0.96 (s,6H,C_{19},C_{21} steroid), 0.69(s,3H,C_{18} steroid), 2.4-0.94(m,52H, all others); FABMS $(M+H)^+$=978.

7) DPH was excited at 357nm and its fluorescence was recorded at 430nm. see also: K. Iwamoto, J. Sunamoto, K. Inoue, T. Endo, and S. Nojima, *Biochim. Biophys. Acta*, **691**,44 (1982); E.W.J. Mosmuller, E.H.W. Pap, A.J.W.G. Wisser, and J.F.T. Engbersen, *ibid.*, **1189**, 45 (1994).

8) Because of the rigid steroid ring, the assembly state of **1** in DPPC/**1** vesicles is the entire membrane-spanning arrangement. Our results of probe bolaamphiphile of this type confirm this expectation (data not shown).

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