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Communication: Rigidification of a lipid bilayer by an incorporated *n*-alkane

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Towards a greater understanding of the effects of organic molecules in biomembranes, the effects of a flexible alkyl chain on the morphologies of phospholipid vesicles are investigated. Vesicles composed of 1,2-dipalmitoyl-*sn*-glycerol-3-phosphocholine and tetradecane (TD) rupture during cooling from the liquid-crystalline phase to the gel phase. A model calculation based on the size-dependent rupture probability indicates that the bending rigidity of the bilayer in the gel phase is more than 10 times higher than that without TD, resulting in the rupture arising from elastic stress. The rigidification is caused by the denser molecular packing in the hydrophobic region by TD. There is little change of the rigidity in the liquid-crystalline phase. Additionally, the rigidification produces a characteristic morphology of the ternary giant vesicles including TD. Reported thermal behaviors imply that molecules with a linear and long alkyl chain, such as trans fatty acids, universally exhibit a similar effect, in contrast to rigid and bulky molecules, such as cholesterol. © *2016 AIP Publishing LLC.* [http://dx.doi.org/10.1063/1.4941059]

Biomembranes in all cells are mainly composed of a phospholipid bilayer. Various small organic molecules, such as sterols, steroids, and isoprenoids, are included in the hydrophobic region of the membrane. These organic molecules have been shown to regulate the functions of biomembranes.[1] For a greater understanding of the regulation mechanism, the investigation of the effects of these molecules on the physicochemical properties of a phospholipid bilayer is crucial. However, these effects are still unclear except for cholesterol, the most typical intramembranous molecule.

An important effect of the intramembranous molecules is the control of the phase behavior. Phospholipid bilayers exhibit a phase transition between the ordered gel (L_β) phase and the disordered liquid-crystalline (L_α) phase depending on their composition and/or external conditions, such as temperature. Cholesterol affects the phase behavior to make the ordered gel phase more disordered (when the cholesterol concentration is higher than approximately 25 mol. %, the disordering effect results in the transition to the liquid-ordered L_o phase[2,3]) and the disordered liquid-crystalline phase more ordered (sometimes termed as the liquid-disordered L_d phase, but it is essentially the same as the liquid-crystalline phase).[2,3] The effect is bidirectional, so the difference in the molecular order between the two phases becomes smaller. The transition temperature (T_M) between these phases is lowered by incorporation of cholesterol.[2,3]

Another important effect of the intramembranous molecules is on the morphology of the bilayers. Changes in the morphology of the bilayers are involved in the cell division, endocytosis, autophagy, and so forth.[1] The bending rigidity is the key quantity to characterize the deformability of the bilayers.[4] Owing to the "bidirectional effect," a bilayer in the gel phase becomes less rigid by the incorporation of cholesterol, while a bilayer in the liquid-crystalline phase becomes more rigid.[4,5] The ratio of the bending rigidity of the liquid-ordered phase is just 1.25 times higher than that of the liquid-crystalline phase with cholesterol,[6] while the ratio is about 10 without cholesterol.[7] The "bidirectional effect" on the morphology of a bilayer vesicle results in an interesting situation when cholesterol is mixed with two lipids that are in the gel and liquid-crystalline phases, respectively. The liquid–liquid phase separation between the liquid-ordered and liquid-crystalline phases is observed on the ternary vesicles, as opposed to the case without cholesterol where the solid–liquid phase separation is observed.[8] The liquid–liquid phase separation is considered to be involved in the raft formation in biomembranes.[9]

In contrast to cholesterol, there have only been a few reports on the effects of other hydrophobic molecules on phase behavior[10–16] and the effect on the morphology has only scarcely been examined. Researchers have, therefore, assumed that other molecules exhibit similar effects on the bilayer morphologies as cholesterol because the incorporated molecules can be treated as nothing but an "impurity" for a lipid bilayer.

Herein, we report the entirely different effect on the bilayer morphologies from that of cholesterol for *n*-alkane. It is found that *n*-alkane makes the lipid bilayer in the gel phase more ordered resulting in drastic changes in the morphology of lipid vesicles, that is, rupture of the vesicles and emergence of a characteristic morphology of ternary vesicles. The observed changes in the morphologies are consistent with the effect of *n*-alkanes on the phase transition behavior.[10,11] This consistency implies that linear and flexible molecules have a similar effect on the bilayer morphology. Indeed, biomembranes include various molecules having linear and long chains,

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such as trans fatty acids, in their hydrophobic region. Since cholesterol is a rather rigid and bulky molecule, the effect on the bilayers seems to be much different. The findings reported here will provide a better understanding of the effect of the incorporated molecules in lipid membranes.

1,2-Dipalmitoyl-*sn*-glycerol-3-phosphocholine (DPPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) for bilayer lipids and tetradecane (TD, >99%) as a typical *n*-alkane were purchased from Wako Pure Chemical Industries and Sigma-Aldrich Co. LLC, respectively. For the observation using a confocal microscope, Rhodamine B 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoetanolamine (Rhodamine-DHPE), triethylammonium salt was purchased from Setareh Biotech LLC, and N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (NBD-PE), triethylammonium salt was purchased from Invitrogen (Molecular Probes).

As the samples for phase contrast microscopy, each of DPPC and TD was dissolved in an organic solvent (chloroform:methanol = 2:1 v/v) in a concentration of 10 mM. The DPPC solution was dropped onto a glass tube, and the organic solvent was dried under vacuum for over 12 h. On the DPPC dry film, the TD solution was dropped so the molar ratio of DPPC and TD was 6:4, and the organic solvent was dried under vacuum for about 30 min to avoid the evaporation of TD. Ultrapure water (MilliQ, 18.2 MΩ cm) was added on the mixed dry film of DPPC and TD so that the lipid concentration was 1 mM. The solution was stored without shaking for over 4 h at 65 °C. Inclusion of TD up to 40 mol. % in a DPPC bilayer was confirmed by differential scanning calorimetry (DSC), using a commercial calorimeter Q200 (TA Instruments), (supplementary material[17]). For confocal microscopy, the sample preparation was similar to that for phase contrast microscopy. Fluorescent molecules (Rhodamine-DHPE and NBD-PE) were added to the DPPC/DOPC dry film so that their molecular ratios were 0.01–0.001 to the total lipids. For wide-angle X-ray diffraction (WAXD), the powder of DPPC and the powder of pure TD were mixed and ultrapure water was added. The lipid concentrations were approximately 150 mM. Samples were sonicated for over 2 h at 50 °C prior to measurement.

For phase contrast microscopy, an IX-71 (Olympus) microscope was used with a 40× objective lens (UPlanFL N 40x/0.60, Olympus) and camera (WAT-213S2, Watec Co., Ltd.). The temperature of the samples was controlled with a hot stage (MATS-55R30, Tokai-Hit). For confocal microscopy, a FV1000-D (Olympus) microscope was used with a 60× objective lens (UPLSAPO 60XS, Olympus). Rhodamine-DHPE and NBD-PE were excited by 559 and 473 nm lasers, respectively. Observations were performed at room temperature (25 °C). WAXD measurement was performed at the BL10C beamline, Photon Factory, KEK, Japan, using a PILATUS3 200K detector (Dectris Ltd.). The wavelength of the X-ray was 0.9864 Å. The sample-to-detector distance and the tilt angle of the detector were calibrated with a standard sample (silver behenate). The measurements were performed at room temperature (22 °C).

Figure 1 shows the typical changes in the morphology of two giant vesicles ((a) and (b)) of DPPC with 40 mol. % of TD under cooling from the liquid-crystalline phase to the gel phase observed by phase contrast microscopy. DSC results (supplementary material[17]) show that the main transition temperature between these phases is approximately 44 °C for this sample. At 50 °C (in the liquid-crystalline phase), the DPPC/TD bilayer forms closed vesicles. During the cooling to 30 °C (in the gel phase), a number of the vesicles rupture. The cooling took a few minutes and the rupture was observed at approximately 40 °C. Since the ruptured bilayers deform to flat plates, the rupture is likely to arise from the elastic stress caused by the rigidification of the bilayers during cooling. As shown in Fig. 1(b), large bilayers sometimes rupture to form the shape where several flat plates are connected. In contrast, the vesicles without TD remain spherical upon cooling and do not exhibit such deformation.

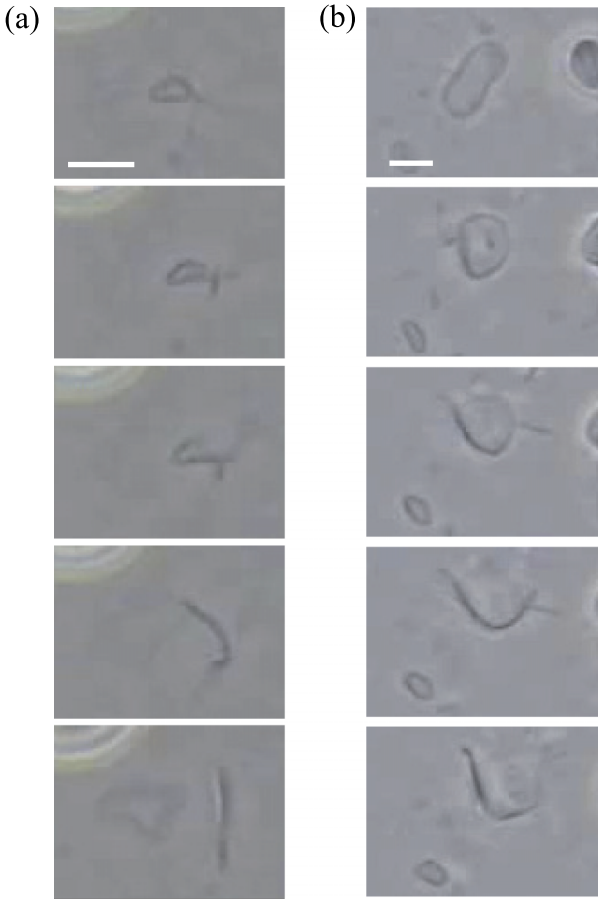

FIG. 1. (From upper to lower figures) Rupture process of DPPC/TD giant vesicles during cooling from 50 °C to 30 °C (from the liquid-crystalline phase to the gel phase) observed by phase contrast microscopy. (a) and (b) show the processes of two different vesicles. Bars are 10 μm.

In Fig. 2(a), the size distributions of closed vesicles are shown for the same sample in both phases (at 50 °C and 30 °C). For both distributions, the lengths of the long axis of over 800 closed vesicles were measured from the microscopy images (excluding the ruptured bilayers in the gel phase). The probability distribution at 50 °C, P(50 °C), has a peak at approximately 2–3 μm. After cooling the sample, the peak of the size distribution of the remaining closed vesicles, P(30 °C), shifts to a larger size. Since the cross-sectional area per lipid is smaller in the gel phase,[18] the distribution should

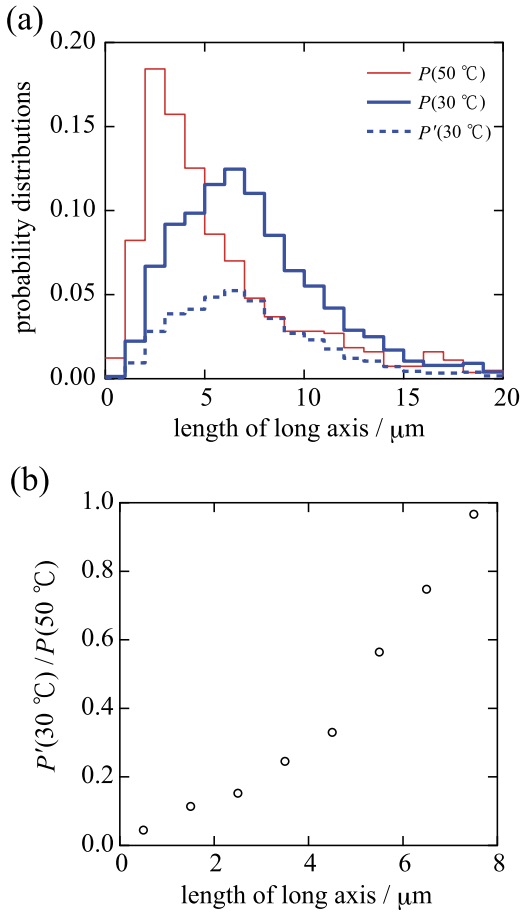

FIG. 2. (a) Size distributions of closed vesicles before and after cooling from 50 °C to 30 °C. The probability distribution is obtained by dividing the number of vesicles within each long axis length range by the total number of vesicles (over 800 for P). P'(30 °C) is obtained by multiplying by a factor that guarantees that P(30 °C) does not exceed P(50 °C). (b) Remaining probability of the closed vesicles after cooling.

shift to a smaller size if the rupture probability is independent of the vesicular size. The result means that smaller vesicles rupture more easily, confirming that an elastic stress arising from the rigidification of the bilayers in the gel phase causes the rupture. To compare the distributions on the basis of the number of vesicles, P(30 °C) is re-scaled down to P'(30 °C), so as not to exceed P(50 °C) at every range of lengths of the long axis. P'(30 °C)/P(50 °C) (Fig. 2(b)) is the largest estimate of the remaining probability during cooling. According to this re-scaling, the vesicles with a length of the long axis of 5 μm rupture with 50% probability, which means the free energies of a closed vesicle and a flat plate coincide with each other at this limit size R_{lim}. Note that P'(30 °C) can be smaller and R_{lim} can be larger than those values assumed here.

The bending rigidity of the DPPC/TD bilayer in the gel phase can be estimated from the limit size as follows. The theoretical model established for the budding of a phase-separated domain in a binary bilayer, which discusses the morphology of the domain with consideration of the bending rigidity κ and the line tension Σ at the domain interface, states

$$\frac{F}{2\pi} = \kappa L^2 C^2 + L\Sigma\sqrt{1-\left(\frac{LC}{2}\right)^2}, \qquad (1)$$

where L is the diameter when the domain is flat and C is the curvature of the domain. When the line tension is dominant for the free energy, the domain buds spherically, while the domain becomes flat when the bilayer is sufficiently rigid. In the present case, the domain is regarded to be freely suspended in water exposing their hydrophobic chains to water.

When the domain forms a complete sphere with diameter R, $\pi L^2 = 4\pi(R/2)^2$, and $C = 2/R$, then the free energy of the sphere is $F_s = 8\pi\kappa$. When the domain is a flat disc, $C = 0$, the free energy is $F_f = 2\pi L\Sigma = 2\pi R\Sigma$. At the limit diameter, these free energies coincide with each other ($F_s = F_f$), leading to $\kappa = R_{\mathrm{lim}}\Sigma/4$. Substituting $R_{\mathrm{lim}} = 5$ μm and $\Sigma = 0.95 \times 10^{-11}$ N (the typical line tension of a DPPC bilayer in the gel phase whose hydrophobic part is exposed to water[19]), the bending rigidity is calculated as $\kappa = 1.2 \times 10^{-17}$ J (ca. $3000k_{\mathrm{B}}T$). Since the bending rigidity of pure DPPC bilayer in the gel phase is $\kappa = 1.0 \times 10^{-18}$ J,[7] the bilayer of DPPC with 40 mol. % TD is more than 10 times as rigid as the pure bilayer in the gel phase. Note that this estimate is a lower boundary of the bending rigidity because the limit size may be larger, as described before.

In the liquid-crystalline phase, the effect of TD on the bending rigidity of DPPC bilayers was evaluated as 1.4×10^{-19} J at 50 °C through analysis of the shape fluctuation of the spherical giant vesicle[20] observed by phase contrast microscopy (detailed results are provided in the supplementary material[17]). Since κ is approximately 1.5×10^{-19} J without TD,[7] the effect of TD on κ in the liquid-crystalline phase is much smaller than that in the gel phase. This small effect is consistent with our previous result from small-angle X-ray scattering.[10]

The molecular origin of bilayer rigidification in the gel phase is investigated using WAXD. In the gel phase, the acyl chains of lipids form a near-hexagonal lattice in plane.[21] From the averaged lattice constant s observed by WAXD, the averaged chain-chain distance $d_c = 2s/\sqrt{3}$ (as an average for the acyl chain-acyl chain, acyl chain-TD, and TD-TD distances) is obtained as a function of the TD concentration C_{TD} as shown in Fig. 3. The chains are packed more densely with an increasing TD concentration

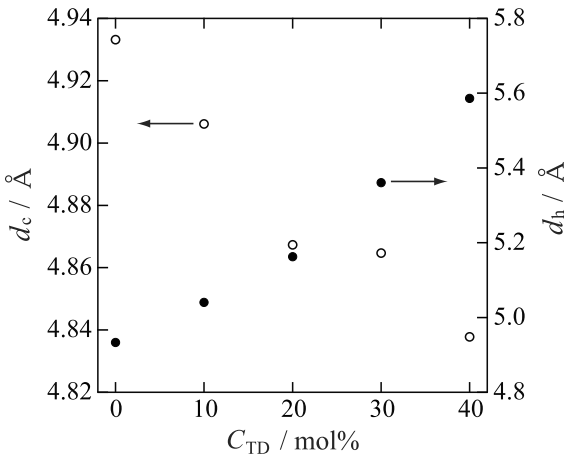

FIG. 3. In-plane chain-chain distance d_c and head-head distance d_h in the DPPC bilayer with TD obtained by WAXD. The d_c is the averaged value for the acyl chain-acyl chain, acyl chain-TD, and TD-TD distances.

though the mean head-head distance, $d_h = d_c\sqrt{(2+x)/2}$, where $x = C_{TD}/(100 - C_{TD})$ is expanded by the addition of TD. At 40 mol. %, the area per single acyl chain (or a TD molecule) is reduced by 4% from that without TD. The decrease in d_c by the addition of *n*-alkane is consistent with the reported results for other lipids.[10] This dense molecular packing in the hydrophobic region is considered to cause the rigidification of the bilayer. In the hydrophobic region of pure phosphatidylcholine bilayers, it has been accepted that gaps exist between the lipids.[22] Incorporated molecules, such as cholesterol, are considered to locate in the gap (the phosphatidylcholine head groups act like an umbrella).[23] It is thus reasonable to assume that TD fits into the gap and the bilayer becomes more stable.

As described in the Introduction, the effect of cholesterol becomes obvious when ternary lipid vesicles are formed. The ternary giant vesicles with DPPC, DOPC, and TD are thus prepared at room temperature (25 °C), as shown in Fig. 4. DOPC is in the liquid-crystalline phase at this temperature. Red fluorescence (Rhodamine-DHPE) is observed in the DOPC-rich liquid-crystalline phase, while green fluorescence (NBD-PE) is in the DPPC-rich gel phase. The ternary vesicles exhibit a characteristic morphology. The DPPC-rich domain forms a flat domain, which looks very rigid, while the DOPC-rich domains flexibly connect the flat domains. This morphology is much different from the case without TD, where the lipids form spherical vesicles. The flat domain is formed by the rigidification of the gel phase caused by TD. Although the content of TD is only 20 mol. % in this case, the rigidification effect is strong. From the microscopic view, the persistence length of the flat domain is estimated to be larger than 40 μm. This seems consistent with the bending rigidity of approximately $3000k_BT$. To the best of our knowledge, this characteristic morphology with very flat domain has never been reported. Detailed phase separation behaviors of ternary vesicles including *n*-alkanes will be reported elsewhere.

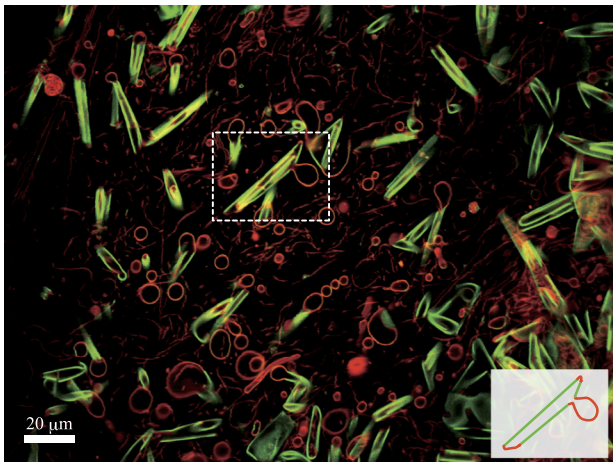

FIG. 4. Confocal microscopy image of the ternary giant vesicles of DPPC, DOPC, and TD (=4:4:2 in molar ratio). An image of the vesicle inside the dashed square is illustrated (bottom right). Red fluorescence (Rhodamine-DHPE) is observed in the DOPC-rich domain in the liquid-crystalline phase, while green fluorescence (NBD-PE) is observed in the DPPC-rich domain in the gel phase.

The effect of the *n*-alkane (rigidification) is found to be entirely contrary to that of cholesterol, which softens the bilayer in the liquid-ordered phase (for example, $\kappa = 0.6 \times 10^{-18}$ J for DMPC with 30 mol. % of cholesterol[5]). Our estimate indicates that, with 40 mol. % of TD, the bending rigidity in the gel phase is higher by about two orders of magnitude than that in the liquid-crystalline phase, while the ratio is approximately 10 without any additives and only 1.25 with cholesterol. That is, the difference between two phases becomes larger with TD, while it becomes smaller with cholesterol. The present study clearly demonstrates that the effects of incorporated molecules in lipid bilayers are not similar but strongly dependent on the incorporated molecule.

The bending rigidity and the phase behavior of lipid bilayers are linked to each other.[24] In our previous study,[10] it was shown that the dense molecular packing in the hydrophobic region by *n*-alkane causes the reduction of the enthalpy in the gel phase, inducing the increase in the main transition temperature T_M between the gel and liquid-crystalline phases. Not only *n*-alkane, but other incorporated molecules with a linear and long alkyl chain of alcohols or fatty acids (e.g., 1-tetradecanol, palmitic acid, and so forth) are also known to increase T_M.[12–14] The increase in T_M is in contrast to the decrease observed for rigid and bulky molecules, such as cholesterol or stilbene.[2,3,16] These results imply that molecules with a linear and long alkyl chain have a similar effect on lipid bilayers: those molecules would cause dense molecular packing of the bilayer in the gel phase resulting in the increase in T_M, and the dense packing would lead to the rigidification of the bilayers. Indeed, the dense packing of a DPPC monolayer is reported for palmitic acid.[25] Therefore, trans fatty acids are expected to exhibit a similar effect on lipid bilayers. Finally, it is noteworthy that palmitoleic acid, which has an unsaturated chain of *cis* form, decreases T_M.[26] This implies that the effect is different when the molecule is not linear.

In summary, DPPC vesicles with 40 mol. % of TD rupture owing to the elastic stress in the gel phase while they form closed vesicles in the liquid-crystalline phase. From the rupture probability as a function of the vesicle size, the bending rigidity is estimated to be over 10 times higher than that without TD. However, in the liquid-crystalline phase, the bending rigidity hardly changes with TD. The dense molecular packing in the hydrophobic region caused by TD is the origin of the rigidification effect. The dense packing is also known to lead to the increase in the phase transition temperature between these two phases,[10] implying the possibility of a similar effect for the incorporated molecules with a linear alkyl chain, such as trans fatty acids. Owing to the rigidification effect of TD in the gel phase, the ternary giant vesicle of DPPC/DOPC/TD exhibits a characteristic morphology. DPPC-rich flat domains are connected by flexible DOPC-rich domains. TD is found to make the bilayer more ordered in the gel phase, while it minimally affects the bilayer in the liquid-crystalline phase, resulting in the enhanced difference in the molecular order of the bilayer between the two phases. This is contrary to the effect of cholesterol, which makes the difference smaller. The present finding indicates that the effect of incorporated molecules in the hydrophobic region of a bilayer is not similar to that of cholesterol in contrast to the previous belief. It is

suggested that the effect can be classified on the basis of the molecular structures of the additives. Trends of the effects should be further investigated more precisely in the future.

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