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Title:

Morphological Estimation of DMPC/DHPC Self-Assemblies in Diluted Condition: Based on Physicochemical Membrane Properties

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ABSTRACT

Self-assembly membranes, composed of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (DHPC), were characterized at the total lipid concentration below 20 mM. The sizes of the assemblies varied depending on the molar ratio of DMPC and DHPC (q = [DMPC]/[DHPC]). The small assemblies with diameter of ca. 10 nm were formed at $q \leq 2.0$ at 20 ºC (below phase transition temperature of DMPC). The physicochemical membrane properties were then studied using fluorescence probes, 1,6-diphenyl-1,3,5-hexatriene and 6-dodecanoyl-*N*,*N*-dimethyl-2-naphthylamine, upon the dilution. DHPC micelle showed a higher membrane fluidity, while the DMPC/DHPC membranes at $q \geq 0.5$ showed lower membrane fluidities as well as DMPC vesicle in gel (ordered) phase. Upon dilution, the ordered membrane properties were maintained while the solution turbidities increased, implying the morphological change of the self-assembly, bicelle to the vesicle in gel phase. Based on the obtained results, a phase diagram of DMPC/DHPC binary system (at 20 ºC) is described: (i) the bicelle suspension is transparent and the membrane is in ordered state, (ii) the micelle suspension is transparent and the membrane is in disordered state, (iii) the vesicle suspension is turbid and the membrane is in ordered state.

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1. INTRODUCTION

Phospholipids form a self-assembled membrane in aqueous solution to reduce an exposure of hydrophobic acyl chain groups. Depending on the chemical structure of phospholipids, the self-assemblies show various morphologies such as vesicle, micelle, and so on. In general, the self-assembly behaviors of amphiphilic molecules are discussed based on the critical packing parameter, which can be estimated from mean head group area, length of hydrocarbon chain, and volume of molecule [1, 2]. One of important characteristics of those self-assembled membranes is to possess both hydrophobic section at inner lipid bilayer and hydrophilic section at surface of membrane. By utilizing such properties, the phospholipid assemblies are applied as a platform to integrate various nano-materials [3-6].

In aqueous solution, long-chained saturated phospholipids (e.g., 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC, C14:0); melting temperature T_m = 23 ºC) often form bilayer membranes, that finally construct spherical vesicles due to the thermodynamic stability of the bilayers [6]. When short-chained phospholipid 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (DHPC, C6:0) is excessively added to a DMPC suspensions, they form micelle-like small assemblies at the total lipid concentration above the critical micelle concentration (CMC): for DHPC, the CMC is 15 mM [7, 8]), in which the small bilayer morphology is also maintained [7, 9-16]. The morphology of small self-assemblies, such as micelle and bilayer-micelle (bicelle), can be dependent on the total lipid concentration, and on the lipid mixing ratio in lipid binary system [7]. Upon the dilution of the small assembly, it has been reported that the bicellar structures are transformed to vesicle structures, because the CMC value of DHPC is higher than that of DMPC (6 nM). The small assembly with bicellar structure has been often employed to reconstruct the

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biomacromolecules and to organize the functional ligands on the lipid membrane [5,17,18]. Usually, a higher concentration of lipid (1-10%, w/w) has been employed to maintain the bicelle structure. Thus, it is beneficial to investigate the phase behaviors of bicelles.

As membranous information, not only the structural information (size and shape) but also the orientation of lipid molecules and phase separation behavior of membranes are important to develop the lipid membrane as functional material [6, 19]. Furthermore, the membrane properties, such as membrane fluidity and polarity, are quite important to characterize the self-assembly membranes [4]. In bilayer systems (vesicles), the phase states, ordered (gel) phase or disordered (liquid crystalline (LC)) phase, can be investigated based on the membrane fluidity and polarity. Below the T_m, saturated phospholipids form ordered bilayer (gel phase). While above the T_m, the hydrocarbon chains are loosely-packed (LC phase). In the case of bicelle (such as DMPC/DHPC systems), however, the physicochemical membrane properties, such as membrane fluidity and membrane polarity, are hardly characterized in previous works, especially, at the lower (or diluted) lipid concentration region (less than 1% lipids, ca. 20 mM) in which there is a phase transition. According to the above, the membrane properties of DMPC/DHPC self-assemblies can be also investigated: it is assumed that the coexistence of excess DHPC destabilizes DMPC bilayer, which will disrupt the ordered membrane properties of vesicle and bicelle assemblies.

The aim of this study is to characterize the DMPC/DHPC self-assemblies, focusing on structure and physicochemical membrane property. The formation of small self-assembly was confirmed by using dynamic light scattering (DLS). At different DMPC/DHPC ratios (q-values) and total lipid concentrations, the membrane properties

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were analyzed by using spectroscopic methods [4, 20, 21]. Based on the membrane properties obtained, the phase behaviors of DMPC/DHPC assemblies at diluted conditions were discussed.

2. MATERIALS AND METHODS

2.1. Materials.

1,2-Dihexanoyl-*sn*-glycero-3-phosphocholine (DHPC) and 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). 1,6-Diphenyl-1,3,5-hexatriene (DPH) and 6-dodecanoyl-*N*,*N*-dimethyl-2-naphthylamine (Laurdan) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium dihydrogenphosphate (anhydrous) and disodium hydrogenphosphate were purchased from Wako Pure Chemical (Osaka Japan), and were used to prepare phosphate buffer (50 mM, pH 7.0). Ultrapure water was prepared with the Millipore Milli-Q system (EMD Millipore Co./Direct-Q® UV3). Other chemicals were used without further purification.

2.2. Membrane Preparation by Thin-Film Hydration Method.

A chloroform solution of phospholipids was dried in a round-bottom flask by rotary evaporation under vacuum. The obtained lipid films were dissolved in chloroform once more, and the solvent was evaporated. This operation was repeated at least twice. The obtained lipid thin film was kept under high vacuum for at least 3 h, and then hydrated with phosphate buffer at room temperature. The phospholipid mixtures (20 mM) were prepared over the CMC of DHPC (15 mM). Since the phase transition temperature (T_m) of DMPC bilayer is 23 ºC, the obtained DMPC/DHPC suspension was frozen at −80 ºC

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and then thawed at 40 ºC (over the T_m of DMPC); this freeze-thaw cycle was repeated five times. Figuring the composition of DMPC/DHPC membranes, the molar ratio of DMPC to DHPC (q) was employed: q = [DMPC]/[DHPC]. The suspensions were prepared with a total lipid concentration 20 mM, and were diluted by the buffer in the measurements of membrane properties. Notably, the DMPC vesicle suspensions were extruded through 2 layers of polycarbonate membranes with a mean pore diameter of 100 nm, using as extruding device (Liposofast; Avestin Inc., Ottawa, Canada). The prepared unilamellar vesicles were kept in 4 ºC until use.

2.3. Dynamic Light Scattering.

The apparent sizes of mixtures (total lipid concentration: 20 mM) were determined by dynamic light scattering (DLS). Measurements were performed with Particle Size Analyzer (LB-500, HORIBA, Kyoto, Japan). The average diameters were calculated based on a number-average diameter.

2.4. Evaluation of Membrane Fluidity.

Physicochemical membrane properties of each self-assembly were investigated, based on previously described methods [20]. A fluorescent probe DPH was added to DMPC/DHPC mixture and vesicle suspensions with a molar ratio of lipid/DPH = 250/1. The fluorescence polarization of DPH (Ex. = 360 nm, Em. = 430 nm) was measured using a fluorescence spectrophotometer (FP-8500, JASCO, Tokyo, Japan) after incubation for 30 min. The sample was excited with vertically polarized light (360 nm), and emission intensities both perpendicular ($I_\perp$) and parallel ($I_\parallel$) to the excited light were recorded at 430 nm. The polarization (P_{DPH}) of DPH was then calculated by using the following

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equations:

$$P_{DPH} = (I_{\parallel} - GI_{\perp})/(I_{\parallel} + GI_{\perp})$$

$$G = i_{\perp}/i_{\parallel}$$

where $i_{\perp}$ and $i_{\parallel}$ are emission intensity perpendicular and parallel to the horizontally polarized light, respectively, and G is the correction factor. The membrane fluidity was evaluated based on the reciprocal of polarization, $1/P_{\mathrm{DPH}}$.

2.4. Evaluation of Membrane Polarity.

Fluorescent probe Laurdan is sensitive to the polarity around itself, which allows the local polarity in lipid membranes to be determined [22, 23]. Laurdan emission spectra exhibit a red shift caused by solvent attack and by solvent relaxation, thus its emission spectrum reflects the polarity (hydration state) of the self-assembly membrane. The Laurdan emission spectra were measured with an excitation wavelength of 340 nm, and the general polarization (GP_{340}), the membrane polarity, was calculated as follows:

$$GP_{340} = (I_{440} - I_{490})/(I_{440} + I_{490})$$

where I_{440} and I_{490} are the emission intensities of Laurdan excited with 340 nm light.

2.5. Turbidity Measurements for Morphology Analysis of Assemblies during Dilution.

To assess the morphological stability of assemblies, the turbidities of DMPC/DHPC mixtures at 500 nm (OD_{500}) were monitored with decrease concentration of the total lipid. A thin quart cell (light path length: 1 mm) was employed to record the varied turbidity. The morphological transition from bicellar assemblies to vesicles can be assessed by measuring the trend of the optical density (UV-1800 Spectrophotometer, SHIMAZU,

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Kyoto, Japan): an increased turbidity in the suspension could be an evidence of growing assembly [24-26].

3. RESULTS AND DISCUSSION

3.1. Membranous Properties of DMPC/DHPC Mixtures.

3.1.1. Structural Properties

The DMPC/DHPC mixtures with total lipid concentration of 20 mM were prepared at several [DMPC] to [DHPC] ratio (q-value). In preliminary experiments, the suspensions of DMPC/DHPC mixtures were confirmed to have high transparency. The size distribution of the self-assemblies formed in the DMPC/DHPC mixtures was first analyzed by using DLS measurement (**Fig. 1**). The observed sizes of DMPC/DHPC mixtures at q = 1.5 and 0.5 were 14.7±3.2 nm and 6.4±1.9 nm, respectively, suggesting that the size of self-assembly dominantly depends on q values. Relevantly, the turbidity (optical density, OD_{500}) of each suspension also depended on the q values. At 20 mM (no dilution), the suspension of DMPC vesicles (i.e. $q = \infty$), prepared by extrusion method (d = 100 nm), was milky turbid, while the suspension of DMPC/DHPC at q = 2 were almost transparent at 20 mM (**Fig. 2**), like DHPC micelle. This suggests that small self-assemblies were formed at $q \leq 2$. Here, DMPC-rich condition, e.g. at q = 3.0 and 5.0, the assemblies showed high turbidities, suggesting the formation of larger assemblies (**Fig. 2**). Such a varied turbidity can be correlated to the phase transition. According to previous report, the turbidities of DMPC/DHPC suspensions can be dependent on phase states, such as micelle (transparent), cylindrical micelle or holey lamellar (turbid) [7]. Thus, an increase in the ratio of long-chained DMPC molecules caused the formation of larger self-assemblies. At least, the morphology of DMPC/DHPC assemblies can be roughly

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indicated by the turbidity: the formation of small-sized self-assemblies can be confirmed by the transparency of the suspension ($q \leq 2$).

3.1.2. Membrane Fluidity and Polarity

To investigate the physicochemical membrane properties of the DMPC/DHPC assemblies, the fluorescence probes, such as DPH and Laurdan, were used to monitor the membrane fluidity ($1/P_{DPH}$) and the membrane polarity (GP_{340}), respectively. The polarized fluorescence of DPH reflects the localized membrane fluidity (microscopic viscosity around DPH). Laurdan is sensitive to the polarity of the environment in the membranes because its chromophore is located near the glycerol moiety of the phospholipids in membranes, and its emission spectrum is changed depending on the surrounding water molecules in the membrane [23]. These parameters were employed to estimate membranous phase state (**Fig. 3**). Based on Cartesian diagram analysis [4, 27], the phase state (ordered phase or disordered phase) of the membrane can be investigated from the position in the diagram [4]. DMPC molecules form a bilayer structure in ordered phase at 20 ºC ($T < T_m$ of DMPC). The DMPC membranes showed a lower membrane fluidity ($1/P_{DPH} < 6$) and a positive membrane polarity ($GP_{340} > 0$), which appeared in the second quadrant of the Cartesian diagram [4]. On the contrary, the DHPC membranes showed a significantly higher membrane fluidity and a low GP_{340} value, which appeared in the first or fourth quadrant. This reveals that the micelle membrane is in disordered state, and the vesicle in LC phase show a higher fluidity. Thus, using DPH and Laurdan, the membrane properties of DMPC/DHPC assemblies were first investigated, and the obtained membrane properties were compared with those of well-studied self-assemblies. As shown in **Figure 3**, the DMPC/DHPC mixtures (20 mM) at q = 1.5 and q = 0.5 were

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present in the second quadrant, suggesting that these self-assemblies had ordered bilayer as well as DMPC vesicle in gel phase.

In contrast, at q = 0.3, the plot position was in the first quadrant: the membrane fluidity was higher as well as DHPC micelle, while the membrane polarity indicated rather dehydrated state. Because DHPC is considered as surfactant, the DMPC/DHPC mixture can form the structure like mixed micelle, a solubilized lipid bilayer that is coated by micelle-forming surfactant [28, 29]. In the mixed micelle, the hydrophobic interaction between protein and lipid can be significant, suggesting that mixed micelle possesses a hydrophobic region. At q = 0.3, the differences in membrane properties suggested that the assemblies were in disordered phases. It could be difficult to distinguish the size-differences of bicelle and micelle (or mixed micelle), while their membrane fluidities ($1/P_{DPH}$) and membrane polarities (GP_{340}) were clearly different. Thus, the DMPC/DHPC assemblies at q = 0.3 could be in disordered phase, wherein DMPC molecules did not form (ordered) bilayer. Thus, the membrane property characterization using DPH and Laurdan enables us to analyze the ordered phase in small assemblies. Considering the size and membrane properties, the bicellar structure of DMPC/DHPC can be re-defined as that small self-assembly with a size of 7-20 nm, which possess a bilayer region in the ordered state as well as gel-phase.

Assuming that bicelle-like discoidal structure was formed in DMPC/DHPC at q =1.5, the membrane composition in the rim and center regions could be different: it is expected that the membrane properties of rim and center could be similar to that of DHPC micelle and DMPC vesicle, respectively. While those at q > 0.3 possessed the ordered bilayer region, which could satisfy the concept of bicelle. In the DMPC/DHPC systems, the gel-phase bilayer is necessary to form the bicelle structure. These physicochemical analytical

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methods can be used to distinguish between bicellar-micellar assemblies. The characteristics of DMPC/DHPC mixtures are summarized in **Table 1**. Considering turbidity, membrane fluidity and membrane polarity have revealed the differences of physicochemical membrane properties, and hopefully, these parameters can be used to categorize other self-assemblies.

3.2. Effect of Dilution on Membrane Properties of DMPC/DHPC Mixtures.

3.2.1. Effect of Dilution on Structural Properties

The morphological stability of the DMPC/DHPC assembly was determined through dilution process, herein OD_{500}, $1/P_{DPH}$ and GP_{340} values were measured for each diluted sample. To assess the morphological change at macroscopic view, the turbidity, which roughly reflects the size of vesicle-like self-assembly, was monitored during the dilution operation. The vesicle suspensions, prepared by extrusion method (sub-micrometer size), are usually white and turbid, whereas micelle or bicelle suspensions are transparent [7] because the assembly size is less than 30 nm. In some of self-assembly systems, the morphological changes can be induced by dilution. The stability of the bicellar self-assembly depends on the solubility of each membrane component [25, 30, 31]. Thus, the increase in turbidity could be evidence of disruption of small self-assemblies, alternatively, larger self-assemblies such as vesicles could be formed in diluted condition. At q = 1.5, the increase of the turbidity was induced by dilution from 1.5 to 2 times (13 to 10 mM of the total lipid concentration) (**Fig. 4a**), in which a part of bilayers transformed to multilamellar vesicles [7, 9]. Takajo *et al.* reported the effect of DHPC that can destabilize DMPC bilayer [16]. Therefore, the morphological stability of the DMPC bilayer can be affected by the existence of DHPC. In dilution process, DHPC

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molecules subsequently moved out from bicelle membrane, and then remained DMPC molecules were re-assembled as vesicle membrane.

In the DMPC/DHPC at different q-values, the OD_{500} values were significantly varied in the same range of lipid concentration (**Fig. 2**). The higher OD_{500} values at the q-values above 3.0 indicated the formation of large assemblies such as vesicle or lamellar phase [7]. For these samples, the OD_{500} values monotonically decreased by dilution, but the decreasing tendency was not linear. Although the dilution might induce the transformation to other phase state (lamellar phase, and so on), it is difficult to identify the phase state of DMPC/DHPC self-assemblies by the turbidity. In these conditions ($q \geq 3.0$), the amount of DHPC molecules was not enough to disperse the DMPC layer into small pieces. At $q \leq 2$, on the contrary, DHPC molecules act as surfactant, which leads DMPC bilayer into dispersed small pieces (bicelle). In the bicelle membrane, the phase state is considered as heterogeneous: rim region (DHPC) is disordered while the bilayer region (DMPC) is ordered. Although smaller sized bilayer domain (ordered phase) could increase line tension, and such smaller domains are energetically unstable [32]. According to molecular dynamics simulation studies, the lipid molecules existing near the rim of phospholipid bilayers could form a micelle-like structure in water [33], which will contribute to the stability of bicelle. In conclusion, the self-assembly state of DMPC/DHPC can be roughly estimated by monitoring the trend of turbidity, wherein the turbidity jump (from transparent to turbid (or translucent) could be an evidence of phase transition during dilution process.

3.2.2. Effect of Dilution on Physicochemical Membrane Properties

Then, the effect of dilution on physicochemical membrane properties were also

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evaluated at 20 °C (**Fig. 4b**, and **4c**). The $1/P_{DPH}$ and the GP_{340} values at q = 0.5 were slightly changed, whereas the membrane properties at q = 1.5 remained constant. These results meant that the ordered membranes ($1/P_{DPH} < 6$, $GP_{340} > 0.2$ [4]) were maintained at q = 0.5 and 1.5, during the dilution. At $q \geq 3.0$, no significant difference could be expected in the values of the membrane properties in the DMPC-enriched bilayer membranes. The $1/P_{DPH}$ and the GP_{340} values at 1 mM were almost similar, and diluted samples were turbid, revealing that DMPC/DHPC assemblies finally transformed to vesicle (gel phase), by 20 times dilution. These results suggest that the morphological changes can happen in the DMPC/DHPC mixtures with lower q values ($q < 0.5$) by dilution. At q = 0.3 (total lipid concentration: 20 mM), DPH indicated higher fluidity like DHPC micelle, whereas Laurdan indicated rather hydrated state of the membrane. After dilution, the $1/P_{DPH}$ decreased but it was slightly higher as compared to DMPC vesicle, suggesting that some of DHPC molecules could be remained and fluidize the membrane (total lipid concentration: 1 mM). The results indicate that the increased DHPC ratio could disperse the small bilayer assemblies at 20 mM total lipids. Based on the physicochemical membrane property analysis, the difference of micellar assembly and bicellar assembly becomes more significant (**Fig. 3**). For the case of micellar assembly (q = 0.3), the phase transition point can be monitored by the decrease of the membrane fluidity, in dilution process.

3.3. Discussion for Phase Diagram of DMPC/DHPC Self-Assemblies

From the above results, the optimal conditions for the formation of DMPC/DHPC bicellar self-assembly was estimated as $0.5 \leq q \leq 2.0$, at total lipid concentration of 20 mM. The phase diagram of DMPC/DHPC mixtures at different lipid concentration is

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shown in **Figure 5**. With an excess DMPC ($q \geq 3.0$), the DMPC/DHPC mixtures formed vesicles independent to DHPC concentration. During dilution, the DMPC/DHPC bicelle at $q = 1.5$ transformed into vesicles at the total lipid concentration below 10 mM (turbidity increase was observed at 10 mM (**Fig. 4a**)). In the case of bicelle or discoidal assembly in TEM picture [7], the self-assembly can be observed as two different patterns of shapes, such as face-on (sticky image) and edge-on (discoidal image). Finally, the self-assembly morphology for DMPC/DHPC at $q = 1.5$ (20 mM) was estimated by TEM image (**Fig. 6a**). Both shapes (face-on and edge-on) were observed in our systems, revealing the formation of bicelle-like discoidal self-assembly at this experimental condition (**Fig. 6b**). Furthermore, the transparency of DMPC/DHPC (at $q = 1.5$) suspension was maintained in the range of lipid concentration 13-20 mM. The turbidity variations were carefully checked by eye during dilution, and the threshold (phase transition concentration) of DMPC/DHPC bicelle at $q = 1.5$ was estimated as 12 mM. In DHPC-rich condition, DHPC molecules as detergent make membranes fluid and act to make smaller assemblies (mixed micelle). From this investigation, the morphological tendency of DMPC/DHPC self-assembly could be clear in the lower concentration range.

4. CONCLUSIONS

We characterized the self-assemblies composed of DMPC/DHPC at total lipid concentration less than 20 mM (< 1wt% lipid). Based on the analyses of suspension turbidity, membrane fluidity (DPH), and membrane polarity (Laurdan), two important aspects were obtained for gel-phase bicelles: 1) bicelle suspension is transparent because of its small size, and 2) the bilayer region is in ordered (gel) state. These findings strongly reveal the previous findings that the DMPC/DHPC bicelle structure can be observed only

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below 23 ºC ($T < T_m$ of DMPC). The transparency of the suspension is a good indicator to judge the size of self-assembly roughly; when the suspension is transparent, the phase state can be bicelle or micelle (mixed micelle). Furthermore, the difference of bicelle ($0.5 \leq q \leq 2.0$) and micellar assembly (micelle or mixed micelle, $q \leq 0.3$) can be more significant: the former one shows gel-phase characteristics while the later one shows disordered phase characteristics. Based on these findings, a phase diagram of DMPC/DHPC at 20 ºC was investigated. As these morphological properties are significantly important in application of bicelles, our findings will accelerate the usage of bicellar self-assemblies.

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Author Contributions:

S.T. and K.S. performed experiments. S.T., K.S., K.H., Y.O., H.N., and H.U. wrote paper. S.T. and H.U. directed the research.

Conflicts of Interest:

There are no conflicts of interest to declare.

Abbreviations:

DMPC	1,2-dimyristoyl-sn-glycero-3-phosphocholine
DHPC	1,2-dihexanoyl-sn-glycero-3-phosphocholine
Tm	phase transition temperature
CMC	critical micelle concentration
LC phase	liquid crystalline phase
DLS	dynamic light scattering
DPH	1,6-diphenyl-1,3,5-hexatriene
Laurdan	6-dodecanoyl-N,N-dimethyl-2-naphthylamine
GP	general polarization
OD	optical density

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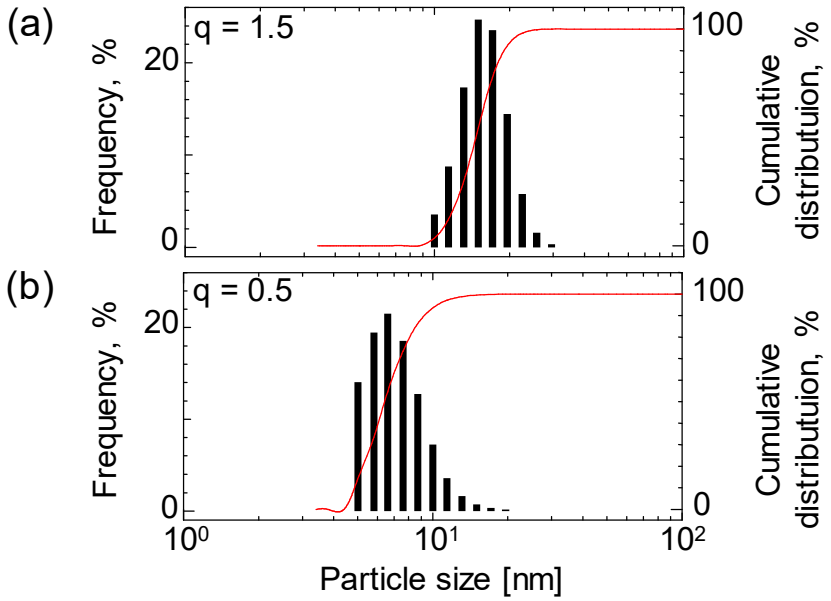

Figure 1 Size distributions of 20 mM DMPC/DHPC ratio (q); (a) 1.5, and (b) 0.5. The median diameters of the mixtures at q = 1.5 and 0.5 were 14.7±3.2 nm and 6.4±1.9 nm, respectively. The red curves were cumulative distributions of the mixtures.

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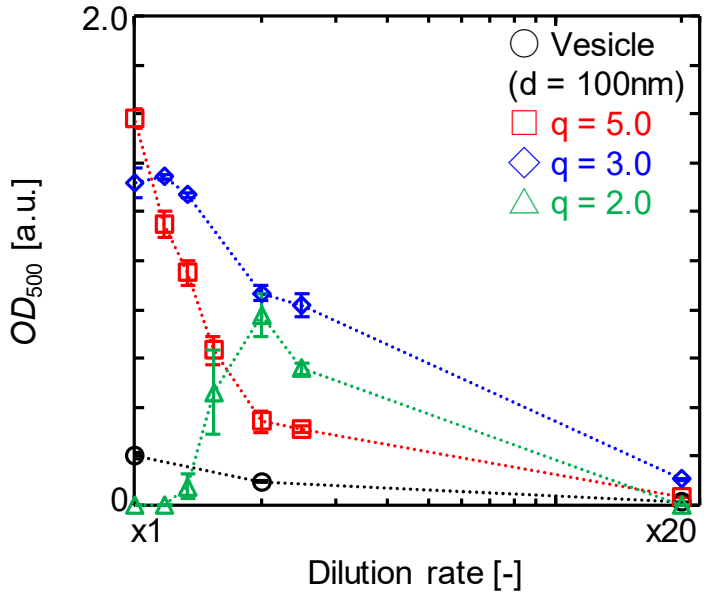

Figure 2 Trends of turbidities (OD_{500}) each constitution of DMPC/DHPC mixture in dilution process at 20 ºC; (circle) DMPC vesicle at 100 nm diameter, (square) q = 5.0, (diamond) q = 3.0, and (triangle) q = 2.0. The dilution took place total lipid concentration from 20 mM (x1) to 1 mM (x20).

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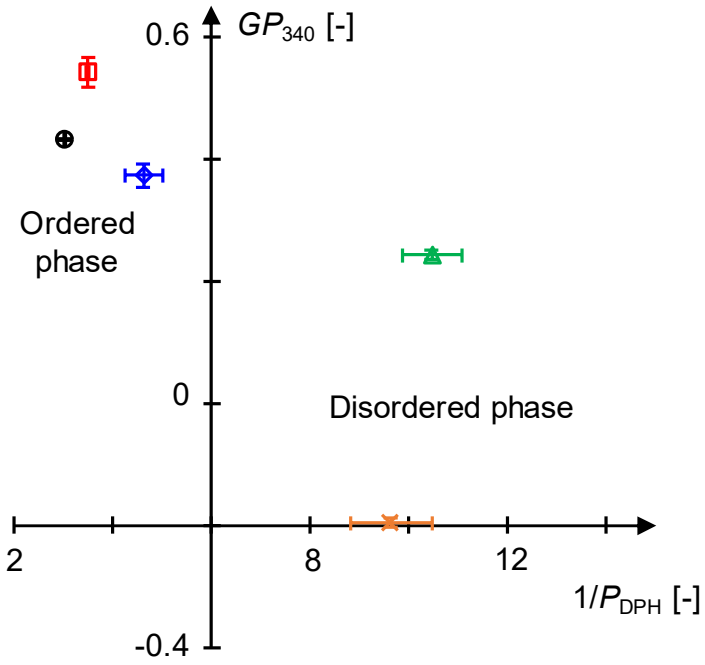

Figure 3 Cartesian diagram of membranes at 20 mM (20 ºC); DMPC vesicle (circle), DMPC/DHPC at q = 1.5 (square), at q = 0.5 (rhombus), at q = 0.3 (triangle), and DHPC micelle (cross). The position plotted in the Cartesian diagram indicates the phase state of each membrane, the first and fourth quadrants: disordered (liquid crystalline) phase, the second quadrant: ordered (gel) phase.

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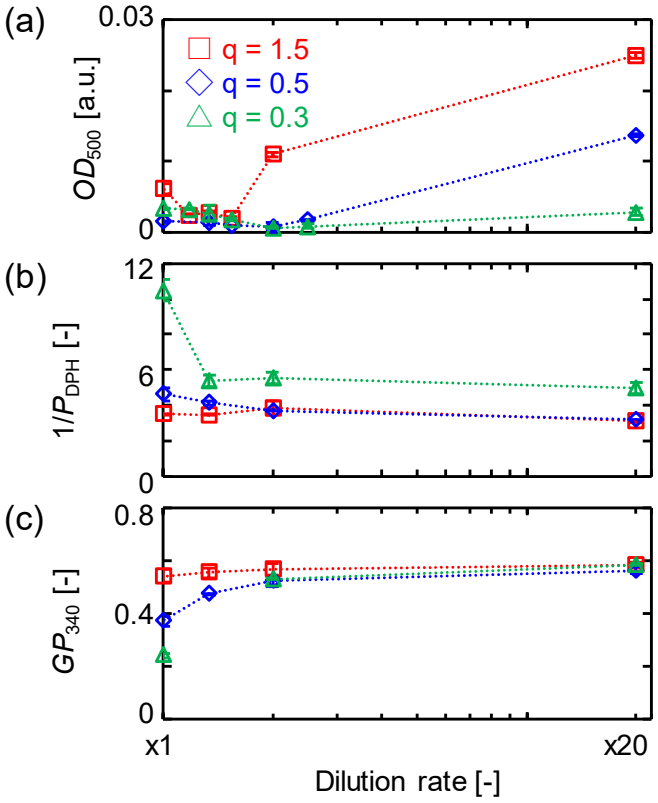

Figure 4 Membrane properties of DMPC/DHPC at dilution process 20 to 1 mM in the total lipid concentration ([DMPC] + [DHPC]) at 20 ºC. The *x* axis indicates dilution rate from 1 to 20 times. (a) turbidites of assemblies, (b) membrane fluidity, $1/P_{DPH}$, and (c) membrane polarity, GP_{340}; (square) q = 1.5, (diamond) q = 0.5, and (triangle) q = 0.3.

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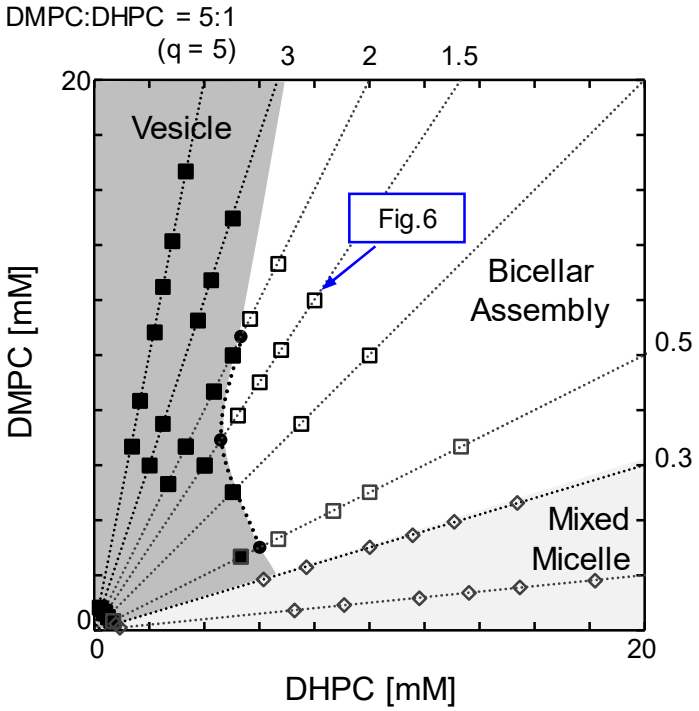

Figure 5 Phase states of DMPC/DHPC mixtures at 20 ºC based on macro- and micro-properties of membranes. The bold filled circles showed the increasing turbidities each q-values as the morphological change. The phase boundary concentrations of q = 0.5, 1.5, and 2, are 9 mM, 12 mM, and 16 mM, respectively (described in total lipid concentration)

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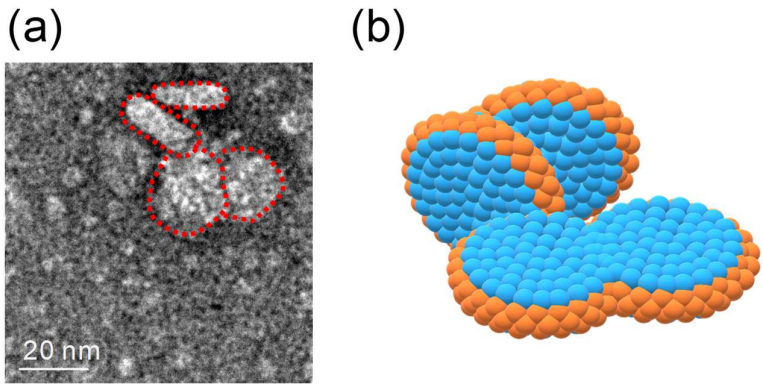

Figure 6 TEM images of DMPC/DHPC mixture; (a) q = 1.5, 20 mM, and (b) the image of the bicelle like structure.

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Table 1 Membrane Properties of DMPC/DHPC mixtures (20 mM) as vesicle, bicellar piece, and micelle at 20 ºC (< T_m).

	Macro-properties		Micro-properties	
	Size distribution (DLS)	Turbidity, OD_{500}	Membrane polarity, GP_{340}	Membrane fluidity, $1/P_{DPH}$ [-]
Vesicle (DMPC)	≥ 50 nm	High	Ordered[a]	Ordered[a]
Bicellar assembly (DMPC/DHPC at 0.3 < q < 3.0)	7-20 nm	Low	Ordered[a]	Ordered[a]
Micelle (DHPC) Mixed micelle (q < 0.3)	< 7 nm	Low	Disordered[a]	Disordered[a]

[a] The phase states, such as ordered and disordered, are judged based on $1/P_{DPH}$ and GP_{340} values, according to Suga *e* (2013) [4].