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Citation	Langmuir , 30 (41) : 12315 - 12320
Issue Date	2014-10-02
DOI	10.1021/la503732q
Self DOI	
URL	http://ir.lib.hiroshima-u.ac.jp/00045809
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Relation	


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[70]Fullerenes Assists the Formation of Phospholipid Bicelles at Low Lipid Concentrations

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ABSTRACT: The incorporation of neutral [70]fullerenes (C_{70}) led to the bicelles formation at relatively low lipid concentration range from neutral lipid mixtures (DMPC/DHPC). Furthermore, the C_{70} addition resulted in the formation of large bicelles with a radius of *ca*. 100 nm in contrast to C_{70}-free bicelles that were formed from anionic lipid mixtures (DMPC/DHPC/DMPG). The stabilization of these bicelles was attributed to C_{70} incorporation into the membranes.

INTRODUCTION

Bilayer micelles or bicelles are small disk-like lipid membranes with a diameter of several tens of nanometers, usually formed from a mixture of amphiphilic lipids with different molecular geometry.[1–3] A typical composition for bicelle formation is a mixture of phospholipids with different length of acyl chains such as the combination of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC)/1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (DHPC), where DMPC forms the disk's flat face and DHPC locates at the edges (Figure 1A).[1] Bicellar systems have been widely employed as a minimal model cell membrane in diverse conformational studies of membrane proteins and peptides using NMR according to their useful property of aligning in a magnetic field as well as their high dispersity in water.[4–6] Other than naturally-derived biomacrobmolecules, the interaction of small incorporated molecules with membrane has been extensively studied using bicelles. For example, Maniero and Ferrarini *et al*. have used bicellar systems to elucidate the positional and orientational distributions of fullerene derivatives in lipid membranes.[7] Although the usefulness of bicelles is well recognized, their biological and nano-material applications are still challenging, because DMPC/DHPC mixtures reversibly change the morphology of the aggregates in water depending on the lipids' concentration.[8–10] Several attempts have been made to overcome the instability of bicelles by the addition of metal ions,[9–11] cholesterol,[12] and charged-cholesterol, -surfactants, and -lipids[13–15] as well as by coating with ceramic layers formed by a sol-gel reaction between alkoxysilyl headgroups of the organic-inorganic hybrid lipid.[16] Most of these attempts were performed under relatively high lipid concentration conditions, whereas studies of bicelles from diluted conditions have been very limited. For example, bicelles formed under concentrated conditions and subsequently

encapsulated in liposomes, remained unaltered under dilute conditions.[17] That is to say, these bicelles were remained to be concentrated in the liposomes after dilution.

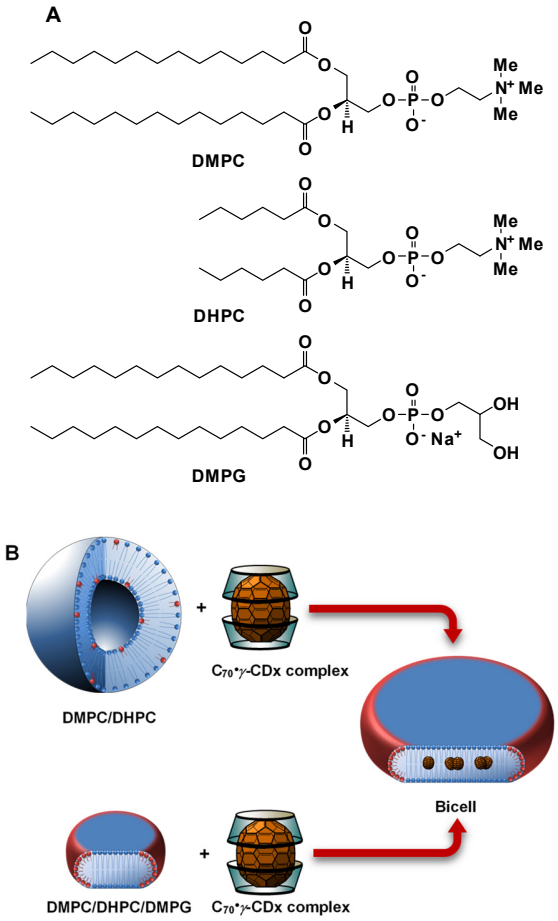

Figure 1. (A) Structures of lipids and (B) schematic representation of the C_{70}-exchange method from the γ-CDx-bicapped C_{70} to liposomes and/or bicelles

Lipid membrane-incorporated C_x ($LMIC_x$: x = 60 or 70) complexes have been prepared by a fullerene exchange reaction from the C_x•γ-cyclodextrin (γ-CDx) complex to liposomes.[18] In this group, the location of C_{60} in prepared $LMIC_{60}$ has been experimentally determined by an exchange reaction method.[19] The results indicated that C_{60} units are located in the hydrophobic core of the lipid bilayer membrane in these liposomes, based on thermal analysis by DSC and ^{13}C NMR

measurements. C_{70} units were expected to be located in a similar fashion in the liposome because of similar results from DSC measurements.[18] Cryogenic transmission electron microscopy (cryo-TEM) images of $LMIC_{70}$ have revealed that most of these liposomes possessed humps in their lipid membranes (C_{70}/DMPC = 10 mol%) and the humps became larger and more planar as the amount of C_{70} increased (C_{70}/DMPC = 30 mol%), indicating that self-aggregation of C_{70} grows in a two-dimensional direction.[18] In this paper, we report unique effect of lipid-C_{70} interaction that lowers the required lipid concentration for the bicelle formation (Figure 1B).

RESULTS AND DISCUSSION

Bicelles (and/or liposomes) were prepared by simple hydration of a lipid mixture consisting of the long-chain phospholipid DMPC and short-chain phospholipid DHPC in the presence or absence of anionic lipid 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol (DMPG) (Figure 1A). $LMIC_{70}$ in bicelles were prepared via a C_{70}-exchange reaction from water-soluble γ-CDx-bicapped C_{70} to bicelles (and/or liposomes), according to a previously described procedure. The C_{70}-exchange reaction was supported by the UV-vis spectroscopy, which demonstrated that the absorption spectral change was similar to that observed with $LMIC_{70}$ in DMPC-liposomes (Figure S1).[18]

The morphology of the DMPC/DHPC mixture was observed in the absence and presence of C_{70} by cryo-TEM (Figures 2 and S2), enabling visualization of supramolecular assemblies in water *in situ*. TEM micrographs showed that the DMPC/DHPC mixture in the absence of C_{70} existed in the form of small unilamellar vesicles (diameter, *ca*. 40–60 nm; Figures 2A and S2A). This result is consistent with the previous report that DMPC/DHPC mixture forms bicelles only at high lipid concentration.[10] Under the conditions of the present study, lipids at 10 mM and DMPC/DHPC at 3.2/1.0 (mol/mol), liposome formation was consistent with a related, published phase diagram.[10]

In contrast, although a few liposomes remained (Figures 2B and S2B, blue arrow), addition of C_{70} resulted in the formation of bicelles, which displays edge-on or face-on projection in the Cryo-TEM image (Figures 2B and S1B, white and red arrows, respectively), indicating that incorporation of C_{70} lowered the lipid concentration required to form bicelles. By tilting the sample holder at 10°, the shape of the objects corresponding to the projection of bicelles changed from rod-like (Figure 2C) to ellipsoidal (Figure 2D), definitely confirming the bicelles' discoidal shapes.

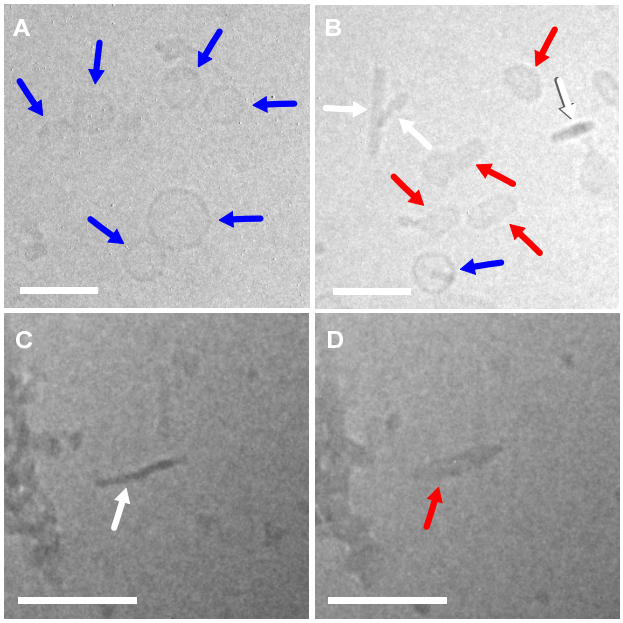

Figure 2. Cryo-TEM images of DMPC/DHPC mixtures (A) in the absence and (B) in the presence of C_{70}. Magnified images of a DMPC/DHPC mixture acquired at a tilting angle of (C) 0° and (D) 10° around the horizontal axis. Lipids, 5.0 mM; DMPC/DHPC, 3.2/1.0 (mol/mol); C_{70}/lipids, 10 mol% in water; scale bars, 100 nm; blue arrows, liposomes; and white and red arrows, bicelles edge-on and face-on, respectively.

On the other hand, by the addition of anionic lipid 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol (DMPG) (Figure 1A), bicelles can be formed in relatively wider range of the lipid

concentrations compared with the DMPC/DHPC system.[10,20] Therefore, we extensively evaluated the effect of C_{70} on the formation of DMPC/DHPC/DMPG bicelles under 10-fold diluted conditions.[21] The lipid assembly in DMPC/DHPC/DMPG mixtures was also visualized by cryo-TEM (Figures 3 and S3). In the absence of C_{70}, very small bicelles and a small amount of liposomes were observed (Figures 3A and S3A, white and blue arrows). These small bicelles and liposomes changed to larger bicelles after addition of C_{70} (Figure 3B). Concurrently, the projected images of the assemblies changed from rod-like (Figure 3C, right disc) to ellipsoidal (Figure 3D, right disc), as seen by tilting the sample holder at 10°, indicating definitely that the bicelles also had a discoidal structure.

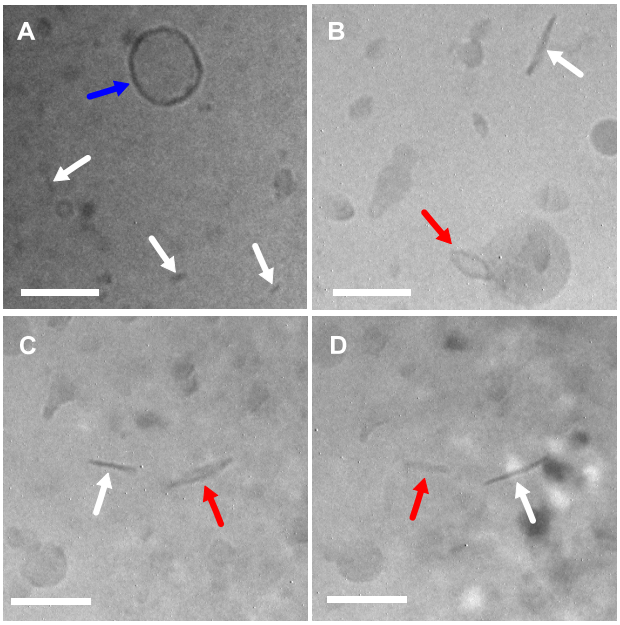

Figure 3. Cryo-TEM images of DMPC/DHPC/DMPG mixture (A) in the absence of C_{70} and (B) in the presence of C_{70}. Magnified images of a DMPC/DHPC/DMPG mixture acquired at a tilting angle of (C) 0° and (D) 10° around the horizontal axis. Lipids, 0.5 mM; DMPC/DHPC/DMPG,

3.4/1.0/0.21 (mol/mol); C_{70}/lipids, 10 mol% in water; scale bars, 100 nm; blue arrows, liposomes; white and red arrows, bicelles edge-on and face-on, respectively.

To further quantify the effect of C_{70} on bicelle formation, we analyzed the size distribution of bicelles based on the TEM images. The histogram is shown in Figure 4. In the DMPC/DHPC system in the absence of C_{70}, no bicelles were observed in the cryo-TEM images. In contrast, the addition of C_{70} produced bicelles with an average diameter of *ca*. 60 nm (Figure 4A). On the other hand, C_{70} increased the diameter of bicelles formed with DMPC/DHPC/DMPG. In particular, their diameter changed roughly form 15–50 nm to 60–110 nm upon the addition of C_{70} (Figure 4B). These results clearly shows that incorporation of C_{70} enabled (i) the lowering the lipid concentration required to bicelle formation and (ii) formation of relatively larger bicelles.

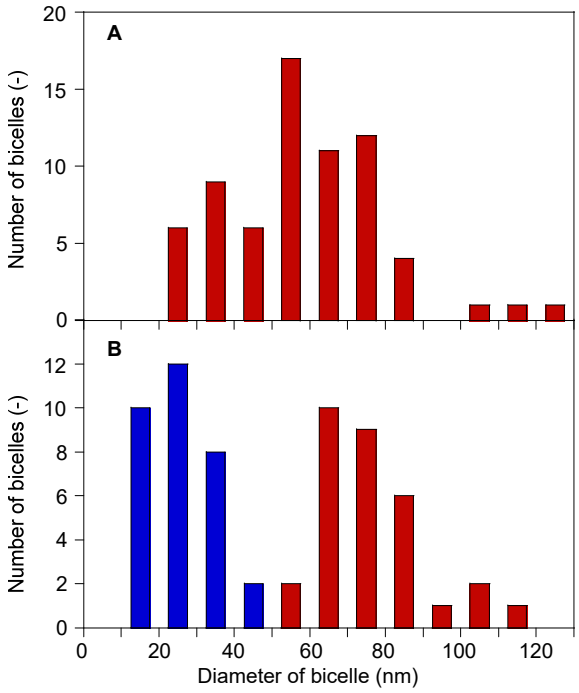

Figure 4. Diameter distribution histograms of bicelles (A) in DMPC/DHPC mixtures in the presence of C_{70} and (B) in DMPC/DHPC/DMPG mixtures in the absence and presence of C_{70} (blue and red bar, respectively) by TEM micrographs.

Further evidence regarding morphological changes in the assemblies of DMPC/DHPC and DMPC/DHPC/DMPG mixtures was confirmed by measuring the size distribution of lipid assemblies by dynamic light scattering (DLS). DMPC/DHPC mixture in the absence of C_{70} showed a wide distribution of hydrodynamic diameter D_{hy} at 62.0 nm with a half width of 16.9 nm (Figure 5A), suggesting that here most DMPC/DHPC formed liposomes.[10] These DLS results were consistent with observations from cryo-TEM images (Figure 2A). On the other hand, the average D_{hy} in the presence of C_{70} revealed a narrow peak at 20.1 nm with a half width of 4.9 nm (Figure 5A). This average D_{hy} was much smaller than that observed by TEM (Figure 4A) because estimated disk diameters tend to be smaller when the mean hydrodynamic radii are calculated using the standard Stokes-Einstein equation for a spherical particle.[22] As the diameters were smaller than in the absence of C_{70}, these results supported bicelle formation.

The DLS results from a DMPC/DHPC/DMPG mixture in the absence of C_{70} showed a narrow peak at 5.6 nm with a half width of 1.4 nm (Figure 5B). As the average D_{hy} in the presence of C_{70} exhibited a narrow peak at 12.7 nm with a half width of 3.6 nm (Figure 5B), the average D_{hy} with C_{70} was larger than that without. These values do not seem to be consistent with the observations from the cryo-TEM images (Figure 3), because the average D_{hy} was estimated by assuming isotropic spherical particles,[22] while both results have the tendency to increase the size of bicelles upon addition of C_{70}. Although the determination of the detailed mechanism of large bicelle stabilization by C_{70} doping was beyond the scope of this report, the observed results might have originated from reduction of the lipid membrane curvature induced by C_{70}. Our previous study on the interaction of C_{70} with phospholipid vesicles clarified that C_{70} induced the local deformation of vesicular membranes to form planar membranes due to C_{70} clustering in a two-dimensional manner.[18] This suggested that the presence of C_{70} in the membrane reduced the intrinsic curvature

of the lipid bilayer to form a zero-curvature membrane. Accordingly, by reducing the membrane's curvature stress, C_{70} contributed to stabilizing large bicelles rather than vesicles with a curvature that was usually formed at low lipid concentrations. Large bicelles required relatively small amounts of short-chain lipid compared with small bicelles that additionally contributed to lower the critical lipid concentration for bicelle formation.

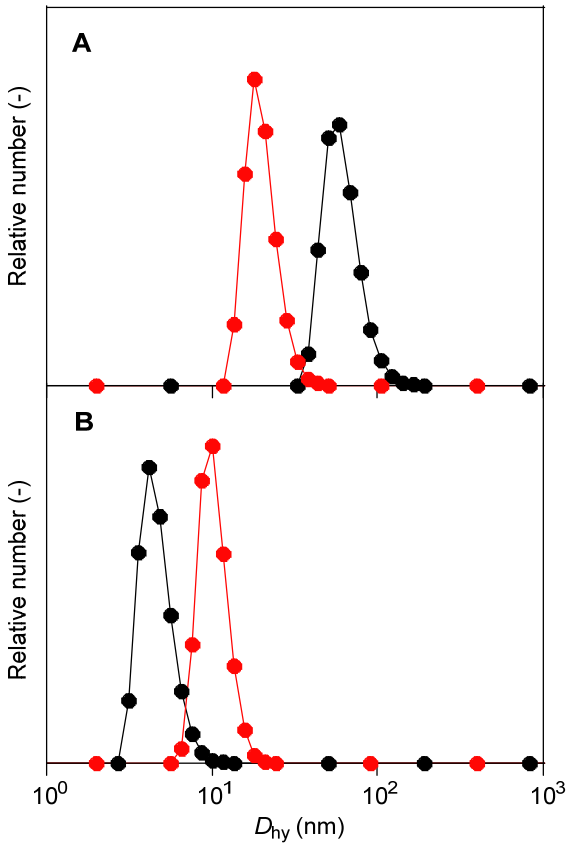

Figure 5. Hydrodynamic diameters (D_{hy}) in mixtures of (A) DMPC/DHPC and (B) DMPC/DHPC/DMPG in the absence and presence of C_{70} (black and red line, respectively) from DLS measurements. (A) Lipids, 5.0 mM, DMPC/DHPC, 3.2/1.0 (mol/mol), and C_{70}/lipids, 10 mol%; (B) Lipids, 0.5 mM, DMPC/DHPC/DMPG, 3.4/1.0/0.21 (mol/mol/mol), and C_{70}/lipids, 10 mol% at room temperature.

It should be noted that a previous molecular dynamics (MD) study is consistent with the experimental observations made in the present study, though in the MD study C_{60} instead of C_{70} was used in vesicles made of DMPC.[23] Namely, laterally expanded C_{60} aggregates were observed near the center of the lipid membranes, which increased the membranes' rigidity. The portion containing a C_{60} aggregate in a vesicle reduced the curvature to some extent, implying the aggregate flattened the membrane.[23] This observation provides a reasonable explanation to the stabilization of bicelles by the C_{70} addition in the present experiments.

Here, we conducted comparative MD simulations of a lipid aggregate in the presence or absence of fullerenes using the SDK coarse-grained (CG) force field,[24,25] to examine the fullerenes' effect on the preferred structure of the lipid assembly. To simplify the calculation, we used C_{60} again as fullerenes and DMPC alone as lipids. Figure 6 shows the morphological change in a DMPC lipid aggregate in the presence or absence of C_{60} in a course of CG-MD. These two simulations started from the same initial configuration: a random lipid aggregate surrounded by plenty of water. The difference was that the C_{60}s were removed from one of the two systems at the beginning of the MD simulation. Vesicle formation was observed in the absence of C_{60}, though a bicelle was obtained in the presence of C_{60}. There was no other stable structure for the lipid aggregates with this size.[26] Once a lipid aggregate formed either of vesicle or bicelle, no further transformation was observed during another 1μs-MD run. Of course, the trial MD runs should show the membrane morphology depending on the initial configuration of the lipid aggregate. We repeatedly conducted similar sets of MD simulations from different initial configurations and confirmed the preference of the bicelle in the presence of C_{60}. The change of the preferred morphology is understood within the framework of the Fromhelz theory,[27] assuming C_{60} aggregates increased bending rigidity. In the MD runs, we observed laterally expanded aggregation of C_{60} near the center of the lipid

membranes, which led to the flattened-membrane bicelle. The results suggest that the bicelle stabilization by the C_{60} addition is attributed to the fact that C_{60} aggregates with a rigid and planar structure grow along the bilayer in the lipid membrane.

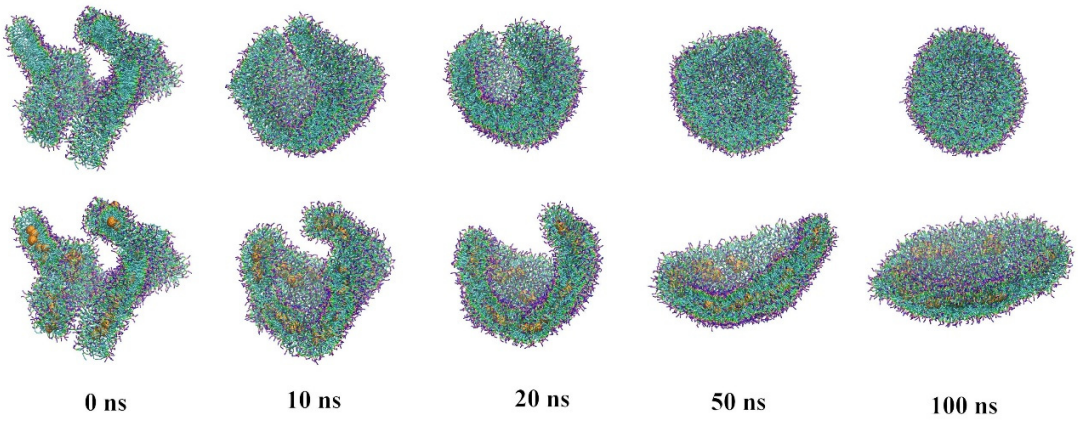

Figure 6. Transformation observed in MD simulations from a random DMPC aggregates in the absence (upper panels) and presence (lower panels) of C_{60}. The headgroups are shown in red (phosphate) and blue (choline) and hydrocarbons are shown in cyan. C_{60} are shown in orange.

CONCLUSION

C_{70} addition led to bicelle formation from neutral lipid mixtures in a low concentration range as well as increasing their sizes when formed from an anionic lipid mixture. Both phenomena may occur because of promotion of a rigid and planar structure by the C_{70} aggregates in the membranes. These results are theoretically supported by the simulation with C_{60}. These bicelles have potential applications in the field of nanobiomaterials because of the presence of C_{70}, an efficient photosensitizer for photodynamic therapy.[18]

EXPERIMENTAL SECTION

Materials. γ-CDx was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) from NOF Corp. (Tokyo, Japan), 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (DHPC), 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol (DMPG) from Avanti Polar Lipids, Inc. (Alabaster, AL), and C_{70} (> 99%) from MER Corp. (Tucson, AZ, USA).

Preparation of bicelles and/or liposomes. Appropriate amounts of DMPC/DHPC and DMPC/DHPC/DMPG (3.2/1 and 3.4/1/0.21 mol/mol, respectively) were dissolved in chloroform, the solvent evaporated under nitrogen gas flow and the residual trace solvent completely removed in vacuo. Hydration of the resulting thin lipid films on the vials' walls was performed above the phase transition temperature with an appropriate amount of water. The resulting bicelles and/or unilamellar vesicles were obtained by five freeze-thaw cycles (-195 and 50 °C). The concentrations were 10 mM for DMPC/DHPC mixtures (7.6 and 2.4 mM, respectively) and 1.0 mM for DMPC/DHPC/DMPG mixtures (0.37, 0.11, and 0.04 mM, respectively). These solutions were mixed with the same volume of C_{70}•γ-CDx solution or water to obtain the samples.

Preparation of the C_{70}•γ-CDx complex. C_{70} (5.00 mg, 5.95×10^{-6} mol) and γ-CDx (61.40 mg, 4.73×10^{-5} mol) were placed in an agate capsule with two agate-mixing balls and then mixed vigorously at 30 Hz for 20 min using a high-speed vibration mill (MM 200; Retsch GmbH, Haan, Germany). The solid mixture was suspended in saline (1.5 mL), yielding a black emulsion. Following a period of centrifugation (18,000g, 25 °C and 20 min), the non-dispersed C_{70} was removed from the solution. The C_{70} concentration in the C_{70}•γ-CDx complex was determined by measuring the solution's absorbance at 380 nm (the molar absorption coefficient for the water-soluble C_{70}•γ-CDx complex, $\varepsilon_{380} = 3.80 \times 10^4$ dm^3 mol^{-1} cm^{-1})[28] and was found to be 1.66 mM.

Preparation of the $LMIC_{70}$ in bicelles. $LMIC_{70}$ in bicelles was prepared using an exchange reaction between the bicelles and/or liposomes and the C_{70}•γ-CDx complex by heating at 50 °C for 20 min, as described previously.[18] Final concentrations of the respective components were 5.0 and 0.5 mM lipids for DMPC/DHPC and DMPC/DHPC/DMPG mixtures, respectively (C_{70}/lipids, 10 mol%).

Cryo-TEM. Cryo-TEM samples were prepared using a universal cryofixation and cryopreparation system (Leica EM CPC, Leica Microsystems GmbH, Wetzlar, Germany). Humidifying the isolation chamber to near saturation levels prior to sample introduction prevented water evaporation from the sample. An aliquot sample (2–3 μL) was placed on a microperforated cryo-TEM grid and the water subsequently absorbed using filter paper, resulting in the formation of thin liquid films of 10–300 nm thickness that freely spanned the micropores in the carbon-coated lacelike polymer layer supported by a metal mesh grid. Following a minimum 30-s holding time, the sample grid assembly was rapidly vitrified with liquid ethane at its melting temperature (-163 to -170 °C). The holding time was specifically selected to relax any possible flow deformations resulting from the blotting process. The vitreous specimen was then held under liquid nitrogen until being loaded into a cryogenic sample holder (Gatan 626.DH, Gatan, Inc., Pleasanton, CA, USA). Imaging was performed with a JEM-3100 FEF system (JOEL Ltd., Tokyo, Japan) operating at 300 kV. The use of a minimal dose system was facilitated by probing a sample's electron radiation sensitivity. Images were recorded using a Gatan 794 multiscan digital camera and processed with Digital Micrographs (Ver. 3.8.1, Gatan, Inc.). Optical density gradients in the background, which are normally ramp-shaped, were digitally corrected using a custom-made subroutine compatible with Digital Micrographs. The cryo-TEM images for all samples were examined within 6 h after the exchange reaction.

Molecular Dynamics (MD) Simulations. Molecular dynamics simulation was carried out using the LAMMPS software.[29] A random lipid aggregate was made in the same manner as in a previous paper.[23] We employed the SDK CG force field.[24,25] The system consisted of 1512 DMPC molecules and 151200 water particles together with/without 100 C_{60} molecules. NPT ensemble was used. The temperature was set to 310 K using the Nosé-Hoover thermostat[30,31] and the pressure was controlled at 1 atm using the Andersen barostat.[32] The LJ interaction was truncated at 1.5 nm, while the Coulomb interaction was estimated using the PPPM scheme.[33] The time step size was 10 fs.

ASSOCIATED CONTENTS

Supporting Information

Further experimental data, including other cryo-TEM images (S1 and S2) and DLS measurements for the mixtures of DMPC/DHPC and DMPC/DHPC/DMPG in the absence and presence of C_{70} (S3), are provided. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENT

This work was supported by JSPS KAKENHI a Grant-in-Aid for Scientific Research (B) (Grant No. 25288037), a Grant-in-Aid for Challenging Exploratory Research (Grant Nos. 24655128 and 25650053), and a Grant-in-Aid for Young Scientists (A) (Grant No. 24681028). We deeply thank Ms. S. Fujita for her assistance in TEM observations.

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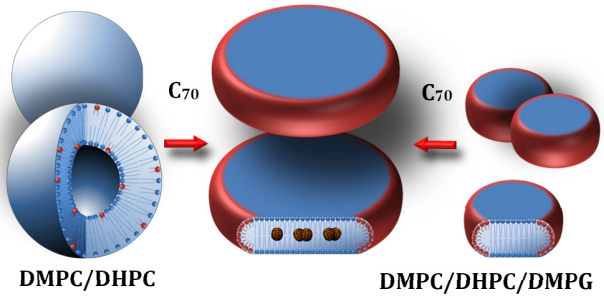

Supporting Information

[70]Fullerenes Assists the Formation of Phospholipid Bicelles at Low Lipid Concentrations

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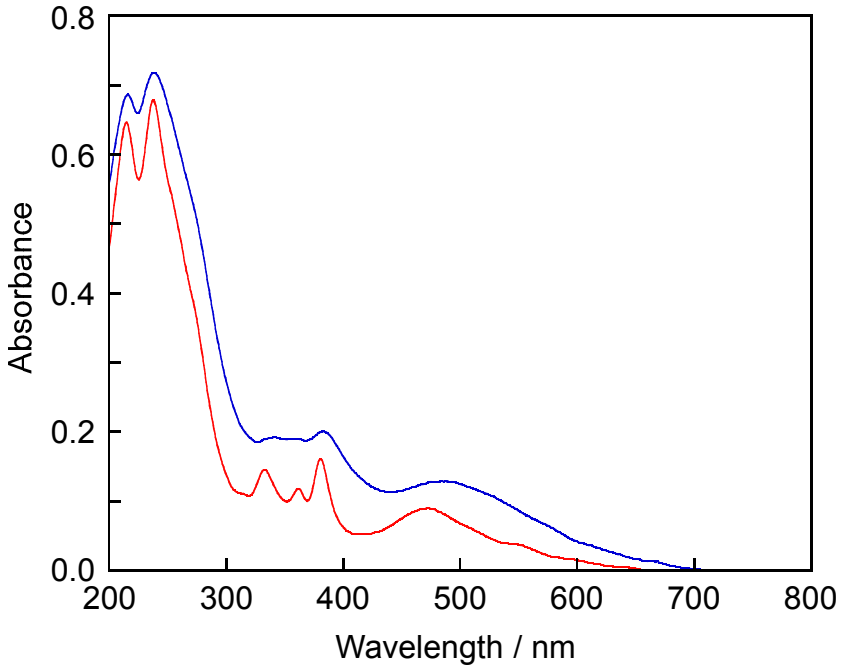

Figure S1. UV-vis absorption spectra of the C_{70}•γ-CDx complex (red line, [C_{70}] = 0.05 mM) and DMPC/DHPC mixtures after the C_{70}-exchange reaction (blue line, [C_{70}] = 0.05 mM).

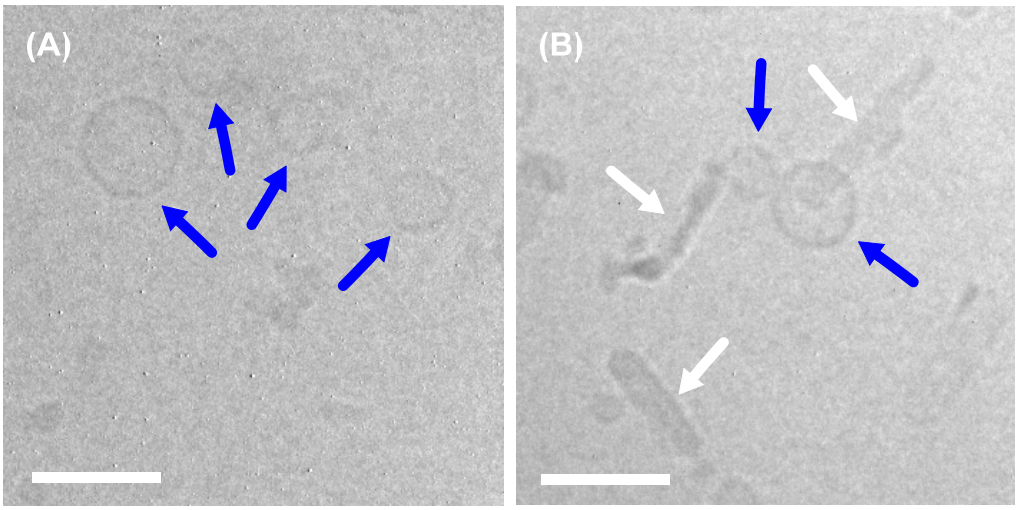

Figure S2. Cryo-TEM images of DMPC/DHPC mixtures (A) in the absence and (B) in the presence of C_{70} in other areas of the image in Figure 2.

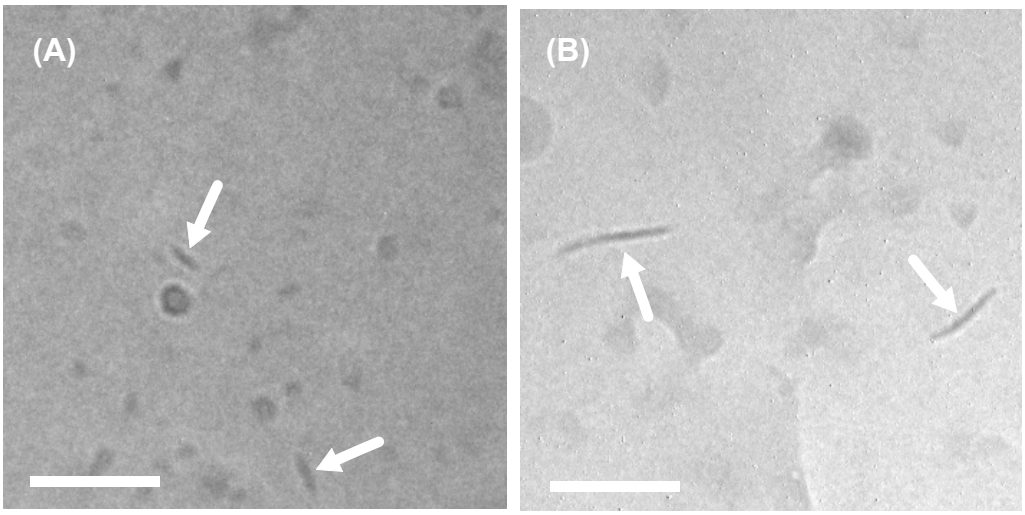

Figure S3. Cryo-TEM images of DMPC/DHPC/DMPG mixtures (A) in the absence and (B) in the presence of C_{70} in other areas of the image in Figure 3.