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**PAPER**

# Formation and regulation of fullerene-incorporation in liposomes under the phase transition temperature†

**Atsushi Ikeda,*[a] Yoshihiko Kawai,[a] Jun-ichi Kikuchi,[a] Motofusa Akiyama,[a] Eiji Nakata,[b] Yoshihiro Uto[b] and Hitoshi Hori[b]**

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The fullerene-exchange reaction from a cyclodextrin cavity to liposomes represents one of the best methods to prepare lipid membrane-incorporated [70]fullerenes ($C_{70}$). The $C_{70}$-exchange reaction occurred completely at temperatures above the phase transition temperature ($T_m$) of the liposomes; however, lowering the temperature to below the $T_m$ led to $C_{70}$ aggregation outside the liposomes. This observation has limited the development of more functional $LMIC_{70}$ using a variety of liposome compositions. In this paper, this reaction was found to occur efficiently by the addition of small amounts of lipids bearing a π-moiety. The π-moieties act as a gate when hydrophobic $C_{70}$ migrates into the hydrophilic liposome surface. Therefore, the π-moieties should exist in the polar head groups of the lipids and the $C_{70}$-exchange reaction can be controlled by pH.

## Introduction

Water-solubilised fullerenes ($C_x$: $x = 60$ or 70) have received recent attention as potential photosensitisers for photodynamic therapy because fullerenes are efficient visible-light triplet-sensitisers and have high photoproduction ability for $^1O_2$ (energy transfer) and anion radicals (electron transfer).[1–17] In particular, the photodynamic activity of water-solubilised $C_{70}$ using a liposome as a solubilising agent is 4.7-fold higher than water-soluble $C_{60}$ for the same photon flux (>400 nm).[13] However, examples of water-solubilised $C_{70}$ are very limited when compared with those for $C_{60}$, because the lower solubility of $C_{70}$ in most solvents renders treatment difficult.[18–20] Recently, we demonstrated that lipid membrane-incorporated $C_{70}$ ($LMIC_{70}$) can be readily prepared by the fullerene-exchange reaction from the γ-cyclodextrin (γ-CDx) cavity to vesicles of dimyristoylphosphatidylcholine (DMPC, phase transition temperature ($T_m$) = 23 °C) at 30 °C.[11] It is known that a number of liposomal dugs consist of lipids with $T_m$ over 37 °C, such as dipalmitoylphosphatidylcholine (DPPC; $T_m$ = 41 °C) or hydrogenated soya phosphatidylcholine (HSPC; $T_m$ = 52 °C) in DOXIL.[21] Furthermore, liposomes consisting of saturated phospholipids, *e.g.*, DPPC and HSPC, are known to have higher retentivity and stability in blood than those consisting of unsaturated phospholipids, *e.g.*, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC).[22–26] On the other hand, when $LMIC_{70}$ is used as a photosensitiser, there is the concern that the liposomes involving unsaturated phospholipids are stabilised due to carbon–carbon double bond oxidation by the generated $^1O_2$. Therefore, we chose DPPC as the lipid in this manuscript. The $C_{70}$-exchange reaction occurred completely above the $T_m$ of liposomes; however, lowering the temperature below the $T_m$ led to $C_{70}$ aggregation outside the liposomes.[16] That is, the $C_{70}$-exchange reaction did not occur in liposomes composed of DPPC at 30 °C.[16] Nevertheless, if the exchange reaction was performed in the presence of 1,10-dioctadecyl-3,3,30,30-tetramethylindocarbocyanine perchlorate (**5**) with a π-moiety, it was not influenced by $T_m$.[16] In this paper, we report on the added effect of various lipids with a π-moiety on the $C_{70}$-exchange reaction using a liposome composed of DPPC with a gel phase membrane, at 30 °C. We evaluated the $C_{70}$-exchange reaction by measuring the stabilities of $LMIC_{70}$ following centrifugation. The results showed that the presence of π-moieties in the neighbourhood of the hydrophilic liposome surface plays an important role in migrating hydrophobic $C_{70}$ into liposomes.

## Result and discussion

### Effect of mixing lipids with a π-moiety on the phase transition temperature

To confirm whether the $T_m$ decreased to under 30 °C due to the mixing of lipids with a π-moiety, we used differential scanning calorimetry (DSC) to determine the $T_m$ for the liposomes consisting of DPPC-lipids with a π-moiety ([**1**, **2**, **5** or DPPE]/[lipids] = 2.5 mol%) (Scheme 1). Slightly broader DSC peaks were observed

[a]*Graduate School of Materials Science, Nara Institute of Science and Technology, 8916-5 Takayama, Ikoma, Nara 630-0192, Japan. E-mail: aikeda@ms.naist.jp; Fax: +81 743 72 6099; Tel: +82 743 72 6091*
[b]*Department of Life System, Institute of Technology and Science, Graduate School, The University of Tokushima, 2 Minamijosanjimacho, Tokushima 770-8506, Japan*

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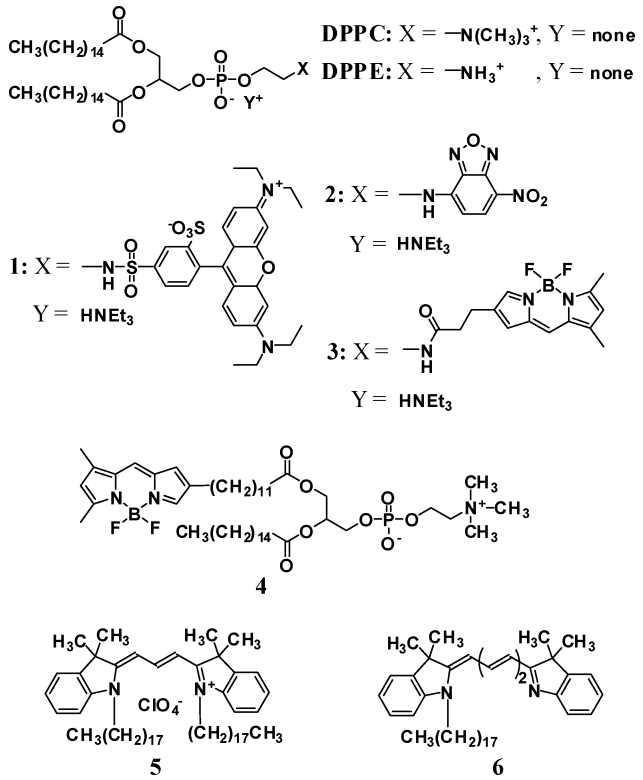


**Scheme 1** Structures of materials used in this study.

for all the liposomes examined; however, there was negligible change in the $T_m$ values (Fig. 1), indicating that the $T_m$ remained essentially unchanged following the addition of all lipids and that the $C_{70}$-exchange reactions are not influenced by the $T_m$.

### Effect of the addition of lipids with a π-moiety

$LMIC_{70}$ ([$C_{70}$]/[lipids] = 10 mol%) was prepared by the molecular exchange reaction from water-soluble γ-CDx-bicapped $C_{70}$[27] to liposomes composed of DPPC and lipids with a π-moiety ([total lipids] = 1.0 mM, [**1**, **2**, **5** or DPPE]/[lipids] = 0.25 or 2.5 mol%) by mixing at 30 °C for 10 min according to the previously described procedure.[13] We confirmed that there was negligible change in the $T_m$ value of $LMIC_{70}$ **1** ([**1**]/[lipids] = 2.5 mol%, [$C_{70}$]/[lipids] = 10 mol%) (Fig. 1, purple line).

The $^1$H NMR peaks assignable to the $C_{70}$·γ-CDx complex (Fig. 2a) were absent in the spectrum after the $C_{70}$-exchange reaction (Fig. 2b–g), indicating that all $C_{70}$ was released from the γ-CDx cavities in the presence of the liposomes. If the released $C_{70}$ is incorporated into the liposomes, the solution is stable, whereas if $C_{70}$ forms aggregates outside the liposomes, the solution is unstable and precipitation should be observed after centrifugation.[11] To confirm whether all $C_{70}$ was transferred into liposomes, aggregated $C_{70}$, which was not incorporated into the liposomes, was eliminated from the solutions of $LMIC_{70}$ by centrifugation at 4500 *g* for 10 min after each 2, 24, 48 and 72 h incubation period. In the absence of lipids with a π-moiety, the peak intensity at 380 nm (absorption maximum of $C_{70}$ in the $C_{70}$·γ-CDx complex) decreased considerably following 2 h incubation (Fig. 3a, black line and Fig. S1a†) and precipitation was observed. The results suggest that the majority of $C_{70}$ was not transferred from the γ-CDx cavity

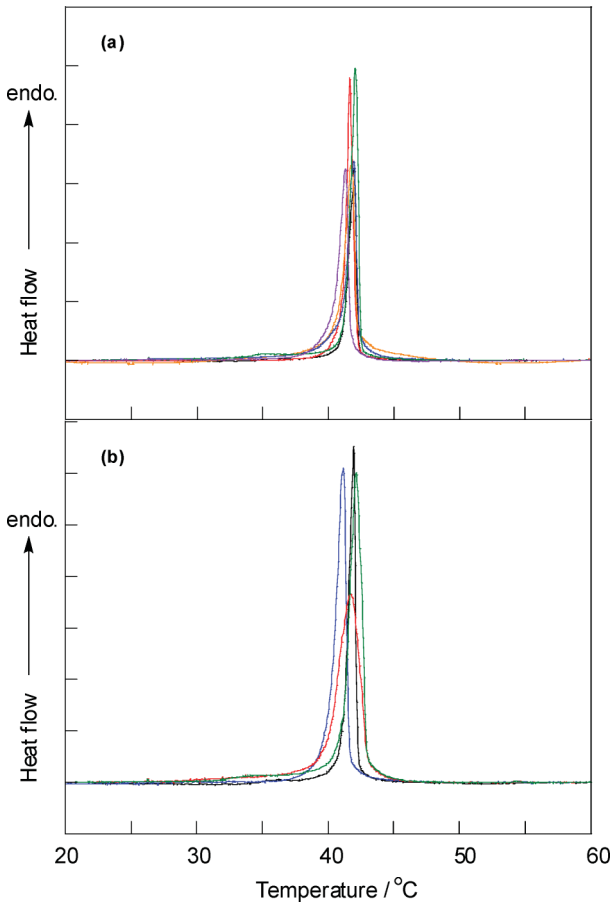


**Fig. 1** DSC curves of (a) DPPC- (black line), DPPC·**1**- (blue line), DPPC·**2**- (orange line), DPPC·**5**- (red line), DPPC·DPPE-liposomes (green line) and $LMIC_{70}$ **1** (purple line) and (b) DPPC- at pH 6.6 (black line) and 2.4 (green line) and DPPC·**6**-liposomes at pH 6.6 (blue line) and 2.4 (red line). [**1**, **2**, **5**, **6** or DPPE]/[lipids] = 2.5 mol%.

to the lipid membranes of DPPC because of the tight packing of the hydrophobic chains in the gel phase of DPPC.[16] A similar result was obtained for the addition of DPPE without a π-moiety (Fig. 3a, green line and Fig. S1b†). The finding shows that the $C_{70}$-exchange reaction did not take place only when mixing with a lipid without a π-moiety. On the other hand, in the presence of lipids with a π-moiety at high concentrations ([**1**, **2** or **5**]/[lipids] = 2.5 mol%), precipitation and absorption spectral changes were hardly observed in the solution of $LMIC_{70}$ in DPPC-liposomes after 72 h incubation and centrifugation (Fig. 3a, blue, orange and red lines and Figs. S2a, S3 and S4†). The order of $LMIC_{70}$ stability is **1** > **2** > **5**.

Furthermore, for low concentrations of lipids with the π-moiety ([**1**]/[lipids] = 0.25 mol%, [$C_{70}$]/[lipids] = 10 mol%), the absorbance of $C_{70}$ slightly decreased in $LMIC_{70}$ **1** (Fig. 3b, blue line and S2b†). How do the lipids with a π-moiety incorporate $C_{70}$? The amounts of lipids with the π-moiety of **1** and **2** used were too small to form π-complexes with $C_{70}$ in lipids ([**1** or **2**]:[$C_{70}$] = 1 : 40 mol/mol). Alternatively, the high stabilities of $LMIC_{70}$ **1** and **2** did not result from the π-complexes between the π-moiety of **1** or **2** and $C_{70}$ in the lipids after the incorporation of $C_{70}$. Many researchers have previously presumed that $C_{60}$ exists between two membranes of liposomes or among alkyl chains as determined

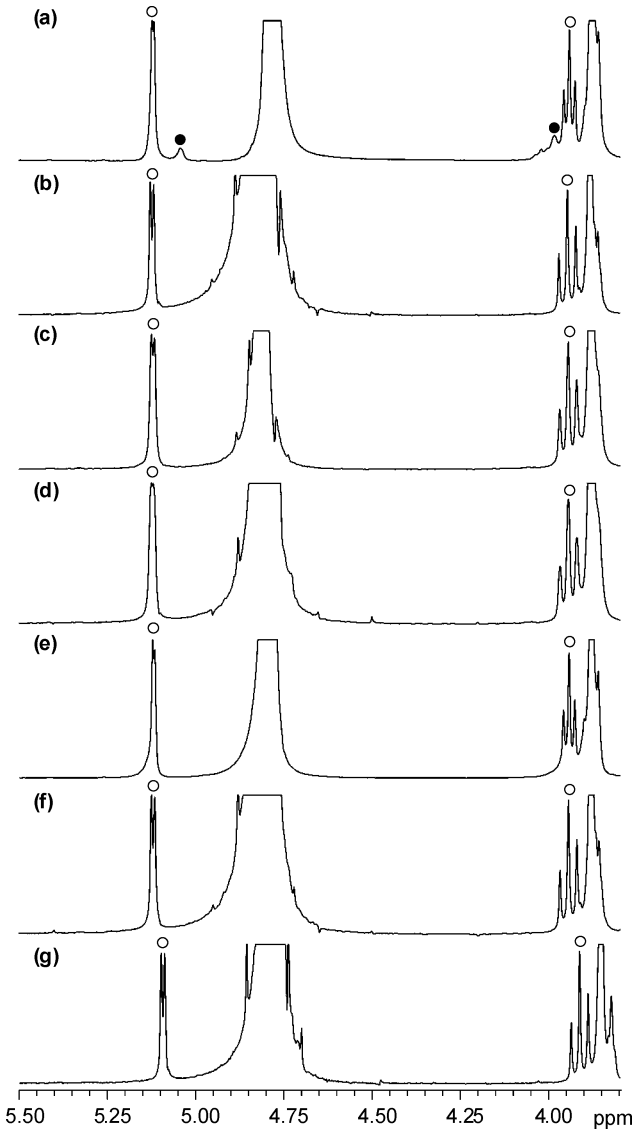


**Fig. 2** $^{1}$H NMR spectra at 400 MHz in $D_2O$ at 25 °C of the $C_{70}$·γ-CDx complex (a) before and after the addition of (b) DPPC·DPPE-liposome, (c) DPPC·**1**-liposome, (d) DPPC·**2**-liposome, (e) DPPC·**5**-liposome, (f) DPPC·**6**-liposome at pH 7.0 and (g) DPPC·**6**-liposome at pH 2.4 (○: free γ-CDx, ●: the $C_{70}$·γ-CDx complex). [total lipids] = 1.0 mM, [**1**, **2**, **5**, **6** or DPPE]/[lipids] = 2.5 mol% and [$C_{70}$]/[lipids] = 10 mol%.

by molecular dynamics simulations of fullerenes inside the lipid bilayer.[28–32] Although there is no example for $C_{70}$, $C_{70}$ in liposomes should show similar behaviour to $C_{60}$. Therefore, the π-moieties of **1**, **2** and **5**, which exist in the hydrophilic liposome surface, are postulated to act as a gate when hydrophobic $C_{70}$ migrates into the hydrophilic liposome surface. However, because the π-moieties of **1**, **2** and **5** are hydrated and can imperfectly coat the $C_{70}$ surfaces, $C_{70}$ is predicted to be separated from the π-moieties and to move into the hydrophobic lipid bilayer membrane by desolvation as a driving force.[33]

### Effect of location of the π-moiety

To confirm the importance of the function as a gate, we carried out comparative experiments examining the difference in stabilities

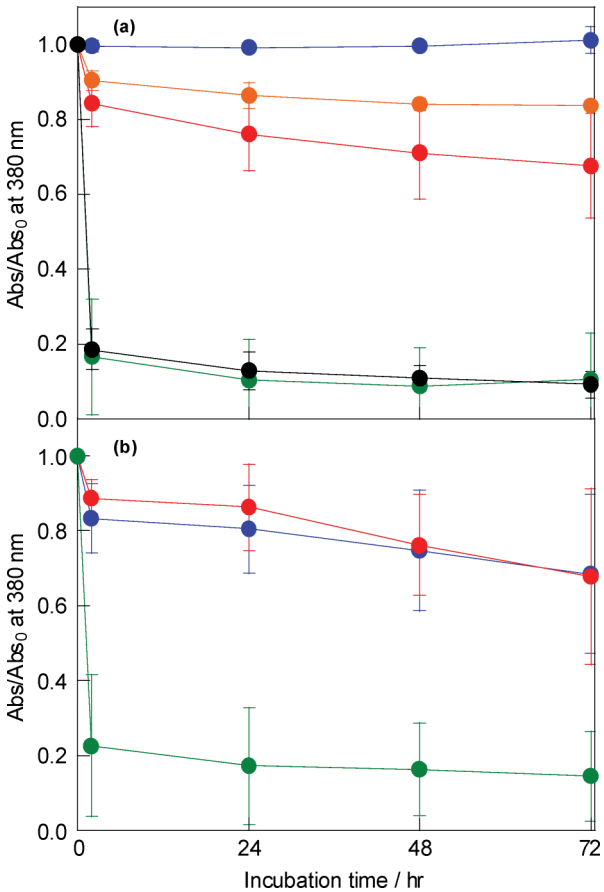


**Fig. 3** Incubation time-dependence of the absorbance at 380 nm of $LMIC_{70}$ in DPPC liposomes with 10 mol% $C_{70}$ in the absence and presence of lipids with a π-moiety: (a) DPPC only (black line), **1** (blue line), **2** (orange line), **5** (red line), and DPPE (green line); 2.5 mol% lipids with π-moiety, and (b) **1** (blue line), **3** (red line) and **4** (green line); 0.25 mol%. Each of these experiments was performed in triplicate.

between $LMIC_{70}$ **3** with a boron dipyrromethene (BODIPY) moiety in the polar head groups and $LMIC_{70}$ **4** with a BODIPY moiety in the alkyl chain groups. As shown in Fig. 3b (red and green lines) and S5†, the solution of $LMIC_{70}$ **3** is much more stable than that of $LMIC_{70}$ **4**, because the $C_{70}$-exchange reaction proceeded in the presence of **3**, indicating that it is important that the π-moiety exists in the polar head groups (*i.e.*, hydrophilic liposome surface).

### Control of the exchange reaction by varying of the pH

Based on the results of **3** and **4**, we carried out the $C_{70}$-exchange reaction using **6** ([**6**]/[lipids] = 2.5%) to control the progress of the reaction by pH. By changing the pH, the hydrophilicity and hydrophobicity of **6** was varied (Fig. 4). Under neutral conditions (pH = 6.6–7.0), the peak intensity at 380 nm decreased considerably after 2 h incubation (Fig. 5, blue line and S6a†) and precipitation was observed (Fig. 4a). The results suggest that the majority of $C_{70}$ was not transferred from the γ-CDx cavity to the lipid membranes of DPPC because the π-moiety of **6** was expected to be located among the acyl chains of liposomes or between two membranes in liposomes due to the hydrophobicity of **6**. Under acidic conditions (pH = 2.4–2.9), a new peak appeared at 652 nm,

 

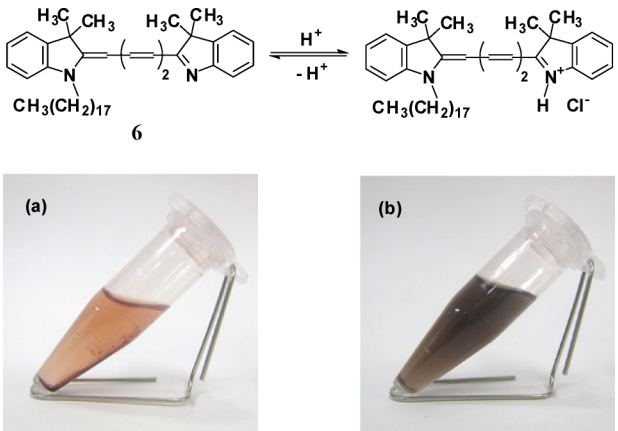


**Fig. 4** Protonation and deprotonation of **6**, and photographs of $LMIC_{70}$ in DPPC·**6**-liposome at (a) pH 6.6 and (b) pH 2.4 after 2 h incubation. [DPPC] = 1.0 mM, [**6**]/[lipids] = 2.5 mol% and [$C_{70}$]/[lipids] = 10 mol%.

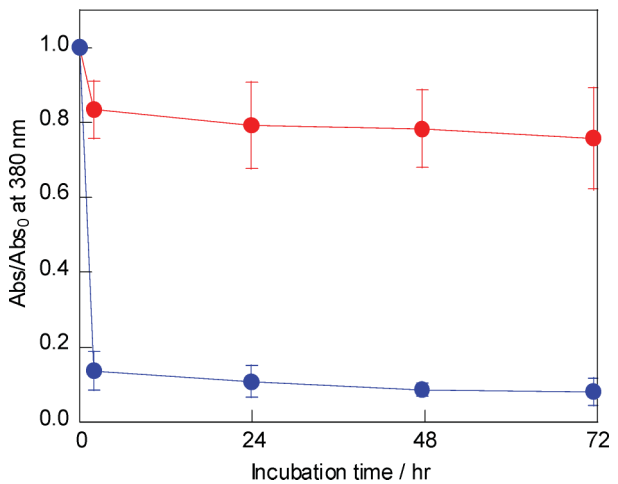


**Fig. 5** Change in the absorbance at 380 nm of $LMIC_{70}$ in DPPC liposomes with 10 mol% $C_{70}$ in the presence of 2.5 mol% **6**: pH 6.6–7.0 (blue line) and 2.4–2.9 (red line). Each of these experiments was performed in triplicate.

indicating that **6** was protonated (Fig. S6b†). Since precipitation and absorption spectral changes were negligible in the solution of $LMIC_{70}$ in DPPC·**6**-liposomes after 2 and 72 h incubation and centrifugation at pH = 2.4–2.9 (Fig. 4b and 5, red line and Fig. S6b†), the protonated **6** assists the incorporation of $C_{70}$ into the liposomes, because the π-moiety of **6** is located in the head groups of the lipids. In contrast, under acidic conditions (pH = 2.5), the peak intensity at 380 nm decreased considerably after 2 h incubation (Fig. S7†) and precipitation was observed. This result clearly indicated that the pH effect is not because of the swelling of DPPC but due to the action of **6** as a gate. These results are consistent with the results of **3** and **4**.

### Change of particle size by the exchange reaction

Dynamic light scattering (DLS) measurements before centrifugation gave further information about the particle sizes of the liposomes following the addition of the $C_{70}$·γ-CDx complex (Table 1). Average particle sizes were estimated to be around 100 nm in DPPC·**1**-, DPPC·**2**-, DPPC·**3**-, DPPC·**5**- and DPPC·**6** (acid pH)-liposomes, which formed stable $LMIC_{70}$ during the

**Table 1** Average particle sizes and polydispersity index of liposomes without and with lipids with a π-moiety before and after the exchange reaction and before centrifugation

| | Before the exchange reaction | | After the exchange reaction | |
|---|---|---|---|---|
| Liposomes | Average particle size/nm[e] | PDI[f] | Average particle size/nm[e] | PDI[f] |
| DPPC | 97 ± 23 | 0.115 | 567 ± 330[g] | 0.599 |
| DPPC·DPPE[a] | 121 ± 14 | 0.141 | 657 ± 224[g] | 0.696 |
| DPPC·**1**[a] | 115 ± 8 | 0.066 | 83 ± 4 | 0.157 |
| DPPC·**1**[b] | 85 ± 8 | 0.061 | 96 ± 22 | 0.171 |
| DPPC·**2**[a] | 117 ± 9 | 0.078 | 108 ± 14 | 0.107 |
| DPPC·**3**[b] | 90 ± 18 | 0.075 | 74 ± 12 | 0.119 |
| DPPC·**4**[b] | 89 ± 9 | 0.087 | 380 ± 257[g] | 0.620 |
| DPPC·**5**[a] | 102 ± 4 | 0.076 | 121 ± 37 | 0.219 |
| DPPC·**6**[a,c] | 111 ± 8 | 0.086 | 394 ± 59[g] | 0.180 |
| DPPC·**6**[a,d] | 113 ± 42 | 0.077 | 96 ± 9 | 0.154 |

[a] [**1**, **2**, **5** or **6**] = 2.5 mol%, [b] [**1**, **3** or **4**] = 0.25 mol%, [c] pH = 2.4–2.9, [d] pH = 6.6–7.0, [e] Each experiment was carried out three times, [f] PDI means Polydispersity index, [g] The precipitates were observed.

$C_{70}$-exchange reaction. Conversely, larger average particle sizes were observed in DPPC-, DPPC·DPPE-, DPPC·**4**- and DPPC·**6** (neutral pH)-liposomes (over 350 nm), suggesting that $C_{70}$ forms aggregates outside the liposomes after release from the γ-CDx cavities.

## Experimental

### Materials

γ-CDx was purchased from Wako Pure Chemical Industries Ltd. (Tokyo, Japan). Dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylethanolamine (DPPE) were purchased from NOF Corp. (Tokyo, Japan). Lissamine rhodamine B 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (**1**), *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (**2**), *N*-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-propionyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (**3**), 2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-dodecanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphocholine (**4**) and 1,10-dioctadecyl-3,3,30,30-tetramethylindocarbocyanine perchlorate (**5**) were obtained from Molecular Probes, Inc. (Eugene, OR). $C_{70}$ (>99%) was bought from MER Co. (Tucson, AZ).

### 1-Octadecyl-3,3,30,30-tetramethylindocarbocyanine (6)

With stirring, a solution of *N*-((1*E*,3*E*)-3-(phenylimino)prop-1-enyl)-*N*-(prop-1-en-2-yl)aniline bromide[34,35] (590 mg, 2.24 mmol) in dry methanol (5 mL) was added dropwise to a solution of 2,3,3-trimethyl-1-octadecyl-3*H*-indolium bromide[36] (922 mg, 2.24 mmol) and 2,3,3-trimethyl-3*H*-indole (357 mg, 2.24 mmol) in dry methanol (15 mL) with AcONa (1.35 g, 18.0 mmol) and refluxed for 17 h. The solvent was removed by evaporation, and the residue was dissolved in diluted $CH_2Cl_2$ and was washed with sat. $NaHCO_3$ aq. and brine followed by drying over $Na_2SO_4$. The solvent was removed in vacuo, and the residue was purified

by column chromatography on silica gel ($CH_2Cl_2$–$Et_2O$: a linear gradient from 1 : 0 to 1 : 1(v/v)) to give compound **6** as a red powder in 1.1% yield (14.8 mg, 24 μmol): $\delta_H$ (400 MHz; $CDCl_3$) 0.88 (3 H, t, *J* 6.7), 1.22–1.35 (2 H, m), 1.38 (6 H, s), 1.58 (6 H, s), 1.62–1.66 (2 H, m), 3.57 (2 H, t, *J* 7.3), 5.44 (1 H, d, *J* 12), 6.18–6.29 (2 H, m), 6.62 (1 H, d, *J* 7.6), 6.86 (1 H, t, *J* 7.3), 7.13–7.31 (6 H, m), 7.55–7.65 (2 H, m); *m/z* (EI-HRMS) calcd for $C_{43}H_{62}N_2$ 606.4913, found 606.4908 $[M]^+$.

### Preparation of liposomes

Each liposome was composed of DPPC only, or DPPC and lipids **1–6** and DPPE ([**1–6** or DPPE]/[lipids] = 0.25 or 2.5 mol%. All liposomes were prepared by stepwise extrusion through 0.05 μm pores using an extruder. The prepared liposomes were uniform in size with *ca*. 80 nm diameter.

### Preparation of $LMIC_{70}$ by fullerene exchange reaction

All $LMIC_{70}$ samples were prepared using an exchange reaction between the liposomes and the $C_{70}$·γ-CDx complex[27] at 30 °C for 10 min, as described previously.[11] The final concentrations of the respective components were evaluated using integrated intensities of their $^1$H NMR spectra, where [γ-CDx] = 0.91 mM, $[C_{70}]$ = 0.10 mM and [lipids] = 1.00 mM (thus γ-CDx/$C_{70}$/lipids = 9.1 : 1 : 10).

### Differential scanning calorimetry (DSC)

The phase transition behaviour of the lipid bilayer vesicles was determined with a differential scanning calorimeter (VP-DSC, MicroCal, Inc., Northampton, MA). The concentrations of the respective components were [**1–6** or DPPE]/[lipids] = 2.5 mol%. The measurements were performed between 10 and 60 °C at 0.5 °C $min^{-1}$ heating rate. The phase transition temperature ($T_m$) was evaluated.

## Conclusions

The $C_{70}$-exchange reactions were observed to take place when small amounts of lipids with a π-moiety were used. Lipids with a π-moiety in the hydrophilic head groups assisted the $C_{70}$-exchange reactions, whereas lipids with a π-moiety located in the acyl chains did not aid the exchange reaction. Therefore, hydrophobic **6** at neutral pH was inefficient in facilitating the $C_{70}$-exchange reaction because the π-moiety of **6** was positioned among the alkyl chains or between two membranes in the liposomes. However, hydrophilic **6** protonated at acidic pH did facilitate the $C_{70}$-exchange reaction because the π-moiety of **6** may be located in the neighbourhood of the head groups of the lipids according to the result of the DSC experiments. Consequently, the exchange reaction can be controlled by pH. The exchange reaction performed at room temperature should be suitable for preparing liposomal drug delivery systems with a conjugated antibody or protein such as transferrin without protein denaturation. Furthermore, these results are very important in the development of liposome formulations as novel carriers for water-insoluble drugs with low thermal stabilities.


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