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Budding and Fission of Cationic Binary Lipid Vesicles Induced by the Incorporation of Pyranine

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Pyranine, a multivalent anionic fluorescent dye, triggered the budding and production of small vesicles from giant vesicles that consisted of zwitterionic and cationic lipids. Calorimetric analysis and microscopic observations revealed that the binding of pyranine induced domain formations of the lipid bilayer followed by membrane fission.

In biological systems, membrane trafficking plays important roles in the inter- and intracellular transport of bioactive molecules and signals.[1] Membrane trafficking consists of several different dynamic processes such as membrane fission,[2] vesicle propagation,[3] and membrane fusion.[4] The detailed mechanism of each process has been clarified by biochemical and biophysical studies.[2a,5] However, the artificial reconstitution of membrane trafficking has not been achieved.

We have been studying artificial cell-based membrane trafficking using liposomes to establish bioinspired communication systems. Here, a series of molecular switches were designed and synthesized to induce stimuli-responsive membrane dynamics such as gemini peptide lipids for the selective propagation of vesicles triggered by ionic[6] and photonic[7] stimuli, and a photoresponsive cholesterol derivative for the manipulation of microdomain structures on a membrane.[8] In this report, we present an example of chemical trigger-induced fission of giant vesicles. Currently, few chemical triggers for membrane fission have been reported[9] which contrasts the large number of fusion triggers reported.[10] Consequently, a variety of fission triggers are required to handle diverse biological information in artificial membrane traffic systems.

N,*N*-Dihexadecyl-*N'*-(3-(trimethylammonio)propyl)urea bromide (**1**) (Chart 1) was synthesized through the condensation of dihexadecylamine with 3-bromopropylisocyanate and followed quaternization with trimethylamine (26% yield, details in Supporting Information).[11,15] Giant unilamellar vesicles (GUVs) were prepared by the gentle hydration of a thin lipid film, which consisted of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and **1** (7:3 molar ratio), in a 12 mM sucrose solution at 40 °C for 12 h.

First of all, we used a microscopy approach to observe the morphological changes of the GUVs induced by the addition of 8-hydroxy-1,3,6-pyrenetrisulfonic acid trisodium salt (pyranine, Chart S1).[15] To evaluate the effect of pyranine in a time-dependent manner, we used a special sample chamber, which can gently introduce the aqueous solution of pyranine to the suspension of GUVs by free diffusion (Figure S1).[15] Since pyranine displays green fluorescence at 510 nm by the excitation at 400 nm in an aqueous solution,[12] fluorescence microscopy can directly visualize the localization of pyranine on the lipid membranes. The present procedure of GUV preparation quantitatively yielded GUVs with a diameter of ca. 5 µm. The addition of pyranine led to the membranes of the GUVs displaying a uniform green fluorescence (Figure 1A), indicating that the pyranine molecules homogeneously adsorbed onto the surface of the lipid membranes. However, over time the pyranine molecules gradually localized to form domain structures (Figures 1B and 1C). The morphology of the GUV subsequently changed to an asymmetric pear-like shape to increase the local curvature of the pyranine-rich domain on the lipid membrane (Figure 1D). The shape change of the GUV continuously took place until a small vesicle budded from the mother giant vesicle (Figure 1E) and the small vesicle was eventually released (Figure 1F). We observed the present budding and fission of giant vesicles with good reproducibility (see Supporting Information). In addition, morphological changes to GUVs described above were not observed if the GUVs were prepared with either DMPC or **1** lipids (Figure S4),[15] suggesting that the coexistence of at least two different lipid components is one of the requirements for the membrane fission.

The effect of pyranine on membrane properties was investigated in detail using differential scanning calorimetry (DSC) of DMPC/**1** liposomes. Here, the concentration of pyranine (Figure 2) was changed and the obtained data was analyzed by the deconvolution of overlapping peaks (Table 1, Figure S5[15]). DSC has been widely used to prove lateral phase separation of lipid membranes.[13] If an additive induces phase separation by accumulating a specific lipid, each lipid phase displays an independent transition temperature (T_m) closer to that of the pure lipid. In the absence of pyranine, we observed a broad endothermic peak at 25.4 °C for the phase transition from

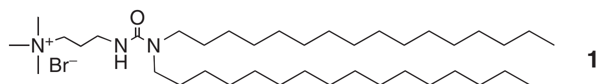

Chart 1.

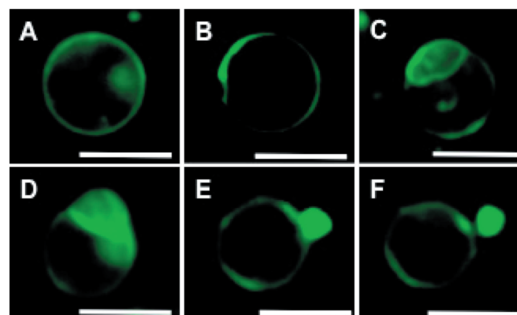

Figure 1. Morphological changes of a GUV induced by pyranine. (A)–(F) were acquired at 2, 5, 10, 12, 15, and 15.1 min after the addition of pyranine, respectively. [DMPC] = 350 µM, [**1**] = 150 µM, [pyranine] = 25 µM in a sucrose solution (12 mM), at 25 °C. Bar: 5 µm. The present morphological change was observed for more than 10 samples.

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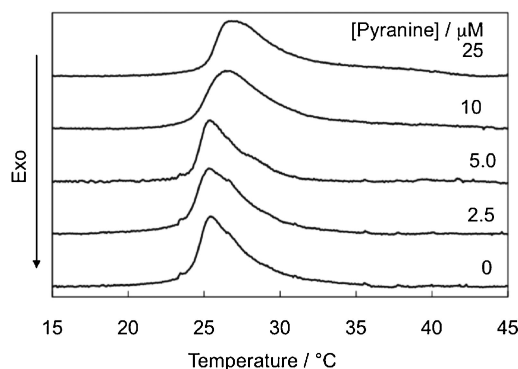

Figure 2. DSC thermograms of liposomes consisting of DMPC and **1** in the presence of pyranine at various concentrations. [DMPC] = 350 µM, [**1**] = 150 µM, in a sucrose solution (12 mM). Pyranine concentration in each liposome suspension was individually shown in the thermogram.

Table 1. Estimated temperature (T_m) and enthalpy of the phase transition (ΔH) of DMPC/**1** liposomes in the presence of pyranine in a sucrose solution (12 mM)

[Pyranine]/µM	T_{m1}/°C	ΔH_1/ kJ mol^{-1}	T_{m2}/°C	ΔH_2/ kJ mol^{-1}
0	25.4	25.5	—[a]	—[a]
2.5	25.2	24.7	—[a]	—[a]
5.0	25.3	23.4	29.5	3.30
10	26.9	22.6	32.9	10.0
25	27.4	20.5	33.6	13.8

[a]An endothermic peak was not observed.

the gel to liquid crystalline state of the lipid bilayer, indicating that two lipid components were miscible with each other. In contrast, addition of pyranine above 5 µM led to a broadening of the endothermic peak with the concomitant appearance of a shoulder at higher temperatures. The temperature of the shoulder was close to the T_m of pure **1** (32.9 °C), which showed no significant shift in the presence of pyranine. Thus, at the observation temperature (25 °C), the GUV forms a phase-separated structure between DMPC-rich and **1**-rich domains. In addition, by increasing the concentration of pyranine, the enthalpy with higher T_m (ΔH_2) increased, reflecting the increase in the number of lipid molecules involved in the phase transition of the higher T_m. These results strongly suggest that the interaction of pyranine with the DMPC/**1** membrane promotes the formation of a **1**-rich domain in the membrane by forming pyranine–**1** complexes.

To evaluate the ionization state of pyranine, we measured fluorescence spectra since the excitation spectrum of pyranine reflects the ionization state of the 8-hydroxy group. The excitation maximum displays a significant red shift when the 8-hydroxy group of pyranine is ionized.[12] Upon the addition of liposomes, the excitation maximum of pyranine shifted from 406 to 470 nm (Figure S6),[15] indicating that the 8-hydroxy group of pyranine is deprotonated by binding to the liposome. This result is consistent with a previous report that showed that the surfaces of cationic liposomes display significantly higher pH values than the bulk phase.[14] Thus, pyranine interacts with the cationic lipid membrane as a tetravalent anion, which is able to form a complex with up to four molecules of monovalent cationic lipid **1**.

In summary, we propose a simple mechanism of membrane fission induced by pyranine as follows. Initially pyranine homogeneously binds to the surface of the positively charged lipid membrane. Subsequently, the cationic lipids gradually accumulate to form a domain structure through electrostatic interactions with pyranine. Formation of the complex between **1** and pyranine alters the molecular packing of the lipid molecules in the **1**-rich domain with a concomitant decrease of local fluidity. Finally, the **1**-rich domain is excluded from the surrounding lipid membrane by the formation of a small vesicle. The detailed mechanism of the present budding and subsequent fission behavior and the application to artificial membrane traffic systems are currently under investigation.

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15 Supporting Information is available electronically on the CSJ-Journal website, http://www.csj.jp/journals/chem-lett/index.html.

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