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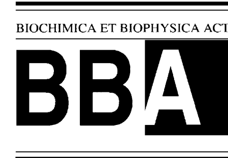

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Rapid report

Stability of giant unilamellar vesicles and large unilamellar vesicles of liquid-ordered phase membranes in the presence of Triton X-100

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Abstract

We have investigated the stability of giant unilamellar vesicles (GUVs) and large unilamellar vesicles (LUVs) of lipid membranes in the liquid-ordered phase (lo phase) against a detergent, Triton X-100. We found that in the presence of high concentrations of Triton X-100, the structure of GUVs and LUVs of dipalmitoyl-PC (DPPC)/cholesterol (chol) and sphingomyelin (SM)/chol membranes in the lo phase was stable and no leakage of fluorescent probes from the vesicles occurred. We also found that ether-linked dihexadecylphosphatidylcholine (DHPC) membranes containing more than 20 mol% cholesterol were in the lo phase, and that DHPC/chol-GUV and DHPC/chol-LUV in the lo phase were stable and no leakage of internal contents occurred in the presence of Triton X-100. In contrast, octylglucoside solution could easily break these GUVs and LUVs of the lo phase membranes and induced internal contents leakage. These data indicate that GUVs and LUVs of the lo phase membranes are very valuable for practical use.
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Keywords: Liquid-ordered phase; DPPC/cholesterol; Sphingomyelin/cholesterol; DHPC/cholesterol; Triton X-100; Octylglucoside

Giant unilamellar vesicles (GUVs) or giant liposomes of lipids such as phosphatidylcholine (PC) whose diameters are more than 10 μm have been used for physical and biological investigations, such as elastic properties of phospholipid membranes [1,2], shape change of vesicles [3–6], interaction of cytoskeleton proteins with the membranes [7], membrane fusion [8], and reconstitution of artificial cells [9,10]. These studies are helpful to understand dynamics of biomembranes. Recently, it is considered that GUVs are useful as a vessel with a small-confined volume for chemical reaction and crystallization of proteins [10,11]. However, GUVs of phospholipid membranes in the liquid-crystalline (Lα) phase have some technical drawbacks; one is that they are easily disrupted by detergents or surfactants [12], and the other is that hydrolysis of phospholipids in water prevents long usage of these GUVs in water.

In this report, we prepared GUVs and large unilamellar vesicles (LUVs) of lipid membranes in the lo phase and investigated their stability against a nonionic detergent, Triton X-100. The lo phase of lipid membranes is formed in binary mixture membranes of cholesterol and saturated PCs such as DPPC, and also of cholesterol and SM [13–18]. DPPC/chol membranes containing ≥25 mol% cholesterol and SM/chol membranes containing ≥30 mol% cholesterol are in the lo phase. In the lo phase, hydrocarbon chains of PC have high orientational order, but the lateral diffusion coefficient of lipids in the membrane is relatively high [15]. The rafts in cell membranes, which are considered to be in the lo phase, have been isolated from cell membranes based on the resistance to the solubilization by Triton X-100 at 4 °C [17], and it is recently shown from the light scattering experiments that DMPC/cholesterol membrane in the lo phase is much more resistant to solubilization by the

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nonionic detergent C12E8 than the Lα-phase membranes [19]. We found that GUVs and LUVs of the DPPC/chol and SM/chol membranes in the lo phase were stable and no leakage of fluorescent probes from the vesicle occurred in the presence of Triton X-100. Next, we investigated effect of cholesterol on membrane of an ether-linked dialkylphospholipid, i.e., dihexadecylphosphatidylcholine (DHPC), which is in the interdigitated gel phase at neutral pH at 20 °C [20,21], but is in the bilayer gel phase in the presence of cholesterol (≥3~5 mol%) [22]. We consider that DHPC membrane can be used in water for a long period due to the difficulty of its hydrolysis. We found that GUVs and LUVs of DHPC/chol membranes in the lo phase were stable against Triton X-100.

DPPC, 1,2-dioleoyl-*sn*-glycero-3-phospocholine (DOPC), SM from brain, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[poly (ethylene glycol) 2,000] (PEG2K-DPPE), and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[poly (ethylene glycol) 2000] (PEG2K-DOPE) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). DHPC was purchased from Fluka Chemie AG. Cholesteryl-BODIPY-FL-C_{12} (BODIPY-chol), 8-aminonaphthalene-1,3,6-trisulfonic acid-disodium salt (ANTS), and *p*-xylene-bis-pyridinium bromide (DPX) were purchased from Molecular Probes (Eugene, OR, USA). Cholesterol and polyoxyethylene (10) octylphenyl ether (Triton X-100) were purchased from Wako Chemical (Tokyo, Japan). Octylglucoside (OG) and calcein were purchased from Dojindo Laboratory (Kumamoto, Japan).

GUVs were prepared by the natural swelling of dry lipid films as follows [5,6]. One hundred microliters of 1 mM lipids (DPPC/chol, SM/chol, DHPC/chol, DOPC) in chloroform in a small glass vessel was dried by N_2 gas, and then the solvent was completely removed by placing the sample in a vacuum desiccator connected to a rotary vacuum pump for more than 12 h. Ten-microliter water was added into this vessel, and it was incubated at 45 °C for a few minutes (prehydration). Then, 1 ml of 0.1 M sucrose in water was added, and incubated at 37 °C for 2 h. GUVs were observed by a fluorescence, phase-contrast microscope using the standard method described in our previous paper [5,6]. Fluorescence intensities of these GUVs containing 0.02 to 0.05 mol% BODIPY-chol using the fluorescence microscope with the high sensitive EB-CCD camera were much lower than those from oligo-lamellar vesicles. Various kinds of concentrations of Triton X-100 or OG in 0.1 M glucose aqueous solution were added into the neighborhood of a GUV through a 10-μm diameter glass micropipet which position was controlled by a micromanipulator (Narishige) [5,6]. In some cases, GUVs were incubated in various kinds of bulk concentrations of Triton X-100 or OG (in 0.1 M glucose) in the chamber to demonstrate the stability of vesicles in the detergent solutions. GUVs containing a water soluble fluorescent probe, calcein, were prepared as follows. It is very difficult to prepare GUVs of PC membranes in a buffer and in the presence of high salt concentrations. To prepare DPPC/chol-GUV, DHPC/chol-GUVs, and DOPC-GUVs in a buffer, we incorporated a small amount (1.0 mol% of the total lipid) of a poly-(ethylene glycol) [PEG]-grafted phospholipid, PEG2K-DPPE (PEG-lipid) in these membranes (for DOPC-GUV, PEG2K-DOPE), and succeeded in the large production of these GUVs in 10 mM HEPES buffer (pH 7.0) containing 1 mM calcein and 0.1 M sucrose. As discussed in our previous paper [10], hydrophilic polymers attached on the surface of electrically neutral lipid membranes by incorporating a small amount of PEG-lipid in PC membrane increase the formability of GUVs of PC membranes in buffers containing high concentrations of salts. The procedure of the formation of GUVs was the same as that for other GUVs described above. To remove the untrapped calcein, at first the GUV suspension was centrifuged (14,000×*g*, 10 min at 20 °C; Tomy, MR-150) to remove multilamellar vesicles (MLVs), and then its supernatant was eluted through a Sephadex G-75 column with 10 mM HEPES buffer (pH 7.0) containing 0.1 M glucose. To investigate the effect of temperature on diameter of GUVs, we controlled the temperature of the solution containing GUVs in the hand-made chamber installed in the inverted phase-contrast microscope using the stage-warmer (MP100DM, Kitazato, Tokyo, Japan). We decreased temperature from 50 to 30 °C at a rate of 1 °C/min, and measured diameters of a few directions of a GUV and obtained their average value.

LUVs of DPPC/chol such as DPPC/40% chol, DHPC/chol such as DHPC/30% chol, and DOPC were prepared by the extrusion method using 200-nm pore-size membrane. The appropriate amounts of lipids in chloroform were mixed and dried by N_2 gas, and then the solvent was completely removed by placing the sample in a vacuum desiccator connected to a rotary vacuum pump for more than 12 h. To prepare MLVs, appropriate amount of 10 mM PIPES buffer (pH 7.0) containing a fluorescence probe ANTS and its quencher DPX was added to this dry lipid film in excess water, and the suspension was vortexed for about 30 s several times at room temperature (~20 °C). Then, we performed freeze-thawings of the MLV solution; it was frozen in liquid N_2 for 2 min, and then thawed at room temperature for 20 min. After the 5× freeze-thawings of the MLV suspension, the resultant solution was extruded through 200-nm pore-size membrane using the LiposoFast apparatus (LF-1, Avestin, Ottawa, Canada) until the solution became transparent. The leakage of internal contents from LUVs was measured using the ANTS/DPX method [23,24]. Briefly, LUVs were prepared in 10 mM PIPES buffer (pH 7.0) containing 12.5 mM ANTS, 45 mM DPX, 22.5 mM NaCl. To remove the untrapped ANTS and DPX, the LUV suspension was eluted through a Sephadex G-75 column with 10 mM PIPES buffer (pH 7.0) containing 100 mM NaCl. For fluorescence measurement, a Hitachi F3000 spectrofluorimeter was used. Fluorescence intensities of samples were measured at room temperature (~20 °C). The excitation wavelength of ANTS was 352 nm, and its

emission wavelength was 517 nm. Excitation band pass and emission band pass were 5 nm. At 1 min and 3 h after mixing the LUV suspensions with various concentrations of Triton X-100 in 10 mM PIPES buffer (pH 7.0) containing 100 mM NaCl, fluorescence intensities of samples were measured. In the case of OG, at 15 min after mixing the LUV suspension with OG in 10 mM PIPES buffer containing 100 mM NaCl, the fluorescence intensities were measured. Concentrations of the total phospholipids in the samples for the measurement of the fluorescence were in the range of 0.04–0.4 mM, which were determined by the Bartlett method. The fluorescence intensity of DOPC-LUV suspension in the presence of 2.0% (v/v) Triton X-100 at 1 min after the mixing was taken as the 100% leakage of internal contents of the DOPC-LUV. On the other hand, the fluorescence intensities of DPPC/30% chol-LUV and DHPC/30% chol-LUV suspensions in the presence of 90 mM OG at 15 min after the mixing were taken as the 100% leakage of internal contents.

It is well known that GUVs of lipid membranes are easily disrupted by detergents or surfactants as well as small-size vesicles such as LUVs [12]. We investigated effect of detergent solution on GUVs of DPPC/chol membrane and SM/chol membrane in the lo phase. As a detergent, we used Triton X-100, which is one of the most popular nonionic detergents to disrupt vesicles and cells rapidly and also to solubilize lipids and membrane proteins. At first, we investigated effect of Triton X-100 on DOPC-GUVs, which is a typical GUV of the L_α phase-membrane [5,6]. 0.1% (v/v) Triton X-100 (=1.7 mM; larger than its CMC <0.24 mM>) was added near a DOPC-GUV through a micropipet at 20±2 °C. Phase-contrast microscope images show that it was easily disrupted and disappeared. On the other hand, when 0.1% (v/v) or 1.0% (v/v) Triton X-100 was added near DPPC/30% chol-GUVs for 5 min to 3 h, these GUVs were not disrupted and their shapes did not change. We observed the same phenomenon in 12 GUVs among 12 examined ones (*n*=12). Similarly, when 0.1% (v/v) or 1.0% (v/v) Triton X-100 was added near SM/40% chol-GUVs for 5 min to 3 h, these GUVs were not disrupted (*n*=20). We also investigated the leakage of a small fluorescent probe, calcein from GUVs using the fluorescent microscope. Fig. 1A shows a typical change of fluorescence microscope image of a DOPC-GUV containing 1 mM calcein induced by addition of 0.1% Triton X-100 through a micropipet near the GUV. It indicates that calcein leaked away from the DOPC-GUV quickly. In contrast, addition of 1% Triton X-100 through a micropipet near the DPPC/30% chol-GUV for 5 min did not induce leakage of calcein (Fig. 1B(2)–(3)), and fluorescence microscope images of DPPC/30% chol-

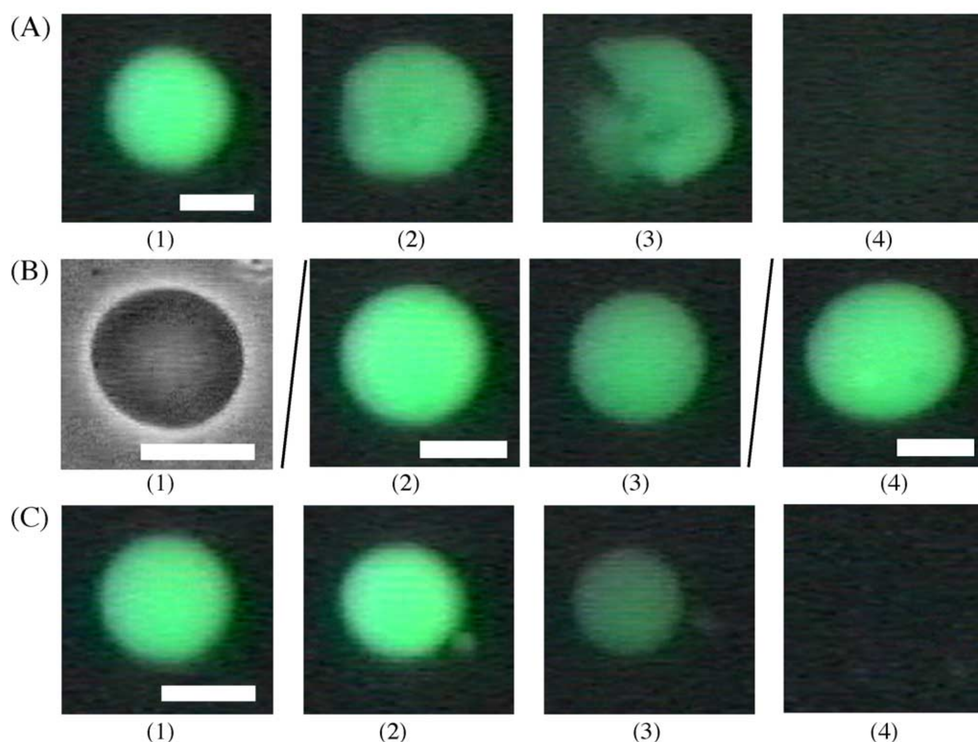

Fig. 1. (A) Fluorescence microscopy images of 1% PEG-lipid/99% DOPC-GUV containing 1 mM calcein. Its image change was induced by the addition of 0.1% Triton X-100 at 20 °C. The time after starting addition through the micropipet is (1) 0 s, (2) 2 s, (3) 4 s, and (4) 6 s for the pictures. (B) (1) A phase-contrast image of a DPPC/30% chol-GUV. (2), (3) A fluorescence microscopy image of a 1% PEG-lipid/69% DPPC/30 % chol-GUV before addition of 1% Triton X-100 (2) and at 5 min after the addition of 1% Triton X-100 through the micropipet (3). (4) A fluorescence microscopy image of another 1% PEG-lipid/69% DPPC/30% chol-GUV in 1% Triton X-100 (where 1% is the bulk concentration in the chamber) incubated for 3 h. (C) Fluorescence microscopy images of 1% PEG-lipid/69% DPPC/30% chol-GUV containing 1 mM calcein. Its image change was induced by the addition of 50 mM OG at 20 °C. The time after starting addition through the micropipet is (1) 0 s, (2) 5 s, (3) 14 s, and (4) 18 s for the pictures. All scale bars in the figures are 10 μm.

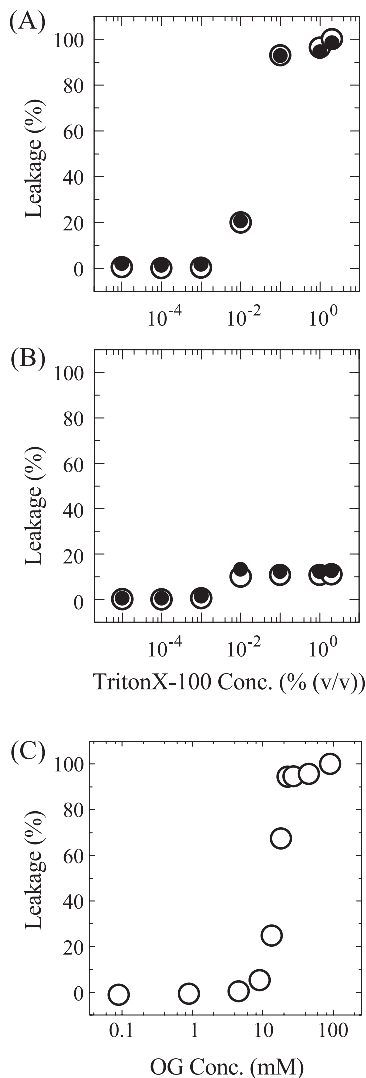

Fig. 2. Triton X-100-induced leakage of internal contents from DOPC-LUV (A) and from DPPC/40% chol-LUV (B): (○) at 1 min and (●) at 3 h after mixing the LUV suspension with various concentrations of Triton X-100. (C) OG-induced leakage of internal contents from DPPC/30% chol-LUV (at 15 min after mixing the LUV suspension with various concentrations of OG).

GUVs in 1% Triton X-100 (where 1% is the bulk concentration in the chamber) did not change after 3-h incubation (Fig. 1B(4)). These results indicate that DPPC/chol-GUV and SM/chol-GUV in the lo phase were stable, i.e., not disrupted in the presence of Triton X-100 at 20 °C. The strong resistance of GUVs of the lo phase membrane to Triton X-100 solution is useful for practical usage of GUVs. On the other hand, when 50 mM OG (larger than its CMC <25 mM>) was added near DPPC/30% chol-GUVs, they were easily disrupted and disappeared. Fig. 1C shows a typical change of fluorescence microscope image of a DPPC/30% chol-GUV containing 1 mM calcein induced by addition of 50 mM OG through a micropipet near the GUV. Calcein leaked away from the DPPC/30% chol-GUV quickly, indicating that OG disrupted this GUV.

We also investigated the effect of Triton X-100 on LUVs of DPPC/chol membrane and SM/chol membrane in the lo phase. To investigate the stability of LUV against Triton X-100 solution, leakage of internal contents of the LUVs into the external solution was measured using a mixture of the fluorescent probe ANTS and its quencher DPX as the internal contents. At first, we investigated effect of Triton X-100 solution on DOPC-LUV. As shown in Fig. 2A, at and above 0.1% Triton X-100 (> its CMC), extent of the leakage was almost 100%. In contrast, for DPPC/40% chol-LUV, even at 1.0% Triton X-100, extent of the leakage was about 10% after 3-h incubation (Fig. 2B), and it did not increase after 24-h incubation. It is considered that a small amount of leakage comes from the LUVs whose stability was low at their formation. These results show that LUVs of the lo phase membranes were not disrupted by Triton X-100, indicating that these LUVs have high resistance against detergents such as Triton X-

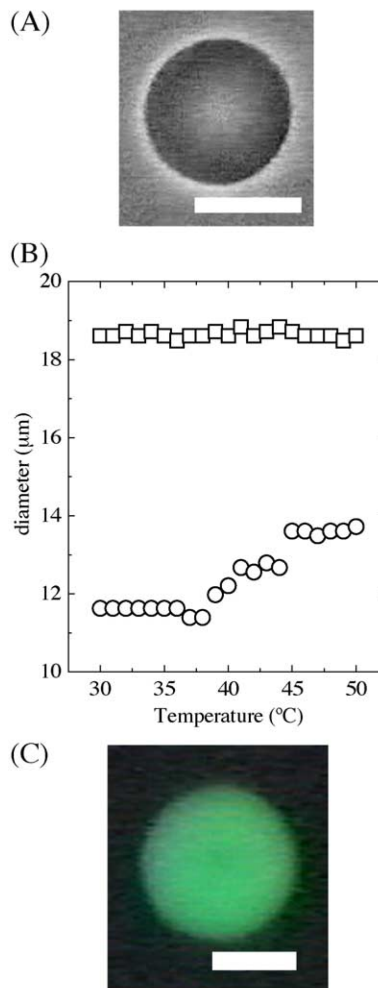

Fig. 3. (A) A phase-contrast image of DHPC/30% chol-GUV. Bar is 20 μm. (B) Temperature dependence of diameter of DHPC/30% chol-GUV and 0.2% PEG-lipid/DHPC-GUV from 50 to 30 °C. (□) DHPC/30% chol-GUV, (○) 0.2% PEG-lipid/DHPC-GUV. (C) A fluorescence microscopy image of another 1% PEG-lipid/69% DHPC/30% chol-GUV in 1% Triton X-100 incubated for 3 h. Bar is 10 μm.

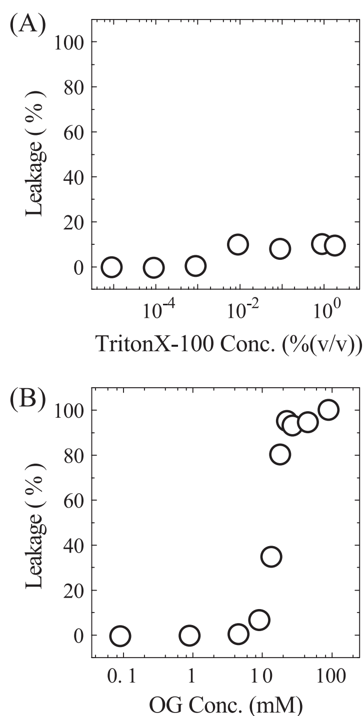

Fig. 4. (A) Triton X-100-induced leakage and (B) OG-induced leakage of internal contents from DHPC/30% chol-LUV (15 min after mixing the LUV suspension with various concentrations of Triton X-100 and OG).

100. It supports the above conclusion of the high stability of GUVs of the lo phase-membrane against Triton X-100. In contrast, OG induced a large leakage from DPPC/30% chol-LUV (Fig. 2C); at 15 mM OG, the leakage became evident, and at ≥23 mM OG the extent of leakage was almost 100%.

Next, we investigated stability of DHPC/chol-GUV. At first, we investigated effect of cholesterol on chain-melting phase transition of DHPC-MLV using DSC [21]. In the absence of cholesterol, there was a large endothermic peak due to the chain-melting phase transition at T_m=43.4 °C [21]. In contrast, in the presence of high concentration of cholesterol (more than 20 mol%), there were no sharp endothermic peaks, but very broad small peaks in DSC heating curves. These data agree with Laggner's data [22]. Moreover, we could produce GUVs of DHPC/chol membranes containing more than 20 mol% cholesterol in water at 20−37 °C, which is lower than T_m of the DHPC membrane (Fig. 3A). In the case of DPPC/chol membranes, we could not produce any GUVs of 100% DPPC membrane and DPPC/10% chol membrane, but in contrast, we could produce many GUVs of DPPC/30% chol membrane and those of DPPC/40% chol membrane which are in the lo phase. Thereby, we can conclude that GUVs can be formed from the membranes in the lo phase, although they cannot be formed from the membrane in the gel phase. Therefore, the above data on DHPC/chol membranes suggest that the DHPC/chol membranes containing more than 20 mol% cholesterol are in the lo phase. To confirm that membranes of the DHPC/chol-GUVs are in the lo phase, we investigated temperature dependence of diameter of DHPC-GUVs and that of DHPC/chol-GUV from 50 to 30 °C. At first, we tried to produce DHPC-GUV in water at 50 °C, but failed. Thereby, to prepare DHPC-GUV, we incorporated 0.2 mol% PEG2K-DPPE (PEG-lipid) in DHPC membrane, and succeeded in the large production of 0.2% PEG-lipid/DHPC-GUV at 50 °C. Fig. 3B (○) shows that the diameter of 0.2% PEG-lipid/DHPC-GUV began to decrease at 44 °C. It is reasonable to consider that this result was due to the chain-melting phase transition of the DHPC membrane. In contrast, as shown in Fig. 3B (□), the diameter of DHPC/30% chol-GUV was almost constant, indicating that no chain-melting transition occurred. This data also supports that the DHPC/chol-GUVs were in the lo phase. When 0.1% (v/v) or 1.0% (v/v) Triton X-100 was added near DHPC/30% chol-GUVs for 5 min to 3 h, these GUVs were not disrupted and their shape did not change (n=6). We also investigated the leakage of calcein from GUVs. Fig. 3C shows a fluorescence microscope image of DHPC/30% chol-GUVs containing 1 mM calcein in the presence of 1% Triton X-100 (where 1% is the bulk concentration in the chamber) after 3-h incubation. This result indicates that calcein did not leak from the DHPC/30% chol-GUV in the presence of 1% Triton X-100. In contrast, when 50 mM OG was added near DHPC/30% chol-GUVs, they were easily disrupted and disappeared (data not shown). We also investigated the effect of Triton X-100 and OG solution on LUVs of DHPC/chol membrane in the lo phase using the ANTS/DPX leakage method. For DHPC/30% chol-LUV, even at 1.0% Triton X-100, extent of the leakage was about 10% after 3-h incubation (Fig. 4A), indicating that these LUVs have high resistance against Triton X-100. In contrast, OG induced a large leakage from DHPC/30% chol-LUV (Fig. 4B); at 15 mM OG, the leakage became evident, and at ≥23 mM OG the extent of leakage was almost 100%.

These results indicate that GUVs and LUVs of the lo phase-membranes were stable and no leakage of internal contents occurred in the presence of Triton X-100, and in contrast, OG solution could easily break these GUVs and LUVs. Thereby, we can separate GUVs of the Lα phase-membrane and GUVs of the lo phase-membranes and also the contents in these GUVs using Triton X-100 and OG. Moreover, GUVs and LUVs of the DHPC/chol membranes in the lo phase can be used in water for a long time, because DHPC is the ether-linked dialkylphospholipid and thereby its hydrolysis does not occur easily.

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