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Inhibition of Intervesicular Aggregation of Phospholipid Vesicles by Incorporation of Dialkyl Oligosaccharide Glycerol

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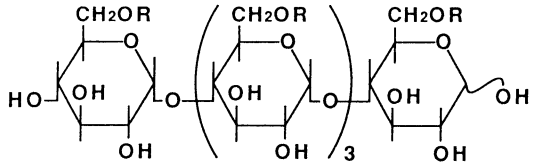
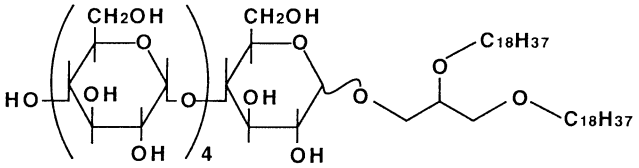
1,2-Di-*O*-octadecyl-3-*O*-α(β)-maltopentaosyl-*rac*-glycerol (DOMPG) was incorporated into lipid vesicles to modify the surface of the vesicles. The aggregation of small unilamellar vesicles below the gel-to-liquid crystalline phase transition temperature and the aggregation of negatively-charged vesicles by the addition of calcium ion were effectively inhibited.

Phospholipids dispersed in water assemble to form lipid bilayer vesicles. Vesicles have potential applications as microcapsules for drugs, proteins or functional water-soluble or -insoluble molecules.[1] However, the aggregation and fusion of vesicles and the leakage of entrapped molecules are serious problems in such applications. Polymerization of the bilayer membrane is one effective method to stabilize vesicles.[2,3] Polymerization sometimes degrades other important properties[4] such as membrane fluidity, phase transition, dynamic phase separation, biodegradability, *etc*. To realize both stability and such vesicular functions, the coating of vesicles with polymers, such as polysaccharides, was reported.[2,5] Though a polymer coating enhances the membrane barrier against the release of entrapped molecules, problems about the aggregation and the precipitation of vesicles have not yet been solved. On the other hand, Wu *et al.* incorporated a series of monosaccharide-type glycolipids into the bilayer membrane of phospholipid vesicles, and reported that some glycolipids depending on their structure had an effect of suppressing the aggregation of vesicles.[6]

The attractive inhibition effect on the aggregation of vesicles was developed using oligosaccharide-type glycolipids.[7,8] We synthesized maltopentaose monopalmitate (MPMP) and maltopentaose dipalmitate (MPDP). MPMP was found to inhibit the aggregation and fusion of vesicles, while MPDP did not show the effective inhibition of intervesicular aggregation. Since the introduction of palmitic acid into oligosaccharides having many reactive OH-groups was non-selective, many isomers of MPDP showed different effects on the



MPMP : 4R = -H , R = $-COC_{15}H_{31}$
MPDP : 3R = -H , 2R = $-COC_{15}H_{31}$



DOMPG

dispersion state of vesicles.[8] Some showed similar inhibition effects as MPMP, while the others induced aggregation. Some structural effects on the aggregation of vesicles were considered to exist from these results, but could not be clarified because the isomer structures had not been determined. In this study, we designed an oligosaccharide derivative, *i.e.*, a maltopentaose derivative having dioctadecyl glycerol at the anomeric position of the oligossacharide through the ether bond, and compared the effects with cases of MPMP and MPDP.

Maltopentaose was acetylated by a reaction with acetic anhydrate in pyridine and washed with an aqueous solution of sodium hydrogencarbonate. Acetylated maltopentaose recrystallized 4 times from 2-propanol was mixed with 1,2-di-*O*-octadecyl-*rac*-glycerol[9] in 1,2-dichloroethane in the presence of molecular sieve 4A powder. Trimethylsilyl trifluoromethanesulfonate was added slowly at room temperature and the resulting mixture was reacted for 60 h. α- and β-Anomers of acetylated products were purified by subjecting to Silica gel 60 column (Merck, toluene/ethyl acetate = 1/1, v/v as eluent). Deacetylation was carried out by refluxing the methanol solutions of α- or β-anomer in the presence of a little amount of triethylamine and water, and then crude materials obtained were recrystallized twice (total yield: 11%). ^{1}H-NMR of DOMPG ($(CD_3)_2SO$, TMS) (in ppm): 0.7-1.0 (6H, -C**H**$_3$), 1.1-1.2 (64H, -(C**H**$_2$)$_{16}$CH$_3$), 2.8-5.6 (60H, maltopentaose, -OC**H**$_2$CH$_2$, glycerol). ^{13}C-NMR (in ppm): 103 (anomer carbon, cf. 92 for anomer carbon of maltopentaose). R_f values on Silica gel 60 TLC plates (Merck, $CHCl_3$/MeOH = 2/1, v/v): 0.10 (α-anomer), 0.16 (β-anomer).

A methanol solution of the oligosaccharide lipid (0.2 mol%) was mixed with chloroform solutions of DPPC. A thin lipid film prepared on the inner wall of a flask was dispersed into pure water, and extruded through polycarbonate membrane filters (final pore size: 0.1 μm). The solution was stored at 4 °C for 24 h to equilibrate the system. In order to measure the incorporation ratio of the oligosaccharide lipid into the bilayer membrane, the vesicle suspension (DPPC: 0.5 g/dl) was centrifuged (300000g, 60 min) to separate the oligosaccharide lipid in the exogenous aqueous phase of the vesicles from the incorporated one. The amount of oligosaccharide lipid in the supernatant was quantitatively analyzed by a phenol-sulfuric acid method.[10] Seventy-two percent of DOMPG was found to be incorporated into the bilayer membrane of small unilamellar DPPC vesicles (78 ± 18 nm). While MPMP showed 52%, which was almost the same as the previous result (50%) measured by gel permeation chromatography (Sepharose CL-4B).[7,8] MPDP showed 90% in this experiment. The order of the ratio should represent the hydrophilic-hydrophobic balance of the lipid structure. Oligosaccharide lipids having two alkyl chains showed a higher incorporation ratio, and MPDP having two alkyl chains at different positions on the oligosaccharide chain should be incorporated more preferentially than DOMPG. It is considered that MPDP stably binds to the surface of the bilayer membrane by two anchoring groups, while DOMPG binds to the membrane by one anchoring group having two alkyl chains at the end of oligosaccharide molecule.

Mixed lipid vesicles (1,2-bis(2,4-octadecadienoyl)-*sn*-glycero-3-phosphorylcholine (DODPC) / cholesterol / palmitic acid / oligosaccharide lipid = 7/7/2/0.5 by mol) were prepared (0.5 g/dl) in saline (pH = 7.4). After incubation at 4 °C for 24 h, the incorporation ratio of each oligosaccharide lipid was measured in the same manner as already mentioned. The incorporation ratio of DOMPG was significantly raised to 95% while MPMP to 92%. In this case, the average diameter of the vesicles is 217 ± 71 nm. Larger size vesicles and the incorporation of cholesterol resulted in the improvement of lipid packing, leading to the high incorporation ratio of oligosaccharide lipids. It is noted that there was no difference in incorporation ratios between the two isomers (α- and β-anomers).

Small unilamellar vesicles (DPPC) prepared with Extruder (Nuclepore, final pore size of membrane

filter = 0.05 μm) tended to aggregate below the gel-to-liquid crystalline phase transition temperature (T_c) because of their constrained lipid packing in the bilayer membrane with high curvature.[11] Preparation of vesicles was carried out at 50 °C, which is above the T_c (43 °C) of DPPC vesicles. The turbidity change (ΔO.D.) of the solution due to the aggregation and fusion of vesicles was monitored at 4 °C as the change in absorbance at 800 nm (Shimadzu MPS-2000). As shown in Fig. 1, DPPC vesicles readily aggregated and fused, and the ΔO.D. saturated at ca. 0.2, while the vesicles of which surface was modified with the oligosaccharide maintained good stability in dispersion of vesicles. DOMPG restricted the aggregation of vesicles more effectively than MPMP because the larger amount of DOMPG was incorporated into the surface of the vesicles. There was no difference between the α- and β-anomers of DOMPG.

Lipid vesicles were prepared with Extruder (final pore size =0.2 μm) from lipid mixture of DODPC /cholesterol/stearic acid /oligosaccharide lipids (7/7/2/0.5 by mol.). Since lipid packing of the vesicles is improved due to the smaller curvature of the vesicles, the introduction of cholesterol, and the electrostatic repulsion between vesicles induced by an ionic charge on the surface, aggregation is suppressed. Therefore, the mixtures of phospholipid/cholesterol/negatively-charged lipid are usually used to provide stable vesicles. However, this kind of vesicles easily aggregates in the presence of Ca^{2+} ion because Ca^{2+} binds on the negatively charged surface and crosslinks vesicles.[12] The aggregation of vesicles was analyzed by viscometry with a cone-plate rotation viscosimeter. A viscometric

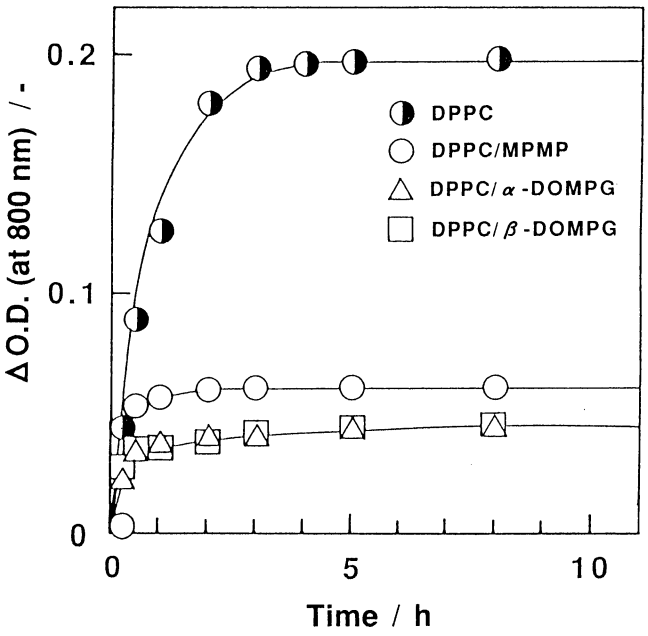


Fig. 1. Inhibition effect of oligosaccharide lipids on the aggregation of DPPC small vesicles (0.5 g/dl) at 4 °C.

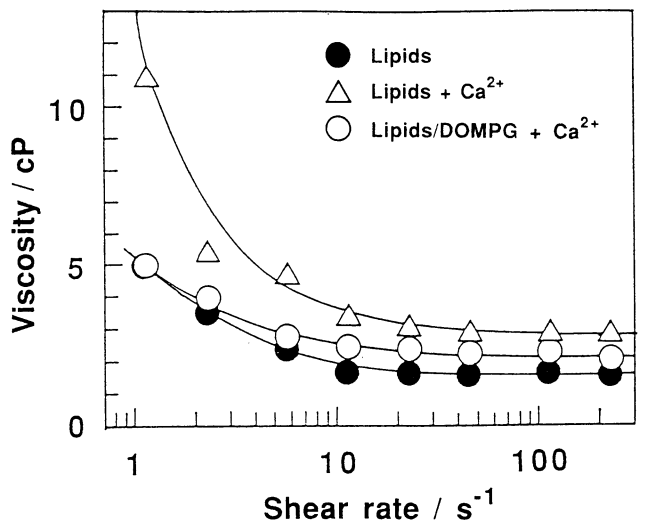


Fig.2. Inhibition effect of DOMPG on the aggregation of (DODPC/cholesterol/stearic acid) vesicles (4.2 g/dl) induced by Ca^{2+} adding (4.8 mM) at 37 °C.

measurement makes it possible to analyze the dispersibility of vesicle suspensions at a high concentration (5.0 g/dl). An increase in the viscosity at low shear rates in the presence of Ca^{2+} means the aggregation of vesicles. This was confirmed for vesicles without DOMPG in Fig.2. On the other hand, vesicles modified with DOMPG showed no increase in the viscosity even after Ca^{2+} addition.

The inhibition mechanism of vesicle aggregation is proposed such that oligosaccharide chains extending from the surface of the vesicles into the bulk aqueous phase should inhibit the access of the vesicles due to the steric repulsion. Therefore, DOMPG is one of the ideal structures with high incorporation ratios that efficiently prevent the aggregation of vesicles.

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