CHEMISTRY LETTERS, pp. 1891-1894, 1993.

Cryoprotection of Synthetic Glycolipids for Phospholipid Vesicles

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The synthetic glycolipid; *N*-hexadecylmaltopentaonamide (HDMPA), was introduced as a component of a phospholipid vesicle (ϕ, *ca.* 100 nm) of 1,2-dipalmitoyl-*sn*-glycero-3-phosphorylcholine (DPPC) to modify the surface of the vesicle with oligosaccharide. After freeze-thawing, the size of the vesicles does not change, and the leakage of 5(6)-carboxyfluorescein encapsulated into the vesicles is suppressed, indicating that the glycolipid possesses a cryoprotective activity.

Phospholipid vesicles have been studied as carriers of drugs, proteins, or functional water-soluble or -insoluble molecules.[1] Some cosmetic products utilizing the vesicles are now commercially available, and recently, some pharmaceutical products are being approved for clinical trials. The long term storage of the vesicles is, however, difficult because the vesicles tend to aggregate and fuse after freeze-thawing or freeze-drying and rehydration. At the same time, leakage of entrapped molecules is inevitable. Dehydration of lipids occurs on freezing because the vesicles are expelled from the crystals of water molecules,[2] and the resulting concentrated and deformed vesicles are unstable. Addition of cryoprotectants such as glycerol[3] and disaccharides[4] is a conventional method to protect the vesicles against freezing. It was reported that hydroxyl groups of these cryoprotectants associated with unfreezable water molecules would bind to the phosphoric groups of lipids through hydrogen bondings.[5] The networks of the cryoprotectants and water molecules on the surface of the vesicles would contribute to stabilization of the bilayer membrane of the vesicles. However, the high osmotic pressure and the high viscosity of the vesiclular suspension due to a large amount of cryoprotectants are serious problems. Baldeschweiler and Huang reported that the introduction of synthetic maltosyl and isomaltotriosyl glycolipids into vesicles provided the vesicles with a cryoprotective activity;[6,7] however, more than 30 mol% of the glycolipid and the addition of disaccharides were necessary for this effect.

We reported on the inhibition of intervesicular aggregation and fusion by introducing various synthetic glycolipids with oligosaccharide chains into the bilayer membrane of a vesicle.[8-10] Excellent stability would be derived from the excluded volume effect of the oligosaccharide chains extended from the vesicular surface which inhibits the access of other vesicles. Based on research of the cryoprotection of saccharides, our glycolipids are considered to possess an excellent cryoprotective activity owing to the oligosaccharide chains. This was examined with conventional methods such as the measurement of diameter change and an increase in the fluorescent intensity of 5(6)-carboxyfluorescein (CF) leaked from the vesicles after freeze-thawing.

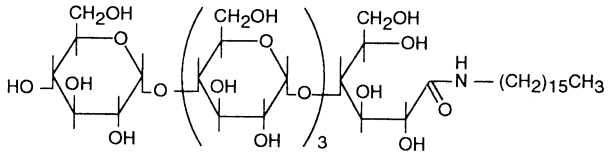
N-Hexadecylmaltopentaonamide (HDMPA) was selected as a glycolipid because of its uniform structure well-characterized and relatively high solubility in organic solvents such as methanol. One structural characteristic of HDMPA is that the terminal glucose unit which is coupled onto the alkyl chain is in an opening

form.[11] This will act as a spacer between the rigid oligosaccharide chain and the alkyl chain to promote the mobility of the oligosaccharide chain on the vesicular surface. The simplicity of synthesis is also an advantage. Maltopentaose lactone obtained by oxidation with IO_4^- was mixed with hexadecylamine in methanol and the reaction mixture was refluxed for 3 h. After washing with acetone and hexane, the crude product was purified by HPLC with a reversed-phase column.

DPPC (NOF Co., 100 mg) and HDMPA (≤ 17.52 mg (12 mol%)) were dissolved in methanol/chloroform (5/95 by vol) and evaporated by a rotary evaporator to form a thin lipid film on an inner wall of a flask. A CF (Kodak Co.) solution (10 ml, 50 mM) buffered by 20 mM Tris-HCl (pH 7.4) was added to each flask and incubated at 50 °C to form multilamellar vesicles. The resulting suspensions were extruded at 50 °C through polycarbonate membrane filters (Nucrepore Co., final pore size: 0.1 μm) producing vesicles with a homogeneous diameter analyzed with a sub-micron particle analyzer (Coulter Co., model N4-SD, equipped with the size distribution processor). The exogenous CF was removed by gel filtration (Sepharose CL-4B) using an isotonic 20 mM Tris-HCl buffered saline as an eluent. After the concentration of DPPC was adjusted to 2.0 mM (0.15 wt%), the suspensions (1 ml each) were quickly frozen in a dry ice/methanol coolant and allowed to stand for 10 min. Then the samples were incubated in water (20 °C) for 30 min for thawing. The leakage of CF from the vesicles was measured as an increase in the fluorescence intensity at 520 nm with a fluorescence spectrometer (JASCO FP-550) with the excitation at 330 nm. The fluorescence intensity at 100% leakage was measured after the addition of Triton X-100 (20 μl) and vortex mixing.

In Fig. 1 are shown the size distributions of DPPC and DPPC/HDMPA ([HDMPA]: 4 mol%) vesicles before and after freeze-thawing. Before the procedure, all the vesicles show a well-controlled diameter owing to the extrusion through the polycarbonate membrane filter (ϕ, 0.1 μm). Extruded vesicles are known to be more stable than the sonicated vesicles due to the better lipid packing.[12] After freeze-thawing, another distribution appears at *ca.* 550 nm. This indicates that some vesicles are fused to form bigger vesicles. Freeze-thawing is known as a method used for the preparation of large unilamellar vesicles by fusion of small unilamellar vesicles.[13] Whereas, the vesicles containing more than 4 mol% of HDMPA show no change in diameter.

Usually, deformation of vesicles is suppressed by the addition of more than 100 mM of disaccharides such as trehalose.[7] This



N-hexadecylmaltopentaonamide (HDMPA)

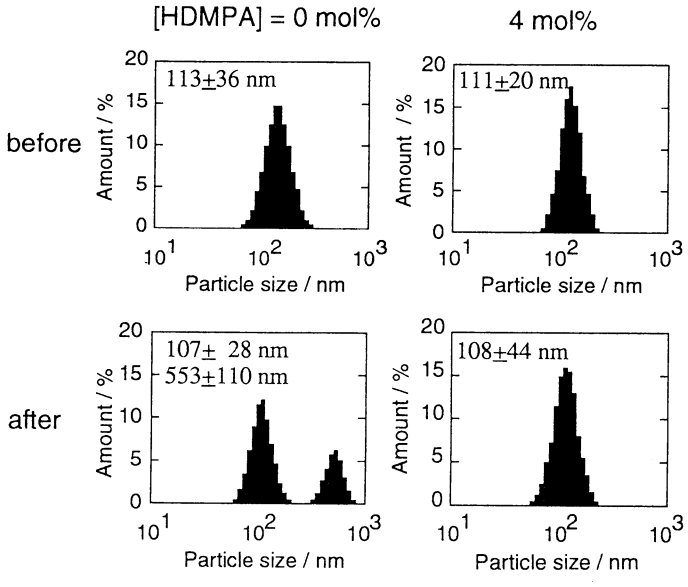


Fig. 1. Size distributions of DPPC and DPPC/HDMPA ([HDMPA] : 4 mol%) vesicles before and after freeze-thawing.

concentration is extremely larger than that in our system; the concentration of the oligosaccharide chain in the vesicle suspension ([HDMPA]: 4 mol%) is only 0.08 mM. HDMPA showed a high incorporation ratio (> 98.5%) in spite of its high solubility in water (> 0.5 g/ml). The amide group of HDMPA will contribute to the hydrogen bonding with surrounding phospholipids as in the same manner with a ceramide group in a sphingolipid,[14] which results in the effective introduction of HDMPA into the vesicle. This direct fixation of oligosaccharides to the vesicular surface is a key point to enhance the cryoprotective activity. No free disaccharide is necessary. The critical amount of HDMPA (4 mol%) needed to maintain the structure is less than that in the systems reported by Baldeschwieler *et al.* ([maltosyl glycolipid]: 30 mol%).[6] This difference is considered to be due to the structural difference in glycolipids. We reported that the aggregation of vesicles in a solution was suppressed by the excluded volume effect of oligosaccharide chains, and a larger effect was obtained when the oligosaccharide chain was longer.[9] During freezing, the vesicles are concentrated because of the crystallization of bulk water. The oligosaccharide chains would also inhibit the access of other vesicles.

Figure 2 shows the amount of CF leaked from the vesicles after freeze-thawing. Without HDMPA, 60% CF was leaked out of the vesicles. The leakage decreased with an increasing amount of HDMPA introduced into the bilayer membrane. The vesicles containing 4 mol% HDMPA leaked 40% CF and the vesicles with more than 8 mol% HDMPA leaked only 6%. These results did not correspond to the diameter change of the vesicles shown in Fig. 1; that is, the vesicles with 4 and 6 mol% HDMPA showed no change in diameter in spite of 40 and 30% CF leakages after freeze-thawing, respectively. CF would leak out through small cracks in the bilayer membrane formed by the stress of ice crystals, the dehydration or the partial osmotic pressure difference during freezing and thawing. More than 4 mol% HDMPA is enough to maintain the vesicular structure, but the critical concentration of HDMPA to prevent leakage of CF is 8 mol%. Above this concentration, leakage was maintained at a low level (≤ 6%). The introduction of HDMPA above this concentration would diminish the formation of cracks and stabilize the bilayer membrane.

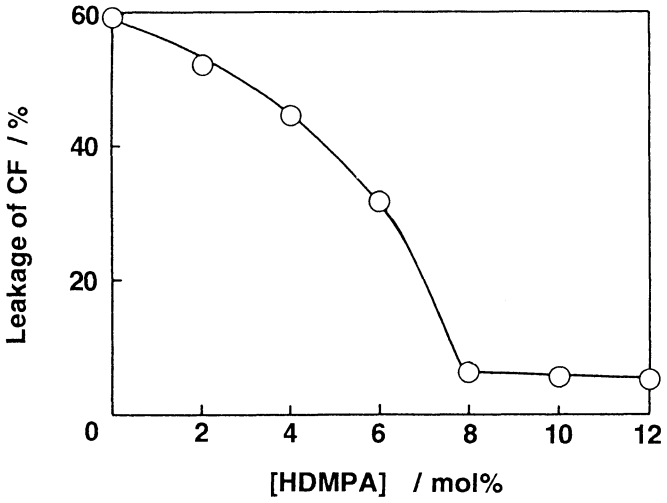


Fig. 2. Leakage of encapsulated CF from DPPC vesicles after freeze-thawing was decreased by introducing HDMPA.

The phase transition temperature of the vesicles was measured *in situ* by observing the temperature dependence of the CF leakage.[15] On raising the temperature from 25 to 50 °C at 5 °C/min, the vesicles released CF rapidly at around 41 - 42 °C, irrespective of the HDMPA content (0 - 12 mol%). DSC thermograms (Seiko SSC5200, DSC120) of multilamellar DPPC vesicles showed a phase transition temperature at 40 °C, and those of DPPC/HDMPA vesicles ([HDMPA]: ≤ 12mol%) at the same temperature (40 - 41 °C) within experimental error. It is indicated that HDMPA does not influence the phase transition of the matrix bilayer membrane under the conditions employed. Therefore, this stability would be derived from the converted surface properties by the effective fixation of oligosaccharide chains onto the vesicular surface by introducing

HDMPA. The percentage of the surface covered with the oligosaccharide chains ([HDMPA]: 8 mol%) was calculated approximately to 17%. The existence of unfreezable water molecules bound to maltopentaose (7.2 mol per glucose unit mol[16]) should be considered for this explanation. Further investigation is necessary to clarify the mechanism of the cryoprotection.

In conclusion, the glycolipids with oligosaccharide chains not only have an inhibitory effect on intervesicular aggregation but also a cryoprotective activity. It is demonstrated that glycolipids are important not only for biochemical functions such as molecular recognition, but also for physicochemical stability.

This work was partly supported by a Grant-in-Aid from the Ministry of Education, Science, and Culture, Japan (No. 05650930). The authors are grateful to Chiba Flour Milling Co., Ltd. for supplying HDMPA.

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(Received July 30, 1993)