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Effect of Nonlamellar-Prone Lipids on Protein Encapsulation in Liposomes

Taeho Ahn*

Department of Biochemistry, College of Veterinary Medicine, Chonnam National University, Gwangju 500-757, Korea

Youn-Tae Chi and Chul-Ho Yun*

School of Biological Sciences and Technology, Chonnam National University, Gwangju 500-757, Korea

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Abstract: We investigated the effect of two nonlamellar-prone lipids, phosphatidylethanolamine (PE) and dioleoylglycerol (DOG), on the efficiency of protein encapsulation in liposomes. When the phosphatidylcholine (PC) matrix was replaced with PE or DOG during liposome formulation, the amounts of glutathione *S*-transferase and bovine serum albumin entrapped in the vesicles increased with increasing PE or DOG concentration. The presence of PE and DOG synergistically affected protein entrapment. These results suggest that protein encapsulation can be enhanced by the presence of nonlamellar lipids and/or lipid-induced membrane properties.

Keywords: protein encapsulation, nonlamellar-prone lipids, liposomes.

Introduction

Lipid vesicles (liposomes) are widely used in biology, medicine, and biotechnology as tools for research and application. Especially, liposomes have been utilized as carriers for material transfer to life on the basis of their advantages over other molecular delivery methods: they can be formulated using natural lipids to increase 'bioavailability' and 'bioacceptability' by evading the immune systems. Liposomes, which are biodegradable and essentially non-toxic, are also able to encapsulate both hydrophobic and hydrophilic materials.[1]

Various methods for the preparation of liposomes have been developed, which are particularly dependent on vesicle diameter and entrapped aqueous volume.[2,3] Popular methods include the reverse-phase evaporation of solvent,[4] the removal of detergent from lipid-detergent micelles,[5] and the extrusion of freeze-thawed lipid-film.[6] To encapsulate molecules in liposomes, similar formulation procedures to the vesicle preparations have been also used.

In this study, we investigated the effect of lipid composition on the encapsulation efficiency of proteins using three different methods mentioned above. Anionic phospholipids such as phosphatidylserine (PS), phosphatidylglycerol (PG), phosphatidic acid (PA), and nonlamellar-prone lipids such as phosphatidylethanolamime (PE) and dioleoylglycerol (DOG) were incorporated in lipid vesicles during the preparation, which these lipids have been known to regulate functional roles of cellular membranes and to change the physicochemical properties of lipid bilayers. The amounts of entrapped proteins were assayed using glutathione *S*-transferase (GST) and bovine serum albumin (BSA) to determine the encapsulation efficiency of protein.

*Corresponding Authors. E-mails: thahn@chonnam.ac.kr or chyun@chonnam.ac.kr

Experimental

Materials. Fatty acid-free BSA and carboxyfluorescein (CF) were purchased from Sigma-Aldrich (St. Louis, MO). GST was expressed in *Escherichia coli* and purified using the pGEX-2T vector (GE Healthcare Bio-Sciences, Piscataway, NJ) according to the manufacturer's instructions. All phospholipids and DOG were purchased from Avanti Polar Lipids (Alabaster, AL). 1,6-Diphenyl-1,3,5-hexatriene (DPH) was obtained from Invitrogen (Carlsbad, CA).

Methods.

Preparation of Liposomes: Liposomes were prepared using the reverse-phase evaporation (REV),[4] freeze-thawing/extrusion (FTE),[7] and dialysis of detergent (DD) methods.[5] In all methods, initial phospholipid concentrations of 1~4 mM were used to encapsulate proteins. After mixing an appropriate amount of lipids in chloroform, the solvent was evaporated under a stream of argon gas and the residual chloroform was removed by centrifugal lyophilization. The dry lipids were hydrated with 1 mL of buffer A (25 mM Tris-HCl, pH 7.4, 100 mM NaCl, 0.1 mM dithiothreitol, 0.5 mM EDTA) containing approximately 56 μg GST or 132 μg BSA to set the lipid to protein ratios (L/P, by molar concentra-

tion) of 500~2,000. In all experiments, 100% 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC) liposomes were used as standard vesicles. When the REV method was used, the dry lipids were dissolved with 3 mL of diethyl ether and then 1 mL of buffer A containing GST or BSA was placed under the organic solvent phase. The resulting two-phase system was sonicated on ice using a Branson Sonifier 250 instrument until the mixture became a single-phase dispersion. The organic solvent was evaporated under a stream of argon gas. Residual diethyl ether was removed by rotary evaporation. When the FTE method was used, the hydrated lipid solution was frozen and thawed 5 times and extruded 20 times through a polycarbonate membrane with a pore size of 200 nm using an extrusion apparatus (Avestin, Ottawa, Canada) to obtain homogeneous unilamellar vesicles. To encapsulate the proteins by the DD method, the dry lipids were hydrated with 1 mL of buffer B (buffer A containing 1.5% (w/v) CHAPS) containing the protein. The mixtures were dialyzed for 4 h at 25 °C against an excess volume of buffer A. The dialysis step was repeated for 4 h. The average diameters of resulting liposomes were determined as previously described.[8] Phospholipid concentrations were determined with the phosphorus assay[9] and DOG concentration was determined with the ester assay using cholesterol acetate as a standard.[10] The membrane stability was checked using the emission fluorescence of CF entrapped in liposomes. To prepare the CF-containing liposomes, the same procedures for the protein encapsulation were used with 20 mM CF dissolved in buffer A as described above. After vesicle formulation, external CF, which was not entrapped in liposomes, was removed by desalting column chromatography (Thermo Fisher Scientific, Rockford, IL).

Proteolytic Digestion of Liposomes: After liposome formulation, proteinase K (Calbiochem, San Diego, CA) was added to 1 mL of the liposome suspension to yield a proteinase K:GST or proteinase K:BSA ratio of 0.02 (w/w). After incubation for 10 min at 25 °C, the reaction was terminated by addition of 1 mM phenylmethylsulfonyl fluoride and liposomes were then ultracentrifuged (Beckman TLA100.2 rotor, 80,000 rpm for 40 min at 25 °C). GST activity and BSA concentration were determined with pellet and supernatant fractions. As a control, the proteolysis was performed with soluble GST and BSA in the absence of liposomes. The experiment showed that GST and BSA were almost digested under the reaction conditions when assayed by the enzyme activity and SDS-PAGE followed by silver staining.

Quantification of Protein Encapsulation: GST activity toward 1-chloro-2,4-dinitrobenzene (CDNB) was used to determine the encapsulation efficiency as described.[11] The catalytic activity was assayed with the liposome pellet and supernatant fractions under the same buffer conditions (25 mM Tris-HCl, pH 7.4, 500 mM NaCl and 1% CHAPS) at 25 °C after ultracentrifugation of prepared liposomes. The absorbance at 340 nm was monitored with a spectrophotometer every 10 s for 5 min. GST activity was expressed as unit (U)/mg, where 1 U is the amount of enzyme producing 1 mmol of CDNB-GSH conjugate/min. GST concentrations in liposomes were also estimated with a bicinchoninic acid procedure (Pierce, Rockford, IL) to determine the encapsulation efficiency of protein. The specific enzyme activity and protein concentration of soluble GST in the absence of liposomes was set to 100% as a control and the encapsulation efficiency was calculated. Free soluble GST was also incubated for the time required for liposome formulation. The encapsulation efficiency of BSA was also measured by the same method as the GST by determining the protein concentration.

Fluorescence Measurement: The florescence emission intensities of CF were measured with a Shimadzu RF-5301 PC spectrofluorometer at 25 °C. The emission wavelength of 520 nm was used with excitation wavelength of 490 nm. To measure steady-state fluorescence polarization (P) of DPH, excitation wavelength at 350 nm and emission at 452 nm were selected with monochromators and P values were calculated by the following equation.[12]

$$P = (I_{\parallel} - I_{\perp}) / (I_{\parallel} + I_{\perp})$$

$I_{\parallel}$ and $I_{\perp}$ are intensities measured with polarizers parallel and perpendicular to the vertically polarized exiting beam, respectively.

Statistical Analyses: The results were presented as mean ±S.E. of three or five independent experiments and Student's t tests were used.

Results and Discussion

Comparison of the Protein Encapsulation Among Three Different Methods. First, the average vesicle diameters were examined after GST encapsulation into liposomes with three different procedures. The REV, FTE, and DD methods produced liposome diameters of approximately 250, 220, and 340 nm, respectively. These values were very similar to those of liposomes formulated in the absence of GST (results not shown). Encapsulation efficiencies were then compared by the catalytic activity of GST and the protein concentration determined with a colorimetric assay. The REV and FTE procedures were both approximately 32~37% efficient in terms of GST activity and 40%~45% efficient in terms of protein concentrations when 100% PC were used at the L/P ratio (M/M) of 1,000 during the liposome formulation (Figure 1(A)). These efficiencies were higher than that of the DD technique, which had approximately 19% and 26% efficiencies as an enzyme activity and a protein concentration, respectively. These results indicate that the dialysis method produced liposomes of the largest diameter that contained the least amount of GST. When the L/P ratio was enhanced by increasing the amount of lipid under a fixed GST concentration, the amount of entrapped GST was

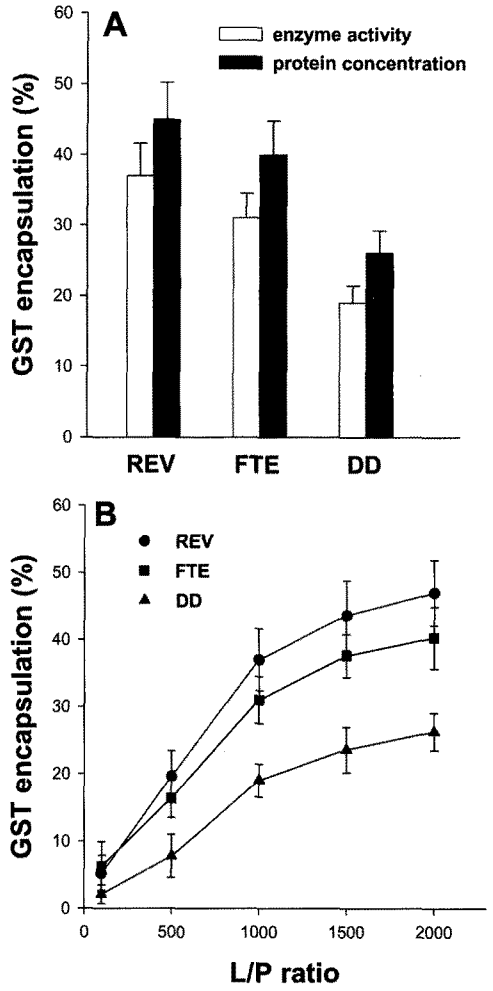

Figure 1. Encapsulation efficiencies of GST among the different methods for vesicle preparation. (A) The amount of entrapped GST in liposomes consisting of 100% PC was assayed by a catalytic activity and a protein concentration at the *L/P* of 1,000. (B) The efficiency was also determined with increasing the ratio (B) by the GST enzyme activity during liposome formulations. Data points represent mean ± S.E. of five independent experiments.

also enhanced, as expected (Figure 1(B)). As a control experiment, soluble GST was mixed with prepared liposomes consisting of 100% PC and the enzyme activity was measured with the membrane fraction obtained by ultracentrifugation. GST activity was not detected with the liposomes (results not shown), suggesting that free soluble GST did not bind to the liposome surface and that the results described in Figure 1 resulted from enzyme activity present in the interior of liposomes. The effect of CHAPS on GST activity was also examined to validate the present assay conditions in which the detergent was used to solubilize the liposomes. The purified recombinant GST showed a specific activity of approximately 13.6 U/mg in the absence of any lipid and detergent components. The catalytic activity increased gradually with increasing CHAPS concentration and the activity was enhanced by approximately 2-fold at 5% CHAPS (Supplemental Figure S1A), which coincides well with a previous result.[13] In order to validate the present assay for protein encapsulation, time-dependent activity changes of soluble GST were also investigated in the absence of lipid and detergent components. The result showed that any discernable changes of GST activity were not observed for about 24 h (Supplemental Figure S1B). Therefore, we could exclude the possibility that the required time for liposome formulation affects the enzyme activity assay.

Effects of Anionic Phospholipids and PE on the Encapsulation Efficiencies of Proteins. To examine the effect of phospholipid compositions on the protein encapsulation efficiency, the anionic phospholipids 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylserine (POPS), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidic acid (POPA), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylglycerol (POPG), and the neutral phospholipid 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylethanolamine (POPE) were individually included at the expense of POPC in the liposome preparation, and GST activity was determined using the FTE method. POPG had little discernable effect on the amount of entrapped GST in liposomes, while both POPA and POPS decreased the protein content with increasing the lipid concentration up to 50 mol% at the *L/P* ratio of 1,000 when the FTE method was used (Figure 2). This result suggests that the tested anionic phospholipids had no or negative effects on GST encapsulation. In contrast, when a neutral and nonlamellar-prone phospholipid POPE were incorporated into the lipid bilayer

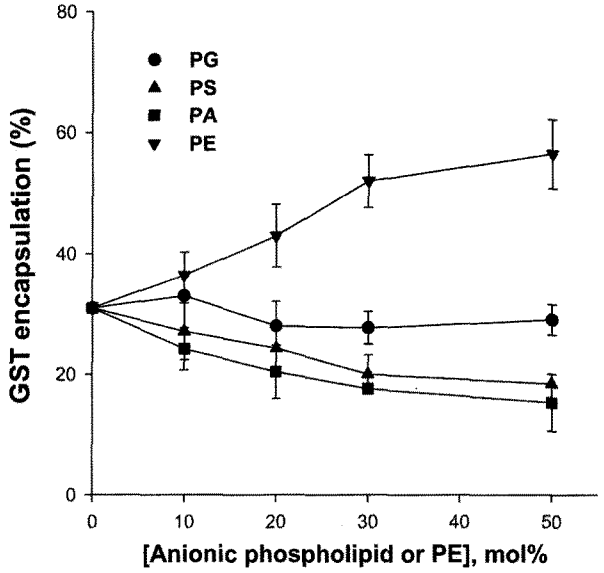

Figure 2. Effect of anionic phospholipids and POPE on the encapsulation efficiency of GST. The FTE method was used for vesicle preparation and the encapsulation efficiency was determined by GST activity. 1-Palmitoyl-2-oleoyl-phosphoglycerides were used in all phospholipids.

instead of PC, the encapsulation efficiency increased in a POPE concentration-dependent manner and approximately 55% GST was entrapped in liposomes at 50 mol% POPE.

In relation to this result, the influence of phospholipid compositions including POPS and POPE on protein encapsulation has been investigated using acetylcholinesterase (AChE) as a model protein, which PS decreased and PE slightly increased the AChE encapsulation in liposomes.[14] The unremarkable effect of PE may be due to low PE concentration in liposomes (5 mol%). The present study suggests that a relatively high PE concentration is required to induce a significant enhancement of protein encapsulation. When the experiment was repeated using the REV method, similar results to those of FTE could be obtained (results not shown).

Diameters of liposomes formed using the different preparation methods with anionic phospholipids or POPE were measured after protein encapsulation. Vesicle sizes were marginally changed when the liposomes were prepared in the presence of 50 mol% PG and PS (Figure 3). However, the POPA and POPE-containing liposomes formulated by all three methods displayed 20 to 30% smaller diameters compared to that of 100% PC. Therefore, the POPE-induced increases in encapsulation efficiency (Figure 2) are not directly related to liposome diameter. The PE effect was examined in more detail with dioleoyl-PE (DOPE), which possesses different acyl chains and lamellar to hexagonal II phase transition (L-H_{II}) temperature (T_H) compared to POPE.[15,16] DOPE was incorporated into lipid lamellars and GST encapsulation was measured under the same conditions as with POPE. Both PE displayed similar efficiencies of GST encapsulation (results not shown). The T_H of PE is dependent on the acyl chain composition of the phospholipid.[17]

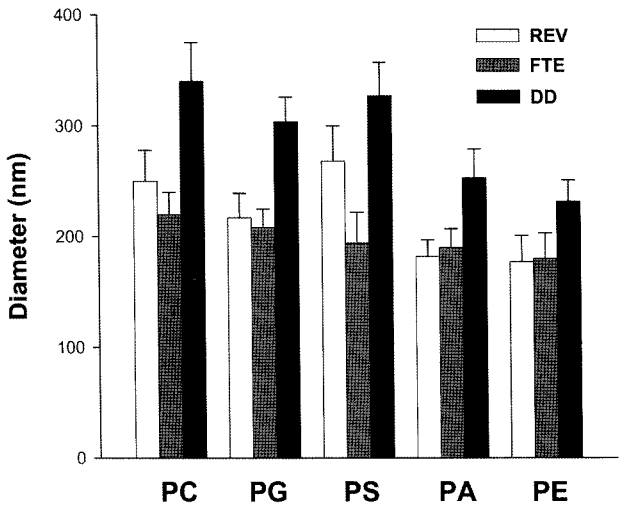

Figure 3. Changes in vesicle diameter dependent on lipid composition. After liposome formulations by three different methods, the vesicle diameters were determined using the dynamic light scattering method as previously described.[7]

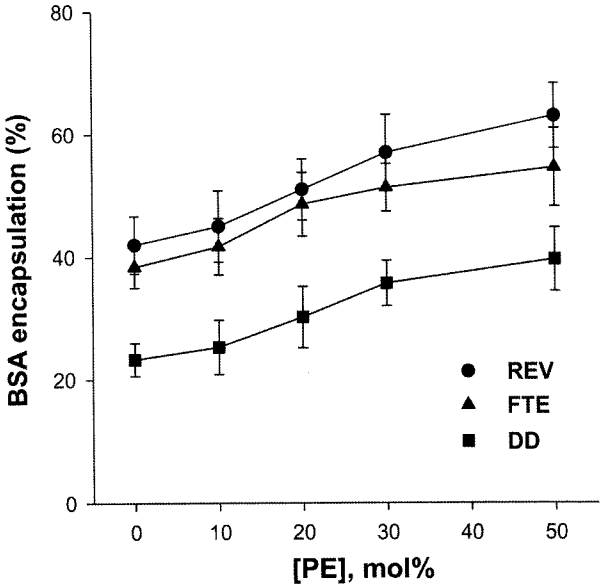

Figure 4. Effect of POPE on BSA encapsulation. The amount of entrapped BSA was determined as a function of POPE concentrations using the REV, FTE, and DD methods as described. Data points represent mean ± S.E. of five independent experiments.

Therefore, the encapsulation efficiencies should be tested with other PE types with varying transition temperatures to ascertain the relationship between the efficiency of protein encapsulation and the transition temperature. As a control experiment, lipid concentrations were measured with pellet and supernatant fractions after the liposome formulation and ultracentrifugation in order to validate the protein encapsulation assay. Approximately 7%, 9%, and 5% of the total lipid concentration were assayed in the supernatant fractions for REV, FTE, and DD methods, respectively (Supplemental Figure S2). More importantly, the lipid concentrations in the supernatants were marginally changed by the lipid compositions used for the liposome preparation although diameters for PA or PE-containing membranes were reduced compared to that of 100% PC vesicle. The possible activity loss of GST during liposome formulations was also checked. The present methods for liposome preparation reduced the specific activity of GST by approximately 13-22%. REV had the most significant effect on the activity decrease (Supplemental Figure S3). However, lipid compositions had little effect on the activity loss.

The entrapment experiments were repeated using BSA instead of GST with increasing the POPE content in liposomes. Protein contents in the vesicles were determined with colorimetric assay. BSA encapsulation efficiency increased from approximately 23-42% in 100% PC membranes to 35-60% depending on the liposome preparation method (Figure 4), confirming that POPE stimulates the encapsulation of protein into liposomes. The result also suggests that POPE-

induced increases in GST encapsulation did not result from specific interactions between PE and the particular protein. As a control experiment, GST or BSA was externally added to preformed liposomes without any protein component and the proteolytic cleavage was performed. However, when the enzyme activity and the protein concentration for GST and BSA were determined with liposomes precipitated by ultracentrifugation, no GST or BSA remained in the membrane fractions (results not shown). This result indicates that the observations described above resulted from protein encapsulation and not from protein binding to the external membrane surface during liposome formulation although BSA might bind to PE specifically.[18]

Effect of DOG on the Encapsulation Efficiencies of Proteins. Diacylglycerol is a neutral lipid that can significantly lower the L-H_{II} temperature of PE[19] and has strong propensity towards formation of nonlamellar membranes.[20] The diacylglycerol DOG was incorporated during liposome preparation and the amount of entrapped GST was determined to correlate nonlamellar-prone lipids with increases in protein encapsulation efficiency. DOG stimulated the GST encapsulation with increasing the lipid concentration and the efficiency reached up to 66% at 5 mol% DOG (Figure 5(A)). This was very similar to the amount of entrapped BSA in the presence of 50 mol% PE and was also dependent on the formulation method. The membrane-bound GST was assayed by mixing soluble GST and prepared liposomes in all experiments. However, GST did not show any considerable vesicle binding regardless of the presence or absence of the anionic phospholipids, POPE, and DOG (results not shown). These results excluded the possibility that the membrane binding of GST had an effect on the determination of the protein encapsulation in liposomes.

Because DOG has been known as a potent promoter of nonlamellar phases in various phospholipid systems, the protein encapsulation efficiency was examined with ternary system consisting of DOG, POPE, and POPC. When DOG concentration increased up to 5% in the presence of 10 mol% PE, the amount of entrapped GST increased and the values were higher than arithmetic sums of POPE or DOG-induced increase in the protein encapsulation (Figure 5(B)). This result implies that POPE and DOG exert a synergistic effect on the GST encapsulation regardless of the methods for liposome preparation. The result also suggests that the nonlamellar propensity of membranes is important in the stimulation of protein entrapment. As a control experiment, time-dependent membrane leakage was also measured using the entrapment of carboxyfluorescein (CF) with liposomes prepared by the REV method to demonstrate vesicle stability. CF fluorescence is self-quenching when the dye is entrapped in liposomes at high concentrations and the fluorescence increases when the dye is released into medium. Membrane leakage was determined as described previously.[21] All vesicles used for this work were apparently stable for at least 2 days at 25 °C. The fluorescence intensities were changed slightly regardless of the lipid compositions (Supplemental Figure S4). The membranes prepared by FTE and DD methods showed similar features of CF leakage (results not shown). To obtain more insights into structural stability changes of liposomes by the nonlamellar-prone lipids in relation with their micro-fluidity, we measured the steady-state fluorescence polarization of DPH incorporated into membranes.

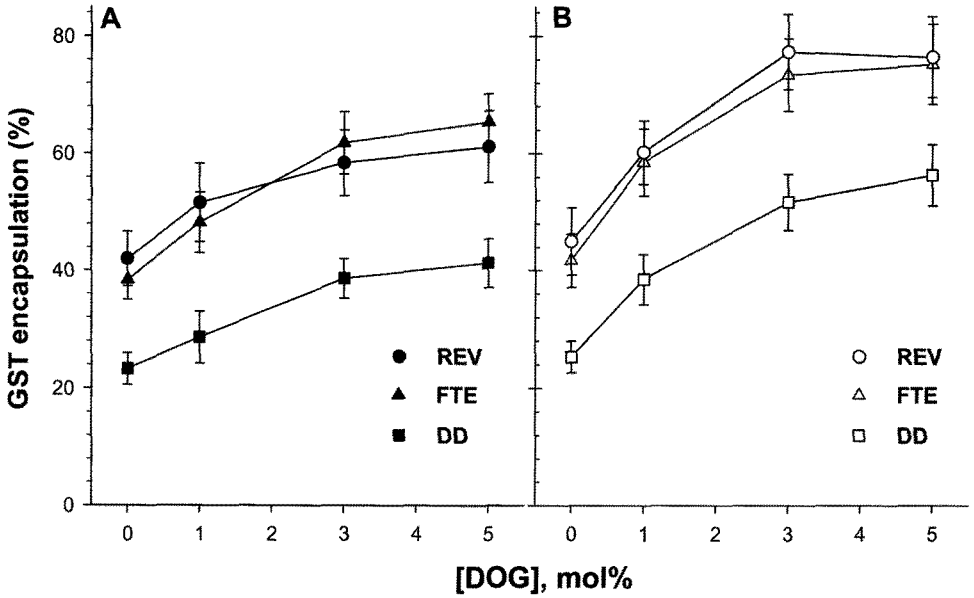

Figure 5. DOG-induced increases in the encapsulation efficiencies of GST. Different methods were used during vesicle preparation and GST activity was assayed (A). To examine a synergistic effect of POPE and DOG, the experiment was repeated in the presence of 10 mol% POPE with increasing DOG concentration (B).

Supplemental Figure S5A shows that the polarization changes were not significant upon increasing the PE concentration up to 30 mol% in the absence of encapsulated proteins regardless of the liposome preparation methods. In contrast, the *P* values were remarkably enhanced by further incorporation of PE contents indicating that micro-viscosity and/or acyl chain order of lipids in membranes decreased, which parallel the previous observations.[12] We anticipate that these results are ascribed to the mixing properties of phospholipids in PE/PC, which are non-ideal and lipid domains are formed as suggested previously.[12] Very similar *P* values and features of DPH polarization to those without protein could be obtained with increasing PE concentration in the presence of entrapped GST and BSA (results not shown). These results, thus, support indirectly that the specific interaction between PE and BSA (and GST) is not present as described above. In contrast, DOG induced gradual decreases in the polarization with increasing its concentration (Supplemental Figure S5B). However, the decrease levels were not significant, which the *P* value was reduced by approximately *10% at 5 mol% of DOG compared to that for 100% PC* membrane. Therefore, these results suggest that the involvement of nonlamellar lipids has little effect on the microfluidity of membranes under the present experimental conditions although nonlamellar lipids have been known to lower gel to liquid-crystalline transition temperature of membranes.[22-24] However, we can not exclude the possibility that DPH only reflects the local properties of its microenvironment rather than those of macroscopic flow. The change in the turbidity measured with a spectrophotometer at 450 nm was also within marginal ranges during experimental time suggesting little effect of nonlamellar lipids on the integrity and/or stability of the used liposomes (results not shown).

Liposomes have been used as model systems for studying many cellular processes, including viral infection, endocytosis, excocytosis, cell fusion, and transport phenomena. In addition to the importance for basic research in biology, liposomes have attracted considerable attention in practical fields as vehicles for drug application,[25] gene transfer in medical therapy[26] and as a micropolymer in the food industry.[27] As a tool for delivering macromolecules such as proteins and DNA, however, the entrapment efficiency of materials into lipid vesicles has been an obstacle to their wide application. Therefore, the present strategy may be an alternative way to efficiently encapsulate proteins in vesicles by incorporating nonlamellar-prone lipids during vesicle formulation.

Conclusions

The incorporation of nonlamellar-prone lipids, like POPE and DOG, into liposomes during formulation enhanced the entrapment of GST and BSA in the aqueous phase of vesicles as a function of lipid concentrations. These results suggest that the effects of nonlamellar-prone lipids do not result from specific interactions between the lipids and proteins or changes in vesicle diameter by the lipid incorporation. At the moment, we do not have a satisfactory explanation for the increased protein encapsulation by an incorporation of nonlamellar lipids during liposome formulation. In relation to the present work, it was previously suggested that the entrapment efficiency of cisplatin was significantly increased by using PE-liposomes compared to PC-liposomes.[28] The detailed role of nonlamellar-prone lipids in the encapsulation of molecules is unclear. Other lipid systems with nonlamellar properties and other proteins and enzymes of varying molecular weight should be examined as well to generalize the effects of nonlamellar-prone lipids as a further work.

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Supporting Information: The materials are available *via* the Internet at http://www.polymer.or.kr.

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