# High-Efficiency Entrapment of Superoxide Dismutase into Cationic Liposomes Containing Synthetic Aminoglycolipid

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**A monofatty acid ester of glucosamine (PGlcN) was synthesized to provide liposomal membranes with a positive charge, and the trapping efficiency of negatively charged substances (superoxide dismutases, SODs) into cationic liposomes containing PGlcN or stearylamine (SA) prepared by various methods was compared to find the most efficient trapping method. We demonstrated that cationic liposomes, which were prepared in a buffer of low ionic strength containing sorbitol by a simple hydration method, could entrap a large amount of negatively charged SODs which retained their activity, as compared with cationic liposomes prepared in a buffer of high ionic strength. We also showed that a reverse-phase evaporation method entrapped a large amount of SODs. However, SODs were inactivated during the preparation; therefore, this method was not suitable to entrap the enzyme. Freeze-thaw method induced the formation of cationic liposomes which were smaller than extruded liposomes and could entrap the SODs in a buffer of low ionic strength. Dehydration–rehydration method with a buffer of low ionic strength also entrapped a large amount of SODs, indicating that the integrity of liposomes was lost in the lipid bilayer after freeze-drying and the SODs were entrapped in the reconstruction of liposomes during rehydration. These findings showed that the hydration method based on electrostatic attraction with a buffer of low ionic strength was simple and the most effective for entrapping SODs without loss of their activity.**

**Key words** cationic liposome; superoxide dismutase; entrapment; electrostatic interaction

Superoxide dismutases (SODs) are metalloenzymes that protect against oxidative stress through the decomposition of superoxide radicals, particularly superoxide anion ($O_2^-$). SOD can be useful drugs to suppress inflammatory disease[1] and ischemic myocardial injury.[2] However, SODs with molecular weights ranging from 32000 to 80000[3] are rapidly filtered by the kidney and disappear from the circulation (half-life in the circulation is $<6$ min); this phenomenon limits their therapeutic use. The circulation lifetime can be greatly increased by coupling the SOD with various polymers, such as dextrans or poly(ethylene glycol) (PEG), to increase their molecular weight.[4] However, these chemical modifications of the proteins may cause loss of their activities.

The encapsulation of biologically active substances into liposomes is an efficient method since biologically active substances can be protected from inactivation *in vivo* and the toxicity of anticancer drugs, such as cytosine arabinoside and adriamycin, can be decreased.[5–7] Turrens *et al.* demonstrated that when SOD was entrapped in liposomes, its half-life increased from 6 min to 4.2 h in circulating blood.[3] In the therapeutic use of liposomes, efficient encapsulation and retention of drugs is required before successful delivery can be achieved. However, the encapsulation into liposomes is low, since liposomes, especially small unilamellar vesicles (SUVs), have a low volume of entrapped aqueous space per mole of lipid. Therefore, reverse-phase evaporation[8] and pH gradient (or membrane potential) loading methods[9,10] have been developed to entrap large amounts of substances into liposomes. However, these methods cannot be applied to entrap protein enzymes into liposomes since the contact with organic solvent and/or the sonication in the reverse-phase evaporation procedure may inactivate the enzymes. In addition, pH gradient loading can be applied only to small lipophilic molecules that can pass through the lipid membranes.

Cu, Zn-SOD from bovine erythrocytes has an isoelectric point at 4.95 and thus bears a negative charge at physiological pH. We attempted to entrap SODs into cationic liposomes using an electrostatic attraction[11] and found that these liposomes could entrap a large amount of SODs. We also found that cationic glucosamine modified liposomes can escape from the reticuloendothelial system (RES) and remain in the circulating blood.[12] The present study evaluated the trapping efficiency of bioactive enzyme SOD into liposomes at a constant lipid concentration by various liposome preparation methods. We attempted to prepare liposomes that avoided RES and contained a large amount of biologically active substances without losing their activity.

## Experimental

**Materials** Egg yolk L-α-phosphatidylcholine (EPC) was obtained from Asahi Kasei Co., Ltd. (iodine value 65, Tokyo, Japan). SOD (Cu, Zn-SOD) from bovine erythrocytes, 3000 units/mg, xanthine oxidase (XOD) from buttermilk and stearylamine (SA) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other chemicals from Wako Pure Chemical Ind., Ltd. (Osaka, Japan) were of special grade, and cholesterol (Chol) was used after recrystallization from ethanol. Calcein was obtained from Dojindo Laboratories (Kumamoto, Japan). Water was distilled twice.

**Synthesis of 2-Amino 6-Palmitoyl Glucoside** Methyl-2-amino-6-palmitoyl-D-glucoside (PGlcN) was synthesized in a similar manner to that previously described.[11] The synthesized compound was characterized by measurement of its $^1$H-NMR spectrum in dimethyl sulfoxide (DMSO)-$d_6$ using tetramethylsilane (TMS) as a reference on a spectrometer (JMN-GX400, JEOL), by elemental analysis, infrared and mass spectra.

**SOD Activity Assay** The SOD activity was determined by the nitrite method with a small modification.[13] The sample (0.1 ml), reagent A (0.2 ml, 65 mM $KH_2PO_4$, 35 mM $Na_2B_4O_7$ and 0.5 mM diethylenetriamine pentaacetic acid (DTPA), and reagent B (0.2 ml, 0.5 mM hypoxanthine

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and hydroxylamine-*O*-sulfonic acid) were mixed and incubated for 15 min at 37 °C. Reagent D (0.2 ml, 0.025 unit/ml XOD) was added to this mixture and incubated for 30 min at 37 °C. Then the mixture was added to 2.0 ml of coloring reagent E (30 mM *N*-1-naphthylethylenediamine, 3 mM sulfanilic acid and 25% acetic acid). The final solution was allowed to stand for 1 h at room temperature, and the optical absorption was measured at 550 nm. The 50% inhibitory dilutions ($ID_{50}$) were obtained for different dilutions of samples; the amount of SOD in the samples was then calculated from the $ID_{50}$ compared with the 50% inhibitory concentration ($IC_{50}$) of the standard SOD solution, assuming no loss of the activity during liposomal preparation.

**Protein Assay** The SOD content was also measured by the Lowry method with a small modification.[14]

**Preparation of SOD Entrapped Liposomes** 1) Hydration Method: Multilamellar vesicles (MLVs) were prepared by extrusion.[15] The lipid mixture in chloroform (EPC : Chol : PGlcN or SA in the desired molar ratios) was dried in a rotary evaporator to form a lipid film on the wall of a round-bottomed flask. The thin lipid film was left under reduced pressure for at least 12 h to completely remove the solvent. The dried lipid was then dispersed with a 100 $\mu$g/ml SOD solution, which contained 10 mM Tris–HCl/150 mM NaCl (pH 7.4) as a buffer of high ionic strength or 10 mM Tris–HCl/300 mM sorbitol (pH 7.4) as a buffer of low ionic strength. The suspensions were successively extruded 5 times each through polycarbonate filters with pore sizes of either 0.6 or 0.2 $\mu$m ($VET_{200}$: Vesicles with a diameter of 200 nm prepared by extrusion technique). The total lipid concentration of liposomal solutions was kept constant at 10 mM.

Unentrapped SOD was removed by gel filtration (Bio-Gel 1.5 m, 2 cm × 35 cm, eluted with 10 mM Tris–HCl/150 mM NaCl (pH 8.4)). The liposomes containing SOD were assayed for SOD activity and the amount of phospholipid after the addition of Triton X-100. The addition of Triton X-100 hardly inhibited the SOD activity assay. The concentration of phosphatidylcholine was determined by Bartlett's method.[16] The trapping efficiency was expressed as a SOD g/lipid mol. Ten grams of SOD/mol lipid corresponded to 100% of the trapping efficiency.

2) Reverse-Phase Evaporation Method: Reverse-phase evaporation vesicles (REVs) were prepared according to Szoka and Papahadjopoulos.[8] The thin lipid films (20 $\mu$mol lipids) were dissolved in 6 ml of diethyl ether. Two milliliters of 100 $\mu$g/ml SOD solutions were added to the lipid solutions and sonicated under nitrogen in a bath-type sonicator (BRANSON 2200) at 25 °C for 5 min. After evaporation at 350—400 mmHg to remove ether, the suspensions were vortexed, then again evaporated at 680—700 mmHg. The suspensions were then extruded 5 times through polycarbonate filters of a 0.2 $\mu$m pore size.

3) Freeze-Thawing Method: The "empty" liposomes ($VET_{200}$) were prepared in the buffer described above. The liposomal suspensions were added to SOD solutions (final SOD concentration 100 $\mu$g/ml, lipid concentration 10 mM). The suspensions were then frozen with liquid $N_2$ and dissolved in a water bath at 25 °C. These processes were repeated 5 times.[17] The suspensions were also extruded 5 times through polycarbonate filters of a 0.2 $\mu$m pore size.

4) Dehydration–Rehydration Method: The "empty" liposomes ($VET_{200}$) prepared in the buffer described above were frozen in liquid $N_2$ and lyophilized on a KYOWA vacuum engineering model RL-10NB overnight. The dry samples were rehydrated to their original volumes with 100 $\mu$g/ml SOD solutions (lipid concentration 10 mM).[18] The suspensions were then extruded 5 times through polycarbonate filters of 0.2 $\mu$m pore size.

**Liposome Size and Zeta Potential Measurement** The size of the liposomes was measured by dynamic light scattering on a Photal laser particle analyzer LPA-3100 connected to a photon correlater LPA-3000 (Otsuka Electronics Co., Osaka, Japan).

The zeta potential of liposomes with a diameter of about 200 nm was calculated by Smoluchowski's equation[19] from their electrophoretic mobility in 10 mM Tris–HCl/0 mM NaCl (as a substitute for 300 mM sorbitol) or 10 mM Tris–HCl/150 mM NaCl buffer (pH 7.4) obtained at 25 °C, from a Zetasizer 4 electrophoretic light scattering spectrophotometer; a ZET 5104 wide capillary cell was used for a sample in 10 mM Tris–HCl and a ZET 5103 narrow capillary cell for a sample in 10 mM Tris–HCl/150 mM NaCl (Malvern Instruments, Worcs., U.K.).

## Results and Discussion

**Characterization of Liposomes** The diameters of $VET_{200}$ were about 200 nm with homogenous distribution as shown in Table 1. Zeta potential values of liposomes composed of EPC, Chol, PGlcN or SA were positive values in a buffer solution at 25 °C, as shown in Table 1. The glucosamine group of our synthetic ester is probably charged due to the $+NH_3$ groups in our buffer solution (pH = 7.4), and their incorporation into liposomal bilayers led to the positive charging of the liposomal membranes. The difference of $pK_a$ between PGlcN– and SA–liposomes reflected the difference of their zeta potentials. The $pK_a$ of PGlcN–liposomes was approx. 7.7 and that of SA-liposomes was over 10, as determined by the zeta potentials of liposomes prepared with buffer at varied pH (data not shown). The zeta potentials of liposomes containing SA obtained in 10 mM Tris buffer containing no NaCl (pH 7.4) were higher than those obtained in 10 mM Tris/150 mM NaCl (pH 7.4) buffer. This was due to the shielding of the positive charge by the ionic atmosphere surrounding the liposomes. The diameter of liposomes prepared by the other various methods followed by extrusion was also about 200 nm with homogenous distribution, except for cationic liposomes prepared by the freeze-thawing method (data not shown).

**Trapping Efficiency of SOD in Liposomes Prepared by Hydration** The trapping efficiency of SOD into liposomes prepared by various methods was determined by a SOD nitrite assay, as shown in Table 2. The neutral liposomes composed of EPC and Chol (8 : 2 in molar ratio) could entrap about 1% of SOD at 10 mM of lipid concentration in both buffers. The trapped volume of $VET_{200}$ was determined as 1.80 ± 0.18 l/mol of total lipid using fluorophore calcein as an entrapped substance. The trapping efficiency of $VET_{200}$ was calculated as 1.8% from their trap volume under experimental conditions at 10 mM as the lipid concentration, and the entrapment efficiency of SOD determined by the nitrite method roughly agreed with the calculated value. The amount of SOD entrapped into cationic liposomes containing 10 mol% PGlcN in the buffer of high ionic strength was 2 times larger than neutral liposomes by the usual hydration method. Furthermore, cationic PGlcN–liposomes prepared in the buffer of low ionic strength could entrap ten times greater amounts of

Table 1. Diameter and Zeta Potential of Liposomes Prepared by Extrusion Method

| Liposomal lipid composition (in molar ratio) | Diameter (nm) | Zeta potential (mV) |
|---|---|---|
| (In 10 mM Tris/300 mM Sorbitol) | | |
| EPC : Chol (8 : 2) | 223.1 ± 24 | −3.94 ± 0.32 |
| EPC : Chol : PGlcN (7 : 2 : 1) | 204.5 ± 48 | 28.94 ± 0.26 |
| EPC : Chol : PGlcN (6 : 2 : 2) | 225.8 ± 25 | 41.12 ± 1.14 |
| EPC : Chol : PGlcN (5 : 2 : 3) | 232.3 ± 24 | 48.84 ± 0.46 |
| EPC : Chol : SA (7 : 2 : 1) | 236.8 ± 45 | 53.68 ± 0.67 |
| EPC : Chol : SA (6 : 2 : 2) | 202.6 ± 44 | 54.70 ± 1.14 |
| EPC : Chol : SA (5 : 2 : 3) | 213.2 ± 38 | 66.58 ± 0.44 |
| (In 10 mM Tris/150 mM NaCl) | | |
| EPC : Chol : PGlcN (7 : 2 : 1) | 222.1 ± 31 | 6.59 ± 0.67 |
| EPC : Chol : PGlcN (6 : 2 : 2) | 215.6 ± 45 | 15.89 ± 0.32 |
| EPC : Chol : PGlcN (5 : 2 : 3) | 227.1 ± 28 | 23.34 ± 1.38 |
| EPC : Chol : SA (7 : 2 : 1) | 224.0 ± 31 | 13.00 ± 0.83 |
| EPC : Chol : SA (6 : 2 : 2) | 219.2 ± 25 | 31.40 ± 0.97 |
| EPC : Chol : SA (5 : 2 : 3) | 216.5 ± 27 | 42.92 ± 0.30 |

Table 2. Entrapment Efficiency (%) of SOD into Liposomes Determined by SOD Activity Assay

| Liposomal lipid composition (in molar ratio) | Buffer medium | Method of vesicle preparation | | | |
|---|---|---|---|---|---|
| | | Hydration | Reverse-phase evaporation | Freeze-thaw | Dehydration–rehydration |
| EPC : Chol (8 : 2) | 10 mM Tris/150 mM NaCl | 0.9±0.1 | 2.4±0.1 | 3.2±0.1 | 1.1±0.1 |
| | 10 mM Tris/300 mM sorbitol | 0.7±0.4 | 2.9±1.1 | 0.9±0.0 | 0.7±0.1 |
| EPC : Chol : PGlcN (7 : 2 : 1) | 10 mM Tris/150 mM NaCl | 1.7±0.2 | 2.8±0.4 | 2.8±0.1 | 0.8±0.4 |
| | 10 mM Tris/300 mM sorbitol | 8.5±1.0 | 7.1±4.6 | 6.8±0.0 | 10.9±0.6 |
| EPC : Chol : SA (7 : 2 : 1) | 10 mM Tris/150 mM NaCl | 1.3±0.1 | 2.9±0.3 | 3.6±0.1 | 2.7±0.3 |
| | 10 mM Tris/300 mM sorbitol | 21.3±1.2 | 32.0±2.8 | 12.8±0.4 | 21.9±0.9 |

Liposomes were prepared by various methods at a total lipid concentration of 10 mM as described in the text. Amount of SOD in liposomes was determined by the nitrite method after removal of untrapped SOD by gel filtration assuming no loss of its activity. Values are expressed as mean±S.D. ($n=3$).

Table 3. Diameters of Liposomes before and after Freeze-Thaw and Freeze-Drying

| Method | Liposomes | EPC : Chol (8 : 2) | | EPC : Chol : PGlcN (7 : 2 : 1) | | EPC : Chol : SA (7 : 2 : 1) | |
|---|---|---|---|---|---|---|---|
| | Buffer medium | Before | After | Before | After | Before | After |
| Freeze-thaw | 150 mM NaCl | 237.2±36 | 555.9±319 | 210.9±32 | 185.7± 87 | 226.9±36 | 175.8± 52 |
| | 300 mM sorbitol | 228.8±32 | 233.8± 45 | 221.4±36 | 170.4±157 | 226.4±32 | 128.3± 54 |
| Dehydration–rehydration | 150 mM NaCl | 219.1±19 | 893.0± 93 | 206.3±20 | 877.0±109 | 211.3±32 | 862.3±234 |
| | 300 mM sorbitol | 233.1±24 | 460.1±126 | 204.5±48 | 375.3±100 | 210.8±37 | 488.8±154 |

Liposomes sizes after freeze-thawing or freeze-drying were measured before extrusion. Values are expressed as mean diameters±S.D. (nm).

SOD. Cationic SA–liposomes also showed high entrapment efficiency when prepared in the buffer of low ionic strength. These findings are probably due to enhanced electrostatic interactions between the negatively charged SOD and the positively charged lipid membrane surface since the zeta potential of cationic liposomes in buffer containing sorbitol was higher than that in buffer containing NaCl.

**Trapping Efficiency of SOD in Liposomes Prepared by Reverse-Phase Evaporation** REVs have a high volume of entrapped aqueous per lipid.[8] Neutral REVs could entrap about three times larger amounts of SOD compared with neutral extruded liposomes. Cationic PGlcN–REVs in the buffer of high ion strength buffer entrapped larger amounts of SOD than did cationic PGlcN extruded liposomes. The entrapment efficiency of cationic PGlcN–REVs in the buffer of low ionic strength determined by the nitrite method (SOD activity assay) was roughly similar to that of cationic PGlcN extruded liposomes; however, the amount of entrapped SOD in PGlcN–REVs determined by the Lowry method was the highest, and almost 100% of SOD was found in liposomes (data not shown). This indicated that more than 90% of SODs entrapped into liposomes lost their activity during preparation of REVs. The other methods did not inactivate SOD because the SOD activity in liposomal suspensions before gel filtration was retained at the same level as that in the initial buffer. This REV method was not suitable for entrapping enzymes since organic solvent and/or the sonication step in the preparation of liposomes probably inactivated the enzyme.[20–22] SODs alone in buffer were sonicated to confirm whether the sonication process inactivated SOD or not. The sonicated SOD retained its activity (data not shown); therefore, contact with organic solvent inactivated SOD. Cationic SA–REVs in the low-ionic strength buffer was also able to entrap large amounts of SOD, about 30% as determined by the nitrite method and 100% as determined by the Lowry method (data not shown). In this case, 70% of the SODs was inactivated during liposome preparation. This may be the reason why SA–liposomes have a larger positive charge than PGlcN–liposomes, why SOD is immediately trapped into liposomes and why the time of contact with organic solvent is short.

**Trapping Efficiency of SOD in Liposomes Prepared by Freeze-Thawing** In the freeze-thawing method, the trapping efficiency of SOD of neutral liposomes prepared in the high-ionic strength buffer was three times greater than that of neutral extruded liposomes prepared in the same buffer. However, the trapping efficiency of neutral liposomes in sorbitol buffer was the same as the that of neutral extruded liposomes. Table 3 shows the diameters of the liposomes before and after freeze-thawing without extrusion. In a buffer containing NaCl, membrane fusion occurred, liposome size increased and liposomes could effectively encapsulate the SOD during freeze-thawing. In the presence of sorbitol which prevents membrane fusion from liposomes,[23,24] the neutral liposome size did not change during freeze-thawing. Therefore, SOD cannot be entrapped into liposomes in buffer containing sorbitol by the freeze-thawing method.

However, in the case of cationic PGlcN– or SA–liposomes prepared in sorbitol buffer, a large amount of SODs was entrapped into liposomes. The diameter of cationic liposomes after freeze-thawing was smaller than that of cationic $VET_{200}$. Westman *et al.* reported that freeze-thaw induced the formation of liposomes which are smaller than those that had been only vortexed.[25] In the freeze-thawing procedure, liposomal membranes are disrupted by ice, form fragmented lipid assemblies during freezing, and then are reconstituted from these lipid assemblies during thawing. Sorbitol prevents liposomes

from contacting other liposomes; therefore, fragmented lipid assemblies derived from SUVs will form SUVs during reconstitution, since liposomes which are smaller than SUVs ($d \cong 20$ nm) cannot be formed due to the stress arising from the high curvature of the membrane[26]; thus the size of SUVs will not change. [23,24] In the case of MLVs, fragmented lipid assemblies derived from one liposome can form some liposomes during reconstitution since liposomes with a diameter of 80 nm or more are stable and have the same gel to liquid crystalline phase transition temperature as multilamellar vesicles.[27,28] The reason for the formation of small liposomes is unclear; however, one reason may be an increase in entropy. In the charged liposomes, the electrostatic repulsion between fragmented lipid assemblies would induce the formation of small liposomes. SODs could be effectively entrapped into cationic liposomes through electrostatic attraction during reconstitution of the liposomes.

**Trapping Efficiency of SOD in Liposomes Prepared by Dehydration–Rehydration** Using the rehydration–dehydration method, the trapping efficiency of SOD was similar to that by the hydration method. Cationic liposomes prepared in sorbitol buffer entrapped a large amount of SOD by the electrostatic interaction between SOD and the cationic bilayer membrane. This finding indicated that the bilayer structure of liposomes after freeze-drying was not left intact. The diameter of liposomes after freeze-drying increased as shown in Table 3, indicating that the bilayer structure of the liposomes broke down. After freeze-drying, the liposomes may lose their integrity in the lipid bilayer, and the SODs may be entrapped into the reconstruction of liposomes during rehydration.[29,30]

**Effect of Amount of Incorporated Positively Charged Lipid** From the findings described above, it was clear that the hydration method followed by extrusion in a buffer of low ionic strength was simple and could entrap a large amount of SODs. Thus, we investigated the effect of the amount of incorporated positively charged lipids on the trapping efficiency. Figure 1 shows the trapping efficiency of SOD by the hydration method followed by extrusion as a function of the mol% of basic lipids (PGlcN or SA) in liposomes prepared in NaCl or sorbitol buffer. In the buffer of low ionic strength containing sorbitol, the entrapment of SOD into cationic liposomes drastically increased following the increase of basic lipid concentration in the liposomal membrane. The hydration method using lipid film containing a high mol% of basic lipid with the buffer of low ionic strength was most effective in entrapping the enzyme without loss of its activity. However, trapping efficiency of SOD into PGlcN- or SA-liposomes in the buffer of high ionic strength containing NaCl did not appreciably increase compared with liposomes in the buffer of low ionic strength. This may have been the result of a decrease in the electrostatic interaction of the lipid bilayer containing basic lipids with SOD due to the increase in ionic strength.

Figure 2 shows the entrapment of SOD *versus* the zeta potential of liposomes. The entrapment of SOD into liposomes prepared in the buffer of low ionic strength increased following the zeta potential of liposomes, indicating that electrostatic interaction is important for

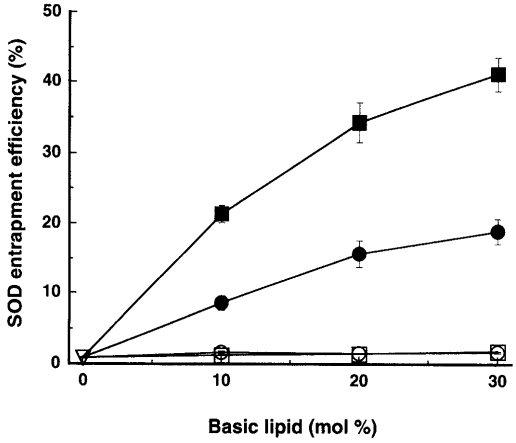


Fig. 1. The Trapping Efficiency of SOD into Liposomes Determined by the Nitrite Method as a Function of the mol% of Basic Lipid

Liposomes were prepared by extrusion at 10 mM lipid concentration as described in the text. (▽) liposomes (EPC : Chol = 8 : 2 in molar ratio) in 10 mM Tris/300 mM sorbitol (pH 7.4); (●) PGlcN-liposomes in 10 mM Tris/300 mM sorbitol (pH 7.4); (○) PGlcN-liposomes in 10 mM Tris/150 mM NaCl (pH 7.4); (■) SA-liposomes in 10 mM Tris/300 mM sorbitol (pH 7.4); (□) SA-liposomes in 10 mM Tris/150 mM NaCl (pH 7.4).

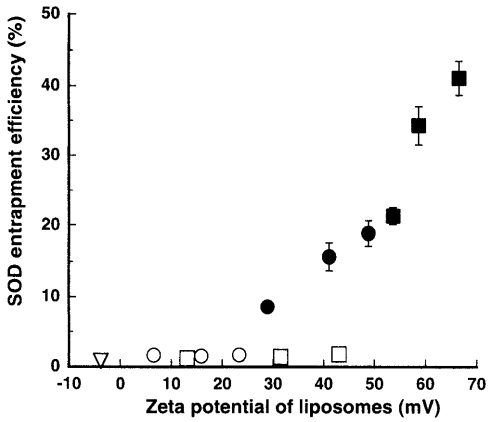


Fig. 2. Zeta Potential *versus* SOD Entrapment

(▽) liposomes (EPC : Chol = 8 : 2 in molar ratio) in 10 mM Tris/300 mM sorbitol (pH 7.4); (●) PGlcN-liposomes in 10 mM Tris/300 mM sorbitol (pH 7.4); (○) PGlcN-liposomes in 10 mM Tris/150 mM NaCl (pH 7.4); (■) SA-liposomes in 10 mM Tris/300 mM sorbitol (pH 7.4); (□) SA-liposomes in 10 mM Tris/150 mM NaCl (pH 7.4).

effective entrapment. Despite the zeta potential of SA-liposomes (*i.e.*, EPC : Chol : SA = 6 : 2 : 2 or 5 : 2 : 3) prepared in NaCl buffer being high, the entrapment was lower than that of PGlcN-liposomes (EPC : Chol : PGlcN = 7 : 2 : 1 or 6 : 2 : 2) prepared in sorbitol buffer. One reason is the shielding of the negative charge of SOD due to the increase in ionic strength and the electrostatic interaction with lipid membrane decrease.

In conclusion, we demonstrated that cationic liposomes could entrap a large amount of negatively charged substances (SODs) by means of usual hydration with a buffer of low ionic strength. The trapping efficiency increased with an increase in the basic lipid concentration in liposomal membrane, and such cationic liposomes containing a high mol% of basic lipid achieved high trapping efficiency. These methods using the electrostatic interaction effectively entrapped the anionic compounds.

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