Stable Positively Charged Liposome during Long-Term Storage

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The effect of taurine, as an isotonic solute, and of benzalkonium chloride (BZC), as a positive membrane component, on the long-term stability of liposomal suspensions was investigated by measuring surface potential. The surface potential, which introduced electrostatic repulsion to liposomes against aggregation, increased dose-dependently with the addition of BZC, which gave a positive charge. However, a further addition of BZC caused unexpected aggregation during storage, so the optimum addition of BZC was defined. On the other hand, taurine, which forms a zwitter ion in an aqueous solution, did not reduce the surface potential, suggesting that taurine is of possible utility as an isotonic solute. As the result of stability testing, the liposomal system using taurine and BZC was stable against aggregation during 6 months at 40 °C. We were successful in developing a stable, positively charged liposomal system during long-term storage, and our liposomal system is believed to be of wide utility as a drug carrier for therapeutic drugs applied topically to negatively charged mucosal tissues.

We applied the Derjaguin–Landau–Verwey–Overbeek (DLVO) theory to estimate the colloidal stability of liposomes. As a result of stability testing, positively charged liposomes had a good correlation between maximum total repulsive energy (V_T(max)/kT) between two liposome particles and colloidal stability, suggesting that the V_T(max)/kT value is useful for estimating stability and for designing liposomal preparations containing some ionic substances.

Keywords liposome; taurine; benzalkonium chloride; surface potential; positive charge; long-term storage

The colloidal stability of positively or negatively charged liposomes has been increased by decreasing the ionic strength and by increasing the surface charge density.[1–4] A stable liposome requires a surface potential high enough to contribute to colloidal stability through the avoidance of aggregation and fusion. Therefore, the charged membrane component and the isotonic solute in a liposomal suspension will determine its stability during long-term storage.

Positively charged liposomes are thought to be available as drug carriers for therapeutic drugs applied topically to negatively charged mucosal tissues such as the eye and nasal passages.[5–7] As positive membrane components of liposomes, stearyl amine and quaternary ammonium salts are generally used. However, a stable positively charged liposomal system during long-term storage has not yet been established. We tried to apply benzalkonium chloride (BZC) as a positive membrane component for the stabilization of liposomes. BZC, which itself can be applied to mucosal tissues as an antiseptic, is a cationic surfactant and a homologous benzyldimethyltetraalkylammonium salt. Some reports on the interaction between liposomes and quaternary ammonium salts which possess strong fungicidal activity suggested that these salts could insert rapidly into the membrane and introduce electrostatic repulsion to liposomes.[8–10]

Amino acids, including taurine (a sulfur-containing β-amino acid) in an aqueous solution, dissociate into electrically neutral zwitter ions at the isoelectric point. These zwitter ions might act near a charged membrane surface similarly to a nonionic solute such as mannitol, which is generally used as an isotonic solute of liposomes. Taurine and some amino acids are thought to play a role in preventing damage to cell structure during freezing stress.[11,12] Furthermore, it was reported that taurine may play a role as an organic osmolyte; it is present in relatively high concentrations among free amino acids in the cells of a variety of marine species,[13] invertebrate cells,[14] or mammalian cells such as lymphocytes,[15,16] retina cells,[17] and astrocytes.[18] We examined the possibility of using taurine as an isotonic solute for liposomal suspensions.

Colloidal stability is affected by various factors, such as surface potential, particle size, ionic strength, and so on.[3,4] We used the maximum total repulsive energy (V_T(max)/kT) between two liposome particles, calculated from these factors according to the Derjaguin–Landau–Verwey–Overbeek (DLVO) theory,[19] to estimate colloidal stability.

In the present study, we investigated the effect of taurine and BZC on the long-term stability of liposomal suspensions by measuring the surface potential of liposomes. Our new positively charged liposomal system is discussed below.

Materials and Methods

Materials Hydrogenated soybean phosphatidylcholine (H-SPC) came from Nippon Fine Chemical Co. BZC, of Japanese Pharmacopeia XI grade, was obtained from Kao Co., Ltd. Benzyldimethylstearylammonium chloride (BDSA) was purchased from Tokyo Kasei Kogyo Co., Ltd. Taurine used in this study was a standardized product (51 A.M., No. 796) synthesized in our laboratory. The other amino acids: glycine, β-alanine, γ-amino-*n*-butylic acid, 5-aminovaleric acid, 6-amino-*n*-caproic acid, 8-aminocaprylic acid, and L-leucine, were purchased from Wako Pure Chemical Industries, Ltd. Mannitol was from Towa Kasei Kogyo Co., Ltd. The other chemicals used in this study were reagent-grade commercial products.

Preparation of Liposomes H-SPC powder was added to 240 mM of a taurine solution (pH 6.5) containing various concentrations of NaCl or to 154 mM of a NaCl solution kept at 65 °C, and was hydrated by stirring for 5 min. The total lipid concentration was adjusted to 30 mM. For preparation of homogeneously-sized liposomes, hydrated lipid suspensions were extruded through a polycarbonate membrane with an 80-nm pore size. Various amounts of BZC were added into taurine suspension solutions or 154 mM NaCl either before the hydration or after the extrusion.

A 30 mM multilamella vesicle composed of 95 mol% H-SPC and 5 mol% BDSA was prepared in 173 μM of a NaCl suspension solution by the conventional method introduced by Bangham *et al.*,[20] and was extruded

through a polycarbonate membrane having an 80-nm pore size. The mean particle sizes of liposomes were determined by dynamic light scattering with a Submicron Particle Analyzer (Nicomp Model 370). Surface potentials of the liposomes were measured with a Coulter Delsa 440 (Coulter Electronics, Inc.). Here, the surface potentials of liposomes were approximated by the measured zeta potential.

Effect of Zwitter Ions on Surface Potential of Positively Charged Liposomes Each amino acid was added to 173 μM of a NaCl aqueous solution, and 10—170 mM amino acid suspension solutions were obtained. Two-hundred μl of 30 mM positively charged liposomes containing 5 mol% BDSA was added into 10 ml of various amino acid suspension solutions, and the surface potentials of the liposomes were measured. Mannitol and KCl suspension solutions were used as controls.

Colloidal Stability of Positively Charged Liposomes as a Function of Surface Potential Thirty-mM homogeneously-sized H-SPC liposomes were diluted with the same taurine suspension solution (240 mM taurine and 17.1 mM NaCl) into 3 mM liposomal suspensions containing various concentrations of BZC. The surface potential and turbidity (at 600 nm) of these liposomal suspensions were measured, and $V_T(\max)/kT$ values between two approaching liposome particles were calculated according to the DLVO theory.

Long-Term Stability Various liposomal suspensions in glass ampoules were stored for 6 months at 40 °C. The stability of the liposomes during storage was checked by their visual appearance with respect to aggregation and mean particle sizes.

Results and Discussion

Figure 1 shows the effect of various amino acids on the surface potential of positively charged liposomes. Taurine, L-leucine and glycine ($n=1$), β-alanine ($n=2$), γ-amino-*n*-butylic acid ($n=3$), and 6-amino-*n*-caproic acid ($n=5$) among the rod-like amino acids ($H_2N(CH_2)_nCOOH$) did not reduce the positive surface potential as well as a mannitol. On the other hand, 5-aminovaleric acid ($n=4$) and 8-aminocaprylic acid ($n=7$) apparently reduced the positive surface potential. Potassium chloride (KCl) that dissociates into monovalent ions decreased the positive surface potential dose-dependently. The order of effectiveness for reducing the positive surface potential among the rod-like amino acids was $n=4>n=7>n=1, 2, 3, 5$. Since the pH of the suspension solutions was not adjusted in this examination, all amino acids are thought to dissociate into electrically neutral zwitter ions at the isoelectric point. Electrically neutral zwitter ions were thought to act like a nonionic solute because their two charges, $+e$ and $-e$ (e is the elementary elecric charge), are associated to each other. However, two rod-like amino acids ($n=4$, 7) were found to reduce the positive surface potential. This is because the decrease of association between the $+e$ charge and the $-e$ charge as the distance between the two charges at their ends increased resulted in each charge acting like a monovalent ion in some manner topically near the positive charged membrane surface. Reduction of the surface potential was not necessarily in proportion to n, suggesting that the distance between the two charges, $+e$ and $-e$, of rod-like amino acids in aqueous solutions are not necessarily in proportion to n. Here, n is the number of methylene groups in rod-like amino acids ($H_2N(CH_2)_nCOOH$). These results indicated that electrically neutral zwitter ions do not always act near a charged membrane surface as effectively as a nonionic solute. Taurine was determined to maintain an effective positive surface potential against the aggregation of liposomes. Furthermore, taurine, which can actually be used as a component of eye drops, is a safe substance to mucosal tissues. Taurine is thought to be of possible utility as an isotonic solute in a liposomal suspension.

When BZC was added into a liposomal suspension, it inserted rapidly into the membrane and the positive surface potentials increased dose-dependently as shown in Fig. 2. Various amounts of BZC were added into homogeneously sized liposomes, either after size control by extrusion or into the hydration solution before the hydration of H-SPC. Surface potentials of the former liposomes exhibited higher values than the latter, suggesting that BZC inserts into the outer hemileaflet of the membrane in the former liposomes or into both hemileaflets of the membrane in the latter liposomes, respectively. We could easily give a positive

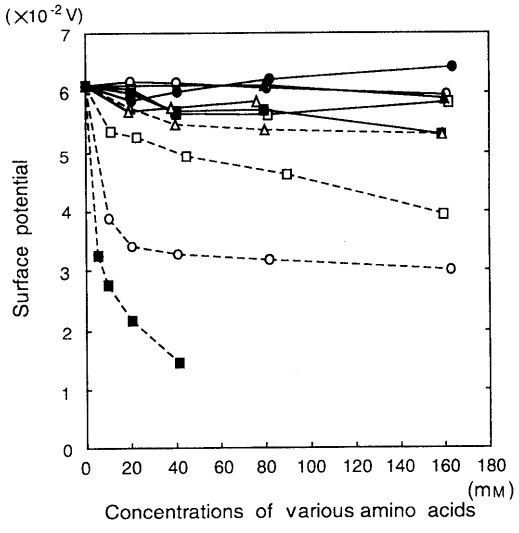

Fig. 1. Effect of Various Amino Acids on Surface Potential of Positively Charged Liposomes

Positively charged liposome was composed of 95 mol% H-SPC and 5 mol% BDSA. The particle size and concentration were 93.5 nm and 3 mM, respectively. Each amino acid solution was contained in 173 μM NaCl: —●—, taurine ($H_2N(CH_2)_2SO_3H$); —○—, glycine (H_2NCH_2COOH); —□—, β-alanine ($H_2N(CH_2)_2COOH$); —■—, γ-amino-*n*-butylic acid ($H_2N(CH_2)_3COOH$); ---○---, 5-amino-valeric acid ($H_2N(CH_2)_4COOH$); ---△---, 6-amino-*n*-caproic acid ($H_2N(CH_2)_5COOH$); ---□---, 8-aminocaprylic acid ($H_2N(CH_2)_7COOH$); —△—, L-leucine ($(CH_3)_2CHCH_2CH(NH_2)COOH$). Mannitol (—▲—) and KCl (---■---) suspension solutions were used as positive and negative controls.

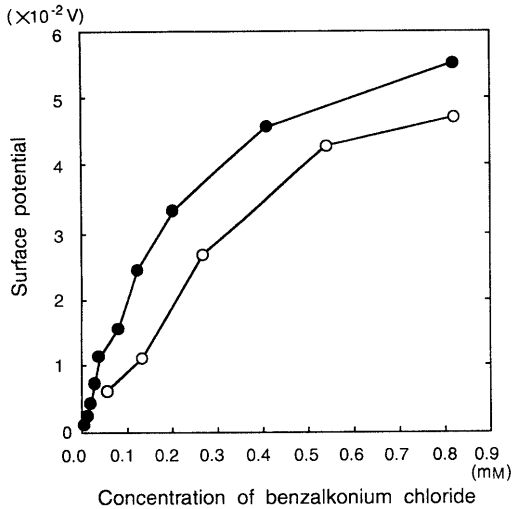

Fig. 2. Surface Potential of Liposomes in Taurine Suspension Solution as a Function of Benzalkonium Chloride Concentrations

Three-mM liposome was prepared in 240 mM taurine and 17.1 mM NaCl suspension solution (pH 6.5) and extruded through a polycarbonate filter with an 80-nm pore size. Various amounts of BZC were added into the hydration solution before the hydration of H-SPC (○) or to homogeneously-sized liposomes after the size control by the extrusion (●).

charge and introduce electrostatic repulsion to uncharged liposomes by adding BZC into liposomal suspensions.

In order to analyze the experimental data, we employed the DLVO theory[19] of colloidal stability. This theory explains the stability of the system of colloidal particles on the basis of two types of interaction forces acting among the particles, that is, the electrostatic repulsion due to overlapping of the electric double layers around the particles, and the long-range van der Waals attraction. The magnitude of the former force is characterized mainly by the particle surface potential ψ_s and the latter by the Hamaker constant A. The interacting energy between two approaching liposome particles was calculated according to the DLVO theory using $V_T = V_R - V_A$ or

$$V_T = \frac{64000\pi N_A C \gamma^2 e^{-\kappa H} a}{\kappa^2} - \frac{Aa}{12H} \quad (1)$$

$$\kappa = \left[\frac{2000 N_A C v^2 e^2}{\varepsilon_r \varepsilon_0 kT}\right]^{1/2} \quad (2)$$

$$\gamma = \tan h \frac{ve\psi_s}{4kT} \quad (3)$$

where V_T, V_A, and V_R are the total, attractive, and electrostatic repulsive energies, respectively. In Eq. 1, a is the radius of the liposomes; H is the distance between the bilayer surfaces; κ is the Debye–Huckel parameter of the electrolyte solution; N_A is the Avogadro number and C is the concentration of the $+e$ (e is the elementary electric charge). Here, V_A is calculated with a simplified equation for solid spheres and $H \ll a$. In Eq. 2, v is the atomicity of ions, ε_r is the relative permittivity of the electrolyte solution, and ε_0 is the permittivity of a vacuum. In Eq. 3, the liposomal surface potential ψ_s can be approximated by the measured zeta potential ζ, if the slipping plane coincides with the liposome surface. These calculations are estimates, because the effective Hamaker constants for H-SPC liposomes are not precisely known. Ohshima *et al.* reported a value of $(3.6 \pm 0.8) \times 10^{-21}$ J for a dipalmitoyl phosphatidylcholine membrane.[21] We chose 4×10^{-21} J for our caclualtions. From the results of Fig. 1, the concentration of taurine that did not reduce the surface potential should not be included in the ionic strength in the calculation. The relationship between the aggregation and the total repulsive energies (V_T) of solid particles is represented as follows:

$$\text{probability of aggregaion } (P) = \exp(-V_T/kT) \quad (4)$$

If the physicochemical property of a liposome membrane is not changed much by the addition of BZC and the liposome particle behaves like a complete solid particle, the colloidal stability of liposomes is supposed to increase with increasing values of V_T against aggregation.

Figure 3 shows the calculation of total repulsive energies (V_T) between two liposome particles according to the DLVO theory as a function of interparticle distance. Values of maximum total repulsive energies ($V_T(\max)/kT$) shown in Fig. 3 increased with increasing concentrations of BZC, and in the low ionic strength of 0.0007 M (240 mM taurine, pH 6.5) at 0.135 mM BZC its value was able to reach to 44.9. On the other hand, $V_T(\max)/kT$ values decreased with increasing ionic strength and in 0.162 M NaCl (0.9% NaCl) at 0.135 mM BZC its value decreased below 0. The increase

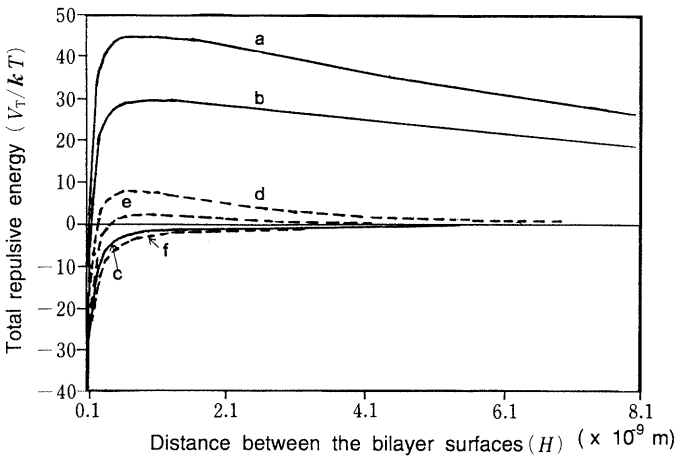

Fig. 3. Calculation of Total Repulsive Energies (V_T/kT) between Two Liposome Particles According to The DLVO Theory as a Function of Interparticle Distance

Three-mM liposome was prepared in 240 mM taurine and various concentrations of NaCl or 0.9% NaCl. Various amounts of BZC were added into 3 mM liposome after extrusion with a polycarbonate filter having an 80-nm pore size. The Hamaker constant is 4×10^{-21} J, based on data reported by Ohshima *et al.*[21] Key [surface potential(V)/ionic strength(M)/BZC concentration (mM)]: (a) 0.0366/0.0007/0.135; (b) 0.0296/0.0007/0.0675; (c) 0.0017/0.0007/0; (d) 0.0205/0.018/0.135; (e) 0.0157/0.0346/0.135; (f) 0.0105/0.162/0.135.

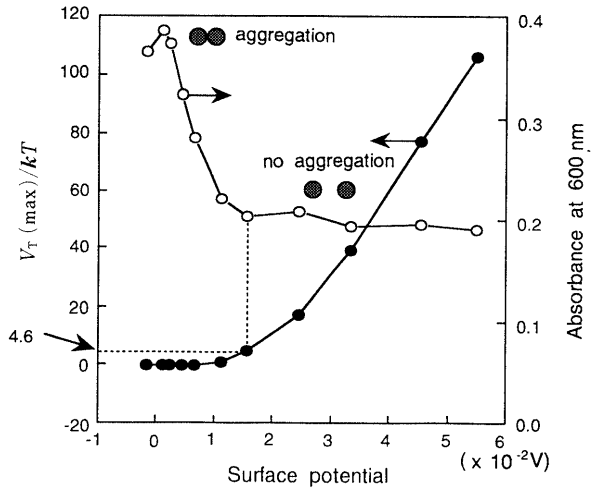

Fig. 4. Colloidal Stability of Positive Charged Liposomes as a Function of Surface Potential

Three-mM liposome was prepared in 240 mM taurine and 17.1 mM NaCl suspension solution (pH 6.5) and extruded through a polycarbonate filter with an 80-nm pore size. Various amounts of benzalkonium chloride were added into 3 mM liposomal suspensions after extrusion with a polycarbonate filter having an 80-nm pore size. Surface potential and turbidity of these liposomal suspensions were measured at 600 nm.

of $V_T(\max)/kT$ values theoretically suggests an increase in the colloidal stability of liposomes. Colloidal particles are thought to be sufficiently stable at $V_T/(\max)/kT \geqq 10$, when the probability of aggregation is $\leqq 0.000045$ as calculated from Eq. 4. Therefore, at a lower ionic strength and or a higher positive charge density, liposomes should be stable.

We examined the relationship between colloidal stability and surface potentials (Fig. 4). The turbidity decreased with increasing surface potentials, and at a surface potential $\geqq 0.0162$ V the turbidity became constant, suggesting that liposomes should be stable against aggregation. On the other hand, values of $V_T(\max)/kT$ increased with increasing zeta potentials. When the surface potential is 0.0162 V, the

Table I. Effect of Benzalkonium Chloride on Colloidal Stability of Liposomes During Long-Term Storage

Rp.[a]	BZC (mM)	Surface potential (V)	V_T (max)/kT	Particle size (nm)			Appearance[b]	
				0 month (M)	3 M	6 M	3 M	6 M
1	0	0.0017	−0.12	104	99	—[c]	△	△
2	0.135	0.0099	0.11	77	77	211	○	△
3	0.27	0.0186	6.0	76	78	122	○	○
4	0.486	0.0271	18	76	83	318[d]	○	△
5	0.67	0.033	40	77	109	1166[e]	○	△

a) Suspension solution of 3 mM liposomes was 240 mM taurine–17 mM NaCl aqueous solution (pH 6.5). Various amounts of BZC were added into the hydration solution before the hydration of H-SPC. *b*) The visual appearance of liposomes during storage at 40 °C was checked by looking for no change (○) or aggregation and/or precipitation (△). *c*) The particle size was not measured. *d*, *e*) Distribution of particle size of *d*) and *e*) exhibited two peaks (59.4, 427 nm) and three peaks (4.8, 36.6, 1894 nm), respectively.

Table II. Effect of Ionic Strength on Colloidal Stability of Positively Charged Liposomes During Long-Term Storage

Rp.[a]	Ionic strength (M)	Surface potential (V)	V_T (max)/kT	Particle size (nm)			Appearance[b]	
				0 month (M)	3 M	6 M	3 M	6 M
6	0.0007	0.018	13	100	99	116	○	○
7	0.0094	0.015	5.0	96	104	134	○	○
8	0.018	0.0096	0.01	94	91	257	○	△
9	0.0346	0.0069	−0.14	93	98	424	○	△
10	0.156	0.0023	−0.30	94	209	1688[c]	△	△

a) Suspension solution (pH 6.5) in Rps. 6—9 contained 240 mM taurine and various amounts of NaCl. All 3 mM liposomal suspensions contained 0.136 mM BZC which was added before the hydration of H-SPC. *b*) The visual appearance of liposomes during storage at 40 °C was checked by looking for no change (○) or aggregation and/or precipitation (△). *c*) Distribution of particle size exhibited two peaks (259, 1780 nm).

V_T(max)/kT value is 4.6 as calculated from Eqs. 1—3. A V_T(max)/kT value of 4.6 is thought to be reasonable as a lower-limited value under the stable condition of liposomes. A V_T(max)/kT value greater than 4.6 gives a value of less than 0.01 in the probability of aggregation calculated from Eq. 4. We discussed whether the experimental results could be predicted by the DLVO theory on the colloidal stability of positive liposomes, as follows.

The effect of BZC on the colloidal stability of liposomes during long-term storage is shown in Table I. In this test of stability, we used the suspension solution of pH 6.5 in consideration of the report by Grit *et al.*, that the optimum stability of DSPC-liposomes is observed at pH 6.5.[22] Liposomes in recipe 1 (Rp. 1) were aggregated reversibly at 3 months of storage because they had a low V_T(max)/kT value, −0.12. Liposomes in Rp. 2 were stable at 3 months of storage in spite of a low V_T(max)/kT value, 0.11. Liposomes in Rp. 3, Rp. 4, and Rp. 5 were stable as expected during 3 months of storage and had a good correlation between the V_T(max)/kT value and colloidal stability. However, at 6 months of storage, irreversible aggregations were found unexpectedly in Rp. 4 and Rp. 5, but not in Rp. 3 despite its having V_T(max)/kT values higher than 4.6. Apparently, factors other than electrical repulsion control stability. Peaks with a smaller particle size than the initial size of liposomes during the storage were observed in Rp. 4 and Rp. 5, indicating that liposome membranes may be partially solubilized by BZC at high concentrations. An increase of V_T(max)/kT was obtained with the addition of BZC, which gave a positive surface potential, while the further addition of BZC produced an unexpected aggregation. If the physicochemical properties of liposomes and membrane components were not changed and the liposome particle behaved like a complete solid particle during storage, we could obtain a much better correlation between the V_T(max)/kT values and colloidal stability. By taking into account the optimum addition of BZC, we were able to obtain stable, positively charged liposomes during 6 months of storage at 40 °C.

Table II shows the effect of ion strength on the colloidal stability of positively charged liposomes during long-term storage. Positively charged liposomes in Rp. 6, 7 were stable during 6 months of storage at 40 °C. A decrease in V_T(max)/kT values was caused by an increase in ionic strength, and liposomes in Rps. 8, 9 and 10, with low values (0.01, −0.14, −0.3, respectively), were aggregated irreversibly at 6 months of storage. These positive liposomes showed a good correlation between the V_T(max)/kT value and colloidal stability. Liposomes in Rp. 8, Rp. 9 and Rp. 2 as shown in Table I, were stable against aggregation during 3 months of storage in spite of V_T(max)/kT values much lower than 4.6. This unexpected stability against aggregation could be ascribed to the hydration forces and the phase transition temperature (49.7 °C) of H-SPC being higher than the temperature of storage, 40 °C. Leneveu[23] and Rand[24] *et al.* indicated that the absence of electrostatic repulsion between two liposome particles will not necessarily result in aggregate formation or fusion of the colloidal particles, since many uncharged liposomes are fully stabilized by hydration energies. Although the colloidal stability of liposomes cannot be explained only in terms of repulsive energy, V_T(max)/kT values are useful for estimating the stability and for designing liposomal preparations containing some ionic substances.

In this study, we established a stable, positively charged liposomal system using taurine and BZC. This liposomal system may be able to entrap various drugs, especially lipophilic drugs. As a result of actual storage stability tests of liposomes consisting of 80 mol% H-SPC and 20 mol% vitamin E acetate using our liposomal system, stability

against aggregation and reduction of the drug was sufficiently retained during 6 months of storage at 40 °C (data not shown). Szulc *et al.* reported that a further increase in the entrapment of vitamins A and E was obtained with the addition of BZC, which produced positive liposomes.[25] Moreover, it was reported that positive liposomes may be of wide utility for therapeutic drugs applied topically to mucosal tissues, such as the eye and nasal passages.[5-7] Our positively charged liposomal system will hopefully be useful for therapeutic drugs applied topically to mucosal tissues.

Conclusion

We were able to develop a stable, positively charged liposomal system during 6 months of storage at 40 °C, and we found out that taurine and BZC are respectively useful as an isotonic solute and a positive membrane component. Our liposomal system may be able to entrap various drugs, especially lipophilic drugs, and it is believed to be of wide utility for therapeutic drugs applied topically to mucosal tissues.

As a result of applying the DLVO theory to estimate colloidal stability, we suggested that the $V_T(\max)/kT$ value is useful for estimating such stability and for designing liposomal preparations containing some ionic substances.

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