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Studies for the Osmotic Parameter of Liposomes

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Abstract □ By using the former equation (8), we modified the equation which can show the dissimilar osmotic behavior of liposome with composition change. The slope of the new equation was presented as the ratio of osmotically active volume (V_{act}) to the total volume($V_{total} = V_{act} + V_{dead}$; V_{dead} is osmotically inactive volume) of liposomes. We defined it as a Z-value, which can elucidate the dissimilarity of the osmotic activity of multilamellar liposomes with the change of phospholipid composition and the differences of physico-chemical properties of liposomes. Z-value was applied for studying the physico-chemical properties of liposomal membrane. The factor that affects on the Z-value was not the lipid concentration of liposome stock dispersion but the lipid composition of liposomal membrane. As the content of dicetylphosphate, the negative charged phospholipid, was increased, the osmotic activity, represented by Z-value, of multilamellar liposome was decreased. Under the hypertonic conditions (shrinking region), Z-value steadily increased and reached a maximum at 10 mole percent cholesterol with increasing the cholesterol content.

Keywords □ Multilamellar liposome, Osmotic behavior, Osmotic activity, Z-value, Dicetylphosphate, Cholesterol

Liposomes were known to be a useful model system for investigating membrane-related phenomna, especially for studying the lipid barrior function of a biological membrane. Multilamellar vesicles of phospholipids including a small amount of charged lipids were shown to be osmotically active and the osmotic behaviors were investigated using by turbidimetry [1,2]. The total volume of liposomes is reciprocally proportional to the turbidity at UV-450 nm. Turbidity is easily measured and the sensitivity of the method is high enough to follow a rapid volume change of liposomes without any pertubation of the system. Hence, it has also been used to investigate the permeability of water or solute through the liposomal membrane [3-6]. It has been determined experimentally that the turbidity of intact mitochondrial and bacterial suspension is reciprocally proportional to the two-thirds power of the particle volume [7]. It has been also reported that this relationship is also applicable to multilamellar liposomal system [8]. However, the dissimilarity of the osmotic activity of liposomes has not previously been determined according to the change of the phospholipid composition and the physico-chemical properties. We establish a parameter which can elucidate the dissimilarity of osmotic activity of liposomes, and utilize it for studying the physico-chemical properties of liposomal membrane in this experiment.

EXPERIMENTAL METHODS

Materials

Egg-yolk phosphatidylcholine(PC) was purchased from Merck Co., Darmstadt, and purified by alumina column chromatography. Cholesterol (Ch) and stearylamine(SA) were purchased from Nakarai Chemicals, Ltd., Kyoto, Japan and used without further purification. Dicetylphosphate (DCP) was purchased from Sigma Co., St. Louis, Mo. All other reagents were commercial and of an analytical grade.

Preparation of multilamellar liposomes

Stock dispersions for turbidimetric measurements were prepared as follows. Appropriate amount of egg-yolk PC with or without charged phospholipid(DCP or SA) and cholesterol in chloroform was put into a round-bottomed flask. The solvent was removed by a rotary evaporator to dryness for 30 min. and further dried in vacuum for 2hrs. Aqueous solution containing 50 mM glucose, 2 mM EDTA, and 10 mM Tris-HCl buffer, pH 7.5, was added to this thin lipid film. The lipid on the surface of the flask was suspended by a vortex mixer for 5 min. The final lipid concentration of liposome stock dispersion was 15 mM. Unless noted

otherwise, all glucose solution used in this studies contained the same buffer system as stock dispersion.

Optical measurement

A 0.1 m*l* of liposome stock dispersion formed in 50 mM glucose solution, which is regarded as inner concentration, C_{in}, of liposome, was diluted with 3 m*l* of glucose solution with various concentration, outer concentration, C_{out}. $C_{in}1C_{out}$ ratio used in this experiments was varied from 0.2 to 2.0 at regular intervals, 0.2. It was incubated for 1 h to achieve equilibrium before the measurement at room temperature. Absorbance of the dispersion was measured at 450 nm on a PYE-UNICAM SP 1750 Ultra-Violet Spectrophotometer.

RESULTS

The osmotic behavior of multilamellar liposome.

The osmotic gradient across the liposomal membrane was formed by the use of glucose as mentioned above. This osmotic gradient induces a volume change of liposomes due to the uptake or release of water. When the multilamellar liposome acts as a perfect osmometer, the total average volume of liposomes change according to the Boyle-van't Hoff's law,

$$V_{total} = V_{act}(C_{in}/C_{out}) + V_{dead} \quad (1)$$

where V_{act} and V_{dead} are the osmotically active and osmotically inactive volumes of liposome, respectively. C_{in}/C_{out} is the ratio of glucose concentration of inner part to that of outer part of liposome. If $C_{in}/C_{out} = 1$, liposomes are under isotonic condition and if $C_{in}/C_{out} > 1$ or $C_{in}/C_{out} < 1$, liposomes are under hypotonic or hypertonic condition, respectively. The volume change of liposome can be easily detected by the turbidimetric method using the equation derived by Yoshikawa et al.[8].

$$V_{total} = K(1/A_{450})^{3/2} \quad (2)$$

where A is the UV_{450}-absorbance and K is constant.

From the combination of Eqs.1 and 2, following equation would be derived.

$$(1/A_{450})^{3/2} = 1/K[V_{act}(C_{in}/C_{out}) + V_{dead}] \quad (3)$$

Eq.3 shows that the osmotically active multilamellar liposomes qualitatively have a linear relationship between the reciprocal 3/2 power of UV-absorbance at 450 nm and the osmotic gradient across liposomal membrane(C_{in}/C_{out}).

Fig. 1 shows the osmotic behaviors of a pure egg-yolk PC liposomes, positively charged liposomes with 5 mole percents of SA, and negatively charged liposomes with 5 mole percents of DCP. Charged liposomes of egg-yolk PC/SA or PC/DCP showed a good linear relationship in accordance with the Eq.3. On the contrary, neutral liposomes of pure egg-yolk PC did not show the linear relationship. It can be inferred that the multilamellar liposomes need the positive or negative charged phospholipid in their bilayer composition so as to have osmotic activity. However, the slope of straight line in the swelling region ($C_{in}/C_{out} > 1.0$) was somewhat different from those in the shrinking region($C_{in}/C_{out} < 1.0$). This can be illustrated by the fact that under hypotonic conditions over $C_{in}/C_{out} = 2.0$, the plots gradually deviate from the linear relationship owing to the release of glucose form liposomes[8]. Hence, the osmotic gradient, C_{in}/C_{out}, were varied from 0.2 to 2.0 in this experiment.

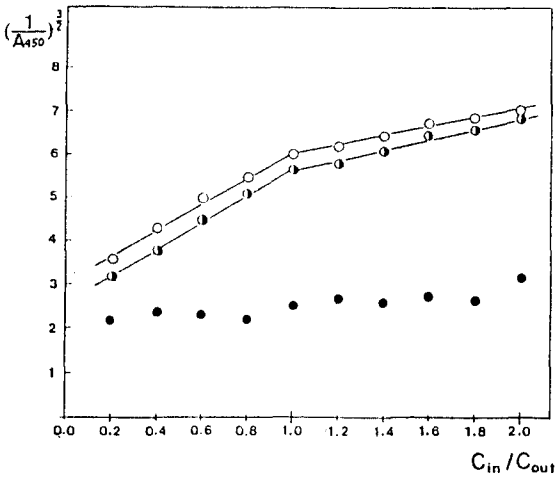

Fig. 1. The reciprocal 3/2s power of absorbance at 450 nm of multilamellar liposomes prepared from phosphatidylcholine only (●), phosphatidylcholine/stearylamine (95:5, molar ratio) (◑) and phosphatidylcholine /dicetylphosphate (95:5, molar ratio) (○).

Establishment of osmotic activity parameter (Z-value)

Using Eqs. 1, 2 and, 3, we modified the equation which can determined the dissimilarity of the osmotic activity of liposomes as follows. From Eqs. 1 and 2, the volume of multilamellar liposomes under the isotonic condition, V_{iso}, is given by

$$V_{iso} = K(1/A_{450})^{\frac{3}{2}}_{iso} = V_{act}(C_{in}/C_{out})_{iso} + V_{dead} = V_{act} + V_{dead} \quad (4)$$

and the volume of multilamellar liposomes according to the various osmotic gradient, V*n*, is also given by

$$V_n = K\,(1/A_{450})_n^{3/2} = V_{act}\,(C_{in}/C_{out})_n + V_{dead} \quad (5)$$

(Eq.5-Eq.4)/Eq.4 yields

$$\frac{V_n - V_{iso}}{V_{iso}} = \frac{(1/A_{450})_n^{3/2} - (1/A_{450})_{iso}^{3/2}}{(1/A_{450})_{iso}^{3/2}}$$

$$= \frac{V_{act}}{V_{act}+V_{dead}}\left[(C_{in}/C_{out})_n - (C_{in}/C_{out})_{iso}\right] \quad (6)$$

In Eq.6, we put

$(1/A_{450})_n^{3/2} - (1/A_{450})_{iso}^{3/2} = \Delta\,(1/A_{450})^{3/2}$ and

$(C_{in}/C_{out})_n - (C_{in}/C_{out})_{iso} = \Delta\,(C_{in}/C_{out})$

then, the Eq. 6 can be simplified in the following equation,

$$\frac{V_n - V_{iso}}{V_{iso}} = \frac{\Delta\,(1/A_{450})^{3/2}}{(1/A_{450})_{iso}^{3/2}} = \frac{V_{act}}{V_{act}+V_{dead}}\,\Delta\,(C_{in}/C_{out}) \quad (7)$$

If $\Delta\,(1/A_{450})^{3/2}/(1/A_{450})_{iso}^{3/2}$ is plotted versus (C_{in}/C_{out}) based on the Eq. 7, the slope of straight line is presented as $V_{act}/(V_{act}+V_{dead})$, i.e. the ratio of osmotically active volume to the total volume of liposome, which implies the quantity of volume changed per unit volume of liposomes when a certain amount of osmotic gradient is given to the liposomal membrane. It can be said that the slope of Eq. 7 may be considered to be a parameter which elucidates the dissimilarity of the osmotic activity of multilamellar liposomes with the change of their phospholipid composition and differences of physico-chemical environment. We defined the slope of Eq. 7 as Z-value, which varies between 1 and 0.

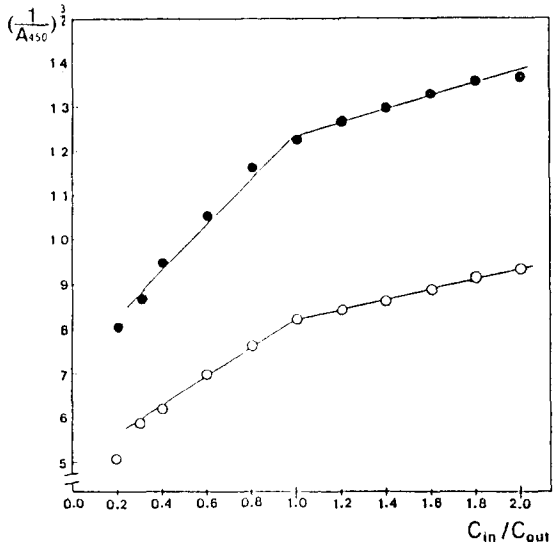

Fig. 2. Osmotic behavior of multilamellar liposomes prepared from phosphatidylcholine/dicetylphosphate (90:10, molar ratio).
The lipid concentration of liposome stock dispersion was 15 mM (●) and 20 mM (○), respectively

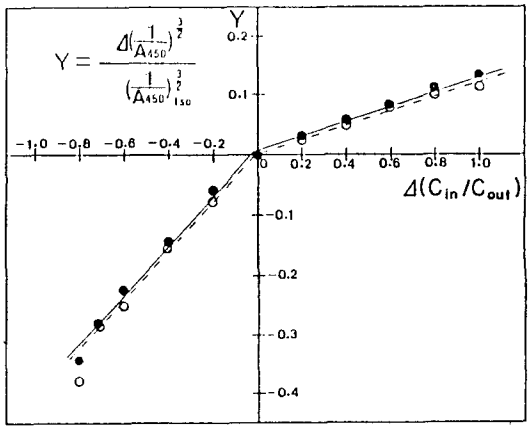

Fig. 3. The plot of the changing ratio in reciprocal 3/2 power of absorbance at 450 nm versus the change in the concentration gradients for phosphatidylcholine/dicetylphosphate (95:5, molar ratio) multilamellar liposomes.
The concentration of liposome stock dispersions were 15 mM (●) and 20 mM (○), respectively.

Fig. 2 shows the osmotic behaviors of egg-yolk PC/DCP (90;10, molar ratio) multilameller liposomes. The lipid concentration of liposome stock dispersion was 5 mM and 20 mM, respectively. Both of two liposomes had good linear relationships between C_{in}/C_{out} and $(1/A_{450})^{3/2}$, but the slopes of two straight lines were different from each other. In case of plot of $\Delta(1/A_{450})^{3/2}(1/A_{450})^{3/2}$ versus $\Delta(C_{in}/C_{out})$, the slopes of the straight lines, Z-value, were nearly same each other (Fig. 3). The Z-value at the shrinking region of 15 mM was 0.3757 and that of 20 mM was 0.3791, and at the swelling region of 15 mM was 0.1216 and that of 20 mM was 0.093.

Fig. 4. shows the plots of $\Delta(1/A_{450})^{3/2}/(1/A_{450})^{3/2}$ versus $\Delta(C_{in}/C_{out})$ for egg-yolk PC/DCP liposome was remarkably different from that of

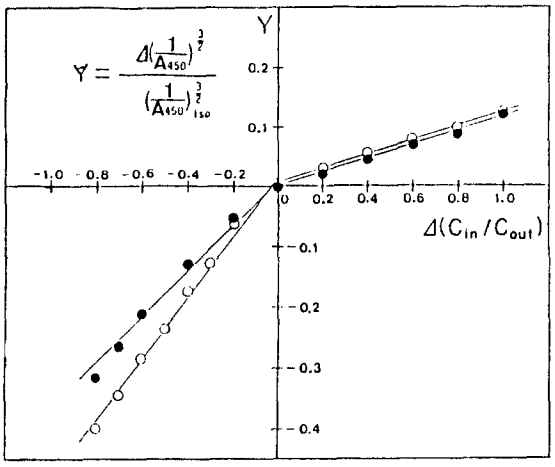

Fig. 4. The plot of the changing ratio in reciprocal 3/2 power of absorbance at 450 nm versus the change in the concentration gradients for phosphatidylcholine/dicetylphosphate (90:10, molar ratio) (●) and phosphatidylcholine/dicetylphosphate/cholesterol (80:10:10, molar ratio) (○) multilamellar liposomes.

egg-yolk PC/DCP/Ch liposome, though the Z-values were nearly same at the swelling region. Therefore, it can be suggested that especially the Z-value at the shrinking region is a significant parameter which can elucidate the dissimilarity of the osmotic activity of multilamellar liposomes with their phospholipid composition.

The effect of the content of DCP, the negatively charged phospholipid, and cholesterol on the osmotic activity parameter, Z-value

The effect of varing molar percentage of anionic DCP incorporated on the Z-value of egg-yolk PC liposomes at the shrinking region is presented in Fig. 5. As the content of DCP in lipid bilayer was increased, the osmotic activity, Z-value, was decreased.

As shown in Fig. 6, the Z-value at shrinking region was increased as the cholesterol content is increased up to 10 mole percents. And when the cholesterol contents were higher than 10 mole percents, the Z-value was decreased with the increase of cholesterol content. At swelling region, i.e. under hypotonic condition, there was no significance in Z-value change.

DISCUSSION

The linear relationship based on the Eq. 3 has been applied for the studies of many membrane related phenomena, especially for studying the permeability properties of the barrier function of biological membrane and the interaction of membrane with drugs such as Ginseng Saponin [9], Sea Cucumber Saponin [10], Glycyrrhizin [11], and polyene antibiotics [12], etc, which have a strong lytic effect on the biological membrane.

The Eq. 3 which has been used for investigating the osmotic phenomena of multilamellar liposomes can not elucidate the dissimilarity of the osmotic activity of liposomes according to the change of the phospholipid composition and the differences of physico-chemical environment, except only the recognition of the existence of osmotic activity. Therefore, we established a parameter named Z-value, which means the quantity of volume changed when a certain amount of osmotic gradient is given to the liposomal membrane. This parameter represents the easiness of the osmotic shrinking or swelling.

As shown in Figs. 2 and 3, the osmotic activities of liposomes at 15 mM and 20 mM were very similar to each other, even though those seemed to be different from Fig. 4. It implies that the osmotic activity remains constant on the same composition of miltilamellar liposome in spite of the change of lipid concentration of the stock dispersion.

Fig. 4 shows that with lipid composition change the Z-value of shrinking region also changes significantly, but that of swelling region has little change. The osmotic activity change due to composition changes seems to be no longer significant under hypotonic condition (the swelling region). Thus, we used the Z-value of shrinking region to investigate the effect of composition change.

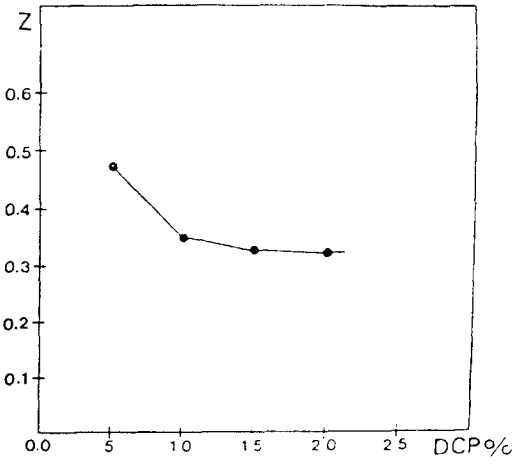

Fig. 5. Effects of dicetylphosphate (DCP) content on the Z-value of liposome under the hypertonic condition.

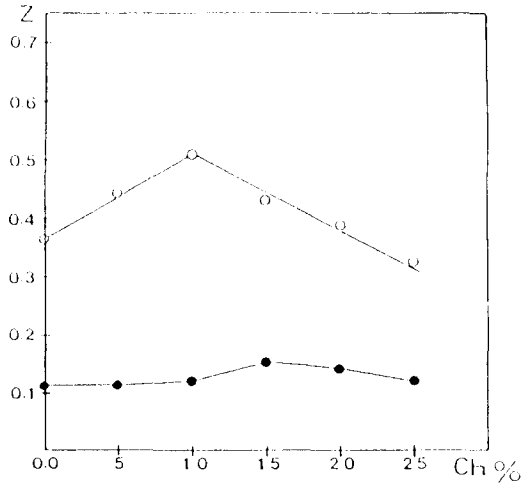

Fig. 6. Effects of cholesterol (Ch) content on the Z-value of liposome prepared from phosphatidylcholine/dicetylphosphate/cholesterol under hypertonic (○) and hypotonic (●) conditions.
Total lipid concentration of liposome stock dispersion was 15 mM and molar percentage of DCP was always 10.

Dicetylphosphate (DCP) is one of the charged phospholipids that contribute the osmotic activity to liposomes. But the increase of DCP content diminished the osmotic activity in Fig. 5. It can be inferred that the difference of chemical structure between DCP and egg-yolk PC makes the conformation of the lipid bilayer unstable. On the other hand, the repulsive forces between the head group of DCP molecules may be considered to hinder the shrinkage of liposomes.

With increasing cholesterol content, as shown in Fig. 6, there was no significant change in Z-value at swelling region, but at shrinking region, Z-value increased initially and reached a maximum at 10 mole percent cholesterol. This implies that multilamellar liposomes of egg-yolk PC and 10 mole percent of DCP have maximum osmotic activity at about 10 mole percent of cholesterol. This is consistent with the suggestion that cholesterol has a condensing effect on lipid bilayer by formation of complex with adjacent phospholipids [13]. It can be suggested that the condensing effect of cholesterol at 10 mole percent content renders the most stable structure to the liposomal membrane so as to have a high osmotic activity, and above 10 mole percent, the liposomal membrane is too rigid to have a high osmotic activity.

In conclusion, results described in this report demonstrate that the Z-value in this experiment is a parameter which can elucidate the dissimilarity of the osmotic activity of multilamellar liposomes with composition change, and also the multilamellar liposomes must have an appropriate amount of charged phospholipids and proper fluidity of lipid bilayer so as to have a high osmotic activity.

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