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Stability and Drug Release Properties of Liposomes Containing Cytarabine as a Drug Carrier

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Abstract □ Liposomes were studied as a drug delivery system. Multilamellar vesicles, small unilamellar vesicles and large unilamellar vesicles containing cytarabine were prepared using egg yolk lecithin and cholesterol. Large unilamellar vesicles showed the highest encapsulation efficiency of all and their encapsulation efficiency increased as the buffer volume decreased. Cholesterol increased the stability of liposomal drug products as drug carriers and reduced the permeability of drug across the liposomal membrane. The release rate of cytarabine increased with incubation temperature and decreased with cholesterol incorporation in liposomal membrane. The release mechanism of cytarabine from large unilamellar vesicles in vitro was chiefly due to simple diffusion across the liposomal membrane rather than liposomal rupture.

Key words □ Liposomes as drug carriers, encapsulation efficiency, stability, cytarabine, multilamellar vesicles, small unilamellar vesicles, large unilamellar vesicles, osmotic behavior.

There has been much attention about the special drug delivery system which can carry drug molecules to specific target site in order to maximize the therapeutic effect and minimize the severe side effect.[1-4] Many attempts have been made to increase the selectivity of drug molecules by entrapping them in drug carriers. Drug carrier should be nontoxic, biocompatible and biodegradable.[6] On considering these criteria, liposomes which are very similar to biomembrane may be useful as drug carriers. To develop liposomes as drug carriers, encapsulation efficiency, stability and target specificity must be considered.

Liposomes are multilamellar, small unilamellar and large unilamellar type vesicles according to the method of preparation. The encapsulation efficiency of each type is quite different.[7] Up to now large unilamellar type prepared by reverse phase evaporation method is known for the highest encapsulation efficiency.[8-10]

The stability of liposomal drug product during storage is mainly depends on the physico-chemical properties of the lipid membrane. The stability of liposomes is usually enhanced by cholesterol incorporation[11-13] or phospholipid polymerization.[14-15] Phospholipid has its own transition temperature at which liquid crystalline state and solid gel state coexist. At this transition temperature, the liposomal membrane is very fluid and releases many entrapped drug molecules. Thus, the stability of liposomal drug products and/or content uniformity of drug in liposomes are highly affected by the temperature.

In this paper, encapsulation efficiency, release rate and release mechanism of cytarabine from liposomes were studied under the current experimental conditions.

EXPERIMENTAL METHODS

Materials

Egg yolk lecithin(PC) was purchased from Sigma Co. and purified by alumina column chromatography. The purity was established by thin layer chromatography. Cholesterol(CH) was purchased from Sigma Co. and cytarabine was supplied by Choong Wae Pharm. Co. They were used without further purification. Alumina(Hayashi) and Sephadex G-50(Sigma Co.) were used. All other chemicals used were of reagent grade and solutions were prepared in distilled water.

Apparatus

Rotary vacuum evaporator(Tokyo Rikaikai), probe type ultrasonicator(MSE) and UV spectrophotometer(Pye-Unicam SP 1750) were used.

Preparation of liposomes

Multilamellar vesicle(MLV), small unilamellar vesicle(SUV) and large unilamellar vesicle(LUV) were prepared. MLV was prepared by the following way; Lecithin and cholesterol(9:2 molar ratio) were dissolved in appropriate amount of chloroform and the chloroform was evaporated by rotary vacuum evaporator to make lipid thin film inside a glass tube. The lipid thin film was suspended in phosphate buffer containing cytarabine by vortex mixing for 5 min.

SUV was prepared by ultrasonicating the MLV for 30 min with probe type ultrasonicator.

LUV was prepared by reverse phase evaporation method as follows:[9-11] Lipid thin film prepared was dissolved in 3 ml of ether, and 1 ml of phosphate buffer containing cytarabine was added to this ether solution. This binary solution was sonicated briefly to make inverted micelles. Aqueous phase containing cytarabine was entrapped in the inverted micelles and ether was excluded outside. Later, ether was evaporated by rotary vacuum evaportor until homogeneous suspension was obtained.

Measurement of drug encapsulation efficiency

The entrapped cytarabine in liposomes was separated from non-entrapped free cytarabine by passing through a Sephadex G-50 column(2.5 cm × 40 cm). Total amount of non-entrapped cytarabine was measured by assaying spectrophotometically at 274 nm. The entrapped cytarabine is the total cytarabine minus the non-entrapped free cytarabine separated from liposomes. Encapsulation efficiency was calculated as follows;

$$\text{Encapsulation efficiency (\%)} = \frac{\text{total cytarabine} - \text{free cytarabine}}{\text{total cytarabine}} \times 100$$

Stability of liposomes

To examine the thermal stability of liposomes, liposomal suspension (LUV) separated from non-entrapped free cytarabine by Sephadex G-50 gel chromatography was divided into three aliquots and incubated at 4, 23 and 38 °C, respectively. Every 12 hours, the cytarabine molecules released from LUVs were separated by dialysis membrane and the amount of cytaratine released from LUVs was measured by assaying spectrophotometically at 274 nm.

To examine the effect of cholesterol on the stability of liposomes, liposomes with different molar ratios of cholesterol to lecithin were prepared and the release rate of cytarabine from each LUV was studied. Liposomal suspension (LUV) separated from free cytarabine by gel chromatography was incubated in a dialysis cell at 35 °C and the cell was shaken continuously. Every one hour the amount of cyarabine released from LUVs was measured by assaying spectrophotometrically at 274 nm.

Osmotic behavior of LUV in hypertonic solution

To examine the effect of cholesterol on the permeability of drug out of liposome and to determine the drug release mechanism, the osmotic behavior of LUV in hypertonic dextrose solution was observed as follows; dextrose was added to liposomal suspension (LUV) to make osmotic pressure gradient across the liposomal membrane. The volume change of the liposome due to shrinkage in hypertonic dextrose solution was expressed as turbidity change of the suspension by determining absorbance at 450 nm. Then, the change of release rate from LUVs in hypertonic dextrose solution was measured.

RESULTS AND DISCUSSION

Drug encapsulation efficiency

Drug encapsulation efficiencies of MLV, SUV and LUV are compared in Table 1. The encapsulation efficiency of LUV prepared by reverse phase

Table 1. Comparison of drug encapsulation efficiency of various liposomes

Type of liposomes	Drug encapsulation efficiency
Multilamellar vesicle	2.8%
Small unilamellar vesicle	1.0%
Large unilamellar vesicle	11.0%

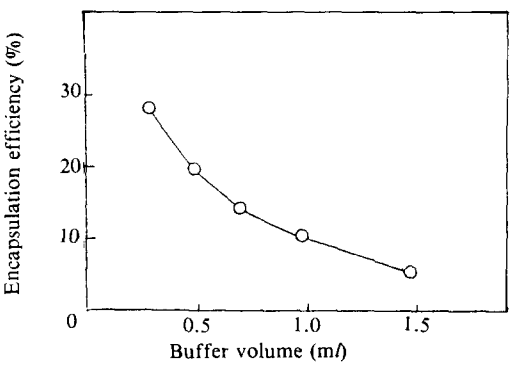

Fig. 1. Effect of buffer volume on the encapsulation efficiency of large unilamellar vesicles composed of lecithin and cholesterol (9:2 molar ratio).

evaporation method was shown to be the highest of all. The effect of buffer volume on the encapsulation efficiency of LUV was also examined. As the volume of buffer solution decreased, drug encapsulation efficiency of liposomes increased as shown in Figure 1. Since the entrapped volume of buffer solution could be assumed to be constant while the volume of buffer solution outside the liposome was reduced, the volume fraction of the buffer solution inside the liposomes might be increased as reduced amount of buffer solution introduced.

Stability of liposomes

Liposome has its own transition temperature (Tc) at which the liposomal membrane is so fluid that the entrapped drug molecules can be released easily. However, at temperature above the Tc, the release rate is expected to be proportional to temperature. The effect of temperature on the release rate of cytarabine is shown in Figure 2. As the temperature rose, the release rate of cytarabine increased. Thus, this indicates that liposomal products should be stored at lower temperature for the stability assurance of liposomes as a drug delivery system.

Cholesterol is known to stabilize liposomes by reducing the fluidity of liposomal membrane. LUVs with different cholesterol content were incubated at 35 °C. The resulting release rates of cytarabine were compared in Figure 3. This figure shows that as the cholesterol content increased, the release rate of cytarabine decreased. That is to say, cholesterol reduces the permeability of cytarabine across the liposomal membrane and stabilizes the liposomes as a reservoir of drug.

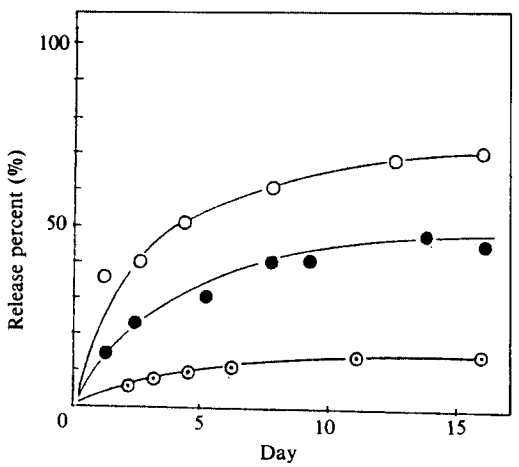

Fig. 2. Release profiles of cytarabine from large unilamellar vesicles at various temperature. lecithin: cholesterol = 9:2 molar ratio.
Key: ○, 38 °C; ●, 23 °C; ⊙, 4 °C.

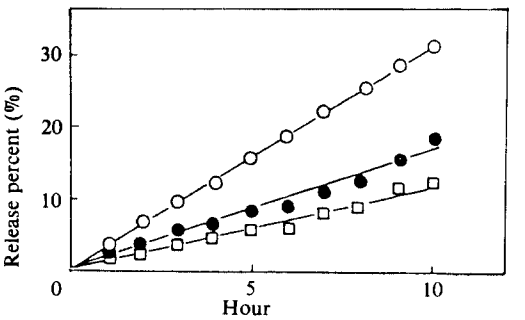

Fig. 3. Effect of cholesterol on the release rate of cytarabine from large unilamellar vesicles at 35 °C.
Key: ○, lecithin only; ●, lecithin : cholesterol = 9:2 molar ratio; □, lecithin : cholesterol = 9:8 molar ratio.

Osmotic behavior of LUV

The volume change of liposomes in hypertonic dextrose solution implies that the liposomal membrane is complete in structure as a semipermeable membrane. If water permeability across the liposomal membrane is reduced by cholesterol incorporation, the volume change due to osmotic pressure gradient may be smaller than that of liposomes composed of lecithin only. The effect of cholesterol on the osmotic behavior of liposomes (LUVs) is shown in Figure 4. Since the conditions of

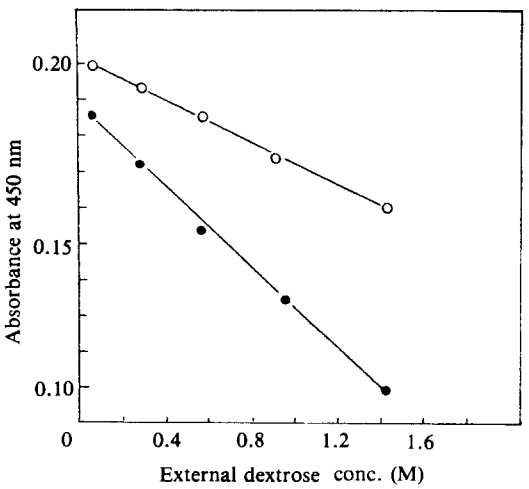

Fig. 4. Effect of cholesterol on the osmotic behavior of large unilamellar vesicles.
Key: ●, lecithin only; ○, lecithin : cholesterol = 9:2 molar ratio.

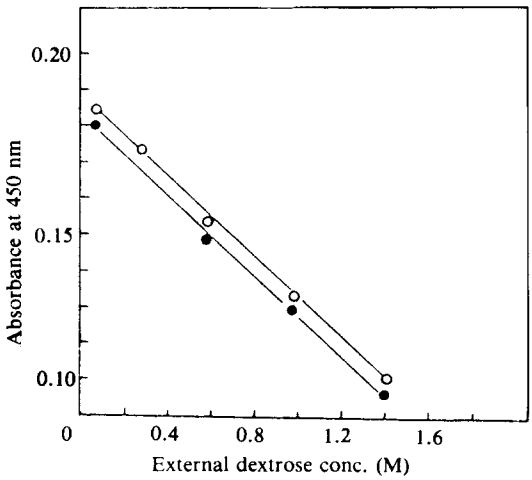

Fig. 5. Osmotic behavior of large unilamellar vesicles composed of lecithin only.
Key: ●, large unilamellar vesicles freshly prepared; ○, large unilamellar vesicles that have released about 50% of entrapped cytarabine at 35 °C.

preparation are identical, the liposomes composed of lecithin only can be thought to be as large as the liposomes composed of lecithin and cholesterol. The turbidity change of liposomal suspension due to volume change of liposomes was proportional to the osmotic pressure gradient as can be seen from the linearity of the lines in Figure 4. Comparing the slopes of the two lines indicates that the turbidity change due to volume change of liposomes composed of lecithin and cholesterol in hypertonic dextrose solution is smaller than that of liposomes composed of lecithin only. This implies that cholesterol reduced the water permeability across the liposomal membrane.

The osmotic behavior of LUVs which have released about 50% of cytarabine at 35 °C is shown in Figure 5 and 6. Figure 5 shows the osmotic behavior of LUVs composed of lecithin only. Figure 6 shows the osmotic behavior of LUVs composed of lecithin and cholesterol (9:2 molar ratio). Freshly prepared LUVs were thought to be so complete in structure as to respond to osmotic pressure gradient. Freshly prepared and aged vesicles were identical liposomes of the same batch except that aged vesicles had released about 50% of entrapped cytarabine. Since liposomes were aggregated during incubation, the initial turbidity (in other words, the highest turbidity point in these graphs) of liposomal suspension that have released about 50% of entrapped cytarabine was greater than that of liposomal

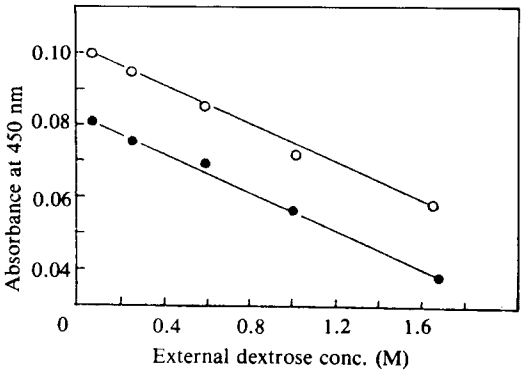

Fig. 6. Osmotic behavior of large unilamellar vesicles composed of lecithin and cholesterol (9:2 molar ratio).
Key: ●, large unilamellar vesicles freshly prepared; ○, large unilamellar vesicles that have released about 50% of entrapped cytarabine at 35 °C.

suspension freshly prepared. But the slopes of the two lines representing the turbidity changes due to volume changes are the same in two cases. These facts imply that even if 50% of entrapped cytarabine was released, the liposomes were as yet complete in structure as semipermeable membrane.

The entrapped cytarabine was released linearly from LUVs in isotonic and hypertonic dextrose solution(570 mM) as shown in Figure 7. Thus, it indicates that the release mechanism of cytarabine is simple diffusion across the liposomal membrane rather that liposomal rupture.

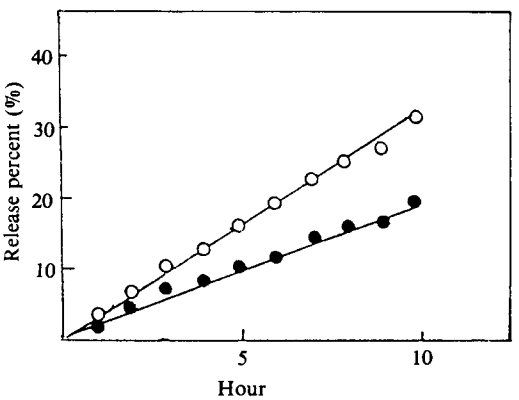

Fig. 7. Release of cytarabine from large unilamellar vesicles incubated in isotonic and hypertonic dextrose solutions, respectively.
Key: ○, isotonic solution; ●, hypertonic dextrose solution (570 mM).

To consider all these things, it may be deduced that entrapped cytarabine does not perturb the liposomal membrane, even if the composition of liposomal membrane is changed. In other words, the fact that release mechanism of cytarabine is simple diffusion insinuates that cytarabine does not damage the liposomal membrane.

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