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Effect of Bovine Serum Albumin on the Stability of Methotrexate-encapsulated Liposomes

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Abstract □ The effect of bovine serum albumin (BSA) on the encapsulation efficiency and stability of liposomes containing methotrexate (MTX) having different surface charges and cholesterol contents were investigated. The encapsulation efficiency of MTX was lower and the release of MTX was faster by the addition of BSA. The leaking of MTX from lipid bilayer depends upon the BSA concentrations. These results may be derived from the interaction of BSA with lipid bilayers. The dynamic structural changes of BSA were monitored indirectly using circular dichroism spectra. Observed dynamic structural changes of BSA with liposomes are presumed to reflect the interaction of BSA with liposomes. Negatively charged liposomes have more strong interaction with BSA than neutral and positively charged liposomes. BSA attacks lipid bilayers whether it is at the inner or at the outer phase of lipid bilayer and induces leakage of entrapped MTX. Especially, negatively charged liposomes are more sensitive than others. The inclusion of cholesterol in the lipid layers inhibits the interaction of BSA with liposomes and shows protective effect against BSA-induced leakage of MTX. To endure the attacking of BSA, liposomes as drug carriers should be made using cholesterol.

Keywords □ Liposomes, drug carrier, BSA, MTX, interaction, encapsulation efficiency, drug release, structural change, CD, charged liposomes.

Many attempts have been made to utilize liposomes as anticancer drug carriers since liposomes have been known to be nontoxic, biocompatible and biodegradable[1,2]. To develop liposomes as drug carriers, some problems such as encapsulation efficiency, release property of lipid bilayer, stability and target specificity must be considered. Large unilamellar vesicles (LUV) formed by reverse phase evaporation method has been known to have the highest encapsulation efficiency[3,4]. For liposomes to be carriers to deliver the entrapped drug molecules to specific target organs through the blood stream, they must retain their integrity until they reach the target tissues[5,6]. The stability of liposomes can be enhanced through cholesterol incorporation and phospholipid polymerization[7-9]. Phosphatidylcholine liposomes containing cholesterol and liposomes prepared with phospholipids having higher phase transition temperature than 37℃ might be expected to be stable in the plasma[10]. It was also reported that phosphatidylcholine liposomes containing unsaturated acyl chains released their entrapped materials in the presence of whole blood and serum albumin *in vitro*[11]. Thus the effects of lipid composition and blood constituents on the stability of liposomes under physiological conditions should be evaluated prior to develop a liposomal drug carrier.

In this paper, methotrexate or methotrexate with serum albumin was encapsulated in various phospholipid liposomes having different surface charges and cholesterol contents. Physical parameters such as drug encapsulation efficiency, stability of liposomes (index of leakage) in the presence of BSA, interaction of liposomes with serum albumin and

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timed-release properties of methotrexate from liposomes were studied.

EXPERIMENTAL

Chemicals and reagents

Egg phosphatidylcholine (PC), cholesterol (CH), dicetylphosphate (DCP), stearylamine (SA) and bovine serum albumin (BSA, fraction V) were purchased from Sigma Co. (St. Louis, MO., USA) and PC was purified by alumina column chromatography. Methotrexate (MTX) was kindly supplied from Choong-Wae Pharmaceutical company (Seoul, Korea). All other chemicals used were of reagent grade and used without further purification. Solutions were prepared in double distilled water.

Preparation of liposomes

Multilamellar vesicles (MLV): Lipid was dissolved in chloroform and the solvent was completely evaporated to make a thin film inside a glass tube. The thin lipid film was suspended in a buffer solution containing MTX by vortexing them for 5 min. Small unilamellar vesicles (SUV) were prepared by sonicating the MLV for 30 min with probe type ultrasonicator (MSE, MK2, Sussex, England).

Large unilamellar vesicles (LUV): LUV were prepared by the following method described in the previous report[12,13]. The prepared thin lipid film was dissolved in 3 m*l* of ether, and then 1 m*l* of aqueous phase containing 1 mg of MTX was added. This binary solution was vortexed for 2 min to make inverted micelles. Aqueous phase containing MTX was entrapped in the inverted micelles and then ether was evaporated by the rotary vacuum evaporator at 200 rpm until a homogeneous suspension was obtained. In the same way, LUV encapsulating MTX with BSA were made using solution of MTX with BSA instead of MTX only.

Determination of encapsulation efficiencies

After formation of the liposomes, they were sedimented through ultracentrifuging (MSE, Europa 65, Zürich, Switzerland) at 100,000 g for 30 min kept at 5 ℃ and washed 3 times with buffer solution containing 2 mM TES, 0.1 mM EDTA and an adequate amount of NaCl. The MTX concentrations in collected supernatant were determined using UV-spectrophotometer at 370 mm. The amount of MTX encapsulated in liposomes was determined by substracting the amount of MTX in supernatant separated from liposomes from the total amount of MTX added as follows.

$$\text{Encapsulation effeciency (\%)} = \frac{\text{Total MTX} - \text{supernatant MTX}}{\text{Total MTX}} \times 100$$

Stability of liposomes

To examine the effect of BSA on the permeability of liposomal membrane, the release of MTX from freshly prepared LUV was examined. Liposomes were suspended in 5 m*l* of buffer of pH 7.4 (containing 2 mM histidine, 2 m*l* TES, 0.1 mM EDTA and an adequate amount of NaCl) or BSA solution (0.5 mg/m*l* and 1.0 mg/m*l*). To determine the effect of BSA on the release of MTX, the amount of MTX released from the liposomes containing MTX with BSA was compared with those containing only MTX. The LUV was suspended in 5 m*l* of buffer solution. Three kinds of LUV, PC : CH: DCP (7 : 1 : 2 in molar ratio), PC : CH : SA (7 : 1 : 3 in molar ratio) and only PC were prepared to investigate the effects of surface charges on the stability of liposomes. These liposomes were suspended in BSA solution (0.5 mg/m*l*). To determine the effects of CH on the release of MTX, the molar ratio of PC to CH was adjusted to 7 : 3 and 9 : 1, respectively. Also the amount of MTX released from LUV in the presence of BSA (0.5 mg/m*l*) was determined. After various liposomes were suspended in PBS, the concentrations of liposomes were adjusted to be equivalent to 39 μM as lipid for all the release studies.

While liposomes were incubated in a water bath for 40 hr kept at 37 ℃, aliquots were removed at designed time intervals. After centrifugation, the clear supernatant was analyzed for the determination of released MTX using UV-spectrophotometer at 370 nm. Structural changes of BSA in liposome-suspended solution after the incubation of BSA with liposomes and the ellipticity of BSA with liposomes were monitored to examine the interaction of BSA with liposomes (*i.e.*, to examine the effect of BSA on the stability of liposomes). All circular dichroism spectra were obtained with Jasco Model J-200 spectropolarimeter (Tokyo, Japan). All BSA solutions dissolved in pH 7.4 buffer solution were scan-

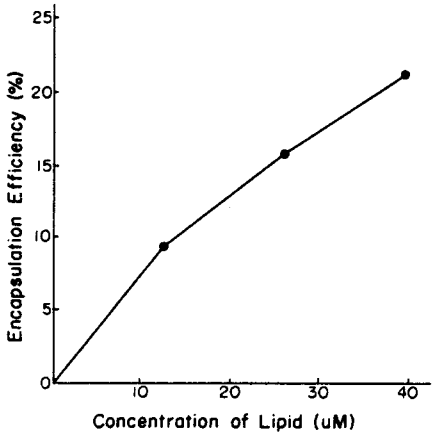

Fig. 1. Effect of lipid content on the encapsulation of methotrexate.
Each liposomes were prepared with various amounts of lipid and 1 m*l* of methotrexate solution (1 mg/m*l*). The amount of methotrexate was 1 mg and lipid content ranged from 13 to 39 μM.

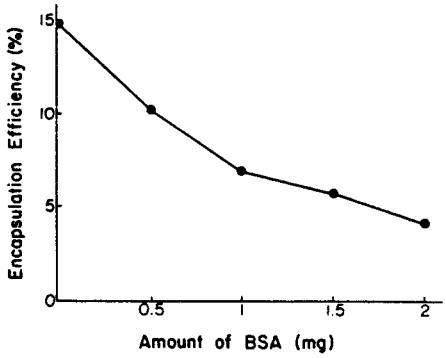

Fig. 2. Effect of BSA on the encapsulation of MTX.
Each lipc ome was prepared with 26 μM of lipid and 1 m*l* of MTX (1 mg) solution containing various amount of BSA (0.5, 1.0, 1.5, 2.0 mg).

ned in 10 mm cells from 220-260 nm at 25 ℃. The concentrations of BSA and lipid were 0.5 mg/m*l* and 1.5 μM/m*l*, respectively. Spectropolarimeter was adjusted as follows; (gas: N_2, flow rate: 4-5 kg/cm·min, chart speed: 2 cm/min, time constant: 4, wave expansion: 10 mm/cm, scale: 20 mdeg/cm, flow rate of cooling water for light source: 2 liter/min).

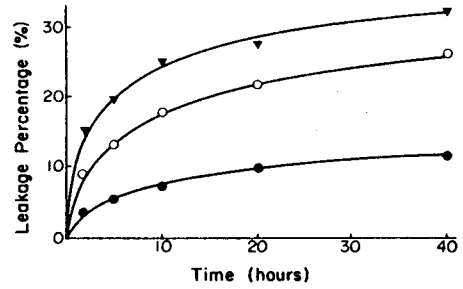

Fig. 3. Induced leakage of methotrexate from LUV composed of phosphatidylcholine (39 μM) at 37°C by BSA.
Key; (●) : LUV was suspendend in pH 7.4 buffer solution
(○) : LUV was suspended in BSA solution (0.5 mg/m*l*),
(▼) : LUV was suspended in BSA solution (1.0 mg/m*l*).

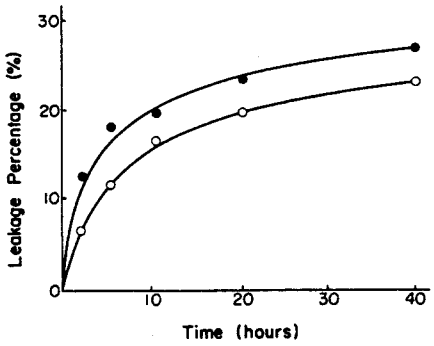

Fig. 4. Leakage of methotrexate from LUV composed of phosphatidylcholine (3 μM) at 37°C in pH 7.4 buffer solution.
Key; (○) : liposomes entrapping methotrexate only,
(●) : liposomes entrapping methotrexate with BSA.

RESULTS AND DISCUSSION

Encapsulation efficiency

The encapsulation efficiencies of MTX in liposomes such as MLV, SUV and LUV were 3.9, 2.1 and 14.6 %, respectively. The encapsulation efficiency was highest for LUV prepared by the reverse phase evaporation method. The encapsulation effi-

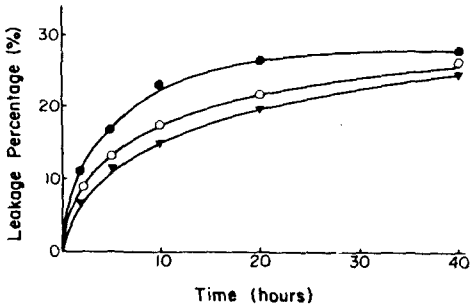

Fig. 5. Effect of surface charge on the leakage of methotrexate from LUV at 37 °C in the BSA solution (0.5 mg/ml). After the liposomes were suspended, concentration of liposomes was adjusted to be equivalent to 39 μM of lipid.

Key; (○) : neutral liposomes,
(●) : negatively charged liposomes,
(▼) : positively charged liposomes.

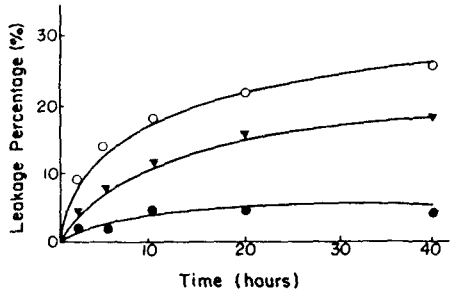

Fig. 6. Effect of cholesterol on the leaking of methotrexate from LUV at 37°C in the BSA solution (0.5 mg/ml). After the liposomes were suspended, concentration of liposomes was adjusted to be equivalent to 39 μM of lipid.

Key; (○) : liposomes made using only phosphatidylcholine,
(●) : liposomes made using phosphatidylcholine and cholesterol (7 : 3, molar ratio),
(▼) : liposomes made using phosphatidylcholine and cholesterol (9 : 1, molar ratio).

ciency was a function of lipid concentration. Fig. 1 shows that encapsulation of MTX increased as the lipid concentration increased. Thus, when we prepare liposomes, optimal concentration of lipid should be determined prior to the preparation.

Fig. 2 shows that the encapsulation of MTX with BSA was inversely proportional to the BSA concentration. This suggests that BSA shows inhibitory effect on the encapsulation of MTX. Since total inner aqueous volume of liposomes is constant and less amount of large molecules such as BSA are usually entrapped in liposome, MTX highly bound with bulky BSA was less entrapped than only MTX (usually 50 % of MTX is bound to the plasma protein). It is also possible that BSA molecules attacking the lipid bilayer result in inducing the leakage of MTX from the liposomes during the preparation.

Release of MTX from liposomes

The stability of liposomes can be monitored by measuring the release rate of the entrapped drug molecules. Fig. 3 shows that leakage of MTX from liposomes is dependent on the BSA concentration added to liposomal suspension. In buffer solution, only 10 % of MTX was released over a period of 40 hr. But in the presence of BSA (0.5 mg/ml) and BSA (1.0 mg/ml), 13 and 20% of MTX were leaked out within 5 hr, and 27 and 32 % of MTX were leaked out within 40 hr, respectively. As a result, when the liposomes are administered into the body, more drug suppose to be released than the amount calculated from *in vitro* test, since 4.4% of serum albumin is existed in the plasma. The leakages of MTX from liposomes containing only MTX and MTX with BSA are shown in Fig. 4. Because the release rate of MTX from liposomes containing MTX with BSA is faster than those containing only MTX, we can assume that BSA molecules have an interaction with lipid bilayers and the membrane is attacked and injured.

Fig. 5 shows that the leaking of MTX from negatively charged liposomes was slightly faster than from the positively charged or neutral ones. This may suggests that when the surface charge of the liposomes is negative, BSA attacks liposomes more strongly and thus induces the leaking of MTX. Managing the permeability of liposomes in the circulation of the injected animals or in the presence of serum is very important. Fig. 6 shows that cholesterol decreases the leakage of MTX by approximately 2-3 folds in the presence of BSA. It is probably due to its protective effect against BSA-induced leaking resulted from the tightening of the lipid bilayer as cholesterol contents increase[12]. These results show that stability and integrity of liposomes may be effectively controlled by adjusting their cholester-

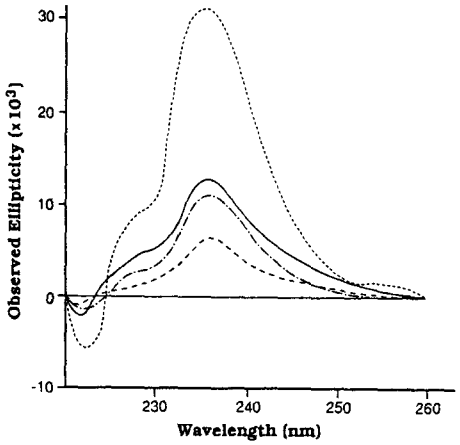

Fig. 7. Circular dichroism spectra of BSA in liposome suspended solution at 37°C.
Key; (- - -) : neutral liposomes,
(——): positively charged liposomes,
(—) : negatively charged liposomes,
(···) : BSA in buffer solution.

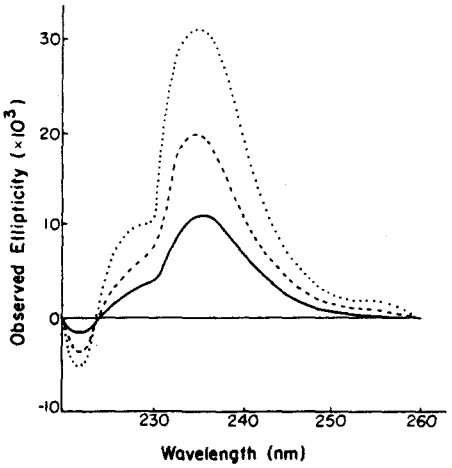

Fig. 8. Circular dichroism spectra of BSA in liposome suspended solution at 37°C.
Key; (——): liposomes made using only phosphatidylcholine,
(—) : liposomes made using phosphatidylcholine and cholesterol (7 : 3, molar ratio),
(···) : BSA in pH 7.4 buffer solution.

ol content.

Structural change of BSA in liposome suspended solution

Dynamic structural change of BSA with liposomes was monitored by circular dichroism spectra. Fig. 7 shows that the change of observed ellipticity of BSA with negatively charged liposomes was greatest of all formulations and that with neutral liposomes was greater than that with positively charged liposomes. It suggests that negatively charged liposomes have more strong interaction with BSA and is easily attacked by BSA molecules than neutral and positively charged liposomes. This result agrees well to the higher leakage of drug from negatively charged liposomes incubated with BSA solution than from the neutral or positively charged liposomes (Fig. 5). Fig. 8 shows that BSA interacts less with cholesterol containing liposomes than with liposomes made using only lecithin. We might expect that the interaction between BSA and liposome is decreased as the contents of cholesterol in liposomal membrane increase.

CONCLUSION

In conclusion, liposomes interact with BSA and change the dynamic structure of BSA. Particularly, negatively charged liposomes have more strong interaction with BSA than neutral and positively charged liposomes. BSA attacks lipid bilayers whether it is at the inner or at the outer phase of lipid bilayer and induces leakage of entrapped materials. Especially, negatively charged liposomes are more sensitive than others. In addition, we confirm that the inclusion of cholesterol in the lipid bilayers inhibits the interaction of BSA with liposomes and also shows protective effect against BSA-induced leakage of MTX. The stability and integrity of liposomes may be controlled by adjusting the cholesterol content.

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