DRUG DEVELOPMENT AND INDUSTRIAL PHARMACY, 20(8), 1411-1423 (1994)

INCREASE OF LIPOSOME STABILITY BY INCORPORATION OF BOVINE SERUM ALBUMIN

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ABSTRACT

The incorporation of the bovine serum albumin in the liposomal membrane to prevent leakage of the entrapped contents was studied. Adsorption of bovine serum albumin was found on the liposomes. After the adsorption of bovine serum albumin, by the addition of glutaraldehyde, the serum albumin molecules cross-linked to themselves and stiffened the membrane structure of the liposomes. It was found that the particle size of the liposomes increased after the treatment of glutaraldehyde. However, there was no significant change in particle size for the liposomes with the stirring effect. Adriamycin, methotrexate, carboxyfluorescein and mitoxantrone were entrapped into this liposome system. The loading efficiency demonstrated a similar value for the liposomes with and without the treatment of glutaraldehyde. However, for the entrapment of

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Presented in part at the Seventh Annual Meeting of American Association of Pharmaceutical Scientists held in San Antonio, TX, November 1992.

adriamycin, there showed an increase in loading efficiency with the effect of glutaraldehyde. For the leakage examination, a decrease of the release rate of the adriamycin, methotrexate and carboxyfluorescein from the glutaraldehyde treated liposomes was demonstrated; whereas, the prevention of leakage for the mitoxantrone was not significant.

INTRODUCTION

Stability of liposomes concerning leakage of the entrapped contents to the enviroment is one of the problems challenged the liposome formulator. The major reason for leakage is due to the characteristics of fluidity of the liposomal membrane. Since the phospholipid bilayer structure of liposome is fluid-like and mobile, it is easy for the entrapped solutes to transport across the liposomal membrane, especially under the harsh condition of the biological enviroment. Therefore, methods to stiffen the bilayer structure or to reduce the mobility of the membrane have been introduced to prevent leakage. This can be modified by incorporation of cholesterol into the bilayers, or by polymerization of the phospholipid molecules (1-3). Another reasons for the leakage of the liposomes are due to the tightness of the interaction of the entrapped materials to the phospholipid bilayers, and the size of the entrapped molecules related to their penetrability to the bilayer membrane (4).

The interaction of the serum albumin with the liposomal membrane demonstrated an increase in permeability of the liposomes (5). Also, the serum albumin molecules were found to adsorb and coat on the liposomes (6, 7).

In the present study, an attempt was made to develop a liposome system with less leakage by incorporation of bovine serum albumin into the phospholipid bilayers and with the treatment of glutaraldehyde, to stiffen the liposomal

membrane. Entrapment of drugs of adriamycin, methotrexate, carboxyfluorescein and mitoxantrone was introduced into this system to investigate their stability.

MATERIALS AND METHODS

Materials

Crystallized and lyophilized bovine serum albumin (essentially fatty acid and globulin free), phosphatidylcholine (type XI-E: from fresh egg yolk), dioleoyl phosphatidylcholine, glutaraldehyde (grade I: 25% aqueous solution) and carboxyfluorescein were purchased from Sigma Chemical Co (St. Louis, MO). Adriamycin was obtained from Farmatalia, Carlo Erba (Italy). Methotrexate was purchased from Lederle Laboratories (American Cyanamid Co., Pearl River, NY). Mitoxantrone was supplied by Kingdom Pharmaceutical Co. (R.O.C.). General chemicals were of analytical grade.

Preparation of Liposomes

Phosphatidylcholine or dioleoyl phosphatidylcholine was dissolved in chloroform and dried to a thin flim on the wall of a round-bottom flask under reduced pressure at 37°C in a rotary evaporator. Buffers or drug solutions were added to the flim, and liposomes were formed by constant vortexing (Maxi Mix II; Thermolyne) for 5 min and sonicating (Bransonic 220; Branson) for 1 min. The lipid concentration of the liposome was 2 mg/ml. The liposome dispersion was hydrated at room temperature for 2 hr.

Adsorption of Bovine Serum Albumin on Liposomes

5 ml of 0.1% bovine serum albumin solution was incubated with 4 ml of liposome dispersion and equilibrated at room temperature with mild shaking for

90 min. Previous analytical procedures of Lowry method and microelectrophoretic technique were used to examine the adsorption of bovine serum albumin on liposomes (6, 7).

Preparation of Liposomes Incorporated with Bovine Serum Albumin

After the adsorption equilibrium, 1 ml of 2.5% glutaraldehyde was added to the bovine serum albumin-liposome mixture dropwise with constant stirring at 1,000 rpm for 15 min and standing for 45 min. In some cases, constant stirring was not applied, but only vortexing was given during the addition of the glutaraldehyde, and the standing time was 1 hr. The dispersion was centrifuged at $2.8 \times 10^5 \times g$ for 20 min and washed with buffer for 4 times. The resultant liposome dispersion was reconstituted to the required concentration for use.

Loading Efficiency Measurement

The entrapped drug concentrations were determined by lysis of the liposomes with absolute alcohol. One volume of liposomes was mixed well with 3 volumes of absolute alcohol and sonicated for 3 min to obtain a clear solution for drug concentration measurement. No interference by the absolute alcohol and the bovine serum albumin was found with the analysis. The entrapped drug concentration in the liposomes was expressed as loading efficiency in umole drug per mmole phospholipid.

Leakage Measurement

Adriamycin, methotrexate, carboxyfluorescein and mitoxantrone were entrapped in the liposomes for leakage measurement. The liposomes were sealed in the glass test tubes and incubated at 37°C. At intervals, the samples were

taken, centrifuged at 2.8 X 10^5 X g for 20 min, and the supernatants were analyzed for drug concentration. The medium for the liposome preparation and for the drug leakage study was water for adriamycin, pH 7.4 phosphate buffer for methotrexate and carboxyfluorescein, and 0.9% sodium chloride solution for mitoxantrone.

Drug Concentration Analysis

Fluorimetric method was made for the determination of the concentration of adriamycin with an excitation maximum at 470 nm and an emission maximum at 550 nm, and carboxyfluorescein with an excitation maximum at 494 nm and an emission maximum at 515 nm. Spectrophotometric method was used to measure the concentration of methotrexate and mitoxantrone at the wavelenght of 303 and 242 nm respectively.

Particle Size Analysis

The particle size of the liposomes was estimated by a Laser Particle Analyzer system (LPA-3000, Photal, Otsuka Electronics, Japan).

RESULTS AND DISCUSSION

The result of the incubation of bovine serum albumin with liposomes demonstrated a profile similar to that from the previous study (6, 7). This showed that the properties of the bovine serum albumin were imposed on the liposomes which confirmed the adsorption of the bovine serum albumin on the liposomes. The adsorption was attributed to the hydrophobic effect between the serum albumin molecules and the phospholipid bilayers of the liposomes. It was suggested that the bovine serum albumin molecules may penetrate or anchor into

the phospholipid bilayers, and the nonpenetrated moiety protrudes outside the phospholipid bilayer and coats on the liposomes (6, 7). Therefore, the introduction of the glutaraldehyde into the bovine serum albumin-liposome mixture was used to fix the adsorbed serum albumin molecules. Since the serum albumin molecules can cross-link to themselves and stiffen the membrane structure of the liposomes, it is expected to reduce the fluidity of the liposomal membrane. Consequently, the leakage of the entrapped contents of the liposomes can be reduced.

Although the presence of serum albumin can induce the permeability of liposomes (5), in this system, the liposomes were prepared and the adsorbed bovine serum albumin was fixed in the enviroment of the drug solution. Therefore, the effect of the induction of the permeability of the entrapped drugs by the bovine serum albumin can be minimized.

Table 1 shows the results of the change of the particle size of the liposomes with adsorbed bovine serum albumin after the treatment with glutaraldehyde and without glutaraldehyde. Two methods were involved in the preparation of liposomes i.e. with and without stirring after the addition of the glutaraldehyde. It is clear that under the stirring condition, empty liposomes or liposomes entrapped with adriamycin and carboxyfluorescien demonstrated no significant change in particle size in the presence and absence of glutaraldehyde; whereas, mitoxantone loaded liposomes showed a small increase in particle size. However, the liposomes prepared without stirring after the treatment of glutaraldehyde showed a dramatic increase in particle size. This may be due to the cross-linking effect of the adsorbed serum albumin molecules in between the surfaces of the liposomes or the free serum albumin bridged between the adsorbed serum

TABLE 1

Particle size of liposomes adsorbed with bovine serum albumin after treatment with and without glutaraldehyde.

Liposome Entrapped with Drug	Particle Size (μm)		
	Without Glutaraldehyde	With Glutaraldehyde	
		With Stirring	Without Stirring
Empty	1.1±0.7	1.0±0.5	11.8±15.8
Adriamycin	8.4±1.7	10.9±1.2	–
Mitoxantrone	6.4±0.1	10.1±1.6	46.7±8.7
Carboxyfluorescein	4.5±3.1	5.1±5.4	–

albumin molecules on the surfaces of the liposomes. The effect of stirring during the process was to reduce the contact of the particles for bridging and prevent precipitation of the liposomes to form aggregates.

Table 2 shows the loading efficiency of adriamycin, methotrexate, carboxyfluorescein and mitoxantrone on the glutaraldehyde treated and untreated liposomes. It is apparent that the loading efficiency of the methotrexate, carboxyfluorescein and mitoxantrone on liposomes was not affected by the treatment of the glutaraldehyde. In other words, the loading efficiency demonstrated a similar value for the liposomes with and without the cross-linked serum albumin. However, for the loading efficiency of adriamycin on the glutaraldehyde treated liposomes, there resulted in a loading concentration of

TABLE 2

Loading efficiency of liposomes adsorbed with bovine serum albumin after treatment with and without glutaraldehyde.

Liposome Entrapped With Drug	Loading Efficiency (umole drug/mmole phospholipid)	
	Without Glutaraldehyde	With Glutaraldehyde
Adriamycin	63	360
Methotrexate	134	139
Carboxyfluorescein	0.070	0.074
Mitoxantrone	87	84

360 mmole adriamycin/mmole phospholipid; whereas, the loading concentration for the liposomes treated without the glutaraldehyde was 63 umole adriamycin/mmole phospholipid. A 5.7-fold increase in the loading efficiency for the liposomes with the cross-linked serum albumin was shown. Since the adriamycin contains an amino group on the daunosamine portion of the molecule, there may be a reaction on the glutaraldehyde cross-linking between the amino groups of the adriamycin and the serum albumin (9). As a result, more adriamycin molecules will couple to the serum albumin molecules leading to an increase of loading efficiency. There are also amino groups on the methotrexate or mitoxantrone molecules. It is likely that these amino groups were not reactive as that of the adriamycin (10). Therefore, little effect on the increase of loading efficiency was found for the methotrexate and mitoxantrone entrapped into the liposomes.

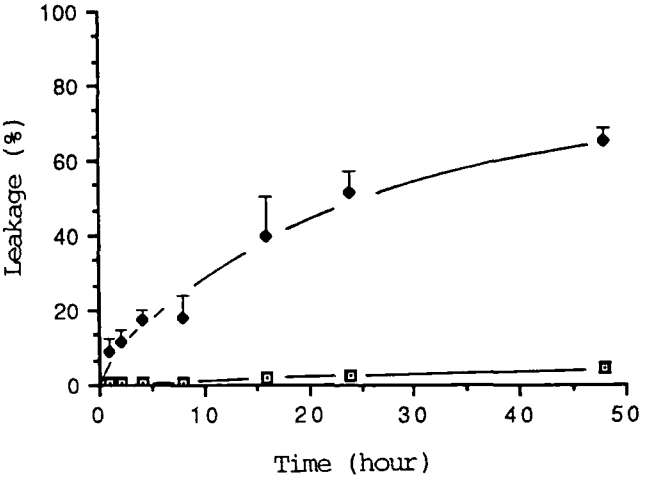

FIGURE 1

Leakage of adriamycin from liposomes adsorbed with bovine serum albumin after treatment with (□) and without (◆) glutaraldehyde.

This liposome system entrapped with adriamycin, carboxyfluorescein, methotrexate and mitoxantrone was compared to the liposome system entrapped with the same drugs and the adsorbed serum albumin but without the treatment of glutaraldehyde for cross-linking of the serum albumin, to see their leakage characteristics. 65% of the entrapped adriamycin was released from the liposomes without the treatment of glutaraldehyde after 48 hr of incubation; whereas, 5% was released from the glutaraldehyde treated liposomes (FIGURE 1). The methotrexate-containing liposomes released about 13% of its content for treatment without glutaraldehyde, and 6% in the case with the treament of glutaraldehyde after 48 hr (FIGURE 2). The carboxyfluorescein-containing liposomes released about 60% of the entrapped carboxyfluorescein for treatment without glutaraldehyde, and 39 % for the liposomes with the treatment of glutaraldehyde after 48 hr (FIGURE 3). It is clear that the cross-linked serum

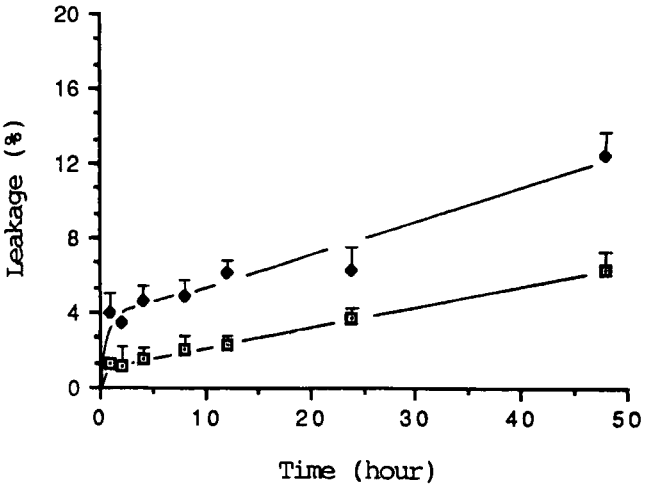

FIGURE 2

Leakage of methotrexate from liposomes adsorbed with bovine serum albumin after treatment with (□) and without (◆) glutaraldehyde.

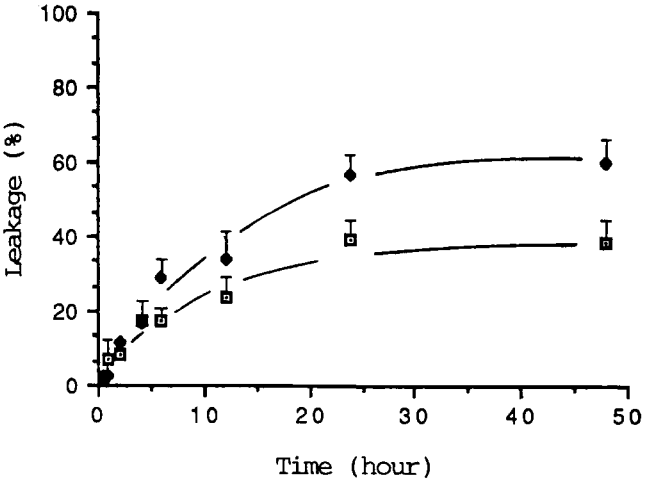

FIGURE 3

Leakage of carboxyfluorescein from liposomes adsorbed with bovine serum albumin after treatment with (□) and without (◆) glutaraldehyde.

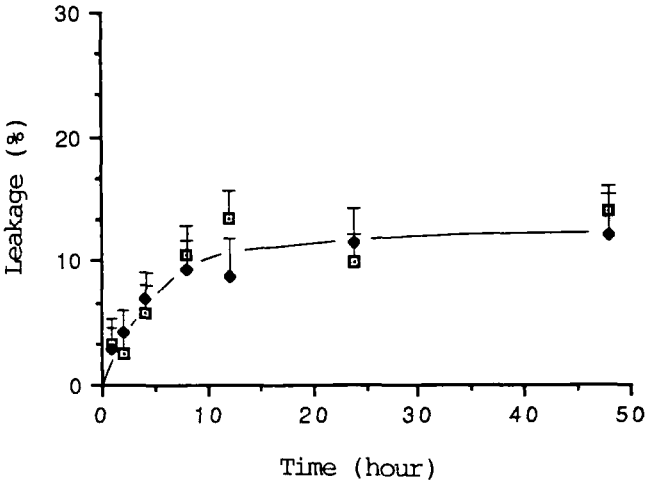

FIGURE 4

Leakage of mitoxantrone from liposomes adsorbed with bovine serum albumin after treatment with (□) and without (◆) glutaraldehyde.

albumin liposomes containing adriamycin, methotrexate and carboxyfluorescein showed a 13, 2 and 1.5-fold decrease in leakage respectively, when compared to those results obtained from the liposome system without the cross-linked serum albumin. Due to the binding of the adriamycin to the bovine serum albumin as mentioned above, this may also play a role in the decrease of the leakage of the adriamycin from the liposomes. The results of the leakage of the mitoxantrone from the liposomes with cross-linked serum albumin and without cross-linked serum albumin are shown in FIGURE 4. This indicated that the prevention of the leakage of the mitoxantrone from the serum albumin cross-linked liposome system was not significant. Since most of the mitoxantrone molecules bind tightly into the phospholipid bilayers of the liposomes, only a few mitoxantrone molecules are entrapped into the aqueous compartment (8). The bound mitoxantrone in the phospholipid bilayers dominates the leakage characteristics

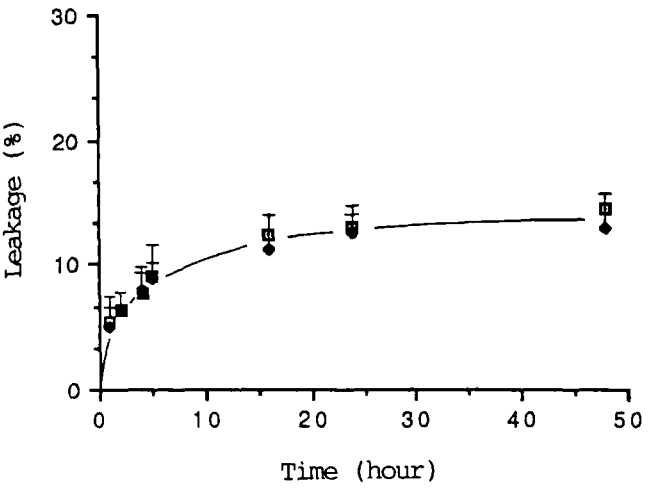

FIGURE 5

Leakage of mitoxantrone from dioleoyl phosphatidylcholine liposomes adsorbed with bovine serum albumin after treatment with (□) and without (◆) glutaraldehyde.

of the mitoxantrone from the liposomes. Although the cross-linked serum albumin coats on the liposomes, it is likely that there is little effect on the rate of the release of the bound mitoxantrone. The effect of dioleoyl phosphatidylcholine on the leakage of mitoxantrone from the glutaraldehyde treated and untreated liposomes is shown in FIGURE 5. This demonstrated no difference between the leakage characteristics of the glutaraldehyde treated and the untreated liposomes. Also, the release result of the mitoxantrone from the dioleoyl phosphatidylcholine liposomes showed a profile similar to that of the phosphatidylcholine liposomes.

ACKNOWLEDGMENTS

This study was supported by the National Science Council R.O.C. (NSC 81-0412-B-075-03 and NSC 79-0204-B075-03)

REFERENCES

1. C. Kirby, J. Clark and G. Gregoriadis, Biochem. J., 186, 591 (1980).
2. G. Scherphof, J. Damen and D. Hoekstra, in "Liposomes: From physical structure to therapeutic applications," C.G. Knight, eds., Elsevier/North-Holland Biomedical Press, Amsterdam, 1981, p. 310.
3. D.S. Johnston and D. Chapman, in "Liposome technology, Volume I, Preparation of liposomes," G. Gregoriadis, eds., CRC Press, Inc., Florida, 1984, p. 123.
4. S.L. Law, W.Y. Lo and G.W. Teh, Drug Devel. Ind. Pharm., 14, 143 (1988).
5. H.K. Kimelberg, Mol. Cell. Biochem., 10, 171 (1976).
6. S.L. Law, W.Y. Lo, S.H. Pai, G.W. Teh and F.Y. Kou, Intern. J. Pharm., 32, 237 (1986).
7. S.L. Law, W.Y. Lo, S.H. Pai and G.W. Teh, Intern. J. Pharm., 43, 257 (1988).
8. S.L. Law, P. Chang and C.H. Lin, Intern. J. Pharm., 70, 1 (1991).
9. E. Hurwitz, R. Levy, R. Maron, M. Wilchek, R. Arnon and M. Sela, Cancer Res., 35, 1175 (1975).
10. D.A. Robinson, J.M. Whiteley and N.G.L. Harding, Biochem. Soc. Trans., 1, 722 (1973).