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Increased circulatory half-life of liposomes after conjunction with dextran

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Abstract. Dextran was covalently coupled to neutral unilamellar liposomes. Dextran conjugated liposomes were cleared from the circulation at a much slower rate than unconjugated liposomes. The uptake of dextran conjugated liposomes by liver and spleen was also decreased. The amount of dextran on the surface of liposomes was found to be a determining factor for their stability in circulation. Dextran conjugated liposomes therefore may be a more effective way of controlled drug release.

Keywords. Unilamellar liposomes; dextran conjugate; drug delivery.

Introduction

Liposomes are becoming increasingly important as carriers of biologically important molecules in living system (Tyrell *et al*., 1976; Papahadjopoulos, 1978; Gregoriadis, 1980; Roerdink *et al*., 1981). Introduction of a specific sugar onto the liposomal surface either by specific glycolipids or by coupling *p*-aminophenyl glycosides helps in directing the liposome towards different cell types of liver (Ghosh and Bachhawat, 1980; Ghosh *et al*., 1981a, 1982). Glycoside-bearing liposomes have been used to deliver therapeutic substances in galactosamine induced hepatitis (Ghosh *et al*., 1981b). Although drugs in general usually must quantitatively arrive at their target, drug-bearing liposomes, however, which have extremely long half-lives in the circulation could enhance the treatment of some of the diseases involving lack of serum factors or can serve as intravascular sustained release of antitumor drugs and other agents. Dextran has long been used as drug carrier to confer greater chemical and biological stability to dextran-drug complex (Molteni, 1979). Dextran remains in the blood stream for periods of time proportional to its molecular weight. In this paper we have conjugated dextran with

Abbreviations used: Chol, Chlesterol; EL., egg lecithin; PE, phosphatidylethanolamine; ConA, concanavalin A

liposomes and have shown that such dextran conjugated liposomes can serve the dual purpose of extending the duration of drugs in the circulation considerably and of protecting the drug from the hostile environment.

Materials and methods

Chemicals

Egg lecithin, cholesterol and phosphatidylethanolamine were obtained from CSIR Centre for Biochemicals, New Delhi. Sepharose-6B, bovine γ-globulin and dextran of mol. wt. 70,000 were purchased from Sigma Chemical Company, St. Louis, Missouri, USA. Carrier-free Na [^{125}I] was obtained from BARC, Bombay. Concanavalin A (Con A) was prepared according to the method of Surolia *et al*. (1973). All other chemicals used were of analytical grade.

Radioiodination of γ-globulin

Radioiodination of γ-globulin was done by the Chloramine-T method of Hunter (1978) using carrier-free Na [^{125}I] . The iodinated protein was separated from free iodide by gel filtration on Sephadex G-25. The specific activity of iodinated protein was $2{\cdot}2 \times 10^6$ cpm/μg.

Preparation of liposome

[^{125}I]-Labelled γ-globulin entrapped unilamellar liposomes were prepared with egg lecithin (EL), cholesterol (Chol) and phosphatidylethanolamine (PE) as described earlier (Surolia *et al*., 1975).

Covalent coupling of dextran to PE-liposome

Covalent coupling of dextran to the available amino groups on PE-liposome was done by a modification of the method described for dextran-protein conjugates (Marshall and Rabinowitz, 1976). Cyanogen bromide (30–35 mg) was added to a stirred solution of dextran (250 mg) in 5 ml of distilled water, adjusted to pH 10·7 with 1 M NaOH, followed by a second addition of cyanogen bromide (30–35 mg), 30 min later. The pH was maintained at 10·7 by 1 M NaOH throughout the activation. Thirty minutes after the second addition, cyanogen bromide (20–25 mg) was again added and allowed to react for another 20 min at pH 10·7. The reaction mixture was then dialysed at 4°C for 1 h against 0·15 M NaCl, pH 9·0, adjusted with Na_2CO_3. The dialysate was added to 3–4 ml of liposome preparation containing, entrapped [^{125}I]-γ-globulin and the mixture was stirred overnight at 4°C. The excess unreacted dextran was blocked by the addition of 0·2 ml of dilute ethanolamine (1:25). After stirring for another 2 h, the conjugate was purified by gel filtration on Sepharose-6B.

Animal experiments

Male Swiss albino mice (IICB strain) weighing approx. 25–30 g were used throughout the experiment. Each pentobarbital anaesthesized mouse received a single intravenous

injection of 0·25 ml liposome suspension (1–1·5 mg lipids) containing 3–6 × 10^4cpm entrapped [^{125}I]-γ-globulin. After time intervals of 2·5, 5, 15, 30, 60, and 120 min, samples of blood were withdrawn from the femoral artery of groups of three mice. Subsequently they were sacrificed and their livers, kidneys, spleens and lungs were removed. Each tissue was washed with saline and blotted with filter paper. The whole liver was digested with 5 ml of 30 % KOH solution and 2 ml of the liver-digest was taken for radio-active counting. Other tissues were digested in 1 ml of 30% KOH solution and counted. Radioactivity was measured in a Prias Scintillation gamma counter.

Results and discussions

We have investigated the effect of dextran conjugation to small unilamellar liposomes on the rate of clearance of their entrapped solute contents from the blood and also the uptake by different tissues of the injected animals. [^{125}I]-γ-Globulin was taken as the entrapped marker.

Dextran-liposome conjugate

The dextran liposome conjugate was freed from any unreacted dextran by gel filtration on Sepharose-6B and subsequently characterized by its agglutination by Con A. Different amounts of PE (10, 18 and 30 mol%) were taken for liposome preparation and were conjugated with the same amount of dextran (250 mg). When all types of conjugates were separately agglutinated by Con A followed by centrifugation, the radioactivity present in the supernatant fractions was negligible showing thereby that the conjugate preparation does not contain any unreacted liposome. This can be explained due to the use of excess dextran over the available amino groups on the PE liposome. However, as expected the conjugate preparations differ significantly in their dextran content and this was monitored by the per cent of total amino groups available even after the coupling, as estimated by the titrations of the liposomal PE amino groups with trinitrobenzesulphonic acid in the presence of 0·1% Triton X-100 (Torchillin *et al*., 1978). In addition, when the purified liposome-dextran conjugate in 6 M urea was rechromatographed on Sepharose-6B to remove contaminated noncovalent material, if any, no peak other than the conjugate was obtained. Therefore, the presence of any non-specifically adsorbed dextran on liposomal surface is quite unlikely.

Blood clearance and tissue uptake studies

Figure 1 shows the rates of clearance of entrapped protein from the circulation of mice. Dextran conjugated PE-liposomes were found to be much more efficient in retaining the radioactivity in blood stream compared to control liposomes (PE-liposomes). About 80% of the control liposomes are removed from the circulation after 30 min of injection, whereas only 50% of the injected dextran-liposomes are cleared during this period. The difference in the retention in circulation of dextran-liposome is found to be 2-fold higher even after 2h of intravenous injection (18% and 8% respectively).

In another control experiment, mice were injected with 0·25 ml of a mixture of

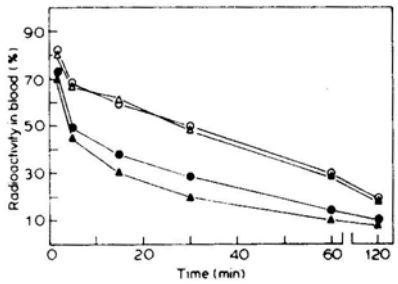

Figure 1. Clearance of different types of liposomes from the circulation of mice. Mice were injected with liposomes containing [^{125}I]-γ-globulin (3–6 × 10^4 cpm). Radioactivity was measured in the blood, the volume of which in all the experiments described here was taken as 2·0ml per 25 g body wt. and was expressed as a percentage of the injected radioactivity per total volume of blood. Each point is an average value obtained from three mice. (▲), Liposome-PE (control); (●), Liposome-PE (10 mol%)-dextran; (○), liposome-PE (18 mol%)-dextran; (Δ), liposome-PE (30 mol%)-dextran.

250 mg of dextran (uncoupled) and radio-labelled PE-liposomes (3 ml) and no significant effect was observed on the clearance of the latter as compared with PE-liposomes alone (data not shown).

Since the amount of dextran on the surface of liposome may be a determining factor for the retention in circulation, varying amounts of PE were taken for the preparation of dextran-liposome conjugates. The extent of dextran coupling is proportional to the number of free amino groups of PE present on the outer surface of liposomes. Thus an increase in the mol % of PE will reflect a corresponding increase in the amount of dextran molecules coupled with the PE liposomes. It was observed that the rate of disappearance of the conjugated liposome from the circulation decreases as the amount of PE in the liposome increases up to a critical concentration of 18 mol%, above which the rate of clearance remains unaltered. Therefore, this could be due to saturation of dextran binding sites on the surface of liposomes, resulting from a steric hindrance caused by the bound dextran molecules.

Differences in the survival of liposomal encapsulated marker in the circulation caused by coupling with dextran should reflect in the extent of uptake by liver and spleen. Tissue distribution of liposome entrapped γ-globulin at different time intervals is presented in table 1. The amount of radioactive marker taken up by the liver and spleen is greater for control liposomes than for dextran liposomes. In both the cases, the maximum uptake by the liver occurred 15 min after the administration of liposomes, after which there was a gradual decrease in the amount of radioactivity in the liver. This may be due to the possible lysosomal degradation of the protein marker in the liver.

Presumably dextran does not cross the cell membrane (Molteni, 1979) which could explain why proteins or haptens coupled to dextran remain in circulation for longer times. Though there are reports (Richter and Hedin, 1982) of dextran hypersensitivity on protracted use in a few patients, the use of clinical grade dextran may considerably reduce the chances of such toxicity in most of the individuals. A sensitivity test for dextran may be performed to avoid any possible side effect *in vivo*. However, the

Table 1. Distribution of [^{125}I]-γ-globulin in mice tissues at different time intervals after injection of liposome entrapped [^{125}I]-γ-globulin.

Liposomal lipid composition	Molar ratio of lipids	Time interval (min)	Liver	Kidney	Spleen	Lung
EL/Chol/PE	7:2:2	2·5	21·1 ± 2·1	0·8 ± 0·1	1·1 ± 0·1	1·1 ± 0·3
		5	30·8 ± 3·1	0·9 ± 0·2	1·2 ± 0·2	0·9 ± 0·3
		15	36·9 ± 2·5	1·2 ± 0·2	2·8 ± 0·4	0·8 ± 0·2
		30	21·0 ± 1·6	1·1 ± 0·1	3·1 ± 0·5	0·6 ± 0·1
		60	11·3 ± 1·2	0·9 ± 0·3	1·6 ± 0·2	0·5 ± 0·1
		120	4·7 ± 0·9	0·6 ± 0·1	0·9 ± 0·1	0·3 ± 0·1
EL/Chol/PE-dextran	7:2:1	2·5	18·3 ± 1·9	0·9 ± 0·2	0·9 ± 0·3	1·2 ± 0·4
		5	26·1 ± 1·7	1·1 ± 0·1	1·2 ± 0·2	1·1 ± 0·3
		15	30·8 ± 2·2	1·3 ± 0·3	2·3 ± 0·4	0·8 ± 0·2
		30	16·9 ± 0·9	1·4 ± 0·2	2·6 ± 0·4	0·8 ± 0·2
		60	9·2 ± 1·1	1·2 ± 0·3	1·8 ± 0·3	0·7 ± 0·1
		120	3·9 ± 0·8	0·9 ± 0·4	1·1 ± 0·1	0·5 ± 0·1
EL/Chol/PE-dextran	7:2:2	2·5	11·3 ± 1·1	0·8 ± 0·1	0·7 ± 0·2	1·3 ± 0·3
		5	15·6 ± 1·4	0·9 ± 0·2	1·0 ± 0·2	1·4 ± 0·4
		15	20·1 ± 2·1	1·1 ± 0·1	2·0 ± 0·4	1·2 ± 0·1
		30	13·2 ± 1·3	1·6 ± 0·2	1·8 ± 0·6	1·0 ± 1·0
		60	8·3 ± 1·0	0·9 ± 0·4	1·3 ± 0·3	0·7 ± 0·2
		120	3·8 ± 0·7	0·8 ± 0·3	1·0 ± 0·1	0·5 ± 0·2
EL/Chol/PE-dextran	7:2:4	2·5	13·5 ± 1·5	0·9 ± 0·2	0·9 ± 0·2	1·3 ± 0·1
		5	17·6 ± 2·1	1·0 ± 0·1	1·1 ± 0·2	1·2 ± 0·2
		15	22·7 ± 2·2	1·3 ± 0·3	2·1 ± 0·4	0·8 ± 0·2
		30	14·9 ± 1·3	2·0 ± 0·1	1·8 ± 0·6	0·7 ± 0·1
		60	10·8 ± 1·4	1·3 ± 0·2	1·6 ± 0·4	0·5 ± 0·2
		120	5·8 ± 1·1	1·0 ± 0·1	1·2 ± 0·2	0·5 ± 0·1

Values are mean per cent of injected [^{125}I]-γ-globulin ± S.D. Each group contained three mice.

optimal molecular size of coupled dextran and its role in diminishing the liposomal disappearance from blood remain to be determined.

Regardless of the mechanism by which dextran promotes the stability of liposome *in vivo*, dextran conjugated liposome should provide an effective means for controlled drug release over longer periods. Such controlled release may be of particular importance in diseases where the slow release of a drug is essential.

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References

Ghosh, P. and Bachhawat, B. K. (1980) *Biochim. Biophys. Acta*, **632,** 562.
Ghosh, P., Bachhawat, B. K. and Surolia, A. (1981a) *Arch. Biochem. Biophys*., **206,** 454.
Ghosh, P., Das, P. K. and Bachhawat, B. K. (1981b) *Biochem. Soc. Trans*., **9,** 512.
Ghosh, P., Das, P. K. and Bachhawat, B. K. (1982) *Arch. Biochem. Biophys*., **213,** 266.
Gregoriadis, G. (1980) *Nature (London)*, **283,** 814.
Hunter, W. M. (1978) in *Handbook of Experimental Immunology*, 3rd edn (ed. D. M. Weir) (Oxford: Blackwell Scientific Publications) p. 14.1.
Marshall, J. J. and Rabinowitz, M. L. (1976) *J. Biol. Chem*., **251,** 1081.
Molteni, L. (1979) in *Drug Carriers in Biology and Medicine* (ed. G. Gregoriadis) (New York: Academic Press) p. 107.
Papahadjopoulos, D. ed. (1978) *Annal. N.Y. Acad. Sci*., **308,** 1.
Richter, A. W. and Hedin, H. I. (1982) *Immunol. Today* **5,** 132.
Roerdink, F., Dijkstra, J., Hartman, G., Bolscher, B. and Scherphof, G. (1981) *Biochim. Biophys. Acta*, **677,**79.
Surolia, A., Parkash, N., Bishayee, S. and Bachhawat, B. K. (1973) *Indian J. Biochem. Biophys*., **10,** 145.
Surolia, A., Bachhawat, B. K. and Podder, S. K. (1975) *Nature (London)*, **257,** 802.
Torchillin, V. P., Goldmacher, V. S. and Smirnov, U. N. (1978) *Biochem. Biophys. Res. Commun*., **45,** 1841.
Tyrell, D. A., Heath, T. D., Colley, C. M. and Ryman, B. E. (1976) *Biochim. Biophys. Acta*, **457,** 259.