

Stability of liposomes in circulation is markedly enhanced by structural modification of their phospholipid component

A. Bali, S. Dhawan and C.M. Gupta*

Division of Biophysics, Central Drug Research Institute, Lucknow-226001, India

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Replacement of the C-2 ester group in phosphatidylcholine by the carbamyloxy function rendered its liposomes completely stable and longer living in the circulation of rats.

Drug delivery

*Modified liposomes
Blood clearance*

*Plasma-induced leakage
Tissue uptake*

Lipid transfer

1. INTRODUCTION

The successful application of liposomes as drug carriers in therapy [1,2] is largely restricted by their major uptake in liver and spleen, and also their structural disintegration in blood [3]. Disruption of liposomes in the circulation is mainly caused by the transfer of their lipid constituents to the high density fraction of plasma lipoproteins [4,5]. This effect of the lipoprotein is reduced by increasing the CH content of the liposomes [6,7] or by an appropriate choice of the phospholipid component [8,11].

In an attempt to design liposomes that would be stable in blood, we have recently modified phosphatidylcholines by introducing one NH residue adjacent to the carbon atom of their C-2 ester group [12]. This change rendered the resulting phospholipids selectively resistant to phospholipase A₂ [12], without adversely affecting the physico-chemical properties of the liposomes

Abbreviations: PC, phosphatidylcholine; MPC, 1-palmitoyl-2-heptadec-10-*cis*-enylcarbamyloxy-*sn*-glycero-3-phosphorylcholine; CH, cholesterol; 6-CF, 6-carboxy-fluorescein; HDL, high density fraction of plasma lipoproteins

* To whom correspondence should be addressed

[12,13]. Further studies with these lipids have revealed that complete replacement of egg PC in liposomes by MPC prevents serum-induced leakage of the entrapped solutes and also inhibits the lipid transfer from liposomes to serum proteins [14]. We now report the results of our studies on the in vivo stability of the liposomes consisting of MPC and CH. Our data show that these liposomes are completely stable and longer living in the circulation of the injected animals.

2. MATERIALS AND METHODS

Egg PC, MPC, egg [*methyl*-¹⁴C]PC (35 μ Ci/ μ mol) and [*methyl*-¹⁴C]MPC (15 μ Ci/ μ mol) were prepared as in [12]. All these phospholipids exhibited single spots on silica gel G-60 thin-layer chromatography (TLC) plates. The assay of radioactive isotopes was carried out as in [14]. The amounts of 6-CF were measured in the absence and in the presence of Triton X-100 (1% final concentration, pH 7.4) on an Aminco SPF-500 fluorimeter using excitation and emission wavelengths of 490 and 520 nm, respectively.

2.1. Liposomes

Small unilamellar liposomes were prepared from 40 μ mol of phospholipid, traces of the corresponding ¹⁴C-labeled phospholipid (¹⁴C, ~100 μ Ci),

20 μ mol of CH and 6-CF (0.2 M) in 2.0 ml Tris-buffered saline (10 mM in 0.9% NaCl, pH 7.4) by probe sonication [12], and fractionated by centrifugation at $105\,000 \times g$ (Ti-50 fixed angle rotor) for 30 min at 10°C. Only the liposomes found in the top two-thirds of the supernatant were used. Free and liposomal 6-CF were separated by gel filtration of the fractionated liposomes on a Sephadex G-50 column (1.5 \times 20 cm). The column was eluted with the Tris buffer. The liposome-rich fractions were pooled together and used in further experiments within 1 h after the gel filtration. Homogeneity of the liposome preparations was routinely analysed by column chromatography on Bio-Gel A-50m. Both egg PC/CH and MPC/CH liposomes invariably eluted with the included volume of the Bio-Gel column, and had similar size distributions. The outer diameter of liposomes was about 30–60 nm, as determined by negative staining electron microscopy.

2.2. Clearance of liposomes from the circulation

Liposomal preparations (P, 5–7 μ mol; 14 C, ~10 μ Ci) in buffered saline (1.0 ml) were injected into tail vein of male rats (albino; average weight 200 g). Blood was drawn by retro orbital puncture into heparinized microcentrifuge tubes at specified time intervals. A measured aliquot (0.2 ml) of blood was suspended in the Tris buffer (1.0 ml) and centrifuged at 3000 rev./min (4°C). Measured volumes of the diluted plasma were assayed for radioactivity and also for free and total (free + liposomal) dye. The amounts of radioactivity and the liposomal dye remaining in the circulation at specified time periods were calculated by assuming 2 min values as 100%. The amounts of 6-CF that remained entrapped at 2 min were 90–98% of the trapped amounts prior to the injection.

2.3. Uptake of liposomes in liver and spleen

Blood (~10 μ l) from rats was drawn into heparinized capillary pipettes immediately (2 min) after injecting liposomes in them, and transferred to preweighed scintillation vials. It was weighed and treated with 0.1 M NaOH (0.1 ml) followed by H₂O₂ (30%, w/v; 0.1 ml). The sample was kept at room temperature for 30–40 min. It was dissolved in the scintillin and analysed for radioactivity. The animals were killed 24 h after the injection and their livers and spleens were taken out. These

were washed with the Tris buffer, blotted and weighed. The tissues were minced and homogenised in the buffered saline (10%, w/v). A measured portion of each homogenate was assayed for radioactivity. The amounts of radioactivity present in liver and spleen were calculated as percentages of the total injected radioactivity. The total radioactivity was calculated from 2 min blood sample by assuming the total blood volume as 8% of the body weight. Just before sacrificing the animals, the radioactivity and 6-CF levels in total plasma were ~30% for MPC/CH liposomes. In case of egg PC/CH liposomes, <7% radioactivity and no 6-CF were detected in the plasma samples.

3. RESULTS AND DISCUSSION

Small unilamellar liposomes were prepared from phospholipids and 50 mol % CH by sonication, and fractionated by centrifugation. These liposomes were labeled with 14 C by including trace amounts of the corresponding 14 C-labeled phospholipid in the lipid mixture. 6-CF (0.2 M) was entrapped in liposomes as the model solute. This dye remains completely latent when entrapped at a high concentration (0.05–0.25 M), and fluoresces only upon its leakage and subsequent dilution in the surrounding medium [15]. Measurements of the amounts of the latent dye in plasma should, therefore, allow accurate monitoring of the liposomal 6-CF in the circulation of the injected animals.

Time-dependent disappearances of free 6-CF from blood was determined by injecting its dilute solutions (0.02 M) in rats. More than 95% of the total injected dye was cleared in 60 min. The clearance time of 6-CF markedly increased when administered after encapsulating it in MPC/CH liposomes (fig. 1A, solid circles), which was quite unlike that observed in egg PC/CH liposomes (fig. 1A, solid triangles). The faster clearance of 6-CF from the egg PC/CH liposomes may result from the plasma-induced leakage of the entrapped dye [7]. This is evident from the differences between the rates of clearance of the liposomal 14 C and the latent 6-CF (fig. 1A,B solid triangles).

The plasma-induced leakage of the entrapped 6-CF from the liposomes was ascertained by comparing the ratios of liposomal 14 C to latent 6-CF at a given period of time and 2 min after the injection.

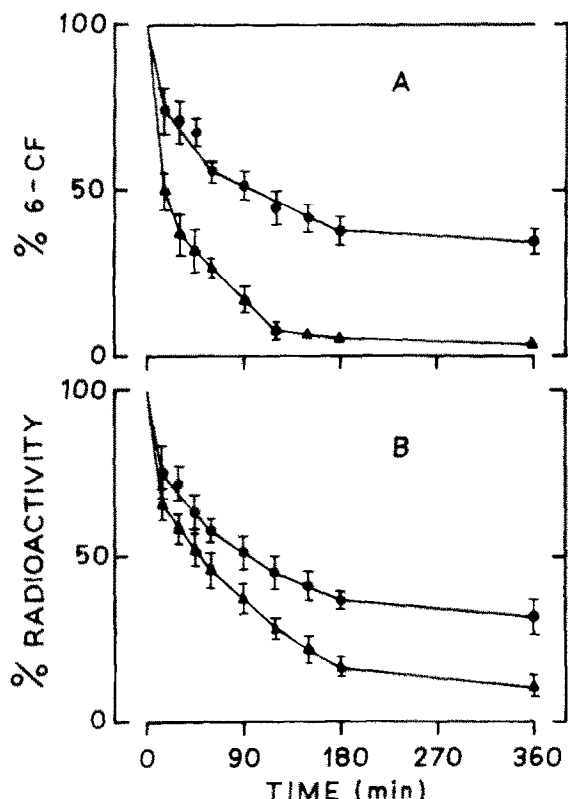


Fig. 1. Clearance of liposomal 6-CF (A) and radioactivity (B) from the circulation of rats after injecting liposomes. Each point is the mean of 4-6 animals. Bars, SD; solid triangles, egg PC/CH liposomes; solid circles, MPC/CH liposomes.

tion. Fig. 2 shows that this ratio for MPC/CH liposomes remains unaltered at least up to 6 h in the circulation, suggesting that the blood components do not induce any change in the permeability behavior of these liposomes. This is quite in accordance with our earlier observation that the incubation of MPC/CH liposomes with serum fails to accelerate the release of the entrapped solutes [14]. As observed with serum [14], the 6-CF efflux from egg PC/CH liposomes was markedly enhanced in blood (fig. 2). To confirm that this enhanced leakage from egg PC/CH liposomes is due to the lipid transfer to plasma proteins [4,5], we have measured the phospholipid transfer from liposomes to these proteins (fig. 3). The results given in table 1 indicate that from egg PC/CH liposomes considerably larger amounts (18-21%, 60 min) of the phospholipid were trans-

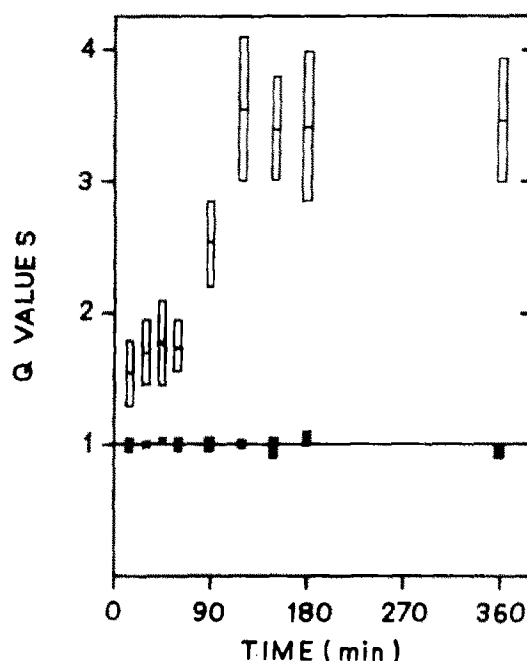


Fig. 2. The plasma-induced leakage of 6-CF was calculated from the ratios of the liposomal ^{14}C to latent 6-F at a given time period (t_x) and 2 min (t_0) after injecting liposomes in rats. Q is a quotient of $[\text{}^{14}\text{C}/6\text{-CF}]_{t_x}$ and $[\text{}^{14}\text{C}/6\text{-CF}]_{t_0}$. When Q -values were close to one, the liposomes were regarded as stable in the circulation. The values greater than one reflected plasma-induced leakage of liposomal 6-CF. The maximum Q -value obtained for MPC/CH liposomes up to 6 h in the circulation was 1.12 whereas this value for egg PC/CH liposomes was 4. Leakage rates of 6-CF from MPC/CH liposomes in buffer (pH 7.4) at 37°C were almost identical to those from egg PC/CH liposomes (5-8%, 6 h). Bars represent means of 4-6 animals \pm SD. Open bars, egg PC/CH liposomes; solid bars, MPC/CH liposomes.

ferred to plasma proteins, as compared to that from the MPC/CH liposomes (3-6%, 60 min). Also, the major amount of protein-associated phospholipid eluted in fractions which match the elution profile of HDL (fig. 3).

These results suggest that a minor but specific structural modification of the phospholipid component can significantly enhance the stability and survival times of the liposomes in the circulation. The structural change that was introduced in PC virtually had no adverse effect on the physicochemical properties of the liposomes [12,13]. In-

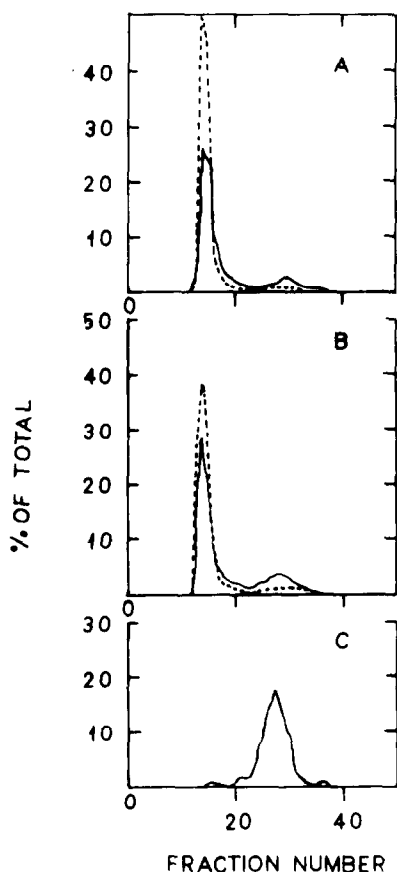


Fig. 3. Liposomes were injected in rats, and blood (~1.0 ml) was drawn into heparinized glass tubes at 15 min and 60 min after injection. The blood samples were centrifuged and a portion of plasma (0.3–0.5 ml) was chromatographed over Sepharose 6B column (1.4 × 35 cm). Fractions were analysed by measuring absorbance at 280 nm on a Spectronic 710 spectrophotometer and also by scintillation counting of ^{14}C . (A) and (B) are elution patterns of plasma samples obtained at 15 min and 60 min after the injection, respectively. Solid line, egg PC/CH liposomes; broken line, MPC/CH liposomes. In one experiment, plasma obtained from blood of rats that were not injected liposomes was also chromatographed as above (C). When liposomes were incubated with buffer at 37°C and a portion of this mixture was passed through the column, >95% of the total radioactivity eluted in fractions 13–17. Pure HDL from this column was eluted in fractions 26–32. Recoveries of radioactivity and protein from the column were at least 90%. The phospholipid transfer from liposomes to plasma proteins was calculated from areas under the respective peaks. The areas under the peaks were expressed as percentages of the total eluted ^{14}C (or protein). A summary of data for phospholipid transfer is given in table 1.

Table 1

Transfer of phospholipids from liposomes to plasma proteins

Liposomes	Phospholipid transfer (%)	
	15 min	60 min
Egg PC, CH	14.61 ± 0.29	19.51 ± 1.90
MPC, CH	3.21 ± 0.89	4.30 ± 1.52

Values are the means of 4–6 animals ± SD

Table 2

Uptake of liposomes in livers and spleens of rats 24 h after injection

Liposomes	% of initial (2 min) amount of radioactivity in blood	
	Liver	Spleen
Egg PC, CH	10.88 ± 2.22	0.40 ± 0.11
MPC, CH	16.45 ± 0.80	0.68 ± 0.20

Values are the means of 3–5 animals ± SD

stead, this change seems to offer the following advantages:

- The phospholipase A_2 -resisting property of MPC could have reduced the capture and lysis of the liposomes in liver [16]:
- Introduction of the NH residue adjacent to the carbon atom of the C-2 ester group leads to a more ordered phospholipid packing in PC bilayers [13] either due to the interlipid H-bonding [13] or because of the stabilization of the phospholipid conformation [17–19], which in turn might have reduced the lipid transfer to HDL [6,11].

To examine the validity of these suggestions, we have determined the amounts of uptake of liposomes in liver and spleen, and also the phospholipid transfer to HDL. The data shown in table 2 indicate that in case of MPC/CH liposomes, relatively higher amounts of radioactivity were present in liver and spleen. However, these amounts should not reflect the higher uptake but could arise from the reduced rates of lysis of the liposomes in these organs [16]. Furthermore, when both MPC/CH and egg PC/CH liposomes were separately incubated with HDL (3 h, 37°C), no transfer of MPC to the protein was observed. On

the contrary, ~50% egg PC was transferred under identical conditions (A. Bali, S. Dhawan, and C.M. Gupta, unpublished results).

This study clearly demonstrates that an appropriate tailoring of the phospholipid component can significantly minimize the basic drawbacks associated with liposomes as drug carriers. The phospholipase A₂-resisting property of MPC will not result in its long-term toxicity, because this lipid would readily be degraded by other phospholipases [12]. The MPC/CH liposomes may, therefore, find some useful applications as drug carriers in therapy.

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REFERENCES

- [1] Gregoriades, G. (1979) in: *Drug Carriers in Biology and Medicine* (Gregoriadis, G. ed) pp. 287-341, Academic Press, London, New York.
- [2] Papahadjopoulos, D., ed (1978) *Ann. NY Acad. Sci.* 308, 1-462.
- [3] Finkelstein, M. and Weissmann, G. (1978) *J. Lipid Res.* 19, 289-303.
- [4] Krupp, L., Chobanian, A.V. and Brecher, P.I. (1976) *Biochem. Biophys. Res. Commun.* 72, 1251-1258.
- [5] Scherphof, G., Roerdink, F., Waite, M. and Parks, J. (1978) *Biochim. Biophys. Acta* 542, 296-307.
- [6] Kirby, C., Clarke, J. and Gregoriadis, G. (1980) *FEBS Lett.* 111, 324-328.
- [7] Kirby, C., Clarke, J. and Gregoriadis, G. (1980) *Biochem. J.* 186, 591-598.
- [8] Mauk, M.R. and Gamble, R.C. (1979) *Proc. Natl. Acad. Sci. USA* 76, 765-769.
- [9] Gregoriadis, G. and Senior, J. (1980) *FEBS Lett.* 119, 43-46.
- [10] Hwang, K.J., Luk, K.S. and Beaumier, P.L. (1980) *Proc. Natl. Acad. Sci. USA* 77, 4030-4034.
- [11] Allen, T.M. (1981) *Biochim. Biophys. Acta* 640, 385-397.
- [12] Gupta, C.M. and Bali, A. (1981) *Biochim. Biophys. Acta* 663, 506-515.
- [13] Curatolo, W., Bali, A. and Gupta, C.M. (1982) *Biochim. Biophys. Acta* 690, 89-94.
- [14] Gupta, C.M., Bali, A. and Dhawan, S. (1981) *Biochim. Biophys. Acta* 648, 192-198.
- [15] Weinstein, J.N., Yoshikami, S., Henkart, P., Blumenthal, R. and Hagins, W.A. (1977) *Science* 195, 489-492.
- [16] Deshmukh, D.S., Bear, W.D., Wisniewski, H.M. and Brockerhoff, H. (1978) *Biochem. Biophys. Res. Commun.* 82, 328-334.
- [17] Büldt, G., Gally, H.U., Seelig, A., Seelig, J. and Zaccari, G. (1978) *Nature* 271, 182-184.
- [18] Gupta, C.M., Costello, C.E. and Khorana, H.G. (1979) *Proc. Natl. Acad. Sci. USA* 76, 3139-3143.
- [19] Pearson, R.H. and Pascher, I. (1979) *Nature* 281, 499-501.