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Role of liposome size and RES blockade in controlling biodistribution and tumor uptake of GM_1-containing liposomes *

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We have examined the effect of liposome size on liposome circulation time in the blood. Liposomes composed of phosphatidylcholine, cholesterol and ganglioside GM_1 were prepared in the various size range. Optimal circulation activity (55% injected dose at 4 h post injection) of GM_1-containing liposomes, which correlated with a relatively high uptake of liposomes by EMT6 tumor in mouse, was obtained with a size range of 70 to 200 nm in diameter. Increasing the diameter of liposome to greater than 200 nm resulted in an enhancement of the spleen uptake and decrease of the blood level. For liposomes with a diameter of less than 70 nm, 70% of the injected dose were taken up by the liver, presumably by the parenchymal cells. In contrast, the biodistribution of phosphatidylserine-containing liposomes was relatively insensitive to changes in liposome size; most of the injected dose was found in the liver. The effect of RES blockade on the circulation time of large ($d > 300$ nm), GM_1-containing liposomes was also studied. Dextran sulfate 500, a commonly used blockade reagent for Kupffer cells, had no effect. On the other hand, preinjection of a large dose of liposomes with a diameter greater than 500 nm showed variable results depending on the lipid composition of the blocking liposomes. Preinjection of liposomes containing GM_1, phosphatidylinositol or (*N*-polycthyleneglycol) phosphatidylethanolamine effectively reduced the spleen uptake of the large GM_1-containing liposomes, whereas liposomes containing phosphatidic acid showed no effect. These results indicate that only spleen homing liposomes can be used as a blocking reagent to prolong the circulation time of the large GM_1-containing liposomes.

* This paper is dedicated to Karl J. Hwang, deceased January 16, 1991, who has made major contributions to our understandings of liposome biodistribution.

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Abbreviations: Chol, cholesterol; DTPA-SA, distearylamide of diethylenetriaminepentaacetic acid; GM_1, monosialoganglioside; PA, phosphatidic acid; PC, phosphatidylcholine; PEG-PE, *N*-(monomethoxypolyethyleneglycol succinyl) dioleoylphosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; RES, reticuloendothelial system.

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Introduction

Liposomes, or phospholipid vesicles, have been studied for their potential use as drug carriers for many years (for a recent review, see Ref. 1). Systemically administered liposomes are rapidly taken up by the cells of the reticuloendothelial system (RES), primarily the resident macrophages in the liver and the spleen (for a review, see Ref. 2). Although such a property is an advantage for delivering drugs to the macrophages, it is a problem for those delivery efforts with target other than the RES. Strong affinity for the RES is particularly problematic for liposomes designed to bind to the target cells via attached targeting ligands such as antibody [3–5]. Several investigators have shown that immunoliposomes are just as rapidly taken up by the RES as the nontargeted liposomes such that only a

small fraction of the liposomes can reach the target cells (for a review, see Ref. 4).

In the last few years, efforts have been made to reformulate the liposome composition for a reduced affinity to the RES. It has been found that liposomes containing ganglioside GM_1 [4,7,8], hydrogenated phosphatidylinositol [7,9] or *N*-(polyethyleneglycol) phosphatidylethanolamine [10–12] are not readily taken up by the macrophages in the RES and hence stay in the circulation for a relatively long period of time. The potential application of the liposomes in chemotherapy as drug carrier has been directly demonstrated by the observations that their uptake by the solid tumor is considerably higher than that of the ordinary liposomes [7]. This may be due to the fact that long-circulating liposomes have a better chance to penetrate the leaky vasculature of the solid tumor [7]. Unfortunately, the long-circulating liposomes normally have a low encapsulation capacity particularly for the macromolecular drugs due to their small size. Formulations or methods designed for maximizing the drug carrying capacity of the long-circulating liposomes would be especially useful.

In this report, we describe the size dependency of the biodistribution of GM_1-containing liposomes, emphasizing the size range which gives rise to the maximal circulation half-life of liposomes. We have also demonstrated the correlation between the uptake by solid tumor and blood level of liposomes in the context of liposome size. Furthermore, we have studied whether prolonged circulation half-life of GM_1-containing liposomes with diameters over 600 nm can be achieved by the method of RES blockade. The results of the study bear relevance to the efficient delivery of macromolecular drugs to the target cells.

Materials and Methods

Materials

Egg phosphatidylcholine (PC), egg phosphatidic acid (PA), bovine brain phosphatidylserine (PS) and bovine liver phosphatidylinositol (PI) were purchased from Avanti Polar Lipids; ganglioside GM_1 was from Supelco; cholesterol (Chol) and dextran sulfate (M_r 500000) were from Sigma; *N*-(monomethoxypolyethyleneglycol succinyl)-dioleoylphosphatidylethanolamine (PEG-PE) (M_r of PEG was 5000) was synthesized according to Ref. 10. ^{111}In-DTPA-SA was prepared as described previously [13].

Liposome preparation

Liposomes composed of PC/Chol/x (x represents the third lipid component) with a mole ratio of 10:5:1 were prepared by extrusion of hydrated lipid dispersions through polycarbonate filters. Briefly, lipids with a trace amount of ^{111}In-DTPA-SA as liposome marker were evaporated under N_2 gas and vacuum desiccated for at least 30 min. The dried lipid film was hydrated in PBS (pH 7.4) overnight at room temperature. The lipid suspension was then extruded through polycarbonate filters (Nuclepore) of defined pore size. Sometimes, two layers of filter were used in order to obtain the desired size of vesicles. The diameter of the liposomes was monitored by dynamic light scattering using a submicron particle analyzer (Colter N4SD). Liposomes with diameter less than 70 nm were prepared by sonication followed by chromatographical fractionation using a gel filtration column (Sepharose 4B-CL). The fractions with desired average size were used for biodistribution assays. The average diameter of liposomes was confirmed by negative stain electron microscopy [14].

Biodistribution of liposomes

Liposomes (1 μmol lipid in 100 μl PBS, 10^4–10^5 cpm in ^{111}In radioactivity) were injected via the tail vein into mice (Balb/c, 6–8 week old, male). Four hour post-injection, mice were bled at the retro orbital sinus and killed by cervical dislocation. Blood and all organs (including spleen, liver, lung, heart and kidney) and carcass were weighed and their radioactivities in each organ were counted in a gamma-counter. Data were expressed as % injected dose in each organ. Correction factors for the blood content in various organs were determined by examining the distribution of ^{51}Cr-labeled erythrocytes 30 min after i.v. injection. Briefly, freshly isolated mouse red blood cells were incubated for 1 h at 37°C with $Na_2{}^{51}CrO_4$ ($2 \cdot 10^7$ cells in 1 ml, 50 μCi ^{51}Cr). Free ^{51}Cr was removed from the cells by centrifugation. The ^{51}Cr-labeled cells were then injected (i.v.) into mice for determination of the blood content of various organs. The amount of liposome radioactivity in each organ was corrected for blood contaminations and expressed as percent injected dose. The weight of blood was assumed to be 7.4% of the total body weight of the mouse [15].

Size-dependent tumor accumulation of liposomes containing GM_1

4–6-week old female Balb/c mice were inoculated subcutaneously at the left hind leg with EMT6 tumor cells [16]. Mice were tested 2–3 weeks after tumor inoculation when the implanted tumor weighed between 0.5 and 2.0 g. Mice were injected via the tail vein with ^{111}In-labeled GM_1-containing liposomes with various diameters at a dose of 1 μmol lipid per mouse. Different organs including tumor were collected and counted for ^{111}In-radioactivity as described above. The tumor uptake was expressed as % injected dose per gram of tumor weight.

RES blockade with liposomes or dextran sulfate

Unlabeled liposomes extruded through filter with pore size of 1 μm were injected at various lipid doses in a volume of 400 μl per mouse. Thirty minutes after injection, test liposomes composed of PC/Chol/GM_1 (10:5:1) containing ^{111}In-DTPA-SA were administered through the same route. In the case of dextran sulfate, test liposomes were injected 2 h after the injection of dextran sulfate (1.5 mg/mouse). The biodistribution of test liposomes were analyzed 4 h later.

Results

Effect of liposome size on the disposition of liposome

Fig. 1 shows the effect of liposome size on the distribution of liposomes containing GM_1. Clearly, liposome biodistribution was liposome-size dependent. Liposomes with a diameter less than 70 nm mainly accumulated in the liver. Spleen uptake became predominant when the liposome diameter exceeded 300 nm or greater. The bell-shaped curve of blood concentration of GM_1-containing liposomes shows that the optimal size range of liposome for high blood concentration is from 70 to 200 nm (Fig. 1A), with 55% of injected dose still in the blood 4 h after injection. Accumulation of liposomes in other tissues including lung, heart, kidney and carcass was relatively size independent; about 20% of injected dose was found in these tissues (Fig. 1A). In contrast, the size-dependent biodistribution of liposomes containing PS was different from that of GM_1-containing liposomes (Fig. 2). The majority of liposomes (80%) was found in the liver for liposomes with size range from 30 to 450 nm in diameter. There was a slightly elevated spleen uptake of liposomes for liposomes with the average diameter greater than 70 nm. In contrast to GM_1-containing liposomes, slightly higher blood concentration (about 10%) of PS-containing liposomes was obtained if the liposome diameter was less than 70 nm. This is compared to 1% of injected dose found in the blood for liposomes with a diameter greater than 70 nm.

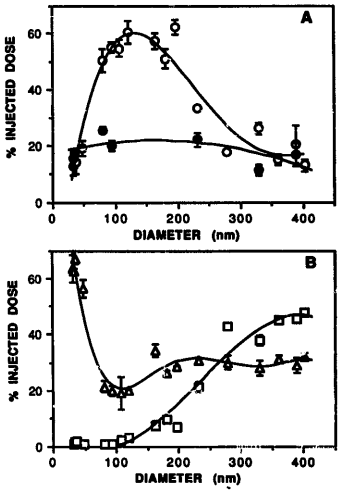

Fig. 1. Size-dependent biodistribution of GM_1-containing liposomes. ^{111}In-labeled liposomes (PC/Chol/GM_1 = 10:5:1) with defined diameter were injected i.v. at a dose of 1 μmol lipid per mouse. The mice were killed 4 h after injection and the radioactivity in each organ was analyzed. Data represent the mean (S.D.) values of three mice. (A) (○) blood, (●) others including all tissues of animal except blood, spleen and liver. (B) (△) liver, and (□) spleen.

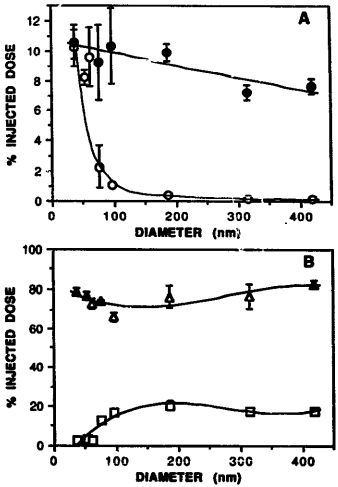

Fig. 2. Size-dependent biodistribution of PS-containing liposomes (PC/Chol/PS = 10:5:1). The conditions of the experiment and the symbols used are same as in Fig. 1.

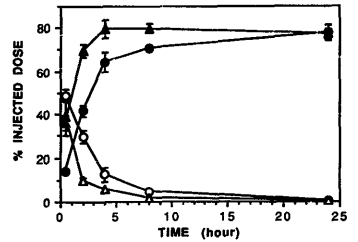

Fig. 3. Kinetics of a small liposome distribution in blood and RES (liver and spleen). Liposomes with average diameter of 36 nm were injected i.v. (1 μmol lipid per mouse). Mice were killed at different times and the radioactivity in each organ was analyzed. Data represent average (S.D.) of three mice. Open symbols are for blood levels and close symbols for RES levels. Circles are for liposomes composed of PC/Chol/GM_1 (10:5:1) and triangles for liposomes composed of PC/Chol/PS (10:5:1).

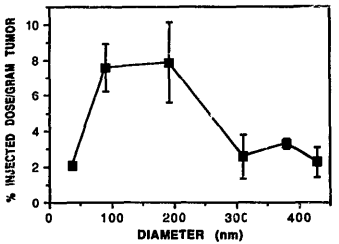

Fig. 4. Size-dependent liposome accumulation in tumor. ^{111}In-labeled liposomes (PC/Chol/GM_1 = 10:5:1) were injected i.v. (1 μmol lipid per mouse) into mice bearing EMT6 tumor. Mice were killed 24 h after injection and the tumors were removed and weighed. ^{111}In radioactivity in the tumor was presented as percent injected dose per gram of tumor weight. Data points represent average (S.D.) values of three mice. Data point of 40 nm is significantly ($P < 0.01$) different from those of 90 and 190 nm.

Time-dependent distribution of small liposomes containing either GM_1 or PS

It is evident from the data in Fig. 1A that the activity of GM_1 in prolonging the liposome circulation time is greatly reduced when liposomes are small. This is opposite from the results with PS-containing liposomes (Fig. 2A). In order to explore the mechanism of this interesting observation, we have investigated the kinetics of biodistribution for both types of small liposomes ($d = 36$ nm). It is clear from the data in Fig. 3 that the blood clearance and the RES (liver and spleen) uptake of the GM_1-containing liposomes were similar to those of the PS-containing liposomes. However, liposomes containing GM_1 showed slightly longer half-life in the blood than the PS-containing liposomes. For example, at 2 h post-injection, there was about 30% injected dose of the GM_1-containing liposomes remaining in the blood compared with only about 10% injected dose left in the blood for the PS-containing liposomes.

Effect of liposome size on tumor uptake of GM_1-containing liposomes

Gabizon and Papahadjopoulos [7] have shown that GM_1-containing liposomes accumulate efficiently in the solid tumor. We have investigated whether the size of liposomes affects the level of tumor uptake for these liposomes in the EMT6 solid tumor model. Fig. 4 shows that liposomes with a diameter ranging from 90 to 200 nm accumulated in the tumor more readily than those with a diameter of 40 nm or larger than 200 nm. About 8% of injected liposome dose per gram of tumor weight was observed with liposomes of this intermedi-

TABLE I

Effect of preinjection of dextran sulfate on the distribution of liposomes

^{111}In-labeled liposomes (1 μmol lipid/mouse) with defined size and composition were i.v. injected into mice 2 h after the injection of dextran sulfate (1.5 mg/ml) or PBS (control). Mice were sacrificed 4 h later and the ^{111}In radioactivity in each organ was counted. Data represent average values (S.D.) of three mice.

Liposome composition	Liposome diameter (nm) (mean (S.D.))	% injected dose							
		blood		spleen		liver		others	
		control	treated	control	treated	control	treated	control	treated
PC/Chol/PS (10:5:1)	35 (13)	5.6 (0.4)	6.5 (0.5)	4.4 (1.2)	8.2 (0.6)	75.2 (4.3)	34.1 (5.6)	9.1 (1.1)	41.3 (4.3)
	110 (29)	0.8 (0.0)	17.1 (0.1)	17.7 (0.7)	27.0 (7.2)	72.9 (2.3)	26.7 (0.3)	2.3 (0.0)	17.0 (3.0)
	747 (broad)	0.1 (0.0)	2.0 (1.0)	10.3 (1.1)	55.5 (10.8)	82.9 (11.9)	27.1 (5.4)	0.8 (0.1)	7.1 (1.3)
PC/Chol/GM_1 (10:5:1)	36 (15)	12.6 (3.2)	15.9 (1.6)	0.8 (0.1)	5.2 (0.4)	63.7 (4.8)	43.5 (3.0)	10.4 (3.0)	30.3 (0.7)
	107 (30)	54.8 (2.8)	48.0 (2.5)	2.1 (0.4)	5.1 (1.1)	23.3 (5.8)	20.7 (2.9)	15.1 (0.9)	21.4 (1.4)
	555 (broad)	8.9 (0.8)	14.6 (1.6)	50.7 (2.5)	46.7 (2.5)	33.9 (10.5)	22.7 (2.2)	4.1 (0.5)	11.8 (1.2)

ate size. The size dependent distribution of liposome in other organs of tumor bearing mice was similar to that found in the normal mice (data not shown). Therefore, relatively high level of tumor accumulation of the GM_1-containing liposomes correlated very well with the relatively high concentration of liposomes in the blood (see Fig. 1A).

Effect of RES blockade on the size-dependent liposome biodistribution

Since liver and spleen play important roles in determining the biodistribution of liposome, we have investigated the effect of RES blockade on the disposition of liposomes containing either GM_1 or PS. Dextran sulfate 500, an effective reagent for RES blockade, was injected into mice 2 h prior to the injection of ^{111}In-labeled liposomes. Data in Table I show that the biodistribution of PS-containing liposomes was very sensitive to dextran sulfate treatment. For example, 70-fold increase of liposome ($d = 110$ nm) concentration in the blood with concomitant large decrease in the liver uptake (from 73 to 27%) was obtained in mice preinjected with dextran sulfate. For large ($d = 747$ nm) PS-containing liposomes, a 5-fold increase (from 10 to 53%) of the spleen uptake was observed in the treated mice. Dextran sulfate treatment had also increased (9 to 41%) accumulation of small ($d = 35$ nm) liposomes in other organs, mainly in the carcass. While these significant changes were observed with the PS-containing liposomes, there was no significant difference observed with the GM_1-containing liposomes of all sizes tested. Only a slight increase in blood concentration of large ($d = 555$ nm) liposomes, accompanied by a 10% decrease in the liver uptake, was obtained in the dextran sulfate treated mice. There were also some increases of liposome uptake in the carcass of the dextran treated mice.

Effect of preinjection of liposomes on the blood concentration of large GM_1-containing liposomes

It is important to increase the circulation time of large liposomes because of their relatively large capacities for drug entrapment. RES blockade by dextran sulfate was inefficient for large liposomes containing either GM_1 or PS (Table I). Since the uptake of large GM_1-containing liposomes was mainly by the spleen, we reasoned that preinjection of a large dose of splenotropic liposomes might block the uptake mechanism of this organ, thereby prolonging the circulation time of subsequently injected GM_1-containing liposomes. Unlabeled liposomes of different compositions were preinjected into mice 30 min before the injection of ^{111}In-labeled liposomes composed of PC/Chol/GM_1 (10:5:1) with a diameter of about 600 nm. The blocking liposomes were prepared by extruding hydrated lipids through filters of 1 μm pore size. Their average diameters were about 600 nm in diameter. Table II shows a dose-dependent effect of the preinjected liposomes on the blood concentration of large GM_1-containing liposomes. Clearly, the blood concentration of ^{111}In-labeled liposomes increased with increasing amount of the blocking liposome preinjected. The effectiveness of the blockade was liposome-composition dependent. The most effective blocking was observed with liposomes of the same composition as the ^{111}In-labeled liposomes, followed by liposomes containing PI, PEG-PE and PG. Liposomes composed of PC/Chol (10:5) or PC/Chol/PA (10:5:1) were not effective. There was only about 6% of ^{111}In-labeled liposomes found in the blood of mice preinjected with 20 μmol of liposomes composed of PC/Chol. This is compared to about 55% injected dose found in the blood of mice preinjected with the same amount of liposomes containing GM_1.

TABLE II

Effect of preinjection of liposomes on the blood concentration of large GM_1-containing liposomes 4 h post-injection

Liposomes were prepared by extruding hydrated lipids through 1.0 μm polycarbonate filter for five times and injected intravenously into mice 30 min before the administration of ^{111}In-labeled PC/Chol/GM_1 (10:5:1) liposomes (1 μmol/mouse). Four hours later, the blood concentration of ^{111}In radioactivity was measured. Data represent average values (S.D.) of three mice.

Liposome preinjected	Preinjected dose (μmol/mouse)	% of injected dose of liposomes (PC/Chol/GM_1) in blood
None	–	2.4 (0.5)
PC/Chol/GM_1	5	21.0 (2.2)
	10	33.4 (6.2)
	20	54.7 (0.5)
PC/Chol/PI	5	17.0 (2.2)
	10	24.6 (10.0)
	20	34.1 (5.6)
PC/Chol/PEG-PE	5	8.5 (1.0)
	10	14.4 (1.7)
	20	36.1 (4.6)
PC/Chol/PA	5	1.6 (0.1)
	10	4.7 (2.3)
	20	10.2 (2.1)
PC/Chol	5	1.7 (0.6)
	10	4.3 (0.3)
	20	6.3 (0.2)

Discussion

It is well established that the mononucleophagocytes of the RES, principally the Kupffer cells of the liver and secondarily the splenic macrophages, are responsi-

ble for the clearance of liposomes from the circulation [17]. Liposomes containing GM_1 show a marked resistance to RES uptake and stay in the circulation for a prolonged period of time [6–8]. The original observations by Allen and Chonn [6] and by Gabizon and Papahadjopoulos [7] were made with liposomes of approx. 100 nm in diameter. We have previously reported that large GM_1-containing liposomes ($d > 300$ nm) do not show prolonged circulation time and accumulate in the red pulps and marginal zones of the spleen [18]. We have attributed this finding to the fact that these large liposomes are retained by the splenic sinusoidal filter [19]. Normally, only a small fraction of the blood passes through the spleen at any given time. Only liposomes with reduced affinity for the liver Kupffer cells would have a sufficient chance to circulate through the spleen and encounter the filter. Or, when the Kupffer cells are blocked by reagents such as dextran sulfate, large liposomes containing PS would also be retained by the spleen (Table I) despite the fact that PS is well known to exhibit opsonin-like activity [20]. Thus, these studies as well as the data shown here (Fig. 1) have established the upper size limit (≈ 200 nm in diameter) of the long-circulating liposomes containing GM_1.

Data in Fig. 1 further indicate that there is a lower size limit (≈ 70 nm in diameter) for the long-circulating liposomes. Small GM_1-containing liposomes were cleared from the blood circulation almost as fast as the small PS-containing liposomes (Fig. 3). These results are not surprising in view of the previous reports by Scherphof et al. [21], Rahman et al. [22] and Roerdink et al. [23] that small unilamellar liposomes could extravasate and accumulate in the parenchymal cells of the liver. This is because the fenestrae of the liver sinus contains holes of average diameter 100 nm [24]. Furthermore, liposomes used in these studies were prepared from phospholipids having a high melting temperature, exhibiting high rigidity and thus relatively prolonged circulation compared to less rigid liposomes [25]. Presumably, the liver Kupffer cells may have reduced affinity for these small rigid liposomes, thus accumulating in the liver parenchymal cells. Small GM_1-containing liposomes, which should also be very resistant to uptake by the Kupffer cells and not retained by the splenic filter, could easily extravasate and be taken up by the liver parenchymal cells. Indeed, data in Fig. 1B and Fig. 3 indicate an efficient liver uptake (60–70% injected dose in 4 h) of these liposomes. Thus, the activity of GM_1 in prolonging the circulation time of liposomes is limited to a relatively narrow size range, i.e. 70–200 nm. Activities of other amphiphiles (PEG-PE, PI, sulfatide) in prolonging the liposome circulation time are also likely to be dependent on the liposome size, although detailed experiments are yet to be done. We have previously reported that large liposomes containing PEG-PE are retained by the spleen [11].

The size limitation of the long-circulating liposome bears some practical significances as shown by the data in Fig. 4. Elevated tumor uptake of the GM_1-containing liposomes also exhibits an optimal liposome size range which coincided with the range shown in Fig. 1A for the prolonged liposome circulation. Therefore, effective tumor uptake of liposomes depends on the prolonged circulation time of liposomes, a conclusion previously made by Gabizon and Papahadjopoulos [7]. The amount of blood circulating through a solid tumor is probably only a small fraction of the total and is likely to depend on the age, location and nature of the tumor. Only liposomes with a prolonged circulation time will have a sufficient chance to encounter the leaky vessels of the tumor. Data in Fig. 4 also clear up a misconception that small liposomes should be better suited than the larger ones to penetrate the tumor vessels. In reality, small GM_1-containing liposomes accumulate less efficiently in tumor (Fig. 4) due to their efficient accumulation in the liver and thus rapid clearance from the circulation (Fig. 1B).

Preinjection of dextran sulfate, although significantly altering the biodistribution of the PS-containing liposomes of all sizes, had little effect on the biodistribution of the GM_1-containing liposomes (Table I). This is because the Kupffer cells are not the principal cells taking up these liposomes and dextran sulfate only blocks the activity of the Kupffer cells [26]. For the large GM_1-containing liposomes, only a preinjection of a large dose of the same liposomes could effectively prolong the circulation time of these liposomes (Table II). Although the effect was pronounced, this treatment is unlikely to be used for drug delivery studies because of the relatively high cost of the GM_1 lipid. Liposomes containing other less expensive lipids which have been shown to prolong the liposome circulation time, i.e. PI and PEG-PE, were less effective (Table II). To our knowledge, no effective blockade reagent has been reported for the macrophages of the spleen. Until such a reagent is available, it is unlikely that large liposomes containing GM_1 or other lipids of similar activity can be used as an effective drug carrier (other than for the delivery to the spleen).

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