Biochimica et Biophysica Acta, 1103 (1992) 94–100

BBAMEM 75483

The role of surface charge and hydrophilic groups on liposome clearance *in vivo*

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(Received 1 May 1991)
(Revised manuscript received 24 September 1991)

Key words: Liposome; Blood clearance; Drug delivery; Surface charge; Membrane fluidity

The effect of negative surface charge and hydrophilic groups on liposome clearance from blood was investigated in mice using liposome-entrapped ^{67}gallium-deferoxamine as a label. The presence of negatively-charged lipids may retard or accelerate liposome clearance. Physicochemical features contributing to optimal retardation of liposome clearance include a hydrophilic carbohydrate moiety and a sterically hindered negatively-charged group. The relevance of the negative charge steric effect is suggested by the finding that phosphatidylinositol phosphate (PIP) and trisialoganglioside (G_{T1}) are less effective than phosphatidylinositol (PI) and monosialoganglioside (G_{M1}), respectively, in retarding liposome clearance. The need for negative charge in addition to the carbohydrate group for optimal effect on retardation of clearance is indicated by the observation that asialoganglioside (AG_{M1}) is less effective than G_{M1} in this respect. The negative charge effect is observed with liposome bilayers having both low and high temperature phase-transitions. Increasing the molar fraction of negatively-charged lipid (hydrogenated PI derived from soya) from 23 to 41% resulted in a dramatic acceleration of liposome clearance. The clearance-accelerating effect of the high negative charge was specifically directed to the liver with selective reduction of spleen uptake. Increasing liposome size also had an accelerating effect on clearance but in this case it was accompanied by a non-specific concomitant increase of both liver and spleen uptake.

Introduction

The presence of negative surface charge in liposomes has long been associated with an accelerated clearance rate of liposomes administered intravenously (for a review, see Ref. 1). Investigations published in recent years have indicated that this is not a general phenomenon and that some negatively charged lipid molecules can actually cause the opposite effect, namely retardation of *in vivo* liposome clearance [2–5]. The mechanism behind these diverse effects is still obscure and the hypotheses proposed range from sophisticated cell recognition phenomena to physical properties derived from specific molecular structures. For example, it has been proposed that PS accelerates liposome clearance by a specific recognition mechanism involved in the scavenging of aged erythrocytes [6,7], and that G_{M1} retards liposome clearance by a steric conformation effect preventing interaction of plasma proteins (opsonins) with the carboxyl group of the sialic acid moiety [3]. A generalized effect on steric stabilization has also been proposed recently [8]. In an attempt to achieve a comprehensive and critical analysis of the factors involved, we present data in this report describing the effect of a variety of negatively charged lipids on liposome clearance.

Abbreviations: PA, phosphatidic acid; PS, phosphatidylserine; C, cholesterol; EPC, phosphatidylcholine derived from egg yolk; EPG, phosphatidylglycerol derived from EPC; DSPC, distearoylphosphatidylcholine; HSPC, hydrogenated soya PC; DPPG, dipalmitoylphosphatidylglycerol; SM, sphingomyelin; HSPI, hydrogenated phosphatidylinositol derived from soya; PI, phosphatidylinositol derived from soya; PIP, phosphatidylinositol phosphate; G_{M1}, monosialoganglioside; G_{T1}, trisialoganglioside; CS, cerebroside sulfate; AG_{M1}, asialoganglioside G_{M1}.

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Materials and Methods

Sources of materials and liposome preparation were as follows: egg phosphatidylcholine (EPC), phosphatidic acid (PA), phosphatidylglycerol (EPG), bovine brain phosphatidylserine (PS), sphingomyelin (SM), bovine liver phosphatidylinositol (PI), hydrogenated soybean PI (HSPI), distearoylphosphatidylcholine (DSPC), were all from Avanti Polar Lipids (Birmingham, AL); dl-α-tocopherol, trisialoganglioside (G_{T1}) from Supelco (Bellefonte, PA); cholesterol (C), cerebroside sulfate (CS), phosphatidylcholine (EPC), phosphatidylinositolmonophosphate (PIP), monosialoganglioside (G_{M1}), asialoganglioside G_{M1} (AG_{M1}), and γ-hydroxyquinoline (oxine) sulfate, from Sigma; hydrogenated soybean PC from Nattermann Chemie (Cologne, Germany); ^{67}Ga citrate from New England Nuclear; deferoxamine mesylate from CIBA-Geigy; acetate form of AG 1-X2 resin from Bio-Rad.

Liposome preparation. Liposomes were prepared as described before [3] by thin lipid film hydration with an isotonic solution of NaCl (pH range, 6.0–7.0) containing deferoxamine mesylate (25–35 mM). The ratio of neutral phospholipid to cholesterol was 2:1, and the negatively charged lipid was 10–11% of the neutral phospholipid unless otherwise specified. α-Tocopherol was included at a ratio of 1% of neutral phospholipid. Multilamellar vesicles were formed by Vortex mixing the lipid dispersions at room temperature or, in the case of high-phase-transition lipids (DSPC), at 55°C for approx. 30 min. Liposomes with homogenous size distribution were obtained by extrusion of the Vortex mixed preparation through double polycarbonate membranes of 0.08- or 0.05-μm pore size (Nucleopore) as before [3]. When high-phase-transition phospholipids were used, the extruder device was preheated to 55°C.

Liposome size was determined by dynamic light scattering with a submicron particle analyzer (Coulter Electronics, Hialeah, Fl., model N4; or Nicomp model 200, Hiac/Royco, Silver Springs, MD). The mean size obtained was in the range of 70–120 nm, with a standard deviation not larger than 30% of the Gaussian mean. In cases in which a second vesicle population of larger size was present, or the Gaussian mean was 130 nm or greater, the liposomes were repeatedly re-extruded and/or spun for 20 min in a microcentrifuge at $10\,000 \times g$. Unencapsulated deferoxamine was removed by gel filtration of the liposome suspension through Sephadex G-50 or G-75 (Pharmacia). Phospholipid concentration was determined by a phosphate assay [3]. Liposomes were stored either under argon or in vacuum-sealed tubes at 5°C and tested within 1 week after preparation.

Labeling procedure. The method of labeling preformed liposomes with ^{67}Ga-labeled deferoxamine has been described in detail [9]. Briefly, between 100 and 200 μCi of ^{67}Ga citrate was adjusted to a vol of 0.5–1.0 ml with a solution (1 mg/ml) of oxine sulfate in 0.9% NaCl. The solution was incubated for 1 h at 50°C. Liposome suspensions were incubated overnight at 4°C with 1–2 μCi of ^{67}Ga-labeled oxine per μmol of phospholipid. This results in formation of ^{67}Ga-labeled deferoxamine complex in the aqueous interior of liposomes [9]. Removal of unencapsulated ^{67}Ga-labeled oxine was achieved by passing the liposome suspension through an anion-exchange resin (AG 1-X2), acetate form, 200–400 mesh).

Biodistribution in mice. Two- to 3-month-old Swiss-Webster (SW) female mice (Simonsen Laboratories, Gilroy, CA) were injected i.v. with liposomes containing 1 μmol of phospholipid per mouse each weighing approx. 20 g. Retroorbital bleeding (approx. 1 ml) under ether anesthesia, killing by cervical dislocation, and complete animal dissection followed at 4 and 24 h after injection. Encapsulated ^{67}Ga-labeled deferoxamine served as a marker to determine the distribution of liposomes in the various tissues. All tissues were weighed and their radioactivity was quantitated in a γ-counter. Blood volume and correction factors for the blood content of various tissues were determined in age- and sex-matched SW mice by examining the tissue distribution of ^{111}In-oxine-labeled syngeneic erythrocytes 10–15 min after i.v. injection. Labeling of erythrocytes was performed as before [3]. Total recovered dose and percentages of recovered and injected dose per organ and per g of tissue were calculated.

Results and Discussion

Table I shows the tissue disposition of radiolabeled liposomes 4 h after liposome injection for a variety of formulations, all with a particle diameter in the range of 70 to 100 nm (Gaussian mean). On the basis of the blood and liver values and their ratios, and using PC liposomes as the baseline, we can recognize two types of formulations in addition to those behaving similarly to PC: those in which the inclusion of a charged lipid (PS, PA, G_{T1}, and PG) enhances liposome clearance and liver uptake, and those in which the inclusion of a charged lipid (PI, G_{M1}) or uncharged AG_{M1} inhibits liposome clearance and liver uptake. As previously reported [3], the % recovered dose in blood and liver were inversely correlated. In addition, we noticed high skin values with G_{M1} and AG_{M1} liposomes, suggesting that liposomes with slow clearance are able to localize to a significant extent in the vascularized dermis. No such correlation was evident in the case of carcass. This last observation, however, is not particularly instructive since carcass includes a heterogeneous group of tissues (bone, bone marrow, muscle) which may behave differently in terms of liposome uptake.

The presence of 10% PS, PA, and PG accelerated liposome clearance by decreasing order of magnitude (Table I). The fact that PA, the phospholipid with the simplest headgroup, accelerated liposome clearance in a dose-dependent manner (compare 5% PA and 10% PA in Table I) supports the contention that an electrostatic interaction at the level of the negatively charged phosphate group, is involved in the initial process leading to liposome clearance, rather than a receptor recognition mechanism. One possibility related to this is that Ca^{2+} may interact simultaneously with the charged liposome group and with specific opsonizing proteins. The extraordinarily fast clearance of PS-containing liposomes would suggest the parallel involvement of a receptor recognition mechanism, but an alternative explanation is that Ca^{2+}-mediated nonspecific opsonization of PS vesicles is greatly facilitated by the high affinity of PS for Ca^{2+} and by the terminal position of the carboxyl group of PS on the liposome surface. The avid interaction of Ca^{2+} with PS and PA vesicles facilitating intermembrane contact and fusion, and the lack of same effect with PI vesicles have already been well documented in earlier studies [10,11].

As reported in a previous study [3], the presence of PI or G_{M1} inhibited liposome clearance (Table I). Interestingly, replacing PI with PIP, in which a charged phosphate group is added to the inositol molecule in position 4, cancelled the inhibitory effect of PI on liposome clearance. A more drastic change was seen by replacing G_{M1} with G_{T1}, in which two additional sialic acid molecules with exposed carboxyl groups are present. These results are consistent with the hypothesis that a shielding effect (steric hindrance) of the sugar moieties on the negative charge plays a key role in the prolongation of liposome circulation in blood [3]. This hypothesis predicts that the addition of 'exposed' charged groups would have the opposite effect as was found to be the case for PIP and G_{T1}. However, an alternative explanation for the latter observations should also be considered: the changes may be merely due to an absolute increase of the surface charge density on the liposome surface.

Replacing G_{M1} with its uncharged counterpart AG_{M1} was found to reduce the recovered dose in blood and to increase the uptake in liver at 4 h (Table I); however, the resulting liposomes accumulate at much lower levels in the liver and higher levels in the skin compared to PC-C. These results suggest that both the negative charge and the sugar moiety are contributing to an optimal inhibition of liposome clearance by the liver. In the case of G_{M1} the negative charge is 'sterically hindered' or shielded by the carbohydrate group and possibly not available for interaction with proteins (either circulating or on cell surfaces). Still, the overall negative surface charge density on the liposome surface is likely to inhibit liposome aggregation and to prevent lateral phase separation of neutral glycolipids. As a result, the presence of such charge is expected to

TABLE I

Tissue distribution of EPC-containing liposome formulations 4 h after liposome injection

To facilitate inter-formulation comparisons we chose to present the percentages of the recovered dose (%RD), instead of the injected dose (%ID), since the former are not affected by the % injected dose recovered in whole body (total body recovery). The %RD values can be interconverted to %ID values by multiplying by the total body recovery and dividing by 100. Data are given for blood, liver, spleen, skin and carcass (bones, muscles, and appendages) which account for most of the dose recovered. For other organs not shown, such as lungs, heart, kidneys and gastrointestinal tract, the %RD were low and did not vary significantly across the liposome formulations tested. Each formulation was tested in 3–6 mice. Formulations are ranked by the liver/blood ratio. Note that the phospholipid to cholesterol ratio was 2:1 in all formulations and, therefore, cholesterol (C) was not a variable in these experiments.

Liposome composition (molar ratio)	% of recovered dose (%RD) per organ (S.D.)					% of injected dose recovered in whole body	Liver/ Blood (ratio)
	blood	liver	spleen	carcass	skin		
PS-PC-C (1:10:5)	0.5 (0.0)	75.0 (1.1)	10.4 (0.8)	7.7 (0.5)	2.5 (0.9)	80.8 (1.6)	150.0
PA-PC-C (1:10:5)	4.1 (1.1)	63.3 (2.6)	5.6 (0.3)	10.8 (0.8)	3.4 (0.6)	61.3 (3.4)	15.4
G_{T1}-PC-C (1:10:5)	10.6 (1.4)	54.2 (8.1)	3.0 (0.5)	15.1 (2.4)	3.4 (0.5)	45.3 (6.8)	5.2
PG-PC-C (1:10:5)	9.1 (4.8)	43.4 (0.9)	1.1 (0.2)	16.2 (1.6)	6.8 (1.1)	84.8 (1.5)	4.8
PA-PC-C (0.5:10:5)	15.1 (1.0)	50.5 (2.3)	5.5 (0.8)	13.4 (1.6)	5.1 (0.8)	84.3 (3.7)	3.3
PC-C (10:5)	30.8 (5.0)	34.5 (0.4)	2.5 (0.3)	9.4 (1.5)	4.0 (0.9)	90.7 (6.6)	1.1
PIP-PC-C (1:10:5)	35.9 (1.6)	36.2 (2.7)	2.7 (0.7)	9.6 (0.8)	3.8 (0.6)	65.5 (1.6)	1.0
CS-PC-C (1:10:5)	33.6 (1.2)	30.5 (2.4)	3.2 (0.9)	18.2 (2.8)	5.9 (0.4)	61.0 (0.9)	0.9
AG_{M1}-PC-C (1:10:5)	32.4 (9.1)	17.9 (3.3)	1.2 (0.1)	12.0 (1.9)	17.2 (9.5)	34.5 (9.4)	0.6
PI-PC-C (1:10:5)	45.9 (4.2)	22.8 (2.6)	1.9 (0.3)	11.2 (1.7)	5.7 (0.9)	88.4 (7.7)	0.5
G_{M1}-PC-C (1:10:5)	44.3 (10.8)	9.7 (4.6)	0.6 (0.3)	13.2 (3.8)	18.3 (7.5)	65.6 (13.4)	0.2

prolong clearance by inhibiting an increase in particle size which usually produces faster clearance (see Fig. 1 below).

Steric hindrance of a negatively-charged group by hydrophilic moieties on the liposome surface may interfere with opsonization by inhibiting both the electrostatic interactions (possibly involving divalent cations), and subsequent adsorption onto the bilayer. As to the additional role of the uncharged surface carbohydrate provided by AG_{MI}, it is quite likely that it may also be inhibiting the opsonization of liposomes by plasma proteins. A possible mechanism for this effect is discussed below, in relation to bilayer fluidity. In this case, the bulkier the group, the more effective the inhibition. The use of synthetic lipids with bulkier hydrophilic head groups, such as polyethylene glycol recently reported by several groups [4,12–16] may provide a more effective protection against opsonization.

Table II shows a significant prolongation of circulation time when phospholipids of high phase-transition temperature are used instead of EPC. Although the differential effect on clearance related to the negatively charged component is in agreement with the observations made with phospholipids of low phase-transition temperature, the differences are much smaller. This is illustrated by the relative differences in liver-to-blood ratio which can be taken as a parameter of clearance. For example, the liver-to-blood ratio of PG-PC-C is 9-fold greater than that of PI-PC-C and 24-fold greater than that of G_{MI}-PC-C (Table I). By comparison (Table II) the ratio for DPPG-DSPC-C is only 2-fold greater than for HPI-HPC-C and only 4-fold greater than for G_{MI}-DSPC-C. These smaller differences indicate that the negative charge of PG in rigid liposomes (DPPG-DSPC-C) is not nearly as effective in enhancing clearance rate as when in fluid ones (EPG-EPC-C).

Besides the relative distribution of the recovered dose among the various organs of the mouse, another important variable is the percent of injected dose recovered in the whole animal. This depends not only on the rate of leakage of radiolabel from the liposome in blood and interstitial fluid, but also on the rate of cellular metabolism and excretion of the radiolabel after liposome endocytosis. The total body recovery (Table I) tends to be lower when the liposomes are taken up faster by the liver probably due to earlier exposure of liposomes and their contents to metabolic and excretory pathways. A lower recovery also occurs when lipids with different phase transition temperatures are used (for example, SM and DSPC in Table II), a phenomenon that may be related to phase separation with a subsequent increase in leakage rate [17]. Higher body recoveries are observed when lipids with fully saturated acyl chains are used, whether blood clearance is faster (DPPG-DSPC-C) or slower (G_{MI}-DSPC-C) (Table II). Thus, in this case, the slow rate of liposome breakdown in tissues, rather than the kinetics of tissue uptake, appears to be the major factor determining the amount of liposome label recovered.

We have also investigated the effect of increasing the surface charge density and vesicle size on the clearance and tissue distribution of PI-containing liposomes. Fig. 1 shows the tissue distribution results 24 h after injection of 1 μmol phospholipid per mouse. The increase of the negatively charged component (hydrogenated PI) from 9% (column c) to 23% (column d) did not have much effect, but an increase from 23% (column d) to 41% (column e) brought about marked changes in tissue distribution, such as an increase in liver uptake and a decrease in spleen and skin uptake. The diameter of these liposomes varied only from 75 to 92 mμ. A similar phenomenon has been reported in tumor-bearing mice [18] and, more recently observed by *in vitro* studies, where the increased percentage of PI and PG results in a much higher uptake of liposomes by a mouse macrophage-like cell line [19]. In contrast, the use of vesicles with the same fraction of PI (9%) but of a larger size resulted in an increased uptake by both liver and spleen, and a decreased uptake by skin (Fig. 1, compare columns a and b with c). The effect of vesicle size on liposome clearance has

TABLE II

Tissue distribution of DSPC-containing liposome formulations 4 h after liposome injection

For experimental details, see Footnote to Table I and text.

Liposome composition (molar ratio)	% of recovered dose (%RD) per organ (S.D.)					% of injected dose recovered in whole body	Liver/Blood (ratio)
	blood	liver	spleen	carcass	skin		
DPPG-DSPC-C (1:10:5)	51.7 (6.7)	21.2 (2.0)	12.3 (0.6)	8.4 (1.4)	4.4 (0.8)	88.6 (6.2)	0.4
HSPI-DSPC-C (1:10:5)	52.8 (1.6)	18.2 (3.5)	1.4 (0.5)	14.0 (4.2)	7.3 (2.9)	78.7 (1.6)	0.3
G_{MI}-DSPC-SM-C (1:8:2:5)	56.4 (4.3)	12.5 (4.8)	1.1 (0.5)	4.2 (1.4)	5.0 (1.3)	80.3 (3.5)	0.2
G_{MI}-DSPC-C (1:10:5)	72.5 (2.6)	7.8 (0.9)	2.5 (0.2)	3.9 (0.9)	5.3 (1.1)	85.2 (2.4)	0.1

been well documented since the initial report of Juliano and Stamp [20] and more recently re-examained by Liu et al. [5]. All liposome compositions are susceptible to this effect. Large and/or aggregated vesicles are probably removed by non-specific mechanisms such as the sieving effect of sinusoids. However, an alternative explanation for the size effect could be based on low affinity, multi-ligand binding between liposomes and cells. In this case, it is possible that the small, non-aggregated liposomes with a comparatively small contact area per vesicle at the cell surface, have substantially diminished chances for a multiligand interaction conducive to binding and/or internalization.

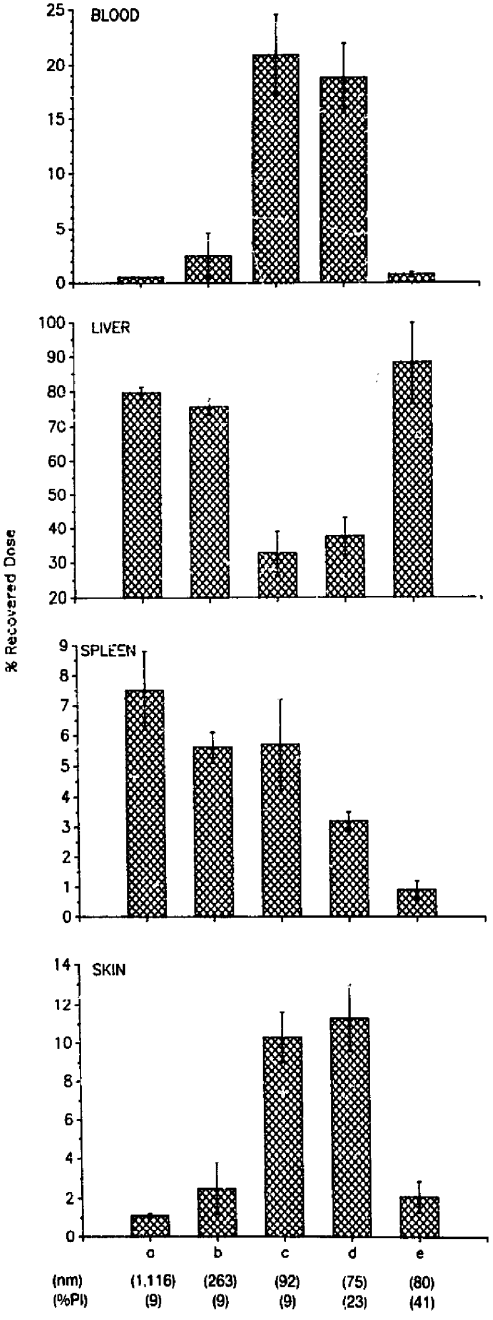

Fig. 1. Comparative effect on liposome clearance by an increase in surface charge density and an increase in vesicle size. In this experiment HSPI-HSPC-C liposomes were used. HSPC fatty acid composition consists of 80% stearic acid and, as reported previously [3] can replace DSPC without any significant changes in the pharmacological properties of liposomes. Each liposome preparation was tested in 3–6 mice at a dose of 1 μmol phospholipid per mouse. Mice were killed 24 h after liposome injection. Respective molar ratios and mean sizes for liposomes shown in Fig. 1 are: a, 1:10:5 (HSPI 9%), 1116 nm±768 (unextruded); b, 1:10:5 (HSPI 9%), 263 nm±68 (extruded through 0.2 μm pore membranes); c, 1:10:5 (HSPI 9%) 92 nm±26 (extruded through 0.05 μm pore membranes); d, 2.3:7.7:5 (HSPI 23%), 75 nm±21 (extruded through 0.05 μm pore membranes); e, 4.1:5.9:5 (HSPI 41%), 80 nm±25 (extruded through 0.05 μm pore membranes).

Differences between this and other reports [5,13] on the percent of injected dose recovered may be related to the nature of the radiolabel used. In this study we used ^{67}Ga-deferoxamine, a complex which is physiologically stable (i.e. no tranfer to transferrin) and is rapidly excreted through the urine if released from the liposomes [9]. In other studies [5,13,21] the use of weaker complexes may result in binding of the radiolabel to proteins and retention in the body long after dissociation of the radiolabel from liposomes.

General discussion and conclusions

The results presented here suggest at least two (possibly complementary) mechanisms involved in the clearance of liposomes besides the effect of size. They both depend on recognition and binding to resident macrophage cells in the liver and spleen and possibly in other sites within the reticuloendothelial system. One of these mechanisms depends on absorption of plasma proteins, which are in turn recognized by cell surface receptors [22]. The other mechanism depends on direct interaction of the liposomes with cell surface ligands. In both cases, binding could be electrostatic and/or hydrophobic and would be controlled by the physicochemical configuration of the head group of the negatively (or positively) charged lipids and the fluidity of the lipid bilayer. Both of these parameters may favor or interfere with adsorption of plasma (or cellular) proteins and the subsequent removal of liposomes from the circulation. At present it is not clear which proteins

are responsible for interactions with liposomes that affect their clearance rate. Although several plasma proteins have been reported to adsorb on liposome surfaces [23,24], it is important to note that in addition to the classical 'opsonins' (including IgG and complement factors) that may act as signals for clearance by macrophage cells [22], liposome surface groups may be recognized specifically by some cells as some recent *in vitro* work indicates [19,25].

It appears that the fluidity or rigidity of the bilayer is in itself an important factor controlling the clearance rate, a fact recognized earlier [26]. The slower clearance of rigid liposomes has been found to correlate with increased stability in plasma [27]. Cholesterol has been known to increase stability in plasma [28–30] resulting in increased antitumor effect with encapsulated cytosine arabinoside [28]. Cholesterol has been recognized to decrease the permeability of liposomes [31] and also to inhibit penetration of proteins into the bilayer [32]. Liposome instability in plasma is known to be related to interactions with various circulating proteins and lipoproteins [1,33]. Such interactions may involve penetration of hydrophobic side-chains into the lipid bilayer. Moreover, penetration is likely to be inhibited in rigid bilayers, which have a low partition coefficient for hydrophobic molecules, and this can be achieved by either addition of cholesterol [32] or/and use of lipids with high phase transition temperatures [34]. Therefore, we suggest that the clearance of liposomes depends on the partial penetration of plasma proteins (and/or cell surface proteins) within the lipid bilayer, and that this interaction (hydrophobic contacts) is enhanced by some electrostatic interactions at the liposome surface. Ca^{2+} could also play an important role, as indicated by experiments examining the *in vitro* uptake of liposomes by Kupffer cells [35]. This interaction can be inhibited either by lowering the partition coefficient for penetration (such as in a rigid bilayer) or by providing steric hindrance to such penetration by bulky carbohydrate groups [8], as discussed above. This could explain why the addition of AG_{MI} prolongs the circulation time of PC-C liposomes. In addition, the rigidity of the bilayer may also inhibit the insertion of plasma free fatty acids within the bilayer, thus decreasing the chances for electrostatic interactions at the liposome surface that could enhance opsonization.

Besides the nature of the charged headgroup, the amount of surface charge also plays a critical role in liposome clearance. The latter effect depends on the recognition of a high surface charge density either through direct interaction with a receptor present in liver Kupffer cells and absent in spleen macrophages [36], or through a liver-specific plasma opsonin [37,38]. This process would be different from red cell scavenging, since the latter occurs mainly in the venous sinuses of the spleen red pulp [39]. As shown here, the effects on liposome clearance which depend on surface charge density and on particle size, clearly operate through different pathways, although the end-result in both cases is an accelerated clearance from circulation.

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