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International Journal of Pharmaceutics 156 (1997) 163–174

international journal of pharmaceutics

Effects of positive charge density on the liposomal surface on disposition kinetics of liposomes in rats

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Received 18 November 1996; received in revised form 20 February 1997; accepted 29 May 1997

Abstract

The effects of positive charge density on the liposomal surface on the disposition kinetics of liposomes in rats were investigated. The cationic liposomes with zeta potentials of about + 15 mV remained in the blood longer than did the neutral liposomes, and the hepatic uptake of these liposomes decreased. The blood clearance of the liposomes with zeta potentials under + 10 mV was comparable to that of the neutral liposomes. In contrast, the blood circulation of the liposomes with a higher positive charge density, above + 25 mV, was shortened and their hepatic uptake was almost the same as that of the neutral liposomes. The optimum value of positive charge density on the liposomal surface to prolong the residency of liposomes in the blood circulation was thus determined. A liver perfusion experiment showed that the uptake of cationic liposomes with a zeta potential of about + 15 mV was effectively suppressed in the presence of erythrocytes, while that of liposomes with a higher zeta potential were little affected. Thus, above + 15 mV, the suppressive effect of erythrocytes on the hepatic uptake of cationic liposomes decreased with the increase of the positive charge on the liposomal surface. These results cannot be explained by the binding model, and we therefore propose the ionic atmosphere model: Cationic liposomes surround the erythrocytes with a negative surface charge like the ion atmosphere of the Debye-Hückel theory. The cationic liposomes with a suitable positive charge surround the erythrocytes as an ionic atmosphere and could then escape the reticuloendothelial system (RES). The higher positively charged liposomes were taken up by the liver probably due to the shield of the negative charge of erythrocytes provided by the cationic liposomal atmosphere. © 1997 Elsevier Science B.V.

Keywords: Cationic liposome; Rat; Positive charge density; Amino-glycolipid; Liver perfusion; Blood cell

Abbreviations: Chol, Cholesterol; EPC, Egg yolk phosphatidylcholine; PGlcN, Methyl-2-amino-2-deoxy-6-*O*-palmitoyl-D-glucoside; RES, Reticuloendothelial system; SA, Stearylamine.

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PII S0378-5173(97)00184-1

1. Introduction

Liposomes have been studied over the past 30 years as both biomembrane models and as drug carriers. Liposomes have various advantages as drug carriers, being biodegradable, having low toxicity and being able to encapsulate a hydrophilic substance within an aqueous environment, lipophilic material within the lipid phase and amphipathic drugs. However, there are inevitable drawbacks to the use of liposomes in vivo. It is well established that the liver and spleen are largely responsible for the clearance of injected liposomes from the blood circulation, and uptake by the reticuloendothelial system (RES) is one of the major barriers to the use of liposomes for drug delivery (Gregoriadis, 1988; Gregoriadis and Allison, 1980). As one approach to avoid the phagocytosis of liposomes by RES, coating the liposomes with chains of polyethylene glycol (PEG) has been used to prolong the circulation time of liposomes, based on a sterical hindrance or an increase of liposomal surface hydrophilicity (Allen et al., 1991; Blume and Cevc, 1993; Illum et al., 1986; Papahadjopoulos et al., 1991). Cationic stearylamine (SA) liposomes also remain in the blood longer than do neutral or acidic liposomes (containing phosphatidylserine or phosphatidic acid) (Gregoriadis and Neerunjun, 1974), and positively charged 'lipofectin'™ liposomes containing DOTMA (*N*[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-triethylammonium chloride) have been studied as tools for the delivery of plasmid DNA and RNA into cells (Felgner et al., 1987; Felgner and Ringold, 1989; Smith et al., 1993).

We have reported that the cationic liposomes can escape phagocytosis and remain in the blood circulation for a longer time than can other liposomes (Aoki et al., 1995). We also proposed that the mechanisms of the RES-avoiding nature of the cationic liposomes involve an interaction with blood cells (Aoki et al., 1997). However, Litzinger et al. recently reported that the cationic lipofectin™ liposomes were rapidly cleared by the liver (Litzinger et al., 1996). Their findings disagree with ours. We suspected that the rapid clearance of lipofectin™ liposomes may be due to its lipid composition, i.e. a 50 mol% of charged lipid. In the present study, therefore, we examined the effect of charge density on the biodistribution of cationic liposomes in vivo following their intravenous injection. We also examined the effect of charge density on the hepatic uptake of cationic liposomes using a liver perfusion system in the presence of blood components to elucidate the mechanisms of liposome uptake.

2. Materials and methods

2.1. Materials

Egg yolk L-α-phosphatidylcholine (EPC) was kindly provided by Asahi Kasei (Iodine value 65; Tokyo, Japan). SA was purchased from Sigma (St. Louis, MO). [1,2(n)-^{3}H]-cholesteryl hexadecyl ether, [^{3}H]CHE was purchased from Daiichi (Tokyo, Japan). Tissue solubilizer Soluene-350® was purchased from Packard (Meriden, CT). Clear-sol I was obtained from Nacalai Tesque (Kyoto, Japan). All other chemicals from Wako (Osaka, Japan) were of special grade. Cholesterol (Chol) was used after recrystallization from ethanol. Water was glass-distilled twice.

2.2. Synthesis of methyl-2-amino 6-palmitoyl glucoside

Methyl - 2 - amino - 6 - palmitoyl - D - glucoside (PGlcN) was synthesized in the manner described previously (Miyajima et al., 1993).

2.3. Preparation of liposomes

Liposomes were prepared basically as described (Aoki et al., 1995). Multilamellar vesicles (MLVs) were prepared by extrusion (Olson et al., 1979). Liposomes composed of EPC and Chol (8:2, molar ratio) were used as controls (Neutral-L) and those composed of EPC, Chol and PGlcN (7:2:1, 8:2:2, 5:2:3 and 4:2:4, molar ratio) or SA (7.5:2:0.5, 7:2:1, 6.5:2:1.5 and 6:2:2, molar ratio) were used as cationic liposomes. The lipid mixtures in chloroform were evaporated to form thin lipid films. To prepare lipid-labeled liposomes, [^{3}H]CHE, as a non-exchangeable and non-degrad-

able marker, was added to the lipid mixture. The thin lipid film was dried overnight in vacuo, then hydrated with phosphate-buffered saline (PBS, pH 7.4). The suspensions were successively extruded five times each through polycarbonate filters with pore sizes of either 0.6 or 0.2 μm. The sizes of the liposomes were measured by dynamic light scattering on a Photal laser particle analyzer (LPA-3100, Otsuka, Osaka, Japan) connected to a photon correlater (LPA-3000). The zeta potential of liposomes with a diameter of approximately 200 nm was calculated using Smoluchowski's equation (Adamson, 1967) from their electrophoretic mobility in PBS (pH 7.4) at 25°C, using an electrophoretic light scattering spectrophotometer (Zetasizer 4; Malvern, Worcestershire, UK).

2.4. Studies in vivo

Liposomes labeled with [^{3}H]CHE (15 μmol of total lipid per kg) were injected into the femoral vein of three Nembutal-anesthetized male Wistar rats weighing from 180 to 200 g. At regular intervals, blood samples were collected from the jugular vein with a heparinized syringe and centrifuged at $2000 \times g$ for 1 min to obtain the plasma. To determine the accumulated amounts of the liposome in each organ at 8 h after the injection, the liver was perfused via the portal vein with isotonic saline to remove the blood.

Thereafter, the liver and spleen were collected and washed with saline. Around 50 mg of tissue samples were dissolved in Soluene-350 and neutralized with HCl, and then Clear-sol I was added. The radioactivity of samples was counted on a scintillation counter (LS5000TA, Beckman, Fullerton, CA). The tissue samples were examined in triplicate.

The plasma liposome concentrations over time data after the intravenous injection showed a bi-exponential decay profile. The time course of the concentration of lipid in plasma was therefore numerically fitted to a two-compartment model using the fitting program MULTI (Yamaoka et al., 1981). A simple analysis variance test method (t-test) was employed to assess the significance of the observed differences in the pharmacokinetics following intravenous injection of liposomes.

2.5. Perfusion of rat liver

The livers of male Wistar rats weighing from 180 to 200 g were perfused by the method of Mortimore (Mortimore et al., 1959) with some modification. The rats were anesthetized with an intraperitoneal injection of Nembutal. The abdomen was opened wide, the common bile duct was cannulated with a polyethylene tube (PE-10) and the hepatic portal vein was cannulated with a polyethylene tube (PE-160). The chest was opened and the inferior vena cava was cannulated through the right atrium with a polyethylene tube (Orion AWG-12). The inferior vena cava immediately above the renal vein was then ligated to prevent leakage of perfusate. The liver was perfused at a rate of 25 ml/min in the physiological direction, using a peristaltic pump (SJ-1215, Atto, Tokyo, Japan) with a combined bubble trap-depulser located between the pump and the portal cannula. The reservoir was 80 ml of liposomal suspension containing 5 μmol of total lipid in 10 mM HEPES/Hanks buffer (pH 7.4); this was stirred gently, maintained at 37°C, and continuously bubbled with 95% O_2/5% CO_2. The liver was initially flushed with 200 ml of buffer before being perfused with vesicle suspension in a closed loop. The perfusion was continued for 1 h, during which time 200 μl aliquots of liposomal suspension were taken from the reservoir at 5 min intervals. For the preparation of the perfusate containing the erythrocytes (Hematocrit, 1%), blood was collected into a heparinized syringe and washed three times with PBS, after which the packed erythrocytes were added to the perfusate. When the reservoir contained the blood or erythrocytes, the samples were decolored by 100 μl of 30% H_2O_2. The liposome concentration in the reservoir was determined by using [^{3}H]CHE as the lipid marker. Finally, the liver was flushed with 150 ml of buffer and collected. To determine the amounts of the liposomes in liver, the radioactivity of tissue samples was determined as described above. The viability of the liver was checked by measurement of its bile flow (> 4 μl/min).

Table 1
The physico-chemical properties of liposomes

Liposome	Lipid composition (in molar ratio)	Diameter (nm)	Zeta potential (mV)
Neutral-L	EPC:Chol (8:2)	236.8 ± 33	-4.3 ± 0.6
PGlcN10-L	EPC:Chol:PGlcN (7:2:1)	212.6 ± 32	6.6 ± 0.7
PGlcN20-L	EPC:Chol:PGlcN (6:2:2)	225.8 ± 25	15.9 ± 0.3
PGlcN30-L	EPC:Chol:PGlcN (5:2:3)	232.3 ± 24	23.3 ± 1.4
PGlcN40-L	EPC:Chol:PGlcN (4:2:4)	219.1 ± 18	28.4 ± 1.1
SA5-L	EPC:Chol:SA (7.5:2:0.5)	214.5 ± 35	8.0 ± 0.7
SA10-L	EPC:Chol:SA (7:2:1)	236.8 ± 45	15.5 ± 1.0
SA15-L	EPC:Chol:SA (6.5:2:1.5)	236.5 ± 25	26.7 ± 0.8
SA20-L	EPC:Chol:SA (6:2:2)	202.6 ± 44	32.1 ± 0.5

2.6. Zeta potential of rat erythrocytes, hepatocytes and Kupffer cells

Rat erythrocytes were washed three times with 10 mM HEPES/Hanks buffer (pH 7.4) and dispersed in the same buffer. Suspensions of rat liver cells were prepared by perfusion of the liver with collagenase (Doolittle and Richter, 1981; Seglen, 1972). The liver perfusion procedure was described above. The liver was perfused first with preperfusion buffer (Ca^{2+} and Mg^{2+}-free HEPES-buffered saline containing 0.5 mM EGTA, pH 7.2) for 10 min and then with HEPES-buffered saline containing 5 mM $CaCl_2$ and 0.05% (w/v) collagenase (type I) (pH 7.5) for 10–20 min. The perfusion rate was maintained at 20 ml/min. Following the discontinuation of perfusion, the liver was excised and the capsule membranes were removed from it. The cells were dispersed in ice-cold 10 mM HEPES/Hanks buffer (pH 7.4). The dispersed cells were filtered through cotton mesh sieves, followed by centrifugation at $50 \times g$ for 1 min. The pellets containing hepatocytes were washed twice with Hanks buffer by centrifugation at $50 \times g$ for 1 min. The supernatant containing nonparenchymal cells was similarly centrifuged twice more. The resulting supernatant was then centrifuged twice at $200 \times g$ for 2 min. Kupffer cells were subsequently purified from the nonparenchymal cells by counterflow centrifugal elutriation (SRR6Y rotor with the himac CR21, Hitachi, Tokyo, Japan) (Knook and Sleyster, 1976). The zeta potential of cells was calculated as described above.

2.7. Binding of liposomes to erythrocytes

Rat erythrocytes were washed three times with 10 mM HEPES/Hanks buffer (pH 7.4) and dispersed in the same buffer. Erythrocyte suspensions were warmed at 37°C, then mixed with the suspension of liposomes labeled with [^{3}H]CHE (hematocrit, 1%). After a 5 min incubation at 37°C, the suspension was centrifuged ($300 \times g$ for 1 min) and the radioactivity level in the supernatants was determined in the Beckman LS5000TA scintillation counter. Erythrocyte suspensions with a hematocrit of 1% contained 1×10^8 erythrocytes/ml.

3. Results

3.1. Properties of liposomes

Table 1 shows the diameter and the zeta potential of the liposomes studied here. According to the dynamic light scattering, the mean diameter of the liposomes was approximately 200 nm, with homogeneous distribution. All the liposomes containing basic lipid showed positive zeta potential values. The difference of zeta potentials of the liposomes containing the same mol% of PGlcN and SA arises from the difference of their p*K*a. The p*K*a values of PGlcN and SA were about 7.7 and above 10, respectively; these values were obtained from their zeta potentials in various pH buffers (data not shown).

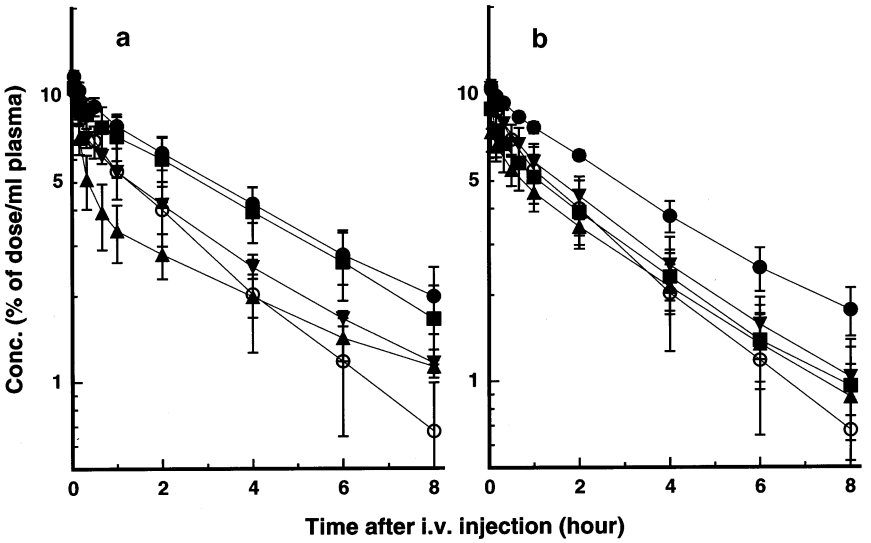

Fig. 1. Plasma clearance of PGlcN-liposomes (a) and SA-liposomes (b) in rats after an intravenous injection. The dose was 15 μmol of total lipid per kg. The liposomes with a diameter of 200 nm were labeled with [^{3}H]CHE. Each value is expressed as a percentage $\pm$ S.D. of the administered [^{3}H]CHE radioactivity per ml of plasma, $n = 3$. (a) (○), Neutral-L; (▼), PGlcN10-L; (●), PGlcN20-L; (■), PGlcN30-L; (▲), PGlcN40-L. (b) (○), Neutral-L; (▼), SA5-L; (●), SA10-L; (■), SA15-L; (▲), SA20-L.

3.2. Studies in vivo

The radioactivity of liposomes in rat plasma after intravenous injection is shown in Fig. 1. The radioactivity of the liposomes containing 10 mol% of PGlcN (PGlcN10-L) in the plasma was slightly larger than that of the controls (Fig. 1a). The hepatic uptake of PGlcN10-L at 8 h after the intravenous injection was not significantly different from that of controls (Fig. 2a) ($p > 0.05$). The radioactivity of the liposomes containing 20 (PGlcN20-L) or 30 (PGlcN30-L) mol% of PGlcN in the plasma was higher than that of the controls at every point determined (Fig. 1a). The uptakes of PGlcN20-L and PGlcN30-L in the liver were decreased compared with that of the controls (Fig. 2a). The radioactivity of the liposomes containing 40 mol% of PGlcN (PGlcN40-L) was rapidly eliminated from the plasma within 1 h after the injection, and thereafter decreased slowly in the plasma. The uptake of PGlcN40-L in the liver was slightly higher than that of Neutral-L.

In the case of SA, the radioactivity of the liposomes containing 5 mol% of SA (SA5-L) in the plasma was slightly higher than that of the controls. The radioactivity of the liposomes containing 10 mol% of SA (SA10-L) in the plasma was greater than that of the neutral liposomes, and the hepatic uptake of SA10-L was decreased. However, the radioactivity of the liposomes containing 15 (SA15-L) or 20 (SA20-L) mol% of SA in the plasma after the injection was less than that of the controls, and the hepatic uptakes of SA15-L and SA20-L were almost the same as that of the controls.

Fig. 3 shows the zeta potentials of liposomes versus the area under the curve (AUC) and mean residence time (MRT) obtained from a fitting program (MULTI). The estimated AUCs and MRTs of PGlcN10-L and SA5-L were not significantly different from those of the controls ($p > 0.05$). The AUCs and MRTs of PGlcN20-L, PGlcN30-L and SA10-L were about twice as large as those of the controls. The AUCs of PGlcN40-L, SA15-L and SA20-L were comparable to that of the controls, although their MRTs were larger than that of the controls. The maximum points in AUC and MRT were obtained at about 15 mV regardless of lipid (PGlcN or SA). Thus, the AUC and MRT of cationic liposomes with zeta poten-

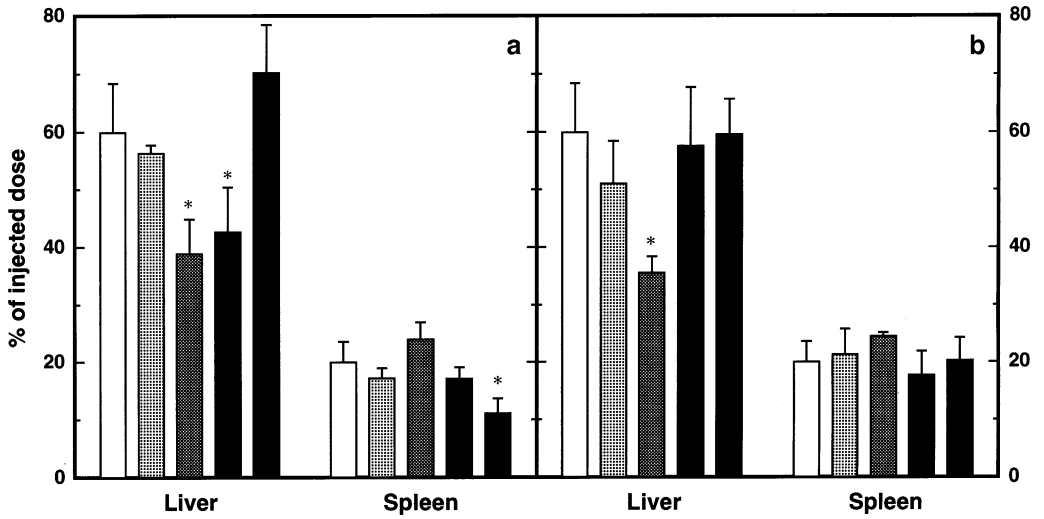

Fig. 2. Tissue distribution of cationic liposomes in the rat 8 h after an intravenous injection. The dose was 15 μmol of total lipid per kg. The liposomes were labeled with [^{3}H]CHE. Each value is expressed as a percentage $\pm$ S.D. of the administered [^{3}H]CHE radioactivity per total organ, $n = 3$. (a) (empty bars), Neutral-L; (light grey bar), PGlcN10-L; (medium grey bar), PGlcN20-L; (dark grey bar), PGlcN30-L; (black bar), PGlcN40-L. (b) (empty bar), Neutral-L; (light grey bar) SA5-L; (medium grey bar), SA10-L; (dark grey bar), SA15-L; (black bar), SA20-L.
* Significant difference from the value of Neutral-L ($P < 0.05$).

tials of about $+15$ mV were largest, and the uptake of these liposomes in the liver was the most strongly suppressed.

3.3. Perfusion of rat liver

Table 2 shows the hepatic uptake of liposomes after 1 h of recirculating perfusion. Neutral liposomes were slightly taken up by the liver, and higher positively charged liposomes showed a larger uptake by the liver in the buffer. We found that erythrocytes are an important factor for the escape from RES uptake of the cationic liposomes (Aoki et al., 1997). We therefore examined the effect of blood components on the hepatic uptake of liposomes. The addition of rat blood into the perfusate caused the low hepatic uptake of all cationic liposomes, but high hepatic uptake of neutral liposomes. The addition of rat serum or erythrocytes into the perfusate caused low hepatic uptake of all cationic liposomes. The liver uptakes of liposomes that have a long circulation in vivo (PGlcN20-L and SA10-L) were effectively suppressed in the presence of erythrocytes in the perfusate. The hepatic uptake of those liposomes in the presence of erythrocytes was about one-tenth compared to that in buffer. In contrast, the hepatic uptake of the liposomes (SA20-L) with a higher positive charge density was depressed only slightly in the presence of rat erythrocytes in the perfusate. Thus, the suppressive effect of erythrocytes on the hepatic uptake decreased with the increase of positive charge (Table 2).

Fig. 4 shows the time courses of liposomes in the perfusate containing erythrocytes during recirculating liver perfusion. The lipid concentrations of the cationic liposomes that have a long circulation in vivo (PGlcN10-L and SA10-L) were hardly decreased in the reservoir. However, that of cationic liposomes (SA20-L) with a high positive charge density decreased rapidly over the first 10–20 min, then decreased gradually.

Fig. 5 shows the effect of the amount of erythrocytes in the reservoir on the hepatic uptake of cationic liposomes. With the addition of the erythrocytes to the reservoir, the hepatic uptakes of

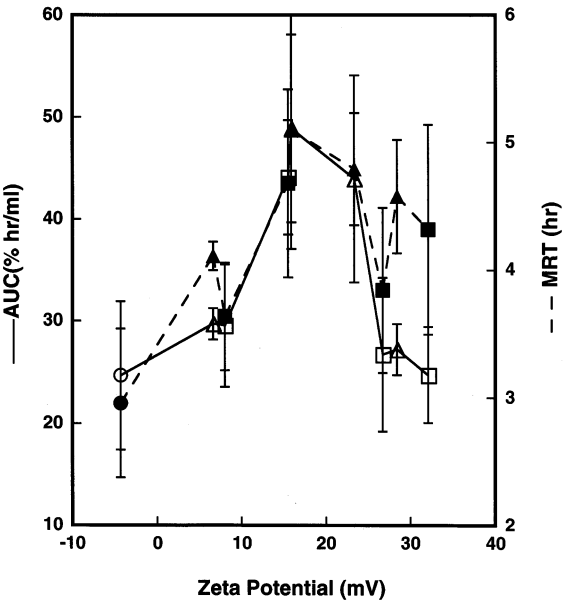

Fig. 3. Zeta potential of liposomes versus pharmcokinetics parameters. The values were obtained from the fitting program MULTI and are expressed as means ± S.D. $n = 3$. (open symbol-straight line), AUC; (closed symbol-dashed line), MRT; (○, ●) Neutral-L; (△, ▲) PGlcN10-L; (□, ■) SA-L.

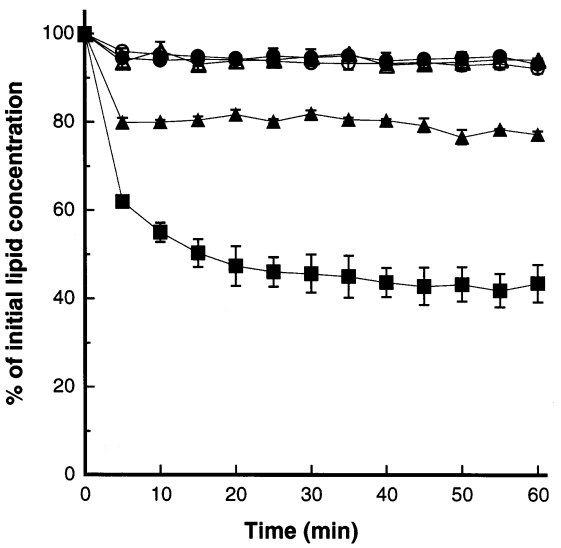

Fig. 4. Time course of the lipid concentration of the reservoir in 10 mM HEPES/Hanks buffer containing rat erythrocytes during the recirculating perfusion of the rat liver with liposomes. The liposomes were labeled with [^{3}H]CHE. The initial lipid concentrations were 62.5 μM, and each value is expressed as a percentage ± S.D. of the initial lipid concentration, $n = 3$. (△), PGlcN10-L; (○), PGlcN20-L; (●), SA10-L; (▲), SA15-L; (■), SA20-L.

PGlcN20-L and SA10-L were effectively suppressed, and the hepatic uptakes were constant at Ht ≥ 1 and 0.25% in the reservoir, respectively. In contrast, the hepatic uptake of SA20-L was little affected in the presence of the erythrocytes.

3.4. Zeta potential of cells

The zeta potential of rat erythrocytes (-20.5 ± 2.6 mV) was lower than those of the liver cells, hepatocytes (-1.7 ± 0.6 mV) and Kupffer cells (-4.3 ± 2.2 mV).

Table 2
Liposomal uptake by the liver in a perfusion system

Liposome	In buffer	+Blood (2.5% v/v)	+Serum (1.25% v/v)	+RBC (Ht = 1%)
Neutral-L	1.5 ± 0.2	6.4 ± 0.1*	5.1 ± 0.2*	ND
PGlcN10-L	6.4 ± 1.1	5.2 ± 1.8	4.0 ± 1.8	3.7 ± 1.1*
PGlcN20-L	24.4 ± 3.1	4.6 ± 0.7*	18.8 ± 5.6	2.3 ± 0.1*
PGlcN30-L	30.8 ± 4.3	6.0 ± 0.6*	19.6 ± 4.6	12.7 ± 1.0*
SA10-L	32.6 ± 3.4	8.0 ± 0.5*	5.9 ± 0.9*	4.4 ± 0.1*
SA15-L	56.2 ± 5.5	10.9 ± 0.4*	23.4 ± 1.2*	22.4 ± 0.8*
SA20-L	63.5 ± 7.9	13.2 ± 2.4*	36.3 ± 4.7*	55.8 ± 3.5

The rat livers were perfused by recirculating for 1 h with liposomes at a dose of 5 μmol lipid/liver. The liposomes were labeled with [^{3}H]CHE. The values are expressed as a % of dose ± S.D. $n = 3$.
ND, Not done.
* Significant difference from the value in buffer ($p < 0.05$).

3.5. Binding of liposomes to erythrocytes

The binding isotherms of the neutral-L, cationic PGlcN20-L and cationic SA20-L are shown in Fig. 6. Cationic, but not neutral liposomes bound to the erythrocytes. The SA20-L bound to the erythrocytes more strongly than did the PGlcN-L. The binding isotherms of the cationic PGlcN20-L and SA20-L assumed a sigmoidal curve. This is because the cationic lipids PGlcN was distributed throughout the erythrocytes and the liposomal surface charge decreased markedly at a low lipid concentration, whereas the liposomal surface charge decreased little at a high lipid concentration (data not shown). Cationic lipid SA is probably distributed throughout the erythrocytes in the same way.

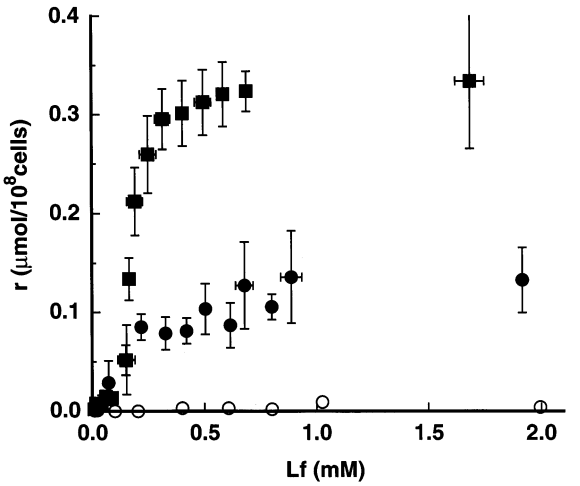

Fig. 6. Binding isotherms of liposomes to rat erythrocytes at 37°C in 10 mM HEPES/Hanks buffer. The liposomes labeled with [^{3}H]CHE were incubated with rat erythrocytes at 37°C for 5 min (hematocrit, 1%), then mixtures were centrifuged ($300 \times g$ for 1 min) and the radioactivity level in the supernatants was determined. (○), Neutral-L; (●), PGlcN20-L; (■), SA20-L.

4. Discussion

The in vivo behavior of the cationic liposomes with a zeta potential below + 10 mV was comparable to that of neutral liposomes. The cationic liposomes with a zeta potential of about + 15 mV remained in the blood longer and accumulated less in the liver than did the neutral liposomes in vivo. However, more positively charged liposomes accumulated more in the liver, comparable to controls. Some investigators have shown that the in vivo behavior of cationic liposomes was the same as that of neutral liposomes (Kirby et al., 1980; Zalipsky et al., 1994). Their cationic liposomes contained only about 5 mol% of charged lipid; consequently, the zeta potential of their liposomes would be small, and the behavior of the cationic liposomes would be not different from that of neutral liposomes. Cationic liposomes containing 10 mol% of SA were reported to remain in the blood longer than neutral liposomes (Abraham et al., 1984; Gregoriadis and Neerunjun, 1974). Their findings agree with ours. Cationic liposomes containing around 50% of basic lipid (such as lipofectin™) were reported to accumulate in the liver rapidly after intravenous injection (Litzinger et al., 1996). Our present experiment also showed that cationic liposomes with a zeta potential of 25 mV or above accumulated in the liver.

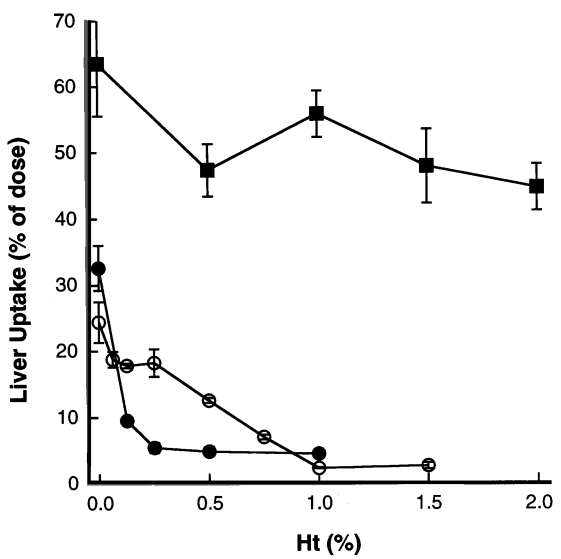

Fig. 5. The hepatic uptake of liposomes in the presence of different rat erythrocyte levels after 1 h of recirculating perfusion. The liposomes were labeled with [^{3}H]CHE. The rat liver was perfused at a dose of 5 μmol of total lipid/rat for 1 h, then the radioactivity level in the liver was determined. Each value is expressed as a percentage ± S.D. of the administered [^{3}H]CHE radioactivity, $n = 3$. (○), PGlcN20-L; (●), SA10-L; (■), SA20-L.

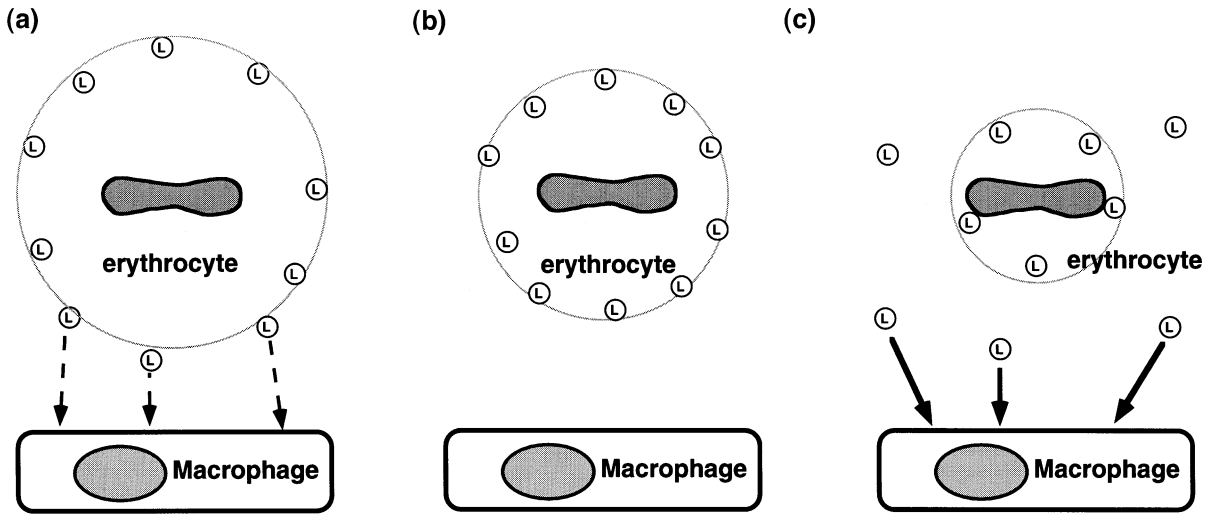

Fig. 7. Schematic diagrams for the proposed model of the interaction of cationic liposomes with erythrocytes and macrophages. (a) cationic liposomes with zeta potentials under +10 mV; (b) cationic liposomes with zeta potentials of about +15 mV, and (c) cationic liposomes with zeta potentials of above +20 mV.

The liver perfusion experiments demonstrated that the hepatic uptake of liposomes increased in the buffer with an increased surface positive charge of liposomes. In general, cell surfaces bear a net negative charge, and the zeta potential of Kupffer cells was −4 mV. These results indicate that the electrostatic attraction between cationic liposomes and liver cells plays an important role in uptake in the buffer (Mutsaers and Papadimitriou, 1988). In the presence of rat whole blood or serum in the perfusate, an enhancement of hepatic uptake of neutral liposomes was observed. We believe that this is due to the action of opsonins (Chonn et al., 1992; Liu et al., 1995; Moghimi and Patel, 1988, 1989; Patel, 1992). However, we observed in the present study that the addition of rat whole blood or erythrocytes to the perfusate caused a decreased hepatic uptake of cationic liposomes containing PGlcN or SA. The zeta potential of rat erythrocytes was −20 mV, lower than that of the Kupffer cells; therefore the cationic liposomes would preferentially interact with erythrocytes and escape the hepatic uptake. The hepatic uptake of cationic liposomes with a zeta potential of about +15 mV was optimal for the depression in the presence of rat erythrocytes, and was one-tenth of the uptake in the buffer, whereas the uptake of liposomes with a higher zeta potential was only slightly affected by the erythrocytes. For example, the hepatic uptake of SA20-L in the presence of erythrocytes was around 88% of that in buffer.

Why does the suppressive effect of erythrocytes on the hepatic uptake decrease with the increase of the surface positive charge of liposomes? Given that cationic liposomes bind to the erythrocytes through electrostatic attraction and that the RES cannot recognize the liposomes bound on the erythrocytes as foreign substances, higher positively charged liposomes are thought to bind to the erythrocytes strongly; the number of free liposomes thus decreases and few are accumulated in the liver. In practice, higher positively charged liposomes (SA20-L) were observed to bind to the erythrocytes more strongly than did PGlcN20-L. However, higher positively charged liposomes (SA20-L) are accumulated in the liver in vivo and in situ. Therefore, the RES-avoiding mechanisms of cationic liposomes cannot be interpreted in terms of this binding model.

We therefore propose the following model: Cationic liposomes surround the erythrocytes with a negative surface charge like the ion atmosphere of the Debye-Hückel theory. The distance

between particles depends on the concentration and surface charge density of cationic liposomes. As seen in Fig. 7a, the liposomes with low positive charge surround the erythrocytes like an ionic atmosphere. The distance of the closest approach is large. In this situation, the liposome can interact with Kupffer cells as do neutral liposomes. In contrast, the liposomes with a high positive charge also surround the erythrocytes; however, the number of liposomes that interact with erythrocytes is small probably due to the shielding of the negative charge of erythrocytes by cationic liposomes, and the distance of the closest approach is small. The number of residual liposomes is therefore large, and the liposomes can interact strongly with Kupffer cells mainly by electrostatic interaction (Fig. 7c). At medium positive charge ($+15$ mV), a fairly large number of liposomes interact with erythrocytes. The number of free liposomes is not so large and the electrostatic interaction between liposomes and Kupffer cells is not as strong as that of the high positive charge density liposomes (Fig. 7b). This may be the reason the liposomes with medium positive charge stay for a relatively long time in the blood circulation. This model is thus based on the concept that the balance of two electrostatic attractions (between liposomes and erythrocytes, and between liposomes and Kupffer cells) is the determining factor of the liver uptake of cationic liposomes.

The maximum points in the AUC and MRT were obtained at about 15 mV. However, the MRTs of the cationic liposomes with a higher zeta potential than $+15$ mV were still greater than that of the controls and significantly different from that of the control liposomes in the in vivo experiment ($p < 0.05$). These phenomena are explicable by the outlined above: i.e. when the higher positively charged liposomes that cannot interact with erythrocytes due to the shield of its negative charge are rapidly taken up by the RES due to the strong electrostatic interaction right after intravenous injection, and the liposomal concentration in the blood circulation decreases, the RES-uptake of residual cationic liposomes that interact with the erythrocytes electrostatically will thereafter be suppressed. Thus, the AUC of higher positively charged liposomes was small due to their rapid decrease right after the intravenous injection, but their MRT was longer than that of the controls due to slow decrease of liposomes at the low concentration in blood. In the liver perfusion experiment using higher positively charged liposomes (SA15-L and SA20-L), the same phenomena were observed; the liposomal lipid concentration rapidly decreased during the first 10 min and thereafter slowly decreased in the reservoir. Thus, the in vivo behavior of cationic liposomes is well explained by our model. The cationic liposomes with a charge lower than 10 mV, such as PGlcN10-L and SAS-L, would not be able to sufficiently interact with erythrocytes and be taken up by the RES, the same as the neutral liposomes.

The hepatic uptake of cationic liposomes was also suppressed in the presence of rat serum by liver perfusion. Serum components (albumin and globulin) prevent the electrostatic attraction of cationic liposomes between cells and cause a decrease in the uptake of the liposomes (Tyrrell et al., 1977). In the case of cationic liposomes, serum components would act as a dysopsonin to decrease the electrostatic attraction. The rat whole blood causes a decrease in the hepatic uptake by a synergistic effect of erythrocytes and serum.

SA and PGlcN were each used as a basic lipid, which is a single-chain acyl compound like a fatty acid, and can leave the liposomal membrane. Therefore, cationic liposomes containing a small amount of DOTMA, which has two long chain fatty acids, or DC-chol (3β-(*N*-(*N*′,*N*′-dimethylaminoethane)carbamoyl)cholesterol), which has a sterol skeleton, would be expected to have the ability to avoid the RES. In a preliminary experiment, cationic liposomes containing 2–3 mol% of DPTAP (1,2-dipalmitoyl-3-trimethylammonium propane) showed long blood circulation.

In conclusion, the liposomal surface charge density is an important factor to determine the fate of liposomes in circulation. It was found that the positive charge density on the liposomal surface of around $+15$ mV as a zeta potential is optimal to prolong the residency of cationic liposomes in the blood circulation. It is speculated that such cationic liposomes would interact with the erythrocytes as an ionic atmosphere and

escape the RES; if cationic liposomes bear a more positive charge, the electrostatic attraction between liposomes and liver cells would increase by the shielding of the negative charge of erythrocytes by a cationic liposomal atmosphere, and the liposomes would be taken up by the liver due to the strong electrostatic interaction. In this way, the hepatic uptake of liposomes can be controlled by the addition of a quantity of positive charge to the liposomal surface.

Acknowledgements

This work was supported in part by a Grant-in-Aid (No. 06772103) for the Encouragement of Young Scientists from the Ministry of Education, Science and Culture of Japan.

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