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Targeted and sustained drug delivery using PEGylated galactosylated liposomes

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Abstract

To achieve a sustained and targeted delivery of liposomes to liver parenchymal cells (PC), we modified distearoyl-L-phosphatidylcholine (DSPC)/cholesterol (Chol) (60:40) (DSPC/Chol) liposomes with a galactosylated cholesterol derivative (Gal-C4-Chol), and polysorbate (Tween) 20 or 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-polyethylene glycol (PEG_x-DSPE). After intravenous injection, DSPC/Chol/Gal-C4-Chol (60:35:5) (Gal) liposomes were rapidly eliminated from the blood circulation and mostly recovered in the liver. The blood elimination of DSPC/Chol/Gal-C4-Chol/Tween 20 (55:35:5:5) (Tween 20-Gal) liposomes was slightly reduced as compared to Gal-liposomes. In contrast, a significant reduction in the blood elimination was observed with DSPC/Chol/Gal-C4-Chol/PEG_{2000}-DSPE (59:35:5:1) (PEG_{2000}-Gal) liposomes. Hepatic uptake of DSPC/Chol/Gal-C4-Chol/PEG_{350}-DSPE (59:35:5:1) (PEG_{350}-Gal) liposomes was intermediate between PEG_{2000}-Gal-liposomes and Tween 20-Gal-liposomes. The uptake of PEG_{350}-Gal-liposomes by liver PC was 7.7-fold higher than that by non-parenchymal cells (NPC). These results suggest that PEG_{350}-DSPE can control the delivery rate of Gal-liposomes to liver PC without losing its targeting capability.
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Keywords: Sustained delivery; Targeting; Galactosylated liposomes; PEGylation; Hepatocytes

1. Introduction

Receptor-mediated drug targeting is a promising approach to selective drug delivery. One particular method exploits the mechanisms of sugar recognition that specific cell types possess (Hashida et al., 1995). Among the various cell types in the body, hepatocytes exclusively express a large number of high affinity cell-surface receptors that can bind asialoglycoproteins and subsequently internalize them to the cell interior. Delivery of drugs using liposomes bound to asialoglycoprotein receptors in a specific manner would provide significant therapeutic benefits in hepatic disease. To this end, extensive studies on chemical modification of liposomes with asialoglycoproteins (Tsuchiya et al., 1986; Hara et al., 1988; Wu et al., 1998) or low-molecular weight glycolipid have been carried out to achieve effective targeting to hepatocytes (Spanjer and Scherphof, 1983; Ghosh and Bachhawat, 1991; Kawakami et al., 1998, 2000a). In our laboratory, we have developed a novel galactosylated cholesterol derivative, i.e. cholesten-5-yloxy-*N*-(4-((1-imino-2-D-thiogalactosylethyl)amino)butyl) formamide (Gal-

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C4-Chol), to modify liposomes with galactose moieties for hepatocyte targeting. We have demonstrated that Gal-C4-Chol-containing liposomes were taken up selectively by hepatocytes via the asialoglycoprotein receptor-mediated mechanism (Kawakami et al., 1998, 2000c).

Although galactosylated liposomes can successfully deliver to the liver, they may not necessarily be effective from the pharmacokinetic point of view. Since the recognition of galactosylated liposomes by the receptors is highly efficient, the liposomes can be rapidly delivered to the target cells. Therefore, the concentration–time profile in the target was rapidly switched to the elimination phase, which in turn, would result in a short duration of drug exposure (Levy, 1987). To avoid frequent repeated administration, the rate of drug delivery to the target needs to be reduced by some means.

The purpose of this study was to examine the feasibility of polyethylene glycol (PEG) coating on galactosylated liposomes to achieve controlled delivery of the liposomes via a receptor-mediated process. Since PEG is known to reduce the interaction of liposomes with biological components (Lasic et al., 1991; Woodle, 1993; Oku and Namba, 1994; Harris et al., 2001; Bhadra et al., 2002), we assumed that coating with PEG retards asialoglycoprotein receptor-mediated uptake of galactosylated liposomes. We report the importance of the chain length of the PEG on controlling the rate of uptake of PEGylated galactosylated liposomes.

2. Materials and methods

2.1. Materials

Distearoyl-L-phosphatidylcholine (DSPC) and cholesteryl chloroformate were purchased from Sigma Chemical Co. (St. Louis, MO). 1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-polyethylene glycol (PEG_x-DSPE), where x represents an average molecular weight of PEG (2000 or 350), was purchased from Avanti Polar Lipids (Alabaster, AL). *N*-(4-Aminobutyl) carbamic acid *tert*-butyl ester was obtained from Tokyo Chemical Industry (Tokyo, Japan). Tween 20, cholesterol (Chol) and Clear-Sol were obtained from Nacalai Tesque (Kyoto, Japan) and Soluene 350 was purchased from Packard (Groningen, The Netherlands). [^{3}H]cholesteryl hexadecyl ether (CHE) was purchased from NEN Life Science Products Inc. (Boston, MA). All other chemicals were of the highest purity available.

2.2. Synthesis of cholesten-5-yloxy-N-(4-((1-imino-2-D-thiogalactosylethyl)amino)butyl) formamide (Gal-C4-Chol)

Gal-C4-Chol was prepared as reported previously (Kawakami et al., 1998). Cholesteryl chloroformate and *N*-(4-aminobutyl) carbamic acid *tert*-butyl ester were reacted in chloroform for 24 h at room temperature. After incubating the reaction mixture with trifluoroacetic acid for 4 h at 4 °C, the solvent was evaporated in vacuo to yield *N*-(4-aminobutyl)-(cholesten-5-yloxyl) formamide. This product was then reacted with an excess amount of 2-imino-2-methoxyethyl-1-thiogalactoside in pyridine containing triethylamine for 24 h at room temperature. After the reaction mixture was evaporated in vacuo, the resultant material was suspended in water, and dialyzed against distilled water for 48 h using a semi-permeable membrane (12 kDa cut-off). After the dialyzate was lyophilized, the crude product was purified three times by recrystallization with ethyl acetate.

2.3. Preparation of liposomes

A control liposome was composed of DSPC and cholesterol at a molar ratio of 60:40. Gal-liposomes contained DSPC, cholesterol, and Gal-C4-Chol at a molar ratio of 60:35:5. One percent of DSPC was replaced by PEG_{350}-DSPE or PEG_{2000}-DSPE to yield PEG_{350}-Gal-liposomes and PEG_{2000}-Gal-liposomes, respectively. Tween 20-Gal-liposomes, used for comparison, were composed of DSPC, cholesterol, Gal-C4-Chol, and Tween 20 at a molar ratio of 55:35:5:5. The lipid mixture was dissolved in chloroform, vacuum-desiccated, and resuspended in 5 ml sterile phosphate-buffered saline (pH 7.4). The suspension was sonicated (200 W, 3 min) and the resulting liposomes were extruded through 200 nm (five times) and 100 nm (five times) polycarbonate membrane filters using an extruder device preheated to 60 °C. The particle sizes of the liposomes were measured in a dynamic light scattering spectrophotometer

(LS-900, Otsuka Electronics, Osaka, Japan). Radiolabeling of the liposomes was done by the addition of [^{3}H]CHE (4 μCi/mg total lipid) to the lipid mixture.

2.4. In vivo distribution study

Five-week-old male ddY mice (25.0–30.0 g) were obtained from Shizuoka Agricultural Co-operative Association for Laboratory Animals (Shizuoka, Japan). [^{3}H]labeled (1.0 μCi/100 μl) liposomes were injected into the tail vein of mice at a dose of 25 mg/kg. At given times, blood was collected from the vena cava under anesthesia, and mice were killed. The liver, kidney, spleen, heart, and lung were excised, washed with saline, blotted dry, and weighed. Ten microliters blood, and a small piece of each tissue were incubated with 0.7 ml Soluene 350 overnight at 45 °C. Following digestion, 0.2 ml isopropanol, 0.2 ml 30% hydroperoxide, 0.1 ml 5N HCl, and 5.0 ml Clear-Sol I were added in this order. The samples were stored overnight and the radioactivity was measured using a liquid scintillation counter (LSA-500, Beckman, Tokyo, Japan).

2.5. Calculation of organ clearance

Tissue distribution data were evaluated using organ distribution clearances as reported previously (Takino et al., 1998). Briefly, the tissue uptake rate can be described by the following equation:

$$\frac{dX_t}{dt} = CL_{uptake} \times C_b \quad (1)$$

where X_t is the amount of [^{3}H]labeled liposomes in the tissue at time t, CL_{uptake} is the tissue uptake clearance, and C_b is the blood concentration of [^{3}H]labeled liposomes. Integration of Eq. (1) gives

$$X_t = CL_{uptake} \times AUC_{(0-t)} \quad (2)$$

where $AUC_{(0-t)}$ represents the area under the blood concentration–time curve from time 0 to t. The CL_{uptake} value can be obtained from the initial slope of a plot of the amount of [^{3}H]labeled liposomes in the tissue at time t (X_t) versus the area under the blood concentration–time curve from time 0 to t ($AUC_{(0-t)}$).

2.6. Hepatic cellular localization

Mice were anesthetized with pentobarbital sodium (40–60 mg/kg) and intravenously injected with [^{3}H]labeled liposomes (0.5–1.0 μCi/100 μl). Their body temperatures were kept at 37 °C with a heat lamp during the experiment. At 24 h after administration, the liver was perfused first with Ca^{2+}, Mg^{2+}-free perfusion buffer (10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 137 mM NaCl, 5 mM KCl, 0.5 mM NaH_2PO_4, and 0.4 mM Na_2HPO_4, pH 7.2) for 10 min and then with perfusion buffer supplemented with 5 mM $CaCl_2$ and 0.05% (w/v) collagenase (type I) (pH 7.5) for 10 min. As soon as the perfusion started, the vena cava and aorta were cut and the perfusion rate was maintained at 3–4 ml/min. Following the discontinuation of perfusion, the liver was excised and its capsular membranes were removed. The cells were dispersed in ice-cold Hank's-HEPES buffer containing 0.1% BSA by gentle stirring. The dispersed cells were filtered through cotton mesh sieves, followed by centrifugation at $50 \times g$ for 1 min. The pellets containing parenchymal cells (PC) were washed twice with Hank's-HEPES buffer by centrifuging at $50 \times g$ for 1 min. The supernatant containing non-parenchymal cells (NPC) was similarly centrifuged two more times. The resulting supernatant was then centrifuged twice at $200 \times g$ for 2 min. PC and NPC were resuspended separately in ice-cold Hank's-HEPES buffer (4 ml for PC and 1.8 ml for NPC). Cell numbers and viability were determined by the trypan blue exclusion method. The radioactivity in the cells (0.5 ml) was determined as for the other tissue samples.

3. Results

3.1. Liposome size

Table 1 summarizes the lipid composition and particle sizes of liposomes prepared. All liposomes were similar in size (a mean diameter of approximately 80 nm). The size of the liposomes remained stable for at least 1 month at 4 °C (data not shown).

3.2. Blood elimination and hepatic accumulation of [^{3}H]labeled liposomes

Fig. 1 shows the blood concentration and liver accumulation of [^{3}H]labeled DSPC/Chol-, Gal-, Tween 20- and Tween 20-Gal-liposomes after intravenous

Table 1
Lipid composition and the mean diameter of liposomes

Liposomes (molar ratio)	Particle size (nm)[a]
DSPC/Chol (60:40)	94.6 ± 6.3
DSPC/Chol/Gal-C4-Chol (60:35:5)	84.0 ± 14.8
DSPC/Chol/Tween 20 (55:40:5)	94.2 ± 22.1
DSPC/Chol/Gal-C4-Chol/Tween 20 (55:35:5:5)	84.7 ± 20.6
DSPC/Chol/PEG_{350}-DSPE (59:40:1)	87.5 ± 16.3
DSPC/Chol/Gal-C4-Chol/PEG_{350}-DSPE (59:35:5:1)	75.8 ± 13.8
DSPC/Chol/PEG_{2000}-DSPE (59:40:1)	68.8 ± 13.3
DSPC/Chol/Gal-C4-Chol/PEG_{2000}-DSPE (59:35:5:1)	75.5 ± 14.3

[a] The particle size of the liposomes was measured using a dynamic light scattering spectrophotometer. Results are expressed as the mean ± S.D. of three experiments.

injection into mice at a dose of 25 mg lipid/kg. DSPC/Chol-liposomes were retained in blood circulation over 1 h, whereas Gal-liposomes were rapidly eliminated from the blood circulation and delivered to the liver. The uptake in the liver reached approximately 80% of the dose for the 10 min post-injection. Tween 20-Gal-liposomes also showed a rapid elimination from the blood circulation in a similar manner to Gal-liposomes.

Fig. 2 shows the blood concentration and liver accumulation of Gal-, PEG_x-, and PEG_x-Gal-liposomes after intravenous injection into mice. PEGylation significantly reduced the blood elimination of Gal-liposomes. PEG_{2000}-Gal-liposomes showed a slower blood elimination rate than PEG_{350}-Gal-liposomes. On the other hand, PEG_{2000}-Gal-liposomes showed a similar distribution profile to that of PEG_{2000}-liposomes, suggesting that surface modification by PEG_{2000} completely abolished galactose-mediated hepatic uptake. In contrast, PEG_{350}-Gal-liposomes exhibited a different distribution profile from that of PEG_{350}-liposomes.

3.3. Pharmacokinetic analysis of biodistribution of [^{3}H]labeled liposomes

Table 2 summarizes the area under blood concentration–time curve and tissue uptake clearances for [^{3}H]labeled liposomes. Gal-liposomes exhibited the lowest AUC and the highest liver uptake clearance among the liposomes investigated. The liver uptake clearance of Gal-liposomes was slightly reduced when Tween 20 was incorporated. In contrast, a marked reduction of liver clearance was observed with PEG_x-Gal-liposomes. The liver uptake clearance of PEG_{2000}-Gal-liposomes was 100 times less than that of Gal-liposomes, where there was no difference between PEG_{2000}-Gal-liposomes and PEG_{2000}-liposomes. The liver uptake clearance of PEG_{350}-Gal-liposomes was about 1/10 of that of Gal-liposomes. Hepatic uptake of PEG_{350}-Gal-liposomes was intermediate between that of Tween 20-Gal-liposomes and PEG_{2000}-Gal-liposomes.

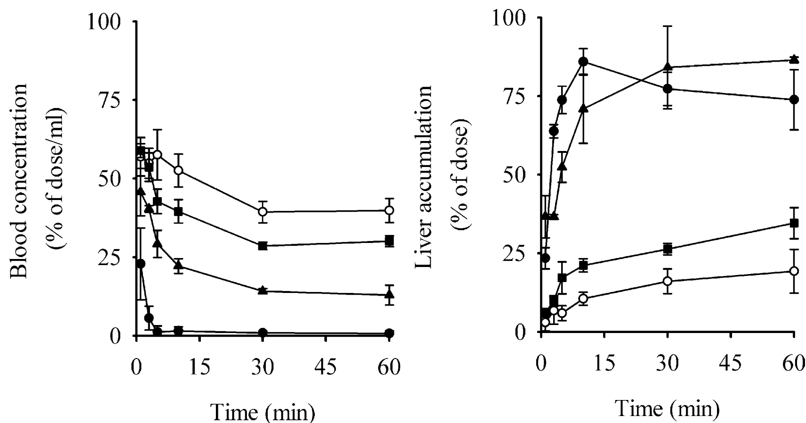

Fig. 1. Blood concentration and liver accumulation of [^{3}H]CHE-labeled DSPC/Chol (○), Gal (●), Tween 20 (■), and Tween 20-Gal (▲) liposomes after intravenous injection into mice. Results are expressed as the mean ± S.D. of three experiments.

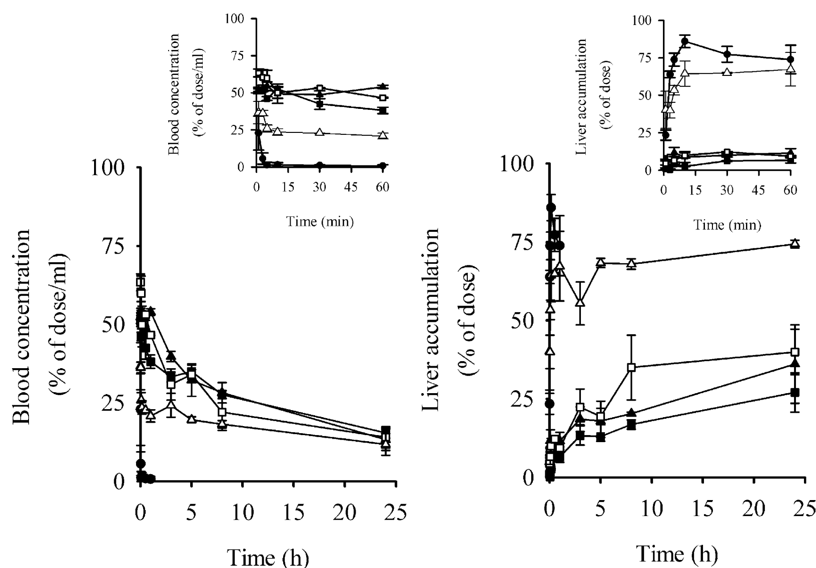

Fig. 2. Blood concentration and liver accumulation of [^{3}H]CHE-labeled Gal (●), PEG_{2000} (■), PEG_{2000}-Gal (▲), PEG_{350} (□), and PEG_{350}-Gal (△) liposomes after intravenous injection into mice. Results are expressed as the mean ± S.D. of three experiments.

Table 2
Area under plasma concentration–time curve and tissue uptake clearance of liposomes after intravenous injection in mice[a]

Liposome	AUC (% h/ml)	Clearance (μl/h)				
		CL_{liver}	CL_{spleen}	CL_{heart}	CL_{lung}	CL_{kidney}
DSPC/Chol-liposome	24.0	749	265.0	21.4	49.5	103.0
Gal-liposome	1.5	51064	2888.0	19.8	33.0	297.0
Tween 20-liposome	20.0	1392	195.0	0.9	46.1	38.2
Tween 20-Gal-liposome	5.9	14372	1702.0	83.0	192.0	291.0
PEG_{350}-liposome	27.0	534	126.0	13.2	43.6	78.6
PEG_{350}-Gal-liposome	13.0	5261	840.0	32.8	87.0	185.0
PEG_{2000}-liposome	24.0	374	104.0	15.2	62.0	163.0
PEG_{2000}-Gal-liposome	25.0	484	66.2	14.9	49.6	64.5

[a] AUC and clearance were calculated for the period until 1 h after injection. An average of three experiments is shown.

3.4. Hepatic cellular localization of [^{3}H]labeled liposomes

Fig. 3 shows the hepatic cellular localization of [^{3}H]labeled PEG_{2000}-liposomes and PEG_{2000}-Gal-liposomes at 24 h after intravenous injection in mice. PEG_{2000}-liposomes and PEG_{2000}-Gal-liposomes were recovered from both PC and NPC, with PC/NPC ratio of 0.9 and 1.7, respectively.

Fig. 4 shows the hepatic cellular localization of [^{3}H]labeled PEG_{350}-liposomes and PEG_{350}-Gal-liposomes at 24 h after intravenous injection in mice. PEG_{350}-liposomes were recovered from both PC and NPC with a PC/NPC ratio of 1.8, whereas PEG_{350}-Gal-liposomes accumulated selectively in PC with a PC/NPC ratio of 7.7.

4. Discussion

Hepatocytes are responsible for the synthesis of a wide variety of proteins and play important physiological roles in the body. In our previous investigations, we developed Gal-C4-Chol as a ligand for hepatocyte

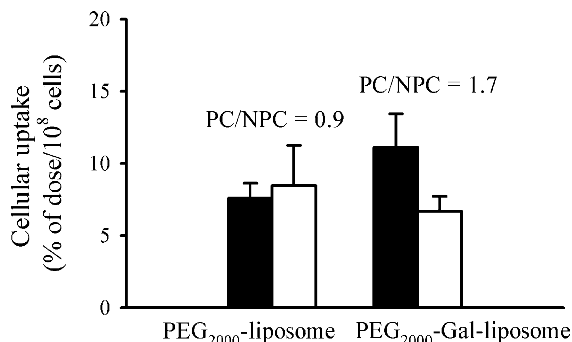

Fig. 3. Hepatic cellular localization of [^{3}H]CHE-labeled PEG_{2000}- and PEG_{2000}-Gal-liposomes after intravenous injection into mice. Radioactivity was determined at 24 h post-injection in PC (■), and NPC (□). Each value represents the mean ± S.D. of three experiments.

targeting of liposomes and examined their feasibility of drug delivery (Hattori et al., 2000; Kawakami et al., 2000b, 2000c, 2001). The biodistribution studies revealed that DSPC/Chol-liposomes modified with 5 mol% of Gal-C4-Chol (Gal-liposomes) were rapidly eliminated from the blood circulation and mainly taken up by liver PC, due to an asialoglycoprotein receptor-mediated uptake mechanism (Kawakami et al., 2000c). We also demonstrated that Gal-liposomes were able to enhance the liver uptake of highly lipophilic drugs such as prostaglandin E_1 (Kawakami et al., 2000b) and probucol (Hattori et al., 2000). Thus, Gal-liposomes appeared to be an effective targetable carrier to hepatocytes.

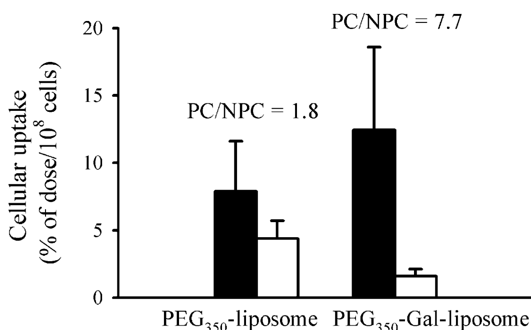

Fig. 4. Hepatic cellular localization of [^{3}H]CHE-labeled PEG_{350}- and PEG_{350}-Gal-liposomes after intravenous injection into mice. Radioactivity was determined at 24 h post-injection in PC (■), and NPC (□). Each value represents the mean ± S.D. of three experiments.

From the pharmacokinetic point of view, however, Levy (1987) pointed out that drug elimination from the site of action following its targeted delivery would be much more rapid than that of a conventionally administered dose, because of the rapid uptake of the drug, and therefore the duration of action of a targeted "bolus" dose would be shorter. Our previous study demonstrated that the rapid uptake of probucol-incorporated emulsion into the liver resulted in a low pharmacological effect (Takino et al., 1998). In some cases, rapid drug delivery by Gal-liposomes might be ineffective in terms of the duration of drug action. In the present study, therefore, we tried to reduce the hepatic uptake rate of Gal-liposomes without losing its targeting ability.

Tween 20, having branched polyoxyethylene chains, has been used as a surfactant to reduce the opsonization of latex particles (Rembaum et al., 1984). In the present study, however, Tween 20 slightly reduced the blood elimination rate of Gal-liposomes by an amount not sufficient to change the liver accumulation profile (Fig. 1). Since each branched chain of Tween 20 has only five monomer units on average, it may be too short to prevent galactose moieties from being recognized by receptors. Another reason may be the instability of Tween 20-liposomes in blood. It has been reported that a derivative of PEG with only one acyl chain was less effective in prolongation a circulation half-life of liposomes than PEG-DSPE (Allen et al., 1991). A single acyl chain may not be enough for these surfactants to be stably incorporated in the liposomes.

PEG-lipids have often been used to prevent liposomes and other lipid-based drug delivery systems from being taken up by the mononuclear phagocyte systems (MPS) (Maruyama et al., 1992; Cullis et al., 1998; Ishida et al., 2001). PEGylated liposomes have been shown to have less protein, including opsonins, adsorbed onto their surfaces (Du et al., 1997). The mechanism by which PEG protects the liposome surface has been attributed to the hydration of the polymer that effectively provides a water shell to the liposome surface; in addition the flexibility of the polymer results in steric stabilization (Lasic et al., 1991).

Two PEG-DSPE with different chain lengths, i.e. PEG_{350}-DSPE and PEG_{2000}-DSPE, were examined in this study, taking into account that the activity of PEG

derivatives in prolonging the circulation time of emulsion depends on the PEG chain length ($PEG_{2000} \geq PEG_{5000} > PEG_{1000}$, Tween 80) (Lui and Lui, 1995). Both PEG_{350}-DSPE and PEG_{2000}-DSPE prolonged a plasma half-life of Gal-liposomes, where effect of PEG_{2000}-DSPE was more remarkable as had been expected (Fig. 2 and Table 2). In addition, PEG_x-Gal-liposomes were remarkably decreased in the liver uptake clearance (Table 2). Thus, PEG-DSPE was able to reduce asialoglycoprotein receptor-mediated hepatic uptake. However, the liver uptake clearance and AUC of PEG_{2000}-Gal-liposomes was similar to that of PEG_{2000}-liposomes (Table 2). PEG_{2000}-DSPE appeared to completely inhibit receptor recognition of Gal-liposomes because of its long PEG chain. In contrast, the liver uptake clearance of PEG_{350}-Gal-liposomes was smaller than that of Gal-liposomes, but higher than PEG_{350}-liposomes (Table 2). The chain length of PEG_{350}-DSPE appeared to be appropriate in controlling receptor recognition of Gal-liposomes, as long as the spacer length of Gal-C4-Chol was conserved.

In intra-hepatic distribution studies, PEG_{350}-Gal-liposomes were taken up selectively by liver PC, with a PC/NPC ratio of 7.7. Thus, even when PEG_{350}-DSPE was incorporated, hepatic uptake of Gal-liposomes appeared to be mediated by asialoglycoprotein receptors. However, it was hard to confirm the involvement of the receptor, because there are no appropriate inhibitors. Although galactosylated bovine serum albumin has often been used as a potent competitive inhibitor (Nishikawa et al., 1995), this model protein was not effective in this case, because it is more rapidly taken up than PEG_{350}-Gal-liposomes even when injected at a high dose. Since a PC/NPC ratio of PEG_{350}-Gal-liposomes was much higher than that of PEG_{350}-liposomes, however, it was likely that PEG_{350}-Gal-liposomes were taken up by a asialoglycoprotein-mediated mechanism.

In summary, the present study revealed that the introduction of polyoxyethylene chains could control interactions of galactose moieties on liposome surfaces with the receptor. Among the surface modifiers investigated, PEG_{350}-DSPE exhibited optimal properties, marking out PEG_{350}-Gal-liposomes as a suitable carrier for hepatic parenchymal cell-specific and sustained targeting.

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