Biodistribution Characteristics of Galactosylated Emulsions and Incorporated Probucol for Hepatocyte-Selective Targeting of Lipophilic Drugs in Mice

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Purpose. Galactosylated emulsions containing cholesten-5-yloxy-*N*-(4-((1-imino-2-D-thiogalactosylethyl)amino)butly)formamide (Gal-C4-Chol) as a "homing device" were developed for hepatocyte-selective drug targeting. The targeting efficiency of galactosylated emulsions was evaluated by a distribution study in mice.

Methods. Soybean oil/EggPC/cholesterol (Chol) (weight ratio, 70:25:5) (bare) emulsions and soybean oil/EggPC/Gal-C4-Chol (weight ratio, 70:25:5) (Gal) emulsions were prepared and labeled with [^{3}H]cholesteryl hexadecyl ether (CHE). [^{14}C]probucol as a model lipophilic drug was incorporated in the emulsions or EggPC/Chol/Gal-C4-Chol (Gal) liposomes. Their tissue and intrahepatic distribution were evaluated following intravenous injection in mice.

Results. After intravenous injection, Gal-emulsions were rapidly eliminated from the blood and accumulated in the liver, in contrast to the bare-emulsions. The liver uptake clearance of Gal-emulsions was 3.2- and 1.2-times greater than that of bare-emulsions and Gal-liposomes, respectively. The uptake ratio in liver parenchymal cells (PC) and nonparenchymal cells (NPC) of Gal-emulsions was higher than that of Gal-liposomes, being 7.4 and 3.0, suggesting that Gal-emulsions are an effective PC-selective carrier. The hepatic uptake of Gal-emulsions, but not that of bare-emulsions, was significantly inhibited by the pre-dosing of not only lactoferrin but also Gal-liposomes, suggesting asialoglycoprotein receptor–mediated endocytosis. Furthermore, [^{14}C]probucol incorporated in Gal-emulsions was efficiently delivered to the liver compared with Gal-liposomes.

Conclusion. Gal-emulsions have been proven to be an alternative carrier for hepatocyte-selective drug targeting.

KEY WORDS: galactosylated emulsions; hepatocytes; lipophilic drug; targeting.

INTRODUCTION

Receptor-mediated drug targeting is a promising approach to cell-selective drug delivery (1). One particular method exploits the sugar recognition mechanisms that specific cell types possess. Receptors for carbohydrates, such as the asialoglycoprotein receptor on hepatocytes and the mannose receptor on several macrophages and liver endothelial cells, recognize the corresponding sugars on the nonreducing terminal of sugar chains. Recently, we synthesized a novel galactosylated cholesterol derivative, that is, cholesten-5-yloxy-*N*-(4-((1-imino-2-D-thiogalactosylethyl)amino)butyl) formamide (Gal-C4-Chol), to modify liposomes with galactose moieties for hepatocytes drug targeting (2). However, for certain lipophilic drugs, liposomal carrier systems are of limited use for delivery because of their restricted solubilizing capacity. Even though a drug carrier exhibits a favorable *in vivo* disposition profile, limited solubility of the incorporated drugs may lead to failure in achieving sufficient therapeutic efficacy.

Lipid emulsions are considered to be superior to liposomes due to the fact that they can be produced on an industrial scale, are stable during storage, are highly biocompatible, and have a high solubilizing capacity as far as lipophilic drugs are concerned (3,4) because lipid emulsions possess an oil phase in particulate form, so that it can dissolve large amounts of highly lipophilic drugs. In this context, lipid emulsions have widely been used as drug carriers, especially as long-circulating drug carriers for passive targeting (5–7). Cell-specific drug targeting is sometimes urgently required for a variety of clinical purposes; however, there are few reports of cell-specific drug targeting using lipid emulsions. Recently, Rensen *et al.* developed novel apo E–associated emulsions for hepatocytes targeting (8,9). These apo E–associated emulsions are reported to be selectively taken up by liver parenchymal cells and are useful for delivery of antiviral drugs, such as iododeoxyuridine, to hepatocytes. However, introduction of apo E to the carrier is rather complicated, and so there can be problems as far as the reproducibility and stability of apo E emulsions are concerned. The lipid emulsion (oil-in-water) surface exhibits aqueous properties; thus, a galactose moiety could be covered on the emulsion surface when Gal-C4-Chol was added because galactose is a hydrophilic molecule, and so the galactose moiety would be fixed on the emulsions surface.

The purpose of this study was to elucidate the biodistribution characteristics of galactosylated (Gal-) emulsions after intravenous administration as a novel drug carrier to hepatocytes. Then, we examined the applicability of probucol, which is a model lipophilic drug, to investigate the usefulness of the drug carrier. The targeting efficiency of probucol incorporated in emulsions was compared with that in EggPC/Chol/Gal-C4-Chol liposomes, which is the optimized lipid composition for the targeted delivery of probucol by Gal-liposomes (10). [^{3}H]Cholesteryl hexadecyl ether (CHE) was used as an emulsion marker (11).

MATERIALS AND METHODS

Chemicals

N-(4-aminobutyl)carbamic acid *tert*-butyl ester was purchased from Tokyo Chemical Industry (Tokyo, Japan). Cholesteryl chloroformate was obtained from Sigma Chemicals (St. Louis, MO, USA). Egg phosphatidylcholine (EggPC) and soybean oil were purchased from Wako Pure Chemicals Industry Ltd. (Osaka, Japan). Cholesterol (Chol) and Clear-Sol I were purchased from Nacalai Tesque (Kyoto, Japan). Soluene 350 was obtained from Packard (Groningen, The Netherlands). [^{3}H]Cholesteryl hexadecyl ether (CHE) was pur-

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chased from NEN Life Science Products Inc. (Boston, MA, USA). [^{14}C]Probucol was purchased from Daiichi Radioisotopes (Tokyo, Japan). All other chemicals were of the highest purity available.

Synthesis of Gal-C4-Chol

Gal-C4-Chol was synthesized by the method described previously (2). Briefly, cholesteryl chloroformate was reacted with *N*-(4-aminobutyl) carbamic acid *tert*-butyl ester in chloroform for 24 h at room temperature and then incubated with trifluoroacetic acid for 4 h at 4°C. *N*-(4-aminobutyl)-(cholesten-5-yloxyl)formamide was obtained after evaporation of the solvent. A quantity of the resultant material was added to an excess of 2-imino-2-methoxyethyl-1-thiogalactoside (12) in pyridine containing triethylamine. After 24 h incubation at room temperature, the reaction mixture was evaporated, resuspended in water, and dialyzed against distilled water for 48 h using a semipermeable membrane (12 kDa cutoff). Finally, the dialyzate was lyophilized.

Preparation of Emulsions and Liposomes

Bare-emulsions consisted of soybean oil, EggPC, and Chol at a weight ratio of 70:25:5. Gal-emulsions contained soybean oil, EggPC, and Gal-C4-Chol at a weight ratio of 70:25:5. Bare-liposomes consisted of EggPC and Chol at a molar ratio of 60:40. Gal-liposomes consisted of EggPC, Chol, and Gal-C4-Chol at a molar ratio of 60:35:5. 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 for 20 min (200 W) under a current of nitrogen. As for liposomes, after hydration, the suspension was sonicated for 3 min (200 W), and the resulting liposomes were passed through 200-nm (5 times) and 100-nm (5 times) polycarbonate membrane filters using an extruder device. The concentration of the emulsions and liposomes was adjusted to 0.5% based on radioactivity measurement so that the total EggPC, Chol, and Gal-C4-Chol content was equivalent to 0.5 g per 100 ml. Radiolabeling of the emulsions and liposomes was performed by addition of [^{3}H]CHE (500 μCi) and/or [^{14}C]probucol (50 μCi) with probucol (13.8 μg) to the lipid mixture before formation of a thin film layer. [^{14}C]Probucol dissolved serum was prepared by addition of mouse serum into a thin film of [^{14}C]probucol (50 μCi) and probucol (13.8 μg). [^{14}C]Probucol dissolved serum was then filtrated through a Mullex HV sterile filter (Millipore, Bedford, USA) before the animal experiments. The particle sizes of the emulsions and liposomes without radioisotope were measured in a dynamic light-scattering spectrophotometer (LS-900, Otsuka Electronics, Osaka, Japan). The zeta potential of emulsions and liposomes without radioisotope was measured electrophoretically using a zeta-potential analyzer (LEZA-500T, Otsuka Electronics). The density of Gal-C4-Chol on emulsions and liposomes was determined by calculating the galactose content of Gal-emulsions and liposomes using the anthrone sulfuric acid method (13).

In vivo Distribution

Five-week-old male ddY mice (25.0–30.0 g) were obtained from Shizuoka Agricultural Co-operative Association for Laboratory Animals (Shizuoka, Japan). All animal experiments were carried out in accordance with the Principles of Laboratory Animal Care as adopted and promulgated by the U.S. National Institutes of Health and the Guideline for Animal Experiments of Kyoto University. [^{3}H]CHE (1.0 μCi/100 μl) and/or [^{14}C]probucol (0.1 μCi/100 μl)-labeled emulsions or liposomes were injected into the tail vein of mice at a dose of 5 mg/kg. In the hepatic uptake inhibition experiments, various compounds were intravenously injected 1 min prior to the intravenous injection of emulsions or liposomes. At given times, blood was collected from the vena cava under anesthesia and mice were then sacrificed. The liver, kidney, spleen, heart, and lung were removed, washed with saline, blotted dry, and weighed. A complete urine collection was obtained by combining the excreted urine and that remaining in the bladder. Ten microliters of blood, 200 μl of urine, and a small amount of each tissue were digested with 0.7 ml Soluene-350 by incubating the samples overnight at 45°C. Following digestion, 0.2 ml isopropanol, 0.2 ml 30% hydroperoxide, 0.1 ml 5 N HCl, and 5.0 ml Clear-Sol I were added. The samples were stored overnight, and the radioactivity was measured using a liquid scintillation counter (LSA-500, Beckman, Tokyo, Japan).

Hepatic Cellular Localization

The separation of liver parenchymal cells and nonparenchymal cells was performed according to collagenase perfusion method (14). Briefly, mice were anesthetized with pentobarbital sodium (40–60 mg/kg) and given an intravenous injection of [^{3}H]CHE (0.5–1.0 μCi/100 μl)-labeled emulsions or liposomes. The body temperatures were kept at 37°C with a heat lamp during the experiment. Then, 30 min 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 followed by 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 by gentle stirring in ice-cold Hank's-HEPES buffer containing 0.1% BSA. The dispersed cells were filtered through cotton mesh sieves, followed by centrifugation at 50 × *g* for 1 min. The pellets containing parenchymal cells (PC) were washed twice with Hank's-HEPES buffer by centrifuging at 50 × *g* for 1 min. The supernatant containing nonparenchymal cells (NPC) was similarly centrifuged twice. The resulting supernatant was then centrifuged twice at 200 × *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). The cell numbers and viability were determined by the trypan blue exclusion method. Then, the radioactivity in the cells (0.5 ml) was determined as for the other tissue samples.

Calculation of Organ Clearance

Tissue distribution data were evaluated using the organ distribution clearances as reported previously (15). Briefly, the tissue uptake rate can be described by the following equation:

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

where X_t is the amount of [^{3}H]-labeled emulsions or 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 emulsions or liposomes. Integration of Eq. (1) gives

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

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 emulsions or liposomes in the tissue at time t (X_t) vs. the area under the blood concentration-time curve from time 0 to t [$AUC_{(0-t)}$].

Statistical Analysis

Statistical comparisons were performed using Student's unpaired t test. $p < 0.05$ was considered to be indicative of statistical significance.

RESULTS

Physicochemical Properties of Emulsions

Sinusoids in the liver lobules have a unique type of endothelial lining consisting of endothelial cells with flattened processes perforated by small fenestrae about 100 nm in size (16). Therefore, emulsions and liposomes with a diameter less than this can readily pass through the fenestration into the Disse space. Accordingly, we prepared emulsions and liposomes less than 100 nm in diameter in order to allow free access to hepatocytes.

Table I summarizes the lipid composition, particle sizes, and zeta potential of the emulsions and liposomes prepared. These emulsions and liposomes were very similar in size with a mean diameter of approximately 100 nm. As shown by the zeta potential, the surface charge of each particle was almost neutral. In addition, the particle size and zeta potential of the emulsions and liposomes were kept constant for a period of at least 2 months at 4°C (data not shown).

Biodistribution of [^{3}H]-Labeled Emulsions

[^{3}H]CHE was selected as a tracer of emulsion (11,17) and liposomes (10,11). Figures 1 and 2 show the blood concentration-, tissue accumulation-, and urine excretion-time course of [^{3}H]-labeled soybean oil/EggPC/Chol (70:25:5) (bare) emulsions, soybean oil/EggPC/Gal-C4-Chol (70:25:5) (Gal) emulsions, EggPC/Chol (60:40) (bare) liposomes, and EggPC/Chol/Gal-C4-Chol (60:35:5) (Gal) liposomes after intravenous injection. In contrast to the bare-emulsions, Gal-emulsions were rapidly eliminated from the blood circulation and mostly recovered in the liver, accounting for 75% of the dose, within 30 min. Gal-liposomes, which have the same Gal-C4-Chol density as that of Gal-emulsions, were also rapidly eliminated from the blood circulation and mostly recovered in the liver, accounting for 60% of the dose, within 30 min.

Pharmacokinetic Analysis of Biodistribution of [^{3}H]-Labeled Emulsions

To compare the disposition profiles of emulsions and liposomes, initial distribution in the early phase up to 10 min, in which the contribution of metabolites can be ignored, was quantified using tissue uptake clearances parameter.

Table II summarizes the area under blood concentration-time curve (AUC) and tissue uptake clearances calculated for the initial 10 min for liver (CL_{liver}), kidney (CL_{kidney}), spleen (CL_{spleen}), lung (CL_{lung}), heart (CL_{heart}), and urine (CL_{urine}) of the [^{3}H]-labeled emulsions and liposomes. The AUC of Gal-emulsions was much lower than that of bare-emulsions. The liver uptake clearance of Gal-emulsions was 3.2-times greater than that of bare-emulsions. Also, the liver uptake clearance of Gal-emulsions was 1.2-times higher than that of Gal-liposomes.

Effect of Gal-C4-Chol Content of Emulsions on Hepatic Accumulation

The amounts of [^{3}H]-labeled emulsions recovered in the liver at 30 min after intravenous injection of Gal-emulsions containing various amounts of Gal-C4-Chol was evaluated. The density of Gal-C4-Chol in the emulsions was calculated to be 3.0×10^{12}, 8.9×10^{12}, and 1.5×10^{13}/unit surface area (cm^2) for Gal-emulsions containing, respectively, a 1%, 3%, and 5% weight ratio of Gal-C4-Chol. All emulsions prepared have almost similar particle sizes (data not shown). As shown in Fig. 3, the liver accumulation of Gal-emulsions increased with the amount of Gal-C4-Chol in the emulsions. The relationship between the liver accumulation and the galactose density of the emulsions on the emulsions surface correlates well, suggesting that the galactose density on the surface of the emulsions is important as far as recognition by the asialoglycoprotein receptors on hepatocytes is concerned.

Table I. Lipid Composition, Mean Diameter, and Zeta Potential of the Tested Emulsions and Liposomes

Formulations	Lipid composition	Mean diameter[a] (nm)	Zeta potential[b] (mV)
Bare-emulsions	Soybean oil:EggPC:Chol (70:25:5) (weight ratio)	100.0 ± 2.3	4.0 ± 1.4
Gal-emulsions	Soybean oil:EggPC:Gal-C4-Chol (70:25:5) (weight ratio)	104.5 ± 3.8	4.7 ± 0.5
Bare-liposomes	EggPC:Chol (60:40) (molar ratio)	93.3 ± 11.2	4.4 ± 0.2
Gal-liposomes	EggPC:Chol:Gal-C4-Chol (60:35:5) (molar ratio)	96.2 ± 5.8	9.0 ± 1.7

EggPC, egg phosphatidylcholine; Chol, cholesterol.

[a] Diameter of emulsions and liposomes was measured by dynamic light-scattering spectrophotometry. Results are expressed as the mean ± SD of three experiments.

[b] Zeta potential of emulsions and liposomes was measured by electrophoretic light-scattering spectrophotometry. Results are expressed as the mean ± SD of three experiments.

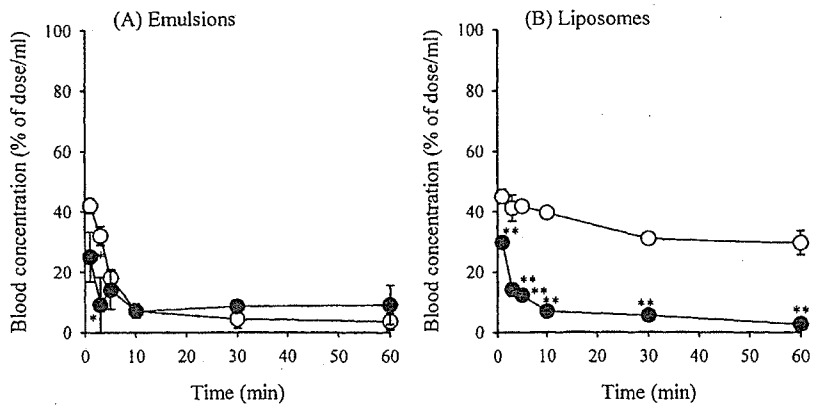

Fig. 1. Blood concentration of [^{3}H]-labeled (A) bare-(○) and Gal-(●) emulsions and (B) bare-(○) and Gal-(●) liposomes after intravenous injection into mice. Each value represents the mean ± SD of three experiments. Statistically significant differences (*$p < 0.05$, **$p < 0.01$) from control group.

Hepatic Cellular Localization of [^{3}H]-Labeled Emulsions

Figure 4 shows the hepatic cellular localization of [^{3}H]-labeled emulsions and liposomes 30 min after intravenous injection. Compared with bare-emulsions, Gal-emulsions accumulated selectively in PC with a PC/NPC ratio of 7.4. Moreover, Gal-liposomes also accumulated selectively in PC with a PC/NPC ratio of 3.0. Thus, the PC selectivity of the Gal-emulsions is higher than that of Gal-liposomes.

Inhibition of Hepatic Uptake of Emulsions by Pre-dosing Various Agents

Figure 5 shows the effect of pre-dosing with various agents on the hepatic accumulation of [^{3}H]-labeled bare- and Gal-emulsions. The liver uptake of Gal-emulsions was significantly inhibited by pre-dosing lactoferrin, which is a ligand of chylomicron remnant receptors on liver parenchymal cells, and Gal-liposomes, which contain a ligand for asialoglycopro-

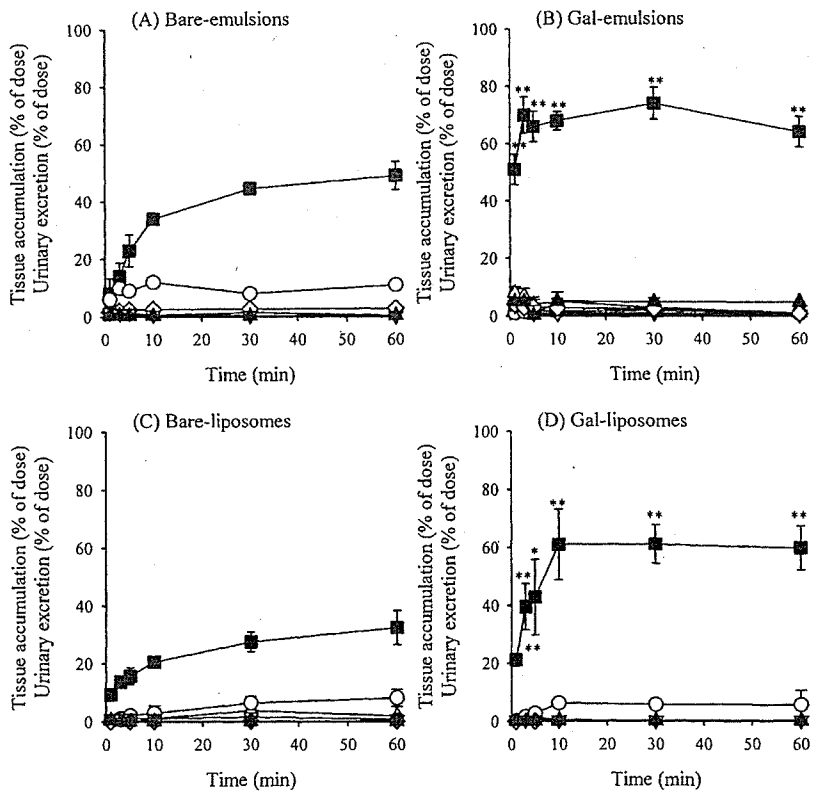

Fig. 2. Tissue accumulation of [^{3}H]-labeled (A) bare-emulsion, (B) Gal-emulsion, (C) bare-liposomes, and (D) Gal-liposomes after intravenous administration into mice. Radioactivity was determined in the liver (■), kidney (△), spleen (○), lung (▽), heart (◇), and urine (▲). Each value represents the mean ± SD of three experiments. Statistically significant differences (*$p < 0.05$, **$p < 0.01$) from each bare emulsion (A vs. B) or bare liposomes (C vs. D).

Table II. Area Under the Blood Concentration-Time Curve (AUC) and Tissue Uptake Clearance of [^{3}H]-Labeled Emulsions and Liposomes After Intravenous Injection Into Mice[a]

Formulations	AUC (% of dose × h/ml)	Clearance[a] (μl/h)					
		CL_{liver}	CL_{kidney}	CL_{spleen}	CL_{lung}	CL_{heart}	CL_{urine}
Bare-emulsions	3.7	9480	201	570	179	31	3
Gal-emulsions	1.9	31,200	1350	1040	239	705	1360
Bare-liposomes	7.0	2960	121	407	152	102	6
Gal-liposomes	2.4	25,300	313	2610	129	76	67

[a] AUC and clearance (CL) were calculated for the period until 10 min after injection. An average of three experiments is shown.

tein receptors on liver parenchymal cells, whereas no inhibition was observed in the case of bare-emulsions.

Blood Elimination and Hepatic Accumulation of [^{14}C]Probucol Incorporated into [^{3}H]-Labeled Gal-Emulsions and Gal-Liposomes

Figure 6 shows the blood concentration and liver accumulation of [^{14}C]probucol dissolved in serum, which represents the original distribution of probucol, [^{14}C]probucol incorporated into [^{3}H]-labeled Gal-emulsions and Gal-liposomes after intravenous injection. [^{14}C]Probucol was dissolved in serum to analyze the inherent distribution of probucol to investigate the controlled distribution of probucol by Gal emulsions and liposomes. Rapid blood elimination of [^{14}C]probucol was observed in Gal-emulsions, followed by Gal-liposomes. Similarly, the fastest blood elimination of [^{3}H]CHE was observed in Gal-emulsions, followed by Gal-liposomes. As for the liver accumulation, rapid liver uptake of [^{14}C]probucol was observed in Gal-emulsions followed by Gal-liposomes. Similarly, the highest hepatic uptake of [^{3}H]CHE was observed in Gal-emulsions, followed by Gal-liposomes.

The Uptake Clearance of [^{14}C]Probucol Incorporated into [^{3}H]-Labeled Gal-Emulsions and Gal-Liposomes

Table III summarizes the AUC and tissue uptake clearances of [^{14}C]probucol incorporated into [^{3}H]-labeled Gal-emulsions and Gal-liposomes. The lowest AUC was observed in Gal-emulsions followed by Gal-liposomes. The liver uptake clearance of [^{14}C]probucol incorporated into [^{3}H]-labeled Gal-emulsions was 1.6-times higher than that into Gal-liposomes.

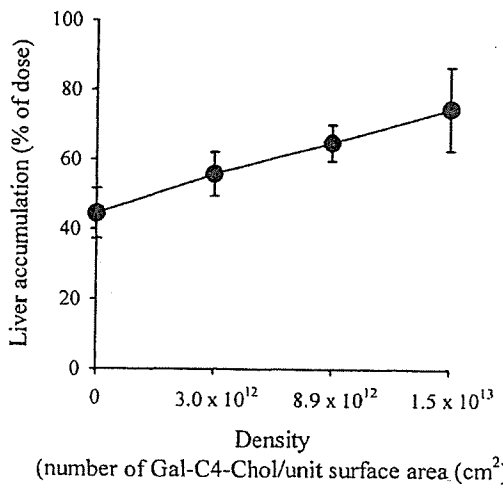

Fig. 3. Liver accumulation of [^{3}H]-labeled Gal-emulsions 30 min after intravenous administration into mice. Gal-emulsions were prepared with various amounts of Gal-C4-Chol. Each value represents the mean ± SD of three experiments.

DISCUSSION

This manuscript summarizes our initial efforts to investigate whether Gal-emulsions would offer a significant advantage as an alternative carrier for drug targeting to hepatocytes. Several investigators have demonstrated that the liposomes that were modified with native glycoproteins (18,19) or synthetic glycolipids possessing tris-galactosides (20) were efficiently recognized by asialoglycoprotein receptors. However, there are several potential problems in using these compounds due to their complicated structures, difficulty in achieving industrial-scale production, and possible antigenicity. Recently, we synthesized Gal-C4-Chol having bifunctional properties of a lipophilic anchor moiety for stable incorporation into liposomes and a galactose moiety for recognition by the asialoglycoprotein receptors (21). Our strategy for efficient targeting of liposomes by glycosylation is to achieve stable fixation of the sugar moiety on the surface of the liposomes under *in vivo* conditions. Therefore, cholesterol was chosen as a hydrophobic anchor, which is stably associated with the liposomal membrane (22,23), and only monogalactoside was introduced to the lipid as a ligand because introduction of many hydrophilic galactose moieties to a lipid anchor would result in their removal from liposomes by interaction with lipoproteins and/or other lipid compartments under *in vivo* conditions (20). In fact, we found that eggPC or

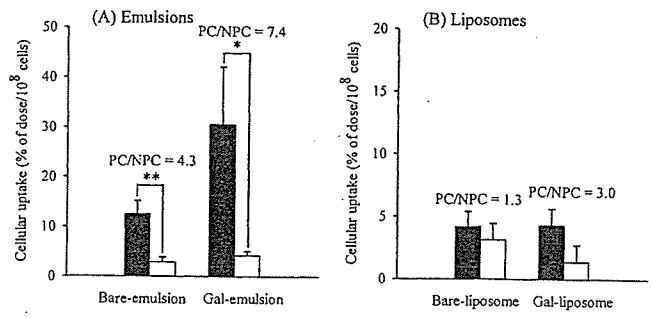

Fig. 4. Hepatic cellular localization of (A) [^{3}H]-labeled emulsions and (B) [^{3}H]-labeled liposomes after intravenous administration into mice. Radioactivity was determined 30 min postinjection in PC (■), and NPC (□). Each value represents the mean + SD of three experiments. Statistically significant differences (*$p < 0.05$, **$p < 0.01$) from control group.

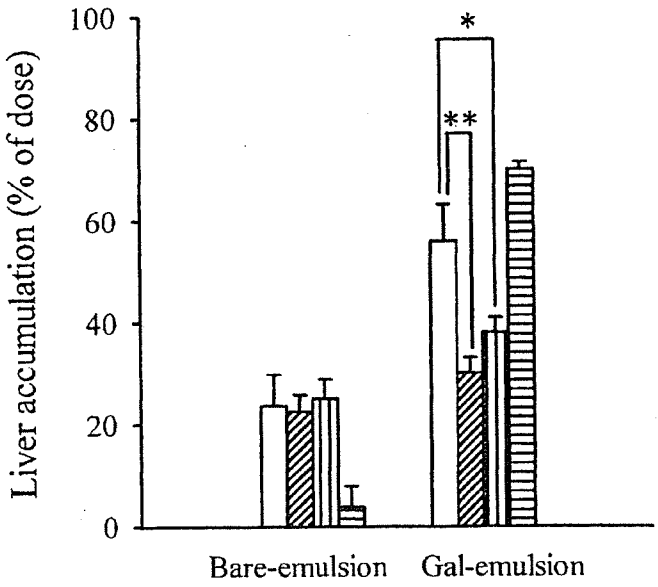

Fig. 5. Inhibition of liver uptake of [^{3}H]-labeled emulsions after intravenous preinjection of various compounds. Liver accumulation was determined at 5 min. Emulsions were injected without (□) or with preinjection of lactoferrin (▨), Gal-liposomes (▥), or an excess of bare-emulsions (▤). Each value represents the mean + SD of three experiments. Statistically significant differences (*$p < 0.05$, **$p < 0.01$) from control group.

DSPC/Chol/Gal-C4-Chol (Gal-) liposomes were rapidly taken up by hepatocytes via asialoglycoprotein receptor–mediated endocytosis (10,21). Thus, the Gal-C4-Chol combination with soybean oil, EggPC, and Chol gave emulsion formulations with suitable pharmaceutical characteristics for targeting under *in vivo* conditions.

As shown in Figs. 2 and 4, [^{3}H]-labeled Gal-emulsions exhibited marked hepatic uptake and rather high PC/NPC ratios, suggesting that Gal-emulsions were more efficiently taken up into hepatocytes compared with bare-emulsions. To investigate the uptake mechanism of Gal-emulsions, we performed a competitive inhibition experiment involving predosing lactoferrin, Gal-liposomes, and bare-emulsions (Fig. 5). Pre-dosing lactoferrin, which is a ligand of chylomicron remnant receptors on liver parenchymal cells (24), significantly inhibited the liver uptake of Gal-emulsions. It has also been reported that lactoferrin can bind to asialoglycoprotein receptors on liver parenchymal cells (24,25); therefore, this result does not make it clear whether the uptake of Gal-emulsions involves chylomycron remnant receptor– or asialoglycoprotein receptor–mediated endocytosis. However, the liver uptake of Gal-emulsions was also markedly inhibited by Gal-liposomes, which contain a ligand of asialoglycoprotein receptors (10), but was not inhibited by bare-emulsions (Fig. 5). Taking these findings into consideration, this suggests that Gal-emulsions were taken up by asialoglycoprotein-mediated endocytosis after intravenous administration.

We have established methods for introducing galactose moieties directly into various molecular species and developed various macromolecular drug carrier systems (26,27), protein derivatives (28,29), and liposomes (30–33) that show superior liver targeting via asialoglycoprotein receptor–mediated endocytosis. As far as the molecular design of the galactosylated protein is concerned, we have demonstrated that the recognition of galactosylated protein by the liver cells is based on the estimated surface density of the galactose residues (34). In this study, we showed that the galactose density on the surface of the Gal-emulsions is an important factor for recognition by the asialoglycoprotein receptors on hepatocytes, suggesting that enhancing the recognition by asialoglycoprotein receptors can be controlled by the amount of added Gal-C4-Chol. This observation correlates well with the effect of the galactose density of galactosylated liposomes, which possess a mono-galactoside, that have been studied by Murahashi *et al.* (35).

In order to achieve drug targeting by a cell-selective approach, the drug release properties from emulsions are important for drug targeting, and so we investigated the relationship between the movement of Gal-emulsions and incorporated probucol. We previously investigated the *in vivo* disposition of drugs with a variety of lipophilicities, incorporated into liposomes, lipid emulsions, and micelles, based on

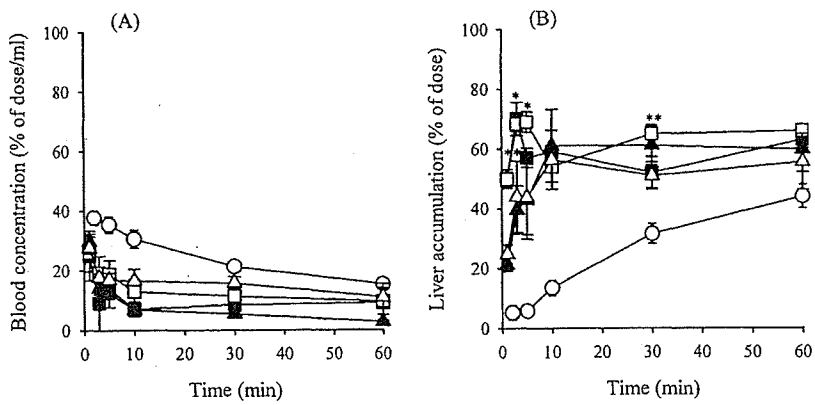

Fig. 6. (A) Blood concentration and (B) liver accumulation of [^{14}C]-labeled probucol dissolved in serum (○) or [^{3}H]CHE-labeled Gal-emulsions (■) and [^{3}H]CHE-labeled Gal-liposomes (▲) or [^{14}C]-labeled probucol incorporated Gal-emulsions (□) and [^{14}C]-labeled probucol incorporated Gal-liposomes (△) after intravenous administration into mice. Each value represents the mean ± SD of three experiments. Statistically significant differences (*$p < 0.05$, **$p < 0.01$) between Gal-emulsions and Gal-liposomes.

Table III. Area Under the Blood Concentration-Time Curve (AUC) and Tissue Uptake Clearance of [^{4}C]probucol Dissolved in Serum, [^{14}C]probucol Incorporated Emulsions and Liposomes After Intravenous Injection into Mice[a]

Formulations	AUC (% of dose × h/ml)	Clearance[a] (μl/h)					
		CL_{liver}	CL_{kidney}	CL_{spleen}	CL_{lung}	CL_{heart}	CL_{urine}
Serum	5.9	2300	60.5	1250	131	55.9	0
Gal-emulsions	2.3	28,700	540	1800	150	403	170
Gal-liposomes	3.2	17,596	680	1620	430	160	145

[a] AUC and clearance (CL) were calculated for the period until 10 min after injection. An average of three experiments is shown.

physicochemical considerations (11,15). We found that the required lipophilicity of drugs for stable incorporation into lipid carries was 10^6 for liposomes and 10^9 for emulsions based on values for the partitition coefficient between *n*-octanol and water (PC_{oct}). Also, we investigated the relationship between the movement of galactosylated liposomes and incorporated drug after intravenous injection in relation to the lipid composition of the liposomes. Thus, probucol, with a PC_{oct} of $10^{10.8}$, was selected as a model lipophilic drug having a suitable lipophilicity for incorporation of both of emulsions and liposomes. In this study, we examined the applicability of probucol to examine the usefulness of Gal-emulsions. As shown in Fig. 6, [^{14}C]probucol incorporated Gal-emulsion exhibited blood concentration and liver accumulation profile similar to that of [^{3}H]CHE emulsions and different from free [^{14}C]probucol, suggesting that probucol was stably incorporated in Gal-emulsions. In contrast, a slight difference was observed between the blood concentration and liver accumulation profiles of [^{3}H]CHE and [^{14}C]probucol in Gal-liposomes, suggesting that probucol was released from liposomes to some extent. Pharmacokinetic analysis demonstrated that the liver uptake clearance of [^{14}C]probucol incorporated into [^{3}H]-labeled Gal-emulsions was 1.6-times higher than that into Gal-liposomes. Thus, these results suggest that Gal-emulsions are more efficient carriers of probucol, which is a model lipophilic drug, for hepatocyte-selective targeting than Gal-liposomes.

In the current study, we showed that introduction of galactosylated cholesterol derivatives into emulsions can be prepared Gal-emulsions. Our current results provide evidence that introduction of ligand-grafted lipids such as mannose (36,37), fucose (38), folate (39,40), and transferrin (41), for cell-selective targeting, to emulsions also allows cell-selective targeting.

In conclusion, we have demonstrated that Gal-emulsions, which incorporate Gal-C4-Chol in emulsions, are alternative hepatocyte-selective carriers for highly lipophilic drugs. We have also demonstrated that the recognition mechanism of Gal-emulsions is mediated by asialoglycoprotein receptors, and the recognition efficiency depends on the galactose density on the emulsion surface. These observations provide information to help in the design of Gal-emulsions for hepatocyte-selective carrier systems for lipophilic drugs.

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In vivo gene transfection via intravitreal injection of cationic liposome/plasmid DNA complexes in rabbits

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Abstract

To optimize the in vivo ocular transfection efficiency of plasmid DNA (pDNA)/cationic liposome complexes, *N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA)/dioleoylphosphatidylethanolamine (DOPE) (1:1 molar ratio) liposomes and DOTMA/cholesterol (Chol) (1:1 molar ratio) liposomes were prepared with varying amounts of pDNA. pDNA/cationic liposome complexes were intravitreally injected (100 μL) in rabbits, and luciferase activity in the cornea, aqueous humor, iris–ciliary body, lens, vitreous body, and retina was measured. Transfection efficiency of pDNA alone did not change with pDNA ranging from 40 to 85 mg. In contrast, transfection efficiency of pDNA complexed with DOTMA/Chol liposomes significantly increased with the amount of pDNA ranging from 40 to 85 μg ($P < 0.05$). pDNA complexed with DOTMA/DOPE liposomes could not be prepared with pDNA greater than 60 μg. Among these experiments, pDNA (85 μg) complexed with DOTMA/Chol liposomes (pDNA:cationic liposome charge ratio (−:+) = 1.0:2.0) showed the highest transfection efficiency in the ocular tissue and its transfection-mediated luciferase activity peaked at 3 days. Among the ocular tissues, the highest gene expression was observed in the aqueous humor.

Keywords: Transfection; Vitreous body; Eye; Cationic liposomes; Plasmid DNA

1. Introduction

Although the ocular transfection efficiency of viral vectors is high, safety concerns have been raised in clinical trials because of their immunogenicity (Reichel et al., 1998). Thus, the use of non-viral vectors has attracted great interest for clinical applications of in vivo gene transfection because such vectors lack some of the risks inherent to viral vector systems (Mahato et al., 1997), and consequently gene transfer to the eye utilizing the non-viral vectors has been widely studied (Masuda et al., 1996; Matsuo et al., 1996; Chaum et al., 1999; Hudde et al., 1999; Abul-Hassan et al., 2000; Urtti et al., 2000; Pleyer et al., 2001). One of the most promising classes of

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non-viral vectors developed so far is cationic liposomes. However, the transfection efficiency of cationic liposomes is lower than that of viral vectors, and thus it is essential to try to enhance the transfection efficiency to the eye.

Recent studies have demonstrated that the optimization of the lipid composition of cationic liposomes and/or the charge ratio (−:+) of plasmid DNA (pDNA) complexed with cationic liposomes greatly enhanced the transfection efficiency after intravenous administration (Huang and Li, 1997; Liu et al., 1997; Song et al., 1997). As for the lipid composition of cationic liposomes, the appropriate selection of neutral lipid types dramatically enhances the transfection efficiency after intravenous administration (Huang and Li, 1997; Liu et al., 1997; Kawakami et al., 2000). However, there is little information about the effects of the physicochemical properties of cationic liposomes or complexes on transfection efficiency to the eye. Once the relationship between the in vivo gene expression and these physicochemical properties is known, it will be possible to design liposomes or pDNA/liposome complexes to enable efficient in vivo gene transfection.

In the present study, we investigated the effect of the neutral lipids of cationic liposomes and the charge ratios of pDNA/cationic liposome complexes on the in vivo ocular transfection efficiency in rabbits. *N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA) was selected as the cationic lipid of cationic liposomes because it is often used as a cationic lipid for gene transfection (Felgner et al., 1987; Zou et al., 1993; Sakurai et al., 2001).

2. Materials and methods

2.1. Materials

DOTMA was obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Dioleoylphosphatidylethanolamine (DOPE) and cholesterol (Chol) were obtained from Avanti Polar-Lipids (Alabaster, AL, USA) and Nacalai Tesque Inc. (Kyoto, Japan). All other chemicals were obtained commercially as reagent grade products.

2.2. Animals

Male Nippon albino rabbits, 1.5–2.5 kg, were used in the study. The rabbits were individually housed in cages in an air-conditioned room and maintained on a standard laboratory diet (ORC4, Oriental Yeast Co., Ltd., Tokyo, Japan) with free access to water. The rabbits were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

2.3. Construction and preparation of pDNA (pCMV-Luc)

pCMV-Luc was constructed by subcloning the Hind III/X ba I firefly luciferase cDNA fragment from pGL3-control vector (Promega, Madison, WI, USA) into the polylinker of pcDNA3 vector (Invitrogen, Carlsbad, CA, USA). pDNA was amplified in *E. coli* strain DH5 α, isolated, and purified using a QIAGEN Plasmid Giga Kit (QIAGEN GmbH, Hilden, Germany). The purified pDNA was diluted in sterile 5% (w/v) dextrose. The concentration of pDNA was measured by UV absorption at 260 nm and adjusted to 2 mg/mL.

2.4. Preparation of cationic liposomes

DOTMA/DOPE (1:1 molar ratio) and DOTMA/Chol (1:1 molar ratio) liposomes were prepared according to a previous report (Kawakami et al., 2001). Briefly, DOTMA (9.5 mg), DOPE (7.9 mg), and Chol (5.8 mg) were dissolved in chloroform. Mixtures of DOTMA/DOPE or DOTMA/Chol were dried as a thin film in a test tube using an evaporator at 25 °C, and then were vacuum-desiccated for approximately 4 h. The film was resuspended in sterile 5% dextrose (5 mL) by vortexing and the dispersions were sonicated at 100 W for 3 min on ice. The resulting liposomes were extruded through a 450 mm polycarbonate membrane filter.

2.5. Preparation of pDNA/cationic liposome complexes

Both pDNA and cationic liposomes were diluted with suitable volumes of sterile 5% dextrose to adjust the charge of pDNA/cationic liposome complexes and

were incubated in sterile 5% dextrose for 30 min at 50 °C. The mixing volume of pDNA and cationic liposomes was 60 mL per experiment (total 120 μL) and 100 μL of pDNA/cationic liposome complexes was used for each intravitreal injection.

2.6. Calculation of theoretical charge ratio

The theoretical charge ratio of pDNA/cationic lipid was calculated as the molar ratio of DOTMA (monovalent) per nucleotide unit (average molecular weight 330) (Li and Huang, 1997).

2.7. In vivo transfection experiments

Rabbits were placed in a restraint box and anesthetized with sodium pentobarbital injected into a marginal ear vein. Naked pDNA (100 μL) or pDNA/cationic liposome complexes (100 μL) were injected directly into the vitreous using a 30-gauge needle attached to a syringe. The rabbits were sacrificed by intravenous administration of an overdose of sodium pentobarbital at 1, 3 or 7 days. After the eyes were enucleated, the aqueous humor (200–300 μL) and vitreous fluid (800–1000 μL) were collected using a syringe inserted and then, the six ocular tissues were dissected so that they could be assayed for gene expression. The cornea, iris–ciliary body, lens, and retina were washed twice with ice-cold saline and homogenized with lysis buffer (0.05% Triton X-100, 2 mM EDTA, 0.1 M Tris–HCl, pH 7.8) (Li and Huang, 1997). The lysis buffer was added at a weight ratio of 4 mL/mg of the sample. After three cycles of freezing with liquid nitrogen for 3 min and thawing at 37 °C for 3 min, the tissue homogenates, aqueous humor, and vitreous body were centrifuged at 15,000 × *g* for 3 min at room temperature. Twenty microliters of supernatant were mixed with 100 μL of luciferase assay buffer (Picagene®, TOYO B-Net Co, Tokyo, Japan) and the light produced was immediately measured using a luminometer (MiniLumat LB9506, EG&G Berthold, Bad Wildbad, Germany). Luciferase activity in the tissue was normalized by relative light units (RLU) per g or mL of the tissue. The gene expression levels of 2×10^3 RLU/g of tissue were considered positive because each tissue mixed with the substrates without the injection of pDNA showed approximately 2×10^3 RLU/g or mL (blank level). The results of each experiment were expressed after the raw results minus the blank level.

2.8. Statistical analysis

Statistical comparisons were performed by analysis of variance and post-hoc test was performed by Turkey–Kramer test. $P < 0.05$ was considered to be indicative of statistical significance.

3. Results

3.1. Effect of neutral lipid of pDNA/cationic liposome complexes on gene expression

Fig. 1 shows the luciferase activity as a measure of transfection efficiency 3 days after intravitreal injection of naked pDNA or pDNA complexed with DOTMA/DOPE or DOTMA/Chol at pDNA doses of 40, 60, and 85 μg in rabbits. The levels of the luciferase activity induced by pDNA (40 μg) complexed with DOTMA/DOPE liposomes in the cornea, aqueous humor, iris–ciliary body, lens, vitreous body, and retina were approximately 3.3, 2.4, 2.5, 2.0, 7.0, and 1.1-fold higher than those obtained with naked pDNA, respectively. At pDNA doses of more than 60 μg, however, it was difficult to prepare the pDNA complexed with DOTMA/DOPE liposomes due to aggregation. On the other hand, the transfection efficiency of pDNA complexed with DOTMA/Chol liposomes was enhanced by increasing the dose of pDNA (Fig. 1(c)). Especially, pDNA (85 mg)/DOTMA/Chol liposomes showed the highest transfection efficiency, producing luciferase activities in the cornea, aqueous humor, iris–ciliary body, lens, vitreous body, and retina that were about 2, 280, 28, 31, 120, and 5-fold higher than those obtained with naked pDNA, respectively. Transfection efficacy by DOTMA/Chol liposomes in the vitreous body was significantly higher than that by naked pDNA ($P < 0.05$). Therefore, pDNA (85 μg)/DOTMA/Chol liposomes was used in the following experiments.

Fig. 2 shows the time dependency of luciferase expression at 1, 3, and 7 days after intravitreal injection of pDNA (85 μg)/DOTMA/Chol in various ocular tissues. Luciferase gene expression was not observed

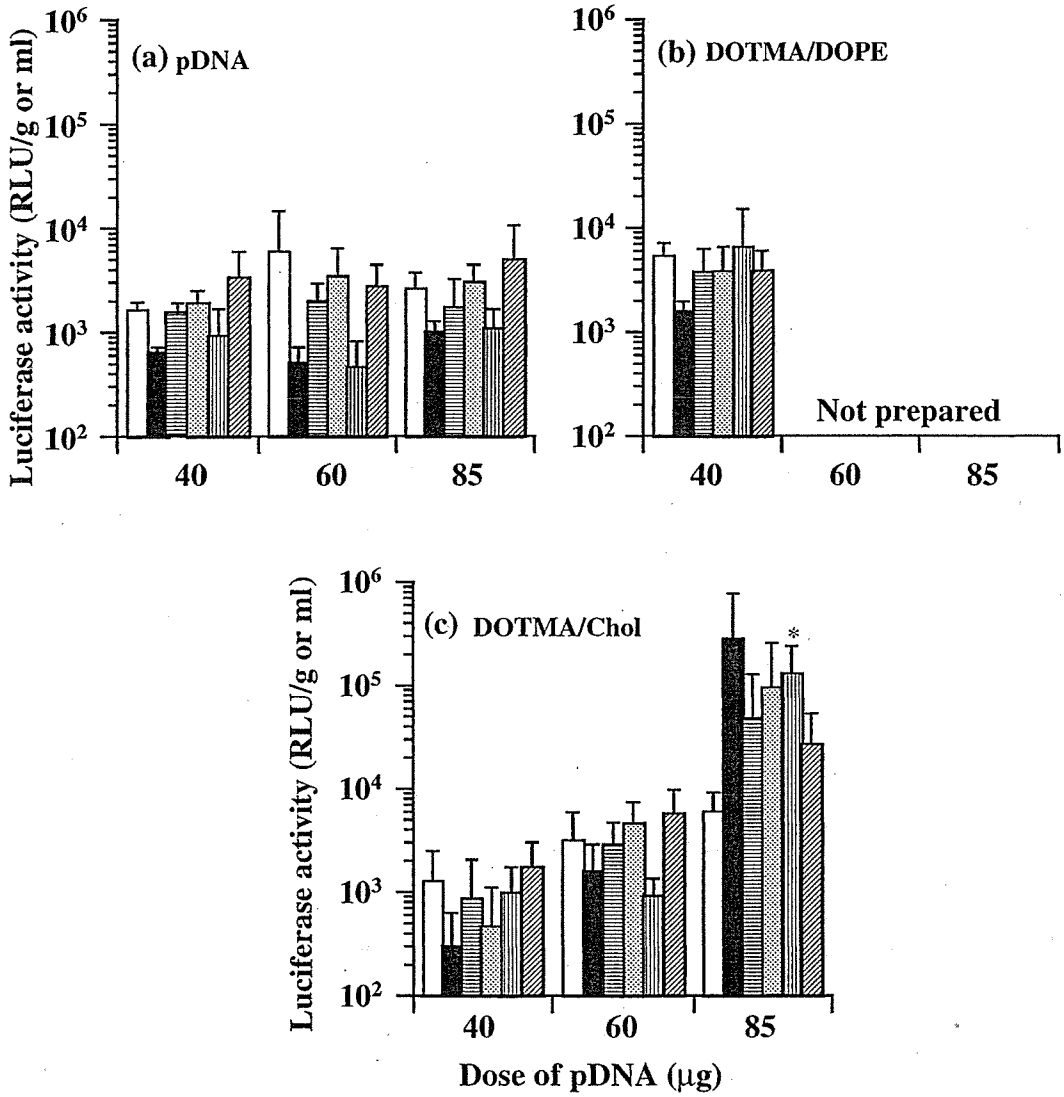

Fig. 1. Transfection efficiency after intravitreal injection of naked pDNA (a), pDNA/DOTMA/DOPE liposomes (b) or pDNA/DOTMA/Chol liposomes (c) at pDNA doses of 40, 60, and 85 μg in rabbits. pDNA was complexed with each cationic liposome at a charge ratio (−:+) of 1.0:2.0. Luciferase activities were measured 3 days post-injection in the cornea (□), aqueous humor (■), iris–ciliary body (▤), lens (▨), vitreous body (▥), and retina (▨), respectively. Each bar represents the mean ± S.D. of three or four experiments. * Significant differences ($P < 0.05$) at pDNA (85 μg).

in the ocular tissues at 0.25 day (6 h). The gene expression in ocular tissues was transient, peaking at 3 days, and dropping to less than 10% of the peak level at 7 days. Transfection efficacy in the cornea and vitreous body at 3 days was significantly higher than that at 7 days ($P < 0.05$).

3.2. Effect of the charge ratio of pDNA/cationic liposome complexes on gene expression

Fig. 3 shows the effect of the charge ratio on transfection efficiency 3 days after intravitreal injection of pDNA (85 μg) complexed with DOTMA/Chol

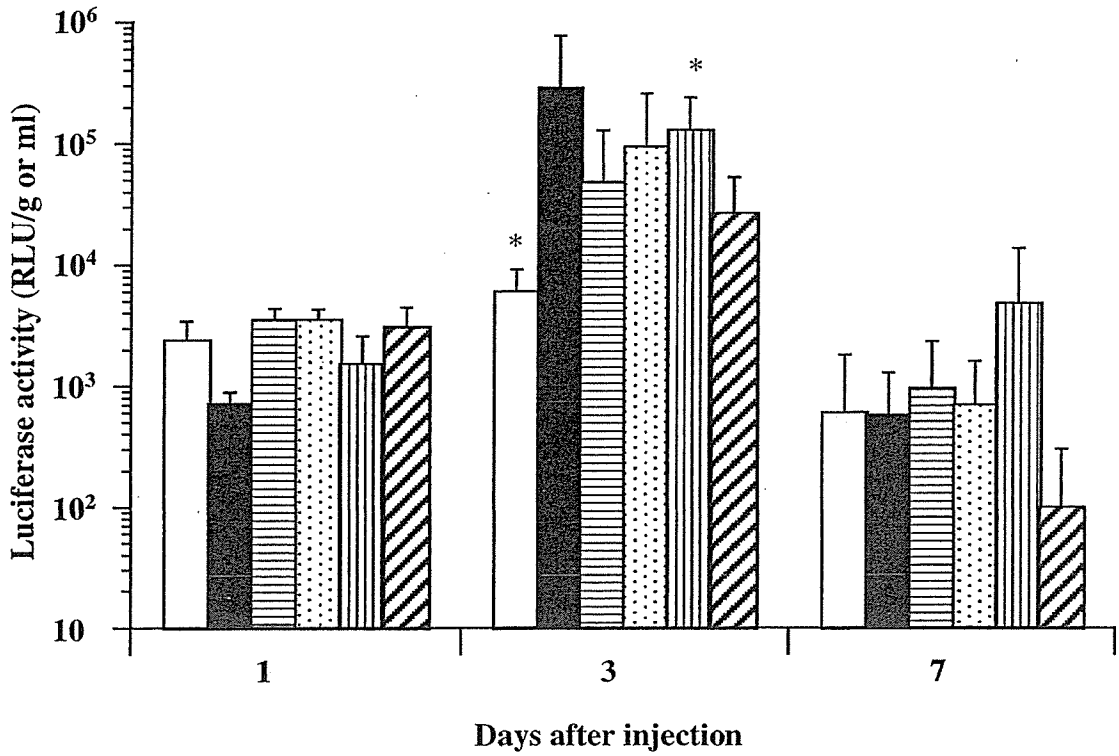

Fig. 2. Time courses of transfection efficiency after intravitreal injection of pDNA complexed with DOTMA/Chol liposomes at a pDNA dose of 85 mg in rabbits. Luciferase activities were measured 1, 3, and 7 days post-injection in the cornea (□), aqueous humor (■), iris–ciliary body (▤), lens (▩), vitreous body (▥), and retina (▨), respectively. Each bar represents the mean ± S.D. of three or four experiments. * Significant differences ($P < 0.05$) at the value of 7 days.

liposomes in rabbits. pDNA (85 μg) was complexed with various amounts of DOTMA/Chol liposomes at the charge ratio (−:+) of 1.0:1.5, 1.0:2.0, or 1.0:2.5. Among these charge ratios, with the charge ratio (−:+) of 1.0:2.0 resulted in the highest transfection efficiency.

4. Discussion

DOPE is often used as the neutral lipid for in vivo ocular gene transfection using cationic liposomes (Matsuo et al., 1996; Masuda et al., 1996; Urtti et al., 2000; Abul-Hassan et al., 2000; Pleyer et al., 2001) because of its pH-sensitive ability to destabilize the lyososomal membranes following endocytosis. Although Chol does not have such a pH-sensitive property, it is well known that liposomal membranes are stabilized by Chol in vivo (Semple et al., 1996; Murao et al., 2002). Recently, Li et al. (1999) reported that DOPE-containing liposome/pDNA complexes were more markedly aggregated by serum exposure than Chol-containing liposome/pDNA complex. It is possible that this ability to stabilize liposomal membranes might also result in stabilization of the pDNA in the vitreous body, resulting in enhancement of the transfection efficiency in the eye. As shown in Fig. 1, pDNA (85 mg) in DOTMA/Chol liposome complexes showed higher transfection efficiency than pDNA in other liposome complexes. These results suggest the potential usefulness of pDNA complexed with DOTMA/Chol liposomes for ocular gene transfection after intravitreal injection. This observation is consistent with previously reported results about systemic administration of pDNA/cationic liposome complexes (Huang and Li, 1997; Sakurai et al., 2001).

In the present study, we found that pDNA complexed with DOTMA/Chol liposomes at a charge ratio

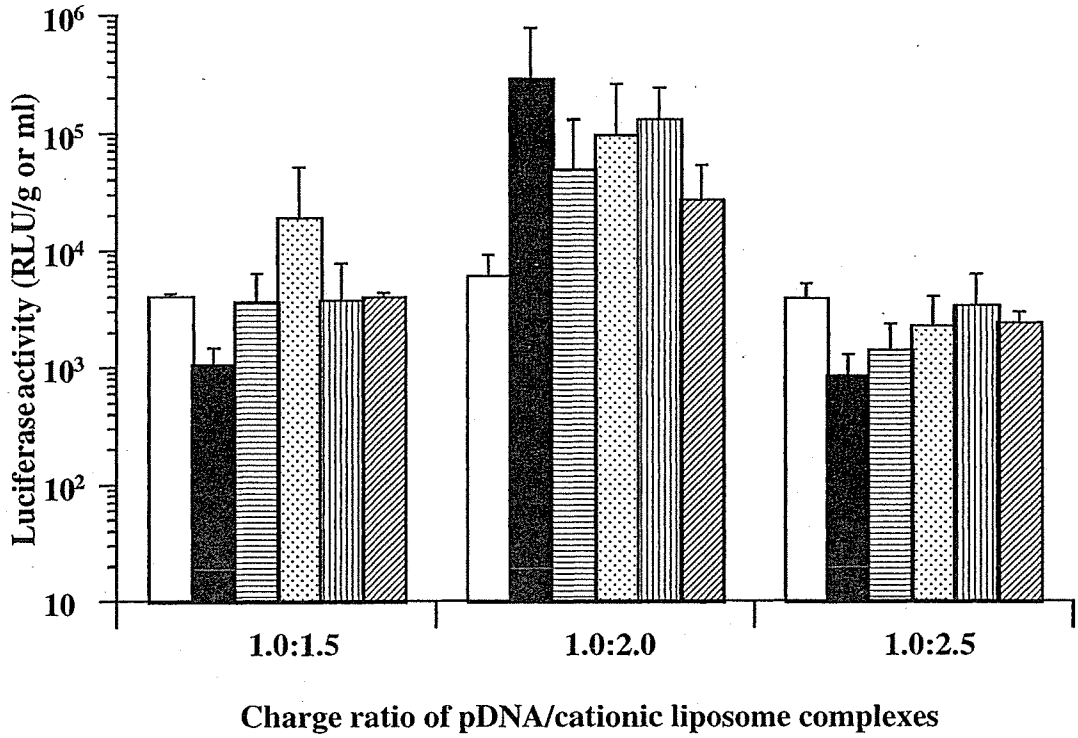

Fig. 3. Effect of charge ratio of pDNA complexed with DOTMA/Chol liposomes on transfection efficiency after intravitreal injection of pDNA/DOTMA/Chol liposomes complex at a pDNA dose of 85 mg in rabbits. pDNA was complexed with cationic liposomes at a charge ratio (−:+) of 1.0:1.5, 1.0:2.0, and 1.0:2.5, respectively. Luciferase activities were measured 3 days post-injection in the cornea (□), aqueous humor (■), iris–ciliary body (▤), lens (▨), vitreous body (▥), and retina (▩), respectively. Each bar represents the mean ± S.D. of three or four experiments.

(−:+) of 1.0:2.0 produced maximal gene expression compared with complexes with other charge ratios (Fig. 3), indicating that the charge of complexes is an important factor with respect to the in vivo ocular transfection efficiency. Cationic liposomes can interact with various types of negatively charged biological components such as serum proteins, probably due to their positive charge after administration into the systemic circulation (Liu et al., 1997). Yang and Huang (1997) reported that the highest transfection efficiency was observed when pDNA was complexed with cationic liposomes at a pDNA–cationic liposome charge ratio (−:+) of 1.0:2.0 in the absence of protein and more than 1.0:4.0 in the presence of protein. Following the intravenous administration of liposome–DNA complexes into mice at a charge ratio of more than 1.0:4.0, high-level, reproducible transgene expression was brought about in various tissues. The discrepancy between the results obtained with intravenous administration and intravitreal administration may be partly due to the fact that the protein content in the vitreous body (about 0.1%) (Ueno et al., 1991) is much lower than that in the blood (about 10%); thus, the characteristics of the transfection efficiency after intravitreal injection might be compatible with those in the absence of protein.

The selection of administration route could be an important issue regarding the efficiency of the transfection of genes into ocular tissues. Thus, various routes of administration such as instillation onto the eye, injection into the anterior chamber, intravitreal injection, and subretinal injection were studied using the pDNA/cationic liposome complexes. Generally, topical administration on the eye is expected to be a convenient and safe method for ophthalmic therapy. It was reported that expression of a transfected gene after topical administration on the eye was found in the corneal epithelium, conjunctival

epithelium, ciliary epithelium, iris stroma, and retinal ganglion cells using pDNA complexed with *N*-(α-trimethylammonioacetyl)-didodecyl-D-glutamate (TMAG)/DOPE/dilauroylphosphatidylcholine (DLPC) liposomes or 3β[*N*-(*N'*,*N'*-dimethylaminoethane)-carbamoyl]cholesterol (DC-Chol)/DOPE liposomes (Matsuo et al., 1996; Masuda et al., 1996). However, our preliminary experiments showed that little luciferase activity was detected even in cornea epithelium after topical administration of 25 mL of pDNA (10 mg) complexed with DOTMA/DOPE or DOTMA/Chol liposomes on to the eye. The cornea, considered to be a major pathway for ocular penetration of topically applied drugs to the eye, is an effective barrier to drug penetration (Sasaki et al., 1996), because the corneal epithelium has annular tight junctions (zonula occludens) that completely surround and effectively seal the superficial epithelial cells. Also, liposomes (Kawakami et al., 2001) like most drugs, are rapidly eliminated from the precorneal area by tear fluid after topical administration to the eye (Yamamura et al., 1999). These features might limit the transgene expression in the eyes following the topical administration of pDNA complexed with DOTMA/DOPE and/or DOTMA/Chol liposomes.

The gene expression in the ocular tissues was transient, with a peak level at 3 days (Fig. 2), even though the pDNA/cationic liposomes were stabilized by the application of DOTMA/Chol liposomes; therefore, more prolonged transgene expression is a current goal for clinical gene therapy because repeated intravitreal administration is painful for the patients. This observation leads us to believe that further studies are needed on the issue of prolonging the gene expression in the eyes.

In conclusion, superior gene transfection in the ocular tissues was observed with the intravitreal administration of pDNA/DOTMA/Chol liposomes, which were an effective gene carrier when delivered by intravenous administration. The use of appropriate neutral lipids and the optimal charge ratio (−:+) of the pDNA/cationic liposome complexes will improve the efficiency of in vivo ocular gene transfection. Our observations based on the physicochemical properties of pDNA/cationic liposome complex can be generalized, so this information will be valuable for designing gene transfection systems for the eye.

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Prednisolone retention in integrated liposomes by chemical approach and pharmaceutical approach

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Abstract

The purpose of this study is to demonstrate a stable retention of prednisolone (PLS) in the unique liposomes integrated by lipophilic derivative approach and PEGylation approach. Palmitoyl prednisolone (Pal-PLS) was newly synthesized and used as a lipophilic derivative. The liposomes were composed of egg phosphatidylcholine (EggPC)/cholesterol (Chol) and L-α-distearoylphosphatidylcholine (DSPC)/Chol with or without L-α-distearoylphosphatidylethanolamine-polyethylene glycol 2000 (DSPE-PEG 2000) or -PEG 5000 (DSPE-PEG 5000). The retentions of PLS and Pal-PLS in the various liposomes were examined by ultrafiltration and gel filtration. Although PLS showed high trapping efficiency by all liposomes after ultrafiltration, low incorporation efficiency was observed in gel filtration. It indicates that PLS was released from the liposomes by a dilution with elution medium in gel filtration. Pal-PLS showed high incorporation into all liposomes after both ultrafiltration and gel filtration. The high incorporation of Pal-PLS into EggPC/Chol liposomes, however, was reduced by incubation with rat plasma in gel filtration. The reducing effect of rat plasma on drug incorporation into liposomes was inhibited by using DSPC and DSPE-PEGs. Thus, we systemically examined the drug retention in various liposomes and demonstrated the high retention of PLS in the liposomes integrated by lipophilic derivative approach and pharmaceutical approach using special lipids.

Keywords: Drug delivery system; Prednisolone; Liposomes; Lipophilic derivative; Polyethylene glycol

1. Introduction

Glucocorticoids are highly potent antiinflammatory and immunosuppressive drugs. However, even at moderate doses, systematic administration of gluco-

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corticoids causes many side effects, such as diabetes, hypertension, Cushing syndrome, and osteoporosis [1]. It is reported that the therapeutic activity of glucocorticoids could be improved through application of liposomal drug carrier technology [2,3]. A major problem, however, exists on the utility of liposomes for delivering drugs because of a rapid removal of liposomes by the mononuclear phagocytic system uptake [4,5]. For improving the pharmacokinetics of liposomes, therefore, the surface-modified polyethylene glycol (PEG) liposomes containing synthetic polyethyleneoxide lipopolymers were introduced and a significant improvement in the liposomes half-lives in the blood could be achieved [6,7]. Even if PEG-modified liposomes exhibited a favorable in vivo disposition profiles, rapid release of incorporated drugs would lead to failure to achieve therapeutic potency. We previously reported that rapid release of incorporated drugs into liposomes was suppressed by chemical modification of drug to lipophilic derivative [8,9]. There has been, however, little information about the unique liposomes of prednisolone (PLS) integrated by chemical modification and pharmaceutical modification. The integrated liposomes of PLS would be useful for drug targeting because of its stable characteristics.

In the present study, we newly synthesized palmitoyl prednisolone (Pal-PLS) and prepared the liposomes with various lipids such as egg phosphatidylcholine (EggPC), L-α-distearoylphosphatidylcholine (DSPC), cholesterol (Chol), L-α-distearoylphosphatidylethanolamine-PEG 2000 (DSPE-PEG 2000) and -PEG 5000 (DSPE-PEG 5000). In addition, the drug retention in the various liposomes was examined by ultrafiltration and gel filtration.

2. Materials and methods

2.1. General procedure

Melting points were determined with a Yanagimoto micromelting point apparatus and are uncorrected. ^{1}H-NMR spectra were recorded on Varian Gemini 300 spectrometers with tetramethylsilane as an internal standard and chemical shifts are given in δ (ppm). Elemental analyses were performed by the Center for Instrumental Analysis, Nagasaki University. Thin-layer chromatography (TLC) was carried out on TLC aluminum sheets precoated with a 0.2-mm layer of Silica gel 60 F_{254} (Merck, Darmstadt, Germany), using the following solvent system: chloroform–methanol–acetic acid (95:4:1, v/v/v).

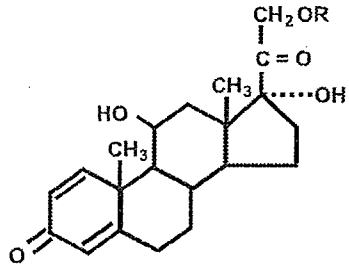
2.2. Materials

PLS was kindly supplied from Shionogi (Osaka, Japan). Palmitoyl chloride was obtained from Nacalai Tesque (Kyoto, Japan). DSPC (COATSOME MC-8080) and EggPC (average molecular weight 773, COATSOME NC-50) were purchased from Nippon Oil and Fats (Tokyo, Japan). DSPE-PEG was a gift from Nippon Oil and Fats. Average molecular weight of PEG chain in the DSPE-PEG was 2000 (SUNBRIGHT DSPE-20H) or 5000 (SUNBRIGHT DSPE-50H). Chol was obtained from Nacalai Tesque. Fluorescein isothiocyanate-dextran (average molecular weight 4400, FD-4) was purchased from Sigma (St. Louis, MO, USA). All other chemicals were of reagent grade and used as obtained commercially. Phosphate-buffered saline (PBS) was prepared by mixing an isotonic phosphate buffer (pH 7.4) with an equal volume of saline.

2.3. Synthesis of Pal-PLS

Palmitoyl chloride (1 ml, 3.3 mmol) was added to PLS (0.5 g, 1.4 mmol) in anhydrous pyridine (10 ml) and stirred at room temperature overnight. The reaction was monitored by thin-layer chromatography. The reaction mixture was dissolved in 1 M HCl (100 ml) and extracted with diethyl ether (50 ml) five times. The organic layer collected was dried, filtered, and evaporated in vacuo to give Pal-PLS as white solid crystals in 70% yield. The structure of synthesized derivative was confirmed by both elemental analysis and ^{1}H-NMR. *Analysis:* Calculated for $C_{37}H_{58}O_6 \cdot 1/4\ H_2O$: C (73.65), H (9.77), O (16.58). Found: C (73.62), H (9.84), O (16.54). ^{1}H-NMR ($CDCl_3$) δ: 0.72–0.98 (H, Pal–CH_3), 0.98 (3H, s, C_{13}–CH_3), 1.09–2.85 (18H, protons belong to B, C, and D rings), 1.36 [26H, s, $-(CH_2)_{14}-$], 2.30–2.50 (2H, m, C_{23}–H_2), 4.52–4.46 (1H, C_{11}–OH), 4.93 (2H, AB-q, C_{21}–H_2), 6.01 (1H, C_4–H), 6.27 (1H, d, J=10 Hz, C_2–H), 7.28 (1H, d, J=10 Hz, C_1–H).

Table 1
Structures and physicochemical properties of PLS and Pal-PLS



Compound	R	Molecular weight	Melting point (°C)	Lipophilic index ($\log k'_0$[a])	Relative lipophilicity
PLS	–H	360.5	235	3.2	1
Pal-PLS	$-CO(CH_2)_{14}CH_3$	598.9	52–56	10.3	1.4×10^7

[a] Lipophilic index was measured by reversed-phase HPLC systems.

2.4. Lipophilic index

The relative lipophilic indices ($\log k'$) were determined by high-performance liquid chromatography (HPLC) employing Eq. (1) [10];

$$\log k' = \log[(t_R - t_0)/t_0] \quad (1)$$

where t_R is the retention time of the solute and t_0 is the elution time of the solvent. The lipophilic indices ($\log k'_0$) were determined by extrapolation of the $\log k'$ values to 0% organic solvent concentration. A mixture of 2-propanol and distilled water was used as the mobile phase at a flow rate of 1.0 ml/min.

2.5. Preparation of liposomes

The liposomes were prepared by sonication method [11]. Both EggPC/Chol and DSPC/Chol liposomes contained phospholipid, Chol, and drug at a molar ratio of 3:2:0.5. In some cases, 1 mol% of DSPE-PEG 2000 or DSPE-PEG 5000 was also added to the lipid mixture. After each mixture of lipids in chloroform was placed in a round-bottom glass tube, chloroform was evaporated. The lipid film obtained was further dried in vacuo in a desiccator for 4 h. The lipid suspension was vortex-mixed followed by ultrasonic radiation for 3 min under nitrogen gas. The liposomes containing DSPC and EggPC were prepared at 60 and 0 °C, respectively. PBS was added to the resulted liposomes, then the lipid was allowed to hydrate for 24 h at 5 °C. FD-4 was selected as a tracer of liposomal fraction in gel filtration. Liposomal formulation was prepared at 20 mmol/l lipid in PBS. All lipid compositions are given as molar ratios unless otherwise indicated.

Table 2
Trapping efficiency of PLS and Pal-PLS by various liposomes in ultrafiltration

Entry	Drug	Lipid composition (molar ratio)	Trapping efficiency (%)
1	PLS	EggPC:Chol (3:2)	97.3 ± 0.4
2	PLS	EggPC:Chol:DSPE-PEG 2000 (3:2:0.05)	98.6 ± 1.2
3	PLS	EggPC:Chol:DSPE-PEG 5000 (3:2:0.05)	96.3 ± 3.3
4	PLS	DSPC:Chol (3:2)	94.5 ± 2.0
5	PLS	DSPC:Chol:DSPE-PEG 2000 (3:2:0.05)	90.5 ± 1.3
6	PLS	DSPC:Chol:DSPE-PEG 5000 (3:2:0.05)	80.0 ± 3.8
7	Pal-PLS	EggPC:Chol (3:2)	99.8 ± 0.2
8	Pal-PLS	EggPC:Chol:DSPE-PEG 2000 (3:2:0.05)	99.8 ± 0.2
9	Pal-PLS	EggPC:Chol:DSPE-PEG 5000 (3:2:0.05)	99.9 ± 0.2
10	Pal-PLS	DSPC:Chol (3:2)	99.9 ± 0.2
11	Pal-PLS	DSPC:Chol:DSPE-PEG 2000 (3:2:0.05)	99.9 ± 0.1
12	Pal-PLS	DSPC:Chol:DSPE-PEG 5000 (3:2:0.05)	99.8 ± 0.1

Each value represents the average ± S.D. of at least three experiments.

2.6. Drug determination

PLS and Pal-PLS were determined using HPLC system (LC-10AD, Shimadzu, Kyoto, Japan) in the reversed-phase mode. The stationary phase used was Cosmosil $5C_{18}$-MS-II packed column (150 ×4.6 mm for PLS and Pal-PLS, Nacalai Tesque). A mixture of 2-propanol, acetonitrile, and water (42:38:20, v/v/v) was used as a mobile phase with a flow rate of 1.0 ml/min for assay of Pal-PLS. A mixture of methanol and ammonium acetate buffer (pH 4) (57.5:42.5, v/v) was used as a mobile phase with a flow rate of 0.55 ml/min for assay of PLS. Retention of drugs was monitored with a variable wavelength ultraviolet detector (wavelength at 240 nm, SPD-10A, Shimadzu).

2.7. Ultrafiltration

Ultrafiltration was performed using Ultrafree®-MC centrifugal filter units (Millipore, Bedford, MA, USA). The drug concentrations in the filtrate and in the applied liposomes were assayed by HPLC. Trapping efficiency was calculated from Eq. (2) [12];

$$\text{Trapping efficiency}(\%) = (D_T - D_F)/D_T \times 100 \quad (2)$$

where D_T is the drug amounts in applied liposomes and D_F is the drug amounts in filtrate, respectively.

2.8. Gel filtration

The plasma was withdrawn from male Wistar rats (230–250 g) after anesthetization with a sodium pentobarbital solution. After liposomes were incubated for 1 min at 25 °C with or without rat fresh plasma (20%, v/v), they were applied to a Sephacryl S-400 column (16 × 2.8 cm) and eluted with PBS at 25 °C.

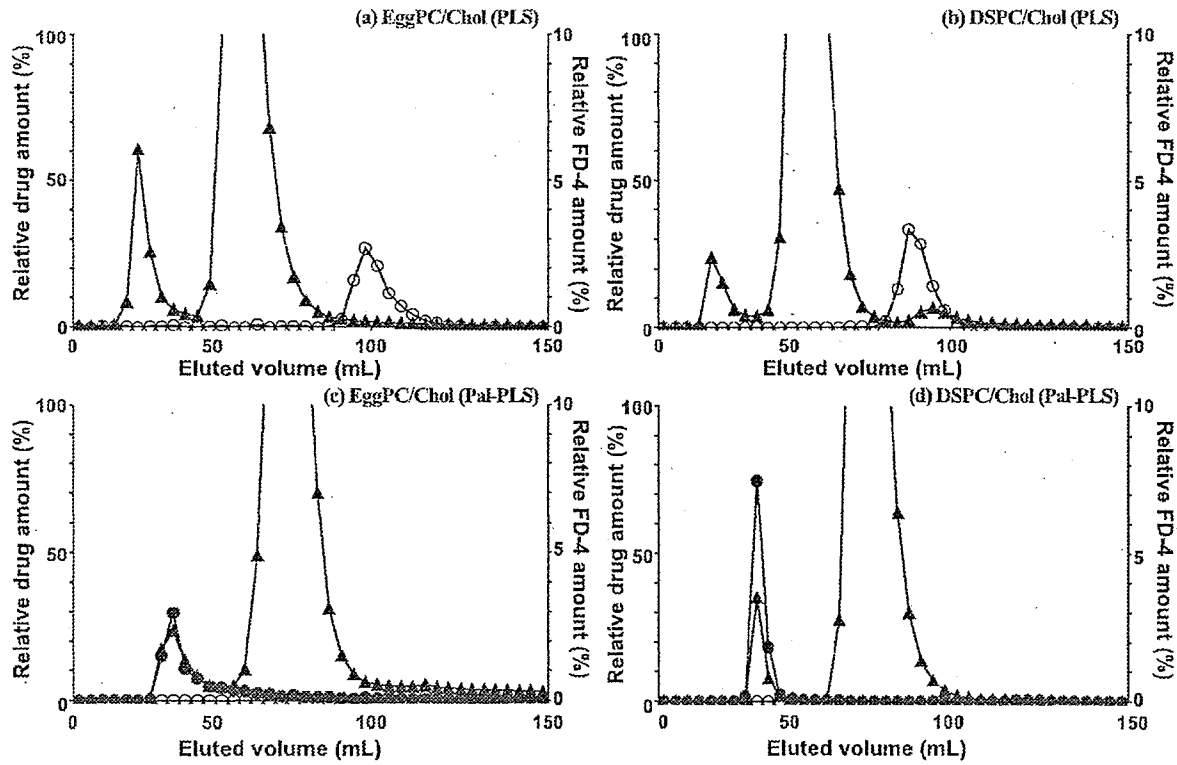


Fig. 1. Typical gel filtration profiles for EggPC/Chol (a and c) and DSPC/Chol (b and d) liposomes incorporating PLS (a and b) and Pal-PLS (c and d) Symbols: (▲) FD-4; (O) PLS; (●) Pal-PLS.