Physical-Chemistry Characteristics and Biodistribution of Poly(ethylene glycol)-Coated Liposomes Using Poly(oxyethylene) Cholesteryl Ether

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Poly(ethylene glycol)-coated liposomes were prepared with poly(oxyethylene) cholesteryl ethers (^{m}PEG-Chol). PEG unit numbers tested were 50, 100 and 200, of which the average molecular weights (m) of PEG were 2200, 4400 and 8800, respectively. Properties of both PEG-coated liposomes and PEG-Chol molecules were investigated. These liposomes exhibited a long circulation time in the blood after i.v. injection in rats, estimated by both the lipid membrane marker, L-α-dipalmitoylphosphatidylcholine [2-palmitoyl-9,10-^{3}H] (^{3}H-DPPC), and an internal aqueous marker, ^{3}H-inulin. Accumulation in the liver and spleen at 8 h-post-injection was significantly reduced compared with conventional liposomes. The percentage of PEG-Chol incorporation in liposomal membranes was also investigated. Liposomes composed of egg yolk phosphatidylcholine (EPC)/PEG-Chol at various molar ratios were separated from free PEG-Chol molecules, which are not incorporated in liposomal membranes by chromatography over Sepharose CL-4B columns. PEG-Chol incorporation reached approx. 14 and 18 mol% of the total lipids with 25% PEG-Chol unit numbers of 200 and 50, respectively. The occupied area per molecule of PEG-Chol was larger than that of Chol, and the fluorescence anisotropy (r) of the initial 25 mol% ^{8800}PEG-Chol liposomes was smaller than that observed for 12.5 mol% Chol liposomes. PEG-coated liposomes containing calcein were incubated at 37 °C in heat-inactivated fetal bovine serum (FBS). In the presence of FBS, calcein leakage was increased with PEG-Chol percentage incorporation and an increase in FBS concentration. The amount released from PEG-coated liposomes represented 60% at maximum and was larger than that of the control liposomes. PEG-Chol molecules are interesting compounds since they can be easily synthesized in a large amount on an industrial scale. The basic physical-chemistry characteristics investigated in this article are critical to assess the pharmacological application of PEG-Chol liposomes as drug delivery systems.

Key words liposome; poly(ethylene glycol); physical-chemistry; critical micellar concentration; biodistribution; drug delivery system

The main disadvantage of liposomes as drug carriers is their high accumulation in the liver and spleen (reticuloendothelial system, RES) after intravenous administration. However, this has been improved with surface modification of the liposomes, *i.e.* using amphiphilic poly(ethylene glycol) (PEG) derivatives incorporated into liposomal membranes.[2] The most common derivatives used so far have been phosphatidylethanolamine conjugates, but others could be suitable.[3]

PEG-coated liposomes have a long lifetime in the bloodstream as well as reduced accumulation in the RES compared with conventional liposomes. It had been proposed that large steric hinderance and/or a hydrophilic barrier caused by the PEG moiety could prevent liposomes from being opsonized by serum proteins.[4] PEG-coated liposome uptake by the phagocytic cells, *i.e.* macrophages in the liver or spleen, could be suppressed as a result of this reduced opsonization, allowing the liposomes to remain in the blood for a longer time. However, the actual mechanisms have not been thoroughly elucidated.

The interaction of liposomes with plasma/serum proteins has been investigated in recent years.[5,6] As for opsonin, as well as dysopsonins interaction, with liposomes, one opsonin bound to negatively charged liposomes, highly phagocytosed, has been shown to be the complement component C3,[6] but no candidate for dysopsonin had been identified yet. PEG chains on liposome surfaces could inhibit protein adsorption onto the liposomal surfaces with relatively low protein specificity.[6,7] This means that the PEG chain may reduce many kinds of protein adsorption, regardless of the presence of opsonins or dysopsonins.

The dependence of PEG molecular weight and the amount of PEG-derivatives, especially PEG-distearoylphosphatidylethanolamine (PEG-DSPE) on liposome behavior had been studied *in vivo*.[7–9] The highest blood level after i.v. injection was achieved with a PEG molecular weight of 1000 to 2000 above 3 mol% PEG-conjugated to phosphatidylethanolamine (PEG-PE) in liposome membranes.[7,9] However, little has been done *in vitro* regarding the influence of PEG on liposome endocytosis by cells. One study by Allen and co-workers shows that a compound which substantially decreases the RES uptake of large unilamellar vesicles, such as the monosialoganglioside (GM1) and ^{1900}PEG-DSPE, also decreases liposomal uptake by bone marrow macrophages in a concentration-dependent manner.[10]

In this report, we studied PEG-coated liposomes with poly(oxyethylene) cholesteryl ether (PEG-Chol), a nonionic surfactant containing a cholesterol group and a poly(oxyethylene) group as lipophilic and hydrophilic group parts, respectively.[11] In order to elucidate the applicability of PEG-Chol liposomes as drug carriers, we first characterized the properties of PEG-Chol molecules in aqueous dispersion state, *i.e.* critical micellar concentration (C.M.C.), incorporation in lipid membranes, stability of the liposomes; then we determined

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in vivo the fate of these liposomes in rats.

Materials and Methods

Materials Egg yolk phosphatidylcholine (EPC) was kindly provided by Asahi Kasei Co., Ltd. (Japan). Cholesterol (Chol) was supplied by Wako Pure Chemical Ind., Ltd. (Japan) and was used after double recrystallizations from ethanol. Inulin-methoxy [Methoxy-^{3}H], (^{3}H-inulin) and L-α-dipalmitoylphosphatidylcholine [2-palmitoyl-9,10-^{3}H], (^{3}H-DPPC) were obtained from Daiichi Pure Chemical Co., Ltd. (Japan). Fetal bovine serum (FBS) was provided by Biowhittaker (Maryland, U.S.A.). Calcein was from Dojin (Japan). PEG-Chol, with an average number of ethylene oxide units of 50, 100 and 200, was synthesized by Nikko Chemical Co. (Japan) using the reaction of ethylene oxide gas and cholesterol in a suitable solvent. Therefore, all these surfactants have the poisson distribution of ethylene oxide chain length, and the average degree of polymerization was determined from the increase in weight of the reaction mixture.[11] These surfactants are white waxy solids and were used without purification. All other chemicals, from Wako Pure Chemical Ind., Ltd. (Japan), were of special grade. Water was purified by double distillation in a glass still.

Physicochemical Properties of PEG-Chol Molecules The C.M.C. value of PEG-Chol in water at 37 °C was determined from the breaking point of surface tension (γ) *vs.* the logarithm of the PEG-Chol concentration (log M) curve. Surface tensions were obtained by the Du Noüy method with a platina ring. The surface area occupied by a molecule (A) was calculated using the Gibbs equation:

$$A = 10^{16}\left(N\frac{-1}{2.303\boldsymbol{R}T}\left(\frac{\partial\gamma}{\partial \log \mathrm{M}}\right)\right)^{-1}$$

where N is the Avogadro number, T the temperature and $\boldsymbol{R}$ the gas constant.[11]

Preparation of Liposomes Multilamellar vesicles coated with PEG-Chol, or uncoated, were prepared as follows: Aliquots of chloroform solutions of EPC, PEG-Chol and/or Chol were mixed into a round-bottomed flask and dried to a thin film with a rotary evaporator under reduced pressure. The remaining solvent was removed *in vacuo* for 2 h. Labeling of the liposome membranes, if necessary, was performed using markers such as ^{3}H-DPPC or 1, 1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) added to the appropriate lipid mixtures prior to solvent evaporation. The lipid film was then hydrated with a buffer solution (pH 7.4). The vesicles were extruded 5 times through Nuclepore filters (Millipore, U.S.A.) of 0.2 μm pore size. The average size of the resulting liposomes ranged from 180 to 220 nm in diameter, as measured by dynamic light scattering using a laser particle analyzer LPA-3100 (Otsuka Electronics, Japan), and was confirmed by electromicroscopy. The quantity of phospholipids was assayed by the Bartlett method.[12]

Separation of Free PEG-Chol and Liposomes Liposomes labeled with 0.2 mol% of DiI were prepared with Hepes buffer (HCMF; NaCl 137 mM, KCl 6 mM, Hepes 10 mM, pH 7.4). In order to separate the PEG-Chol molecules that were not incorporated into the liposomal membranes, 400 μl of liposome suspension (approx. 5 mM) was applied to the chromatography over Sepharose CL-4B columns[3] (1.8 × 20 cm) equilibrated by HCMF buffer. Each fraction (15 drops), collected using a Mini Collector SJ-1410 (Atto, Tokyo, Japan), was assayed for the fluorescence intensity of DiI with excitation and emission wavelengths at 550 and 565 nm, respectively, using the Spectrofluorometer RF-5000 (Shimadzu, Japan), the PEG-Chol content by Bio-Rad Protein assay (Bio-Rad Lab., U.S.A.)[3] and the phospholipid concentration by the Bartlett method.[12]

When free PEG-Chol molecules and liposomes co-existed, 2 peaks were observed in the fraction profile, the former due to liposomes and the latter to the PEG-Chol molecules of the aqueous phase. The percentage of PEG-Chol incorporation in liposomes was calculated from the area under the curve of the Bio-Rad Protein assay profile, corrected by the influence of EPC presence and then expressed as the mol% of total lipids in liposomes.

Fluorescence Anisotropy Measurement 1,6-Diphenyl-1,3,5-hexatriene (DPH) labeled liposomes were prepared by the addition of DPH dissolved in $CHCl_3$ to the lipid mixture of each of the liposomes, in the ratio of 1 to 100 mol lipids, before evaporating the solvent.[13]

Steady-state fluorescence anisotropy of DPH was measured at 25 °C on the spectrofluorometer RF 5000. Excitation wavelength was 360 nm with a 3 nm band width, and emission wavelength was 430 nm with a 5 nm band width. The anisotropy (r) was calculated using the following equation:

$$r = \frac{(I_{vv} - I^s_{vv}) - G(I_{vh} - I^s_{vh})}{(I_{vv} - I^s_{vv}) + 2G(I_{vh} - I^s_{vh})}$$

where I_{vv} and I_{vh} are the observed intensities as measured with polarizers parallel and perpendicular to the vertically polarized exciting beam, respectively. I^s is the contribution of scattered light for an unlabeled liposome suspension of the same composition used as a reference. G is the relative transmission which corresponds to the intensity ratio (I_{hv}/I_{hh}).

Calcein Leakage in FBS and FBS Fractions Calcein-containing liposomes were prepared using a 70 mM calcein solution in water adjusted at pH 7.4 and a lipid concentration of 20 mM. Untrapped calcein was removed by gel-filtration on Sepharose CL-4B columns with HCMF as an eluent. These liposomes were incubated in FBS solution as well as lipoprotein fraction and lipoprotein-deficient fraction. These fractions were prepared by an ultracentrifugation technique. Solid NaBr was added to the heat-inactivated FBS to give a solution density of 1.21 mg/ml. Then FBS was centrifuged at 38000 rpm for 48 h in a Beckman 60Ti rotor at 4 °C. The lipoprotein fraction floated and then was collected by slicing the top of the centrifugation tube. Both fractions were dialyzed for 24 h at 4 °C in at least 50 volumes of HCMF changed 3 times. The protein amount of each fraction was determined by Bio-Rad Protein assay and was adjusted to be equivalent to the amount existing in 30% FBS solution by dilution with a buffer. The lipoprotein concentration in FBS is 0.783 mg/ml.[14] The incubation was carried out by mixing 1 mM of the liposome suspension with Hepes buffer containing FBS or FBS fractions, and was continued up to 4 h. FBS was used after heat-inactivation (56 °C, 30 min). Liposome suspensions and FBS solutions were pre-incubated separately at 37 °C for 15 min prior to mixing.

Samples taken from the incubation mixture at selected times were diluted by 20 with Hepes buffer, and the fluorescence intensities were measured before and after adding Triton X-100 10% (v/v) in HCMF (excitation wavelength at 490 nm and emission wavelength at 520 nm) on a Spectrofluorometer RF-5000. Calcein retention was calculated from the following equation:

$$\%\ \text{release} = \frac{(F_t/F)(F_x - F_0)}{F_t - F_0}$$

where F_0 is the fluorescence intensity of the sample in HCMF at 37 °C at time zero. F_t is the total fluorescence intensity of the liposomes in HCMF after the addition of 10% Triton X-100. F_x is the fluorescence intensity of the liposomes incubated either in HCMF or FBS solutions at time x. F is the total fluorescence intensity of the liposomes in serum after the addition of Triton X-100. F_t/F is a correction term which is used to minimize the pipetting and other systematic errors in the measurement. The value of F_t/F ranged from 0.9 to 1.1.[15]

Fluorescein Isothiocyanate (FITC)–Dextran Retention in 50% Rat Plasma FITC–dextran (M.W. 4400) entrapped liposomes were prepared by a procedure similar to that used for calcein entrapment described above with slight modifications: phosphate buffered saline (PBS, pH 7.4) was used instead of HCMF, and the separation was performed with a Sephadex G-100 column for gel-filtration.

Liposome suspension and the same volume of untreated rat plasma, which was prepared from male Wister rats (Shimizu Laboratory Supplies, Kyoto, Japan), were mixed and incubated at 37 °C. At the indicated time, samples were collected and filtrated over a Sephadex G-100 column. Each fraction was diluted with PBS and mixed with Triton X-100 before fluorescence measurement (excitation wavelength 490 nm and emission wavelength 520 nm). Percent retention was calculated from:

$$\%\ \text{retention} = \frac{A_L}{A_L + A_R}$$

where A_L is the sum of fluorescence intensities of FITC–dextran in liposome fractions. A_R is sum of fluorescence intensities in fractions containing released FITC–dextran.

Animal Experiment ^{3}H-DPPC labeled liposomes were prepared with PBS (pH 7.4). ^{3}H-inulin entrapped liposomes were hydrated with ^{3}H-inulin-containing PBS, and untrapped ^{3}H-inulin was removed by gel-filtration over Sephadex G-100 columns.

Male Wister rats weighing 185—230 g from Shimizu Laboratory Supplies (Kyoto, Japan) were administered liposome suspension (3 μmol

lipid) *via* the femoral vein. During the experiments, rats were under anesthesia after nembutal injection. Blood samples were collected at various times with heparinized syringes, and plasma samples (100 μl) were obtained by centrifugation (2000 × *g* for 1 min). At 8 h post-injection, the liver was perfused with PBS *via* the portal vein in order to remove the remaining blood inside, and it was then excised and weighed. Other selected organs, the spleen, kidney, heart, lung, intestine and lymph, were excised and weighed, too. Samples of each organ were dissolved in tissue solubilizer Soluene®-350, and associated radioactivities were measured with a liquid scintillation analyzer (Beckman LS 5000TA). The results were expressed as a percentage of administered radioactivity accumulated in each organ. Plasma volume was obtained by 2-compartment model calculation on ^{3}H concentration in plasma samples.

Results

Physico-Chemical Characteristics of PEG-Chol PEG-Chol had polyethylene glycol and cholesterol as hydrophilic and lipophilic moieties in its molecule. In this report, PEG units numbering 50, 100 and 200, which have an average molecular weight of about 2200, 4400, 8800 respectively, were studied. They are surface-active and make micelles in an aqueous solution at concentrations above C.M.C. The C.M.C. value and occupied area per molecule of each PEG-Chol in water at 37 °C are shown in Table 1.

In the case of PEG-PE, the C.M.C. value was under 10^{-6} M, and the micellar size at 10 mM was 16 ± 5 nm in diameter.[16] The value was comparable to those of PEG-Chol. Since both PEG-PE and PEG-Chol were water soluble, both derivatives could exist in either the free form or the liposome-associated form depending on the amount of PEG-derivative and its chain length.

Liposome Size Distribution and Free PEG-Chol Separation Based on DLS experiments, we can see that liposome size is reduced in the presence of PEG-Chol (Table 2). However, the chain length had no significant effect on liposome diameter, and increasing the amount of PEG-Chol from 5 to 25 mol% did not induce significant changes, either.

Since PEG-Chol molecules are able to form micelles, it is important to know the amount of PEG-Chol incorporated into liposome membranes. In order to investigate the percentage of PEG-Chol incorporation into the liposome membranes as a function of its initial concentration, PEG-coated liposomes were separated from free PEG-Chol molecules by gel-filtration with a Sepharose CL-4B column.[3]

The elution profiles are given in Fig. 1. In the case of liposomes containing 10 mol% of ^{2200}PEG-Chol, [EPC/^{2200}PEG-Chol (9/1)], PEG-Chol molecules were completely incorporated into the membranes (Fig. 1A). A similar profile was found for ^{4400}PEG-Chol molecules at 10 mol% (data not shown). In contrast, 2 peaks appeared from 10 mol% of ^{8800}PEG-Chol liposomes and for 15 mol% of PEG-Chol with PEG M.W. 2200 and 4400, similar to the one shown in Fig. 1B for EPC/^{8800}PEG-Chol (75/25) liposomes. The first was due to the liposomes and the second to those PEG-Chol molecules which were not in-

Table 1. Physicochemical Characteristics of PEG-Chol in Water at 37 °C

PEG unit number	PEG M.W.	C.M.C. value (μM)	Occupied area (Å^2/molecule)
50	2200	4.0	70
100	4400	3.9	110
200	8800	1.7	120

Table 2. Liposome Diameter after Extrusion

Liposomes	Diameter (nm)			
	Control	^{8800}PEG	^{4400}PEG	^{2200}PEG
EPC only	218.3 ± 7.8			
EPC/Chol (87.5/12.5)	217.8 ± 8.6			
EPC/Chol (75/25)	218.2 ± 10.3			
EPC/Chol/PEG-Chol (75/20/5)		185.1 ± 8.6	184.6 ± 3.3	184.0 ± 6.6
EPC/PEG-Chol (75/25)		178.4 ± 8.7	177.8 ± 9.4	183.9 ± 4.2

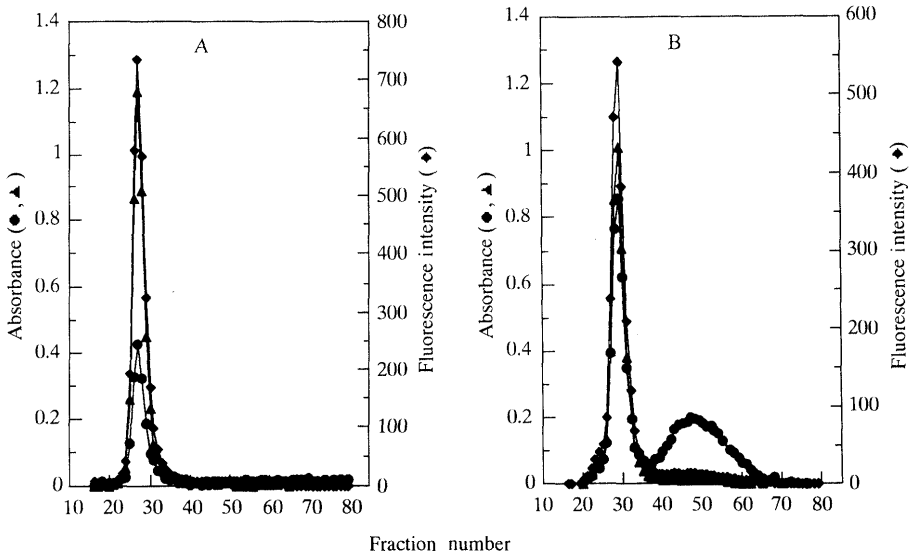

Fig. 1. Elution Profile of DiI Labeled Liposomes Composed of EPC and PEG-Chol

10 mol% ^{2200}PEG-liposomes (A) and 25 mol% ^{8800}PEG-liposomes (B) were gel-filtrated over Sepharose CL-4B columns. Contents of phospholipid (▲) and PEG-Chol (●) and fluorescence intensity (◆) of each fraction are plotted.

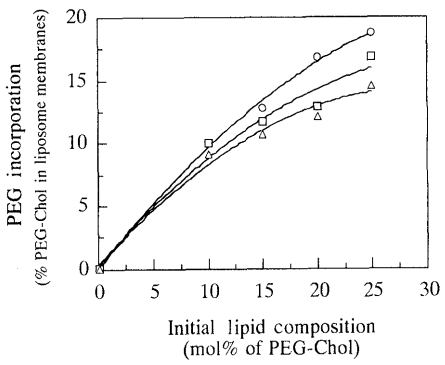

Fig. 2. Dependence of PEG-Chol Incorporation in Liposomal Membranes on Initial Lipid Composition

Values of PEG-Chol incorporation in liposomes is calculated from profiles obtained after filtration on Sepharose CL-4B columns. Liposomes composed of EPC/PEG-Chol are prepared with ^{2200}PEG-Chol (○), ^{4400}PEG-Chol (□) and ^{8800}PEG-Chol (△). The error for each value is less than 10%.

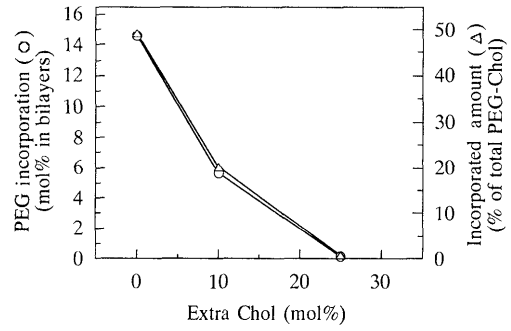

Fig. 3. The Influence of Cholesterol on PEG-Chol Incorporation in Liposomes

Extra cholesterol is added to the lipid composition of EPC/^{8800}PEG-Chol liposomes (75/25). The procedure is described in Fig. 2. The amount of PEG-Chol incorporated (△) is expressed as a percentage of total PEG-Chol added. The percentage of PEG-Chol in membranes (○) is calculated from the fractions profile.

corporated into the membranes. Using ^{2200}PEG-Chol and ^{4400}PEG-Chol, 7—8% of the total amount of EPC was found in the latter peak, but with ^{8800}PEG-Chol, only 2—5%. In the case of the membrane marker, DiI, less than 10% of the total amount was found in the second peak.

Figure 2 shows PEG-Chol incorporation into liposome membranes. With 10 mol% PEG-Chol at the initial lipid composition regardless of PEG M.W., more than 90% of the applied amount was incorporated. The incorporation with an initial concentration of PEG-Chol at 25% reached 18, 16 and 14 mol% for PEG of M.W. 2200, 4400 and 8800, respectively. Due to its large polar head group, PEG-Chol cannot be incorporated to the same extent as Chol, the larger the head group, the smaller the incorporation.

We then investigated the influence of Chol in liposome bilayers on the incorporation of ^{8800}PEG-Chol. An extra Chol amount was added to the lipid composition of EPC/^{8800}PEG-Chol (75/25) prior to making the liposomes. The results are presented in Fig. 3. Incorporated amounts of PEG-Chol into liposome membranes decreased with increases in Chol. Namely, PEG-Chol molecules were replaced by Chol molecules in membranes. That is, Chol molecules should prevent the PEG-Chol incorporation by occupying the site of PEG-Chol molecules in the bilayer.

Fluorescence Anisotropy Measurement Fluorescence

Table 3. Fluorescence Anisotropy of DPH in ^{8800}PEG-Chol Containing Liposomes and Micelles

Liposomes and micelles	Anisotropy (r)
EPC only	0.079±0.001
EPC/Chol (87.5/12.5)	0.109±0.003
plus ^{8800}PEG-Chol micelle	0.119±0.001
EPC/Chol (75/25)	0.147±0.004
EPC/PEG-Chol (75/25)	0.101±0.004
Filtrated	0.085±0.001
EPC/Chol/PEG-Chol (75/20/5)	0.134±0.001
^{8800}PEG-Chol micelle	0.206±0.005

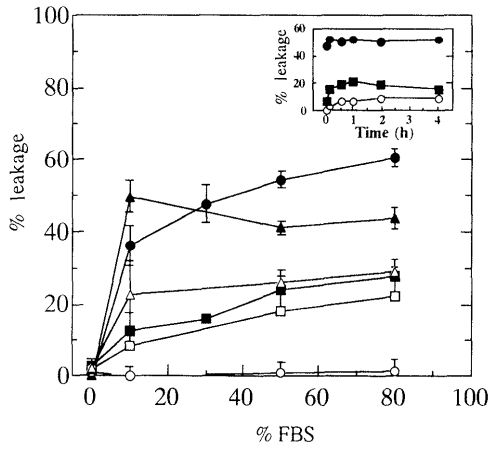

Fig. 4. The Effect of FBS Concentration on Calcein Release after 1 h-Incubation

Liposomes were incubated with various FBS concentrations. FBS is used after heat-inactivation (56°C, 30 min). Control liposomes are composed of EPC (△) or EPC/Chol 75/25 (○) or 87.5/12.5 (□). Liposomes containing 25 mol% PEG-Chol (●, ▲) or 5 mol% PEG-Chol (■) are composed of EPC/Chol/PEG-Chol at 75/0/25 and 75/20/5, respectively. PEG-Chol with PEG M. W. 2200 (▲) or 8800 (●, ■) was investigated. The results were expressed as the mean±S.D. Inset: typical kinetics of calcein release in the presence of 50% FBS for liposomes containing 25 mol% Chol (○), 5 mol% (■) and 25 mol% (●) of ^{8800}PEG-Chol.

anisotropy of DPH in EPC liposomes was increased with the Chol content in bilayers (Table 3). This means that membrane fluidity decreases with an increase in Chol content, as is already known. The anisotropy meaured for liposomes containing 25 mol% PEG-Chol represent an average value for DPH in liposome membranes and in PEG-Chol micelles. When the micelles were removed from 25 mol% PEG-Chol liposomes by gel-filtration, anisotropy decreased since the r value of micelles was high, while it increased when micelles were added to PEC/Chol liposomes. The 25 mol% PEG-Chol liposomes contained 14 mol% PEG-Chol in membranes, but their r value was smaller than that of 12.5 mol% Chol liposomes. Liposomes containing 5 mol% PEG-Chol and a total of 25 mol% of Chol and Chol-derivative show a decrease in the anisotropy value, indicating that PEG-Chol has an opposite effect from Chol in terms of membrane fluidity.

Calcein Leakage in Heat-Inactivated FBS and Its Fractions The percentage of calcein leakage after 1 h incubation in heat-inactivated FBS at 37 °C is shown in Fig. 4. EPC liposomes release 20—30% of their contents in FBS. The presence of Chol in liposomes reduced the leakage to a certain extent, as has already been shown[17] and an increase in Chol content up to 25 mol% totally suppressed the calcein leakage at any serum concentra-

tion. The liposomes containing 25 mol% PEG-Chol released more calcein than EPC liposomes. However, 5 mol% PEG-Chol liposomes released similar amounts of calcein to EPC liposomes. In the absence of serum, calcein release was not significantly observed with all kind of liposomes. Calcein leakage from PEG-Chol liposomes occurred at the initial stage, that is, immediately after mixing the liposome suspension and the FBS solution. No further leakage was observed up to 4 h (Fig. 4 inset). PEG-Chol and Chol had opposite effects on the calcein leakage when incorporated in EPC bilayers.

Lipoprotein fraction and lipoprotein deficient fraction were prepared by an ultracentrifugation technique, and liposomes containing ^{8800}PEG-Chol 25 mol% or 5 mol% were incubated in both fractions. The protein amount in each fraction was equivalent to the amount existing in 30% FBS solution (Fig. 5). Both fractions induced calcein leakage from these liposomes, but the total percentage of release did not represent half of that induced by the 30% FBS solution, indicating a synergistic effect of these two fractions. Lipoproteins (predominantly high-density lipoproteins (HDL); Sigma) from bovine plasma or bovine serum albumin (BSA) did not induce significant calcein leakage from these liposomes (data not shown).

FITC–Dextran and Calcein Retention in Rat Plasma A macromolecular substance, FITC-dextran (M.W. 4400), was used as an internal aqueous phase marker instead of calcein. More than 92% of the dye stayed inside the EPC/Chol (75/25) liposomes up to 8 h (Table 4). No differences in the percentage of retention were observed between EPC/Chol liposomes and 25 mol% ^{8800}PEG-Chol liposomes. Calcein release was similar to that observed for heat-inactivated FBS; however, kinetics were different from each other. Liposome diameter did not change before or after incubation in rat plasma (data not shown).

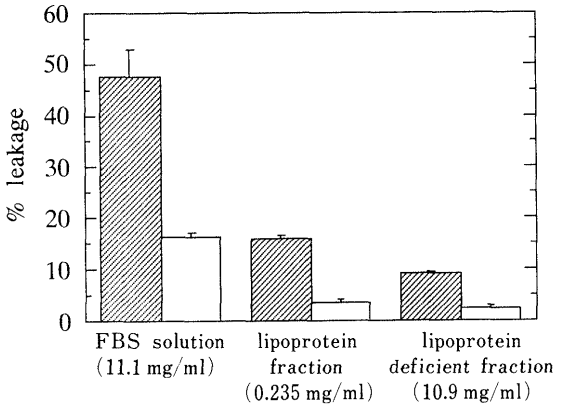

Fig. 5. Calcein Release Induced by Lipoprotein Fraction and Lipoprotein-Deficient Fraction from Heat-Inactivated FBS

Lipoprotein fraction ($d<1.21$ g/ml) and lipoprotein deficient fraction were prepared by an ultracentrifugation technique. The protein amount of each fraction was equivalent to a 30% FBS solution. PEG-coated liposomes are composed of either EPC/Chol/^{8800}PEG-Chol (75/0/25, hatched bars) or (75/20/5, open bars).

Biodistribution of Liposomes in Rats Liposomes were injected into rats and their biodistribution was investigated. The results are shown in Table 5. Significant amounts of ^{3}H-inulin entrapped in the control liposomes (EPC/Chol; 75/25) was accumulated in the liver and spleen (*i.e.* RES), and a small amount remained in plasma (blood). However, when the liposome composition was changed to EPC/PEG-Chol, with 25 mol% of ^{8800}PEG-Chol, ^{3}H-inulin accumulation in the RES was reduced by half. Consequently, a higher plasma (blood) level was achieved, as observed for PEG-PE-containing liposomes.[2] The half life of liposome-entrapped ^{3}H-inulin in plasma was extended from 1.7 to 4.5 h by PEG-Chol incorporation. No significant accumulation of liposomes was observed in any of the other tissues.

Discussion

There are many advantages in using liposomes as drug carriers. Liposomes are generally composed of biodegradable components such as phospholipids and Chol. Both hydrophilic and lipophilic substances can be incorporated. Incorporation in these systems reduces the toxicity and inactivation of the applied drugs. The main disadvantage in the use of liposomes is their high accumulations in the liver and spleen when liposomes are administered intravenously. This high accumulation prevents prolonged circulation in the blood and subsequent delivery to the desired site. However, it was

Table 4. FITC–Dextran and Calcein Retention in Rat Plasma

Liposomes	Calcein % of retention		FITC–Dextran % of retention	
	30 min	8 h	30 min	8 h
EPC/Chol (75/25)	93.3	85.3	94.4	92.1
EPC/^{8800}PEG-Chol (75/25)	63.7	56.9	92.8	92.2

Table 5. The Effect of PEG-Chol Incorporation on the Biodistribution of Liposomes in Rats

Liposome	Composition (EPC/Chol/PEG-Chol)	Marker	*n*	Plasma[a]	Liver	Spleen	% remaining *in vivo*
Control	75/25/ 0	Inulin	3	13.0±1.5	47.9±5.2	18.1±2.4	86.9±6.1
		DPPC	7	8.0±2.2	22.1±2.7	6.4±1.0	
5 mol% ^{8800}PEG	75/20/ 5	DPPC	3	22.1±0.8	15.2±1.5	4.5±0.6	
15 mol% ^{8800}PEG	75/10/15	DPPC	3	30.5±0.4	10.8±0.3	4.9±0.3	
25 mol% ^{8800}PEG	75/ 0/25	Inulin	3	34.5±2.8	19.3±1.2	17.1±0.8	78.4±3.2
		DPPC	6	36.8±3.3	11.4±0.9	4.4±0.3	
25 mol% ^{4400}PEG	75/ 0/25	DPPC	4	25.4±5.4	15.0±2.0	3.8±0.2	
25 mol% ^{2200}PEG	75/ 0/25	DPPC	4	19.2±5.2	20.6±3.3	4.5±0.4	

The amounts of ^{3}H-inulin or ^{3}H-DPPC in plasma and various tissues were determined at 8 h post-injection. Rats (*n*) received 3 μmol of liposomes composed of the indicated lipid composition. The results were expressed as a percent of total injected radioactivity±S.D. *a*) Plasma volume was determined by the 2-compartment model calculation on radioactivity concentration in plasma.

reported that PEG-modified liposomes exhibited reduced accumulation in the RES and longer circulation in the blood. These liposomes were prepared by incorporating amphiphilic PEG derivatives, mainly PEG conjugated to PE (PEG-PE), in liposome membranes.[2)]

In this report, we show that liposomes containing PEG-Chol inhibited the accumulation in the RES and presented a higher blood level compared to EPC/Chol liposomes in rats. The plasma level of PEG-Chol liposomes increased with PEG molecular weight and PEG-Chol content. Inulin is rapidly accumulated in the kidney and excreted into urine when it is released from liposomes into the bloodstream with a half life of <5 min.[18)] Therefore, the % remaining *in vivo* could be an index of liposome stability in the bloodstream.[18,19)] More than 70% of injected ^{3}H-inulin entrapped in the control, or 25 mol% ^{8800}PEG-liposomes remained *in vivo*, indicating that the stability in blood was constant with a change in liposome composition. The ^{3}H-DPPC label which associated with RES was less than that of ^{3}H-inulin. This difference is due to the degradation of ^{3}H-DPPC in RES like other lipid markers[2b,20)] while ^{3}H-inulin did not degrade. Moreover, some ^{3}H-DPPC label in liposomes may migrate to HDL during blood circulation.[19)] However, regardless of the labels, PEG-coated liposomes exhibited a reduced association to the RES and the extended half-life in blood.

All PEG-Chol-coated liposomes showed RES avoidance after i.v. injection and achieved a higher plasma level than the control liposomes. The most effective liposomes among those investigated were 25 mol% ^{8800}PEG-Chol liposomes. RES accumulation of the liposomes composed of PEG-Chol was dependant on PEG M.W. and PEG-Chol content. A longer half life in plasma was observed, the higher the PEG M.W. The plasma level increased in conjunction with the PEG-Chol content in liposome composition.

In the case of PEG-PE, when EPC/Chol (1/1) liposomes containg 6 mol% PEG-PE with a diameter approx. 200 nm were injected in mice, an increase in the molecular weight of PEG over 5000 did not increase the blood level.[9)] The blood level of PEG-PE liposomes increased with the PEG-PE content up to 3.7 or 5 mol%, but thereafter reached a constant value.[7,21)] In the case of PEG-Chol within our experiments, none of these two phenomena were observed. At the present stage, the reason for these differences between PEG-Chol and PEG-PE liposomes is still unknown, although the RES avoiding mechanism of PEG-Chol liposomes may be similar to PEG-PE liposomes, that is, relating to the steric hindrance or hydrophilicity of PEG.

PEG-Chol cannot be totally incorporated in liposomes at a high molar ratio, and the values depend on the PEG chain length. Similar results have been observed in the case of PEG-PE while incorporation reached 6 or 7.1 mol% for ^{1900}PEG-PE or ^{5000}PEG-PE, respectively, with an initial concentration of 10 mol%.[3)] However, higher molar contents can be obtained with PEG-Chol.

Liposome stability in serum was investigated using the calcein leakage index. EPC/Chol liposomes released calcein depending on Chol amount; leakage was decreased with an increase in Chol in EPC bilayers. The leakage was inhibited by the addition of Chol, which stabilizes the fluid EPC bilayers. A similar observation was reported with small unilamellar vesicles (SUVs).[17)] However, as for permeability measurements, we observed that PEG-Chol had less effect on stabilizing EPC membranes and PEG-Chol liposomes had disordered membrane structures. The occupied area per molecule was much larger than that of Chol (Table 1) and the fluorescence anisotropy (r) of initial 25 mol% ^{8800}PEG-Chol liposomes was smaller than that observed for 12.5 mol% Chol liposomes (Table 3), although 25 mol% ^{8800}PEG-Chol liposomes is composed of 2 parameters, namely. DPH in bilayers (PEG-Chol was approx. 14 mol%) and DPH in PEG-Chol micelles (r value was approx. 0.2). Therefore, these observations led us to believe that PEG-Chol cannot rigidify the EPC bilayers due to the presence of the large PEG moiety and no OH group in position 3. Moreover, PEG-Chol liposomes were permeable to calcein due to this disordered structures but not to macromolecular FITC–Dextran. These significant differences implied that lysis or fusion of PEG-Chol liposomes does not occurs *in vitro*. The liposome diameter did not change before or after incubation in rat plasma (data not shown). Liposomes were also stable in the bloodstream after intravenous injection in rats, because approx. 80% of the inulin entrapped in liposomes remained *in vivo* after 8 h (Table 5). PEG-PE liposomes did not leak the entrapped calcein or fluorescein any more than the control liposomes composed of PC/Chol[22)] or distearoylphosphatidylcholine (DSPC),[23)] because the former were stabilized by Chol and the latter were in a gel phase, and the addition of PEG-PE did not change the membrane structures (Maruyama K., personal communication).

PEG-Chol molecules are slightly soluble in water and make micelles at a concentration above C.M.C. These micelles, which co-existed in PEG-coated liposome suspension, may interact with blood components after being administered to rats. In the case of erythrocytes, PEG-Chol with PEG unit numbers 24, 25 or 30 (with molecular weights of 1050, 1100 or 1300, respectively) had hemolytic activity[24–26)]; however, ^{2200}PEG-Chol does not induce hemolysis.[24,25)] ^{4400}PEG-Chol and ^{8800}PEG-Chol were assumed to have no hemolytic activity. It has not yet been shown that PEG-Chol micelles can interact with cells, especially macrophages resident in the liver and spleen, and if so, it will be quite interesting to analyze the effects of PEG-Chol on cellular responses.

In conclusion, PEG-coated liposomes with PEG-Chol were not broken down in either the bloodstream or a serum solution *in vitro*. Furthermore, they escape from the RES after intravenous administration in rats and achieve a long lifetime in blood. Two important points have to be emphasized regarding the choice of these new PEG derivatives. Cholesterol is already known to be a good factor for increasing the half-life of liposomes *in vivo*[27,28)]; moreover, PEG-Chol molecules can be easily synthesized in a large amount on an industrial scale. Therefore, these new PEG-coated liposomes are thought to have suitable properties for use as drug carriers although the optimum lipid composition may change with

applied drugs.

References and Notes

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