Prolongation of Liposome Circulation Time by Various Derivatives of Polyethyleneglycols

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New lipid derivatives of polyethyleneglycol (PEG) have been synthesized and tested for the ability to allow liposomes to evade uptake by the reticuloendothelial system (RES) and to prolong the circulation time of liposomes in mice. Liposomes were prepared from distearoylphosphatidylcholine (DSPC) and cholesterol (CH) (1:1, m/m) containing 6 mol% of various PEG-derivatives. The activity of the CH derivative of PEG (CH-PEG) in prolonging the circulation time of liposomes was proportional to the average molecular weight of PEG, i.e., $4800 > 2600 > 1700 > 800$. α -Methoxy- ω -(1,2-dioctadecenoyloxy glyceryl)polyoxyethylene (DO-PEG) 1000 and α -methoxy- ω -(1,2-ditetradecenoyloxy glyceryl) polyoxyethylene (DT-PEG) 1000, in which PEG is directly linked to glycerol, prolonged the circulation time as effectively as distearoylphosphatidyl-*N*-(methoxy polyoxyethylene succinyl)-ethanolamine (DSPE-PEG). PEG-derivatives with a functional group at the PEG terminal, such as distearoylphosphatidyl-*N*-(3-carboxypropionyl polyoxyethylene succinyl)ethanolamine (DSPE-PEG-COOH) and α -(dipalmitoylphosphatidyl)- ω -hydroxypolyoxyethylene (DPP-PEG-OH), effectively prolonged the circulation time of liposomes. Incorporation of PEG-derivatives did not change membrane fluidity even after treatment with serum. Furthermore, incorporation of PEG-derivatives into liposomes decreased uptake by J774 cells, a murine macrophage-like cell line, in vitro. The newly synthesized PEG-derivatives seem to prevent or reduce the interactions of liposomes with serum protein and macrophages, resulting in enhanced stability and a prolonged circulation time.

Key words liposome; long-circulating liposome; polyethyleneglycol

Recently developed amphipathic polyethyleneglycol (PEG)-coated long-circulating liposomes (PEG-liposomes) have great potential as a drug delivery system. The inclusion of PEG appreciably reduces the reticuloendothelial system (RES) uptake of liposomes and effectively prolongs the circulation time of liposomes.¹⁻⁴⁾ In terms of drug delivery, these so-called long-circulating liposomes have several advantages over traditional liposomal formulations. We have demonstrated that a remarkable accumulation of doxorubicin (DXR) in solid tumor tissue in tumor-bearing mice can be achieved by encapsulation of the drug in PEG-liposomes, since the

vasculature in the tumor tissue is leaky enough for extravasation of small-sized PEG-liposomes.^{5,6)} Enhanced delivery and antitumor activity of DXR were observed in the combined treatment of solid tumors in mice with thermosensitive PEG-liposomes and local hyperthermia.⁷⁾

PEG is particularly useful because of its ease of preparation, relatively low cost, controllability of the molecular weight and linkability to lipid by various methods. PEG is believed to act by forming a hydrophilic coat and by causing steric hindrance at the liposome surface, thus reducing liposome-serum protein interaction and liposome-RES cells interactions. Thus, the lipid

Fig. 1. Structure of Polyethyleneglycol Derivatives

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moiety of the molecule must be sufficiently lipophilic to firmly anchor the hydrophilic coat to the surface.

In this study, we synthesized a new series of lipid derivatives of PEG (Fig. 1) and tested their abilities to prolong the circulation time of liposomes and their influence on membrane fluidity. Furthermore, we tested the hypothesis that the ability of liposomes to be removed from the circulation by the RES *in vivo* could be correlated with the uptake of liposomes by macrophages *in vitro*.

MATERIALS AND METHODS

Materials Distearoylphosphatidylcholine (DSPC) (Coatsome MC-8080), monomethoxy polyethyleneglycol succinimidyl succinate (PEG-OSu), polyethyleneglycol bis(succinimidyl succinate) (PEG-2OSu), α -(dipalmitoylphosphatidyl)- ω -hydroxypolyoxyethylene (DPP-PEG-OH) with an average molecular weight of 1000, α -cholesteryl- ω -hydroxypolyoxyethylene (CH-PEG) with average molecular weights of 800, 1700, 2600 and 4800, α -methoxy- ω -(1,2-dioctadecenoyloxy glyceryl)polyoxyethylene (DO-PEG) with an average molecular weight of 1000 and α -methoxy- ω -(1,2-ditetradecenoyloxy glyceryl)polyoxyethylene (DT-PEG) with an average molecular weight of 1000 were kindly donated by Nippon Oil & Fats (Tokyo, Japan). The average molecular weight of PEG used was measured by gel permeation chromatography (Shodex KF801, KF803 and KF804 combination columns). Cholesterol (CH) and triethylamine were purchased from Wako Pure Chemical (Osaka, Japan). Diphenylhexatriene (DPH) was purchased from Sigma Chemical Co. $^{67}\text{GaCl}_3$ was a kind gift from Dr. I. Umeda (Teikyo University). Other chemicals used were of reagent grade.

Synthesis of DSPE-PEG and DSPE-PEG-COOH Distearoylphosphatidyl-*N*-(methoxy polyoxyethylene succinyl)ethanolamine (DSPE-PEG) and distearoylphosphatidyl-*N*-(3-carboxypropionyl polyoxyethylene succinyl)ethanolamine (DSPE-PEG-COOH) were synthesized as described previously.⁴⁾ In brief, an aliquot of PEG-OSu or PEG-2OSu in CHCl_3 was added to a solution of DSPE in CHCl_3 -methanol (3:1, v/v), followed by addition of triethylamine to give a 3:1:3.5 molar ratio of PEG-2OSu/DSPE/triethylamine. The reaction mixture was stirred overnight at 35°C. Full conversion of the primary amino group in DSPE was confirmed by the negative ninhydrin reactivity after separation of the products by TLC. Phospholipid phosphorus assay⁸⁾ showed the presence of a new phosphate-positive spot at a higher *R_f* value than that of DSPE. A small amount of water was added to the evaporated reaction residues to form micelles. The DSPE-PEG-COOH micelles were dialyzed for 5 d against water using a dialysis bag with large pores (Spectra-Por CE 300000 MWCO, Spectrum Medical, CA) and then lyophilized.

Preparation of Liposomes Liposomes composed of DSPC and CH (1:1 molar ratio) and containing various PEG-derivatives (6 mol%) were prepared according to the method described for reverse-phase evaporation vesicles by Szoka and Papahadjopoulos.⁹⁾ A radiolabeled aqueous marker, ^{67}Ga -deferoxamine (DF), was in-

corporated into the liposomes. The resulting liposomes were extruded ten times through two stacked 200 nm polycarbonate membrane filters (Nuclepore Co., CA). The extrusion was performed at 60°C in a thermostated extrusion device (Extruder, Lipex Biomembrane, Canada). The extruded liposomes were chromatographed on a Bio-Gel A 1.5 m column (Bio-Rad Lab., CA) to remove unencapsulated ^{67}Ga -DF. Liposome size was determined by dynamic laser light-scattering measurements using a Nicomp 370 HPL submicron particle analyzer.

Animal Experiments Male ddY mice weighing 23–27 g were obtained from Sankyo Labo Service (Shizuoka) and were maintained in standard housing. They were injected in the tail vein with 0.5 mg/mouse of lipids in 0.2 ml of saline. At desired times post-injection, mice were lightly anesthetized and bled by eye puncture. Blood was collected and weighed. The mice were killed by cervical dislocation and dissected. Organs were collected, weighed and analyzed for ^{67}Ga -DF radioactivity in an Aloka ARI250 gamma-counter (Aloka Co., Tokyo). ^{67}Ga -DF was used as a nonmetabolizable aqueous marker for liposomes.¹⁰⁾ Any free marker that leaks from the liposomes is rapidly excreted from the animal with a short half-life (less than 5 min). Thus, the marker retained in the blood effectively represents intact liposomes. The results are presented as percentages of the total injected dose for each organ. The radioactivity accumulated in lung, heart and kidneys did not exceed 3% of the injected dose. The total radioactivity in the blood was determined by assuming that the total volume of blood was 7.3% of the body weight.¹¹⁾ Contamination with blood in each organ was determined by examining the distribution of ^{51}Cr -labeled erythrocytes 30 min after i.v. injection.⁴⁾

Measurement of Liposomal Membrane Fluidity Fluorescence anisotropy of DPH, which is a marker for the fluidity of the lipid bilayer, was measured by a reported method.¹²⁾ A solution of DPH in tetrahydrofuran was added to the liposome suspension at a final concentration of 0.2 mol%. Liposomes were incubated with saline or mouse serum for 1 h at 37°C. The excitation and emission wavelengths used for DPH were 360 nm and 430 nm, respectively. The anisotropy was measured at 37°C and calculated using the following equation:

$$r = \frac{I_2 - I_1 \times G}{I_2 + 2I_1 \times G}$$

where I_2 is the fluorescence intensity parallel to and I_1 is the intensity perpendicular to the plane of polarization of the excitation beam. G is the correction factor obtained using horizontally polarized light ($G = I_1/I_2$).

Assay of Liposomal Binding to J774 Cells Cells of J774, a murine macrophage-like cell line, were cultured in RPMI1640 medium (Nikken Inc., Tokyo) with 10% complement-immobilized fetal calf serum (FCS, Filtron Pty Ltd., Australia). J774 cells were plated in wells (1 cm diameter) of microtiter plates at 1×10^6 cells per 1 ml and incubated for 24 h at 37°C in a CO_2 incubator to permit adherence of the cells to the wells. Then the cells were washed with fresh medium and incubated in 1 ml

of medium with 10% FCS for 1 h. Liposomes (500 nmol as phospholipids) containing ^{67}Ga -DF were added to each well, and the plates were incubated for 4 h at 37 °C in a CO_2 incubator or at 4 °C. After incubation, the wells were washed three times with ice-cold saline to remove unbound liposomes. Liposomes associated with cells were analyzed for ^{67}Ga radioactivity in an Aloka 250 gamma-counter.

RESULTS AND DISCUSSION

Biodistribution of PEG-Liposomes in Mice Several laboratories have independently concluded that, in order to use PEG to prepare long-circulating liposomes, it must be covalently linked to a lipid anchor. Each group has developed its own chemical strategy for synthesis of PEG-derivatives, *i.e.*, dioleoylphosphatidylethanolamine-PEG,¹⁾ DSPE-PEG, stearic acid-PEG,¹³⁾ dipalmitoylglycerol-PEG,³⁾ and CH-PEG.^{13,14)} We previously demonstrated that DSPE-PEG with average molecular weights in the range of 1000–12000, when incorporated into the liposome membrane, appreciably reduced the uptake of liposomes by the RES and hence effectively prolonged the residence time in the blood.⁴⁾ We also studied whether circulation time was influenced by difference in lipid composition or termination of PEG.

In this study we synthesized new lipid derivatives of PEG as shown in Fig. 1, and tested their ability to prolong the circulation time of liposomes *in vivo*. We first examined the influence of various derivatives of PEG on the biodistribution of DSPC/CH liposomes (1 : 1, m/m) with an average diameter of 180–200 nm. Liposomes containing 6 mol% of PEG-derivatives were injected into mice *via* the tail vein and the biodistribution was measured 6 h later. Incorporation of 6 mol% of PEG-derivatives was taken as the standard in this experiment for convenience in comparing the data with previous data from our laboratory and other groups. The percent of the injected dose in blood, liver and spleen, the blood/RES ratio and the percent remaining *in vivo* are summarized in Table 1. Incorporation of the PEG-derivatives examined into liposomes appreciably increased the blood level of

liposomes and correspondingly decreased the RES uptake at 6 h after injection. Conventional liposomes without PEG showed low blood levels and high accumulation in liver and spleen, suggesting that liposomes in the size range of 180–200 nm are readily taken up by the RES. Liposomes composed of DSPC/CH and 6 mol% of DSPE-PEG1000 had a substantially elevated blood level of 25.0% of the injected dose. Liposomes containing other PEG-derivatives lacking the phosphatidylethanolamine (PE) residue, such as DO-PEG1000 and DT-PEG1000 had blood levels of 29.4% and 28.5%, respectively, which were not appreciably different from those seen for liposomes containing DSPE-PEG1000. When CH-PEG was incorporated in liposomes, increasing the PEG molecular weight from 800 to 4800 caused an increase in blood level from 7.8 to 32.9%. However, the blood level of CH-PEG4800 at 6 h after injection was similar to that of DSPE-PEG1000. On the other hand, the blood levels of liposomes containing PEG-derivatives which have functional groups at their terminals, such as DPP-PEG-OH1000, DSPE-PEG-COOH2400 and DSPE-PEG-COOH3600, were higher than that of the liposomes containing DSPE-PEG1000 (39.1%, 43.3% and 50.9%, respectively, *versus* 25.0%). Inclusion of these PEG-derivatives in liposomes resulted in longer circulating activity than that of similar molecular weight DSPE-PEG (data not shown).

Parr and co-workers¹⁵⁾ examined the influence of the lipid anchor and linkage chemistry in PEG-derivatives on the circulation lifetime of liposomes. The anchoring capacity of PE anchors is extremely sensitive to the acyl chain composition, and distearoyl PE species are considerably more effective anchors than palmitoyloleoyl species. They tested the chemical stability of four different linkages, including succinate, carbamate and amide linkages between PEG derivatives and the amino head group of PE, as well as a direct link to the phosphate head group of phosphatidic acid, in serum at 37 °C. The succinate linkage is the most labile.

Although the mechanism of action of the PEG-derivatives in prolonging the circulation time of liposomes has not been fully elucidated, increased hydrophilicity¹⁶⁾

Table 1. Effects of Derivatives of Poly(Ethylene Glycol) on Tissue Biodistribution, Blood/RES and Percent Remaining *in Vivo* for DSPC/CH Liposomes in Mice

Liposomal composition	Percentage of dose			Blood/RES	% remaining <i>in vivo</i>
	Blood	Liver	Spleen		
(DSPC/CH)	3.9 (0.7)	48.8 (3.8)	18.1 (3.3)	0.1	71.1 (3.9)
DSPE-PEG1000	25.0 (4.2)	15.1 (0.4)	9.5 (1.3)	1.0	63.0 (0.5)
DO-PEG1000	29.4 (3.0)	14.6 (0.9)	11.3 (2.0)	1.1	59.2 (0.7)
DT-PEG1000	28.5 (9.3)	22.2 (4.0)	12.7 (6.9)	0.8	67.5 (4.4)
CH-PEG800	7.8 (2.7)	35.0 (5.7)	20.9 (0.9)	0.1	66.4 (1.2)
CH-PEG1700	20.4 (2.8)	30.0 (6.1)	13.8 (1.6)	0.5	67.5 (2.6)
CH-PEG2600	27.1 (2.9)	27.8 (5.2)	14.6 (7.4)	0.6	72.1 (7.2)
CH-PEG4800	32.9 (0.5)	21.0 (0.2)	14.1 (0.8)	0.9	73.1 (0.1)
DPP-PEG-OH1000	39.1 (5.3)	14.3 (0.9)	3.9 (0.9)	2.1	62.9 (4.1)
DSPE-PEG-COOH2400	43.3 (6.7)	15.8 (3.5)	3.5 (0.2)	2.2	67.5 (4.6)
DSPE-PEG-COOH3500	50.9 (3.1)	15.0 (4.9)	3.6 (0.3)	2.7	74.9 (5.7)

DSPC/CH (1 : 1 molar ratio) liposomes and those containing various derivatives of PEG were injected *via* the tail vein in mice and their biodistribution was estimated at 6 h after injection. Results are expressed as means (S.D.) ($n=3$).

and/or a steric barrier¹⁷⁾ at the liposomal surface, provided by PEG, play an important role. As shown in Table 1, the blood level of CH-PEG liposome was dependent on the molecular weight of PEG. It was reported by Mori *et al.*¹⁷⁾ that the activity of PEG in prolonging the circulation time of liposomes is directly proportional to the chain length of the PEG. They further examined the steric barrier activity of PEG by a liposome agglutination assay and showed that this activity was directly correlated to the chain length of PEG. PEG is a linear polymer which exhibits considerable conformational flexibility. Torchilin *et al.*¹⁸⁾ reported that the molecular mechanism of PEG protective action is determined by the properties of the flexible polymer molecule (free rotation of individual units around inter-unit linkages) in solution, and involves the formation of a dense polymeric "cloud" over the liposome surface. It was shown that PEG coating of liposomes hinders protein interaction with the liposome surface, whereas liposome-grafted dextran (a more rigid polymer) in similar quantities does not affect the interaction of protein and liposome. The activity of CH-PEG4800 was less effective than DSPE-PEG1000 in spite of the long chain length of PEG. Although the reason for the differences between DSPE-PEG and CH-PEG liposomes in ability to prolong the circulation time of liposomes is still unknown, it is possible that CH-PEG derivatives offer low steric hindrance and the conformational transitions proceed at a slower rate than those of DSPE-PEG, so the cholesterol-anchor may be located deeper in the liposome membrane. PEG-OH and PEG-COOH derivatives were effective in preventing RES uptake, probably because they form high-density conformational clouds over the liposome surface in spite of the negative charge in the PEG-terminal. DSPE-PEG-COOH could be used to prepare novel immunoliposomes carrying monoclonal antibodies at the distal ends of the PEG chains. Attachment of antibodies to the distal terminal of the PEG chain should afford immunoliposomes with both prolonged circulation time and good target binding.¹⁹⁾

PEG-Liposomal Membrane Fluidity Measurement Previous reports on the composition of long-circulating liposomes concluded that liposomes of low membrane fluidity generally showed better long-circulating activities.^{20,21)} It seems that the destabilization of liposomes in blood is related to the association of proteins with liposomes. There are at least two possibilities here: the first is that proteins are attached to the surface of liposomes, and the second is that proteins are inserted into the liposome membrane. In the latter case, the fluidity afforded to the membrane by a PEG-derivative is expected to decrease appreciably. We have examined this possibility by measuring the fluorescence depolarization of a hydrophobic probe, DPH, incorporated into the lipid core of the liposome membrane. The fluorescence depolarization was measured for liposomes suspended in saline, as well as for those which had been treated with 50% mouse serum at 37°C for 1 h and then separated from non-associated serum components by column chromatography. As summarized in Table 2, the values of anisotropy of the liposome membrane, composed of DT-PEG1000, DO-PEG1000 or DSPE-PEG1000, showed similar levels

Table 2. Change of Membrane Fluidity of DSPC/CH Liposomes upon Interaction with Serum

	Fluorescence anisotropy		Saline/serum
	Saline	Serum	
(DSPC/CH)	0.384±0.002	0.227±0.001	1.7
DSPE-PEG1000	0.384±0.002	0.320±0.003	1.2
DO-PEG1000	0.384±0.003	0.329±0.003	1.2
DT-PEG1000	0.378±0.004	0.351±0.009	1.1
CH-PEG800	0.365±0.011	0.240±0.002	1.5

DSPC/CH (1:1 molar ratio) liposomes containing 0.2 mol% DPH and various derivatives of PEG were incubated with an equal volume of either saline or serum for 1 h at 37°C. Results are expressed as means (S.D.) (n=3).

Fig. 2. Uptake of DSPC/CH Liposomes Containing 6 mol% of Various Derivatives of PEG (500 nmol Lipid/10⁶ Cells, Labeled with ⁶⁷Ga-DF) by J774 Cells during 4 h Incubation

Liposome uptake was calculated as specific uptake (uptake at 37°C minus uptake at 4°C) and is shown as a percentage ratio of specific uptake of DSPC/CH liposomes (n=6).

(0.378—0.384) excepting CH-PEG800 (0.365) in saline. The anisotropy of the liposome membrane after the serum treatment was reduced. However, the decrease was small for PEG-liposomes as compared with conventional liposomes. It is clear that there is a correlation between the long-circulating activity and the low fluidity of the liposomes.

PEG-Liposomal Uptake by Macrophages Macrophages play a major role in clearance of foreign particulate matter, including liposomes, from the blood circulation. We have tested PEG-derivatives for their ability to affect the uptake of liposomes by macrophages. The interaction of liposomes with J774 cells was studied by measurement of the ⁶⁷Ga-DF activity associated with the cells at 4°C and 37°C. Cell-associated radioactivity at 37°C represents a combination of liposome adsorption on the cell surface and endocytosed liposomes, whereas at 4°C endocytosis is inhibited and surface adsorption is the main mode of liposome association with the macrophages, as demonstrated earlier.^{22,23)} The effects of various derivatives of PEG on uptake of liposomes by macrophages, as compared to DSPC/CH liposomes, are shown in Fig. 2. DSPE-PEG1000 resulted in a 50% reduction of uptake of liposomes by macrophages. Other PEG-derivatives, DO-PEG1000, DT-PEG1000 or CH-PEG800, caused lower reductions in uptake (30%, 34% or 32%, respectively). CH-PEG, when incorporated into liposomes,

substantially decreased liposome uptake by macrophages in a molecular weight-dependent manner. The incorporation of CH-PEG4800 in liposomes reduced the uptake by as much as 70%, while liposomes containing DSPE-PEG-COOH showed a 60% reduction in uptake. Recently, a good correlation was observed between RES uptake of liposomes *in vivo* in mice and uptake of liposomes by bone marrow macrophages *in vitro*.¹³⁾ The effect of PEG-derivatives at the liposomal surface *in vivo* seems well correlated with the cellular uptake of liposomes *in vitro*. PEG-derivatives appear to increase liposome surface steric hindrance, aiding the avoidance of opsonization by serum protein and consequent recognition by macrophages as a foreign object.

These novel PEG-derivatives can be regarded as the key components of second-generation long-circulating liposomes with a wide range of potential medical applications. Further advances in an understanding of the mechanisms involved would permit even better control of biological interactions and should greatly extend the therapeutic applications.

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