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Phosphatidyl polyglycerols prolong liposome circulation in vivo

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Abstract

To obtain liposomes with longer circulation times in vivo, we newly synthesized dipalmitoylphosphatidylpolyglycerol (DPP-PG). A series of DPP-PGs of different chain lengths was used in this study. The individual derivatives were incorporated into distearoylphosphatidylcholine/cholesterol liposomes (1:1, molar ratio). The effectiveness of DPP-PG derivatives was dependent on the amount and degree of polymerization. Low-polymerized PGs such as diglycerol and tetraglycerol needed a high incorporation rate of 8 mol%, while high-polymerized PGs such as octaglycerol required 4 mol%. The incorporation of 6 mol% of DPP-hexaglycerol was most effective in prolonging the circulation time of liposomes.

Key words: Prolonged circulation; Circulation time; Drug delivery; Liposome

Clearance of liposomes from circulation in vivo depends on their rate of uptake by reticuloendothelial system (RES) cells in the liver and spleen. Incorporation of polyethylene glycol-derived phosphatidylethanolamine (PEG-PE) (Klibanov et al., 1990) or monosialoganglioside (G_{M1}) (Allen and Chonn, 1987) into liposomes has resulted in altered pharmacokinetics of these liposomes, leading to increased blood levels of drug-carrying liposomes and reduced uptake by RES cells. In terms of drug delivery, these so-called long-circulating liposomes have several advantages over traditional liposomal formulations. PEGs are particularly useful because of their ease of preparation, relatively low cost, controllability of molecular weight and linkability to lipids by a variety of methods. The mechanism of action of these coatings is explained by the hydrophilicity and steric hindrance of PEG on the liposome surface, reducing liposome-serum protein interactions and liposome-RES cells interactions (Allen, 1992; Senior, 1992). It is therefore likely that other molecules will be identified and synthesized based on the above functions of PEG. In this study, we newly synthesized a number of polyglycerol (PG)-derived phospholipids and assayed the

Abbreviations: DPPC, dipalmitoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; G_{M1}, monosialoganglioside; PE, phosphatidylethanolamine; LUV, large unilamellar vesicle.

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biodistribution of such coating-modified liposomes in mice.

A series of dipalmitoylphosphatidylpolyglycerols (DPP-PG) were synthesize by phosphatidylation of polyglycerol via phospholipase D (Yang et al., 1967). DPPC (7 mmol) in chloroform was mixed with polyglycerol (180 mmol) in 1 M acetate buffer (pH 5.6) at 40°C. Diglycerol (Mol. Wt 166), tetraglycerol (average Mol. Wt 300), hexaglycerol (average Mol. Wt 500), octaglycerol (average Mol. Wt 600) and decaglycerol (average Mol. Wt 750) purchased from Kashima Chemical (Tokyo) were used as polyglycerol. 200 U of phospholipase D (Toyo Jyozo, Tokyo) was added to this mixture and stirred for 6 h at 40°C. The pH of the reaction mixture was then increased to 6.8 with sodium acetate, and the chloroform layer was separated and evaporated. Purification was achieved by repeated recrystallization with ethanol, and confirmed by thin-layer chromatography with a solvent system of $CHCl_3$/MeOH/H_2O/28% NH_4OH (65:25:4:0.3, v/v). The thin-layer chromatoplate showed it to be in a high state of purity (> 95%). Infrared absorption bands of O-H and ester groups appeared at 3400 and 1735 cm^{-1}, respectively.

Liposomes were prepared from DSPC, cholesterol (1:1, molar ratio), and an appropriate amount of each DPP-PG with various molecular weight values by the reverse-phase evaporation method. The lipid mixture was dissolved in isopropyl ether/chloroform (1:1, v/v), to which was added saline containing a tracer marker, ^{67}Ga-deferoxamine, with a half volume of organic solvent. The mixture was sonicated for 30 s to give a w/o emulsion, and the organic solvent in the emulsion was evaporated slowly at 60°C to form an LUV suspension. Liposomes were extruded through Nuclepore filters to control size (100–150 nm in diameter) then passed through a Biogel A1.5 m column. Liposome size was measured by a Nicomp 370 HPL submicron particle

Table 1
Biodistribution and zeta potential of liposomes containing DPP-PG

Liposomes	mol%	Biodistribution (% injected dose)			Zeta potential (mV)
		Blood ± SD	Liver ± SD	Spleen ± SD	
(DSPC/CH)	–	18.30 ± 1.78	31.27 ± 2.33	13.65 ± 3.01	0.15
DPP-PG(2)	6	24.95 ± 8.13	27.48 ± 7.35	7.90 ± 1.74	
	8	29.47 ± 1.13	24.75 ± 7.95	5.10 ± 0.67	−11.68
	10	19.34 ± 1.40	36.13 ± 7.19	3.02 ± 0.82	
DPP-PG(4)	6	16.40 ± 5.08	38.39 ± 8.32	12.93 ± 2.19	
	8	25.14 ± 5.76	34.94 ± 2.91	9.75 ± 5.25	−13.57
	10	19.75 ± 6.14	40.96 ± 3.93	7.89 ± 3.34	
DPP-PG(6)	2	3.01 ± 1.23	54.85 ± 2.38	16.51 ± 3.19	
	4	31.08 ± 7.80	31.10 ± 2.33	10.89 ± 3.88	
	6	38.11 ± 5.44	16.70 ± 0.64	6.02 ± 2.06	−13.78
	8	5.69 ± 2.75	49.52 ± 2.28	10.21 ± 0.72	
	10	0.36 ± 0.31	66.85 ± 9.28	3.52 ± 1.62	
DPP-PG(8)	?	0.91 ± 0.08	46.23 ± 5.11	20.43 ± 5.04	
	ł	26.85 ± 4.68	31.06 ± 2.51	11.05 ± 3.28	−11.99
	ś	23.63 ± 3.90	28.18 ± 5.74	13.91 ± 3.86	
DPP-PG(10)	2	9.55 ± 5.22	40.54 ± 2.70	14.87 ± 4.77	
	4	13.58 ± 7.26	28.72 ± 2.58	7.69 ± 2.93	−12.70
	6	2.91 ± 0.04	38.76 ± 2.98	15.49 ± 1.83	

Liposomes composed of DSPC/cholesterol (1:1) and various amounts of DPP-PG were labelled with ^{67}Ga-deferoxamine and i.v. injected into mice. Percentage of the injected dose in blood, liver and spleen was measured 6 h post-injection, and represented as the mean ± S.D. of 3–6 mice.

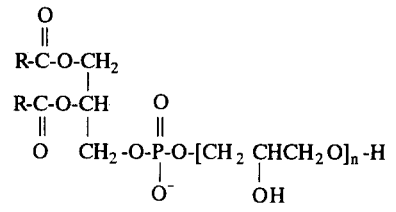

Fig. 1. Dipalmitoylphosphatidylpolyglycerol (R = C16).

analyzer (HIAC Pacific Scientific, CA, U.S.A.). Phospholipid content of the liposome solutions was determined by phosphorus assay.

^{67}Ga-labeled liposome (0.5 mg lipid/mouse) was injected i.v. into male dd/Y mice (20–25 g). At 6 h after injection, mice were bled from the retro-orbital sinus under anesthesia and killed by cervical dislocation. Blood was collected in heparinized tubes. Internal organs including the liver, spleen, kidney, lung and heart were immediately collected. These tissues were lightly rinsed in saline to remove any excess blood, weighed, then cooled in ice. Their radioactivities were immediately measured in a gamma counter (Aloka ARI250). The weight of mouse blood was assumed to be 7.3% of total body weight. Contamination of blood in each organ was corrected (Maruyama et al., 1993). The zeta potential of liposomes was measured by electrophoretic light scattering (ELS-800, Otsuka Electronics) after dilution of the liposomal solution with saline.

The structure of DPP-PG derivatives is shown in Fig. 1 and their ability to prolong liposomal circulation in vivo is summarized in Table 1. Liposomes (118 ± 28 nm as SD) composed of DSPC and cholesterol were rapidly taken up by the RES, and showed a low blood concentration at 6 h after injection. The incorporation of DPP-PG derivatives into DSPC/cholesterol liposomes (117.7 ± 36 nm as SD) appeared to decrease liposome uptake by RES cells and resulted in a prolongation of circulation time. Ability to prolong circulation time was dependent on both the degree of polymerization of PG and their incorporation rate. Low polymerized PGs such as PG(2) and PG(4) needed a high incorporation rate of 8 mol%. In contrast, a low incorporation rate of 4 mol% was sufficient to prolong circulation with the higher polymerized PGs. Incorporation of DPP-PG(6) into liposome with 6 mol% was most effective. DPP-PG(10) had little effect on liposome circulation or RES uptake. It therefore appears that there exists an optimum amount of DPP-PG which can be incorporated into liposomes, and that this amount depends on the degree of polymerization of PG. The DPP-PG-coated liposomes showing the greatest blood residence time in each group were subjected to the measurement of zeta potential. Potentials were between -11.7 and -13.8 mV; these liposomes thus showed similar negativity.

It is well known that large liposomes and liposomal aggregates are rapidly taken up by the RES. In the present study, liposomes lacking PG precipitated during storage in a refrigerator for a few days owing to aggregation. In contrast, DPP-PG-coated liposomes exhibited good dispersion characteristics, and no aggregation was observed even after storage for several weeks. The improved dispersion of these liposomes was considered to be due to the negativity and increased hydrophilicity of the liposome surface. Thus, the incorporation of negatively charged DPP-PG into lipid bilayers and the small size resulted in low aggregation and reduced RES uptake (Table 1). The increased circulation time was attributable primarily to these two factors.

It has been observed that liposomes containing negatively charged phospholipids are removed rapidly from the circulation and concentrated mainly in the RES (Allen et al., 1984). Phosphatidylserine, phosphatidylglycerol and other negatively charged phospholipids are known to enhance liposome uptake by RES cells (Raz et al., 1981; Allen et al., 1988). In contrast, G_{M1}, sulfatides and phosphatidylinositol, which are also negatively charged, inhibit RES uptake and prolong the circulation time of liposomes (Allen et al., 1989). It has been proposed that the negative charge is shielded by surrounding bulky neutral hydrophilic groups. The hypothesis has accurately predicted activity in the prolongation of circulation time of PEG-phospholipid derivatives containing a negatively charged phosphate group shielded by a PEG chain. The observations of Park et al. (1992), however, are not consistent

with this hypothesis; they reported that negatively charged phospholipids with an exposed and unshielded carboxylic group such as *N*-glutaryl PE and *N*-adipyl PE also show considerable activity in prolonging the circulation time of liposomes.

It has been suggested that the PEG chains of phospholipid derivatives create steric hindrance on the liposome surface which prevents opsonization (Lasic et al., 1991). To determine whether DPP-PG can result in steric hindrance on the liposome surface, the effect of these derivatives on liposome agglutination was measured by the liposome agglutination assay (Klibanov et al., 1991). Liposomes were prepared from DSPC/CH/DPP-PG(4), DPP-PG(6), PEG1000-PE or G_{M1} (10:10:1.3, molar ratio) and 2.5 mol% biotinamidocaproyl-PE. 50 μl of liposomes (1 mg lipid/ml) were mixed with 0.5 ml PBS in a microcuvette, to which 5 μg streptavidin was added. Increase in turbidity was monitored as absorbance at 440 nm. It has been previously shown that such measurement sensitively indicates the steric barrier activity of G_{M1} and PEG-PE, such that the longer the chain length of PEG, the greater the steric hindrance created by the PEG; interestingly, G_{M1} provides relatively weak steric hindrance (Klibanov et al., 1991; Mori et al., 1991). As shown in Fig. 2, liposome agglutination mediated by streptavidin was slightly inhibited in the presence of DPP-PG(4) and G_{M1}, and was significantly inhibited by PEG1000. DPP-PG(6) showed an intermediate effect. The results of agglutination assay indicated that DPP-PG(4) and DPP-PG(6) provide weaker steric hindrance than PEG1000-PE. The relationship between the steric hindrance activity of DPP-PG and its activity in prolonging the circulation time of liposomes is therefore ambiguous.

It has been proposed that surface hydrophilicity might play a key role in the phagocytosis of particulate matter (Allen, 1992; Senior, 1992). Illum et al. (1986) have shown that a hydrophilic coating decreases the uptake of colloidal particles by hepatic and peritoneal macrophages. The prolonged circulation half-lives of liposomes containing G_{M1} have also been ascribed to the increase in surface hydrophilicity which the incorporation of G_{M1} provides. A similar mechanism has been

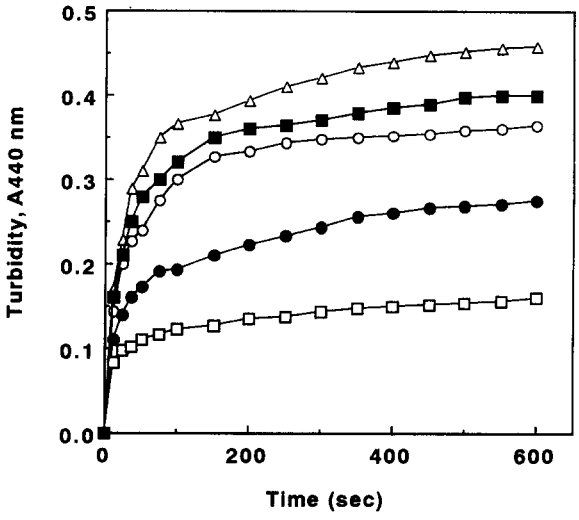

Fig. 2. Effect of DPP-PG on the streptavidin-induced agglutination of liposome containing biotianidocaproyl-PE. Increase in turbidity ($A_{440\ nm}$) was measured with time for liposomes (DSPC/CH (Δ)) containing 8 mol% of PG(4) (○), 6 mol% of PG(6) (●), PEG1000 (□) and G_{M1} (■).

proposed for the effect of PEG on PEG-modified liposomes. A hydrophilic coating would provide sufficient bulk to create a hydrophilic layer which would prevent opsonization of liposomes with plasma compartments involved in the recognition and uptake of the vesicles. Polyglycerol (PG) is a more hydrophilic polymer than PEG. We therefore consider that the reduction of uptake of DPP-PG-coated liposomes by the RES was partly due to the increase in surface hydrophilicity induced by polyglycerol modification.

It has been shown that G_{M1}-liposomes, which have relatively weak steric hindrance, allow immunoliposomes to bind with target cells in a highly efficient manner (Maruyama et al., 1990). The target binding of the PEG-PE-coated immunoliposomes, however, was dependent on the chain length of PEG: PEG with an average molecular weight of 5000 caused a reduction in target binding of immunoliposomes due to its overly strong steric hindrance, whereas PEG with an average molecular weight of 2000 induced high targeting activity which was comparable with that of G_{M1} (Mori et al., 1991). DPP-PG, newly synthesized in this study (Fig. 2), showed weak

steric hindrance, and is expected to provide better binding of immunoliposomes to target cells. Its use in immunoliposomes should be investigated.

In conclusion, DPP-PG-coated liposomes had a longer circulation time than liposomes lacking DPP-PG. There is an optimum amount and degree of polymerization of PG. This effect depends on surface masking by the hydrophilic layer created with PG. Moreover, the inclusion of DPP-PG endows liposomes with sufficient hydrophilicity to protect them from recognition by the RES.

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