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# 1-O-Palmityl-D-glucuronate Endows Liposomes with Long Half-Life In Vivo

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More liposomes containing 1-O-palmityl-D-glucuronic acid (PGA), a synthetic glycolipid, bound to macrophages than those containing phosphatidylglycerol did in vitro; however, PGA-liposomes circulated longer in vivo. PGA-liposomes did not aggregate in the presence of serum, but liposomes containing 1-O-palmityl-D-glucose or myristic acid aggregated rapidly, suggesting that both carbohydrate and carboxyl group of PGA are important for preventing liposomal aggregation in serum. This low agglutinative character may be one of the factors for long circulation of PGA-liposomes in vivo.

Liposomes have been used widely as microcapsules for drug delivery. When liposomes were administered systemically, they tend to be trapped in reticuloendotherial system (RES), such as liver and spleen.[1] When liposomes are used as reservoir for sustained release of drugs, this tendency of liposomes is unfavorable. For prolonging the circulation time of liposomes in blood, many applications have been investigated.[2] There are several factors which affect the circulation time of liposomes, i.e., size, charge, rigidity of liposomal membrane, and the content of sialic acid. Small liposomes tend to escape RES-trapping.[3] The incorporation of gangliosides such as $GM_1$ into liposomal membrane is known to be effective in reducing reticuloendotherial uptake of the liposomes.[2] Since removal of sialic acid from liposomes containing $GM_1$ caused an increase in RES-trapping of liposomes, the presence of surface sialic acid is an important factor for prolonging the half-life of liposomes in circulation. Sialic acid is a typical molecule having carbohydrate moiety and carboxyl group in it, and we predict that such a structure should prevent RES-uptake of liposomes.

Here we report the synthesis of a compound which has carbohydrate moiety and carboxyl group. We synthesized glucuronic acid derivative, 1-O-palmityl-D-glucuronic acid (PGA), and investigated the behavior of liposomes modified PGA in vivo. This derivative has several advantages. It has simple structure of glycolipids; thus the synthesis or mass-production of PGA is easy. The presence of

surface sialic acid is known to prolong half-life of liposomes in circulation. PGA has structural resemblance to sialic acid, namely, carboxylic acid on carbohydrate backbone. Glucuronate is a biomolecule; thus PGA is expected to be biodegradable, non-toxic, and should not have immunogenicity and antigenicity. PGA is readily incorporated into liposomal membranes, and because of its simple structure, it is useful for studying cellular recognition of surface-modified liposomes.

1-O-Palmityl-D-glucuronic acid (PGA) was prepared as follows. 4.1 g of hexadecyl alcohol was dissolved in 100 ml $CHCl_3$ and placed in a 500 ml round bottom flask fitted with an additional funnel. After addition of 6.4 g of silver oxide, 2.3 g of iodine and 60 g of activated $CaSO_4$, 2 g of methyl(2,3,4-tri-O-acetyl-α-D-glucopyranosyl bromide)uronate[4] dissolved in 50 ml $CHCl_3$ was added dropwise to the stirred mixture via the additional funnel. The mixture was stirred for 18 hr, monitoring the progress of the reaction by TLC. After the reaction was over, the mixture was filtered and the organic solvent was removed by evaporation. Product was purified by silica gel chromatography. 2 g of the peracetylated glycolipid was obtained. Acetyl group of the glycolipid was removed by the addition of sodium methoxide at room temperature. The reaction progress was monitored by TLC, and at the end of the deacetylation, 10 ml of potassium hydroxide solution was added to the reaction mixture for hydrolyzing methyl ester. After neutralization of the reaction mixture with ion exchange resin, filtrate was concentrated. Finally, 1.5 g of white powder was obtained. PGA was identified by TLC, NMR, and IR spectrum. Orcin-$H_2SO_4$ reagent was used to confirm the presence of carbohydrate moiety in the obtained sample.

TLC; one spot with Rf of 0.1($CHCl_3$/MeOH=8/2): NMR;0.9(3H,q), 1.3(30H,m), 3.0-4.2 (5H,m) 4.4(1H,d): IR; 3388.7(OH), 2850.8($CH_3$, $CH_2$), 1720.5(COOH)

1-O-Palmityl-D-glucose(PGlc) was prepared as follows. 2,3,4,6-Tetra-O-acetyl-α-D-glucopyranosyl bromide dissolved in $CHCl_3$ was added dropwise to the mixture of hexadecyl alcohol, silver oxide, iodine, and $CaSO_4$. The mixture was stirred for 18hr and filtered, and the organic solvent was removed. After complete deacetylation by sodium methoxide, the reaction mixture was neutralized, and the filtrate was concentrated. Synthesized PGlc was identified by TLC, NMR, and IR spectrum.

Liposomes used in this study were prepared as follows. Dipalmitoylphosphatidylcholine (DPPC, 80 μmol), cholesterol (Chol, 80 μmol), and dipalmitoylphosphatidylglycerol (DPPG, 40 μmol) or PGA (40 μmol) dissolved in $CHCl_3$/MeOH were dried under reduced pressure and stored in vacuo for at least 1 h. For the in vivo study, 30 μCi of cholesteryl [$^{14}$C]oleate was added to the lipid film as a marker, and 2 mol% of N,N'-dioctadecyloxacarbocyanine-p-toluene-sulfonate was added in vitro study. Then the lipid film was hydrated with 1.25 ml of 0.3 M glucose. The resulted multilamellar vesicles were frozen and thawed three times, and extruded through polycarbonate membrane with 100 nm pore size.

First we carried out the in vivo study, and examined circulation time of liposomes in blood. Five rats weighing 250-300 g were injected intravenously (i.v.) with liposomes containing 30 μmol lipids. At time intervals after administration of liposomes, blood plasma was collected and the radioactivity in plasma was measured. Percentage of recovery from blood was calculated. As apparent in the

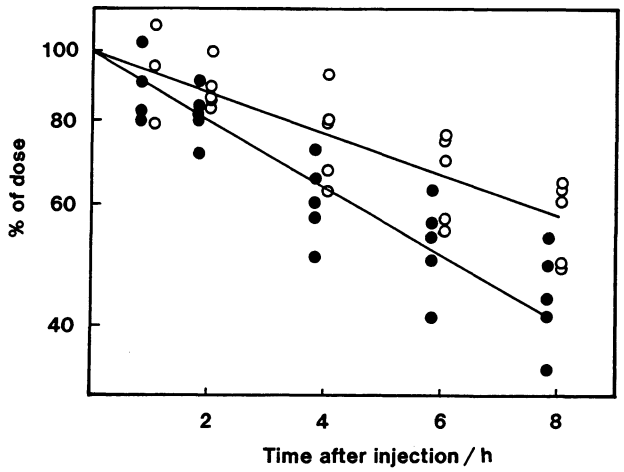


Fig.1. Clearance of liposomes contaning PGA or DPPG from blood stream of rat.

Five rats were injected (i.v.) with 0.5 ml liposomes: DPPC/Chol/PGA (o), or DPPC/Chol/DPPG (●). At time intervals after administration of liposomes, the radioactivity in plasma was measured. Percent of recovery from blood was plotted for each rat.

Table 1. Liposomal binding to mouse peritoneal macrophage-rich cell fraction[a)]

| Liposomes composed of | % bound |
|---|---|
| DPPC/Chol/PGA =45:45:10 | 0.56 |
| DPPC/Chol/PGA =40:40:20 | 1.60 |
| DPPC/Chol/DPPG=45:45:10 | 0.20 |

a) Macrophage-rich cells from ICR mice ($2 \times 10^5$ cells/0.5 ml) were incubated with liposomes containing fluorescent lipid for 30 min at room temperature. After cells were washed with HBSS, fluorescence associated with the cells was measured in the presence of 1% reduced Triton X-100.

Table 2. Turbidity change of liposomes in bovine serum[a)]

| Liposomes composed of | Turbidity ($A_{450}$ x 1000) in 0.3 M glucose | in 70% bovine serum 1 min | 5 min |
|---|---|---|---|
| DPPC/Chol/PGA =45:45:10 | 65 | 24 | 32 |
| DPPC/Chol/PGA =40:40:20 | 96 | 17 | 17 |
| DPPC/Chol/DPPG =45:45:10 | 71 | 41 | 50 |
| DPPC/Chol/DPPG =40:40:20 | 64 | 20 | 22 |
| DPPC/Chol/MA =45:45:10 | 73 | 199 | 288 |
| DPPC/Chol/PGlc =45:45:10 | 232 | 436 | 456 |
| DPPC/Chol/PGlc =40:40:20 | 264 | 560 | 581 |
| DPPC/Chol/MA/PGlc=40:40:10:10 | 98 | 337 | 355 |

a) Liposomes composed as shown in the Table were prepared in 0.3 M glucose and extruded through a polycarbonate filter with 100 nm pore size. Liposomal solution was diluted to 2 mM as lipids in 0.3 M glucose or in 70% bovine serum. Then the turbidity of liposomal solution was measured at 450 nm. MA; myristic acid.

Fig. 1, liposomes containing PGA showed longer half-life in circulation, in comparison with liposomes containing DPPG.

For elucidating why glucuronic acid prolongs liposomal circulation time in vivo, we examined whether PGA-liposomes bind to macrophages. Mouse peritoneal macrophage-rich cell fraction was prepared as follows. ICR mice were injected with 10% polypepton at 4 days before harvest, and cell-rich fluid of peritoneal cavity was collected. Cells were washed three times in Hanks' balanced salt solution (HBSS) and used without removing nonadherent cells. Macrophage-rich cells were incubated with liposomes containing fluorescent lipid in HBSS for 30 min at room temperature, and free liposomes were washed out. Then fluorescence associated with the cells was measured after addition of 1% reduced Triton X-100. As shown in Table 1, unexpectedly, more liposomes containing PGA bound to the cells than control liposomes did. Thus factors other than the recognition by macrophages should be involved in prolonging liposomal circulation time in vivo.

The uptake of liposomes by RES is thought to occur as a result of liposomal aggregation or opsonization by plasma proteins.[5] For examining whether liposomes containing PGA cause aggregation or not, we incubated liposomes in 70% bovine serum. Aggregation was measured by the change in turbidity of liposomes at 450nm: The increase in turbidity indicates the aggregation of liposomes. As shown in Table 2, the turbidity of PGA-liposomes did not increase after a 5 min incubation, whereas that of liposomes containing PGlc or myristic acid increased. This result indicates that the aggregation of PGlc-liposomes or liposomes containing myristic acid occurred but that of PGA-liposomes did not. PGlc has the same sugar moiety as PGA except carboxylic acid. The turbidity of both PGA-liposomes and DPPG-liposomes were decreased, instead of no change, by the addition of serum. The reason for this decrease in turbidity is not clear at this point. The low agglutinability of PGA-liposomes may be one of the factors for prolonging half-life of liposomes in circulation. However, other factors might be important for the long circulatin character, since DPPG-liposomes also did not aggregate during 5 min incubation.

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