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Glucuronate-Modified Liposomes with Prolonged Circulation Time

Yukihiro NAMBA,[a] Toshiyuki SAKAKIBARA,[a] Mikio MASADA,[b] Fumiaki ITO,[c] and Naoto OKU*,[c]

Research Laboratories, Nippon Fine Chemical Co., Ltd.,[a] Takasago, Hyogo 676, Japan, Department of Hospital Pharmacy, Chest Disease Research Institute, Kyoto University,[b] Sakyo-ku, Kyoto 606, Japan and Department of Biochemistry, Faculty of Pharmaceutical Sciences, Setsunan University,[c] Hirakata, Osaka 573-01, Japan. *Received November 13, 1989*

For the purpose of obtaining liposomes with long circulation time in blood, we synthesized 1-*O*-palmityl-D-glucuronic acid (PGlcUA) and incorporated it into the liposomal membranes. The clearance of the PGlcUA–liposomes composed of dipalmitoylphosphatidylcholine (DPPC), cholesterol, and PGlcUA (40 : 40 : 20 as a molar ratio) from blood and their tissue distribution were compared with those of dipalmitoylphosphatidylglycerol (DPPG)–liposomes (DPPC/cholesterol/DPPG = 40 : 40 : 20). When [^{3}H]inulin-loaded PGlcUA–liposomes and DPPG–liposomes were intravenously injected into rats, the half-life of the PGlcUA–liposomes in the blood appeared to be 1.7-fold longer than that of DPPG–liposomes. Radioactivities present in plasma and various tissues were measured 22 h after administration of these liposomes, and radioactivity remaining in the plasma was 2.5-fold greater when PGlcUA–liposomes were injected. The distribution pattern of [^{3}H]inulin in PGlcUA–liposomes was similar to that in DPPG–liposomes. The radioactivity recovered in urine was 25% lower in rats treated with PGlcUA–liposomes than in those treated with DPPG–liposomes. Since both PGlcUA- and DPPG–liposomes exhibited similar size distribution and ζ-potential, glucuronic acid, rather than negative charge, on the liposomal surface appears to endow liposomes with a longer circulation time in the bloodstream.

Keywords liposome; lipid vesicle; glucuronate; drug delivery

Many applications of liposomes as drug carriers and as tools for microinjection have been investigated. Liposomal encapsulation of drugs enhances their efficacy and reduces their side effect. Liposomes have been especially useful as drug carriers against bacteria and parasites that reside in the reticuloendothelial system (RES) or macrophages,[1-3] and as carriers of agents that activate macrophages.[4,5] These application utilize the nature of liposomes to be captured by RES or macrophages. This tendency is unfavorable, however, when the target of liposomes is not the RES, or when liposomes are used as a reservoir for slow release of drugs or biologically active molecules that are normally rapidly removed or degraded in the bloodstream. Monoclonal antibody-modified liposomes are known to bind selectively to desired target cells, but these liposomes are also trapped in the RES and cleared shortly after administration.[6]

Many attempts have been made to prolong the circulation of liposomes. Their clearance from the circulation is greatly affected by their size, and small liposomes with diameters less than 100 nm which can pass through fenestrated endothelium in the liver stay in the bloodstream longer than larger liposomes. Recent studies utilizing liposomes containing monosialoganglioside (G_{M1}) indicated the possibility of reducing the rate and extent of uptake of liposomes into the RES and achieving a prolonged half-life in circulation in mice.[7,8] Furthermore, it is reported that solid liposomes composed of distearoylphosphatidylcholine, cholesterol, and G_{M1} and having diameters less than 100 nm gained longer circulation time due to a reduction of the RES-trapping.[9]

In this paper, we describe the effect of a synthetic glucuronic acid derivative, 1-*O*-palmityl-D-glucuronic acid (PGlcUA), which was incorporated into liposomal membranes, on the behavior of these liposomes *in vivo*. We chose glucuronic acid since it is negatively charged like sialic acid. PGlcUA was readily incorporated into the liposomal membranes, and PGlcUA–liposomes circulated longer in the bloodstream than vesicles lacking the derivative.

Experimental

Chemicals Dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylglycerol (DPPG) were products of Nippon Fine Chemical Co. Cholesterol (Chol) was purchased from Sigma. [^{3}H]Inulin and cholesteryl [^{14}C]oleate were purchased from Amersham.

Preparation of PGlcUA[10] Two grams of methyl(2,3,4-tri-*O*-acetyl-β-D-glucopyranosyl bromide) uronate[11] dissolved in $CHCl_3$ was added dropwise to a reaction mixture containing hexadecyl alcohol, silver oxide, iodine, and activated $CaSO_4$. The mixture was stirred for 18 h at room temperature, and after filtration the organic solvent was removed. The product was purified by silica gel chromatography, and 2 g of peracetylated glycolipid was obtained. Then, deacetylation of the glycolipid was performed by the addition of sodium methoxide, and an aqueous solution of potassium hydroxide was added to hydrolyze the methyl ester. After neutralization with an ion-exchange resin, the filtrate was concentrated. Finally, 1.5 g of white powder was obtained and identified as PGlcUA by thin-layer chromatography (TLC), nuclear magnetic resonance (NMR), and infrared (IR) spectra.

Preparation of Liposomes Liposomes were prepared as follows: DPPC (80 μmol), Chol (80 μmol), trace of cholesteryl [^{14}C]oleate (925 kBq) and DPPG (40 μmol) or PGlcUA (40 μmol) dissolved in $CHCl_3$ were dried under reduced pressure and stored *in vacuo* for at least 1 h. Cholesteryl[^{14}C]oleate was chosen as a liposome marker since it does not exchange rapidly with blood or tissue lipids.[12] The thin lipid film was hydrated with 1.25 ml of 0.3 M glucose containing [^{3}H]inulin (3.7 MBq) to produce multilamellar liposomes. All wares and aqueous solutions were sterilized before use. For preparation of homogeneously-sized liposomes, the multilamellar vesicles were frozen and thawed three times, and then extruded through a polycarbonate membrane with a 100-nm pore size (Nucleopore). Liposomal size was determined by dynamic light scattering with a Submicron particle analyzer (Nicomp Model 370). The mean size obtained was in the range of 135—145 nm, with a standard deviation of about 35 nm. The liposomal solutions were diluted with saline, and untrapped [^{3}H]inulin was removed by centrifugation at 320000 *g* for 30 min. After the liposomes had been washed twice with saline, the liposomal pellet was resuspended in 3 ml of saline. The liposomes were extruded again, this time through a membrane with 200-nm pore size, and centrifuged at 1500 *g* for 5 min prior to administration to rats.

Determination of Hemolytic Activity of PGlcUA in Aqueous Phase or in Liposomes Heparinized blood was freshly drawn from a healthy donor and washed three times with saline. Then, 0.5% (v/v) erythrocytes in 1 ml of saline or 50% bovine serum were incubated for 30 min at 37 °C with various amounts of PGlcUA in aqueous phase or in vesicle bilayers. DPPG vesicles were also used. After centrifugation, released hemoglobin was determined at 541 nm. One hundred percent hemolysis was achieved with hypotonic treatment.

Biodistribution Studies of Liposomes in Rats Five rats weighing

250—300 g were injected intravenously (i.v.) with liposomes containing 30 μmol lipids. At selected time intervals after administration, about 0.2 ml of blood was collected and centrifuged; then the radioactivity in plasma was measured. At 22 h after administration, rats were sacrificed. Liver, spleen, kidney, lung, and brain were removed, washed with saline, and weighed. The radioactivity in each organ was determined by a scintillation counter (Aloka, LSC-3500). The radioactivity of urine accumulated during 0—22 h was also determined. Percentage of recovery from each organ was calculated.

Determination of Calcein Leakage from Liposomes Liposomes were prepared in 0.3 M glucose solution containing 5 mM calcein. After removal of untrapped calcein by centrifugation, liposomes were incubated with 0.5 ml of bovine serum at 37 °C. The amount of calcein released from liposomes was determined fluorometrically (Ex. 488 nm, Em. 520 nm, using a Hitachi F-4000 fluorescence spectrophotometer) after separating liposomes by centrifugation (350000 *g* for 5 min, using a Beckman TL-100 ultracentrifuge). The value for 100% leakage of calcein was determined after destruction of the liposomes by the addition of reduced Triton X-100.

ζ-Potential Measurement ζ-Potential of liposomes with a mean diameter of 200 nm was measured with a Zetasizer 2 (Malvern, U.K.) after dilution of the liposomal solution with distilled water.

Results

Characteristics of Liposomes The ζ-potential of liposomes composed of DPPC, Chol, and PGlcUA (45 : 45 : 10 as molar ratio), and that of those composed of DPPC, Chol, and DPPG (45 : 45 : 10) were determined. Both ζ-potentials were -53.1 mV, and 76.8% of PGlcUA–liposomes and 80.0% of DPPG–liposomes had the potential between -36.0 mV and -69.7 mV; thus the two liposomes showed similar negativity. This result also indicates that PGlcUA is stably incorporated in liposomes.

Next, we determined the stability of liposomes *in vitro*. Table I shows the efflux of preloaded calcein from the liposomes. When liposomes containing 20% or less PGlcUA or DPPG were incubated in bovine serum at 37 °C, they were stable. We used liposomes containing 20% PGlcUA or DPPG for the following experiments.

Interaction of PGlcUA in Aqueous Phase or in Liposomes with Erythrocytes To determine whether or not PGlcUA–liposomes are cytotoxic, we examined the hemolytic activity of PGlcUA–liposomes and DPPG–liposomes. Human erythrocytes were incubated with PGlcUA in the aqueous phase or in liposomal bilayers. As shown in Fig. 1, PGlcUA in the aqueous phase had strong hemolytic activity, whereas PGlcUA–liposomes were far less toxic. PGlcUA in the aqueous phase preincubated with liposomes was as toxic as PGlcUA alone, indicating that exogenous PGlcUA can not be incorporated into liposomes after liposomes have been formed. These results also indicate that PGlcUA that is originally incorporated into the lipid bilayer is stable there.

Table I. Stability of Liposomes Containing PGlcUA or DPPG

Liposomes composed of	Release % of calcein	
	90% bovine serum (3 h, 37 °C)	PBS (1 w, 4 °C)
DPPC : Chol : PGlcUA = 45 : 45 : 10	4.8	1.5
DPPC : Chol : PGlcUA = 40 : 40 : 20	2.3	1.8
DPPC : Chol : DPPG = 45 : 45 : 10	2.1	0.7
DPPC : Chol : DPPG = 40 : 40 : 20	2.1	1.1

Liposomes composed as shown were prepared in 0.3 M glucose containing 5 mM calcein. Liposomes were extruded through a polycarbonate filter having a 100-nm pore size and untrapped calcein was removed by centrifugation. Then, they were incubated in bovine serum at 37 °C for 3 h or in phosphate-buffered saline (PBS), pH 7.0, at 4 °C for 1 week. Calcein released from liposomes was determined fluorometrically and expressed as a percent release, where 100% release was determined after the addition of reduced Triton X-100.

Blood Clearance and *in Vivo* Integrity of Liposomes The blood clearance of PGlcUA–liposomes was compared with that of DPPG–liposomes and is shown in Fig. 2a. Since DPPG–liposomes showed similar size, ζ-potential, and stability to those of PGlcUA–liposomes, they were chosen as control liposomes. In the figure, 100% of injected dose was determined as follows: Blood was collected and the radioactivity in plasma was determined at 5, 10, 15, 30 min

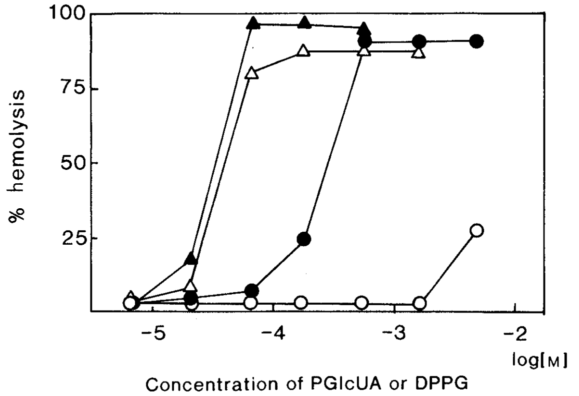

Fig. 1. Hemolytic Activity of PGlcUA in Aqueous Phase or in Liposomes

Liposomes containing PGlcUA or DPPG were obtained after extrusion through a polycarbonate filter with a 100-nm pore size. Human erythrocytes (0.5%) in saline were incubated for 30 min at 37 °C with various amounts of PGlcUA in aqueous phase (▲), PGlcUA–liposomes (○), or DPPG–liposomes (●). Hemolytic activity of PGlcUA in aqueous phase preincubated with DPPC/Chol was also determined (△). After centrifugation, released hemoglobin was determined at 541 nm. One hundred percent hemolysis was achieved with hypotonic treatment.

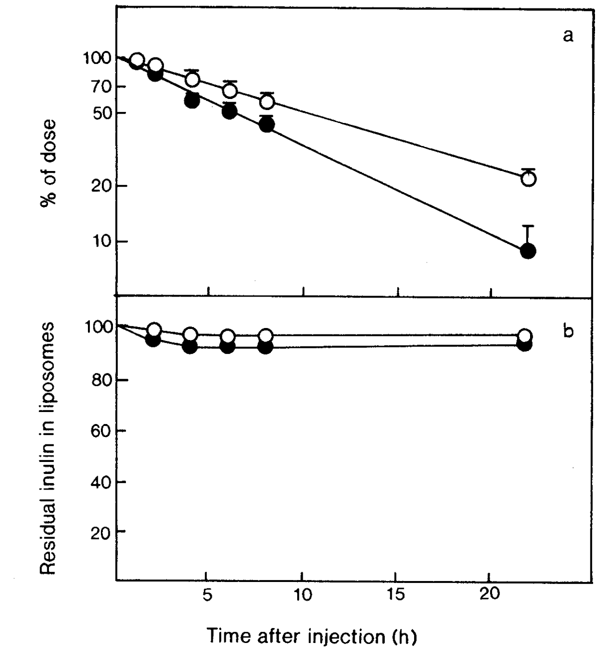

Fig. 2. Clearance and Stability of Liposomes after Intravenous Administration to Rats

a) Rats were injected intravenously with 0.5 ml liposomes composed of DPPC, Chol, and PGlcUA (○), or with those composed of DPPC, Chol and DPPG (●), prepared as described in Experimental. Values at various times are expressed as percent ±S.D. of the administered radioactivity per total blood.

b) Double-labeled liposomes were prepared as described in Experimental, and injected intravenously into rats. The value of $^{3}H/^{14}C$ in plasma was measured at various times after injection of PGlcUA–liposomes (○) or DPPG–liposomes (●).

Table II. Distribution of Liposomes in Various Tissues 22 h after i.v. Injection

Tissue	DPPC : Chol : PGlcUA = 40 : 40 : 20 % injected dose per:		DPPC : Chol : DPPG = 40 : 40 : 20 % injected dose per:	
	Tissue	100 mg	Tissue	100 mg
Liver	29.9 (2.4)	0.414 (0.04)	31.8 (6.4)	0.453 (0.08)
Spleen	23.3 (5.5)	6.85 (1.6)	24.6 (9.4)	8.15 (2.7)
Kidney	1.20 (0.4)	0.064 (0.02)	1.79 (0.2)	0.058 (0.03)
Lung	1.22 (0.5)	0.097 (0.02)	1.98 (1.2)	0.195 (0.11)
Brain	0.10 (0.01)		0.08 (0.02)	
Urine	24.8 (5.3)		33.0 (4.0)	
Plasma[a]	22.2 (2.7)	0.333 (0.04)	8.91 (3.6)	0.123 (0.04)

The amounts of [^{3}H]inulin in plasma and various tissues were determined 22 h after intravenous injection of the liposomes. The data of each organ (total and per 100 mg) are expressed as percent of total administered radioactivity. *a*) In plasma, the percent of dose at 22 h shown in Fig. 2a was used as tissue value, and percent recovery in 100 μl was used instead of 100 mg. Cumulative urinary excretion was also monitored. Numbers in parentheses indicate S.D.

and 1 h after administration. These values were almost equal to each other and zero time value was determined by extrapolation. As shown in the figure, the amount of inulin in PGlcUA–liposomes remained higher in blood than that in DPPG–liposomes at all time points determined, and the half-life of the former was prolonged about 1.7-fold. The amount of radioactivity in plasma at 22 h after administration when PGlcUA–liposomes were used was about 2.5-fold higher than when DPPG–liposomes were injected. To determine the structural integrity of liposomes after i.v. administration, we labeled them with [^{3}H]inulin as a marker of the internal aqueous phase, and cholesteryl [^{14}C]oleate as a marker of the lipid phase. As shown in Fig. 2b, the values of ^{3}H/^{14}C at 2, 4, 6, 8, and 22 h after injection were found to be almost equal. This result indicates that the bilayer structure of both types of liposomes remains intact in the bloodstream, since if liposomes were destabilized they would release inulin, which would disappear from the blood quickly.[13]

Tissue Distribution of Inulin Entrapped in Liposomes We examined the tissue distribution of inulin entrapped in liposomes in the following six compartments: liver, spleen, lung, kidney, brain, and urine. The fate of liposomal contents was followed using [^{3}H]inulin because this polysaccharide is metabolically inert in cells and free inulin is rapidly eliminated from the blood. Table II shows the tissue distribution of inulin that was originally encapsulated in liposomes. With respect to their tissue distribution, inulin mainly distributed to the RES. The ratio of the amount of inulin in the RES (liver and spleen) to that in blood determined by Fig. 2a was 2.74 and 7.25 when PGlcUA– and DPPG–liposomes were injected, respectively. This indicates that the modification of liposomes with PGlcUA changes their behavior *in vivo* and prolongs their half-life in the circulation. The difference in RES/blood ratio was primarily dependent on the difference in recovery from blood. Excretion of [^{3}H]inulin in urine from PGlcUA–liposomes was less than that from DPPG–liposomes. This phenomenon may relate to the difference in the trapping and degradation rate between the two liposomes in liver and spleen, since free inulin generated by degradation of liposomes in these tissues might remain intact and be rapidly excreted in urine after entering the bloodstream.[13,14]

Discussion

In this study, we synthesized a glucuronic acid derivative (PGlcUA) that is easily prepared and readily incorporated into the liposomal bilayer, and investigated the biodistribution of liposomes containing this derivative.

There are several factors that affect liposomal half-life in the bloodstream. When liposomes are modified with specific molecules such as sialic acid, which are involved in cell–cell interaction, their half-lives in blood are changed. Liposomal clearance from blood occurs mainly by RES-trapping. It has been reported that gangliosides such as G_{M1}[7–9] and glycoproteins[15] are effective in reducing the reticuloendothelial uptake of liposomes. When sialic acid was removed from liposomes containing G_{M1} by neuraminidase treatment, RES-trapping of liposomes was increased.[8] Thus the presence of surface sialic acid is one of the most important factors in determining the half-life of liposomes in the circulation. Secondly, composition (or rigidity) of liposomes is another important factor affecting their half-life of liposomes in the blood. A tightly packed bilayer is reported to be an asset in preventing recognition by the RES.[16,17] The activity of ganglioside to prolong the half-life of liposomes in circulation is enhanced when solid liposomes are used.[9] A third factor is the size: liposomes small enough to pass through fenestrations of the RES stay longer in circulation.[18]

Among the above factors, the second and third are easy to manipulate by selecting lipid composition and by sizing liposomes; in this experiment, however, we focused on the first factor. Glucuronic acid was chosen as a modifier of the liposomal surface because of its resemblance to sialic acid, a sugar characterized by a carboxyl group bound to a carbohydrate backbone. A cetyl group was bound to glucuronate at the C-1 position of the sugar to embed this glycolipid into lipid bilayers. We first examined the stability, size distribution, and ζ-potential of PGlcUA–liposomes in comparison with the same parameters for DPPG–liposomes. PGlcUA–liposomes were stable in bovine serum and had a gaussian size distribution after passage through a polycarbonate membrane. Liposomes containing DPPG also showed satisfactory stability in serum and size distribution, but displayed a shorter half-life in the circulation than PGlcUA–liposomes. Thus it is clear that PGlcUA has the ability to prolong the half-life of liposomes. Because of the resemblance of glucuronic acid to sialic acid, the carboxyl group on carbohydrate backbone might be sufficient to prolong the liposomal circulation time. The uptake of liposomes by the RES is thought to be triggered by the opsonization of liposomes by plasma proteins,[19,20] which in turn is thought to be dependent on the hydrophobicity of the liposomal surface.[21] Since the PGlcUA–liposomes have hydrophilic carboxyl and hydroxyl groups on their surface, PGlcUA may prevent the opsonization and RES-trapping of liposomes by reducing surface hydrophobicity.

It is well known that large liposomes or liposomal aggregates are rapidly taken up by RES. We examined whether or not PGlcUA–liposomes cause aggregation. The agglutinability of PGlcUA–liposomes was quite low in the presence of serum. The long half-life of PGlcUA–liposomes in circulation, however, could not be explained by this low agglutinability alone, since DPPG–liposomes also show low agglutinability.(10)

In conclusion, PGlcUA–liposomes had a longer half-life in the bloodstream than DPPG–liposomes. This effect may depend on the structure of the PGlcUA molecule itself. At this point, we are unable to determine whether this is specific for glucuronate or is common to all uronic acid derivatives. One explanation for this effect of PGlcUA-incorporation is that PGlcUA, with its hydroxy groups and carboxyl group, endows liposomes with sufficient hydrophilicity to protect them from recognition by the RES.

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