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Molecular Recogniton of Polysaccharide-Coated Liposomes. Importance of Sialic Acid Moiety on Liposomal Surface

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Conjugation of sialic acid to cholesterol-substituted polysaccharides has been carried out. Liposomes, which are coated with sialic acid-modified polysaccharide, were significantly aggregated by sialic acid-specific lectin, and inhibition of phagocytosis of the liposomes by phagocytic cells was observed. Appropriate treatment of the liposomes with a neuraminidase made the efficient removal of sialic acid from the polysaccharide on the liposomal surface.

Liposomes have gained wide acceptance as a drug carrier to target a specific tissue or cell.[1] Two important problems, however, must be overcome before liposomes are employed in clinical practice. First of all, the greater structural stabilization must be attained, since liposomes are easily lyzed by proteins or enzymes in blood stream after intravenously administered.[2] Secondly, targetability of liposome must be developed, since conventional liposomes themselves do not show any active targetability and are accumulated in mostly reticuloendothelial system (RES) such as liver and spleen when systemically administered.[3]

We have already reported that mannan- or amylopectin-coated liposomes are effectively taken up by phagocytes such as alveolar macrophages, blood monocytes, or neutrophils, and are accumulated at the lungs by receptor-mediated mechanism.[1] These characteristics of polysaccharide-coated liposome are unfavorable for targeting other tissues or cells except RES or phagocytes. From this concept, the development of an improved liposome which is able to escape from RES after systemical administration has been considered.

It is known that sialic acid residue on the liposomal surface is important in cell recognizability. For example, sialoganglioside-incorporated liposome inhibits the liver uptake,[4] liposome bearing sialic acid-modified peptide increases the half-life in the circulation of liposome in blood stream after i.v. injection,[5] and the internalization of human glycophorin-reconstituted liposomes into phagocytes is also significantly inhibited.[6,7]

Based on these previous informations, in this study we newly prepared sialic acid-conjugated polysaccharides to coat the liposomal surface, in order to make

liposomes stable and simultaneously able to escape from phagocytosis or to decrease their accumulation in RES.

Substitution of Cholesterol group to pullulan and amylopectin has been described elsewhere.[8] Polysaccharide derivatives so obtained were coded as CHP-50-1.9, and CHAp-112-1.8. The former is the pullulan ($\overline{Mn}$ = 50000) derivative which is substituted with 1.9 cholesterol moieties per hundred glucose units. The latter is the amylopectin ($\overline{Mn}$ = 112000) derivative which is substituted with 1.8 of cholesterol moieties per hundred glucose units. *N*-Acetylneuraminic acid (NeuNA), which was isolated from edible bird's nest and purified, was introduced to cholesterol-substituted polysaccharides according to the method described in literatures[9,10] with minor modification. Briefly, *N*-acetylneuraminic acid (1.00 g, 3.2 mmol) was reacted with acetylchloride (50 ml, 0.64 mol) under stirring at 25 °C for 48 h. And then, gaseous dry HCl (8 L) was bubbled into the reaction mixture for 24 h at 25 °C. Unreacted acetylchloride and HCl were removed off under reduced pressure to obtain 4,7,8,9-tetra-*O*-acetyl-2-chloro-*N*-acetylneuraminic acid (abbreviated as OAc-Cl-NeuNA):[11] yield, 1.5 g (94%).

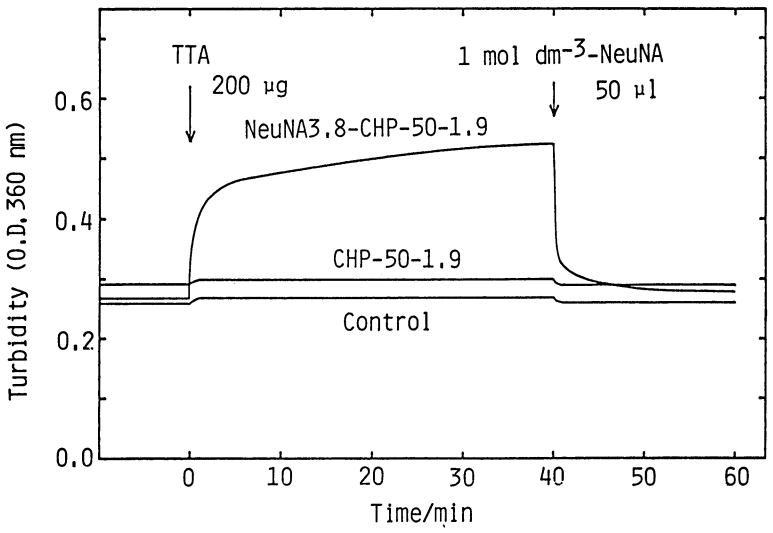
OAc-Cl-NeuNA (397 mg, 0.8 mmol) and CHP-50-1.9 (130 mg, 0.8 mmol of monosaccharide unit) were solubilized in a mixture of 10 ml of dry DMSO and 5 ml of DMF in the presence of silver trifluoromethan sulfonate (308 mg, 1.2 mmol) and Na_2HPO_4 (114 mg, 0.8 mmol). The reaction mixture was stirred under N_2 atmosphere for 4 days at 3 °C in the dark. After removing of precipitates by using hyflo-super cell, the filtrates were poured onto 100 ml of ethanol, and the resulting solution was kept overnight. The precipitates produced were collected by filtration (Wattman No 2), and dissolved in 50 ml of water. The aqueous solution was dialyzed for 2 days against 15 ml of water. After freeze-drying of the resulting solution, the product so obtained was deacetylated with 20 ml of 1 mol dm^{-3} NaOH for 30 min on an ice-bath, the resulting solution was neutralized with 1 mol dm^{-3} HCl, dialyzed for 2 days against 15 L of water, and lyophilized: yield, 82 mg. Substitution degree of sialic acid group per hundred glycopyranose units was determined by periodate resorcinol assay[12] to be 3.8. The obtained compound was coded as NeuNA-3.8-CHP-50-1.9. Conjugation of sialic acid to amylopectin was carried out exactly by the same procedure as that employed for pullulan. The substitution degree of sialic acid per hundred glycopyranose was 4.1 and coded as NeuNA-4.1-CHAp-112-1.8. Sialic acid might be bound to the polysaccharide to give sialosyl α(2→6) glycopyranose.[9,10]

Polysaccharide-coated multilamellar liposomes (MLV) were prepared according to the method previously described.[8] A suspension of MLV in an appropriate buffer was mixed with an aqueous solution of polysaccharide derivatives at the ratio of (polysaccharide)/(phospholipid) = 0.5 by wt. Separation of polysaccharides unbound was accomplished by gel-filtration on a Sepharose 4B column. Coating efficiency was determined from the concentration of egg PC and sialic acid, and estimated to be 50.0% for NeuNA-3.8-CHP-50-1.8 and 43.6% for NeuNA-4.1-CHAp-112-1.9, respectively.

First of all, a specific lectin-induced aggregation of polysaccharide-coated liposomes was investigated by employing Japanese horseshoe crab lectin from Tachypleus tridentatus (TTA, Seikagaku Kogyo). Lectin-induced aggregation of lipo-

some was followed by monitoring the turbidity increase at 360 nm (Fig. 1). Only sialic acid-bearing polysaccharide-coated liposomes were aggregated by TTA. When the turbidity increase was levelled off, a 50 µl of 1 mol dm^{-3} NeuNA aqueous solution was added to the system. By this NeuNA addition, the turbidity was instantaneously diminished and the clearness of the system was recovered (Fig. 1). For conventional and regular polysaccharide-coated liposomes, no significant change in the turbidity was observed even if TTA and free NeuNA were added to the suspensions.

Secondly, interaction between sialic acid-modified polysaccharide-coated liposomes and human blood monocytes was investigated by employing liposomes labelled with [^{14}C]-dipalmitoylphosphatidylcholine. Preparation procedures of isotope-labelled liposomes were previously described.[8] Figure 2 shows a typical result of the interaction between liposomes and human blood monocytes. Conjugation of sialic acid to pullulan or amylopectin showed an effective rejection in the phagocytosis of the liposomes coated with such the modified polysccharides.



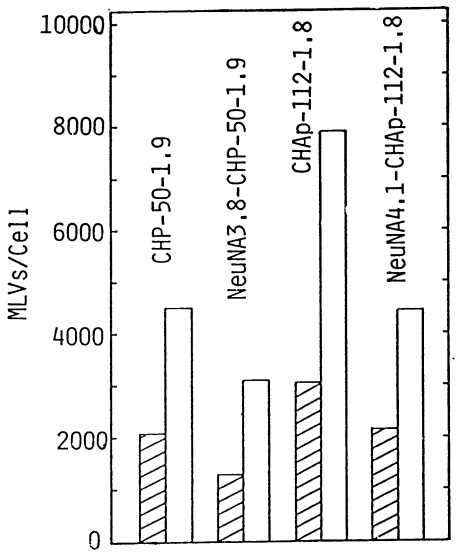


Fig. 1 (left). TTA-induced aggregation of polysaccharide-coated MLV. A liposomal suspension ([egg PC] = 2.1 × 10^{-4} mol dm^{-3}) in 2.8 ml of Tris-HCl buffer containing 200 mmol dm^{-3} NaCl (pH 7.4) was preincubated in a cuvette for 10 min at 25 °C, and 200 µg of TTA dissolved in 200 µl of water was added at the point of the first arrow. After 40 min, 50 µl of 1 mol dm^{-3} NeuNA solution was added at the point of the second arrow.

Fig. 2 (right). Internalization efficiency of various radio-isotope labelled liposomes into human monocytes. Human monocytes were isolated from heparinized blood of healthy adult volunteers by treatment with 5% dextran-saline and Ficoll-Conray. Obtained cell suspension (2.0 × 10^6 cells) in 450 µl of RPMI-1640 supplemented with 10% FCS was incubated with 50 µl of MLV (the number of liposomes was 2.0 × 10^{12}) at 37 °C for 30 min (▨) and 60 min (□). Free liposomes were removed by density-gradient centrifugation (12000 G for 3 min) using a mixed silicon oil (d = 1.021). The number of liposomes internalized into the cells was estimated from the count rate determined on a Aloka III liquid scintillation counter, assuming that one MLV is consisted with 268000 lipids molecules. The diameter of MLV employed was approximately 109 nm, which was estimated from electron microscopic pictures.

Finally, the possibility of enzymatic elimination of sialic acid moieties on the liposomal surface was investigated. Sialic acid-modified polysaccharide-coated liposome was treated with 0.1 U of neuraminidase (EC. 3.2.1.18, from Arthrobacter ureafacients, Seikagaku Kogyo) in 6.1 ml of 200 mmol dm^{-3} MOPS buffer (pH 6.5) containing 0.14 mol dm^{-3} NaCl under N_2 atmosphere at 37 °C. At 0, 0.5, 1, and 24 h after the additon of the enzyme, a 500 µl aliquot of the reacting mixture was pipetted out and, the amount of sialic acid was determined, after the pH adjustment (pH 7) with 0.2 mol dm^{-3} borate buffer. Results are summarized in Table 1. The results apparently indicate that sialic acid substituted to the parent polysaccarides is certainly recognized by the enzyme.

Table 1. Efficiency (%) of removal of sialic acid residues from polysaccharide and human erythrocyte glycophorin on the liposomal surface by using neuraminidase

Sample	Incubation time			
	0	0.5 h	1 h	24 h
NeuNA-3.8-CHP-50-1.9 on MLV[a)]	0%	11.8%	23.5%	52.9%
NeuNA-4.1-CHAp-112-1.8 on MLV[b)]	0%	26.7%	26.7%	53.3%
Glycophorin[c)]	0%	30.0%	46.1%	78.4%

a) [egg PC] = 7.7×10^{-4} mol dm^{-3}. b) [egg PC] = 7.5×10^{-4} mol dm^{-3}.
c) [glycophorin] = 0.063 mg/ml.

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11) IR (KBr disc); 1745, 1670, 1540, 1220, 1120, and 600 cm^{-1}. Found: C, 46.02; H, 5.28; N, 2.82%. Calcd for $C_{19}H_{26}NO_{12}Cl$: C, 46.11; H, 5.37; N, 2.70%.
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