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Physicochemical and Disposition Characteristics of Antisense Oligonucleotides Complexed with Glycosylated Poly(L-lysine)

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ABSTRACT. The disposition characteristics of a 20 *mer* antisense phosphodiester oligonucleotide (PO) and its fully phosphorothioated derivative (PS) alone or complexed with glycosylated poly(L-lysine) (galactosylated polylysine, Gal-PLL; mannosylated polylysine, Man-PLL) were studied in mice in relation to their physicochemical characteristics. Good complex formation was obtained at a ratio of 1:0.6, w/w [oligonucleotides (ODNs)/carrier]. The 1:0.6 weight ratio of ODNs/Gal-PLL and ODNs/Man-PLL complexes had ζ potentials of −27 to −31 mV and mean particle size of 100 to 160 nm. After intravenous injection, ^{35}S-labeled ODNs were eliminated rapidly from the circulation; however, their organ disposition characteristics depended on their type. Complex formation with glycosylated PLL increased the hepatic uptake and decreased the urinary clearance of these ODNs to a great extent. These complexes were taken up by both liver parenchymal cells (PC) and nonparenchymal cells (NPC). However, ODNs/Gal-PLL complexes showed a fairly high PC concentration, whereas ODNs/Man-PLL complexes distributed equally to both PC and NPC. The hepatic uptakes of PS/Gal-PLL and PS/Man-PLL complexes were partially inhibited by prior administration of Gal-BSA and Man-BSA, respectively, suggesting their hepatic uptake via the respective receptor-mediated endocytosis. However, uptake by galactose receptors of Kupffer cells, ζ potential, particle size, and Kupffer cell phagocytosis also seem to influence their uptake process. In conclusion, this study illustrates that ODNs can be delivered to hepatocytes and macrophages via galactose and mannose receptors, respectively. BIOCHEM PHARMACOL 53;6:887–895, 1997.

KEY WORDS. oligonucleotides; pharmacokinetics; hepatic uptake; particle size; zeta potential; macrophage

ODNs† can act sequence specifically to modulate foreign or cellular gene expression. They are used widely as research tools for inhibiting gene expression and are under investigation for possible use as therapeutic agents in diseases where the genetic target and its sequence have been identified. To avoid the degradation of unmodified PO by nucleases, various derivatives have been prepared; among them PS derivatives are being investigated extensively [1–3].

Following systemic administration, ODNs are distributed to all major peripheral organs with liver and kidney accumulating most of the injected ODNs [4–6]. The major limitation for the effective use of ODNs as therapeutic agents is their low cellular uptake and lack of cellular targeting. Several approaches have been proposed to increase their cellular uptake. Substitution of ODNs with lipophilic molecules [7] and association with liposomes [8, 9] or polycations such as PLL [10] have been shown to increase the cellular uptake and biological activities of ODNs. However, their effective use *in vivo* will require a more specific method to deliver them selectively to a target cell population.

Receptor-mediated endocytosis offers the potential to deliver ODNs to the intracellular space of the target cells. Unmodified 18 *mer* ODNs complementary to c-myb mRNA, complexed with transferring-PLL [11] or folic acid-PLL [12] have been shown to be more effective in inhibiting leukemic cell line HL-60 proliferation than do free PO or PS. Complex formation with asialoorosomucoid-PLL has also been shown to enhance the cellular uptake of PS, complementary to hepatitis B virus polyadenylation signal, and to reduce expression of virus surface antigen from virus-transfected Hep G2 cells [13]. Along the same line, ODNs targeted to cancer cells via the epidermal growth factor (EGF) receptor [14] or to macrophages via the mannose receptor [15, 16] have been found to be taken up more

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† *Abbreviations:* ODNs, oligonucleotides; PO, phosphodiester oligonucleotide(s); PS, phosphorothioate oligonucleotide(s); PLL, poly(L-lysine); Gal-PLL, galactosylated poly(L-lysine); Man-PLL, mannosylated poly(L-lysine); PC, parenchymal cells; and NPC, nonparenchymal cells.

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efficiently by the cells than free ODNs. However, systematic information on the *in vivo* disposition characteristics of these complexes is still limited.

Hepatocytes express a galactose-specific receptor on their sinusoidal (blood-facing) surface, whereas Kupffer and liver endothelial cells express a mannose-specific receptor [17, 18]. As a first step towards the use of glycosylated PLL for *in vivo* delivery of ODNs, our intent in this study was to examine the disposition characteristics of ODNs and their glycosylated PLL complexes in mice in relation to their physicochemical properties. We used 20 *mer* PO and its PS derivative, complementary to the human c-myc mRNA, as model antisense molecules.

MATERIALS AND METHODS

Chemicals

The 20 *mer* model antisense PO (3′-TACGGGGAGTT-GCAATCGAA-5′) and its fully phosphorothioated derivative (PS) complementary to the human c-myc mRNA, including the AUG translation initiation codon site, were purchased from GENESET (Paris, France). These ODNs were obtained from the manufacturer as clear solutions with >99% purity and a T_m of about 56–60°. Clear-sol I and T4 polynucleotide kinase (10 U/μL) were purchased from Nacalai Tesque (Kyoto, Japan) and Takara Biomaterials (Otsu, Japan), respectively. [α-^{35}S]dATP (1000–1500 Ci/mmol) and Soluene-350 tissue solubilizer were obtained from the DuPont/NEN Co. (Boston, MA, U.S.A.) and Packard Instrument (Groningen, The Netherlands), respectively. Poly(L-lysine) hydrobromide (mols wt ~35,200) and collagenase (type I) were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). BSA (fraction V), β-D-galactose, and α-D-mannose were obtained from Nacalai Tesque. All other chemicals were obtained commercially as reagent-grade products.

Radiolabeling of Oligonucleotides

All ODNs were labeled by adding [α-^{35}S]dATP to the 3′ end terminal using a Deoxynucleotide Transferase Kit (Promega, Madison, WI, U.S.A.) as described by Desjardins *et al.* [19]. Unincorporated label was removed from radiolabeled ODN by size exclusion chromatography with NAP^{TM}-5 Sephadex G-25 columns (Pharmacia Biotech, Uppsala, Sweden). Specific activity of the labeled ODNs was about 6–9.5 × 10^7 cpm/μg.

Preparation of Glycosylated Poly(L-lysine)

Galactose or mannose was incorporated into amino groups of PLL (mol. wt ~35,200) as described by Lee *et al.* [20]. Briefly, 2-imino-2-methoxyethyl 1-thioglycosides (mol. wt ~403.4, weight ~28.65 mg) were synthesized by treating 0.1 mM cyanomethyl-thioglycoside (CNM-thioglycosides) in absolute methanol with 0.1 equivalent weight of sodium methoxide for 24 hr at room temperature and then were conjugated with 10 mg/mL PLL (50 mg) in 0.05 M borate buffer (pH 9.5) for 3 hr at room temperature. Two types of PLL conjugates with 1-thiogalactose (for Gal-PLL) and 1-thiomannose (for Man-PLL) were synthesized. The resulting derivatives were washed with distilled water, concentrated by ultrafiltration (mol. wt cutoff 10,000), and lyophilized. The number of sugars incorporated into a PLL molecule was determined by colorimetric assay for neutral sugars, in which neutral sugars react with anthrone in the presence of a 72% sulfuric acid solution [21]. Gal-PLL and Man-PLL were determined to contain about 28.0 galactose and 25.0 mannose residues per PLL molecule, respectively.

Formation of ODNs/Glycosylated PLL Complex

A defined amount of ODNs was heated at 98° for 2 min and then dissolved in an aqueous solution of mannitol to get a final concentration of 0.1 μg/μL. The mannitol solution was prepared dissolving mannitol (0.72 M) and NaCl (0.9%) in distilled water and adjusting the pH to 8.00. Similarly, a defined amount of glycosylated PLL was dissolved in the same mannitol solution to get a final concentration of 0.6 μg/μL. Equivolumes of these two solutions were mixed under vigorous vortexing, and complex formation was allowed to proceed for 15 min at room temperature. In studies designed to evaluate the effect of concentrations of the carrier on complex formation and aggregation, various ratios of ODNs/glycosylated PLL were used. An excessive amount of glycosylated PLL (i.e. at the ratios of 1:≥2, w/w) was often found to cause aggregation. Slow vortexing and/or rapid mixing also caused aggregation.

Gel Electrophoresis

Complex formation efficiency was checked by agarose gel electrophoresis, using a procedure similar to that described by Citro *et al.* [12] for analysis of ODNs/folic acid-PLL complex. Briefly, the 1:0.3 and 1:0.6 weight ratio ODNs/glycosylated PLL complexes were prepared by dissolving separately ODNs and glycosylated PLL in 0.72 M mannitol solution, pH 8, as described above. These complexes (0.75 μg equivalent weight of ODNs in each lane) were run at constant voltage (100 V) on 1.5% (w/v) agarose gel prepared in Tris–borate–EDTA buffer. Both naked ODNs and glycosylated PLL were used as controls. The gel was then stained for 30 min in ethidium bromide (10 μg/mL) and photographed under UV light.

ζ Potential and Particle Size

The ζ potential and particle size of ODNs/glycosylated PLL complexes were measured using procedures and equipment similar to those described previously [22]. ODNs/Gal-PLL and ODNs/Man-PLL complexes were prepared at a ratio of 1:0.6 (w/w) (ODNs/carrier) as described above, and the total dilution factor was maintained at 10 for all samples. The electrophoretic mobility of these samples was deter-

mined with an ELS-800 Zetasizer (Otsuka Electronics Co. Ltd., Osaka, Japan). All experiments were performed at 26° using 0.72 M mannitol solution, pH 8. The sample compartment is connected to a glass capillary cell. Under constant voltage, these complexes started to migrate across the capillary on which a beam of a 10 mM helium-neon laser was focused at a constant angle of 15°. The ζ potential was automatically calculated from the electrophoretic mobility based on the Smoluchowski formula [23]. Following the determination of electrophoretic mobility, the samples were subjected to mean particle size measurement.

In Vivo *Distribution*

ODNs alone or complexed with glycosylated PLL at a ratio of 1:0.6 (w/w) (ODNs/carrier) was administered to mice (Shizuoka Agricultural Cooperative Association for Laboratory Animals, Shizuoka, Japan) via the tail vein at a dose of 1 (for naked ODNs) or 0.5 (for the complex) mg ODNs/kg. Blood was collected from the vena cava under ether anesthesia at 1, 2.5, 5, 15, 30, and 60 min, and the mice were killed at each collection time point. Tissues (liver, spleen, kidney, lung, heart, and muscle) were isolated, washed with saline, blotted dry, and weighed. Blood was centrifuged and plasma was separated. Just prior to blood collection, urine was also collected directly from the urinary bladder using a 26-gauge needle syringe. Two hundred microliters of plasma and of urine, and a small amount of each tissue were digested with Soluene-350 (0.5 mL for plasma and urine and 0.7 mL for tissues) through incubation overnight at 45°. Following digestion, 0.2 mL of 2 N HCl (for neutralization) and 5 mL of Clear-sol I (scintillation medium) were added to each tissue, plasma, and urine sample, the samples were stored overnight, and radioactivity was measured using a scintillation counter (LSA-500, Beckman, Tokyo, Japan).

Data Analysis

The tissue distribution data of [^{35}S]ODNs either alone or complexed with glycosylated PLL were analyzed in terms of a clearance and a tissue uptake rate index using biexponential equations as described previously [24]. Radioactivity of each tissue sample was corrected for blood contamination based on the data of ^{111}In-labeled BSA at 10 min after intravenous injection. The change in the amount of radioactivity in a tissue with time can be described as follows:

$$dT(t)/dt = CL_{in}C(t) - K_{out}T(t) \quad (1)$$

where T(t) (% of dose/g) represents the amount of radioactivity in 1 g of the tissue, C(t) (% of dose/mL) is the plasma concentration of radioactivity, CL_{in} (mL/hr/g) is the tissue uptake rate index from the plasma to the tissue, and K_{out} (1/hr) is the rate constant for efflux from the tissue. In the present study, the efflux process can be considered negligible during the initial time points. Hence, Equation (1) integrates to

$$CL_{in} = T(t_1) / \int_0^{t_1} C(t_1)dt = T(t_1) / AUC_0 - t_1 \quad (2)$$

where t_1 (hr) is the sampling time. According to Equation 2, the tissue uptake rate index is calculated using the amount of radioactivity in the tissue at an appropriate interval and the area under the plasma concentration–time curve (AUC) up to the same time point. Then, the organ clearance (CL_{org}) is expressed as follows:

$$CL_{org} = CL_{in} W \quad (3)$$

where W (g) is the total weight of the organ. When the tissue uptake process followed non-linear kinetics, CL_{in} values would represent an average value for the overall experimental period. Total body clearance (CL_{total}) was calculated from AUC for infinite time (AUC_∞) by the following equation:

$$CL_{total} = Dose/AUC_\infty \quad (4)$$

The tissue uptake clearance and index were calculated using the values up to 5 min after injection, assuming that ODNs complexed with glycosylated PLL were fairly stable within this period.

Hepatic Cellular Localization

Each mouse was anesthetized with pentobarbital sodium (40–60 mg/kg) and injected intravenously with [^{35}S]ODNs alone or complexed with glycosylated PLL [1:0.6 (w/w), ODNs/carrier]. The body temperature of the mouse was kept at 37° with a heat lamp during the experiment. At 15 min after administration, the liver was perfused first with perperfusion buffer (Ca^{2+}, Mg^{2+}-free HEPES solution, pH 7.2) for 10 min and then with HEPES solution containing 5 mM $CaCl_2$ and 0.05% (w/v) collagenase (type I) (pH 7.5) for 10–20 min. As soon as perfusion was started, the vena cava and aorta were cut, and the perfusion rate was maintained at 3–4 mL/min. Following the discontinuation of perfusion, the liver was excised and deprived of the capsule membranes. The cells were dispersed in ice-cold Hanks'–HEPES buffer containing 0.1% BSA by gentle stirring. The dispersed cells were filtered through the cotton mesh sieves, followed by centrifugation at 50 *g* for 1 min. The pellets containing PC were washed twice with Hanks'–HEPES buffer by centrifuging at 50 *g* for 1 min. The supernatant containing NPC was similarly centrifuged two more times. The resulting supernatant was then centrifuged twice at 200 *g* for 2 min. PC and NPC were resuspended separately in ice-cold Hanks'–HEPES buffer (4 mL for PC and 1.8 mL for NPC). The cell number and viability were determined by the trypan blue exclusion method. The cells (0.5 mL) were digested with Soluene-350 (1 mL) through incubation overnight at 45°. Following digestion, 0.3 mL of 2 N HCl

and 5 mL of Clear-sol I were added, the mixture was stored overnight, and radioactivity was measured using a scintillation counter. The amount of radioactivity on each cell fraction was calculated as percent of dose per 10^7 cells.

Competitive Inhibition of Hepatic Uptake of [^{35}S]PS/Glycosylated PLL Complex

One minute after intravenous injection of unlabeled glycosylated BSA (Gal-BSA or Man-BSA) into the tail vein of each mouse at a dose of 10 mg/kg, [^{35}S]-labeled PS complex [1:0.6 (w/w), PS/carrier] was also injected similarly at a dose of 0.5 mg ODNs/kg. At 15 min following administration of ODNs, the mouse was killed, blood was collected, and the liver was harvested. Plasma and liver samples were subjected to radioactivity assay as mentioned before.

RESULTS

Gel Electrophoresis

The complex formation efficiency between ODNs and Gal-PLL was checked by agarose gel electrophoresis, followed by gel staining with ethidium bromide and photography under UV light (Fig. 1). The figure shows that the electrophoretic bands of the complexes at 1:0.6 weight ratio ODNs/glycosylated-PLL did not move towards the positive pole. The results also suggest that 1:0.3 weight ratio samples contain ODNs in both free and complexed forms.

ζ Potential and Particle Size

The ζ potential and particle size distribution of these preparations were measured using an electrophoretic light scattering technique. Table 1 summarizes the ζ potential and mean particle size of naked ODNs, glycosylated PLL, and the complexes. Both Gal-PLL and Man-PLL had positive ζ potentials of about 40 to 45 mV, whereas ODNs had negative ζ potentials of about −34 to −40 mV. The 1:0.6 weight ratio ODNs/Gal-PLL and ODNs/Man-PLL complexes also had negative ζ potentials of −27 to −31 mV. These complexes had the mean particle size of 100 to 160 nm.

On the basis of the composition, we also calculated the number of positive charges borne by the carrier molecules and the negative charges borne by the ODN molecules. The carrier molecules (0.6 μg, Gal-PLL or Man-PLL; 0.53 μg PLL equivalent) had 3.6 nmol of positively charged lysines. Similarly, 1 μg of ODNs had about 3.2 nmol of negatively charged nucleotides. This calculation suggests that the net charge density of 1:0.6 (w/w) ODNs/Gal-PLL or ODNs/Man-PLL should be slightly positive, which is not in good agreement with the data obtained from the ζ potential measurement, suggesting that a part of the ODN molecules is exposed to the surface of the complex, resulting in negative ζ potential.

In Vivo *Disposition*

Figures 2 and 3, illustrate the time course of radioactivity in plasma, kidney, liver, urine, spleen, lung, heart, and muscle after intravenous injection of [^{35}S]ODNs alone or complexed with either Gal-PLL or Man-PLL into mice. Both types of ODNs were eliminated rapidly from the circulation; however, the tissue accumulation and urinary clearance depended on their types. PO had wide distribution to all of the tissues tested with high liver and kidney accumulation and rapid renal clearance, whereas PS accumulated

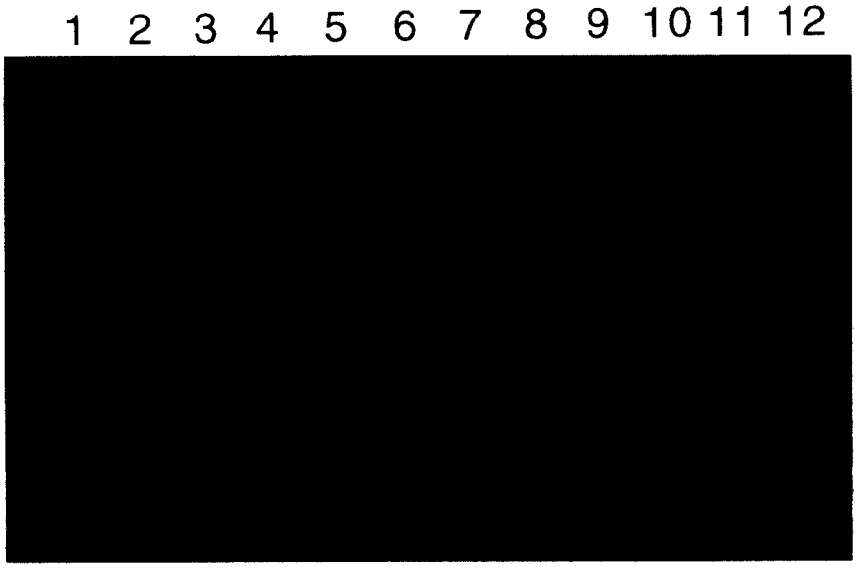

FIG. 1. Assessment, using electrophoresis, of complex formation between oligonucleotides and glycosylated poly(L-lysine). PO:Gal-PLL: lane 1, 1:0.3 (w/w); lane 2, 1:0.6; PO/Man-PLL: lane 3, 1:0.3; lane 4, 1:0.6; PO: lane 5; PS: lane 6; PS/Gal-PLL: lane 7, 1:0.3; lane 8, 1:0.6; PS/Man-PLL: lane 9, 1:0.3; lane 10, 1:0.6; Gal-PLL: lane 11; and Man-PLL, lane 12. Each lane was loaded with 7.5 μg equivalent of ODNs, except for lanes 11 and 12 which were loaded with 7.5 μg of Gal-PLL and Man-PLL, respectively. Samples were separated by electrophoresis on 1.5% agarose gel at 100 V with 1x Tris–borate-EDTA (pH 8) running buffer. The gel was stained with ethidium bromide (10 μg/mL) and photographed under UV light.

TABLE 1. Particle size and ζ potential of oligonucleotides complexed with glycosylated poly (L-lysine)*

Samples	Ratio (w/w)	Particle size (nm)	ζ Potential (mV)
Gal-PLL			42.02
Man-PLL			45.23
PO			−41.92
PS			−34.53
PO/Gal-PLL	1:0.6	150 ± 63	−27.79 ± 2.54
PO/Man-PLL	1:0.6	109 ± 31	−30.90 ± 1.86
PS/Gal-PLL	1:0.6	146 ± 50	−30.72 ± 0.96
PS/Man-PLL	1:0.6	159 ± 71	−31.05 ± 0.76

* Abbreviations: Gal-PLL, galactosylated poly(L-lysine); Man-PLL, mannosylated poly(L-lysine); PO, phosphodiester oligonucleotides; and PS, phosphorothioate oligonucleotides.
† Mean ± SD, N = 3.

largely in the liver and had relatively smaller urinary clearance. Complex formation with glycosylated PLL enhanced the hepatic uptake and reduced urinary clearance of these ODNs to a great extent.

Pharmacokinetic Analysis

Table 2 summarizes the pharmacokinetic parameters such as the total body, hepatic and urinary clearances, AUC_∞, and tissue uptake indices for representative tissues calculated using biexponential equations. Renal and urinary clearances of PS were much smaller compared with those of PO. ODNs/glycosylated PLL complexes had high hepatic clearances and much lower urinary clearances, compared with the naked ODNs. Spleen uptake index of these complexes was also quite high, especially for ODNs/Man-PLL samples.

Hepatic Cellular Localization

Figure 4 shows the distribution of radioactivity in PC and NPC at 15 min after intravenous injection of [^{35}S]ODNs alone or complexed with glycosylated PLL into mice at a dose of 0.5 mg ODNs/kg for the complex preparations and 1 mg ODNs/kg for PS samples. Our previously published data [25] for galactosylated and mannosylated bovine serum albumin (Gal-BSA and Man-BSA) are also shown in this figure for comparison. The radioactivity derived from [^{35}S]PS as well as that from [^{35}S]ODNs/Gal-PLL complexes accumulated in both PC and NPC; however, the concentration in PC was fairly high for PS/Gal-PLL complex. In contrast, PS/Man-PLL complex accumulated equally in PC and NPC.

Mechanism of Hepatic Uptake of [^{35}S]PS/Glycosylated PLL Complex

To test whether the hepatic uptake of ODNs/glycosylated-PLL complex was mediated by a specific receptor-mediated pathway (i.e. galactose and mannose receptors for Gal-PLL and Man-PLL, respectively), we performed competition studies using unlabeled glycosylated BSA. The results indicated that the hepatic uptake of PS/Gal-PLL and PS/Man-PLL complexes was inhibited significantly by prior administration of Gal-BSA and Man-BSA, respectively (Table 3).

DISCUSSION

Systemic delivery of antisense ODNs is promising for the treatment of both genetic and non-genetic disorders [26]. Rational delivery systems should be designed based on the information of disposition characteristics of naked ODNs. Hence, as a first step, we previously studied the stability and pharmacokinetic properties of naked ODNs at whole body and organ levels [27–30]. On the basis of these findings, our intent in the present study was to see whether the disposition characteristics of ODNs can be controlled by complexing them with glycosylated PLL.

Receptor-mediated uptake of macromolecules is determined by their physicochemical properties such as size, charge, and terminal sugar [31, 32]. Hence, we conjugated sugars to PLL molecules using cyanomethylthioglycosides as described by Lee *et al.* [20]. This synthesis procedure was selected because of no gross change in the net charge of the protein after modification. Glycosylation of PLL is generally accomplished by the method developed by McBroom *et al.* [33] using phenylisothiocyanates, which may cause nonspecific hydrophobic interaction and reduction in the net charge of PLL after modification.

In this study, ODNs were labeled on the 3′ end with [α-^{35}S]dATP as described by Desjardins *et al.* [19]. Radiolabeling of ODNs at the 3′ terminal is known to be susceptible to exonuclease in serum and tissues. Hence, the stability of ^{35}S-end-labeled PO and PS was examined in 10% serum containing medium and found to be fairly stable at 2 hr after incubation at 37°. Furthermore, the stability of intravenously injected [^{35}S]PS was checked by autoradiography and found to be fairly stable in all the tissues tested, such as plasma, liver, and kidney at 5 and 30 min after injection (unpublished results). In addition, to avoid the influence of any degradation products that may occur *in vivo*, the organ uptake clearance and rate index were calculated using values only up to 5 min after injection.

Following intravenous administration, PO was widely distributed to most of the tissues examined, with high liver accumulation and rapid urinary excretion, whereas PS had, comparatively, much higher liver accumulation and lower urinary excretion (Figs. 2 and 3). Hnatowich *et al.* [34] also reported that after intravenous administration ^{99m}Tc-labeled 22 *mer* PS had much higher hepatic uptake and retention but lower urinary clearance compared with those of ^{99m}Tc-labeled PO samples. Sands *et al.* [35] reported that ^{3}H-labeled PS accumulated not only in the liver but also in the kidney to a much greater extent than did ^{3}H-labeled PO. The highest uptake of PS by the liver and kidney was

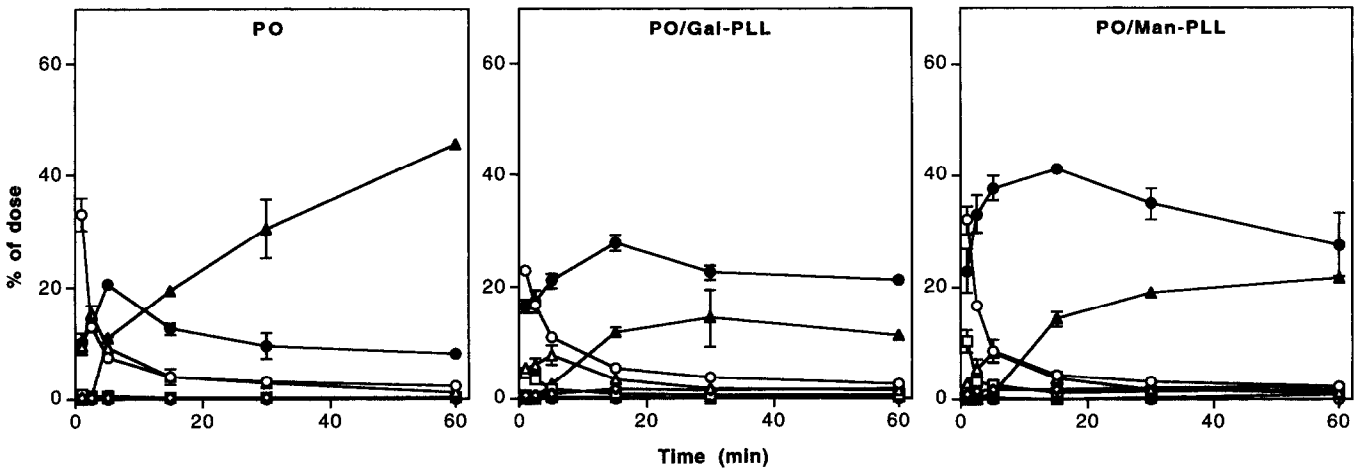

FIG. 2. Tissue accumulation of radioactivity after intravenous injection of ^{35}S-labeled PO administered alone or complexed with glycosylated poly(L-lysine) into mice. Dose: 1 and 0.5 mg PO/kg for naked ODNs and the complex, respectively. Key: (○) plasma, (●) liver, (△) kidney, (▲) urine, (□) lung, (◇) spleen, (■) heart, and (⊕) muscle (mean ± SD, N = 3). Abbreviations: Gal-PLL, galactosylated poly(L-lysine); and Man-PLL, mannosylated PLL.

further supported by the work of Agrawal *et al.* [4] who intravenously injected ^{35}S-labeled 20 *mer* PS into mice. In the present study, the concentrations of radioactivity for these ODN samples were also quite high in both liver and kidney.

Naked PS was nonspecifically taken up by both liver PC and NPC (Fig. 4). This is in good agreement with our previous observation for ODNs in the single-pass rat liver perfusion system [29], in which a partially phosphorothioated derivative of PO was nonspecifically taken up by the liver as an anionic compound, since both large and small anionic molecules inhibited its hepatic uptake. In contrast to these results, Inagaki *et al.* [36] reported that 1 hr after intravenous injection of ^{32}P-labeled 15 *mer* PS into rats, the hepatic uptake of radioactivity was mainly by NPC. Biessen *et al.* [37] also demonstrated that the binding of ^{32}P-labeled 18 *mer* PO to both Kupffer and endothelial cells *in vitro* was mediated by a specific receptor whose characteristics are similar to those of the scavenger receptor. However, the results reported by these authors cannot be correlated directly with our present findings due to differences in the types of ODNs as well as radiolabeling and experimental procedures used.

The disposition data obtained in this study suggest that the *in vivo* behaviors of ODNs can be controlled by the carrier macromolecules (Figs. 2, 3 and 5). By complexing with Gal-PLL or Man-PLL, both PO and PS could be targeted to the liver and their urinary clearance could be decreased to a great extent. Apart from having large hepatic uptake, ODNs/Man-PLL complexes also had a relatively high spleen uptake index, probably due to their uptake by macrophages that recognize mannose-terminated proteins

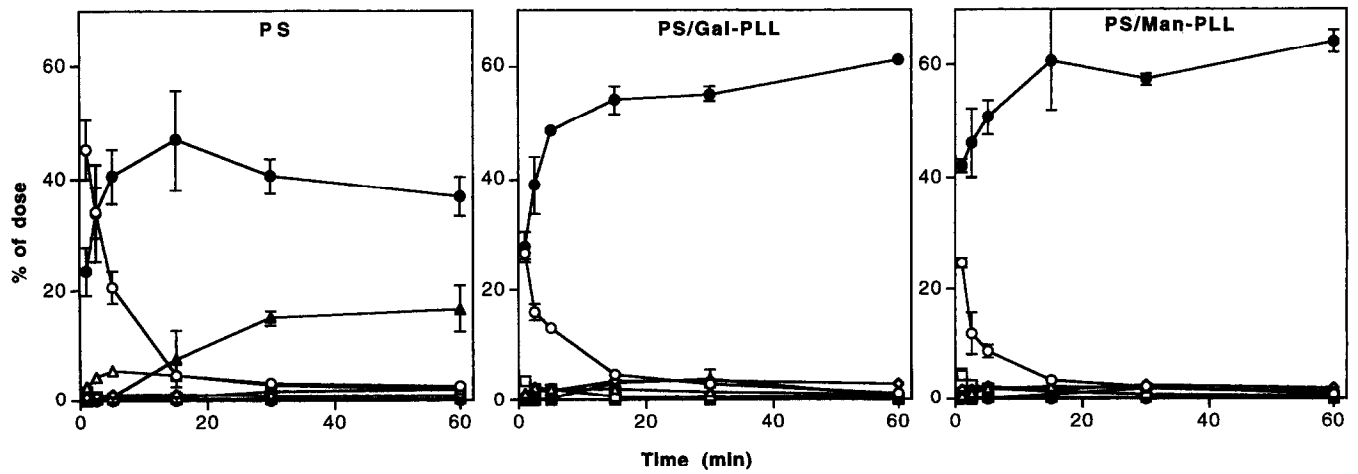

FIG. 3. Tissue accumulation of radioactivity after intravenous injection of ^{35}S-labeled PS administered alone or complexed with glycosylated poly(L-lysine) into mice. Dose: 1 and 0.5 mg PS/kg for naked ODNs and the complex, respectively. Key: (○) plasma, (●) liver, (△) kidney, (▲) urine, (□) lung, (◇) spleen, (■) heart, and (⊕) muscle (mean ± SD, N = 3). Abbreviations: Gal-PLL, galactosylated poly(L-lysine); and Man-PLL, mannosylated PLL.

TABLE 2. Pharmacokinetic parameters of oligonucleotides administered alone or complexed with glycosylated poly(L-lysine) into mice

Samples	Ratio (w/w)	AUC (% of dose · hr/mL)	Clearance (μL/hr)				Tissue uptake rate index (μL/hr/g)					
			Total	Liver	Kidney	Urine	Liver	Kidney	Spleen	Lung	Heart	Muscle
PO		7.17	25,680	12,914	5,799	6,823	8,077	20,237	1,740	2,125	2,061	854
PO/Gal-PLL	1:0.6	7.91	26,050	18,904	6,608	80	11,844	19,234	5,515	25,883	4,081	1,970
PO/Man-PLL	1:0.6	7.67	28,908	23,411	4,367	87	16,684	11,265	12,819	18,304	2,374	1,033
PS		14.06	13,257	10,314	1,008	1,688	7,073	3,325	2,948	920	469	281
PS/Gal-PLL	1:0.6	5.69	31,238	28,849	1,175	144	19,147	3,725	14,505	9,308	1,277	425
PS/Man-PLL	1:0.6	4.26	38,164	35,139	1,376	118	24,499	3,206	24,869	10,795	1,511	373

(Table 2). The extent of aggregation and dissociation that may occur *in vivo* could not be directly estimated, which may also ordain the *in vivo* disposition profiles of these complexes.

The recognition and affinity of the ligand for a specific receptor are important for the delivery of macromolecules. In the liver, both parenchymal and Kupffer cells possess receptors on their plasma membranes that specifically bind and internalize D-galactose-containing materials in a size-dependent manner: small particles are efficiently taken up by hepatocytes, whereas large particles are taken up by Kupffer cells [38, 39]. Complex formation with Gal-PLL enhanced the hepatic uptake of ODNs by both PC and NPC (Fig. 4). Although the uptake of PS/Gal-PLL complex by PC was significantly higher than that of naked PS, the difference between their intracellular distributions was only moderate. The detection of high ^{35}S radioactivity in PC for the naked PS samples may be due to the possible occurrence of intercellular transport during collagenase liver perfusion and the cell separation process. In addition, the hepatic uptakes of both complexed and free PS may have been influenced by sulfur atoms present in the PS molecules.

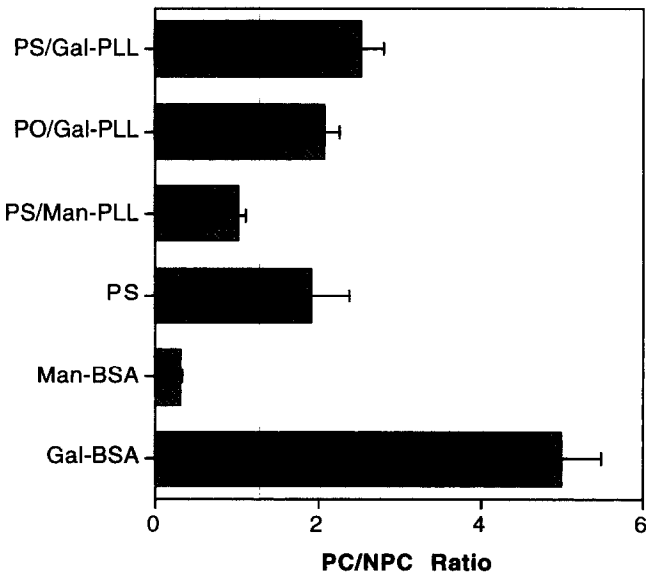

FIG. 4. Hepatic cellular localization of ^{35}S-labeled ODNs administered intravenously alone or complexed with poly(L-lysine) into mice (mean ± SD, N = 3). Dose: 1 and 0.5 mg ODNs/kg for naked ODNs and the complex, respectively. Abbreviations: Gal-PLL, galactosylated poly(L-lysine); and Man-PLL, mannosylated PLL. Data for Man-BSA and Gal-BSA [25] are included for comparison.

The hepatic uptake of PS/Gal-PLL complexes was partially inhibited by prior intravenous administration of excess Gal-BSA, suggesting that the complexes were taken up by the hepatocytes via the galactose receptor-mediated process. Due to the negative ζ potential caused by possible exposure of ODNs to the complex surface and wide particle size distribution (Table 1), a part of the complexes is likely to be recognized by the galactose receptor of the Kupffer cells, by scavenger receptors of the endothelial and Kupffer cells as polyanions [40] and/or being phagocytosed by Kupffer cells. Moreover, sulfur atoms present in the PS molecules may also influence the nonspecific hepatic uptake of its complexes.

We tested the ability of the receptor-mediated process to direct ODNs to other receptors by substituting mannose for galactose in order to target the mannose receptor present in liver macrophages. Complex formation with Man-PLL enhanced the uptake of PS by NPC; however, the uptake by PC was also quite high (Fig. 4). The hepatic uptake of the complex was partially inhibited by prior injection of excess Man-BSA (Table 3). These data clearly suggest that a part of the complexes was taken up via the mannose receptor-mediated pathway, and a part by some of the other mechanisms mentioned above.

TABLE 3. Competitive inhibition of hepatic uptake of [^{35}S]-labeled PS complexed with glycosylated poly(L-lysine)

Samples	Inhibitor	Plasma concentration (% of dose/mL)	Liver accumulation (% of dose)
PS/Gal-PLL	None	3.04 ± 0.08	47.41 ± 4.52
	Gal-BSA	4.77 ± 0.70*	39.91 ± 5.28*
PS/Man-PLL	None	3.27 ± 0.13	53.25 ± 3.60
	Man-BSA	5.54 ± 0.84*	40.25 ± 3.58*

One minute after intravenous injection of unlabeled inhibitor (10 mg/kg), the complex sample (0.5 mg PS/kg) was administered in a similar manner. Plasma concentration and liver accumulation were compared with those of the control samples at 15 min after injection. Values are means ± SD of three animal experiments.

* Statistically significant difference based on one-way ANOVA followed by Dunnett's test for pairwise comparisons, $P < 0.05$.

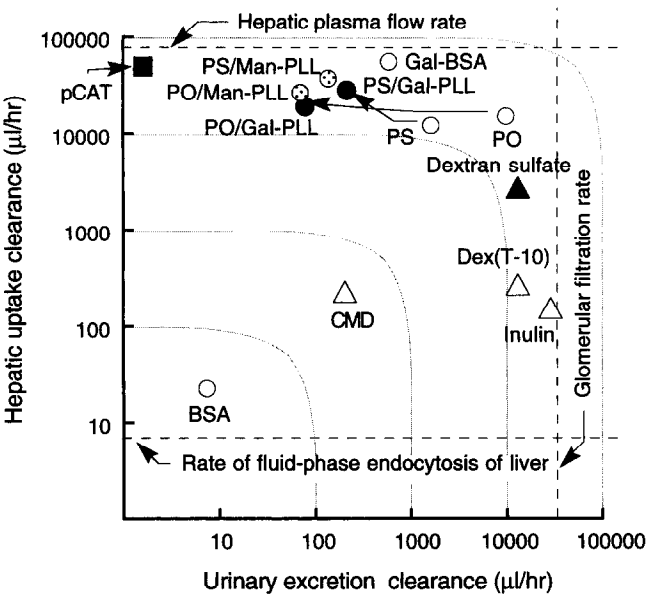

FIG. 5. Hepatic and urinary clearances of macromolecules after intravenous injection into mice. Abbreviations: CMD, carboxymethyl dextran; PLL, poly(L-lysine); Dex(T-10), dextran (mol. wt. ~10,000); PO, phosphodiester oligonucleotides; PS, phosphorothioate oligonucleotides; Gal-PLL, galactosylated PLL; and Man-PLL, mannosylated PLL.

Hepatic uptake of ODNs/glycosylated PLL complexes is greatly influenced by the particle size, ζ potential, sugar substitution level, molecular weights of both PLL and ODNs, and type of ODNs [2]. The data presented in this paper show only modest effects on ODN uptake by PC and NPC due to complex formation with glycosylated PLL. However, this is an important study, demonstrating the importance of particle size and ζ potential of the complex for their hepatic uptake via the receptor-mediated process. Based on these findings, we are now trying to further improve both the physicochemical and disposition characteristics of these complexes by producing electrically neutral complexes of a much smaller size to avoid their nonspecific uptake.

In conclusion, the present study suggests that ODNs can be delivered to the hepatocytes and macrophages *in vivo* after complexing them with Gal-PLL and Man-PLL. However, their disposition characteristics are heavily dependent on the physicochemical properties such as particle size, ζ potential, and surface properties of the complex. Therefore, the physicochemical characteristics of the ODNs/carrier complexes should be examined thoroughly prior to their *in vivo* applications.

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