Targeted Delivery of Plasmid DNA to Hepatocytes In Vivo: Optimization of the Pharmacokinetics of Plasmid DNA/Galactosylated Poly(L-Lysine) Complexes by Controlling their Physicochemical Properties

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ABSTRACT
In vivo receptor-mediated targeting of plasmid DNA to hepatocytes was achieved through optimizing the physicochemical and pharmacokinetic properties of a plasmid DNA/cARRIER complex. Galactosylated poly(l-lysine) (Gal-PLL) was synthesized using PLL with a molecular weight of 1,800, 13,000 or 29,000 without loss of the cationic charge. Plasmid DNA encoding chloramphenicol acetyltransferase was complexed with each Gal-PLL. A larger amount of PLL1800 is required for the complex formation than with PLL13000 and PLL29000, and increasing the number of galactose units on Gal-PLL resulted in reduced binding to plasmid DNA. The particle size and z-potential of the complexes varied depending on the mixing ratio and Gal-PLL used. Then, plasmid DNA/Gal-PLL complexes having diameters of 200 nm or less and a weak negative charge were prepared. After i.v. injection of [32P]plasmid DNA/Gal13-PLL13000 and [32P]plasmid DNA/Gal5-PLL18000 complexes, almost 80% of the radioactivity rapidly accumulated in the liver, preferentially in the parenchymal cells. The hepatic uptake clearances (CLliver) were much greater than any of the other tissue uptake clearances. Compared with these complexes, [32P]plasmid DNA/Gal13-PLL1800 and [32P]plasmid DNA/Gal5-PLL13000 had a smaller CLliver, suggesting that both the molecular weight of PLL and the degree of galactose modification determine the hepatic targeting of plasmid DNA. In vitro and in vivo gene expression studies revealed that plasmid DNA/Gal13-PLL13000 and plasmid DNA/Gal5-PLL29000 complexes are superior to plasmid DNA/Gal5-PLL1900 complex for introducing DNA into cells. These results demonstrated that an optimal design of a DNA/cARRIER complex based on physicochemical properties and a pharmacokinetic analysis of the distribution properties leads to successful in vivo gene delivery.

For the rational design of drug delivery systems, we have emphasized the importance of pharmacokinetic considerations associated with the biodistribution of delivery systems (Takakura and Hashida, 1996; Nishikawa et al., 1996). Pharmacokinetic analyses based on the clearance concept revealed that physicochemical properties such as the molecular weight and electrical charge are important in determining the in vivo disposition profiles of drug-macromolecule conjugates (Atsumi et al., 1987; Hashida et al., 1984, 1997; Nishikawa et al., 1993) and chemically modified proteins (Fujita et al., 1990, 1992; Takakura et al., 1990, 1994). Moreover, these properties affect the targeting efficiency of glycosylated macromolecules to the liver via receptor-mediated mechanisms (Hirabayashi et al., 1996; Nishikawa et al., 1992, 1995a, b). These findings indicate that not only the nature of the ligands grafted to carriers but also the overall physico-
chemical properties of the macromolecular compounds determine the amount delivered to a target after their systemic administration.

The in vivo disposition of plasmid DNA and its complexes is also considered to depend on physicochemical characteristics. Our previous study demonstrates that \( ^{32}P \) plasmid DNA is rapidly eliminated from the circulation after i.v. injection into mice. Extensive uptake by the liver, especially by the nonparenchymal cells, plays a major role in its rapid elimination which is an obstacle to delivering genes to other cells in the body. The strong negative charge and high-molecular weight of plasmid DNA determine its biodistribution after intravenous injection. Therefore, development of a delivery system with suitable physicochemical and biological properties is required for site-directed delivery of plasmid DNA and its efficient in vivo expression.

To achieve selective delivery of plasmid DNA to the PCs of the liver, Gal-PLL was synthesized as a macromolecular carrier possessing the cationic charge necessary for the electrostatic binding between plasmid DNA and galactose residues as the targetable ligand to the cells. In this study, Gal-PLLs with various molecular weights and varying numbers of galactose residues were synthesized to rationally design a suitable delivery system. The relationship involving the physicochemical properties, in vivo distribution and gene expression of plasmid DNA/Gal-PLL complexes was investigated, and factors affecting the targeting and expression of plasmid DNA were identified. Preliminary results on the in vivo distribution of plasmid DNA complex with a type of Gal-PLL have been reported elsewhere (Hashida et al., 1998).

**Methods**

**Chemicals.** Three types of PLL hydrobromide (average molecular weights of 1,800, 13,000 and 29,000 as PLL), collagenase (type I) and BSA (fraction V) were purchased from Sigma Chemical Co. (St. Louis, MO). \([\alpha-^{32}P]dCTP\) and 1-deoxy\([dichloroacetyl-1-^{14}C]\) chloramphenicol were obtained from Amersham (Tokyo, Japan). A polyclonal rabbit anti-CAT antibody was purchased from 5 Prime-3 Prime, Inc. (Boulder, CO). TrueBlue Peroxidase Substrate and Contrast Red Solution were obtained from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, MD). All other chemicals were obtained commercially as reagent-grade products.

**Preparation of plasmid DNA.** A plasmid DNA, pCAT-Control Vector (Promega, Madison, WI), containing CAT gene under the control of the SV40 promoter was selected as a model gene. The pCAT was amplified in the HB101 strain of *Escherichia coli*, extracted by the alkaline lysis technique, and purified by precipitation with ethanol. Purified plasmid DNA was redissolved in normal saline and stored at \(-20^\circ C\) until use. The concentration of plasmid DNA was measured by UV absorption at 260 nm. For the in vivo disposition studies, the plasmid was labeled with \([\alpha-^{32}P]dCTP\) by nick translation (Sambrook et al., 1989). For the in vivo expression studies, plasmid DNA encoding CAT gene that was under the control of the cytomegalovirus promoter, pdcNAS/CAT (Invitrogen Corp., Carlsbad, CA), was used.

**Synthesis of Gal-PLL.** Gal-PLL was synthesized by covalently binding 2-imino-2-methoxyethyl 1-thiogalactoside (IME-thiogalacto-side) to PLL as previously reported (Hashida et al., 1998). Briefly, cyanomethyl 1-thiogalactoside was treated with 0.01 M sodium methoxide at room temperature. The solvent was dried under reduced pressure and PLL dissolved in 50 mM tetraborate buffer (pH 9.5) was added to the resultant syrup of IME-thiogalactoside. The mixture was reacted for 3 hr, subjected to ultrafiltration and lyophilized.

The number of galactose residues and galactose content were determined by the anthrone-sulfuric acid method.

**Formation of plasmid DNA complexes.** Plasmid DNA complexes were prepared by adding various amounts of PLL or Gal-PLL to pCAT followed by brief stirring at room temperature. The complex formation was monitored by gel retardation assay (Sambrook et al., 1989). Complexes were run at a constant voltage (50 V) on a 1% agarose gel prepared in Tris-borate-EDTA buffer and the gel was stained with ethidium bromide and photographed under UV light.

**Particle size and z potential measurements.** The particle size of the plasmid DNA/Gal-PLL complexes was measured by a dynamic light scattering spectrophotometer (LS-900, Otsuka Electronics, Osaka, Japan). The \( z \) potential of the complexes was determined with a laser electrophoresis zeta-potential analyzer (LEZ-A500T, Otsuka Electronics).

**In vivo distribution experiment.** Male ddY mice (25–28 g) were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan) and were maintained on standard food and water under conventional housing conditions. Mice received injections with 20 \( \mu g/\text{kg} \) \( ^{32}P \) plasmid DNA or its complex with PLL derivatives in saline. At appropriate intervals after injection, groups of three mice each were anesthetized with ether and blood was collected from the vena cava to obtain plasma by centrifugation. The liver, kidneys, spleen, and lungs were excised, rinsed with saline and weighed. A piece of each tissue sample was dissolved with Soluene-350 (Packard, Netherlands), then scintillation medium (Clear-sol I, Nacalai Tesque, Kyoto, Japan) was added and the \(^{32}P\)-radioactivity was counted using a LSC-5000 liquid scintillation counter (Beckman, Tokyo, Japan). Radioactivity from the plasma in each tissue sample was corrected for using the distribution data of \(^{111}\text{In-BSA} \) at 10 min after i.v. injection (Nishikawa et al., 1995a), assuming that BSA was not taken up by the tissues during the 10-min period.

**Pharmacokinetic analysis.** Tissue distribution patterns of \(^{32}P\) plasmid DNA and its complexes after intravenous injection were evaluated using the CL\(_{\text{tiss}}\), which was calculated by dividing the amount of radioactivity in an organ at an appropriate time by the AUC up to the same time point (Nishikawa et al., 1996). Because there was little influx of \(^{32}P\)-radioactivity from tissues (including the liver) within the first 5 min after i.v. administration of \(^{32}P\) plasmid DNA complexes, CL\(_{\text{tiss}}\) was calculated using the distribution data obtained 5 min postinjection. The CL\(_{\text{tiss}}\) was calculated by fitting an equation to the plasma concentration data of the \(^{32}P\)-radioactivity-time profile using the nonlinear least-squares program MULTI (Yamaoka et al., 1981).

**Hepatic cellular localization of plasmid DNA complexes.** Groups of three mice each received injections with \(^{32}P\) plasmid DNA or its complexes and anesthetized by peritoneal administration of pentobarbital sodium. At 10 min after injection, the liver was perfused from the portal vein first with preperfusion buffer (Ca\(^{++}\)-, and Mg\(^{++}\)-free HEPES buffer, pH 7.2) for 10 min and then with HEPES buffer (pH 7.5) containing 5 mM CaCl\(_2\) and 0.05% (w/v) collagenase for about 20 min. Then the liver was excised and the cells were dispersed by gentle stirring in ice-cold Hanks'-HEPES buffer containing 0.1% BSA. The dispersed cells were filtered through cotton mesh sieves and centrifuged for 1 min at \(50 \times g\) to sediment the liver PC. The supernatant was removed and kept as the source of NPC. The pellet of PC was resuspended in buffer and centrifuged again to remove other cells. The NPC suspension was centrifuged twice at \(50 \times g\) for 1 min to remove the PC, then subjected to centrifugation at \(200 \times g\) for 2 min to obtain the pellet of NPC. Both PC and NPC fractions were resuspended in buffer and the number of cells was determined by the Trypan blue exclusion method. The radioactivity of both samples was also determined as in the in vivo distribution experiment.

**In vitro gene expression.** Human hepatoma cell line HepG2 cells, purchased from Dainippon Pharmaceutical (Osaka, Japan), were cultured in Dulbecco’s modified eagle medium (DMEM) (Nissui...
Pharmaceutical, Tokyo, Japan) plus 10% heat-inactivated fetal bovine serum (Bio Whittaker, Walkersville, MD) and Penicillin-Streptomycin-Glutamine (GIBCO, Renfrewshire, UK). The cells were seeded in six-well tissue culture plates and grown for 10 days. Then the culture medium was removed and 3 μg plasmid DNA or its complexes with PLL or Gal-PLL was added. After 6 hr incubation at 37°C under 5% CO2, the medium was removed, 2 ml fresh medium were added, and the cells were further incubated for 48 hr at 37°C.

Then the medium was aspirated and the cells were washed with ice-cold PBS, scraped from the plates and centrifuged at 1500 × g for 3 min at 4°C. The pellets were resuspended in 175 μl 0.25 M Tris-HCl (pH 8.0), subjected to three cycles of freezing and thawing, heated for 10 min at 60°C to inactivate endogenous deacetylase, and centrifuged at 13,000 × g for 15 min at 4°C. Aliquots of the supernatants were assayed for their protein content (Bradford, 1976) and chloramphenicol acetyltransferase activity as reported by Gorman et al. (1982).

**Immunohistochemical staining of CAT expression in vivo.**
A total of 6 μg pCAT (free or complex form with PLL derivatives) was repetitively injected into mice via the tail vein four times at intervals of 1 hr. At 48 hr after the first injection, the liver and kidneys were excised, frozen and sliced. Thin tissue sections (8 μm) were run at 50 V on a 1% agarose gel prepared in Tris-borate-EDTA buffer, and the gel was stained with ethidium bromide and photographed under UV light. a, plasmid DNA/Gal5-PLL1800; b, plasmid DNA/Gal13-PLL13000; c, plasmid DNA/Gal26-PLL29000.

**Results**

**Physicochemical characteristics of Gal-PLL and complex formation with plasmid DNA.** Table 1 summarizes the physicochemical characteristics of the synthetic Gal-PLLS: Gal26-PLL13000, Gal26-PLL13000, Gal5-PLL13000, Gal36-PLL130000, Gal25-PLL29000 and Gal44-PLL29000. Complex formation between plasmid DNA and PLL or Gal-PLL was determined by agarose gel electrophoresis, followed by gel staining with ethidium bromide and photography under UV light. The addition of any PLL derivative to plasmid DNA resulted in the formation of complexes that did not move toward the positive pole. The electrophoretic bands of plasmid DNA disappeared when more than 3 μg PLL13000 was mixed with 1 μg plasmid DNA, suggesting that all plasmid DNA was complexed with the polymer at this ratio. When PLL13000 or PLL29000 was used, only 0.6 μg of each was required for complete formation of complexes with 1 μg pCAT. Gal5-PLL1800, Gal13-PLL13000, Gal13-PLL13000 and Gal26-PLL29000 had the same mixing ratio (1:0.6, plasmid DNA:Gal-PLL on a weight basis) as the corresponding unmodified PLLs (fig. 1), suggesting that these PLL derivatives retain their potent ability to electrostatically bind to DNA. However, derivatives which are highly modified with galactose moieties, i.e., Gal36-PLL13000 and Gal44-PLL29000, showed less binding ability than other Gal-PLLS as determined by the amount needed for the retardation of plasmid DNA on the gel (table 2).

**Size and ζ potential of plasmid DNA/Gal-PLL complexes.** Figure 2 shows the size of the plasmid DNA complexes with Gal5-PLL13000, Gal13-PLL13000, and Gal26-PLL29000 in various mixing ratios. The addition of a small amount of Gal-PLL produced plasmid DNA/Gal-PLL complexes whose diameters were smaller than that of naked pCAT (about 200 nm). However, increasing the amount of Gal-PLL resulted in the formation of large complexes whose sizes were 500 nm or more in diameter. The ζ potentials of the complexes correlated with the changes in particle size (fig. 3). Although the particle size of the complexes was similar to or less than that of naked pCAT, the ζ potential was still negative although the intensity of the negative charge was reduced by the addition of any Gal-PLL. An increase in the amount of Gal-PLL resulted in the formation of cationic complexes of plasmid DNA and Gal-PLL.

**Tissue distribution of [32P]plasmid DNA/Gal-PLL complexes after i.v. injection in mice.** Based on the observations above, plasmid DNA/Gal-PLL complexes were

### Table 1
Physicochemical characteristics of Gal-PLL

<table>
<thead>
<tr>
<th>Compound</th>
<th>Molecular Weight of PLL</th>
<th>Degree of Polymerization of PLL</th>
<th>No. of Galactose* (mol/mol PLL)</th>
<th>Galactose Content%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gal26-PLL13000</td>
<td>1,800</td>
<td>43</td>
<td>5.1</td>
<td>33.7</td>
</tr>
<tr>
<td>Gal26-PLL13000</td>
<td>13,000</td>
<td>93</td>
<td>4.5</td>
<td>6.0</td>
</tr>
<tr>
<td>Gal13-PLL13000</td>
<td>13,000</td>
<td>93</td>
<td>13.3</td>
<td>15.9</td>
</tr>
<tr>
<td>Gal5-PLL13000</td>
<td>13,000</td>
<td>93</td>
<td>35.9</td>
<td>33.9</td>
</tr>
<tr>
<td>Gal25-PLL29000</td>
<td>29,000</td>
<td>214</td>
<td>25.9</td>
<td>13.8</td>
</tr>
<tr>
<td>Gal44-PLL29000</td>
<td>29,000</td>
<td>214</td>
<td>43.6</td>
<td>21.2</td>
</tr>
</tbody>
</table>

*The number of galactose residues and galactose content were determined by anthrone-sulfuric acid method.

### Table 2
Mixing ratio required for complete formation of plasmid DNA complex

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mixing Ratio* (DNA:PLL, w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLL13000</td>
<td>1.3</td>
</tr>
<tr>
<td>Gal26-PLL13000</td>
<td>1.3</td>
</tr>
<tr>
<td>PLL13000</td>
<td>1.0</td>
</tr>
<tr>
<td>Gal26-PLL13000</td>
<td>1.0</td>
</tr>
<tr>
<td>Gal26-PLL29000</td>
<td>1.0</td>
</tr>
<tr>
<td>PLL29000</td>
<td>1.0</td>
</tr>
<tr>
<td>Gal26-PLL29000</td>
<td>1.0</td>
</tr>
<tr>
<td>Gal26-PLL29000</td>
<td>1.1</td>
</tr>
</tbody>
</table>

*The mixing ratio was determined by gel retardation assay on a 1% agarose gel.
prepared at mixing ratios of 1:3 for Gal 5-PLL1800 and 1:0.6 for PLL13000 and PLL29000 derivatives. Table 3 summarizes the zeta potential and mean particle size of naked plasmid DNA and the complexes used in the following investigation. All complexes had a weak negative charge and their size was around 200 nm in diameter, which was nearly equal to, or smaller than, that of the naked plasmid DNA.

Figure 4 shows the time-courses of the concentration in plasma and the amount of 32P-radioactivity in the liver after intravenous injection of [32P]plasmid DNA, [32P]plasmid DNA/PLL13000, [32P]plasmid DNA/Gal5-PLL13000, [32P]plasmid DNA/Gal13-PLL13000 into mice. When [32P]plasmid DNA was administered, the radioactivity rapidly disappeared from the plasma and more than 50% of the radioactivity was recovered in the liver within the first 3 min. Complex formation with PLL13000, Gal 5-PLL13000 and Gal 13-PLL13000 increased the amount of 32P-radioactivity recovered in the liver. [32P]plasmid DNA/Gal 13-PLL13000 showed more rapid hepatic accumulation of radioactivity than [32P]plasmid DNA/Gal 5-PLL13000, suggesting that the number of galactose residues is an important factor determining the affinity of the plasmid DNA/Gal-PLL complex for the asialoglycoprotein receptor on hepatocytes.

Figure 5 shows the time-courses of the concentration in plasma and the amount of the radioactivity in the liver after intravenous injection of [32P]plasmid DNA/Gal-PLL complexes into mice. In the case of [32P]plasmid DNA/Gal 26-PLL29000, almost 80% of the radioactivity was accumulated in the liver within the first 3 min, and the profiles of the radioactivity in plasma and liver were similar to those of [32P]plasmid DNA/Gal 13-PLL13000. However, when [32P]plasmid DNA/Gal 5-PLL1800 was injected, the radioactivity was slowly eliminated from the plasma and the profile of the radioactivity in the liver was comparable with that of free [32P]plasmid DNA.

Pharmacokinetic analysis of [32P]plasmid DNA/Gal-PLL after i.v. injection into mice. Table 4 summarizes the pharmacokinetic parameters of [32P]plasmid DNA and its complexes. The hepatic uptake clearances ($\text{CL}_{\text{hep}}$) of [32P]plasmid DNA/Gal 13-PLL13000 and [32P]plasmid DNA/Gal 26-PLL29000 were more than that of [32P]plasmid DNA.

**Table 3**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mixing Ratio (DNA:Gal-PLL, w/w)</th>
<th>Particle Sizea (nm)</th>
<th>Zeta Potentialb (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCAT</td>
<td></td>
<td>197 ± 136</td>
<td>−36.4</td>
</tr>
<tr>
<td>pCAT/Gal5-PLL1800</td>
<td>1:3</td>
<td>208 ± 45</td>
<td>−29.1</td>
</tr>
<tr>
<td>pCAT/Gal13-PLL13000</td>
<td>1:0.6</td>
<td>179 ± 63</td>
<td>−20.6</td>
</tr>
<tr>
<td>pCAT/Gal26-PLL29000</td>
<td>1:0.6</td>
<td>169 ± 36</td>
<td>−12.1</td>
</tr>
</tbody>
</table>

*The particle size of plasmid DNA and its complexes was measured by a dynamic light scattering spectrophotometer.

bThe zeta potential of pCAT and its complexes was determined by a laser electrophoresis zeta potential analyzer.
which is known to be rapidly taken up by liver nonparenchymal cells due to its strong negative charge. The $C_{L_{\text{Liver}}}$ values were much more than the uptake clearances by any other tissues and accounted for more than 70% of the total body clearance of each complex, indicating that these complexes are selectively taken up by the liver. Compared with these complexes, $[^{32}\text{P}]$plasmid DNA/Gal$^{5}$-PLL$^{1800}$ had smaller $C_{L_{\text{Liver}}}$ values. Except for $C_{L_{\text{Liver}}}$, the uptake clearances by all tissues were small and almost identical for the $[^{32}\text{P}]$plasmid DNA complexes investigated.

**Hepatic cellular localization of $[^{32}\text{P}]$plasmid DNA/Gal-PLLs.** Figure 6 shows the localization of the radioactivity in the liver after administration of $[^{32}\text{P}]$plasmid DNA or its complexes with Gal-PLLs. $[^{32}\text{P}]$plasmid DNA and $[^{32}\text{P}]$plasmid DNA/PLL$^{13000}$ were predominantly taken up by NPC compared with PC, although both $[^{32}\text{P}]$plasmid DNA/Gal$^{13}$-PLL$^{13000}$ and $[^{32}\text{P}]$plasmid DNA/Gal$^{26}$-PLL$^{29000}$ were preferentially taken up by PC. However, $[^{32}\text{P}]$plasmid DNA/Gal$^{5}$-PLL$^{1800}$ was recovered in both PC and NPC, indicating that plasmid DNA is not efficiently delivered to PC by Gal$^{5}$-PLL$^{1800}$.

**CAT expression in HepG2 cells.** Figure 7 shows the CAT activity expressed in HepG2 cells treated with plasmid DNA and its complexes. High CAT activity was obtained in the cells treated with plasmid DNA/Gal$^{13}$-PLL$^{13000}$ or plasmid DNA/Gal$^{26}$-PLL$^{29000}$, although little activity was found in the cells treated with plasmid DNA/Gal$^{5}$-PLL$^{1800}$. When naked plasmid DNA or plasmid DNA/PLL$^{13000}$ was applied, CAT activity in the cells was almost negligible.

**CAT expression in vivo.** CAT could be detected in the liver sections of mice injected with plasmid DNA/Gal$^{13}$-PLL$^{13000}$ and plasmid DNA/Gal$^{26}$-PLL$^{29000}$, but was not found in the liver sections of mice treated with plasmid DNA alone or plasmid DNA/Gal$^{5}$-PLL$^{1800}$. Figure 8 shows the liver sections of mice receiving injections with plasmid DNA/Gal$^{13}$-PLL$^{13000}$ and plasmid DNA/Gal$^{26}$-PLL$^{29000}$. Little CAT activity was found in the kidney sections of any group of mice studied.

**Discussion**

In vivo gene expression will take place only at restricted sites that are reached by plasmid DNA in its intact form after administration. To achieve cell-specific gene expression, therefore, the in vivo fate of a plasmid DNA/carrier complex

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**TABLE 4**

Clearances of $[^{32}\text{P}]$plasmid DNA and its complexes with PLL derivatives after i.v. injection in mice

<table>
<thead>
<tr>
<th>Compound</th>
<th>Total Body Clearance* (ml/hr)</th>
<th>Liver</th>
<th>Kidney</th>
<th>Spleen</th>
<th>Lung</th>
</tr>
</thead>
<tbody>
<tr>
<td>$[^{32}\text{P}]$plasmid DNA</td>
<td>51.4</td>
<td>27.8</td>
<td>2.5</td>
<td>0.8</td>
<td>0.4</td>
</tr>
<tr>
<td>$[^{32}\text{P}]$plasmid DNA/PLL$^{13000}$</td>
<td>45.5</td>
<td>30.1</td>
<td>1.9</td>
<td>2.0</td>
<td>1.4</td>
</tr>
<tr>
<td>$[^{32}\text{P}]$plasmid DNA/Gal$^{13}$-PLL$^{13000}$</td>
<td>47.0</td>
<td>35.5</td>
<td>3.0</td>
<td>2.3</td>
<td>0.9</td>
</tr>
<tr>
<td>$[^{32}\text{P}]$plasmid DNA/Gal$^{5}$-PLL$^{1800}$</td>
<td>61.1</td>
<td>47.6</td>
<td>1.4</td>
<td>1.3</td>
<td>0.4</td>
</tr>
<tr>
<td>$[^{32}\text{P}]$plasmid DNA/Gal$^{26}$-PLL$^{29000}$</td>
<td>52.1</td>
<td>32.3</td>
<td>2.5</td>
<td>0.6</td>
<td>0.3</td>
</tr>
<tr>
<td>$[^{32}\text{P}]$plasmid DNA/Gal$^{13}$-PLL$^{13000}$</td>
<td>80.6</td>
<td>59.9</td>
<td>2.4</td>
<td>1.7</td>
<td>0.6</td>
</tr>
</tbody>
</table>

* The total body clearance was calculated by fitting an equation to the plasma concentration data of radioactivity-time profile using a nonlinear least-square method.

† The organ clearances were calculated by dividing the amount of radioactivity in each organ at 5 min after the injection by the area under the plasma concentration of radioactivity-time curve up to the same time point.
should be quantitatively investigated. Once the *in vivo* disposition and gene expression of the complex are linked with its physicochemical and biological properties, it is then possible to design a carrier and a plasmid DNA/carrier complex to enable cell-specific *in vivo* gene transfer.

The addition of polycations to DNA results in formation of a polyelectrolyte complex (Kabanov and Kabanov, 1995). Of the various polycations available, PLL of different molecular weight is widely used as a DNA-binding moiety in developing DNA carriers (Edwards et al., 1996; Wadhwa et al., 1995; Wu and Wu, 1991). The difference in the molecular weight of PLL affects several aspects of the DNA/PLL complex (Erbach et al., 1995; Dash et al., 1997), but the importance of size on the *in vivo* delivery of plasmid DNA to target cells is not yet fully understood. In the present study, the molecular weight of PLL used greatly affected the formation and particulate properties of plasmid DNA complexes. A 5-fold greater amount of PLL1800 was required for complete formation of plasmid DNA-complex than with PLL13000 and PLL29000. The electrostatic binding force of PLL is reported to be virtually independent of the molecular weight of PLL (Afshar-Rad et al., 1987). The results of this study, therefore, suggest that the formation and stability of the complexes do not result from merely an electrostatic interaction of Gal-PLL with plasmid DNA but also involve the entanglement of these polymers.

The increase in the transfection efficiency of plasmid DNA/Gal-PLL complex both *in vivo* and *in vitro* correlates with the increased amount of DNA taken up by the cells of interest. Gal13-PLL1800 hardly altered the *in vivo* disposition of [32P]plasmid DNA when injected as a complex (figs. 5 and 6). In addition, it failed to provide efficient gene transfer in HepG2 cells and in the liver after systemic administration. These results suggest that Gal13-PLL1800, the smallest derivative used in this study, rapidly dissociates from plasmid DNA before the complex reaches the target. In a previous study (Mahato et al., 1997), we investigated the *in vivo* disposition of [35S]oligonucleotide/Gal-PLL or Man-PLL complex. Although the molecular weight of PLL used was about 35,000, larger than that of the PLLs used in this study, the uptake of the complexes by the liver cells was less cell-specific than that of [32P]plasmid DNA/Gal13-PLL13000 or Gal26-PLL29000 complex, suggesting that [35S]oligonucleotide, a very much smaller DNA than plasmid DNA, more rapidly dissociates from the carriers than plasmid DNA. Therefore, it can be concluded that the *in vivo* stability of DNA/carrier complexes is determined by the size of both the nucleic acids and polycationic carriers.

To be effectively recognized by the asialoglycoprotein receptor, carriers should possess a sufficient number of galactose residues in their structures. The hepatic uptake clearance of galactosylated derivatives of poly(l-glutamic acid) showed a good correlation with the number of galactose residues (Hirabayashi et al., 1996). Furthermore, the hepatic uptake of galactosylated proteins of varying molecular weights has been shown to be regulated by the density of galactose residues on their molecular surface (Nishikawa et al., 1995b). Therefore, the hepatic targetability of Gal-PLL will depend on the number of galactose residues grafted. The hepatic uptake clearance of [32P]plasmid DNA/Gal13-PLL13000 was higher than that of [32P]plasmid DNA/Gal13-PLL13000, indicating that the former is more efficiently recognized by the receptor due to the higher degree of galactose-modification of PLL. The involvement of the asialoglycoprotein receptor in their uptake by the parenchymal cells in the liver was confirmed by the finding that preadministration of galactosylated BSA, a well-known ligand of the receptor, reduced the uptake of [32P]plasmid DNA/Gal13-PLL13000 by the cells (Hashida et al., 1998). However, extensive modification of PLL13000 or PLL29000 resulted in a reduced potential to form a
DNA complex (table 2). The electric charge of these derivatives would be the same as on the corresponding native PLLs, because the method for glycosylation used in this study, the attachment of 2-imino-2-methoxyethyl 1-thiogalactoside to amino residues on PLL, is reported not to change the charge of the proteins (Lee et al., 1976). Therefore, these results suggest that a large number of galactose residues somehow interferes with the electrostatic interaction and entanglement of plasmid DNA and Gal-PLL. Based on these considerations, the optimal degree of modification of PLL with galactose can be concluded to be around 14 to 16% (w/w).

The physicochemical properties of DNA/crrier complexes greatly influence their in vivo disposition characteristics. Cationic nature of cationic liposomes or polymers is used in a lot of investigations as a force to electrostatically interact with both plasmid DNA and target cells. Even if a targeting moiety, such as sugars, is grafted on to them, cell-specific delivery of plasmid DNA could not be achieved, because such cationic complexes can distribute to various types of cells after systemic administration (Mahato et al., 1995a, b; Zhu et al., 1993). In addition, Gal-PLL or Man-PLL showed less cell-specific uptake by the parenchymal and nonparenchymal cells, respectively, in the liver than corresponding derivatives of anionic polymer, poly(y-glutamic acid) (Akamatsu et al., in press). In addition, naked plasmid DNA is rapidly eliminated from the blood circulation due to extensive uptake by hepatocytes, therefore, were designed to possess a weak negative charge as a whole, which would emphasize the specific uptake of complexes with hepatocytes via asialoglycoprotein receptor-mediated endocytosis.

In addition to the electrical charge, the size of DNA/carrier complexes is one of the most important determinants for delivery of plasmid DNA to hepatocytes after systemic administration (Mahato et al., 1995b; Zhu et al., 1993). Targeted delivery of human recombinant superoxide dismutase by chemical modification of mono- and poly-saccharide derivatives. J Pharmacol Exp Ther 263: 971–978.

References


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