Master Thesis

Efficiency and site selectivity evaluation of in vivo gene transfer to the liver in mice: a comparison between naked plasmid DNA and gene carriers

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1 Introduction

Gene therapy is a promising candidate therapeutic approach for intractable inherited and acquired diseases. Since the 1970s, a great number of studies have been conducted to validate the concept of gene therapy [1]. These studies are mainly classified into two large categories, i.e., viral and non-viral gene delivery approaches, and both of them have experienced a great deal of progress [2, 3]. However, there are still some critical factors preventing gene therapy from becoming an effective treatment in clinical use. In viral approaches, the key problems are the safety issues such as immunogenicity for all viral vectors and insertion mutagenesis for retroviral vectors [4, 5]. For non-viral methods, the toxicity from the synthesized carriers and the lack of efficiency in gene expression are the main disadvantages. Because the therapeutic genes always encode highly bioactive proteins, for example, cytokines [6], gene expression in non-target tissues may cause unexpected adverse effects. Therefore, target-selective or -specific gene transfer is important for maximal therapeutic action and minimal adverse effects in the clinical use of gene therapy.

Belonging to the non-viral gene delivery approaches, naked plasmid DNA (pDNA) is the simplest since it can be prepared without recombinant viruses or complexing with polymers or lipids, and the safest since it can be used without concerning about the immunogenicity or toxicity. After intravenous injection, however, it is well known that naked pDNA without any carrier is easily degraded by reticuloendothelial cells (liver Kupffer cells, etc.) and DNase in the plasma [7, 8]. The in vivo gene delivery of naked pDNA was reported to be achieved with the help of physical forces, such as electroporation [9, 10], hydrodynamics-based intravenous injection [8, 11, 12] and injection with mechanical massage [13]. However, the most serious disadvantage of those methods is the concern of safety due to the physical hurt to organs or cells.

In our previous studies, naked pDNA was utilized in the organ-surface gene transfer experiments. Following the instillation of naked pDNA onto the organ surface
of liver, stomach, spleen and kidney in mice or rats, we have already demonstrated the
gene transfections with high efficiency [14-19]. This method dramatically improved the
organ-selectivity and is of high safety because of no physical forces against the organs.
However, in the organ surface gene delivery system, there are no reports about the gene
transfer efficiency of gene carriers. Because the gene transfer mechanism may be
different from other gene delivery systems, in the organ surface system, an evaluation of
the gene delivery of gene carriers is necessary. In the first part of the present study,
therefore, I introduced the cationic gene carriers into the liver surface gene transfer
experiments. Then the gene transfer efficiency and organ selectivity of carrier/pDNA
complexes in different experiment conditions were evaluated with a comparison to the
naked pDNA.

The gene carrier utilized in this research is polyethylenimine (PEI), which is a well
known gene vector belonging to synthetic cationic polymers (polycation). This selection
is based on the large amount of applications in gene expression researches in vivo and
in vitro during the last decade [20-22]. The chemistry of PEI can be divided into linear
and branched structures and both of which have various molecular weights [22]. Due to
different physicochemical characteristics, PEI moleculars with different structures or
molecular weights have different gene delivery performance in vitro and in vivo [22,
23]. Because the organ surface gene delivery is a new application for PEI, we chose
both the linear and branched structures in this research. Additionally, with removal of
the residual N-acyl moieties from linear PEI, a new PEI type called linear PEI ‘MAX’
was reported to have enhancements in gene delivery in vivo and invitro [24, 25]. Hence,
linear PEI ‘MAX’ was also chosen as one of the carriers.

In this research, liver is the target organ. Since the liver plays important roles in the
synthesis of serum proteins, regulation of metabolism and maintenance of homeostasis,
the gene therapy targeted to this organ is greatly important and there are a lot of
progresses toward liver-targeted gene therapy. Including our liver surface gene transfer,
the hydrodynamics-based intravenous injection which is mentioned above, and specific injection methods such as injection of naked pDNA or lipoplexes via portal vein also got good selective hepatic uptake and expression [26, 27]. In the first part of this research, we analyzed the characteristics of gene transfer using naked pDNA or complexes in liver surface instillation experiments. Therefore, to clarify whether the gene transfer characteristics are specific only in the liver surface instillation system or not, there is a need to expand the comparison in other administration routes. After concerning the safety issue, in the second part of the research, we performed similar evaluation and comparison in intraportal injection experiments.
2 Materials and methods

2.1 Materials

Three types of PEI with different molecular weights and structures (25 kDa-linear PEI (LPEI), 25 kDa-linear PEI ‘MAX’ (LPEIM) which is fully deacylated from linear PEI, and 1.8 kDa-branched PEI (BPEI)) were obtained from Polysciences (Warrington, PA, USA) and selected as the cationic gene vectors. The structures are shown in Scheme 1.

Scheme 1 Structures of polyethyleneimine (PEI). (A) Linear PEI (LPEI); (B) Linear PEI ‘MAX’ (LPEIM); (C) Branched PEI (BPEI).

PEI were dissolved in 5% glucose solution. The pH was adjusted to 7.0 by hydrochloric acid (LPEI and BPEI) or sodium hydroxide (LPEIM). PEI solutions were stocked at 4°C prior to experiments.

Other reagents were of the highest purity available.

2.2 Animals

Five-week-old Male ddY mice (24-35 g) were purchased from Kyudo Co., Ltd. (Kumamoto, Japan). They were housed in a cage in an air-conditioned room and maintained on a standard laboratory diet (MF, Oriental Yeast, Co., Ltd., Tokyo, Japan) and water ad libitum. All animal experiments were carried out in accordance with the Guidelines for Animal Experimentation of Nagasaki University.
2.3 Construction and preparation of pDNA

pCMV-Luc (Scheme 2) was constructed by subcloning the HindIII/XbaI firefly luciferase cDNA fragment from a pGL3-control vector (Promega, Madison, WI, USA) into the polylinker of a pcDNA3 vector (Invitrogen, Carlsbad, CA, USA). pDNA was amplified in the *Escherichia coli* (E. coli) DH5α, isolated and purified using an EndoFree Plasmid Giga Kit (QIAGEN GmbH, Hilden, Germany). pDNA was dissolved in 5% glucose and stored at -20°C prior to experiments.

![Scheme 2](image)

**Scheme 2** Structure of pCMV-Luc vector (pCMV-Luc) which contains the cDNA encoding firefly luciferase.

2.4 Preparation of complexes

Stock solution of PEI was mixed with pDNA in 5% glucose at equal volumes and samples were incubated at room temperature for 30 min to allow complex formation before experiments.

The N/P ratio of complex was calculated as the molar ratio of the nitrogen groups of PEI to the phosphate groups of nucleic acids.

2.5 Agarose gel electrophoresis

Formation of complex was evaluated by agarose gel electrophoresis. After
preparation (section 2.4), naked pDNA and complexes at various N/P ratios were electrophoresed in a 1% (w/v) agarose gel in tris-acetic acid-EDTA buffer (pH 7.4) at 100 V. The DNA was visualized by an UV illuminator following staining of gels with ethidium bromide (0.5 μg/ml).

2.6 Particle size and zeta potential

Fifty microliters of pDNA (0.6 mg/ml) was mixed with equal volume of PEI in 5% glucose and incubated for 30 min at room temperature to form complexes at indicated N/P ratios. Before measurement, each prepared mixture (100 μl) was diluted by addition of 700 μl of 5% glucose. Particle size and zeta potential were determined by using Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, Worcestershire, UK).

2.7 In vivo gene expression experiments

2.7.1 Left lateral liver lobe surface instillation

Mice were anesthetized with sodium pentobarbital (40-60 mg/kg, intraperitoneal injection). Naked pDNA or complexes were instilled onto the surface of left lateral liver lobe by micropipette. The peritoneum was sutured and mice were kept lying on the back for 1 hour. Subsequently, mice were freed in the cage. Indicated times after instillation, the mice were killed under anesthesia, and the liver (administered lobe and other lobes), stomach, kidneys (left and right), spleen and diaphragm were removed. The tissues were washed twice with saline and homogenized with lysis buffer (0.1 M Tris/HCl buffer, 0.05% Triton X-100, 2 mM EDTA, pH 7.8). Homogenates were centrifuged at 15,000×g for 5 min. Twenty microliters of supernatant was added into 100 μl luciferase assay buffer (Picagene, Toyo Ink Mfg. Co., Ltd) and then the mixture was measured using a luminometer (Lumat LB9507, BERTHOLD TECHNOLOGIES, Bad Wildbad, Germany). The luciferase activity is indicated as the relative light units (RLU) per gram of tissue.
2.7.2 Intraportal injection

Mice were anesthetized with sodium pentobarbital (40-60 mg/kg, intraperitoneal injection). Two hundred microliters of naked pDNA or complexes were injected into the portal vein by a 27 G × 1/2” needle. The dose of pDNA was 30 μg. The peritoneum was sutured and mice were kept lying on the back for 1 hour. Subsequently mice were freed in the cage. Six hours after instillation, the mice were killed under anesthesia, and the liver, stomach, kidneys (left and right), spleen, lung and heart were removed. The treatments of tissues, homogenizations and luciferase assay were same with those in left lateral liver lobe surface instillation described in section 2.7.1.
3 Results and discussion

3.1 Physicochemical characteristics of complexes

The interaction between the polymers and the pDNA at different N/P ratios was analyzed by agarose gel electrophoresis. The results confirmed that the three polymers used could form complex with the DNA (Fig. 1). The lane of naked pDNA (lane of N/P ratio 0) revealed two bands corresponding to the supercoiled and open circular forms of the pDNA. In the lane of N/P ratio 1 of three polymers, a fraction of pDNA was still free to migrate in the gel. All of the three polymers showed pDNA retardation from N/P ratio 3, suggesting formation of complexes.

![Fig. 1 Agarose gel electrophoresis as the confirmation for complex formation at various N/P ratios. After preparation in 5% glucose, naked pDNA and complexes at various N/P ratios were electrophoresed and visualized by a UV illuminator. The lane of N/P ratio 0 represents naked pDNA.](image)

Particle size is an important factor which reflects the condensation status of complex and affects the gene transfer efficiency in vitro and in vivo [28]. Zeta potential can express the affinity of a particle to the negatively charged cell membrane in a certain degree. As to LPEIM, deacylation can change the conformation of linear PEI. Hence the condensation capability may also be changed. Therefore, we determined the particle sizes and zeta potential of the complexes at different N/P ratios (Table 1). At N/P ratio of 6, we failed to measure the particle size of LPEI complex because of severe aggregation. LPEIM complex was larger at N/P ratio 3 (diameter of 194.7 nm) than at N/P ratio 1, 6, 10, 20, 30 and 40 (diameters of 131.0-159.0 nm). BPEI complexes at N/P
ratio 1, 3 and 10 (diameters of 161.4-179.9 nm) were larger than at N/P ratio 20 and 40 (diameters of 129.2 and 126.2 nm). The BPEI complex showed negative zeta potential only at the N/P ratio 1, whereas LPEI and LPEIM complex showed negative zeta potentials at both N/P ratio 1 and 3. In cases of N/P ratios larger than 3, all of the detected complexes showed high positive charges (zeta potential of 45.0-53.1 mV). The zeta potential results may be due to the more primary amines in BPEI structure which make it easier to be protonated than the linear PEI.

Table 1 Particle sizes and zeta potentials of complexes in 5% glucose. Results are shown as mean ± S.D. of triplicates.

<table>
<thead>
<tr>
<th>N/P ratio</th>
<th>1</th>
<th>3</th>
<th>6</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Particle size (d. nm)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPEI</td>
<td>135.3±30.4</td>
<td>147.1±8.1</td>
<td>(Agg.)</td>
<td>135.3±3.7</td>
<td>163.6±3.4</td>
<td>(n.d.)</td>
<td>162.2±3.2</td>
</tr>
<tr>
<td>LPEIM</td>
<td>141.7±33.1</td>
<td>194.7±24.2</td>
<td>143.4±3.1</td>
<td>131.0±3.4</td>
<td>159.0±12.3</td>
<td>153.9±1.3</td>
<td>153.8±16.2</td>
</tr>
<tr>
<td>BPEI</td>
<td>179.9±1.9</td>
<td>169.2±5.1</td>
<td>(n.d.)</td>
<td>161.4±3.0</td>
<td>129.2±7.8</td>
<td>(n.d.)</td>
<td>126.2±4.3</td>
</tr>
<tr>
<td><strong>Zeta potential (mV)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPEI</td>
<td>-38.8±7.5</td>
<td>-25.1±4.2</td>
<td>(Agg.)</td>
<td>47.8±1.4</td>
<td>52.4±0.5</td>
<td>(n.d.)</td>
<td>53.1±1.9</td>
</tr>
<tr>
<td>LPEIX</td>
<td>-31.3±2.1</td>
<td>-19.6±1.1</td>
<td>45.6±1.5</td>
<td>46.7±0.5</td>
<td>46.4±1.4</td>
<td>45.0±0.4</td>
<td>45.0±1.0</td>
</tr>
<tr>
<td>BPEI</td>
<td>-19.8±2.0</td>
<td>30.7±0.4</td>
<td>(n.d.)</td>
<td>47.7±0.8</td>
<td>47.8±0.5</td>
<td>(n.d.)</td>
<td>46.7±0.5</td>
</tr>
</tbody>
</table>

a Aggregation. b Not determined.

3.2 Comparison of gene transfer between naked plasmid DNA and gene carriers after left lateral liver lobe surface instillation

3.2.1 Gene expression comparison in the administrated liver lobe

Following the instillation of naked pDNA to the surface of left lateral liver lobe in mice, we have already demonstrated the liver-selective gene transfections with high efficiency [14]. Because there are no reports about the gene transfer by gene carriers in the organ surface gene delivery system, we initially introduced the cationic gene carrier, PEI, into the organ surface gene transfer experiments. In preliminary experiments, a relatively low dose of 3 μg pDNA in 10 μl 5% glucose was suitable for complex preparation at various N/P ratios, and the gene expression levels were high enough to be detected. This dose was chosen as the instillation dose in most experiments. Ten
microliters of 5% glucose without pDNA was administered as a blank control. Six hours after instillation, luciferase activity in the tissues of administered liver lobe (AL), other liver lobes (OL), stomach, kidneys (left and right), spleen and diaphragm was determined. Because of background luminescence, $10^4$ RLU/g tissue was considered to be the detection limit of gene expression in this study.

The results of gene expression of naked pDNA and complexes in the AL were shown in Fig. 2, 3 and 4.

a) Gene transfection of LPEI complexes

Linear PEI is one of the most common transfection reagents in vivo and in vitro. In Fig. 2, however, the 25 kDa-LPEI did not show any improvement in the gene expression level than that of naked pDNA in AL. The lowest N/P ratio (N/P ratio 1) and the high N/P ratios (N/P ratio 20 and 40) showed relatively better results than the intermediate N/P ratios (N/P ratio 3 and 10). Additionally, we failed to get the complex at N/P ratio 6 of LPEI due to severe aggregation.

![Gene expression of naked pDNA and LPEI/pDNA complexes in the administered liver lobe after liver surface instillation in mice.](image)

**Fig. 2** Gene expression of naked pDNA and LPEI/pDNA complexes in the administered liver lobe after liver surface instillation in mice. pDNA dose was 3 μg/10 μl in 5% glucose solution. Luciferase activities were determined 6 h after instillation. Each bar represents the mean ± S.E. of at least 12 experiments. Statistic method was steel-test with naked pDNA as a control group. *$P < 0.05$, vs. naked pDNA.
b) Gene transfection of LPEIM complexes

With the removal of the residual N-acyl moieties from linear PEI, the LPEIM is reported to have a dramatically enhancement in gene delivery in vivo and in vitro by several orders of magnitude [24, 25]. Since the reports which utilized this commercial product are very limited, and the improvement of gene transfer after tail vein injection was specific in the lung of mice [24], in this research, we initially introduced this polyplex into the liver-targeted gene delivery system. In Fig. 3, LPEIM showed better results than other two polymers, and at the N/P ratio 20 and 30, there were significant improvements of gene expression in AL than naked pDNA. At the ratios other than 20 and 30, the gene expression levels were comparable with that of naked pDNA.

![Gene expression of naked pDNA and LPEIM/pDNA complexes in the administered liver lobe after liver surface instillation in mice. pDNA dose was 3 μg/10 μl in 5% glucose solution. Luciferase activities were determined 6 h after instillation. Each bar represents the mean ± S.E. of at least 12 experiments. Statistical method was steel-test with naked pDNA as a control group. **P < 0.01, vs. naked pDNA.](image)

**Fig. 3** Gene expression of naked pDNA and LPEIM/pDNA complexes in the administered liver lobe after liver surface instillation in mice. pDNA dose was 3 μg/10 μl in 5% glucose solution. Luciferase activities were determined 6 h after instillation. Each bar represents the mean ± S.E. of at least 12 experiments. Statistical method was steel-test with naked pDNA as a control group. **P < 0.01, vs. naked pDNA.


c) Gene transfection of BPEI complexes

The PEI with high branched level was reported as the gene vector earlier than the linear PEI [20]. The low molecular BPEI has lower cell toxicity [22]. In the present study (Fig. 4), however, similar with LPEI, the results of gene expression in AL from BPEI at various N/P ratios did not show any improvement compared with naked pDNA.
Fig. 4 Gene expression of naked pDNA and BPEI/pDNA complexes in the administered liver lobe after liver surface instillation in mice. pDNA dose was 3 μg/10 μl in 5% glucose solution. Luciferase activities were determined 6 h after instillation. Each bar represents the mean ± S.E. of at least 10 experiments.

3.2.2 Organ selectivity comparison

In our previous studies, the specific gene expression in liver was achieved after the instillation of naked pDNA in 30 μl solution onto the left lateral liver lobe in mice [14]. Recently, it is further demonstrated that decreasing the instillation volume to around 1 μl can dramatically improve the organ selectivity in mice or rats after microinstillation onto the gastric serosal surface [29]. In this research, however, because the complexes aggregated very easily in concentrated pDNA solution, and the pDNA with dose of less than 3 μg was difficult to be clearly detected, a relatively small volume of 10 μl was selected as a balance of gene expression level and instillation volume. We evaluated whether there is an improvement of liver selectivity after introducing gene carriers into this gene delivery system. Six hours after administration, simultaneously with instilled liver lobe, we determined the gene expression levels in various tissues in the abdominal cavity, which were described in section 2.7.1.

We compared the gene expression selectivity in these tissues using a relative selectivity value (R.S.), which is a relative luciferase activity in targeted tissue to the tissue under evaluation. The higher the R.S. value, the better the selectivity of target
organ to the selected organ is regarded. The R.S. value was calculated when the gene expression level was above the detection limit (10⁴ RLU/g tissue), otherwise it was considered to be undetectable. In liver surface instillation experiments, the R.S. value equals the ratio of gene expression level in the AL to the tissue under evaluation. The naked pDNA expression in different organs was shown in Fig. 5. Besides the high selectivity in AL, the gene expression was detectable in the OL, stomach, spleen and diaphragm.

![Luciferase activity (RLU/g tissue)](image)

**Fig. 5** Distribution of gene expression of naked pDNA in the liver (AL, OL), stomach, kidneys (left and right), spleen and diaphragm after liver surface instillation. pDNA dose was 3 μg/10 μl in 5% glucose solution. Luciferase activities were determined 6 h after instillation. The values in brackets nearby bars represent relative selectivity values (R.S.), which equal the ratios of gene expression level in the AL to those in the OL, stomach and diaphragm. Each bar represents the mean ± S.E. of 12 experiments.

Similar with naked pDNA, PEI complexes showed high selectivity in the AL. The R.S. values of the three types of complexes at various N/P ratios after instillation and determination were shown in **Fig. 6d, 7d** and **8d**. Including OL, stomach, spleen and diaphragm, the LPEI and LPEIM complexes also showed gene expressions in the kidneys (**Fig. 6** and **7**). As to the BPEI complexes, regardless of the relatively low gene expression in the AL, there was better organ selectivity to the target compared with naked pDNA and other two complexes. The distributions of gene expression of complexes at different N/P ratios were selected and showed as the ones with relatively
better gene expression level in the AL (LPEI: N/P ratio 1, 20, 40, LPEIM: N/P ratio 1, 20, 30 and BPEI: N/P ratio 1, 20, 40) in Fig. 6a-c, 7a-c and 8a-c, respectively. The better liver selectivity of BPEI complexes may be due to the branched molecular structure. The mechanism of the better liver selectivity of BPEI needs to be further clarified.

Fig. 6 Distributions of gene expression (a-c) and the R.S. values (d) of LPEI/pDNA complexes in the liver (AL, OL), stomach, kidneys (left and right), spleen and diaphragm after liver surface instillation. pDNA dose was 3 μg/10 μl in 5% glucose solution. Luciferase activities were determined 6 h after instillation. In (a-c), the values in brackets nearby bars represent R.S. values, which equal the ratios of gene expression level in the AL to the tissue under evaluation. Each bar represents the mean ± S.E. of at least 12 experiments. In (d), the N/P ratio of 0 represents naked pDNA.
**Fig. 7** Distributions of gene expression (a-c) and the R. S. values (d) of LPEIM/pDNA complexes in the liver (AL, OL), stomach, kidneys (left and right), spleen and diaphragm after liver surface instillation. pDNA dose was 3 μg/10 μl in 5% glucose solution. Luciferase activities were determined 6 h after instillation. In (a-c), the values in brackets nearby bars represent R.S. values, which equal the ratios of gene expression level in the AL to the tissue under evaluation. Each bar represents the mean ± S.E. of at least 12 experiments. In (d), the N/P ratio of 0 represents naked pDNA.
Fig. 8 Distributions of gene expression (a-c) and the R. S. values (d) of BPEI/pDNA complexes in the liver (AL, OL), stomach, kidneys (left and right), spleen and diaphragm after liver surface instillation. pDNA dose was 3 μg/10 μl in 5% glucose solution. Luciferase activities were determined 6 h after instillation. In (a-c), the values in brackets nearby bars represent R.S. values, which equal the ratios of gene expression level in the AL to the tissue under evaluation. Each bar represents the mean + S.E. of at least 10 experiments. In (d), the N/P ratio of 0 represents naked pDNA.

3.2.3 Comparison of gene expression in the administrated liver lobe: dose dependency and time course

Because of no need to concerning about the foreign material-degrading pathway in vivo and side effects caused by carrier, naked pDNA can be utilized at a high dose in our previous research [14-17]. Since the LPEIM showed a significant improvement of gene expression in target tissue at certain N/P ratios (20 and 30), we further purposed to evaluate the gene transfection of LPEIM in various pDNA doses. Gene expressions in AL were examined and compared between naked pDNA and LPEIM complex (N/P
ration 20) at different doses from 0.3 μg in 10 μl of 5% glucose (Fig. 9). The transgene expression level was improved by this complex in relatively low doses (0.3, 1, and 3 μg). With the dose increasing to 6 μg, however, the gene expression of the complex decreased. Moreover, the complex began to aggregate in 10 μg and could not be prepared in larger concentrations.

The gene expression of naked pCMV-luciferase pDNA peaked 6 h after instillation [14]. In this study, we performed time course experiments to detect whether the gene expression of LPEIM complex was longer than naked pDNA. We compared the performance of naked pDNA and LPEIM complex (N/P ratio 20) 6, 12, 24 and 48 h after instillation (Fig. 10). As a result, at the time points of 12, 24 and 48 h, there is no significant difference between naked pDNA and complex. In other words, the complex did not prolong the unstable luciferase expression time.
Time course of gene expression in the administered liver lobe after liver surface instillation of naked pDNA and LPEIM/pDNA complex (N/P ratio 20) in 10 μl of 5% glucose solution in mice. Luciferase activities were determined 6, 12, 24 and 48 h after instillation. Each value represents the mean ± S.E. of at least 6 experiments. Statistic method was Wilcoxon rank sum test. ***P < 0.001, complex vs. naked pDNA at the same time point.

3.2.4 Discussion

Since the characteristics of organ surface instillation, i.e., “direct contact with organ surface cells” and “no effect from blood”, are similar to in vitro condition, to some extent, this approach can be considered as an intermediate between the classical in vivo approach and in vitro cell culture. Some reports indicated that the behavior of complexes with different structures were different in vitro and in vivo as follows. For in vitro experiments [23, 30], larger PEI complex particles or aggregates which were formed at N/P ratio around 3 or in high ionic solution, such as saline, were more favorable than smaller particles. This might be due to the sedimentation effect [23, 30]. However, for the intravenous injection experiments, more condensed particles and an excess of polycation are essential for better PEI gene delivery performance [23]. In our results of complexes (Fig. 2, 3 and 4), the complexes at high N/P ratios (>10), which mean excess of PEI, showed better performance than those at intermediate N/P ratios. Additionally, in our research using the saline solution or aggregated complexes, there were nearly no detectable gene expressions of the PEI complexes (data not shown).
Thus, in liver surface instillation, smaller particles with excess polycation showed better transfection efficiency than larger particles and insufficient polycation. Consequently, the gene transfer efficiency in liver surface instillation has a resemblance to the intravenous application rather than in vitro condition.

However, when the transfection efficiency of complexes is compared with naked pDNA, this result is no longer similar with both the intravenous and the cell culture conditions. The gene expression levels were comparable with that of naked pDNA. In our previous research, it was demonstrated that pDNA is taken up and expressed by mesothelial cells on the liver surface [unpublished data] and macropinocytosis/phagocytosis plays a critical role [unpublished data]. In contrast, caveolae-mediated endocytosis was reported to play important role in the gene transfer of unmodified PEI complexes in several cell lines [31, 32]. PEI was reported to improve the gene expression by promoting the endosomal release according to the “proton sponge hypothesis” after cellular entry [20, 33]. Taken together, the comparable gene expression between naked pDNA and PEI complexes may be a result of similar efficiencies of different intracellular pathways. The improved transgene performance of LPEIM may be due to a better promoting effect on the endosomal release than the other two PEI moleculars.

Although the transgene expression of LPEIM at certain N/P ratios was significantly better than naked pDNA, the improved efficiency was limited in relatively low DNA doses. Because of the aggregations, the PEI complexes in this research were limited in the non-ionic solution (5% glucose) and the instillation volume could only be decreased to 10 μl, whereas naked pDNA could be utilized in various solution compositions [34] and the instillation volume could be very small (<1 μl), which would lead to a better organ targeting [29]. The duration of gene expression was also not prolonged by this carrier. Furthermore, taking the toxicity of PEI into account, it is implied that there was no significant improvement in gene delivery from the PEI complexes in the present
study. The naked pDNA showed comparable gene transfection efficiency and organ selectivity with those of PEI complexes.

3.3 Comparison of Gene transfer between naked plasmid DNA and gene carriers after intraportal injection

3.3.1 Gene expression comparison in the liver of mice

Injection via the portal vein is an often used liver-targeted method which is utilized in the experiments of liver cancer therapy and gene delivery [26, 27, 35]. In this part, we chose this improved intravascular injection approach as a positive control of the liver-targeted gene delivery system. The differences between the liver surface instillation and intraportal injection were aimed to be analyzed.

After preliminary experiments, we chose 30 μg of pDNA in 200 μl of 5% glucose as the administration dose. Two hundred microliters of 5% glucose solution without pDNA was administered as a blank control. LPEIM at N/P ratios of 1, 3, 10 and 20, LPEI and BPEI at N/P ratio of 20 were chosen as the complexes to be evaluated and compared with naked pDNA. Six hours after administration, luciferase activity in the tissues of liver, stomach, kidneys (left and right), spleen, lung and heart was determined. Because of background luminescence, $10^4$ RLU/g tissue was considered to be the detection limit of gene expression in this study. The results of gene expression of naked pDNA and complexes in the liver were shown in Fig. 11. Except for the LPEIM in N/P ratio 3 and branched PEI, naked pDNA and complexes had high gene expression in the liver. The results of naked pDNA and BPEI complex were consistent with the published reports [27, 35]. Similar with liver surface instillation, the LPEIM complex at N/P ratio 20 showed a significantly higher gene expression than that of naked pDNA.
Fig. 11 Gene expression in liver after intraportal injection of naked pDNA and PEI/pDNA complexes in mice. pDNA dose was 30 μg/200 μl in 5% glucose solution. Luciferase activities were determined 6 h after injection. Each bar represents the mean ± S.E. of at least 10 experiments. Statistic method was steel-test with naked pDNA as control group. **P < 0.01, vs. naked pDNA.

3.3.2 Gene transfer distribution comparison

Intraportal injection of naked pDNA had better liver distribution than intravenous injection [26]. In intraportal injection experiments, we calculated the R.S. value as the ratio of gene expression level in the liver to the tissue under evaluation. We separately determined the gene expression in left and right kidney (Fig. 12). The gene expression was high in kidneys with R.S. values were 0.2 and 1.6 for left and right kidney, respectively. With considerable interest, there is a significant inter-kidney difference, i.e., a higher gene expression in the left kidney than that in the right kidney.
**Fig. 12** Distribution of gene expression in mice after intraportal injection of 30 μg/200 μl naked pDNA in 5% glucose solution. Luciferase activities were determined 6 h after injection. The values in brackets nearby bars represent relative selectivity values (R.S.), which equal the ratios of gene expression level in the liver to that in the left and right kidney. Each bar represents the mean ± S.E. of 14 experiments.
**Fig. 13** Distributions of gene expression after intraportal injection of PEI/pDNA complexes in mice. pDNA dose was 30 μg/200 μl in 5% glucose solution. Luciferase activities were determined 6 h after injection. The values in brackets nearby bars represent relative selectivity values (R.S.), which equal the ratios of gene expression level in the liver to the organ under evaluation. Each bar represents the mean + S.E. of at least 10 experiments.

Distribution of gene expression of LPEIM at N/P ratio 1 after intraportal injection complexes (**Fig. 13a**) showed R.S. values of 0.1 and 1.1 for left and right kidney, respectively, and the distribution was similar with that of naked pDNA. This may be due to the pDNA not covered into the complex. Distributions of gene expression of other complexes (**Fig. 13b-f**), however, were very different with naked pDNA. Accompanied by the increased expression in the liver (LPEIM N/P 10 and 20), the gene
expression was considerably high in several tissues such as lung, spleen and kidneys.

![Graph showing gene expression data](image)

**Fig. 14** Gene expression in kidneys after intraportal injection of naked pDNA and PEI/pDNA complexes in mice. pDNA dose was 30 μg/200 μl in 5% glucose solution. Luciferase activities were determined 6 h after injection. The values in brackets above bars of right kidneys represent ratios of gene expression level in the left kidney to the right kidney (LK/RK). Each bar represents the mean ± S.E. of at least 10 experiments. Statistic method was Wilcoxon rank sum test. *****P < 0.001, left kidney vs. right kidney.

To further explain the selectivity of gene expression in kidneys, we replotted the kidney data together in **Fig. 14** and compared statistically. The ratio of gene expression level in the left kidney to the right kidney (LK/RK) was used to show the gene expression differences between kidneys. High gene expressions in kidneys were observed in naked pDNA and LPEIM complexes at N/P ratio 1 and 20. The gene expression of naked pDNA in left kidney was significantly higher than the right kidney (LK/RK 6.5). This left kidney-selective gene expression was also observed in LPEIM at N/P ratio 1 (LK/RK 8.7). In the complexes of LPEIM at N/P ratio 3 and 10, LPEI and BPEI at N/P ratio 20, relatively low gene expressions in kidneys were observed with no significant side kidney-selective gene expression.
3.3.3 Discussion

In the intraportal injection experiments, similar with liver surface instillation, naked pDNA and PEI complexes showed a comparable gene delivery performance in the liver. However, the organ distribution characteristics were very different between naked pDNA and PEI complexes. For the first time, we observed a left kidney-selective gene expression from naked pDNA, and the mechanism is of large interest. It was possible that this single kidney-favorable distribution was due to operation procedure, especially a leakage during injection. Therefore, to clarify whether there was a leakage during injection, we administered the naked pDNA of pZsGreen1-N1 (Takara Bio, Shiga, Japan), which encodes reef coral fluorescent protein, by intraportal injection in mice. Twenty-four hours after injection, we observed if the gene expression was on the surface of the kidney or not using a fluorescence stereo microscopy. As a result, the gene expression was detectable and much higher in left kidney than the right kidney. However, most signals were detected below the surface layer of left kidney (data not shown). Consequently, this selective gene expression was not from leakage. More experiments, such as gene expressing cell-type identification, are needed to clarify the mechanism of this phenomenon.

With the removal of the residual N-acyl moieties from linear PEI, the LPEIM/pDNA complex is reported to have an enhancement in gene delivery in the lung of mice in vivo [24]. Other than the lung, the present research demonstrated a good gene expression performance in the liver by LPEIM complex via not only liver surface, but also the portal vein.

In liver surface instillation experiments, the pDNA dose was 3 μg, and the gene expression levels of the naked pDNA and LPEIM complex (N/P 20) were $1.2 \times 10^5$ and $7.4 \times 10^5$ RLU/g tissue, respectively. In intraportal injection experiments, the pDNA dose was 30 μg, and the gene expression levels of naked pDNA and LPEIM complex (N/P 20) were $8.8 \times 10^5$ and $1.1 \times 10^7$ RLU/g tissue, respectively. When comparing this data, the
gene expression levels per dose in two systems are similar. The intraportal injection is an intravascular approach and the transgene might be expressed in the whole liver. On the other hand, the transgene-expressing cells might be limited in the surface layer in the liver surface instillation experiments. Consequently, the density of transgene expression in the surface layer of the liver would be higher than that in intraportal injection experiments, suggesting high effectiveness of liver surface instillation.

After the liver surface instillation of naked pDNA in the present research, although gene expressions in several organs in the abdominal cavity were detectable (Fig. 5), the targeting could be controlled and dramatically improved by decreasing the administration volume [29]. After intraportal injection, however, without any carriers, the naked pDNA was expressed not only in the liver, but also in kidneys (Fig. 12). Taken all together, the targeting of liver surface instillation system is better than the intraportal injection system.
4 Conclusion

In this study, I introduced cationic gene carrier PEI into two gene delivery systems and evaluated the gene transfer efficiency and target selectivity by comparison with naked pDNA. In liver surface instillation, only the LPEIM showed better transgene expression than naked pDNA at certain N/P ratios and relatively low pDNA doses. The duration of gene expression was not prolonged compared with naked pDNA. A similar result was obtained from intraportal injection experiments, in which only the LPEIM at N/P ratio 20 showed a significant better transgene efficiency compared with naked pDNA. Consequently, in both of the liver surface instillation and intraportal injection experiments, the transgene expression levels of complexes in liver were comparable with that of naked pDNA.

The distributions of gene expression of naked pDNA and complexes in the liver surface instillation were comparable, whereas those in intraportal injection were very different. In the liver surface instillation experiments, the distributions of gene expression of BPEI complexes showed better liver selectivity than that of naked pDNA. However, because complexes aggregate easily in concentrated solution, we could not improve the site selectivity by decreasing the instillation volume. After intraportal injection, the gene expression of complexes was detectable in several tissues such as lung, spleen and kidneys. On the other hand, naked pDNA showed high gene expression only in the kidneys, and the gene expression in left kidney was significantly higher than the right kidney. The mechanism of the left kidney-selective gene expression needs to be clarified in the future.

The evaluation and comparison of naked pDNA and PEI complexes in this research provided useful information for better use of naked pDNA and PEI complexes in further research and for designing novel gene carriers in vivo.
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