# Effect of Solution Composition of Plasmid DNA on Gene Transfection Following Liver Surface Administration in Mice

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**We investigated the effect of plasmid DNA (pDNA) solution composition on gene transfection following liver surface administration in mice. Gene transfection experiments *in situ* and *in vivo* were performed using the following pDNA solutions: dextrose solution, NaCl solution, phosphate buffer, phosphate-buffered saline, Tris/HCl buffer with EDTA, Tris/HCl buffer with EDTA and Triton X-100, and water. In *in situ* experiments, we used a glass cylindrical diffusion cell that limited the contact area between the liver surface and the naked pDNA solution. The gene transfection at the site of diffusion cell attachment increased in hypotonic solution, and decreased in hypertonic solution, compared with isotonic solution. In *in vivo* experiments, instillation of naked pDNA solution onto the liver surface using a micropipette caused no significant differences in gene transfection in the applied lobe. These results suggest that it is important to select the optimal pDNA solution composition to control the gene transfection.**

**Key words** gene therapy; solution composition; osmotic pressure; plasmid DNA; liver; mouse

Gene therapy is an attractive method for the treatment of various diseases.[1—9] Lately, its range of application has spread to acute diseases, traumas, and dental treatment.[10—13] Worldwide progress in gene therapy needs the establishment of efficient, simple, safe, and low cost gene transfection techniques. At present, approximately 70% of clinical gene therapy uses viral vectors.[14] These vectors have high transfection efficiencies and/or long-term expression of transgenes.[15,16] However, some patients have suffered from adverse effects, including death and leukemia, because of the dosage of the therapeutic viral vectors used.[17—19] Sufficiently safe use of viral vectors for clinical gene therapy is not yet possible. On the other hand, nonviral vectors are safer than viral vectors.[20] Several gene carriers have been developed to obtain high efficiency nonviral gene transfection.[21—26] Generally, nonviral vector gene transfection procedures use naked plasmid DNA (pDNA). The procedures are simple and low cost, since naked pDNA solution is prepared without complex formation with various gene carriers. Furthermore, the dosage of naked pDNA is safer than that of the pDNA-gene carrier complex, as it is not associated with immunogenicity or cytotoxicity derived from the gene carrier.[27,28] Hence, overcoming low transfection efficiency is extremely important for gene therapy using naked pDNA.

When naked pDNA was administered in the vascular system, it was impossible to achieve effective gene expression in the targeted tissue due to clearance of the pDNA from the systemic circulation by reticuloendothelial cells (liver Kupffer cells, *etc.*) and degradation of the pDNA by nucleases in the blood.[29] To avoid these problems, we developed a method for instilling naked pDNA solution onto the surface of the liver or kidney, and found it resulted in effective organ- and site-selective gene expression.[30—32] This method has the advantage of being noninvasive for the organ, compared with several naked pDNA transfer methods which utilize physical force.[33—38] Moreover, we reported that liver-specific gene expression was achieved by limiting the contact area between the pDNA solution and the liver surface.[39]

Organ surface administration of pDNA will most likely be applied in the treatment of various diseases. It is important to control the level of gene expression for clinical gene therapy as well as disease site-specific gene transfection. In this study, we investigated the effect of pDNA solution composition on gene transfection following liver surface administration in mice.

## MATERIALS AND METHODS

**Materials** All chemicals were purchased from Nacalai Tesque, Inc. (Kyoto, Japan) and were of the highest purity available.

**Animals** Male ddY mice were housed in cages 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 procedures in the present study conformed to the Guidelines for Animal Experimentation of Nagasaki University.

**Construction and Preparation of pDNA** pCMV-luciferase was constructed by subcloning the *Hind*III/*Xba*I firefly luciferase cDNA fragment from a pGL3-control vector (Promega, Madison, WI, U.S.A.) into the polylinker of a pcDNA3 vector (Invitrogen, Carlsbad, CA, U.S.A.). pDNA was amplified in the *Escherichia coli* strain DH5$\alpha$, isolated, and purified using a EndoFree® Plasmid Giga Kit (QIAGEN GmbH, Hilden, Germany).

**Preparation of pDNA Solution** For solutions of pDNA, dextrose solution, NaCl solution, phosphate buffer (PB), phosphate-buffered saline (PBS), TE, TET, and water were used. The compositions of these solutions are shown in Table 1. Dextrose solution is a typical nonionic solution, and NaCl solution, PB, and PBS are typical ionic solutions. TE is a Tris/HCl buffer with EDTA, which has antinuclease activity. TET is a Tris/HCl buffer with EDTA and Triton X-100. Triton X-100 is a strong surfactant and causes membrane destabilization. Water (distilled $H_2O$) was used as a simple solvent. pDNA dissolved in these solutions (1 $\mu$g/$\mu$l) was stored

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Table 1. Solution Composition of pDNA

| Solution | Composition |
|---|---|
| Dextrose | 0.5%, 1%, 5%, 25%, 50% dextrose (w/v) |
| NaCl | 0.09%, 0.9%, 9% NaCl (w/v) |
| PB | Phosphate buffer of 0.15 M $NaH_2PO_4$/0.15 M $Na_2HPO_4$ pH 5.4, 6.4, 7.4, 8.4, 9.2 |
| PBS | Equal mixture (v/v) of PB (pH7.4) and 0.9% NaCl |
| TE | 0.01 M Tris/HCl buffer with 1 mM EDTA |
| TET | 0.1 M Tris/HCl buffer with 2 mM EDTA and 0.05% Triton X-100 (w/v) |
| Water | Distilled $H_2O$ |

at −20 °C prior to the experiments.

***In Situ* Gene Transfection Experiments** Five-week-old male ddY mice (22.0—35.0 g) were anesthetized with sodium pentobarbital (40—60 mg/kg, intraperitoneal injection). The central peritoneum was dissected with a cut of approximately 2 cm and a glass cylindrical diffusion cell (i.d. 6 mm, effective area 28 $mm^2$) was attached to the surface of the left lateral lobe of the liver with a thin film of surgical adhesive (Aron Alpha®, Sankyo Co., Ltd., Tokyo, Japan) (Fig. 1A). Naked pDNA solution (30 $\mu$g/30 $\mu$l) was added directly to the diffusion cell. The top of the diffusion cell was sealed with a piece of aluminum foil to prevent evaporation of the pDNA solution. After 10 min, the pDNA solution was removed from the diffusion cell and the liver surface within the diffusion cell was washed with solution (500 $\mu$l) five times. After the diffusion cell was removed from the liver surface, the peritoneum was sutured. Mice were kept lying in a supine position for 1 h and then freed into the cage. After 6 h, the mice were sacrificed, and the liver, kidney, spleen, heart, and lung were removed. To evaluate the intrahepatic distribution of gene expression, the liver, after freezing with liquid nitrogen, was divided into three sections. Site 1, site 2, and site 3 are sites of diffusion cell attachment (1 cm×1 cm), left lateral lobe excluding site 1, and other lobes, respectively. The tissue was washed twice with saline and homogenized with a lysis buffer, which consisted of 0.1 M Tris/HCl buffer (pH 7.8) containing 0.05% Triton X-100 and 2 mM EDTA. The volumes of the lysis buffer added were 4 $\mu$l/mg for liver sites and 5 $\mu$l/mg for other tissues. After three cycles of freezing and thawing, the homogenates were centrifuged at 15610×***g*** for 5 min. The supernatants were stored at −20 °C prior to the luciferase assays. Twenty microliters of supernatant was mixed with 100 $\mu$l of luciferase assay buffer (Picagene®, Toyo Ink Mfg. Co., Ltd., Tokyo, Japan) and the light produced was immediately measured using a luminometer (MiniLumat LB 9506, BERTHOLD TECHNOLOGIES, Bad Wildbad, Germany). Luciferase activity is expressed as relative light units (RLU) per gram of tissue.

***In Vivo* Gene Transfection Experiments** Five-week-old ddY male mice (22.0—35.0 g) were anesthetized with sodium pentobarbital (40—60 mg/kg, intraperitoneal injection). The central peritoneum was dissected with a cut of approximately 1 cm and the liver exposed. Naked pDNA solution (30 $\mu$g/30 $\mu$l) was then instilled onto the surface of the left lateral lobe of the liver using a micropipette (PIPETMAN®, GILSON, Inc., Villiers-le-Bel, France) (Fig. 1B). After 1 min, the peritoneum was sutured. Mice were kept lying in a supine position for 1 h and then freed into the cage. After 6 h, the mice were sacrificed and the liver removed. The applied lobe (left lateral lobe) was then separated from the other lobes. The subsequent steps were the same as those performed in the *in situ* gene transfection experiments.

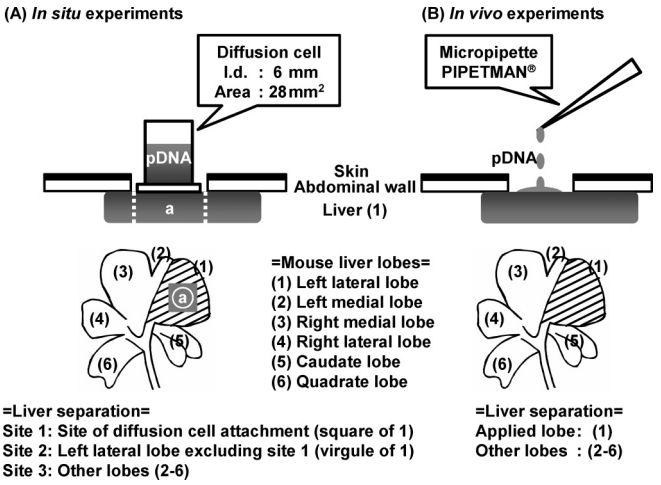


Fig. 1. Experimental Procedure

(A) The glass cylindrical diffusion cell was attached to the left lateral lobe of the liver (circle a) of mice. Naked pDNA (30 $\mu$g/30 $\mu$l) was administered directly into the diffusion cell, and the luciferase activity was measured at site 1, site 2, and site 3. (B) Naked pDNA (30 $\mu$g/30 $\mu$l) was administered onto the surface of the left lateral lobe in the liver using a micropipette, and luciferase activity was measured in the applied lobe and other lobes.

**Statistical Analysis** The Steel–Dwass test was performed to determine the significance of differences. Differences with a value of $p<0.05$ were considered significant.

## RESULTS AND DISCUSSION

**Effect of pDNA Solution on Gene Transfection *in Situ*** To investigate the effect of pDNA solution composition on gene transfection, we examined the *in situ* experiments that used a glass cylindrical diffusion cell that was able to limit the contact area between the liver surface and naked pDNA solution administered in mice. This system enabled us to examine gene uptake from the liver surface without any interference caused by uptake from other organs. We previously reported that liver site-specific gene expression was achieved by the liver surface administration of pDNA solution using a diffusion cell.[39]

Five percent dextrose solution, 0.9% NaCl solution, PB (pH 7.4), PBS, TE, TET, and water were used as solutions for pDNA. Dextrose solution is a typical nonionic solution, and NaCl solution, PB, and PBS are typical ionic solutions. TE has antinuclease activity due to EDTA. TET has antinuclease activity and a membrane destabilizing effect due to EDTA and Triton X-100, respectively. Water was used as a simple solvent. In a previous report,[30] maximum gene transfection in the applied liver lobe following the instillation of pDNA to the liver surface in mice was observed at 6 h. Therefore, the luciferase activity at site 1 was measured 6 h after liver surface administration of pDNA for 10 min using a diffusion cell. No stable gene expression at site 2, site 3, kidney, spleen, heart, or lung was observed in these experiments (data not shown). Figure 2A shows the effect of the different pDNA solutions on gene transfection. Gene transfection in

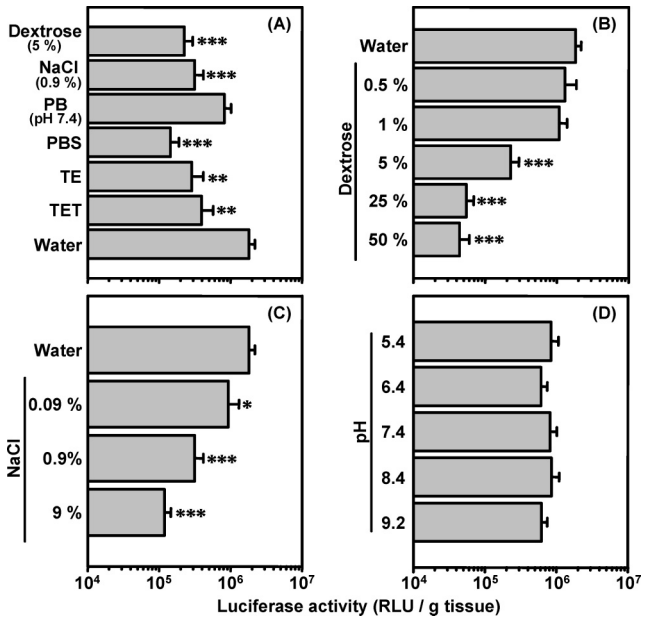


Fig. 2. Effect of pDNA Solution (A), Dextrose Concentration (B), NaCl Concentration (C), and pH (D) on Gene Transfection

pDNA solution (30 $\mu$g/30 $\mu$l) was administered onto the liver surface using the diffusion cell for 10 min. The luciferase activity at site 1 was measured 6 h after administration of pDNA solution. Statistical comparisons were performed using the Steel–Dwass test (* $p<0.05$, ** $p<0.01$, *** $p<0.001$ *vs.* water). Each bar represents the mean ± S.E. of at least ten experiments.

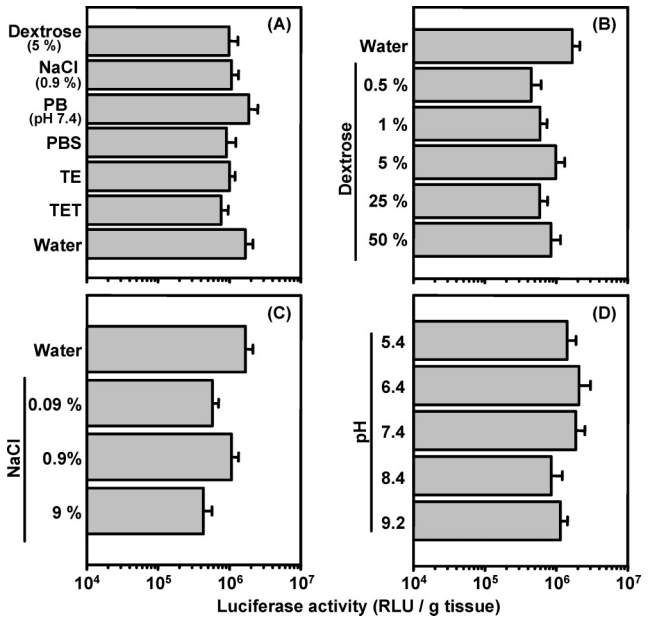


Fig. 3. Effect of pDNA Solution (A), Dextrose Concentration (B), NaCl Concentration (C), and pH (D) on Gene Transfection

pDNA solution (30 $\mu$g/30 $\mu$l) was instilled onto the liver surface using a micropipette. The luciferase activity in the applied lobe was measured 6 h after instillation of pDNA solution. Each bar represents the mean ± S.E. of at least ten experiments.

the case of water was significantly higher than that of the other solutions except for PB.

**Effects of Dextrose and NaCl Concentrations on Gene Transfection *in Situ*** We examined the effects of the concentrations of dextrose and NaCl on gene transfection. Both dextrose solution (Fig. 2B) and NaCl solution (Fig. 2C) decreased gene transfection and gene transfection decreased with an increasing concentration of dextrose or NaCl. The gene transfection at site 1 increased in hypotonic solution, and decreased in hypertonic solution, compared with isotonic solution.

For peritoneal dialysis, water flow from the systemic circulation to the peritoneal cavity occurs due to the presence of hypertonic dextrose solution.[40] This phenomenon is applied in the removal of extra water from dialysis patients. The direction of water flow can be reversed by introducing a hypotonic solution into the peritoneal cavity. Moreover, it is well known that the environment surrounding cells affects ultrastructures inside them because of osmotic pressure and the transition of water.[41] Taking these results into consideration, water flow from the liver surface to blood vessels or cytoplasm might have occurred after the administration of a hypotonic solution onto the liver surface. However, the penetration of water and small solute molecules into blood vessels is thought to be dominated by the three-pore theory.[42—44] pDNA is, therefore, not fully able to penetrate the systemic circulation, given that pDNA (pCMV-luciferase) is a macromolecule with a molecular weight of approximately 4.7 MDa.

**Effect of Solution pH on Gene Transfection *in Situ*** Figure 2D shows the effect of solution pH on gene transfection. The pH of PB was adjusted from 5.4 to 9.2 using a 0.15 M $NaH_2PO_4$/0.15 M $Na_2HPO_4$ buffer system. Gene transfection did not change due to the pH of the pDNA solution.

**Effect of pDNA Solution on Gene Transfection *in Vivo*** We previously reported that liver site-selective gene expression was achieved by liver surface instillation of pDNA solution using a micropipette.[30] The effect of pDNA solution composition on gene transfection following liver surface instillation of pDNA solution using a micropipette was examined. The luciferase activity in the applied lobe was measured 6 h after liver surface instillation of pDNA solution. Figure 3 shows the effect of different pDNA solutions (A), dextrose concentration (B), NaCl concentration (C), and pH (D) on gene transfection. In *in vivo* gene transfection experiments, no significant differences were observed in gene transfection, possibly due to the dilution of pDNA solutions by peritoneal fluid. The volume of peritoneal fluid in mice is 50—60 $\mu$l,[45] so 30 $\mu$l of pDNA solution administered into the peritoneal cavity was diluted approximately 3-fold. We previously reported that continuous microinstillation of a drug solution onto the rat liver surface was effective for delivering the drug to the liver.[46] Hydrogel, collagen sheets, and other methods have also been developed for the dosage form of pDNA.[47—51] Further studies are needed so the pDNA can be delivered to the liver specifically by developing a dosage form and administration device. We are currently performing experiments to obtain specific transfection following continuous microinstillation of pDNA onto the surface of mouse liver.

In conclusion, significant differences in gene transfection in *in situ* experiments were observed by altering the composition or osmotic pressure in pDNA solutions. In *in vivo* experiments, however, no change in pDNA solution composition that affected gene transfection was observed. These results suggest that it is important to select the optimal pDNA solution composition in order to control gene transfection. Additional studies are required to clarify the mechanism of

the effect of composition or osmotic pressure in pDNA solutions on gene transfection.

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