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Biodegradable nanoparticles composed of dendrigraft poly-L-lysine for gene delivery

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Abstract

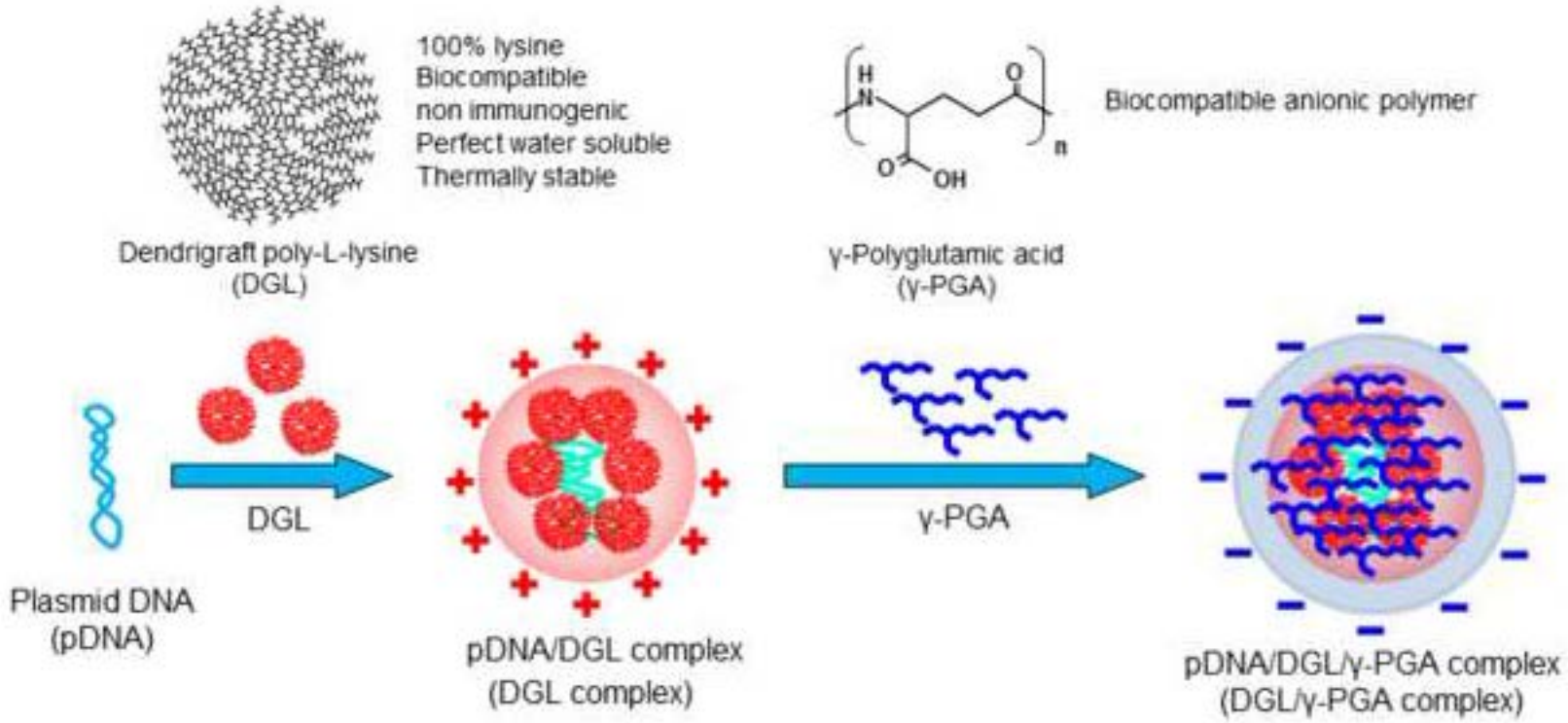
We developed novel gene vectors composed of dendrigraft poly-L-lysine (DGL). The transgene expression efficiency of the pDNA/DGL complexes (DGL complexes) was markedly higher than that of the control pDNA/poly-L-lysine complex. However, the DGL complexes caused cytotoxicity and erythrocyte agglutination at high doses. Therefore, γ-polyglutamic acid (γ-PGA), which is a biodegradable anionic polymer, was added to the DGL complexes to decrease their toxicity. The resultant ternary complexes (DGL/γ-PGA complexes) were shown to be stable nanoparticles, and those with γ-PGA to pDNA charge ratios of >8 had anionic surface charges. The transgene expression efficiency of the DGL/γ-PGA complexes was similar to that of the DGL complexes; however, they exhibited lower cytotoxicity and did not induce erythrocyte agglutination at high doses. After being intravenously administered to mice, the DGL6 complex demonstrated high transfection efficiency in the liver, lungs, and spleen, whereas the DGL6/γ-PGA8 complex only displayed high transfection efficiency in the spleen. Future studies should examine the utility of DGL and DGL/γ-PGA complexes for clinical gene therapy.

Abbreviations: DGL, dendrigraft poly-L-lysine; γ-PGA, γ-polyglutamic acid; PAMAM,

polyamidoamine; PLL, poly-L-lysine; WST-1, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt; 1-methoxy PMS, 1-methoxy-5-methylphenazinium methyl sulfate; DMSO, dimethyl sulfoxide; Rh, rhodamine B isothiocyanate; EDTA, ethylenediaminetetraacetic acid; RLU, relative light units; CPZ, chlorpromazine

Keywords: dendrigraft poly-L-lysine; biodegradable; gene delivery; γ-polyglutamic acid; nanoparticle

Graphical Abstract



1. Introduction

In gene delivery, non-viral vectors, including cationic polymers, have several advantages, e.g., they are non-immunogenic; cause few acute toxicities; and their structural and chemical properties can be tightly controlled, which allows vehicles that are suitable for mass production to be designed [1,2]. Among non-viral vectors, cationic dendrimers are a new class of polymeric vectors in which hyperbranched macromolecules composed of layers of monomer units radiate from a central core. Dendrimers are monodispersed, stable, and of a defined size and are characterized by the presence of numerous ionizable terminal groups, which means that they can efficiently bind to a large amount of genetic material. In addition, the electrostatic compaction of DNA by dendrimers was found to protect it from degradation by nucleases and to enhance the cellular uptake of dendrimer-based DNA-containing particles via adsorptive endocytosis or phagocytosis. Polyamidoamine (PAMAM) dendrimers are commercially available macromolecules that can be used as carriers for plasmid DNA, antisense oligonucleotides, and siRNA [5-8]. However, PAMAM dendrimers are not biodegradable and exhibit strong cytotoxicity [9,10].

Therefore, we examined the utility of dendrigraft poly-L-lysine (DGL) polymers, a recently discovered subset of biodegradable dendritic polymers, which are usually

synthesized by protect/deprotect or activation schemes, as DNA vectors [11]. Although the conventional dendritic poly-L-lysine compounds used for gene delivery are usually prepared from a non-biodegradable core, e.g., hexamethylenediamine or silsesquioxane cores [12-16], DGL, including its central core, consists entirely of lysine, and hence, is completely biodegradable. In addition, DGL is water-soluble, thermally stable, and non-immunogenic. In the present study, we developed a novel gene vector composed of DGL and investigated its efficacy and safety *in vitro* and *in vivo*. During the production of cationic dendrimer molecules, each complete grafting cycle; i.e., the formation of each layer, is referred to as a generation [3,4]. In a previous study examining 1st to 7th generation lysine dendrimers, the 5th and 6th generation lysine dendrimers were found to form stable complexes with pDNA and to have markedly higher gene transfection abilities than the other dendrimers [16]. We used 5th generation DGL polymers in this study.

Unfortunately, it has been found that DGL vectors can induce toxicities due to their cationic properties. However, the cytotoxic and blood component agglutination-inducing effects of cationic vectors such as DGL vectors can be countered by incorporating them in ternary complexes, which neutralizes their cationic charges. In fact, in a previous study we found that coating cationic complexes with a biodegradable

anionic polymer, γ-polyglutamic acid (γ-PGA), decreased their toxicity, without reducing their transgene expression efficiency [17]. Furthermore, the latter ternary complexes were constructed using a pharmaceutical, rather than a chemical, approach, which has several benefits, e.g., it makes manufacturing and sterilization easier. In this study, we also developed γ-PGA-coated DGL vectors in an attempt to decrease the toxicity of DGL vectors.

2. Materials and methods

2.1. Chemicals

Poly-L-lysine compounds (PLL) (mean molecular weight: 22,500) were obtained from Sigma Chemical Co. (St. Louis, MO, USA), and 5th generation DGL compounds (MW: 172,300 Da, 963 lysine groups) were purchased from COLCOM S.A.S. (Montpellier, France). γ-PGA was provided by Yakult Pharmaceutical Industry Co., Ltd. (Tokyo, Japan). Bovine serum albumin (BSA) was purchased from Sigma Aldrich (St. Louis, MO, U.S.A.), and fetal bovine serum (FBS) was purchased from Biological Industries Ltd. (Kibbutz Beit Haemek, Israel). RPMI 1640, Opti-MEM I, antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin), and the other culture reagents were obtained from GIBCO BRL (Grand Island, NY, USA). 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt (WST-1), and 1-methoxy-5-methylphenazinium methyl sulfate (1-methoxy PMS) were purchased from Dojindo Laboratories (Kumamoto, Japan). Rhodamine-PLL (Rh-PLL) and rhodamine-DGL (Rh-DGL) were prepared in our laboratory. Briefly, PLL or DGL and rhodamine B isothiocyanate were dissolved in dimethyl sulfoxide (DMSO) and stirred overnight at room temperature in the dark. The resultant Rh–PLL and Rh-DGL were purified by gel filtration. Almost 1.5% of the PLL and DGL

molecules were labeled with rhodamine B. All other chemicals were of reagent grade.

2.2 Preparation of pDNA, binary complexes, and ternary complexes

pCMV-Luc was constructed by subcloning the Hind III/Xba I firefly luciferase cDNA fragment from the pGL3-control vector (Promega, Madison, WI, USA) into the polylinker of the pcDNA3 vector (Invitrogen, Carlsbad, CA, USA). The resultant pDNA was amplified using an EndoFree Plasmid Giga Kit (QIAGEN GmbH, Hilden, Germany), before being dissolved in 5% dextrose solution and stored at -80 °C until the analysis. The pDNA concentration of the solution was assessed by measuring the absorbance of the solution at 260 nm and adjusted to 1 mg/mL.

To prepare the binary complexes, pDNA solution and PLL solution or DGL solution (pH 7.4) were mixed by thorough pipetting, before being left for 20 min at room temperature. PLL, which like DGL is entirely composed of lysine, was used as a control vector. The charge ratio of the pDNA/PLL complex (PLL complex) was 1:8, whereas pDNA/DGL complexes with charge ratios of 1:2, 4, 6, 8, or 10 (the DGL2, 4, 6, 8, or 10 complexes) were produced. To construct the ternary complexes, γ-PGA was mixed with the DGL6 complex by pipetting to produce complexes with charge ratios of 1:6:2, 1:6:4, 1:6:6, 1:6:8, 1:6:10, and 1:6:12 (the DGL6/PGA2, 4, 6, 8, 10, and 12 complexes),

which were then left for another 20 min at room temperature.

2.3. Physicochemical properties of the complexes

The particle sizes and ζ-potentials of each complex were measured using a Zetasizer Nano ZS (Malvern Instruments, Ltd., Malvern, United Kingdom). Particle size is shown as the number-weighted mean diameter.

To assess complex formation, 20-μL aliquots of each complex solution containing 1 μg pDNA were mixed with 4 μL loading buffer (30% glycerol and 0.2% bromophenol blue) and loaded onto 0.8% agarose gel. Electrophoresis (i-Mupid J; Cosmo Bio, Tokyo, Japan) was carried out at 50 V in running buffer solution (40 mM Tris/HCl, 40 mM acetic acid, and 1 mM ethylenediaminetetraacetic acid (EDTA)) for 60 min, and pDNA retardation was visualized with ethidium bromide staining.

2.4. In vitro gene expression and cellular uptake experiments

B16-F10 cells, a mouse melanoma cell line, were obtained from the Cell Resource Center for Biomedical Research (Tohoku University, Japan). The cells were maintained in RPMI 1640 supplemented with 10% FBS and antibiotics (culture medium) in a humidified atmosphere of 5% CO_2 in air at 37 °C, before being plated on

24-well plates (Becton-Dickinson, Franklin Lakes, NJ, USA) at a density of 1.0×10^4 cells/well and cultivated in 500 µL culture medium. In the transfection experiment, the medium was replaced with 500 µL Opti-MEM I medium after 24 h pre-incubation, and then the cells were treated with each complex (containing 1 µg pCMV-Luc) and incubated for 2 h. After the cells had been transfected, the Opti-MEM I medium was replaced with culture medium, and the cells were cultured for a further 22 h in a humidified atmosphere of 5% CO_2 in air at 37 °C. After 22 h incubation, the cells were washed with phosphate-buffered saline (PBS) and then lysed in 100 µL lysis buffer (pH 7.8; 0.1 M Tris/HCl buffer containing 0.05% Triton X-100 and 2 mM EDTA). Ten-microliter lysate samples were mixed with 50 µL of the luciferase assay buffer (PicaGene; Toyo Ink, Tokyo, Japan), and the amount of fluorescence produced was immediately measured using a luminometer (Lumat LB 9507; EG & G Berthold, Bad Wildbad, Germany). The protein content of the lysate was determined in a Bradford assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA) in which BSA was used as a standard and absorbance was measured using a microplate reader (Sunrise RC-R; Tecan Japan Co., Ltd., Kanagawa, Japan) at 595 nm. Luciferase activity is shown as relative light units (RLU) per mg protein.

To visualize the uptake of the complexes, B16-F10 cells were transfected with

complexes composed of pCMV-Luc and Rh-PLL or Rh-DGL, as described above. After 22 h incubation, the relative levels of Rh-PLL and Rh-DGL in the cells were characterized using fluorescence microscopy (200 × magnification).

2.5. pH titration of PLL complex, DGL complex, and DGL/PGA complex

The PLL, DGL, or DGL/PGA complex was titrated using 2N sodium hydroxide (NaOH). Each solution had its pH adjusted to 3.0, and then the solutions were titrated with 2N NaOH. The resultant pH changes were recorded using a pH meter (LAQUA; HORIBA Ltd., Kyoto, Japan) and plotted to allow the buffering capacities of each complex to be compared.

2.6. WST-1 assay

Tests examining the cytotoxic effects of the various complexes on B16-F10 cells were carried out using a WST-1-based commercially available cell proliferation reagent. The reagent was prepared (5 mM WST-1 and 0.2 mM 1-methoxy PMS in PBS) and filtered through a 0.22 μm filter (Millex-GP; Millipore Co, Bedford, MA, USA) just before the experiments. B16-F10 cells were plated on 96-well plates (Becton-Dickinson) at a density of 3.0×10^3 cells/well in culture medium. Complexes

containing 1 μg pDNA in 100 μL Opti-MEM I medium were added to each well and incubated for 2 h. Then, the medium was replaced with 100 μL culture medium and incubated for another 22 h at 37 °C. The medium was then substituted for 100 μL culture medium, and 10 μL of the WST-1 reagent were added to each well. The cells were incubated for an additional 2 h at 37 °C, and the absorbance of each well was measured at a wavelength of 450 nm (reference wavelength: 630 nm) using a microplate reader. The results are shown as percentages of the value for the untreated cells.

2.7. Inhibition study

To determine the endocytotic pathway used to transport the complexes into the cells, the cells were subjected to 23 h pre-incubation, before being treated with 0.014 mM chlorpromazine (CPZ), as an inhibitor of clathrin-mediated endocytosis; 0.2 mM genistein, as an inhibitor of caveolae-mediated endocytosis; or 1 mM amiloride, as an inhibitor of macropinocytosis, for 1 h. After the cells had been treated in the abovementioned manner, the DGL6 or DGL6/γ-PGA8 complex was added to the media containing each inhibitor, and then the cells were incubated for 2 h at 37 °C. After 2 h transfection, the medium was replaced with culture medium, the cells were cultured for a further 22 h at 37 °C, and then their luciferase activity was determined. The results

are shown as percentages of the values for the untreated cells.

2.8. Animals

All animal care and experimental procedures were performed in accordance with the Guidelines for Animal Experimentation of Nagasaki University after receiving approval from the institutional animal care and use committee. Male ddY mice (5-weeks-old) were purchased from Japan SLC (Shizuoka, Japan). After being delivered, the mice were allowed to acclimatize to their new environment for at least one day before the experiments.

2.9. In vivo study

Two hundred and fifty μL of the DGL6 or DGL6/γ-PGA8 complex (containing 40 μg pCMV-Luc) were intravenously injected into each mouse to examine the transgene expression efficiency of these complexes. Six hours after being injected with the complexes, the mice were sacrificed, and the liver, kidneys, spleen, heart, and lungs were dissected. The tissues were washed twice with cold saline and homogenized with lysis buffer. The resultant homogenates were centrifuged at 15,000 rpm (Kubota 3500) for 5 min, and the associated supernatants were subjected to luciferase assays.

Luciferase activity is indicated as RLU per gram of tissue.

2.10. Agglutination study

Mouse erythrocytes were subjected to three rounds of centrifugation at 5000 rpm at 4 °C (Kubota 3500; Kubota, Tokyo, Japan) for 5 min and then resuspended in PBS (a 2% (v/v) stock suspension was prepared). DGL6 or DGL6/γ-PGA8 complexes were added to samples of the erythrocyte suspension (complex: stock suspension = 1:1), which were then incubated for 15 min at room temperature. Next, 10 μL of each sample were placed on a glass plate, and the extent of agglutination within the samples was assessed by microscopy (200 × magnification).

2.11. Statistical analysis

The statistical significance of differences between two groups was assessed using the Student's *t* test. Multiple comparisons among the groups were performed using Dunnett's pairwise multiple comparisons *t* test.

3. Results

3.1. Physicochemical properties and electrophoretic assay of PLL and DGL complexes

A PLL complex with a PLL to pDNA charge ratio of 8 was used as a control for the comparisons with the DGL complexes. The particle sizes and ζ-potentials of the PLL and DGL complexes are summarized in Table 1. The PLL complex had a particle size of 76.4 ± 12.8 nm and a ζ-potential of 48.4 ± 0.3 mV. The DGL complexes had particle sizes of approximately 30–158 nm and ζ-potentials of 34–46 mV.

During a gel retardation assay, naked pDNA was detected as bands on agarose gel. On the other hand, no such bands were detected in the lanes for the PLL and DGL complexes (Fig. 1). These findings confirmed that both PLL and DGL were able to form stable pDNA-containing complexes.

3.2. In vitro transfection efficiency and buffering capacity of the PLL and DGL complexes

B16-F10 cells were transfected with the PLL complex or one of the DGL complexes, incubated for 22 h, and then had their luciferase activity evaluated. The *in vitro* transgene expression efficiencies of the PLL and DGL complexes are shown in Fig. 2.

The PLL complexes displayed low transgene expression levels. Conversely, the transgene expression levels of the cells that were transfected with the DGL complexes were over 100-fold higher than those seen in the cells transfected with PLL complexes. Among the cells that were transfected with the DGL complexes, luciferase activity increased with the amount of DGL contained within the complex, up to a charge ratio of 6, and then gradually decreased.

Other B16-F10 cells were transfected with PLL or DGL complexes containing Rh-PLL or Rh-DGL to allow the uptake of the complexes to be visualized (Fig. 3). As a result, many red dots representing Rh-PLL or Rh-DGL were observed in most of the transfected cells.

The results of the experiment assessing the buffering capacity of the DGL and PLL complexes are shown in Fig. 4. The DGL complexes exhibited higher buffering capacity than the PLL complex between pH 5 and 7.

3.3. Physicochemical properties and electrophoretic assay of the DGL/γ-PGA complexes

On the basis of our findings regarding the physicochemical properties of the complexes and the results of the *in vitro* transfection experiments, we prepared a

number of ternary complexes containing the DGL6 complex and varying amounts of γ-PGA. The particle sizes and ζ-potentials of these DGL6/γ-PGA complexes are shown in Table 2. DGL6/γ-PGA complexes with γ-PGA to pDNA charge ratios of >8 exhibited particle sizes of ~100 nm, while the DGL6/γ-PGA4 complex tended to aggregate. The addition of γ-PGA decreased the ζ-potential of the DL6 complex in a concentration-dependent manner. The DGL6/γ-PGA complexes with charge ratios >8 had anionic surface charges.

As was done for the PLL and DGL complexes, gel retardation assays were performed to examine whether that the DGL6/γ-PGA complexes were stable DNA vectors (Fig. 5). Once again, no bands representing naked pDNA were detected in the lanes for the DGL6/γ-PGA complexes, indicating that they were stable DNA vectors.

3.4. In vitro transfection efficiency of the DGL/γ-PGA complexes

Next, the DGL/γ-PGA complexes were incubated with B16-F10 cells, incubated for 22 h, and then had their luciferase activity determined. The *in vitro* transgene expression efficiencies of DGL/γ-PGA complexes with various charge ratios are shown in Fig. 6. The transgene expression efficiency of the DGL6 complex exceeded 10^9 RLU/mg protein, whereas that of the DGL6/γ-PGA2 complex was ~10^{10} RLU/mg

protein, and the luciferase activity induced by the complexes decreased as the amount of γ-PGA contained within them increased. The luciferase activity of each DGL6/γ-PGA complex was higher than that of the DGL6 complex. Also, the DGL6/γ-PGA8 complex exhibited greater buffering capacity than the PLL complex between pH 5 and 7 (Fig. 4).

3.5. Cytotoxicity

To evaluate the cytotoxicity of the complexes, each complex was added to B16-F10 cells, and then the viability of the cells was evaluated using the WST-1 assay (Fig. 7). The DGL6 complex displayed significantly greater cytotoxicity than the control ($P < 0.01$). However, the addition of γ-PGA decreased the cytotoxicity of the DGL6 complex, and the DGL6/γ-PGA complexes with charge ratios of 6-10 did not markedly affect the viability of the B16-F10 cells.

3.6. Inhibition study

On the basis of our findings regarding the physicochemical properties of the complexes and the results of the in vitro transfection experiments and the WST-1 assay, the DGL6 and DGL6/γ-PGA8 complexes were subjected to an inhibition study in order

to examine the mechanisms responsible for their uptake. As a result, it was found that endocytotic inhibitors decreased the transgene expression efficiencies of the DGL6 and DGL6/γ-PGA8 complexes (Fig. 8). Namely, CPZ, an inhibitor of clathrin-mediated endocytosis, and genistein, an inhibitor of caveolae-mediated endocytosis, decreased the transgene expression efficiencies of the DGL6 complex to about 25% and 7%, respectively. In addition, the inhibition of macropinocytosis with amiloride decreased the transgene expression efficiency of the DGL6 complex to ~50%. On the other hand, the transgene expression efficiency of the DGL6/γ-PGA8 complex was only significantly suppressed by genistein.

3.7. In vivo study

Next, the *in vivo* transgene expression efficiency of the DGL6 and DGL6/γ-PGA8 complexes was examined in ddY male mice (Fig. 9). The luciferase activity levels of several tissues were determined at 6 h after the intravenous administration of the complexes. The DGL6 complex induced gene expression in the liver, spleen, and lungs. On the other hand, the DGL6/γ-PGA8 complex only induced markedly high gene expression in the spleen.

3.8. Agglutination study

As previous studies have found that DGL vectors induced agglutination, the extent of the erythrocyte agglutination induced by the DGL6/γ-PGA8 complex was compared with that induced by the DGL6 complex (Fig. 10). Microscopic examinations demonstrated that the DGL6 complex caused severe agglutination while the DGL6/γ-PGA8 complex did not induce any agglutination.

4. Discussion

DGL is a type of a dendrigraft polymer composed of naturally occurring amino acid (lysine) monomers, making it favorable for biomedical applications. In this study, we attempted to develop new biodegradable gene vectors composed of DGL. First, it was important to confirm that DGL is able to form stable complexes with pDNA. Electrophoresis demonstrated that DGL formed stable self-assembling pDNA-containing nanoparticles; i.e., no bands for naked pDNA were detected in the lanes for the DGL complexes. In addition, the physicochemical properties of biodegradable gene vectors, such as their particle size and ζ-potential, have important effects on their transfection efficiency. For example, a previous study reported that cells commonly take up complexes measuring fifty to several hundred nanometers in diameter [18]. Thus, the particle sizes of the DGL DNA vectors produced in the present study were measured, and it was found that they varied from 30-158 nm (Table 1), which would put them roughly within the range described above. However, they also exhibited positive surface charges, which might be disadvantageous as cationic particles have been shown to have cytotoxic effects.

Next, we compared the transfection efficiency and cellular uptake of each complex in B16-F10 cells. The gene expression levels induced by the DGL complexes were

100-fold higher than those induced by the control PLL complex (Fig. 2), although strong cellular uptake of both the PLL and DGL complexes was observed during experiments involving complexes containing Rh-PLL or Rh-DGL (Fig. 3). Yamagata et al. also reported that another type of poly-L-lysine induced markedly higher dendrimer gene expression than PLL [12]. Generally, non-viral gene carriers with strong pH buffering abilities are able to promote endosomal escape, resulting in an increase in gene transfection efficiency. In this study, pH titration demonstrated that the DGL complexes had higher buffering capacity than the PLL complex (Fig. 4). Thus, the marked difference between the gene expression levels induced by the DGL and PLL complexes might have mainly been caused by variations in the frequency of endosome escape. However, a previous study also showed that lysine dendrimers facilitate DNA transcription; i.e., a lysine dendrimer was found to cause less pDNA compaction than PLL [12].

Many cationic non-viral gene vectors have been reported to cause cytotoxicity due the strong affinity of positively charged particles for the cellular membrane [19]. In this study, the DGL complexes also exhibited cytotoxicity at high doses. One promising approach to overcoming such cytotoxicity is the addition of anionic polymers to cationic complexes. For example, we previously found that γ-PGA decreased the

toxicity of cationic compounds [17]. Therefore, in the present study various amounts of γ-PGA were added to the DGL6 complex to produce ternary complexes (DGL/γ-PGA complexes).

γ-PGA is known to be biocompatible and is produced by microbial species typified by *Bacillus subtilis* [28]. Moreover, a previous study found that synthesized γ-PGA had negligible toxic effects on the human B-cell line EHRB, even at a concentration of 100 mg/L, whilst another study found that when 1 mg γ-PGA was injected into mice it had no toxic effects and did not cause any immunological or inflammatory reactions [29, 30].

The addition of γ-PGA to the DGL6 complex decreased its ζ-potential in a concentration-dependent manner, and the DGL/γ-PGA complexes with γ-PGA to pDNA charge ratios of >8 exhibited anionic surface charges (Table 2). Interestingly, the particle sizes of the DGL/γ-PGA complexes were not larger than those of the DGL complexes; therefore, γ-PGA might have increased the compaction of the DGL complexes via electrostatic interactions. The negative surface charges possessed by the DGL/γ-PGA complexes with γ-PGA to pDNA charge ratios of >8 indicate that these particles contained γ-PGA on their outer surfaces. Worryingly, some anionic polymers, such as heparin sulfate and heparan sulfate, have been shown to cause pDNA to be

released from pDNA/cationic polymer complexes [20, 21]. However, the addition of γ-PGA to the DGL6 complex did not result in pDNA release (Fig. 5). In addition, the anionically charged DGL/γ-PGA complexes exhibited markedly decreased cytotoxicity compared with the DGL complexes (Fig. 7).

Anionic complexes are generally not taken up by cells because they electrostatically repulse the cellular membrane. However, we previously found that a γ-PGA-coated complex with an anionic charge was taken up via a γ-PGA-specific receptor-mediated energy-dependent process [17]. In agreement with this, the DGL/γ-PGA complexes with anionic surface charges were found to be stronger inducers of transgene expression than the DGL complexes (Fig. 6). In particular, the markedly high transgene expression induced by the DGL6/γ-PGA 2 complex might be worth investigating in a future study. Also, the DGL/γ-PGA complexes continued to display sufficient buffering capacity (Fig. 4).

To clarify the mechanisms responsible for the uptake of the complexes, we compared the uptake pathway of the anionic DGL6/γ-PGA8 complex with that of the cationic DGL6 complex. CPZ, genistein, and amiloride all suppressed the transgene expression efficiency of the DGL6 complex, suggesting that it was taken up by the cells via several mechanisms, including caveolae-mediated endocytosis and

clathrin-mediated endocytosis (Fig. 8A). On the other hand, the transgene expression efficiency of the DGL6/γ-PGA8 complex was only suppressed by the addition of genistein (Fig. 8B). These results suggested that the DGL/γ-PGA complexes were taken up by caveolae-mediated endocytosis, which could be advantageous as it has been reported that caveolae-mediated uptake does not lead to lysosomal degradation [22,23]. Based on these findings, we consider that the charge ratio of DGL to γ-PGA might affect the cellular uptake pathways of DGL/γ-PGA complexes.

A previous study showed that cationic non-viral gene vectors caused the agglutination of blood components such as erythrocytes and albumin because of their strong electrostatic interactions [24]. Such agglutination often leads to adverse effects, including embolisms and inflammatory reactions. In the current study, the DGL6 complex induced transient erythrocyte agglutination at high doses, as shown in Fig. 10. In contrast, the DGL6/γ-PGA8 complex did not cause any agglutination. Thus, changing the polarity of the surface charge of the DL6 complex from positive to negative appeared to have reduced its interactions with blood components.

DGL complexes are biodegradable nanoparticles that exhibit high transfection efficiency. Furthermore, in the present study DGL/γ-PGA complexes exhibited similar transfection efficiency to DGL complexes whilst displaying decreased toxicity. In

addition, after being injected into mice the DGL6 complex induced marked gene expression in the liver, spleen, and lung (Fig. 9A), which makes sense as nanoparticles are taken up by the reticuloendothelial system. On the other hand, the DGL6/γ-PGA8 complex only induced strong gene expression in the spleen (Fig. 9B). Sutherland et al. reported that poly-γ-D-glutamic acid (γDPGA), which is a capsular component of *Bacillus anthracis*, mainly accumulated in the spleen and liver after its intravenous injection into mice [26, 27]. Therefore, the DGL/γ-PGA complexes examined in the present study might have accumulated in the spleen via the same mechanism. We previously reported that a pDNA/polyethylenimine/γ-PGA complex, which also induced strong gene expression in the spleen, was an effective DNA vaccine against malaria [25]. Thus, DGL/γ-PGA complexes might also prove to be useful for such vaccines.

5. Conclusions

We developed novel gene vectors composed of DGL. Whilst the transfection efficiency of the developed DGL complexes was sufficiently high, the addition of γ-PGA to the complexes decreased their cytotoxicity and their tendency to induce erythrocyte agglutination. However, whilst the DGL6 complex induced marked gene expression in the liver, spleen, and lungs of mice, the DGL6/γ-PGA8 complex only induced strong gene expression in the spleen. Future studies should examine whether DGL and/or DGL/γ-PGA complexes have clinical applications.

6. Acknowledgement

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Figure captions

Figure 1. Electrophoretic analysis of the PLL and DGL complexes

The complexes were loaded onto agarose gel, and electrophoresis was carried out. pDNA retardation was visualized using ethidium bromide.

Figure 2. *In vitro* transfection efficiencies of the PLL and DGL complexes

B16-F10 cells were transfected with various PLL- or DGL-containing complexes. Twenty-two hours after being transfected, the cells were lysed, and their luciferase activity was determined. Each bar represents the mean ± S.E. (n = 3). **P <0.01 vs. PLL complex.

Figure 3. Fluorescence microscopy images of transfected B16-F10 cells

B16-F10 cells were transfected with Rh-PLL- or Rh-DGL-containing complexes. Twenty-four hours after the cells had been transfected, the uptake of Rh-PLL (i) and Rh-DGL (ii) was assessed (200 × magnification).

Figure 4. Buffering capacity of the PLL, DGL, and DGL/γ-PGA complexes

The pH of each complex solution was adjusted to 3.0, and then each solution was

titrated using 2N NaOH. ●: PLL8 complex, ○: DGL6 complex, and ◍: DGL6/γ-PGA8 complex.

Figure 5. Electrophoretic analysis of the DGL and DGL/γ-PGA complexes

The complexes were loaded onto agarose gel, and electrophoresis was carried out. pDNA retardation was visualized using ethidium bromide.

Figure 6. *In vitro* transfection efficiencies of the DGL/γ-PGA complexes

B16-F10 cells were transfected with the DGL6 complex or one of the DGL6/γ-PGA-containing complexes. Twenty-two hours after being transfected, the cells were lysed, and their luciferase activity was determined. Each bar represents the mean ± S.E. (n = 3). *P <0.05, **P <0.01 vs. DGL complex.

Figure 7. Cytotoxicity of the DGL6 and DGL/γ-PGA complexes towards B16-F10 cells

The viability of B16-F10 cells treated with the DGL6 complex or one of the DGL6/γ-PGA-containing complexes was measured using the WST-1 assay. The cells were incubated with the complexes for 2 h, and their viability was measured at 22 h

after treatment. Data are shown as percentages of the value for the untreated cells. Each bar represents the mean ± S.E. (n = 8). *P <0.05, **P <0.01 vs. control.

Figure 8. Influence of endocytotic inhibitors on the transgene expression efficiency of the DGL6 (A) and DGL6/γ-PGA8 complexes (B)

B16-F10 cells were transfected with the DGL6 or DGL6/γ-PGA8 complex in medium containing various endocytotic inhibitors. Twenty-two hours after being transfected, the cells had their luciferase activity evaluated. Data are shown as percentages of the value for the untreated cells. Each bar represents the mean ± S.E. (n = 4). **P <0.01 vs. control.

Figure 9. *In vivo* transgene expression efficiency of the DGL6 (A) and DGL6/γ-PGA8 complexes (B) in mice

The complexes were intravenously injected into mice (40 μg DNA per mouse). Six hours after being injected, the mice were sacrificed, and each organ was dissected and had its luciferase activity quantified. Each bar represents the mean ± S.E. (n = 4).

Figure 10. Erythrocyte agglutination induced by the complexes

Erythrocytes were treated with the DGL6 or DGL6/γ-PGA8 complex, and the resultant agglutination was observed by microscopy (200 × magnification). (A) PBS, (B) DGL6 complex, and (C) DGL6/γ-PGA8 complex.

Fig. 1

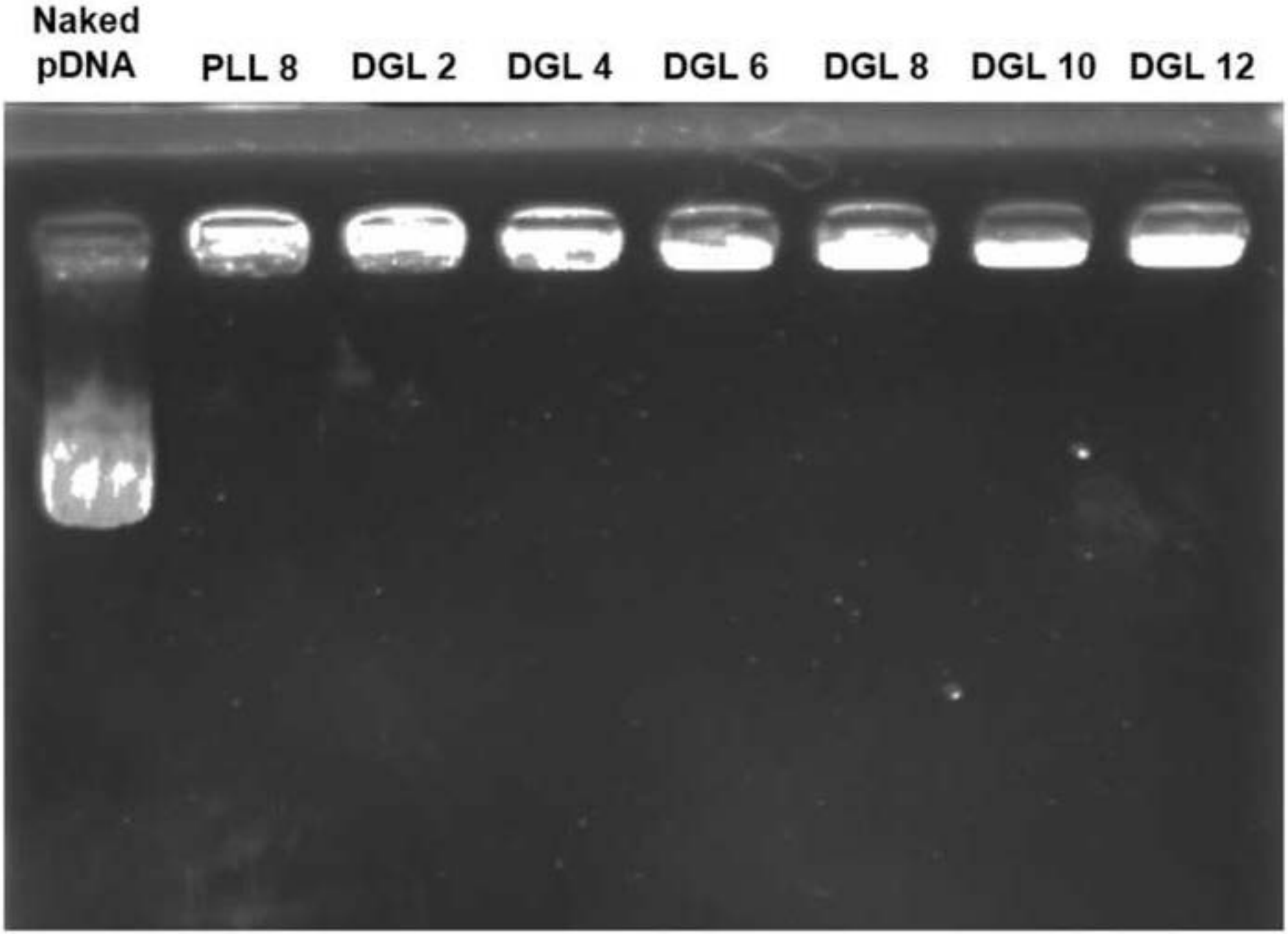


Fig. 2

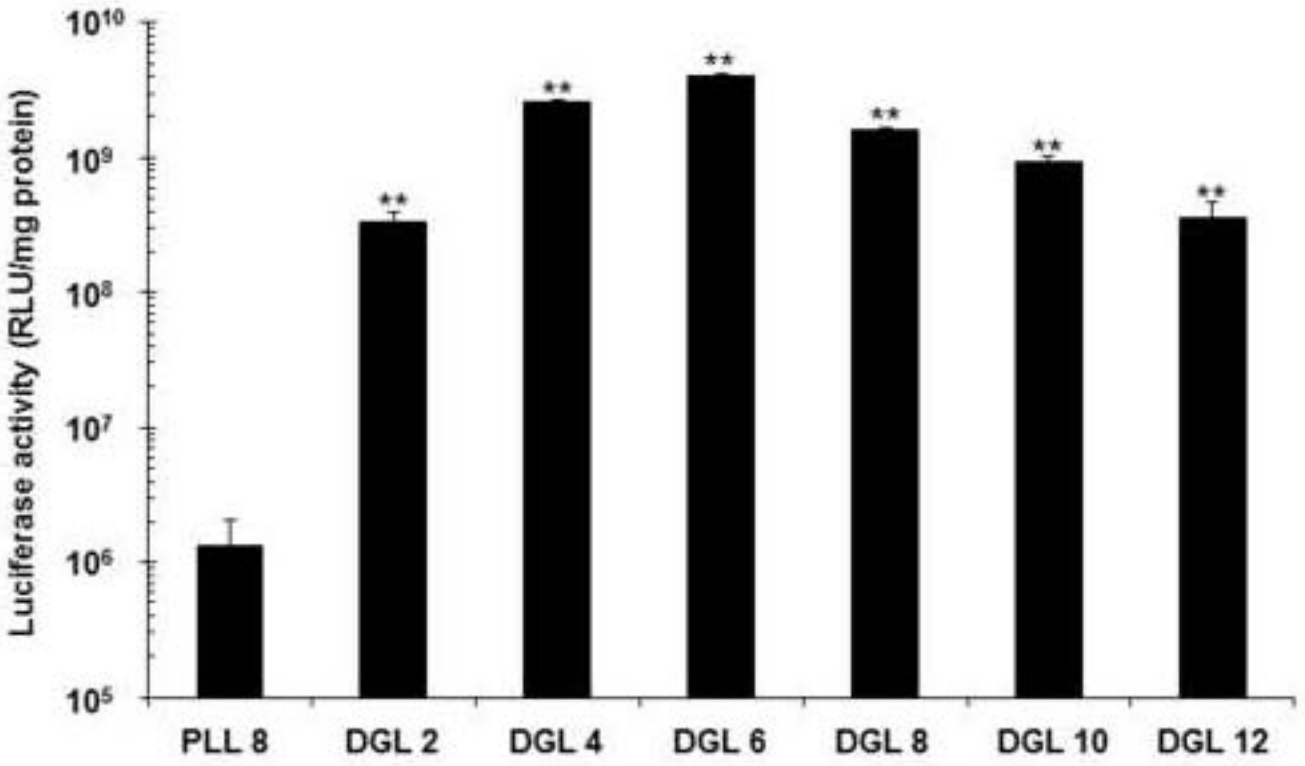
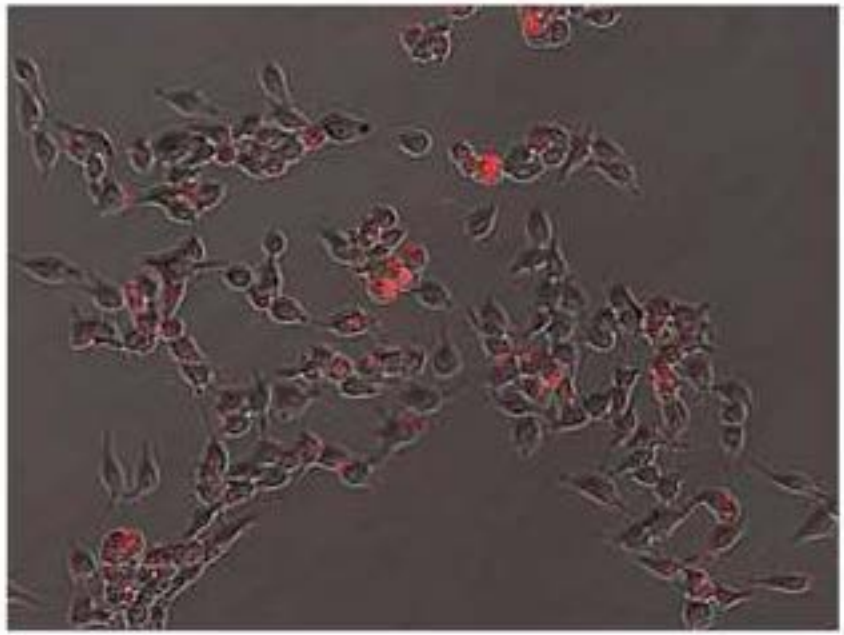

Luciferase activity (RLU/mg protein)
10^10
10^9
10^8
10^7
10^6
10^5
**
PLL 8
DGL 2
DGL 4
DGL 6
DGL 8
DGL 10
DGL 12

Fig. 3

(a)



(b)

Fig. 4

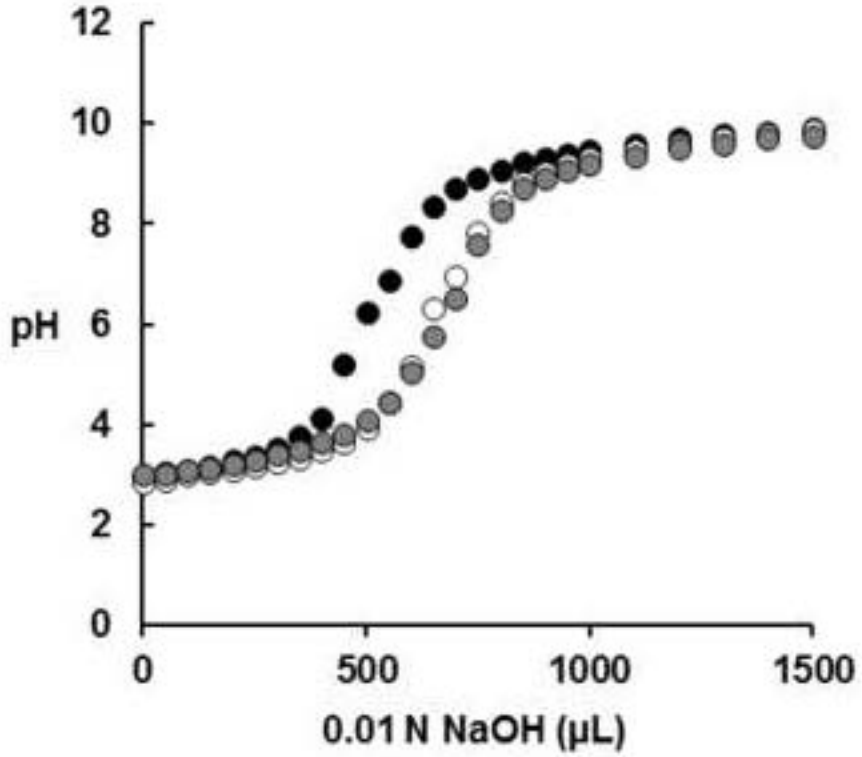


Fig. 5

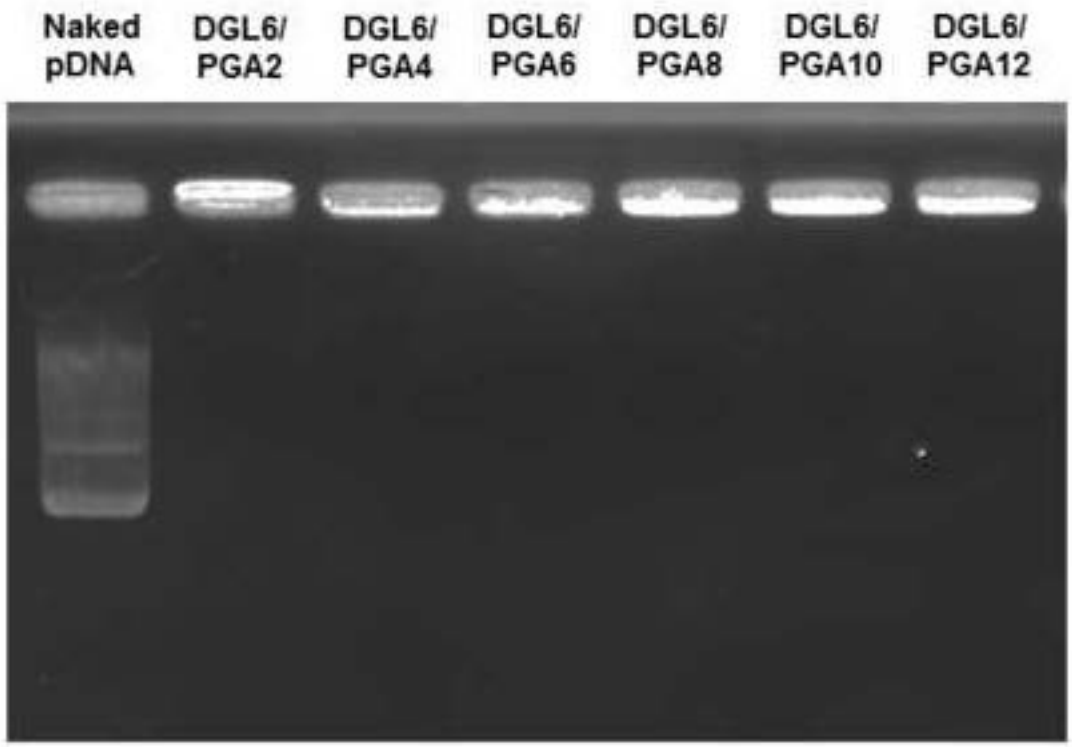

Naked pDNA
DGL6/ PGA2
DGL6/ PGA4
DGL6/ PGA6
DGL6/ PGA8
DGL6/ PGA10
DGL6/ PGA12

Fig. 6

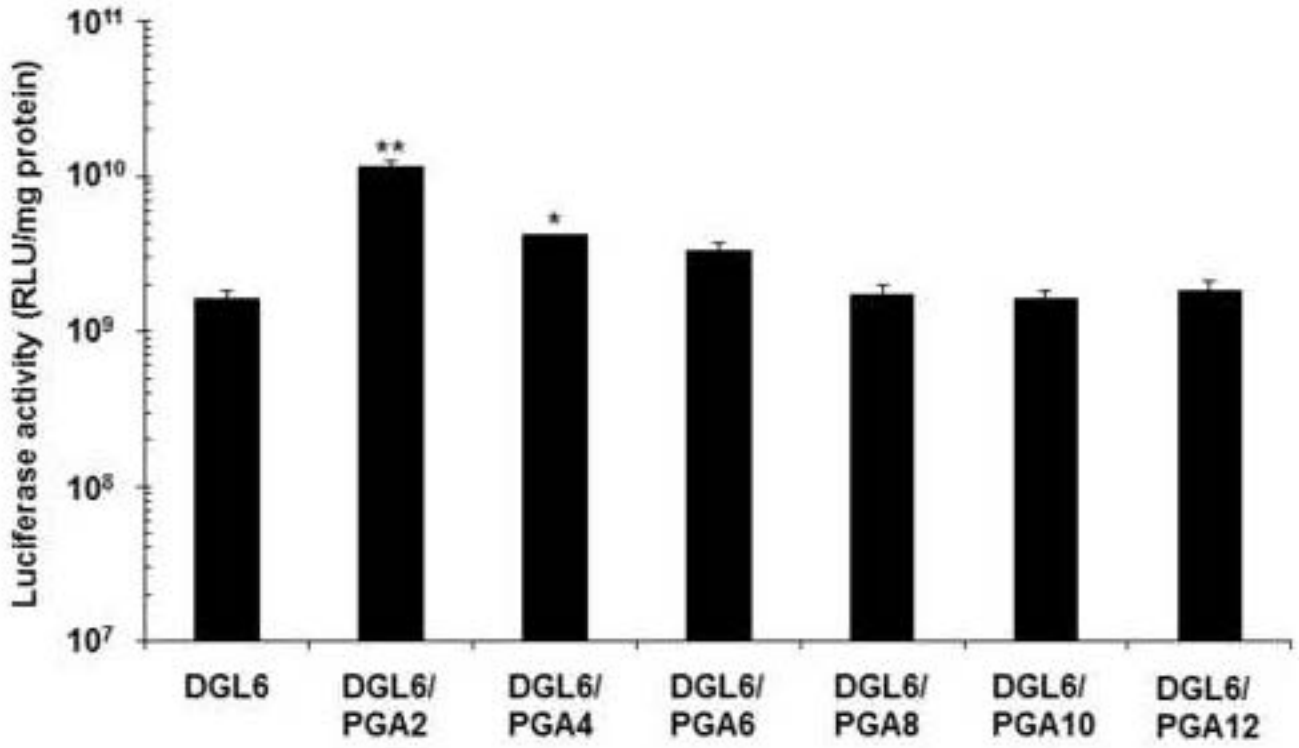

Luciferase activity (RLU/mg protein)
10^{11}
10^{10}
10^{9}
10^{8}
10^{7}
**
*
DGL6
DGL6/ PGA2
DGL6/ PGA4
DGL6/ PGA6
DGL6/ PGA8
DGL6/ PGA10
DGL6/ PGA12

Fig. 7

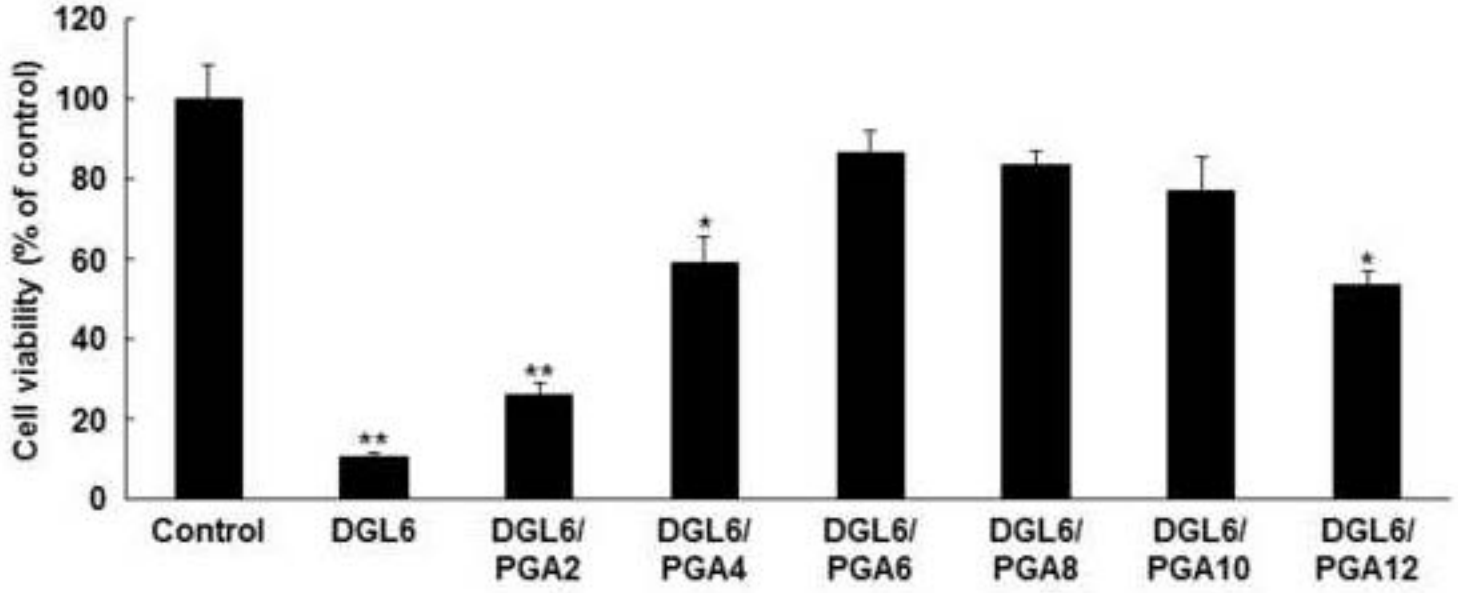

Cell viability (% of control)
120
100
80
60
40
20
0
**
**
*
*
Control
DGL6
DGL6/ PGA2
DGL6/ PGA4
DGL6/ PGA6
DGL6/ PGA8
DGL6/ PGA10
DGL6/ PGA12

Fig. 8

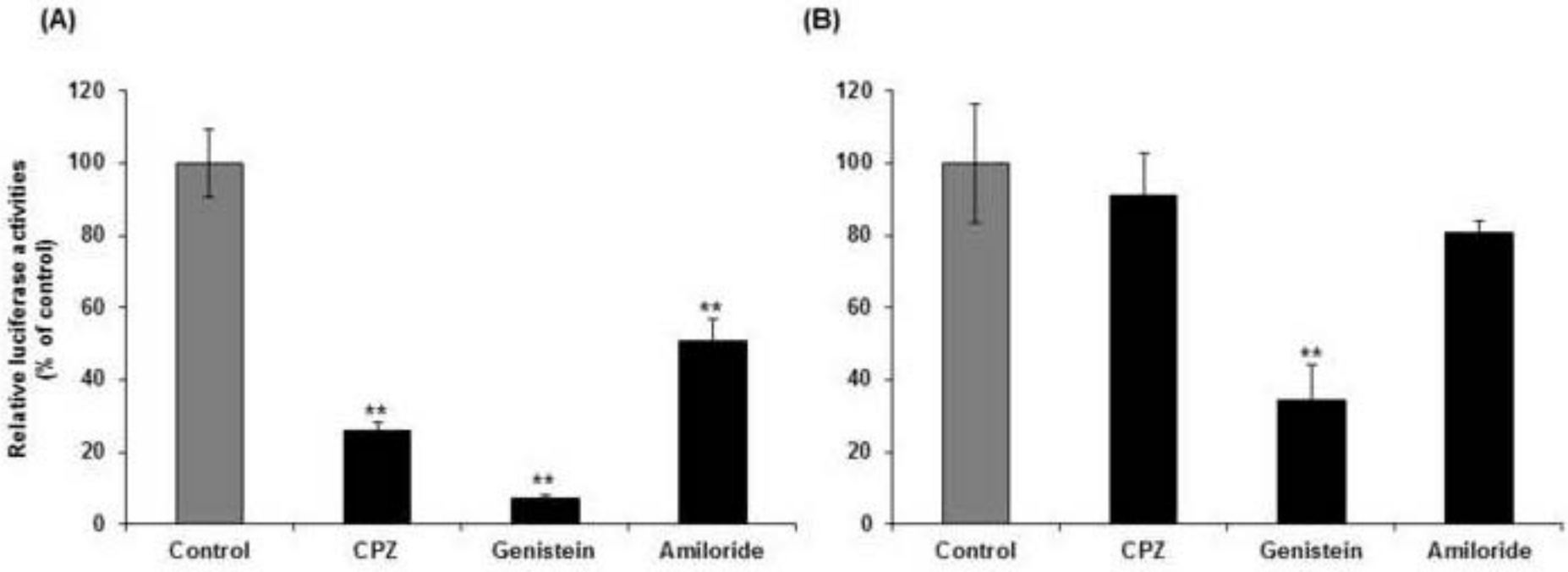

(A)
(B)
Relative luciferase activities
(% of control)
120
100
80
60
40
20
0
**
Control
CPZ
Genistein
Amiloride

Fig. 9

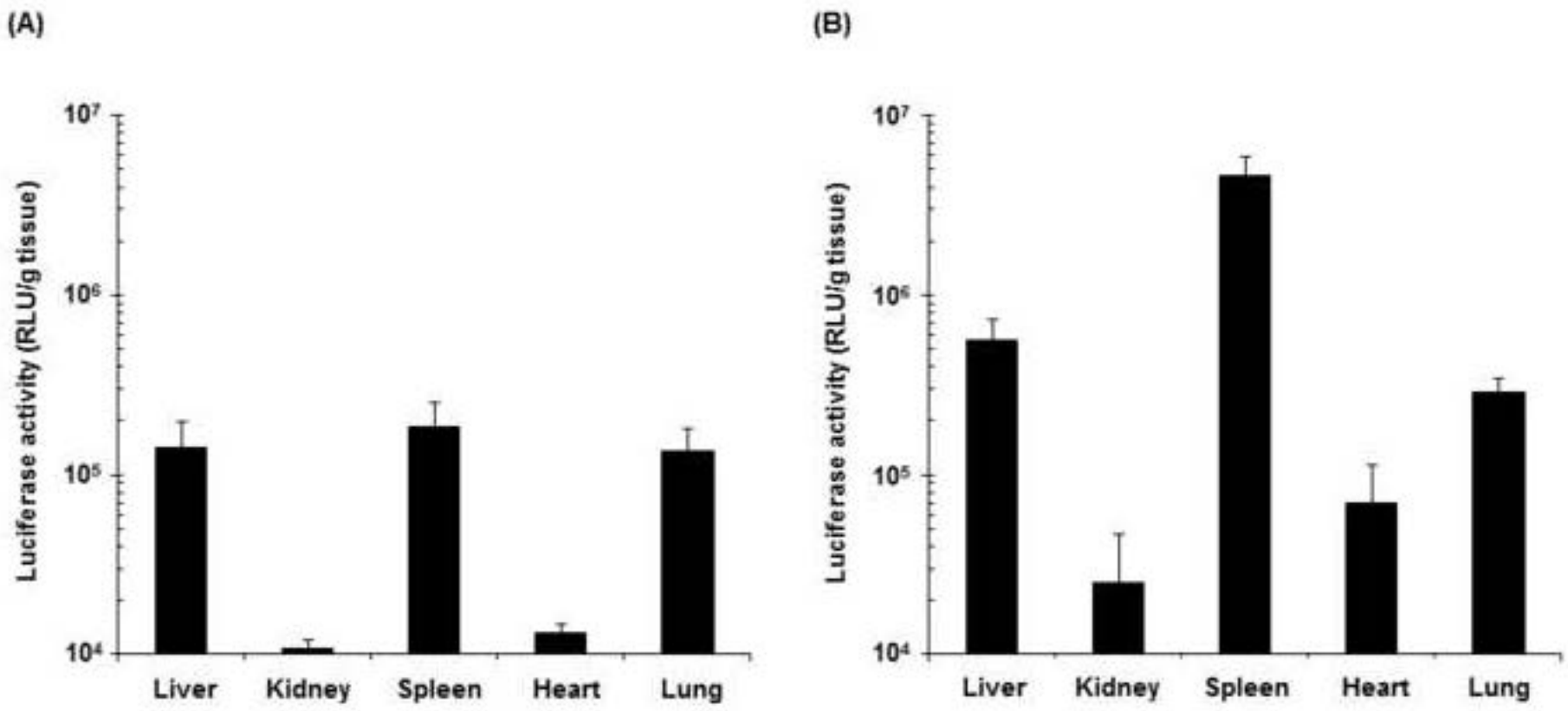

(A)
(B)
Luciferase activity (RLU/g tissue)
10^7
10^6
10^5
10^4
Liver
Kidney
Spleen
Heart
Lung

Fig. 10