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Ternary complexes of pDNA, polyethylenimine, and γ -polyglutamic acid for gene delivery systems

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Abstract

We discovered a vector coated by γ -polyglutamic acid (γ -PGA) for effective and safe gene delivery. In order to develop a useful non-viral vector, we prepared several ternary complexes constructed with pDNA, polyethylenimine (PEI), and various polyanions, such as polyadenylic acid, polyinosinic-polycytidylic acid, α -polyaspartic acid, α -polyglutamic acid, and γ -PGA. The pDNA/PEI complex had a strong cationic surface charge and showed extremely high transgene efficiency although it agglutinated with erythrocytes and had extremely high cytotoxicity. Those polyanions changed the positive ζ -potential of pDNA/PEI complex to negative although they did not affect the size. They had no agglutination activities and lower cytotoxicities but most of the ternary complexes did not show any uptake and gene expression; however, the pDNA/PEI/ γ -PGA complex showed high uptake and gene expression. Most of the pDNA/PEI/ γ -PGA complexes were located in the cytoplasm without dissociation and a few complexes were observed in the nuclei. Hypothermia and the addition of γ -PGA significantly inhibited the uptake of pDNA/PEI/ γ -PGA by the cells, although L-glutamic acid had no effect. These results strongly indicate that the pDNA/PEI/ γ -PGA complex was taken up by γ -PGA-specific receptor-mediated energy-dependent process. Thus, the pDNA/PEI/ γ -PGA complex is useful as a gene delivery system with high transfection efficiency and low toxicity.

Keywords: γ -polyglutamic acid; gene delivery; polyethylenimine; pDNA; ternary complex

1. Introduction

Gene therapy is expected to be an effective method to treat cancer, infection, innate immunodeficiency, and cardiovascular diseases [1, 2]. The success of gene therapy highly depends on the development of effective and secure delivery vectors. Gene delivery vectors are categorized into viral and non-viral vectors.

Among non-viral vectors, polyethylenimine (PEI) is a popular cationic polymer to form a complex with pDNA. The complex has shown high gene expression in *in vitro* and *in vivo* gene delivery [3, 4]. The complex binds non-specifically to negatively charged proteoglycans on cell membranes and agglutinates with blood components, such as erythrocytes and serum albumins. Agglutinations of the complex often leads to its rapid elimination and adverse effects, such as embolism and inflammatory reaction [5-8].

In order to reduce the cytotoxicity and agglutination with blood components, several novel polymers of PEI covalently binding to hydrophilic polymers, polyethyleneglycol (PEG), polyhydroxypropylmethacrylamide (pHPMA), and polyvinylpyrrolidone, have been developed to modify the complex surface [6, 8, 9]. On the other hand, anion polymers themselves can bind to the cationic complex electrostatically to modify the complex surface [10, 11]. In the present study, we investigated the ternary complexes of pDNA/PEI coated by polyanions, such as polynucleic acid: polyadenylic acid (polyA) and polyinosinic-polycytidylic acid (polyIC) and polyamino acid: α -polyaspartic acid (α -PAA), α -polyglutamic acid (α -PGA), and γ -polyglutamic acid (γ -PGA). Among them, we newly

discovered that only the ternary complex coated by γ -PGA showed high gene expression without cytotoxicity and agglutination of erythrocytes.

2. Materials and Methods

2.1. Chemicals

PEI (branched form, average molecular weight of 25,000) and rhodamine B isothiocyanate were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). PolyA, polyIC, α -PAA, and α -PGA were obtained from Sigma (St. Louis, MO, USA). The γ -PGA was provided by Yakult Pharmaceutical Industry Co., Ltd. (Tokyo, Japan). Fetal bovine serum (FBS) was obtained from Biosource International Inc. (Camarillo, CA, USA). RPMI 1640, Opti-MEM I, antibiotics (penicillin 100 U/mL and streptomycin 100 μ g/mL), and other culture reagents were obtained from GIBCO BRL (Grand Island, NY, USA). The 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2*H*-tetrazolium, monosodium salt (WST-1) and 1-methoxy-5-methylphenazinium methylsulfate (1-methoxy PMS) were obtained from Dojindo Laboratories (Kumamoto, Japan). YOYO-1 and Hoechst 33342 were purchased from Molecular Probes (Leiden, The Netherlands). Rhodamine-PEI (Rh-PEI) was prepared in our laboratory. Briefly, PEI and rhodamine B isothiocyanate were dissolved in dimethyl sulfoxide (DMSO) and stirred overnight at room temperature in the dark. Rh-PEI was purified by gel filtration. Almost 1.5% of PEI nitrogen was labeled with rhodamine B. All other chemicals were of the highest purity available.

2.2. Preparation of pDNA 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). Enhanced green fluorescence protein (GFP) encoding the pDNA (pEGFP-C1) was purchased from Clontech (Palo Alto, CA, USA). The pDNA was amplified using an EndoFree[®] Plasmid Giga Kit (QIAGEN GmbH, Hilden, Germany). The pDNA was dissolved in 5% dextrose solution and stored at -80 °C until analysis. The pDNA concentration was measured at 260 nm absorbance and adjusted to 1 mg/mL. For fluorescent labeling, pDNA was mixed with the intercalating nucleic acid stain YOYO-1 using a molar ratio of 1 dye molecule per 300 base pairs for 30 minutes at room temperature in the dark.

For the preparation of ternary complexes, pDNA solution and PEI solution (pH 7.4) were mixed by pipetting thoroughly and left for 15 min at room temperature, and then each polyanion was mixed with pDNA/PEI complex by pipetting and left for another 15 min at room temperature (Fig. 1). In this study, we constructed ternary complexes at a theoretical charge ratio: phosphate of pDNA: nitrogen of PEI: phosphate or carboxylate of polyanion = 1: 8: 6.

2.3. Physicochemical Property of Ternary Complexes

The particle sizes and ζ -potentials of ternary complexes were measured using a Zetasizer Nano ZS (Malvern Instruments, Ltd., United Kingdom). The number-fractioned mean diameter is shown.

To determine complex formations, 10 μ L aliquots of ternary complex solution containing 1 μ g of pDNA were mixed with 2 μ L of loading buffer (30% glycerol and 0.2% bromophenol blue) and loaded onto a 0.8% agarose gel containing 0.03% ethidium bromide.

Electrophoresis (i-Mupid J[®]; Cosmo Bio, Tokyo, Japan) was carried out at 35 V in running buffer solution (40 mM Tris/HCl, 40 mM acetic acid, and 1 mM EDTA) for 80 min. The retardation of the pDNA was visualized using a FluorChem Imaging Systems (Alpha Innotech, CA, USA).

2.4. Interaction with Erythrocytes

Erythrocytes from mice were washed three times at 4 °C by centrifugation at 5,000 rpm (Kubota 3700, Kubota, Tokyo, Japan) for 5 min and resuspension in PBS. A 2% (v/v) stock suspension was prepared for agglutination study. Various complexes were added to the erythrocytes (complexes: stock suspension = 1: 1). The suspensions were incubated for 15 min at room temperature. The 10 μ L suspensions were placed on a glass plate and agglutination was observed by microscopy (400 \times magnification). For hemolysis study, 5% stock suspension was prepared. Various complexes were added to the erythrocytes and incubated for 24 h at room temperature. After incubation, the suspensions were centrifuged at 5,000 rpm for 5 min, and supernatants were taken. Hemolysis was quantified by measuring the absorbance of hemoglobin at a wavelength of 545 nm, using a microplate reader (Multiskan Spectrum, Thermo Fisher Scientific, Inc., Wyman Street Waltham, MA,

USA). Lysis buffer (pH 7.8 and 0.1 M Tris/HCl buffer containing 0.05% Triton X-100 and 2 mM EDTA) was added to erythrocytes and used for the 100% hemolysis sample.

2.5. WST-1 Assay

The mouse melanoma cell line, B16-F10 cells, was obtained from the Cell Resource Center for Biomedical Research (Tohoku University, Japan). B16-F10 cells were maintained in RPMI 1640 supplemented with 10% FBS and antibiotics (culture medium) under a humidified atmosphere of 5% CO₂ in air at 37 °C. Cytotoxicity tests of various complexes on B16-F10 cells were carried out using a WST-1 commercially available cell proliferation reagent. WST-1 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, Franklin Lakes, NJ, USA) at a density of 3.0×10^3 cells/well in the culture medium. Ternary complexes containing 1 µg of pDNA in 100 µL Opti-MEM I medium were added to each well and incubated for 2 h. After incubation, the medium was replaced with 100 µL culture medium and incubated for another 22 h. Medium was replaced with 100 µL culture medium and 10 µL of the WST-1 reagent was added to each well. The cells were incubated for an additional 2 h at 37 °C, and absorbance was measured at a wavelength of 450 nm with a reference wavelength of 630 nm, using a microplate reader. The results are shown as a percentage of untreated cells.

2.6. Transfection Experiments

B16-F10 cells were plated on 24-well plates (Becton-Dickinson, Franklin Lakes, NJ, USA) at a density of 1.5×10^4 cells/well and cultivated in 0.5 mL culture medium. In the transfection experiment, after 24 h pre-incubation, the medium was replaced with 0.5 mL Opti-MEM I medium and each complex containing 1 μ g of pDNA was added to the cells and incubated for 2 h. After transfection, the medium was replaced with culture medium and cells were cultured for a further 22 h at 37 °C.

2.7. Determinations of Uptake of Ternary Complexes and Gene Expressions

To visualize the uptake of the ternary complexes and gene expressions, B16-F10 cells were transfected by various complexes constructed with pEGFP-C1, Rh-PEI, and polyanions as described above. After 22 h incubation, the relative levels of Rh-PEI and GFP expression were characterized using fluorescent microscopy (200 \times magnification).

To determine the uptake of ternary complexes, B16-F10 cells were transfected by various complexes containing Rh-PEI as described above. After 22 h incubation, cells were washed with PBS and then lysed in 300 μ L of lysis buffer. The lysates were placed onto 96-well plates, and the fluorescence of Rh-PEI was measured at an emission wavelength of 590 nm with an excitation wavelength of 540 nm, using a fluorometric microplate reader (Fluostar OPTIMA, BMG LABTECH, Offenburg, Germany).

To determine gene expression, B16-F10 cells were transfected by various complexes containing pCMV-Luc, PEI, and polyanions as described above. After 22 h incubation, the cells were washed with PBS and then lysed in 100 μ L of lysis buffer. Ten microliters of lysate samples were mixed with 50 μ L of luciferase assay buffer (Picagene, Toyo Ink, Tokyo, Japan) and the light produced was immediately measured using a luminometer (Lumat LB 9507, EG & G Berthold, Bad Wildbad, Germany). The protein content of the lysate was determined by a Bradford assay using BSA as a standard. Absorbance was measured using a microplate reader at 570 nm. Uptake of Rh-PEI was indicated as μ g per mg protein, and luciferase activity was indicated as relative light units (RLU) per mg protein.

2.8. Evaluations of Intracellular Distribution of Ternary Complexes

To evaluate the intracellular distribution of complexes, B16-F10 cells were transfected as described above with the pDNA/PEI complex and pDNA/PEI/ γ -PGA complex containing YOYO-1 labeled pCMV-Luc and Rh-PEI. At 21.5 h after transfection, cells were incubated with culture medium containing Hoechst 33342 for 30 min to visualize nuclei and then medium was replaced with PBS, and fluorescence distributions of YOYO-1-labeled pCMV-Luc, Rh-PEI, and Hoechst 33342 were observed with fluorescent microscopy (400 \times magnifications). The tone of each image was adjusted and overlapped to give a merged picture by digital processing.

2.9. Inhibition Study

For hypothermal experiments, B16-F10 cells were plated as described above and pre-incubated for 23.5 h, and the cells were incubated at 4 °C for 30 min in Opti-MEM I medium prior to the addition of complexes to the cells. After incubation, pDNA/PEI/ γ -PGA complex was added to the well and incubated for a further 2 h at 4 °C. After transfection, medium was replaced with culture medium and the cells were cultured for a further 22 h at 37 °C. For experiments using inhibitors, the cells were transfected by pDNA/PEI/ γ -PGA complex in Opti-MEM I medium containing various concentrations of γ -PGA and L-glutamic acid. After transfection, medium was replaced with culture medium and cells were cultured for a further 22 h at 37 °C, and then the uptake of Rh-PEI and luciferase activities were determined as described above.

2.10. Statistical Analysis

Statistical significance among groups was identified by Dunnett's pairwise multiple comparison *t* test.

3. Results

3.1. Physicochemical Characteristics and Electrophoresis Assay

In the preliminary experiment, the ζ -potentials of ternary complex were determined at various charge ratios. The addition of anionic polymer to the pDNA/PEI complex decreased its ζ -potentials and reached a plateau at a charge ratio 1:8:6 of phosphate of pDNA: nitrogen of PEI: phosphate or carboxylate of polyanion; therefore, the ternary complex at a charge ratio of 1:8:6 was used throughout the present study. Table 1 shows the sizes and ζ -potentials of various complexes. The pDNA/PEI complex showed 65.4 ± 15.8 nm particle size and $+55.5 \pm 0.6$ mV ζ -potential. On the other hand, ternary complexes showed anionic charges and had significantly lower ζ -potentials than the pDNA/PEI complex ($P < 0.01$), without much effect on the sizes.

Complex formations were examined by a gel retardation assay (Fig. 2). Naked pDNA was detected as a band on agarose gel. At the same time, bands of pDNA were not detected in any complexes.

3.2. Interaction with Erythrocytes

Various complexes were added to erythrocytes to evaluate agglutination (Fig. 3A). The pDNA/PEI complex showed severe agglutination on microscopy but no agglutination was observed in ternary complexes. Figure 3B shows hemolysis activities of the various complexes. All complexes showed little hemolysis activity, which was lower than 10%.

3.3. Cellular Toxicities

To determine cellular toxicities, each complex was added to B16-F10 cells and cell viability was evaluated by WST-1 assay (Fig. 4). The pDNA/PEI complex showed significantly higher cellular toxicity than the control ($P < 0.01$). On the other hand, little cellular toxicity was observed in the ternary complexes.

3.4. Fluorescent Microscopy

B16-F10 cells were transfected with various complexes containing Rh-PEI and pEGFP-C1 to visualize the uptake of complexes and gene expressions (Figs. 5A and 5B). In the pDNA/PEI complex, the red dots of Rh-PEI and bright green fluorescence of GFP were highly observed in most cells. On the other hand, the ternary complexes coated by polyA, polyIC, α -PAA, or α -PGA showed no red dots and green fluorescence. In the pDNA/PEI/ γ -PGA complex, however, the red dots and green fluorescence were highly observed in most cells.

3.5. Determination of Uptake and Transfection Activities

B16-F10 cells were transfected with various complexes containing pCMV-Luc and/or Rh-PEI to determine uptake and gene expression (Figs. 6A and 6B). The pDNA/PEI complex was highly taken up in the cells, as shown in Fig. 6A. On the other hand, the

uptake of ternary complexes coated by polyA, polyIC, α -PAA, and α -PGA was significantly lower than the pDNA/PEI complex ($P < 0.01$). The pDNA/PEI/ γ -PGA complex, however, showed significantly higher uptake than the pDNA/PEI complex ($P < 0.01$).

Figure 6B shows luciferase activities after application of the complexes containing pCMV-Luc and PEI. The pDNA/PEI complex exceeded 10^{10} RLU/mg protein in transgene efficiency. On the other hand, transgene efficiencies of all ternary complexes were significantly lower than the pDNA/PEI complex ($P < 0.01$). The pDNA/PEI/ γ -PGA complex, however, exceeded 10^{10} RLU/mg protein in transgene efficiency. A commercial transfection reagent, lipofectin, showed only 2.52×10^8 RLU/mg protein under the same conditions.

3.6. Intracellular Distribution of Ternary Complexes

Intracellular distribution of pDNA/PEI and pDNA/PEI/ γ -PGA complexes was visualized using YOYO-1-labeled pCMV-Luc, Rh-PEI, and Hoechst 33342 (Figs. 7A, 7B). In pDNA/PEI complex, green dots of YOYO-1-labeled pCMV-Luc were located mainly in the cytoplasm of most cells together with red dots of Rh-PEI (Figs. 7Aii, iii, and iv). A few dots were observed in nuclei. Synchronized green dots with red dots were seen as orange dots in merged images as shown in Fig. 7Av. In the pDNA/PEI/ γ -PGA complex, the same result was shown.

3.7. Inhibition Study

Inhibition studies were performed with hypothermia or inhibitory agents, such as γ -PGA and L-glutamic acid. Figure 8A shows the uptake of the pDNA/PEI/ γ -PGA complex. Uptake of the complex tended to decrease at 4 °C incubation ($P < 0.1$), and γ -PGA reduced the uptake of pDNA/PEI/ γ -PGA complex concentration-dependently. On the other hand, L-glutamic acid did not affect the uptake of the pDNA/PEI/ γ -PGA complex. Figure 8B shows the gene expression of the pDNA/PEI/ γ -PGA complex. The complex decreased its gene expression at 4 °C incubation ($P < 0.01$). γ -PGA reduced the gene expression of the pDNA/PEI/ γ -PGA complex concentration-dependently, but this reduction was not shown by L-glutamic acid.

4. Discussion

In gene delivery, non-viral vectors, including cationic polymers, have several advantages, including non-immunogenicity, low acute toxicity, and flexibility to design a vehicle with well-defined structural and chemical properties to yield mass production [12, 13].

Polyethylenimine (PEI) is a popular cationic polymer forming a complex with pDNA as shown by high gene expression in *in vitro* and *in vivo* gene delivery [3, 4]. Several advantages of PEI in the process of gene transfection have been reported: condensing pDNA by electrostatic interaction, binding to the cell surface, take up by the endocytotic pathway, and release of pDNA into the cytoplasm, via the so-called 'proton sponge mechanism' [14-16]. PEI, however, is reported to show cytotoxicity and agglutination by its strong cationic charge.

One promising approach for overcoming the disadvantages of PEI is the formation of a ternary complex coated by anionic polymers which modify the surface of the vector. In the present study, we newly discovered that a ternary complex, pDNA/PEI complex coated by γ -PGA, showed high gene expression without cytotoxicity and agglutination of erythrocytes. The gene expression of the complex is superior to a commercial transfection reagent, lipofectin, and is comparable to a pDNA/PEI complex.

We prepared ternary complexes of pDNA/PEI coated by polyanions such as polynucleic acid: polyadenylic acid (polyA) and polyinosinic-polycytidylic acid (polyIC) and polyamino acid: α -polyaspartic acid (α -PAA), α -polyglutamic acid (α -PGA), and γ -polyglutamic acid (γ -PGA). The size and ζ -potential of these complexes were determined and are shown in

Table 1. The addition of polyanions changed the positive ζ -potential of pDNA/PEI complex to the negative although they did not greatly affect the size of the complex. The total charge ratio of ternary complex is + 1; however, the ζ -potential of the ternary complex was apparently negative. This result suggests a concentrated distribution of anionic polymers outside of the particles.

Nucleic acids are rapidly degraded by nucleases and exhibit poor cellular uptake when delivered in aqueous solutions. Non-viral vector plays a role to compact pDNA and protect against its degradation. Naked pDNA was detected as a band on agarose gel and bands of pDNA were not detected in the pDNA/PEI complex, as shown in Fig. 2. The ternary complex also showed no bands of pDNA, despite the addition of anionic polymers. These results support that the ternary complex is stable as self-assembled nano-particles.

The ternary complexes developed in the present study are expected to have low hematotoxicity and cytotoxicity because of their negative ζ -potential. We therefore evaluated the safety of those complexes (Figs. 3A, 3B, and 4). The pDNA/PEI complex severely agglutinated erythrocytes. In contrast, all ternary complexes showed no agglutination activities (Fig. 3A) and low hemolysis activity (Fig. 3B). Furthermore, we evaluated the cytotoxicity of complexes by WST-1 assay. The pDNA/PEI complex showed extremely high cytotoxicity, as shown in Fig. 4. All ternary complexes, however, did not show significant cytotoxicity. A negative surface charge may cause the ternary complex to have increased safety. In fact, the additions of alginic acid and anionic PEG derivatives to

the cationic complexes were demonstrated to reduce agglutination and cytotoxicity [17, 18].

The uptake of these complexes and their gene expression efficiency were visualized in B16-F10 cells using the complexes loading Rh-PEI and pEGFP-C1. Generally, anionic complexes can not be taken up well by cells because they repulse the cellular membrane electrostatically; the ternary complexes coated by polyA, polyIC, α -PAA, and α -PGA did not show any uptake or gene expression (Figs. 5A and 5B); however, the pDNA/PEI/ γ -PGA complex was highly taken up by the cells and high GFP expression was observed even if it had anionic charge. In the same manner, uptake of these complexes and their gene expression efficiency was quantified in B16-F10 cells by using the complexes loading pCMV-Luc and/or Rh-PEI (Figs. 6A and 6B). The pDNA/PEI/ γ -PGA complex showed extremely high uptake and gene expression in the cells although the ternary complexes coated by polyA, polyIC, α -PAA, and α -PGA showed significantly lower uptake and gene expressions than the pDNA/PEI complex ($P < 0.01$). These results indicate that the pDNA/PEI/ γ -PGA complex with a negative charge is taken by cells through a particular mechanism. The pDNA/PEI complex was reported to be taken by cells through endocytosis according to electrostatic interaction with membrane heparan sulfate [19]. Therefore, we visualized and compared the intracellular distribution of the pDNA/PEI/ γ -PGA complex to that of the pDNA/PEI complex in B16-F10 cells by using Rh-PEI, YOYO-1-labeled pDNA, and Hoechst 33342 (Figs. 7A and 7B). Most of the two complexes was located in the cytoplasm without dissociation and a few complexes were observed in the nuclei, although

some efficient non-viral vectors were reported to be mostly located in the cytoplasm, not nuclei [20]. The inhibition experiment for uptake and gene expression of pDNA/PEI/ γ -PGA complex was carried out by hypothermia, γ -PGA, and L-glutamic acid (Figs. 8A and 8B). Hypothermia and the addition of γ -PGA dominantly inhibited the uptake and gene expression of pDNA/PEI/ γ -PGA by the cells, although L-glutamic acid did not affect them. These results strongly indicate that the pDNA/PEI/ γ -PGA complex was taken up by an energy-dependent process and γ -PGA-specific receptor-mediated pathway.

Thus, the pDNA/PEI/ γ -PGA complex is very useful as a gene delivery system with high transfection efficiency and low toxicity. γ -PGA is known to be produced by microbial species typified by *Bacillus subtilis* [21]. Synthesized γ -PGA showed little toxic effect on the human B-cell line EHRB even at high concentration, 100mg/L. It also showed no toxic effect on mice following the injection of 1mg γ -PGA and was not caused by immunoreactions and inflammatory reactions [22-24]. These reports support the safety of the pDNA/PEI/ γ -PGA complex.

Conclusion

We developed the pDNA/PEI/ γ -PGA complex as a promising non-viral vector. The γ -PGA can electrostatically coat the plasmid DNA/PEI complex to form stable anionic particles. The coating of γ -PGA dramatically decreased the toxicities of pDNA/PEI complex; furthermore, the pDNA/PEI/ γ -PGA complex was highly taken by the cells via γ -PGA-specific receptor-mediated pathway and showed extremely high transgene efficiencies. Further studies are necessary to examine the in detailed uptake mechanism and clinical safety as gene delivery vector.

References

1. Verma IM, Somia N. Gene therapy -- promises, problem and prospects. *Nature* 1997;389:239-42.
2. Edelstein ML, Abedi MR, Wixon J. Gene therapy clinical trials worldwide to 2007 -an update. *J Gene Med* 2007;9:833-42.
3. Godbey WT, Wu KK, Mikos AG. Poly(ethylenimine) and its role in gene delivery. *J Control Release* 1999;60:149-60.
4. Kircheis R, Wightman L, Wagner E. Design and gene delivery activity of modified polyethylenimines. *Adv Drug Deliv Rev* 2001;53:341-58.
5. Fischer D, Bieber T, Li Y, Elsasser HP, Kissel T. A novel non-viral vector for DNA delivery based on low molecular weight, branched polyethylenimine: effect of molecular weight on transfection efficiency and cytotoxicity. *Pharm Res* 1999;16:1273-9.
6. Ogris M, Brunner S, Schuller S, Kircheis R, Wagner E. PEGylated DNA/transferrin-PEI complexes: reduced interaction with blood components, extended circulation in blood and potential for systemic gene delivery. *Gene Ther* 1999;6:595-605.
7. Ogris M, Wagner E. Targeting tumors with non-viral gene delivery systems. *Drug Discov Today* 2002;7:479-85.
8. Cook SE, Park IK, Kim EM, Jeong HJ, Park TG, Choi YJ, et al. Galactosylated

- polyethylenimine-graft-poly(vinyl pyrrolidone) as a hepatocyte-targeting gene carrier. *J Control Release* 2005;105:151-63.
9. Oupicky D, Ogris M, Howard KA, Dash PR, Ulbrich K, Seymour LW. Importance of lateral and steric stabilization of polyelectrolyte gene delivery vectors for extended systemic circulation. *Mol Ther* 2002;5:463-72.
 10. Trubetskoy VS, Wong SC, Subbotin V, Budker VG, Loomis A, Hagstrom JE, et al. Recharging cationic DNA complexes with highly charged polyanions for in vitro and in vivo gene delivery. *Gene Ther* 2003;10:261-71.
 11. Ito T, Iida-Tanaka N, Koyama Y. Efficient in vivo gene transfection by stable DNA/PEI complexes coated by hyaluronic acid. *J Drug Target* 2008;16:276-81.
 12. Tang MX, Szoka FC. The influence of polymer structure on the interactions of cationic polymers with DNA and morphology of the resulting complexes. *Gene Ther* 1997;4:823-32.
 13. Mario CF, Nigel CP. Major limitations in the use of cationic liposomes for DNA delivery. *Int J Pharm* 1998;162:159-70.
 14. Boussif O, Lezoualc'h F, Zanta MA, Mergny MD, Scherman D, Demeneix B, et al. A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. *Proc Natl Acad Sci USA* 1995;92:7297-301.
 15. Kichler A, Leborgne C, Coeytaux E, Danos O. Polyethylenimine-mediated gene delivery: a mechanistic study. *J Gene Med* 2001;3:135-44.

16. Itaka K, Harada A, Yamasaki Y, Nakamura K, Kawaguchi H, Kataoka K. In situ single cell observation by fluorescence resonance energy transfer reveals fast intra-cytoplasmic delivery and easy release of plasmid DNA complexed with linear polyethylenimine. *J Gene Med* 2004;6:76-84.
17. Jiang G, Min SH, Kim MN, Lee DC, Lim MJ, Yeom YI. Alginate/PEI/DNA polyplexes: a new gene delivery system. *Yao Xue Xue Bao* 2006;41:439-45.
18. Maruyama K, Iwasaki F, Takizawa T, Yanagie H, Niidome T, Yamada E, et al. Novel receptor-mediated gene delivery system comprising plasmid/protamine/sugar-containing polyanion ternary complex. *Biomaterials* 2004;25:3267-73.
19. Demeneix B, Behr JP. Polyethylenimine (PEI). *Adv Genet* 2005;53:217-30.
20. Hama S, Akita H, Ito R, Mizuguchi H, Hayakawa T, Harashima H. Quantitative comparison of intracellular trafficking and nuclear transcription between adenoviral and lipoplex systems. *Mol Ther* 2006;13:786-94.
21. Sung MH, Park C, Kim CJ, Poo H, Soda K, Ashiuchi M. Natural and edible biopolymer poly-gamma-glutamic acid: synthesis, production, and applications. *Chem Rec* 2005;5:352-66.
22. Prodhomme EJ, Tutt AL, Glennie MJ, Bugg TD. Multivalent conjugates of poly-gamma-D-glutamic acid from *Bacillus licheniformis* with antibody F(ab') and glycopeptide ligands. *Bioconjug Chem* 2003;14:1148-55.

23. Schneerson R, Kubler-Kielb J, Liu TY, Dai ZD, Leppla SH, Yergey A, et al.
Poly(γ -D-glutamic acid) protein conjugates induce IgG antibodies in mice to the capsule of *Bacillus anthracis*: a potential addition to the anthrax vaccine. *Proc Natl Acad Sci USA* 2003;100:8945-50.
24. Prescott AG, Scott LR. Method for producing medical and commercial grade poly- γ -glutamic acid of high molecular weight. US Patent No. 0095679, 2005.

Figures

Fig. 1.

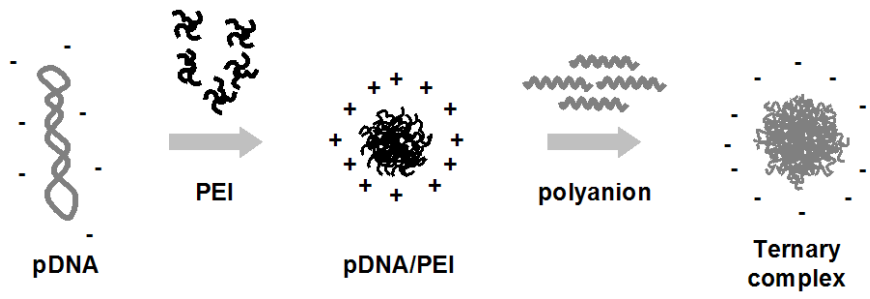


Fig. 2.

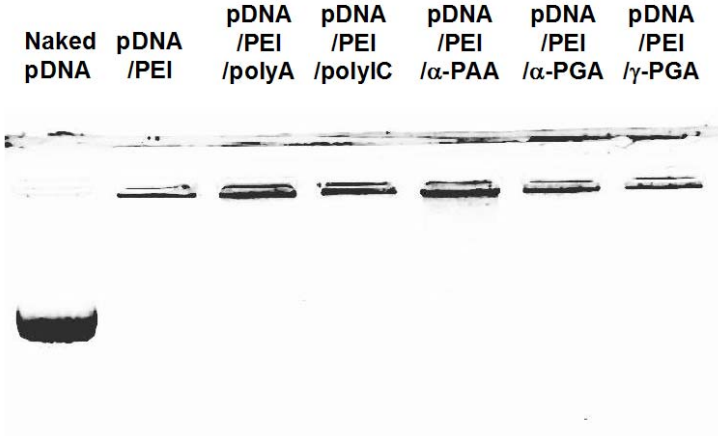
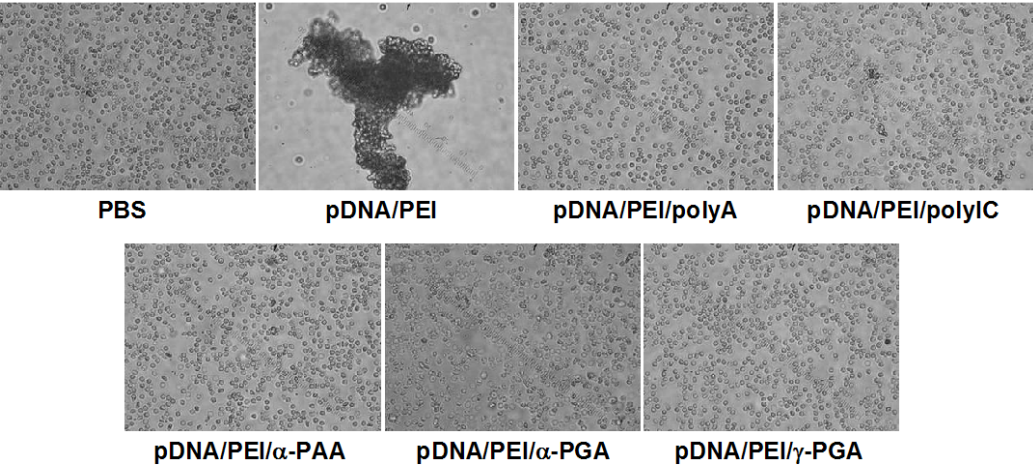


Fig. 3.

(A)



(B)

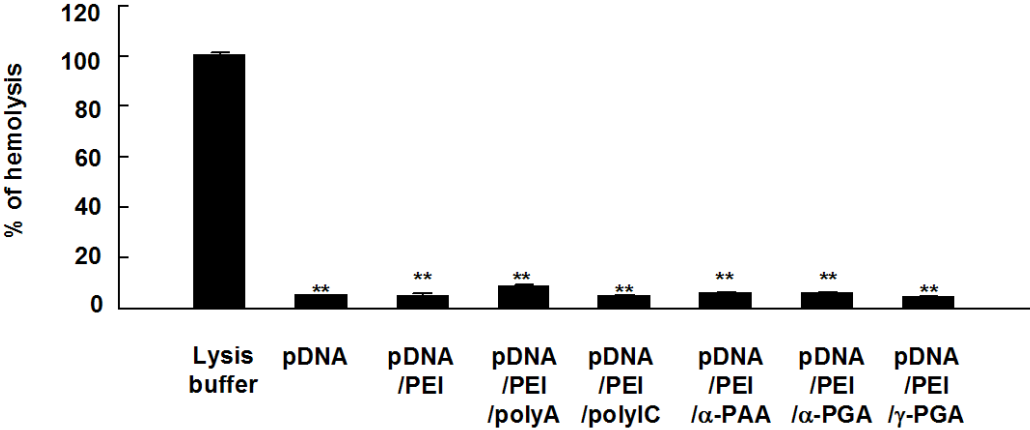


Fig. 4.

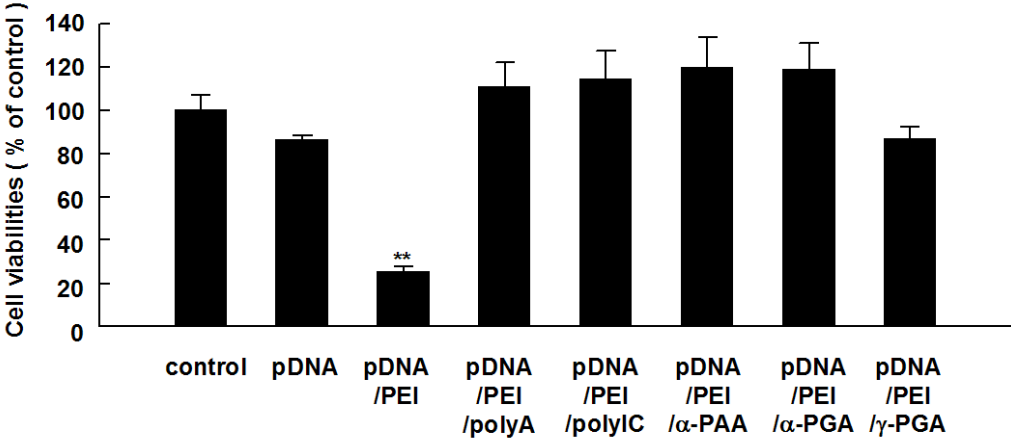
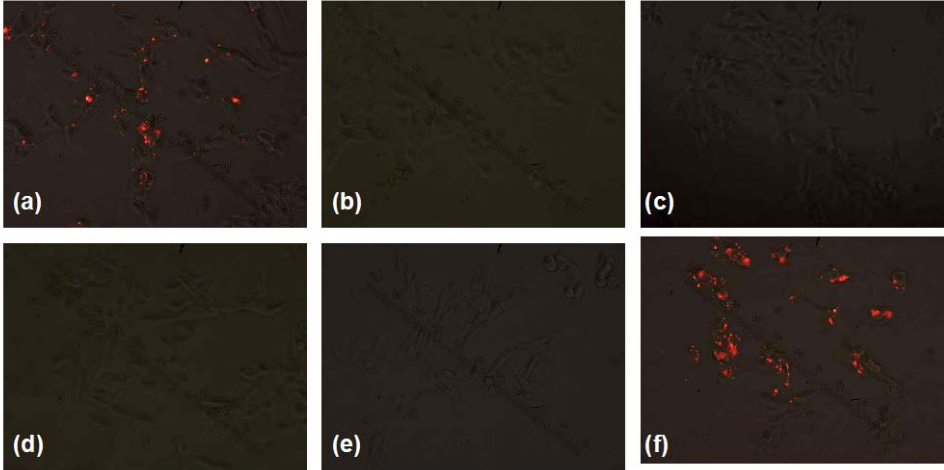


Fig. 5.

(A)



(B)

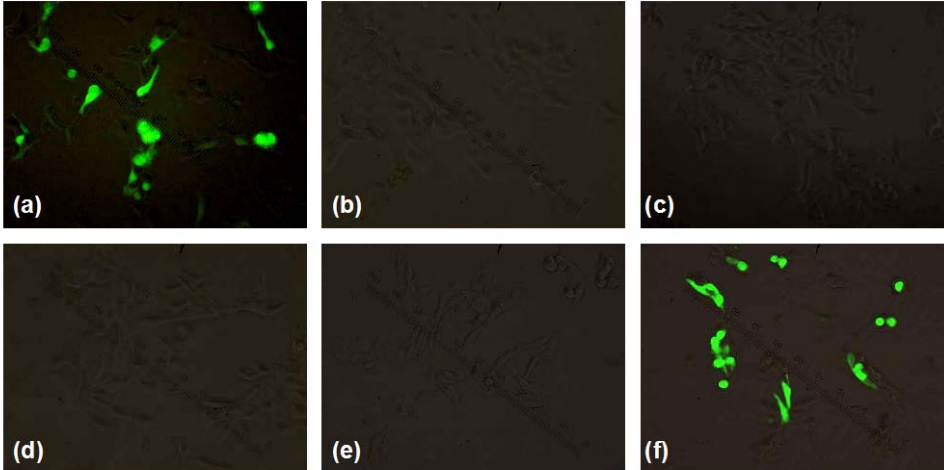


Fig. 6.

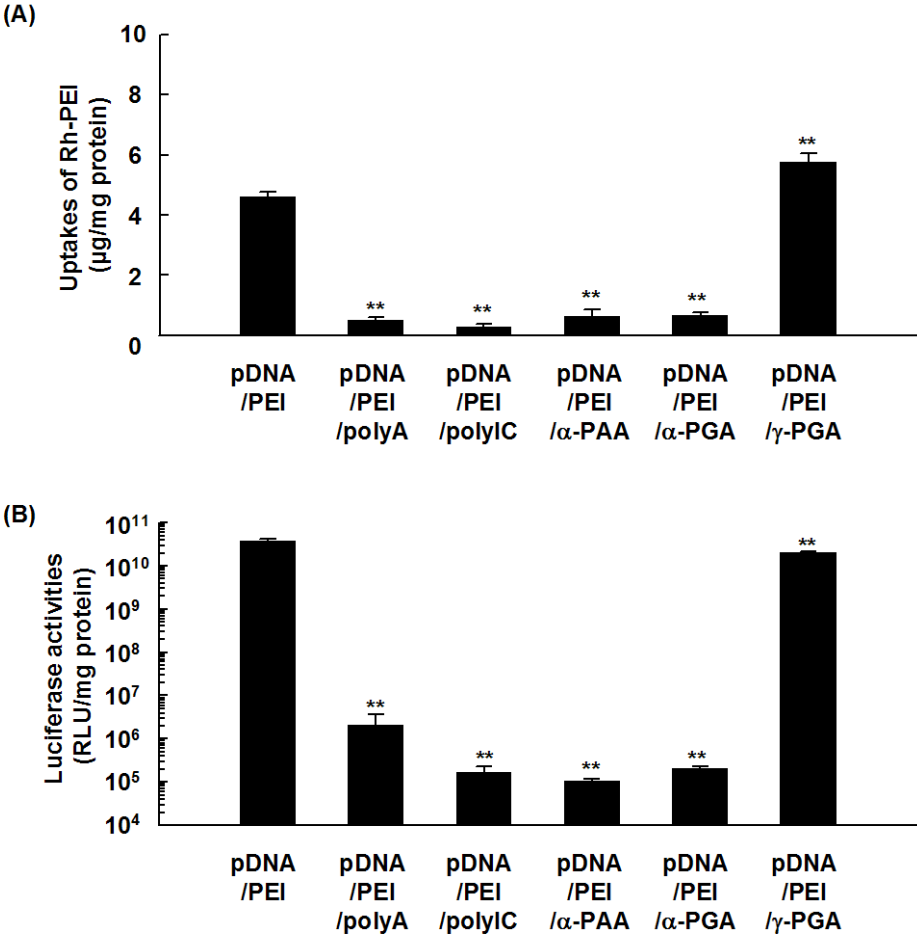


Fig. 7.

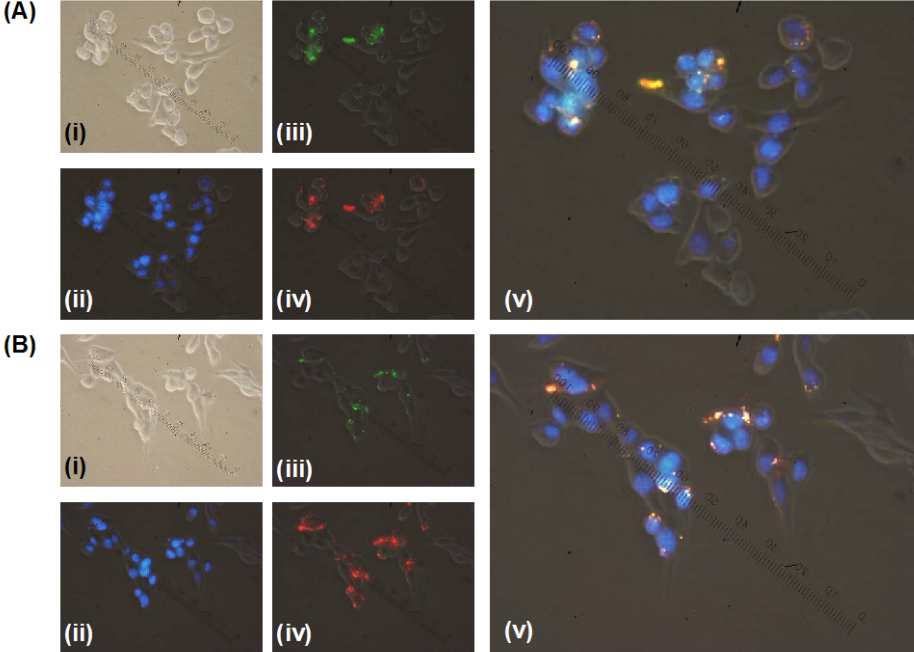


Fig. 8.

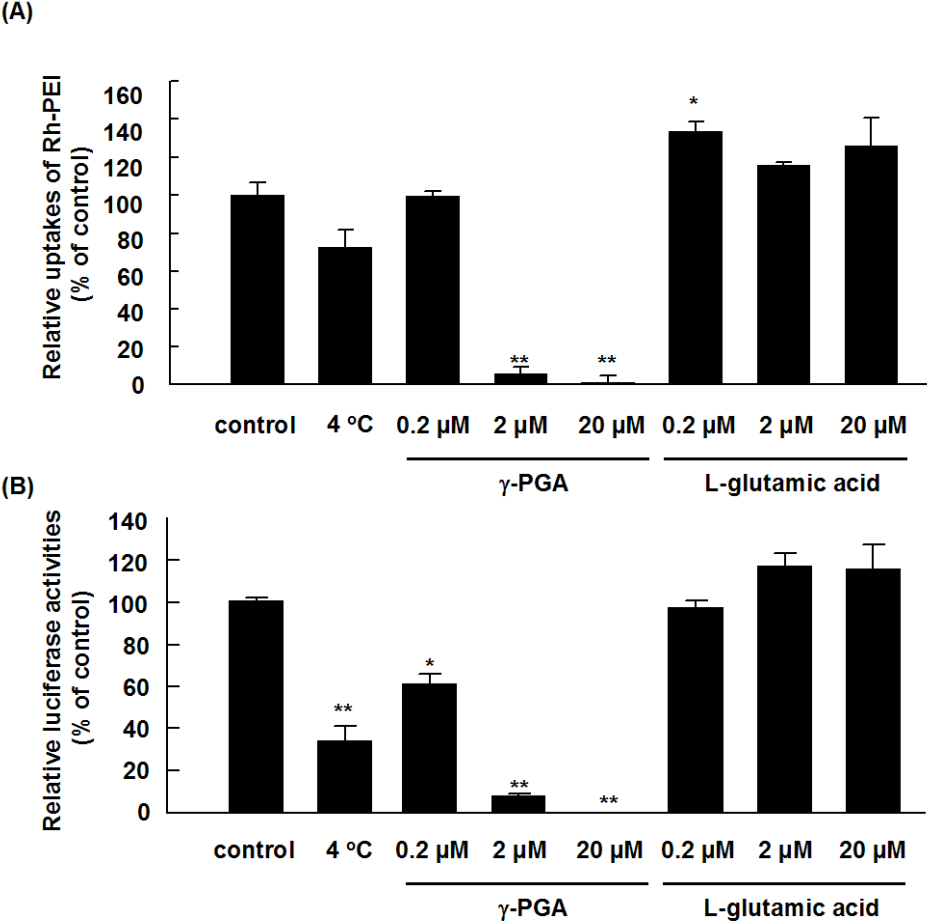


Figure captions and tables

Fig. 1. Formation of pDNA/PEI/polyanion ternary complexes with negative ζ -potential.

Table 1. Particle size and ζ -potential of the complexes.

	Size	ζ -Potential
pDNA/PEI	65.4 \pm 15.8	+55.5 \pm 0.6
pDNA/PEI/polyA	52.0 \pm 14.4	-18.9 \pm 0.4**
pDNA/PEI/polyIC	40.2 \pm 8.3	-19.6 \pm 0.2**
pDNA/PEI/ α -PAA	84.5 \pm 3.2	-39.7 \pm 0.2**
pDNA/PEI/ α -pGA	85.1 \pm 8.0	-22.9 \pm 0.6**
pDNA/PEI/ γ -PGA	88.9 \pm 3.8	-28.4 \pm 1.3**

Each data was represent the mean \pm S.E. (n=3).

**; $P < 0.01$ vs pDNA/PEI

Fig. 2. Effect of each polyanion on electrophoretic migration of pDNA through an agarose gel.

Each complex was loaded onto agarose gel, and electrophoresis was carried out. Retardation of pDNA was visualized using ethidium bromide.

Fig. 3. Interaction of complexes with erythrocytes.

Each complex was added to erythrocytes, and agglutinations (A) and hemolysis activities (B) were assessed.

Agglutination was observed by phase microscopy (400 \times magnification). Hemolysis activity was determined by measuring hemoglobin release at 545 nm. Each bar represents the mean \pm S.E. of three experiments. **: $P < 0.01$ vs lysis buffer.

Fig. 4. Cytotoxicity tests of various complexes on B16-F10 cells.

Cell viability of cells treated with ternary complexes was measured by WST-1 assay. Cells were incubated with various complexes for 2 h and cell viability was measured at 24 h after treatment. Data represent the percentage to untreated cells. Each bar represents the mean \pm S.E. of thirteen experiments. **: $P < 0.01$ vs control.

Fig. 5. Fluorescent microscopy images of B16-F10 cells transfected with ternary complexes.

Cells were transfected with each complex containing pEGFP-C1 and Rh-PEI. Twenty-four hours after transfection, the uptake of Rh-PEI (A) and the expression of GFP (B) were monitored (200 \times magnification). (a): pDNA/PEI; (b): pDNA/PEI/polyA; (c): pDNA/PEI/polyIC; (d): pDNA/PEI/ α -PAA; (e): pDNA/PEI/ α -PGA; (f): pDNA/PEI/ γ -PGA.

Fig. 6. Uptake efficiency (A) and transgene efficiency (B) of ternary complexes.

B16-F10 cells were transfected with complexes containing pCMV-Luc and/or Rh-PEI. Twenty-four hours after transfection, fluorescence of Rh-PEI (A) and luciferase activity (B) were evaluated. Each bar represents the mean \pm S.E. of six experiments. **: $P < 0.01$ vs pDNA/PEI complex.

Fig. 7. Intracellular distribution of pDNA/PEI complex (A) and pDNA/PEI/ γ -PGA complex (B).

Cells were transfected with complexes containing YOYO-1-labeled pDNA and Rh-PEI. Twenty-four hours after transfection, phase contrast image (i), nuclei staining with Hoechst 33342 (ii), YOYO-1-labeled pDNA (iii), Rh-PEI (iv), and merged image (v) are indicated (400 \times magnification).

Fig. 8. Effect of the Inhibitors on the uptake efficiency (A) and transfection efficiency (B) of pDNA/PEI/γ-PGA

complex.

pDNA/PEI/γ-PGA complex was transfected in medium which was at 4 °C or contained various concentrations of

γ-PGA or L-glutamic acid. Twenty-four hours after transfection, fluorescence of Rh-PEI (A) and luciferase activity

(B) were evaluated. Each bar represents the mean ± S.E. of three experiments. *: $P < 0.05$, **: $P < 0.01$ vs

control.