Ocular Gene Delivery Systems Using Ternary Complexes of Plasmid DNA, Polyethylenimine, and Anionic Polymers

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Received August 17, 2012; accepted October 23, 2012

In this experiment, we developed anionic ternary complexes for effective and safe ocular gene delivery. Ternary complexes were constructed by coating plasmid DNA (pDNA)/polyethylenimine (PEI) complex with anionic polymers such as γ-polyglutamic acid (γ-PGA) and chondroitin sulfate (CS). The cationic pDNA/PEI complex showed high gene expression on the human retinal pigment epithelial cell line, ARPE-19 cells. The pDNA/PEI complexes, however, also showed high cytotoxicity on the cells and aggregated strongly in the vitreous body. On the other hand, the anionic ternary complexes showed high gene expression on ARPE-19 cells without such cytotoxicity and aggregation. After intravitreous administration of the complexes, the anionic ternary complexes showed high gene expression in the retina. These results strongly indicate that anionic ternary complexes are suitable for effective and safe ocular gene therapy.

Key words non-viral gene delivery; biomaterial; gene vector; nanoparticle; DNA

Gene therapy is expected to be an effective method for intractable ocular diseases such as retinitis pigmentosa, age-related macular degeneration (AMD), proliferative vitreoretinopathy, and glaucoma.[1–3] The eyes are suitable for topical gene therapy because of their accessibility, ease of monitoring, and their enclosed structure.[2,4,5]

Viral vectors such as adenoviral, lentiviral, and adeno-associated virals are commonly used for ocular gene transfer[6–9]; however, these viral vectors have some crucial problems such as toxicity, immunogenicity, possible genomic integration, limited size of inserted DNA, and difficult preparative procedures.[10,11] Therefore, non-viral vectors such as cationic polymers and cationic liposomes have been focused on because of their non-immunogenicity, low toxicity, well-defined structure, and chemical properties to yield mass production.[5,12,13] The cationic complexes of plasmid DNA (pDNA) with an excess of cationic polymers or cationic liposomes have been reported to show high transgene efficiency in ocular tissues after intraocular injection.[14,15] The cationic complexes, however, cause severe cytotoxicity in retinal pigment epithelial cells and aggregation with the vitreous body in case of intravitreal injection because of their strong cationic surface charge.[16–19] These side effects could decrease visual acuity and even cause blindness.

In the previous study, we investigated the ternary complexes pDNA/polyethylenimine (PEI) coated by polyanions, such as polyadenylic acid, polyinosinic–polycytidylic acid, α-polyaspartic acid, α-polyglutamic acid, γ-polyglutamic acid (γ-PGA), fucoidan, λ-carrageenan, xanthan gum, alginic acid, hyaluronic acid, and chondroitin sulfate (CS).[20,21] Among them, we newly discovered that only the ternary complex coated by γ-PGA or CS showed high gene expression without cytotoxicity after intravenous administration into mice.

In this experiment, we constructed anionic gene delivery vectors, ternary complexes, with pDNA, PEI, and anionic polymers such as γ-PGA and CS and confirmed their usefulness for ocular gene delivery. The ternary complexes showed high transgene efficiencies under *in vitro* and *in vivo* conditions and did not show cytotoxicity or aggregation with the vitreous body.

MATERIALS AND METHODS

Chemicals PEI (branched form, average molecular weight of 25,000) and rhodamine B isothiocyanate were purchased from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). Chondroitin sulfate A sodium salt was obtained from Sigma (St. Louis, MO, U.S.A.). 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, U.S.A.). DMEM-F10, Opti-MEM I, antibiotics (penicillin 100 U/mL and streptomycin 100 μg/mL), and other culture reagents were obtained from GIBCO BRL (Grand Island, NY, U.S.A.). 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). 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.

Preparation of pDNA and Gene Vector pCMV-Luc was constructed by subcloning the Hind III/Xba I firefly luciferase cDNA fragment from the pGL3-control vector (Promega, Madison, WI, U.S.A.) into the polylinker of the pcDNA3 vector (Invitrogen, Carlsbad, CA, U.S.A.). Enhanced green

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fluorescence protein (GFP) encoding the pDNA (pEGFP-C1) was purchased from Clontech (Palo Alto, CA, U.S.A.). 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.

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 anionic polymer was mixed with pDNA/PEI complexes by pipetting and left for another 15 min at room temperature. In this study, we constructed ternary complexes at a theoretical charge ratio: phosphate of pDNA : nitrogen of PEI : carboxylate of γ-PGA or sulfate of CS=1 : 8 : 6.

Physicochemical Property of Ternary Complexes The particle sizes and ζ-potentials of various 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 μL aliquots of various complex solutions containing 1 μg pDNA were mixed with 2 μL loading buffer (30% glycerol and 0.2% bromophenol blue) and loaded onto a 0.8% agarose gel. Electrophoresis (i-Mupid J; Cosmo Bio, Tokyo, Japan) was carried out at 100 V in running buffer solution (40 mM Tris/HCl, 40 mM acetic acid, and 1 mM ethylenediaminetetraacetic acid (EDTA)). The retardation of the pDNA was visualized with ethidium bromide, using a FluorChem Imaging System (Alpha Innotech, CA, U.S.A.).

***In Vitro* Gene Expression Experiment** The human retinal pigment epithelial cell line, ARPE-19 cells, were maintained in DMEM-F10 supplemented with 10% FBS and antibiotics (culture medium) under a humidified atmosphere of 5% CO_2 in air at 37°C. The cells were plated onto 24-well plates (Becton-Dickinson, Franklin Lakes, NL, U.S.A.) at a density 2.5×10^4 cells/well and cultivated in 0.5 mL culture medium. In the transfection experiment for complexes, after 24 h preincubation, the medium was replaced with 0.5 mL Opti-MEM I medium and various complexes containing 1 μg pDNA were then added to each well 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.

To determine the uptake of ternary complexes, ARPE-19 cells were transfected by various complexes constructed with pEGFP-C1, Rh-PEI, and anionic polymers as described above. After 22 h incubation, the relative levels of Rh-PEI and GFP expression were characterized using fluorescent microscopy (200× magnification, ECLPSE TE 200; Nikon, Tokyo, Japan).

To determine transgene efficiency, ARPE-19 cells were transfected by various complexes containing pCMV-Luc, PEI, and anionic polymers as described above. After 22 h incubation, the cells were washed with PBS and then lysed in 100 μL lysis buffer (pH 7.8 and 0.1 M Tris–HCl buffer containing 0.05% Triton X-100 and 2 mM EDTA). Ten microliters of lysate samples were mixed with 50 μL 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 595 nm (Multiskan Spectrum; Thermo Fisher Scientific Inc., Waltham, MA, U.S.A.). Luciferase activity was indicated as relative light units (RLU) per mg protein.

WST-1 Assay Cytotoxicity tests were carried out on ARPE-19 cells using a commercially available WST-1 cell proliferation reagent. ARPE-19 cells were plated onto 96-well plate (Becton-Dickinson, Franklin Lakes, NJ, U.S.A.) at a density of 1.0×10^4 cells/well and cultivated in 100 μL culture medium. Complexes containing 1 μg pDNA in 100 μL Opti-MEM I were added to each well and incubated for 2 h. After transfection, the medium was removed and the cells were cultured for a further 22 h at 37°C with culture medium. After incubation, the medium was replaced with 100 μL culture medium, and 10 μL WST-1 mixture solution (4.95 mM WST-1 and 0.2 mM 1-methoxy PMS) was added to each well and incubated for an additional 2 h at 37°C. The absorbance in each well was measured using a microplate reader at 450 nm with a reference of 630 nm. The results are shown as a percentage of untreated cells (control).

Interaction with Vitreous Body Vitreous body from pigs (Sasebo MeatCenter, Nagasaki, Japan) was centrifuged at 2500 rpm (Kubota 3700; Kubota, Tokyo, Japan) for 10 min and filter through a 0.22 μm filter (Millex-GP; Millipore Co., Bedford, MA, U.S.A.). Various complexes were added at an equal volume to the vitreous body. The solutions were incubated for 15 min at room temperature. For microscopy, the solution was placed on a glass plate and aggregation was monitored (400× magnification). Turbidity was quantified using a microplate reader by measuring the absorbance at a wavelength of 630 nm. The results are shown as a percentage of pDNA/PEI complexes.

***In Vivo* Gene Expression Experiment** Male white Japanese rabbits (KBT Oriental, Tosu, Japan) weighing 2.5–3.0 kg were individually housed in cages under controlled temperature (21°C) and humidity (50±5%) and a 12 : 12 h light/dark cycle at the Laboratory Animal Center for Biomedical Research, Nagasaki University. Each complex containing 20 μg pDNA was injected into the vitreous body of rabbits (100 μL per eye). The rabbits were sacrificed 24 h after the injection. The retina was dissected and homogenized with lysis buffer using a homogenizer (Omni TH-115; Yamato Scientific Co., Ltd., Tokyo, Japan). After the centrifugation of homogenates at 15000 rpm for 5 min, 10 μL supernatant samples were mixed with 50 μL luciferase assay buffer and the light produced was immediately measured using a luminometer. Luciferase activity was indicated as relative light units (RLU) per g tissue.

Animal care and experimental procedures were performed in accordance with the Guidelines for Animal Experimentation of Nagasaki University with approval from the Institutional Animal Care and Use Committee.

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

RESULTS

Physicochemical Characteristics and Electrophoresis Assay The pDNA/PEI complexes with a charge ratio of 1 : 8 had high transgene efficiency under *in vitro* and *in vivo* conditions.[22,23] Ternary complexes (pDNA/PEI/anionic polymer) with a charge ratio of 1 : 8 : 6 showed high transgene efficiency without toxicity *in vitro* in previous experiments

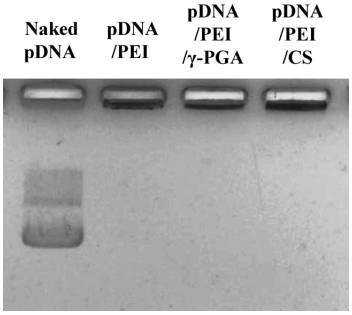

Fig. 1. Gel Retardation Assay

Various complexes were loaded onto agarose gel and electrophoresis was carried out. Retardation of pDNA was visualized using ethidium bromide.

Table 1. Particle Size and ζ-Potential

Complex	Particle size (nm)	ζ-Potential (mV)
pDNA/PEI	55.8±13.1	46.1±0.1
pDNA/PEI/γ-PGA	71.2±16.9	−32.8±0.2**
pDNA/PEI/CS	83.2±2.3	−28.3±1.2**

Each value represents the mean±S.E. (n=3). **p<0.01 *vs.* pDNA/PEI.

and were used throughout the present study.[20,21] The particle sizes and ζ-potentials of these complexes were measured with Zetasizer Nano ZS and are shown in Table 1. The pDNA/PEI complexes showed 55.8±13.1 nm particle size and 46.1±0.1 mV ζ-potential. Ternary complexes, however, showed anionic charge and significantly lower ζ-potentials than the pDNA/PEI complexes (p<0.01), although the addition of anionic polymers did not greatly affect the size of the complexes. Secondary, stability of the complexes was determined by a gel retardation assay (Fig. 1). Naked pDNA was detected as bands on agarose gel. The pDNA/PEI complexes and the ternary complexes did not show any bands of naked pDNA, indicating the formation of stable complexes including pDNA.

Evaluation of Uptake and Gene Expression by Fluorescent Microscopy The uptake of the complexes and their gene expressions were visualized in ARPE-19 cells after transfection with complexes containing Rh-PEI and pEGFP-C1 (Figs. 2A, B). In the pDNA/PEI complexes and pDNA/PEI/γ-PGA complexes, red dots of Rh-PEI and bright green florescence of GFP were highly observed in most cells. On the other hands, few cells showed red dots and bright green fluorescence in the pDNA/PEI/CS complexes.

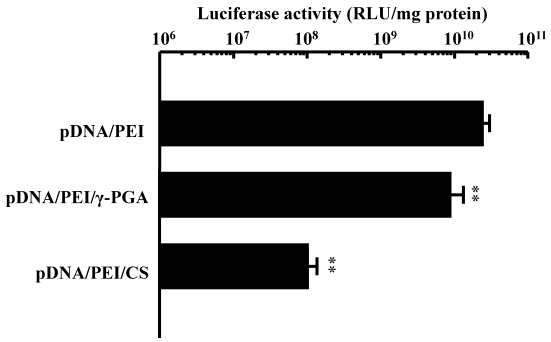

Fig. 3. Transfection Efficiency of Various Complexes

ARPE-19 cells were incubated with the complexes for 2 h. At 22 h after transfection, cells were lysed for quantification of luciferase activity. Each bar represents the mean±S.E. (n=3). **p<0.01 *vs.* pDNA/PEI.

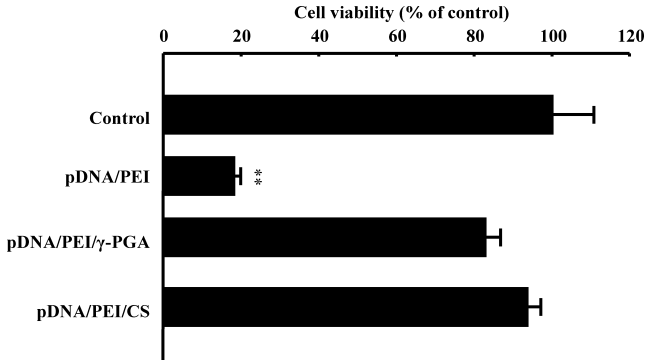

Fig. 4. Cytotoxicity of Various Complexes

Cell viability of ARPE-19 cells was evaluated by WST-1 assay. Cells were incubated with complexes for 2 h and cell viability was measured at 24 h after transfection. Data are the percentage to untreated cells. Each bar represents the mean±S.E. (n=8). **p<0.01 *vs.* control.

Determination of Transgene Efficiency Transgene efficiency was quantitatively determined as luciferase activity in ARPE-19 cells after transfection with various complexes

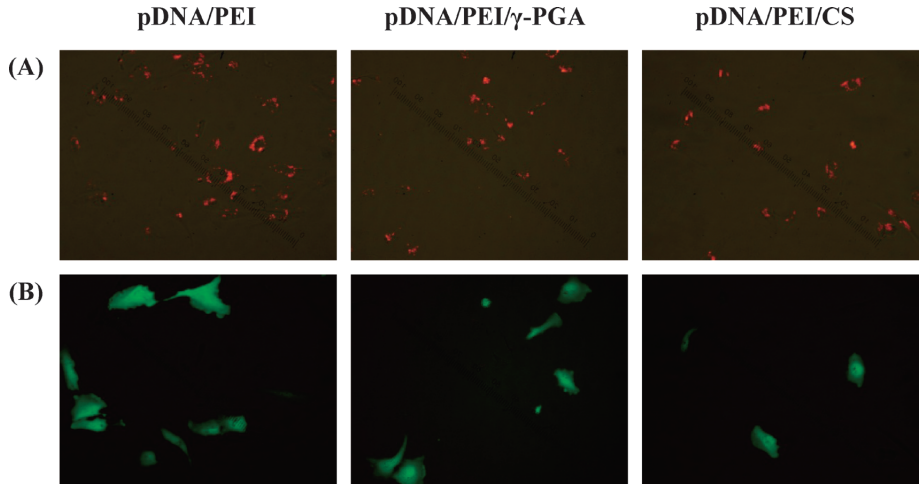

Fig. 2. Fluorescent Microscopy Images of ARPE-19 Cells Transfected with Various Complexes

Cells were transfected with various complexes containing Rh-PEI and pEGFP-C1. Twenty-four hours after transfection, the uptake of Rh-PEI (A) and GFP expression (B) were observed (200× magnification).

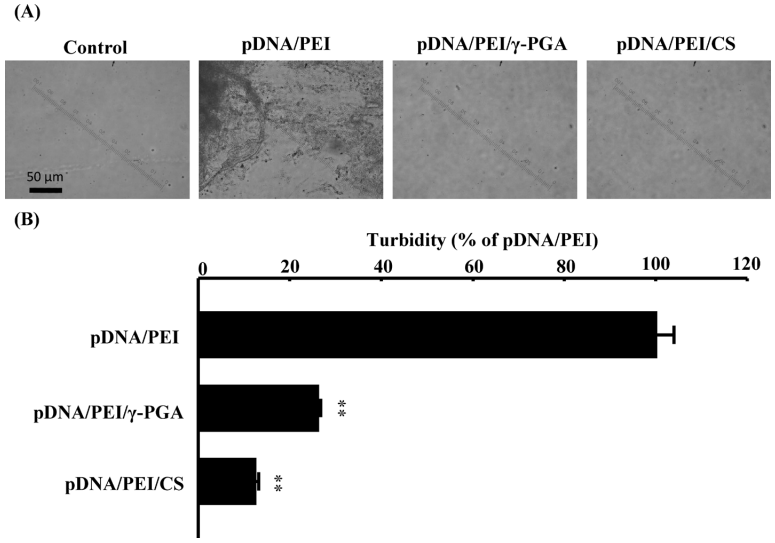

Fig. 5. Interaction of Complexes with Vitreous Body

Various complexes were added to the vitreous body, and agglutination (A) and turbidity of vitreous body (B) was assessed. Agglutination was observed by phase microscopy (400× magnification). Turbidity of vitreous body was determined by measuring the absorbance at a wavelength of 630 nm. Each bar represents the mean±S.E. (n=3). **p<0.01 *vs.* pDNA/PEI.

containing pCMV-Luc (Fig. 3). The pDNA/PEI complexes showed higher transgene efficiency than ternary complexes (p<0.01). The pDNA/PEI/γ-PGA complexes, however, exceeded 10^9 RLU/mg protein in transgene efficiency.

Cytotoxicity Study In order to determine the cytotoxicity of the complexes, the viability of ARPE-19 cells was determined by WST-1 assay after incubation with the complexes (Fig. 4). The pDNA/PEI complexes showed significantly higher cytotoxicity than the control (p<0.01). On the other hand, little cytotoxicity was observed in the ternary complexes.

Interaction with Vitreous Body The aggregation of the complexes in the vitreous body and the turbidity of vitreous body mixed with the complexes were determined to evaluate the suitability of the complexes for the intraocular injection. Aggregation was observed by microscopy after addition of the complexes to the vitreous body (Fig. 5A). The pDNA/PEI complexes showed severe aggregation but no aggregation was observed in ternary complexes. Figure 5B shows the turbidity of the vitreous body mixed with various complexes. The pDNA/PEI complexes increased turbidity; however, ternary complexes showed lower turbidity than pDNA/PEI complexes (p<0.01).

***In Vivo* Gene Expression Experiment** The transgene efficiency of the complexes in the post-vitreous organ, the retina, was determined as luciferase activity 24 h after their intravitreal injection in white male Japanese rabbits (Fig. 6). All complexes showed transgene efficiency over 10^5 RLU/g tissue in the retina. The pDNA/PEI/γ-PGA complexes showed higher transgene efficiency than pDNA/PEI complexes. On the other hand, pDNA/PEI/CS complexes showed lower transgene efficiency than pDNA/PEI complexes.

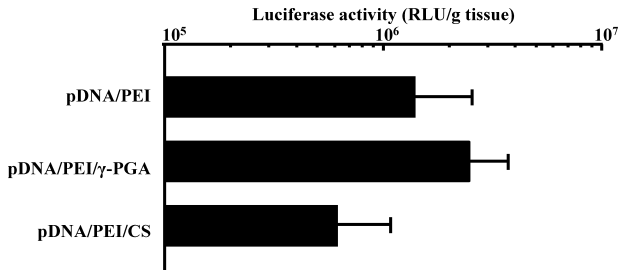

Fig. 6. Transfection Efficiency of Various Complexes in Rabbits

The complexes were injected intravitreally into rabbits (20 μg pDNA per rabbit). At 24 h after injection, rabbits were sacrificed and the retina was dissected for quantification of luciferase activity. Each bar represents the mean±S.E. (n=3).

DISCUSSION

Non-viral vectors such as cationic polymers and cationic liposomes hold great promise for ocular diseases. Among them, PEI is widely used and shows high transgene efficiency *in vitro* and *in vivo* because of specific mechanisms such as condensing pDNA by electrostatic interaction, binding to the cell surface, being taken up by the endocytotic pathway, and releasing pDNA into cytoplasm.[24–28] On the other hand, it is reported that PEI shows high cytotoxicity and binds non-specifically to blood proteins and extracellular matrix components such as proteoglycans.[29,30] To reduce these toxicities, some reports have developed ternary complexes coated with polyethyleneglycol copolymers, human serum, polyacrylic acid, and polysaccharides.[31–34] Hornof *et al.* also demonstrated that pDNA/PEI complexes coated with hyaluronan reduced toxicity in corneal epithelial cells.[35]

In our previous studies, we developed ternary complexes, pDNA/PEI complex coated with γ-PGA or CS, showing high transgene efficiency without cytotoxicity or aggregation with erythrocytes.[20,21] These safe and effective gene delivery vectors were also expected to be applied to intraocular gene delivery for posterior segment diseases. In the present study, we investigated the usefulness of these ternary complexes as ocular gene delivery vectors.

Physicochemical characteristics such as the particle size and ζ-potential of pDNA/PEI complexes and ternary complexes were determined and are shown in Table 1. Generally, cellular uptake and distribution of complexes depend on the size of complexes. Sakurai *et al.* reported that nanoparticles

smaller than 200 nm were observed in retinal cells other than the vitreous cavity and trabecular meshwork where only larger diameter particles were distributed after intravitreal injection.[36] Thus, small particle size is an important factor for effective ocular gene delivery. The pDNA/PEI complexes showed 55.8±13.1 nm particle size. Ternary complexes coated with anionic polymers were as small as pDNA/PEI complexes regardless of the addition of anionic polymer. The addition of anionic polymer can possibly destabilize the electrostatic formation of pDNA/PEI complexes; therefore, we examined the formation of ternary complexes by a gel retardation assay. In all complexes, the band of pDNA was not detected when naked pDNA was detected as bands on agarose gel, as shown in Fig. 1. Furthermore, the ternary complexes had negative ζ-potential, as shown in Table 1. These results indicate that γ-PGA or CS was adsorbed onto the surface of pDNA/PEI complexes, leading to the formation of ternary complexes with negative surface charges.

Firstly, the *in vitro* experiments were carried out with the human retinal pigment epithelial cell line, ARPE-19 cells. Cationic pDNA/PEI complexes have been reported to show high transgene efficiency[20,21,24]; however, they are known to have strong cytotoxicity by interaction with the negative surface of the cellular membrane.[29] In fact, pDNA/PEI complexes were taken up highly by the cells and showed high GFP expression and luciferase activity in ARPE-19 cells (Figs. 2A, B, 3). At the same time, pDNA/PEI complexes also showed high cytotoxicity in the cells (Fig. 4).

On the other hand, anionic complexes are generally considered to have little interaction with negatively charged cell surfaces, suggesting low cytotoxicity and low gene expression, and the anionic ternary complexes did in fact show low cytotoxicity (Fig. 4). The anionic complexes in the present study, however, showed high uptake of Rh-PEI and high GFP expression by fluorescent microscopy of ARPE-19 cells (Figs. 2A, B). The high transgene efficiency of these ternary complexes was also confirmed by their luciferase activity (Fig. 3). We previously reported these specific phenomena in a mouse melanoma cell line, B16-F10 cells, and demonstrated the specific uptake pathways of these ternary complexes.[20,21] The process of gene transfection was involved in condensing pDNA by electrostatic interaction, binding to the cell surface, take up by the endocytotic pathway, and release of pDNA into cytoplasm. The higher gene expression of γ-PGA complexes compared to CS complexes may be explained by functional differences in these processes. Further experiment is necessary to clarify the mechanism.

For effective and safe retina gene delivery, the complexes must diffuse through the vitreous body before they can reach the retina without aggregation with the vitreous body. The vitreous body is a highly hydrated (98% water) transparent bio-gel containing anionic polymers such as hyaluronan (65–400 μg/mL), chondroitin sulfate, and heparan sulfate.[17,37] Cationic gene delivery vectors interact with these components of the vitreous body, and it was reported that cationic liposomes aggregated with the vitreous body *in vitro*.[16] In this experiment, cationic pDNA/PEI complexes also aggregated with the vitreous body and made the vitreous body turbid. Aggregation of the vitreous body might lead to fatal adverse reactions such as visual degradation and blindness. Nevertheless, the anionic ternary complexes showed no aggregation and low turbidity, indicating their biocompatibility and clinical safety in ocular application (Fig. 5).

Based on these results, we examined the *in vivo* transgene efficiency of ternary complexes in the retina as luciferase activity after their intravitreal injection into rabbits. High luciferase activities of the pDNA/PEI complexes and ternary complexes were observed in the retina (Fig. 6). In particular, pDNA/PEI/γ-PGA complexes showed the highest transgene efficiency. Aggregations of pDNA/PEI complexes were immobilized in the vitreous body; consequently, only a small amount of pDNA/PEI complexes might have reached the neural retina or the retinal pigment epithelial cells.

Thus, we have developed a safe and efficient gene delivery vector, ternary complexes, for ocular gene delivery. Ternary complexes will be very useful for the treatment of posterior segment diseases. Further experiments may be necessary to investigate their clinical usefulness.

Acknowledgements This study was supported in part by the Global COE Program, Nagasaki University, Japan and a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

REFERENCES

1) Naik R, Mukhopadhyay A, Ganguli M. Gene delivery to the retina: focus on non-viral approaches. *Drug Discov. Today*, **14**, 306–315 (2009).
2) Peeters L, Sanders NN, Demeester J, De Smedt SC. Challenges in non-viral ocular gene transfer. *Biochem. Soc. Trans.*, **35**, 47–49 (2007).
3) Binley K, Widdowson PS, Kelleher M, de Belin J, Loader J, Ferrige G, Carlucci M, Esapa M, Chipchase D, Angell-Manning D, Ellis S, Mitrophanous K, Miskin J, Bantseev V, Nork TM, Miller P, Naylor S. Safety and biodistribution of an equine infectious anemia virus-based gene therapy, RetinoStat®, for age-related mascular degeneration. *Hum. Gene Ther.*, **23**, 980–991 (2012).
4) Colella P, Cotugno G, Auricchio A. Ocular gene therapy: current progress and future prospects. *Trends Mol. Med.*, **15**, 23–31 (2009).
5) Bloquel C, Bourges JL, Touchard E, Berdugo M, BenEzra D, Behar-Cohen F. Non-viral ocular gene therapy: potential ocular therapeutic avenues. *Adv. Drug Deliv. Rev.*, **58**, 1224–1242 (2006).
6) Acland GM, Aguirre GD, Ray J, Zhang Q, Aleman TS, Cideciyan AV, Pearce-Kelling SE, Anand V, Zeng Y, Maguire AM, Jacobson SG, Hauswirth WW, Bennett J. Gene therapy restores vision in a canine model of childhood blindness. *Nat. Genet.*, **28**, 92–95 (2001).
7) Campochiaro PA. Gene transfer for ocular neovascularization and macular edema. *Gene Ther.*, **19**, 121–126 (2012).
8) Bainbridge JW, Mistry A, De Alwis M, Paleolog E, Baker A, Thrasher AJ, Ali RR. Inhibition of retinal neovascularisation by gene transfer of soluble VEGF receptor sFlt-1. *Gene Ther.*, **9**, 320–326 (2002).
9) Ralph GS, Binley K, Wong LF, Azzouz M, Mazarakis ND. Gene therapy for neurodegenerative and ocular diseases using lentiviral vectors. *Clin. Sci.*, **110**, 37–46 (2006).
10) Cai X, Conley S, Naash M. Nanoparticle applications in ocular gene therapy. *Vision Res.*, **48**, 319–324 (2008).
11) Thomas CE, Ehrhardt A, Kay MA. Progress and problems with the use of viral vectors for gene therapy. *Nat. Rev. Genet.*, **4**, 346–358 (2003).
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.*, **4**, 823–832 (1997).
13) Mario CF, Nigel CP. Major limitations in the use of cationic

liposomes for DNA delivery. *Int. J. Pharm.*, **162**, 159–170 (1998).
14) Kawakami S, Harada A, Sakanaka K, Nishida K, Nakamura J, Sakaeda T, Ichikawa N, Nakashima M, Sasaki H. *In vivo* gene transfection *via* intravitreal injection of cationic liposome/plasmid DNA complexes in rabbits. *Int. J. Pharm.*, **278**, 255–262 (2004).
15) Liao HW, Yau KW. *In vivo* gene delivery in the retina using polyethylenimine. *Biotechniques*, **42**, 285–286, 288 (2007).
16) Pitkänen L, Ruponen M, Nieminen J, Urtti A. Vitreous is a barrier in nonviral gene transfer by cationic lipids and polymers. *Pharm. Res.*, **20**, 576–583 (2003).
17) Peeters L, Sanders NN, Braeckmans K, Boussery K, Van de Voorde J, De Smedt SC, Demeester J. Vitreous: a barrier to nonviral ocular gene therapy. *Invest. Ophthalmol. Vis. Sci.*, **46**, 3553–3561 (2005).
18) Kim H, Robinson SB, Csaky KG. Investigating the movement of intravitreal human serum albumin nanoparticles in the vitreous and retina. *Pharm. Res.*, **26**, 329–337 (2009).
19) Urtti A, Polansky J, Lui GM, Szoka FC. Gene delivery and expression in human retinal pigment epithelial cells: effects of synthetic carriers, serum, extracellular matrix and viral promoters. *J. Drug Target.*, **7**, 413–421 (2000).
20) Kurosaki T, Kitahara T, Fumoto S, Nishida K, Nakamura J, Niidome T, Kodama Y, Nakagawa H, To H, Sasaki H. Ternary complexes of pDNA, polyethylenimine, and gamma-polyglutamic acid for gene delivery systems. *Biomaterials*, **30**, 2846–2853 (2009).
21) Kurosaki T, Kitahara T, Kawakami S, Nishida K, Nakamura J, Teshima M, Nakagawa H, Kodama Y, To H, Sasaki H. The development of a gene vector electrostatically assembled with a polysaccharide capsule. *Biomaterials*, **30**, 4427–4434 (2009).
22) Lee JH, Ahn HH, Kim KS, Lee JY, Kim MS, Lee B, Khang G, Lee HB. Polyethyleneimine-mediated gene delivery into rat pheochromocytoma PC-12 cells. *J. Tissue Eng. Regen. Med.*, **2**, 288–295 (2008).
23) Ahn HH, Lee JH, Kim KS, Lee JY, Kim MS, Khang G, Lee IW, Lee HB. Polyethyleneimine-mediated gene delivery into human adipose derived stem cells. *Biomaterials*, **29**, 2415–2422 (2008).
24) Godbey WT, Wu KK, Mikos AG. Poly(ethylenimine) and its role in gene delivery. *J. Control. Release*, **60**, 149–160 (1999).
25) Kircheis R, Wightman L, Wagner E. Design and gene delivery activity of modified polyethylenimines. *Adv. Drug Deliv. Rev.*, **53**, 341–358 (2001).
26) Boussif O, Lezoualc'h F, Zanta MA, Mergny MD, Scherman D, Demeneix B, Behr JP. A versatile vector for gene and oligonucleotide transfer into cells in culture and *in vivo*: polyethylenimine. *Proc. Natl. Acad. Sci. U.S.A.*, **92**, 7297–7301 (1995).
27) Kichler A, Leborgne C, Coeytaux E, Danos O. Polyethylenimine-mediated gene delivery: a mechanistic study. *J. Gene Med.*, **3**, 135–144 (2001).
28) 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.*, **6**, 76–84 (2004).
29) Lv H, Zhang S, Wang B, Cui S, Yan J. Toxicity of cationic lipids and cationic polymers in gene delivery. *J. Control. Release*, **114**, 100–109 (2006).
30) Ruponen M, Ylä-Herttuala S, Urtti A. Interactions of polymeric and liposomal gene delivery systems with extracellular glycosaminoglycans: physicochemical and transfection studies. *Biochim. Biophys. Acta*, **1415**, 331–341 (1999).
31) Finsinger D, Remy JS, Erbacher P, Koch C, Plank C. Protective copolymers for nonviral gene vectors: synthesis, vector characterization and application in gene delivery. *Gene Ther.*, **7**, 1183–1192 (2000).
32) Trubetskoy VS, Wong SC, Subbotin V, Budker VG, Loomis A, Hagstrom JE, Wolff JA. Recharging cationic DNA complexes with highly charged polyanions for *in vitro* and *in vivo* gene delivery. *Gene Ther.*, **10**, 261–271 (2003).
33) Carrabino S, Di Gioia S, Copreni E, Conese M. Serum albumin enhances polyethylenimine-mediated gene delivery to human respiratory epithelial cells. *J. Gene Med.*, **7**, 1555–1564 (2005).
34) Pathak A, Patnaik S, Gupta KC. Polyethylenimine derived nanoparticles for efficient gene delivery. *Nucleic Acids Symp. Ser.* (Oxf.), **53**, 57–58 (2009).
35) Hornof M, de la Fuente M, Hallikainen M, Tammi RH, Urtti A. Low molecular weight hyaluronan shielding of DNA/PEI polyplexes facilitates CD44 receptor mediated uptake in human corneal epithelial cells. *J. Gene Med.*, **10**, 70–80 (2008).
36) Sakurai E, Ozeki H, Kunou N, Ogura Y. Effect of particle size of polymeric nanospheres on intravitreal kinetics. *Ophthalmic Res.*, **33**, 31–36 (2001).
37) Bishop P. The biochemical structure of mammalian vitreous. *Eye* (Lond.), **10**, 664–670 (1996).