

γ -Polyglutamic acid-coated vectors for effective and safe gene therapy

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Abstract

In the present study, we developed some novel gene delivery vectors, coated cationic complexes with γ -polyglutamic acid (γ -PGA) for effective and safe gene therapy. Cationic complexes were constructed with pDNA and cationic vectors, such as poly-L-arginine hydrochloride (PLA), poly-L-lysine hydrobromide (PLL), N-[1-(2, 3-dioleyloxy) propyl]-N, N, N-trimethylammonium chloride (DOTMA)-cholesterol (Chol) liposomes, and DOTMA-dioleoylphosphatidylethanolamine (DOPE) liposomes. The cationic complexes showed high gene expression with strong cytotoxicity in melanoma B16-F10 cells. The cationic complexes were also strongly toxic to erythrocytes. On the other hand, the γ -PGA was able to coat all cationic complexes and form stable nano-sized particles with negative charges. These γ -PGA-coated complexes had high gene expression without cytotoxicity and toxicities to the erythrocytes. In *in vivo* transfection experiments, polyplexes showed high transfection efficiency over 10^5 RLU/g lung tissue after intravenous injection, although γ -PGA-coated polyplexes showed a high value in the spleen. High transfection efficiency in lipoplexes and γ -PGA-coated lipoplexes was observed in the spleen and lung. Thus, γ -PGA-coated vectors are useful for clinical gene therapy.

Key words: non-viral gene delivery; cationic polymer; cationic liposome; ternary complex; γ -polyglutamic acid.

1. Introduction

Gene therapy, which involves the intracellular delivery of DNA either to block a dysfunctional gene or to deliver a therapeutic gene, has huge potential for treating intractable diseases such as cancer and cardiovascular disorders [1,2]. Gene delivery vectors are categorized as viral or non-viral vectors. In light of recent concerns about safety, non-viral gene delivery has emerged as a promising method, because non-viral vectors have advantages such as much lower immunotoxicity, a clear structure, and easy modeling [3-5].

Among non-viral vectors, cationic polymers and cationic liposomes have often been used to form stable cationic complexes with pDNA as polyplexes and lipoplexes, respectively, and show high gene expression *in vitro* and *in vivo* [6,7]. These cationic complexes, however, have been reported to show cytotoxicity and agglutination by their strong cationic surface charge [8,9].

Recharging cationic complexes with anionic compound was reported to be a promising method for overcoming these adverse effects [10,11]. In a previous study, we discovered an anionic coating, γ -polyglutamic acid (γ -PGA), which decreased the toxicities of cationic complexes without reductions of efficacies [12].

In the present study, we evolved the polyplexes and lipoplexes to the safe and effective gene delivery vectors using γ -PGA for clinical gene therapy. We constructed the cationic cores with pDNA and cationic peptide such as poly-L-arginine hydrochloride (PLA) and poly-L-lysine hydrobromide (PLL) or cationic liposomes such as N-[1-(2, 3-dioleoyloxy) propyl]-N, N, N-trimethylammonium chloride (DOTMA)-cholesterol (Chol) liposomes and DOTMA-dioleoylphosphatidylethanolamine (DOPE) liposomes; and we successfully coated the cationic cores with γ -PGA. Then, γ -PGA-coated vectors showed high efficiencies with markedly low toxicities.

2. Materials and Methods

2.1. Chemicals

PLA (average molecular weight 22500), PLL (average molecular weight 10000), and bovine serum albumin (BSA) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). DOTMA was purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). Chol was obtained from Nacalai Tesque, Inc. (Kyoto, Japan). DOPE was purchased from Nippon Oil and Fats Co. (Tokyo, Japan). γ -PGA was provided by Yakult Pharmaceutical 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). All other chemicals were of the highest purity available.

2.2. Construction of pDNA

pCMV-luciferase (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 pDNA (pEGFP-C1) was purchased from Clontech (Palo Alto, CA, USA). pDNA was amplified using an EndoFree[®] Plasmid Giga Kit (QIAGEN GmbH, Hilden, Germany), dissolved in 5% dextrose solution to 1 mg/mL, and stored at -80 °C until analysis.

2.3. Preparation of Cationic Liposomes

DOTMA-Chol (1:1 molar ratio) and DOTMA-DOPE (1:1 molar ratio) liposomes were prepared according to a previous report [13]. The lipids were dissolved in chloroform and mixtures of each lipid were dried as a thin film in a test tube using an evaporator at 25 °C, and

then vacuum-desiccated for approximately 4 h. The film was resuspended in 5% sterile dextrose. After hydration, the dispersions were sonicated at 100 W (sonicator; Ohtake Works Co., Tokyo, Japan) for 3 min on ice. The resulting liposomes were extruded 11 times through double-stacked 100 nm polycarbonate membrane filters.

2.4. Preparation of Complexes

The theoretical charge ratio of cationic polymers and liposomes to pDNA was calculated as the molar ratio of polymers and liposomes nitrogen to pDNA phosphate. To prepare the binary complexes, appropriate amounts of stock polymer solution or liposome solution were mixed with a diluted solution of pDNA by pipetting thoroughly at a charge ratio of 8 for polyplexes or 2 for lipoplexes, and left for 15 min at room temperature. To prepare γ -PGA-coated complexes, γ -PGA solution was added to binary complexes at a charge ratio of 6, and left for another 15 min at room temperature (Fig. 1). The theoretical charge ratio of γ -PGA to pDNA was calculated as the molar ratio of γ -PGA carboxylate to pDNA phosphate.

2.5. Physicochemical Property and Gel Retardation Assay

The particle size and ζ -potential of the complexes were measured with 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 complex solution containing 1 μ g pDNA were mixed with 2 μ L loading buffer (30% glycerol and 0.2% bromophenol blue) and loaded onto a 0.8% agarose gel containing 0.1 μ g/mL ethidium bromide. Electrophoresis (Mupid[®]-2plus; ADVANCE, Tokyo, Japan) was carried out at 50 V in running buffer solution (40 mM Tris/HCl, 40 mM acetic acid, and 1 mM EDTA) for 60 min. The retardation of the pDNA was visualized using a FluorChem Imaging System (Alpha Innotech, CA, USA).

2.6. Transfection Experiment

The mouse melanoma cell line, B16-F10 cells, was obtained from the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer Tohoku University.

B16-F10 cells were maintained in culture medium (RPMI 1640 supplemented with 10% FBS and antibiotics) under a humidified atmosphere of 5% CO₂ in air at 37 °C. B16-F10 cells were plated on 24-well collagen-containing plates (Becton-Dickinson, Franklin Lakes, NJ, USA) at a density of 1.5×10^4 cells/well and cultivated in 1.0 mL culture medium.

In the transfection experiment, after 24 h pre-incubation, the medium was replaced with 1 mL Opti-MEM[®] I medium and each complex containing 1 µg pCMV-Luc 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. 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 the Bradford assay using BSA as a standard. Absorbance was measured using a microplate reader (Multiskan Spectrum; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 570 nm. Luciferase activity was indicated as relative light units (RLU) per mg protein.

To visualize gene expressions, B16-F10 cells were transfected by complexes containing pEGFP-C1. After 22 h incubation, the relative levels of GFP expression were characterized using fluorescent microscopy (200× magnification).

For experiments using inhibitors, the cells were transfected by the various γ -PGA-coated complexes in Opti-MEM[®] I medium containing 20 µM of L-glutamic acid or γ -PGA. After transfection, medium was replaced with culture medium and cells were cultured for a further 22 h at 37 °C, and then the luciferase activities were determined as described above.

2.7. WST-1 Assay

Cytotoxicity tests of complexes on B16-F10 cells were carried out using a WST-1 commercially available cell proliferation reagent. 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 culture medium. Complexes containing 1 µg 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 μ L culture medium and incubated for another 22 h. Medium was then replaced with 100 μ L culture medium and 10 μ L WST-1 reagent (4.95 mM WST-1 and 0.2 mM 1-methoxy PMS) 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.

2.8. Animals

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. Male ddY mice (5–6 weeks old) were purchased from Japan SLC (Shizuoka, Japan). After shipping, mice were acclimatized to the environment for at least one week before the experiments.

2.9. Interaction with Erythrocytes

Erythrocytes from mice were washed three times at 4 °C by centrifugation at 5000 rpm (Kubota 3700; Kubota, Tokyo, Japan) for 5 min and resuspended in PBS. A 2% (v/v) stock suspension of erythrocytes was prepared for agglutination study. The complexes were added to the erythrocyte suspension and incubated for 15 min at room temperature. The 10 μ L sample was placed on a glass plate and agglutination was observed by microscopy (400 \times magnifications). For hemolysis study, 5% stock suspensions of erythrocytes were prepared. The complexes were added to erythrocytes and incubated for 1 h at room temperature. After incubation, the suspensions were centrifuged at 5000 rpm for 5 min, and supernatants were taken. Hemolysis was quantified by measuring hemoglobin release at 545 nm using a microplate reader. Lysis buffer was added to erythrocytes and used for the 100% hemolysis sample.

2.10. In Vivo Gene Expression Experiment

Complexes, including 40 μ g pCMV-Luc, were prepared for *in vivo* gene expression

experiments. A 300 μ L aliquot of complex solution was injected into mice *via* the tail vein. The liver, kidney, spleen, heart, and lung of mice were dissected 6 h after the injection. The tissues were washed twice with cold saline and homogenized with lysis buffer.

Homogenates were centrifuged at 15000 rpm for 5 min and the supernatants were used for luciferase assays. Luciferase activity was indicated as RLU per gram of tissue.

2.11. Statistical Analysis

Results are expressed as the mean \pm S.E. of at least three experiments. Statistical analysis was performed using Student *t*-test. A multiple comparison test was performed using Dunnett's test with Bonferroni's correction for multiple comparisons. $P < 0.05$ was considered to indicate significance.

3. Results

3.1. Physicochemical Property

The particle size and ζ -potential of each complex are shown in Table 1. Polyplexes had a smaller particle size than lipoplexes, but both polyplexes and lipoplexes had a cationic charge of 41.5–54.4 mV in ζ -potential. The addition of γ -PGA significantly increased the particle size of polyplexes ($P < 0.01$), but did not affect the particle size of lipoplexes. All γ -PGA-coated complexes showed anionic charges and had significantly lower ζ -potential than each binary complex ($P < 0.01$).

3.2. Electrophoresis Assay

Complex formations were examined by a gel retardation assay (Fig. 2). Naked pDNA was detected as bands on agarose gel, but were not detected for all complexes.

3.3. In Vitro Transfection Efficiency

In vitro gene expression efficiency of various complexes was determined with B16-F10 cells (Fig. 3). In polyplexes, PLA/pDNA and PLL/pDNA showed 5.4×10^7 and 2.6×10^6 RLU/mg protein, respectively. Lipoplexes of DOTMA-Chol/pDNA and DOTMA-DOPE/pDNA showed a high gene expression over 10^8 RLU/mg protein. Gene expressions of all γ -PGA-coated complexes were larger than those of each binary complex. In particular, γ -PGA-coated PLA/pDNA showed a significantly higher value than PLA/pDNA ($P < 0.01$).

3.4. Fluorescent Microscopy

B16-F10 cells were transfected with complexes containing pEGFP-C1 to visualize the gene expression (Fig. 4). Bright green fluorescence of GFP was observed in many cells treated with the complexes. γ -PGA-coated complexes showed more cells expressing GFP than each binary complex.

3.5. Inhibition Study

Inhibition studies were performed with L-glutamic acid and γ -PGA (Fig. 5). L-glutamic acid did not affect the transfection efficiencies of γ -PGA-coated complexes. In contrast, transfection efficiencies of γ -PGA-coated complexes were significantly decreased by γ -PGA ($P < 0.05$).

3.6. Cytotoxicity

The complexes were added to B16-F10 cells and cell viability was evaluated by WST-1 assay (Fig. 6). Polyplexes and lipoplexes showed significantly higher cytotoxicity than the control ($P < 0.01$) and the cytotoxicity of lipoplexes was lower than that of polyplexes. On the other hand, γ -PGA-coated complexes showed markedly low cytotoxicity compared with each binary complex.

3.7. Interaction with Erythrocytes

The complexes were incubated with erythrocytes to evaluate agglutination (Fig. 7A). Severe agglutination was observed in binary complexes on microscopy, whereas no agglutination was observed in γ -PGA-coated complexes. Figure 7B shows the hemolysis activities of the complexes, with polyplexes and γ -PGA-coated polyplexes showing little hemolysis (less than 10%). Strong hemolysis of erythrocytes, 79.3% and 67.8%, was observed in lipoplexes of DOTMA-CHOL/pDNA and DOTMA-DOPE/pDNA, respectively; γ -PGA coating of lipoplexes significantly suppressed hemolysis ($P < 0.01$).

3.8. In Vivo Transfection Efficiency

In vivo transgene efficiency of complexes was examined in ddY male mice (Fig. 8). Luciferase activities in several tissues were determined 6 h after intravenous administration of the complexes. Polyplexes showed high gene expression over 10^5 RLU/g tissue in the lung; however, γ -PGA-coated polyplexes showed high gene expression in the spleen. On the other hand, in lipoplexes, high gene expression was observed in the spleen and lung of DOTMA-Chol/pDNA, and in the spleen of DOTMA-DOPE/pDNA. γ -PGA-coated lipoplexes also showed high gene expression in the spleen and lung; however, γ -PGA coating

of DOTMA-Chol/pDNA significantly decreased the gene expression of DOTMA-Chol/pDNA in the lung ($P < 0.05$).

4. Discussion

A wide range of non-viral vectors has been developed, such as cationic polymers and cationic lipids, for successful gene therapy [14,15]. Among cationic polymers, PLA and PLL are considered to have an advantage for clinical use because of their biodegradable peptide structure [16,17]. PLA and PLL formed stable particles as self-assembly complexes with pDNA, and the particle size of PLA and PLL polyplexes showed 33.9 and 50.4 nm with positive ζ -potential of +44.8 and +54.4 mV, respectively (Table 1). The γ -PGA coating of each polyplex significantly increased the particle size. Anionic γ -PGA may weaken the compaction of polymer and pDNA; however, γ -PGA did not release pDNA from the polyplex, as shown in Fig. 2, and the formation of stable ternary complexes was indicated. These γ -PGA-coated complexes showed an apparently anionic surface charge even if their total charge ratio was +1. This result suggested that the anionic coating of γ -PGA was concentrated on the surface of polyplexes.

Among cationic liposomes, DOTMA-DOPE is commercially supplied as a transfection reagent “lipofectin”, which was demonstrated to have high transfection efficiency in cells, and had relatively stable expression in several tissues [18,19]. The lipoplex DOTMA-Chol/pDNA was reported to have higher stability and expression than lipofectin under *in vivo* conditions [20]. DOTMA-Chol and DOTMA-DOPE liposomes also constructed stable nano-particles with 98.7 and 115.0 nm particle sizes and +41.5 and +47.8 mV ζ -potentials, respectively (Table 1). γ -PGA-coating of lipoplexes did not influence particle size, although γ -PGA coating significantly decreased ζ -potentials of lipoplexes. Furthermore, γ -PGA did not release pDNA from lipoplexes, suggesting the anionic coating of γ -PGA on surface of lipoplexes (Fig. 2). It is valuable to note that the addition of γ -PGA to polyplexes and lipoplexes can form stable anionic complexes coated by γ -PGA.

In cationic systems, strong electrostatic interaction with a negatively charged cellular membrane can contribute to high gene expression through the endocytotic pathway [21]. We compared the transfection efficiency of each complex in mouse melanoma cell lines, B16-F10 cells, which are often used to examine the basic gene expression of non-viral vectors [22-24]. Polyplexes and lipoplexes showed high gene expressions although lipoplexes were more

effective than polyplexes (Fig. 3). Itaka *et al.* reported that polyplexes with PLA and PLL showed high gene expression although their gene expressions were lower than the polyplex with PEI [25]. It was also reported that the PLL/pDNA polyplex showed lower transfection efficiency than PEI/pDNA polyplex because of a lack of amino groups allowing endosomolysis [26]. The lower gene expressions of PLA/pDNA and PLL/pDNA may be explained by their weak intracellular trafficking.

Generally, anionic complexes are not taken up well by cells because they repulse the cellular membrane electrostatically, whereas γ -PGA-coated complexes showed higher gene expressions than each binary complex. In particular, γ -PGA coating of PLA/pDNA increased to a 7.4-fold gene expression from PLA/pDNA (Fig. 3). The high gene expressions of ternary complexes were also confirmed as the visualized GFP expressions by the observation of fluorescent microscopy images (Fig. 4). The γ -PGA-coated complexes with an anionic charge must therefore have a different uptake mechanism from polyplexes and lipoplexes with a cationic charge.

For clarifications of the uptake mechanisms, each γ -PGA-coated complex was added to the cells in the medium containing 20 μ M of L-glutamic acid or γ -PGA (Fig. 5). Transfection efficiencies of γ -PGA-coated complexes were significantly decreased by γ -PGA, not L-glutamic acid. These results indicated that γ -PGA-coated complexes with anionic charges were taken by the cells via γ -PGA-specific receptor-mediated pathway. The γ -PGA-coated DOTMA-DOPE/pDNA, however, was less inhibited by γ -PGA than other complexes. The surface of γ -PGA-coated DOTMA-DOPE/pDNA might be different from other γ -PGA-coated complexes.

The strong positive charges of polyplexes and lipoplexes are known to cause severe cytotoxicity by interaction with the negative surface of the cellular membrane [27]. In this experiment, polyplexes and lipoplexes also showed strong cytotoxicity (Fig. 6), although γ -PGA coating markedly decreased the cytotoxicity of each binary complex. The change in the surface charge from positive to negative must decrease the interaction of complexes with the cellular surface. It was also reported that cationic polyplexes and lipoplexes often caused agglutination of erythrocytes and albumin because of their strong electrostatic interaction

[8,9]. These agglutinations often lead to rapid elimination and adverse effects, such as embolism and inflammatory reaction [28-31]. The present polyplexes and lipoplexes severely agglutinated erythrocytes, and lipoplexes showed hemolysis activity (Figs. 7A and 7B). The high hemolytic activities of lipoplexes may be caused by interaction of the lipid component with the erythrocyte membrane. On the other hand, γ -PGA-coated complexes with an anionic charge showed no agglutination activities and γ -PGA-coating significantly reduced hemolysis by lipoplexes ($P < 0.01$).

For the clinical gene therapy, not only high transfection efficiency but also biocompatibility for frequent dosing is indispensable. It was notable that γ -PGA-coating increased the transfection efficiency of each binary complex and markedly decreased their toxicity, indicating their utility as a clinical gene delivery system.

We therefore determined the *in vivo* transgene efficiencies of γ -PGA-coated complexes. After intravenous injection of the complexes into mice, luciferase activities in five organs, the liver, kidney, spleen, heart, and lung, were determined by luciferase assay. Polyplexes and lipoplexes showed high transfection efficiency in the lung (Fig. 8). This high gene expression in the lung may be associated with agglutination, which was suggested to be embolized in the pulmonary vein and showed high accumulation in the lung. γ -PGA-coated complexes decreased the lung gene expression, except for γ -PGA-coated DOTMA-DOPE/pDNA. The low agglutination of γ -PGA-coated complexes can explain the decreased gene expression in the lung.

γ -PGA-coated complexes showed high gene expression in the spleen. Sutherland *et al.* reported that poly- γ -D-glutamic acid (γ DPGA), which is a capsular component of *Bacillus anthracis*, was mainly accumulated in the spleen and liver after intravenous injection into mice [32,33]. In particular, γ DPGA was located in red pulp in the spleen and Kupffer cells and sinusoidal endothelial cells in the liver. γ -PGA-coated complexes may be taken up by splenic macrophage through a similar mechanism of γ DPGA. Non-coating lipoplexes also showed high gene expression in the spleen, although the uptake mechanism of lipoplexes must be different from γ -PGA-coated complexes.

5. Conclusion

In this experiment, we developed some novel vectors for clinical gene therapy. Those vectors showed high transfection efficiencies with extremely low toxicities. Further study is necessary to clarify the uptake mechanism, tissue distribution, and clinical safety of γ -PGA-coated complexes.

Acknowledgements

This study was supported in part by the Global COE Program, Nagasaki University, Japan.

References

- [1] M.L. Edelstein, M.R. Abedi, J. Wixon, Gene therapy clinical trials worldwide to 2007- an update, *J. Gene Med.* 9 (2007) 833-834.
- [2] M. Hedman, J. Hartikainen, M. Syväne, J. Stjernvall, A. Hedman, A. Kivelä, E. Vanninen, H. Mussalo, E. Kauppila, S. Simula, O. Närvänen, A. Rantala, K. Peuhkurinen, M.S. Nieminen, M. Laakso, S. Ylä-Herttua, Safety and feasibility of catheter-based local intracoronary vascular endothelial growth factor gene transfer in the prevention of postangioplasty and in-stent restenosis and in the treatment of chronic myocardial ischemia: phase II results of the Kuopio Angiogenesis Trial (KAT), *Circulation* 107 (2003) 2677-2683.
- [3] F. Liu, L.M. Shollenberger, L. Huang, Non-immunostimulatory nonviral vectors, *FASEB J.* 18 (2004) 1779-1781.
- [4] X. Gao, K.S. Kim, D. Liu, Nonviral gene delivery: what we know and what is next, *AAPS J.* 9 (2007) E92-E104.
- [5] A. Hirko, F. Tang, J.A. Hughes, Cationic lipid vectors for plasmid DNA delivery, *Curr. Med. Chem.* 10 (2003) 1185-1193.
- [6] O. Boussif, F. Lezoualc'h, M.A. Zanta, M.D. Mergny, D. Scherman, B. Demeneix, J.P. Behr, A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine, *Proc. Natl. Acad. Sci. USA.* 92 (1995) 7297-7301.
- [7] M. Matsumoto, R. Kishikawa, T. Kurosaki, H. Nakagawa, N. Ichikawa, T. Hamamoto, H. To, T. Kitahara, H. Sasaki, Hybrid vector including polyethylenimine and cationic lipid, DOTMA, for gene delivery, *Int. J. Pharm.* 363 (2008) 58-65.
- [8] H. Eliyahu, N. Serval, A.J. Domb, Y. Barenholz, Lipoplex-induced hemagglutination: potential involvement in intravenous gene delivery, *Gene Ther.* 9 (2002) 850-858.
- [9] L. Dekie, V. Toncheva, P. Dubruel, S. E.H. chacht, L. Barrett, L.W. Seymour, Poly-L-glutamic acid derivatives as vectors for gene therapy, *J. Control. Release* 65 (2000) 187-202.
- [10] V.S. Trubetskoy, S.C. Wong, V. Subbotin, V.G. Budker, A. Loomis, J.E. Hagstrom, J.A. Wolff, Recharging cationic DNA complexes with highly charged polyanions for in vitro and in vivo gene delivery, *Gene Ther.* 10 (2003) 261-271.

- [11] T. Ito, N. Iida-Tanaka, Y. Koyama, Efficient in vivo gene transfection by stable DNA/PEI complexes coated by hyaluronic acid, *J. Drug Target.* 16 (2008) 276-281.
- [12] T. Kurosaki, T. Kitahara, S. Fumoto, K. Nishida, J. Nakamura, T. Niidome, Y. Kodama, H. Nakagawa, H. To, H. Sasaki, Ternary complexes of pDNA, polyethylenimine, and gamma-polyglutamic acid for gene delivery systems, *Biomaterials* 30 (2009) 2846-2853.
- [13] T. Kurosaki, T. Kitahara, M. Teshima, K. Nishida, J. Nakamura, M. Nakashima, H. To, H. Fukuchi, T. Hamamoto, H. Sasaki, Exploitation of De Novo Helper-Lipids for Effective Gene Delivery, *J Pharm Pharmaceut Sci.* 11 (2008) 56-67.
- [14] Y.B. Lim, S.O. Han, H.U. Kong, Y. Lee, J.S. Park, B. Jeong, S.W. Kim, Biodegradable polyester, poly[alpha-(4-aminobutyl)-L-glycolic acid], as a non-toxic gene carrier, *Pharm. Res.* 17 (2000) 811-816.
- [15] Y. Hattori, S. Suzuki, S. Kawakami, F. Yamashita, M. Hashida, The role of dioleoylphosphatidylethanolamine (DOPE) in targeted gene delivery with mannosylated cationic liposomes via intravenous route, *J. Control. Release* 108 (2005) 484-495.
- [16] D.J. Mitchell, D.T. Kim, L. Steinman, C.G. Fathman, J.B. Rothbard, Polyarginine enters cells more efficiently than other polycationic homopolymers, *J. Pept. Res.* 56 (2000) 318-325.
- [17] G.Y. Wu, C.H. Wu, Receptor-mediated in vitro gene transformation by a soluble DNA carrier system, *J. Biol. Chem.* 262 (1987) 4429-4432.
- [18] P.L. Felgner, T.R. Gadek, M. Holm, R. Roman, H.W. Chan, M. Wenz, J.P. Northrop, G.M. Ringold, M. Danielsen, Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure, *Proc. Natl. Acad. Sci. USA.* 84 (1987) 7413-7417.
- [19] J.H. Felgner, R. Kumar, C.N. Sridhar, C.J. Wheeler, Y.J. Tsai, R. Border, P. Ramsey, M. Martin, P.L. Felgner, Enhanced gene delivery and mechanism studies with a novel series of cationic lipid formulations, *J. Biol. Chem.* 269 (1994) 2550-2561.
- [20] K. Hong, W. Zheng, A. Baker, D. Papahadjopoulos, Stabilization of cationic liposome-plasmid DNA complexes by polyamines and poly(ethylene glycol)-phospholipid conjugates for efficient in vivo gene delivery, *FEBS Lett.* 400 (1997) 233-237.
- [21] M.T. da Cruz, S. Simões, P.P. Pires, S. Nir, M.C. de Lima, Kinetic analysis of the initial steps involved in lipoplex--cell interactions: effect of various factors that influence

transfection activity, *Biochim. Biophys. Acta.* 1510 (2001) 136-151.

[22] R. Kircheis, A. Kichler, G. Wallner, M. Kursa, M. Ogris, T. Felzmann, M. Buchberger, E. Wagner, Coupling of cell-binding ligands to polyethylenimine for targeted gene delivery, *Gene Ther.* 4 (1997) 409-418.

[23] C.W. Pouton, P. Lucas, B.J. Thomas, A.N. Uduehi, D.A. Milroy, S.H. Moss, Polycation-DNA complexes for gene delivery: a comparison of the biopharmaceutical properties of cationic polypeptides and cationic lipids, *J. Control. Release* 53 (1998) 289-299.

[24] V. Piddubnyak, P. Kurcok, A. Matuszowicz, M. Głowala, A. Fiszer-Kierzkowska, Z. Jedliński, M. Juzwa, Z. Krawczyk, Oligo-3-hydroxybutyrates as potential carriers for drug delivery, *Biomaterials* 25 (2004) 5271-5279.

[25] K. Itaka, A. Harada, Y. Yamasaki, K. Nakamura, H. Kawaguchi, K. Kataoka, 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 (2004) 76-84.

[26] T. Merdan, K. Kunath, D. Fischer, J. Kopecek, T. Kissel, Intracellular processing of poly(ethylene imine)/ribozyme complexes can be observed in living cells by using confocal laser scanning microscopy and inhibitor experiments, *Pharm. Res.* 19 (2002) 140-146.

[27] L.T. Nguyen, K. Atobe, J.M. Barichello, T. Ishida, H. Kiwada, Complex formation with plasmid DNA increases the cytotoxicity of cationic liposomes, *Biol. Pharm. Bull.* 30 (2007) 751-757.

[28] M. Ogris, S. Brunner, S. Schüller, R. Kircheis, E. Wagner, PEGylated DNA/transferrin-PEI complexes: reduced interaction with blood components, extended circulation in blood and potential for systemic gene delivery, *Gene Ther.* 6 (1999) 595-605.

[29] M. Ogris, E. Wagner, Targeting tumors with non-viral gene delivery systems, *Drug Discov. Today* 7 (2002) 479-485.

[30] S.E. Cook, I.K. Park, E.M. Kim, H.J. Jeong, T.G. Park, Y.J. Choi, T. Akaike, C.S. Cho, Galactosylated polyethylenimine-graft-poly(vinyl pyrrolidone) as a hepatocyte-targeting gene carrier, *J. Control. Release* 105 (2005) 151-163.

[31] R. Kircheis, S. Schüller, S. Brunner, M. Ogris, K.H. Heider, W. Zauner, E. Wagner,

Polycation-based DNA complexes for tumor-targeted gene delivery in vivo, *J. Gene Med.* 1 (1999) 111-120.

[32] M.D. Sutherland, P. Thorkildson, S.D. Parks, T.R. Kozel, In vivo fate and distribution of poly-gamma-D-glutamic acid, the capsular antigen from *Bacillus anthracis*, *Infect. Immun.* 76 (2008) 899-906.

[33] M.D. Sutherland, T.R. Kozel, Macrophage uptake, intracellular localization, and degradation of poly-gamma-D-glutamic acid, the capsular antigen of *Bacillus anthracis*, *Infect. Immun.* 77 (2009) 532-538.

Figure legends

Fig. 1. Formation of γ -PGA-coated vectors with negative ζ -potential.

Fig. 2. Electrophoresis analysis.

Each complex was loaded onto agarose gel for electrophoresis. Retardation of pDNA was visualized using ethidium bromide.

Fig. 3. Transfection efficiencies of various complexes.

B16-F10 cells were transfected with the various complexes. 22 h after transfection, cells were lysed and luciferase activities were determined. Each value represents the mean \pm S.E. (n= 3-6). **: $P < 0.01$.

Fig. 4. Fluorescent microscopy images of B16-F10 cells transfected with various complexes.

Cells were treated with each complex containing pEGFP-C1. GFP expressions were observed 22 h after transfection by fluorescent microscopy (200 \times magnification). (a): PLA/pDNA; (b): PLL/pDNA; (c): DOTMA-Chol/pDNA; (d): DOTMA-DOPE/pDNA; (e): γ -PGA-coating PLA/pDNA; (f): γ -PGA-coated PLL/pDNA; (g): γ -PGA-coated DOTMA-Chol/pDNA; (h): γ -PGA-coated DOTMA-DOPE/pDNA.

Fig. 5. Effects of L-glutamic acid and γ -PGA on the transfection efficiencies of γ -PGA-coated complexes.

B16-F10 cells were transfected with γ -PGA-coated complexes in the medium containing 20 μ M of L-glutamic acid or γ -PGA. 22 h after transfection, cells were lysed and luciferase activities were determined. Each value represents the mean \pm S.E. (n = 4). **: $P < 0.01$, #: $P < 0.05$ vs. control, ###: $P < 0.01$ vs. control.

Fig. 6. Cytotoxicity test of each complex on B16-F10 cells.

Cell viabilities of B16-F10 cells treated with complexes were measured by WST-1 assay.

Cells were incubated with each complex for 2 h and cell viability was measured 22 h after transfection. Data represent the percentage to untreated cells. Each value represents the mean \pm S.E. (n = 8-12). **: $P < 0.01$, ##: $P < 0.01$ vs. control.

Fig. 7. Interactions of various complexes with erythrocytes.

The complexes were added to erythrocytes, and agglutination (A) and hemolysis activities (B) were assessed.

Agglutination was observed by phase microscopy (400 \times magnification). (a): PLA/pDNA; (b): PLL/pDNA; (c): DOTMA-Chol/pDNA; (d): DOTMA-DOPE/pDNA; (e): γ -PGA-coated PLA/pDNA; (f): γ -PGA-coated PLL/pDNA; (g): γ -PGA-coated DOTMA-Chol/pDNA; (h): γ -PGA-coated DOTMA-DOPE/pDNA.

Hemolysis activity was determined by measuring hemoglobin release at 545 nm. Each value represents the mean \pm S.E. of three experiments. **: $P < 0.01$, ##: $P < 0.01$ vs. lysis buffer.

Fig. 8. Transgene efficiencies of the various complexes in mice.

The complexes were injected intravenously into mice (40 μ g DNA per mouse). At 6 h after administration, mice were sacrificed and each organ was dissected to quantify luciferase activity. Each value represents the mean \pm S.E. (n = 3-5). *: $P < 0.05$. □: non-coating and ■: γ -PGA-coating.

Tables

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

	Complex	Particle size (nm)	ζ - Potential (mV)
Non-coating	PLA/pDNA	33.9 \pm 9.1	+44.8 \pm 0.3
	PLL/pDNA	50.4 \pm 2.5	+54.4 \pm 0.8
	DOTMA-Chol/pDNA	98.7 \pm 2.7	+41.5 \pm 0.2
	DOTMA-DOPE/pDNA	115.0 \pm 6.1	+47.8 \pm 0.4
γ -PGA-coating	PLA/pDNA	92.6 \pm 1.4**	-12.6 \pm 0.5**
	PLL/pDNA	76.1 \pm 1.3**	-28.7 \pm 0.8**
	DOTMA-Chol/pDNA	121.0 \pm 5.6	-40.8 \pm 0.2**
	DOTMA-DOPE/pDNA	115.0 \pm 1.5	-41.5 \pm 0.6**

Each value represents the mean \pm S.E. (n=3).

** : $P < 0.01$ vs. each binary complex

Figures

Fig. 1

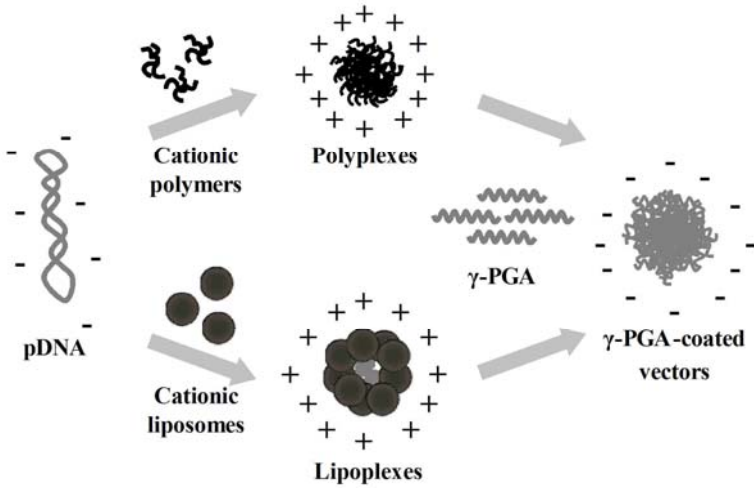


Fig. 2

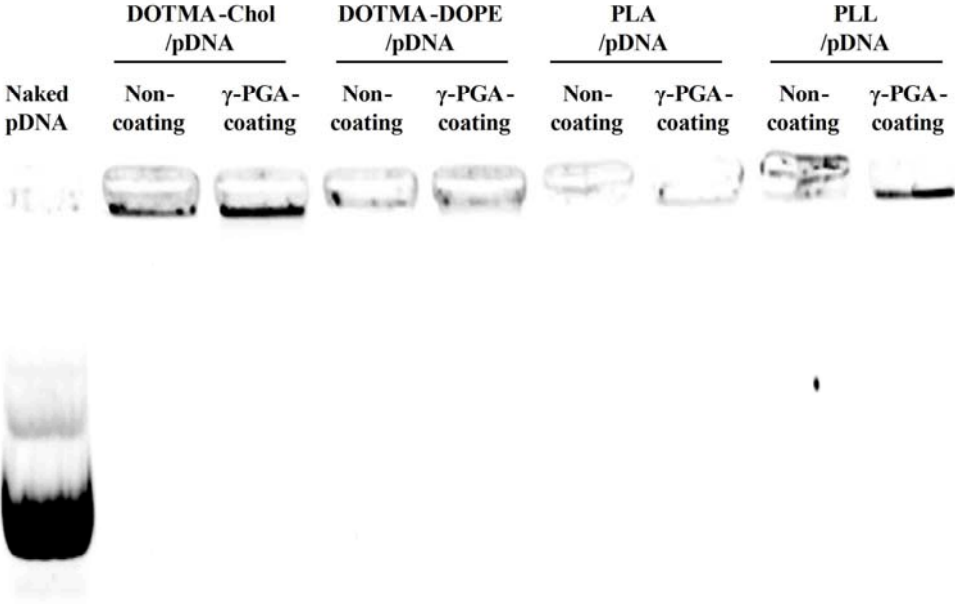


Fig. 3

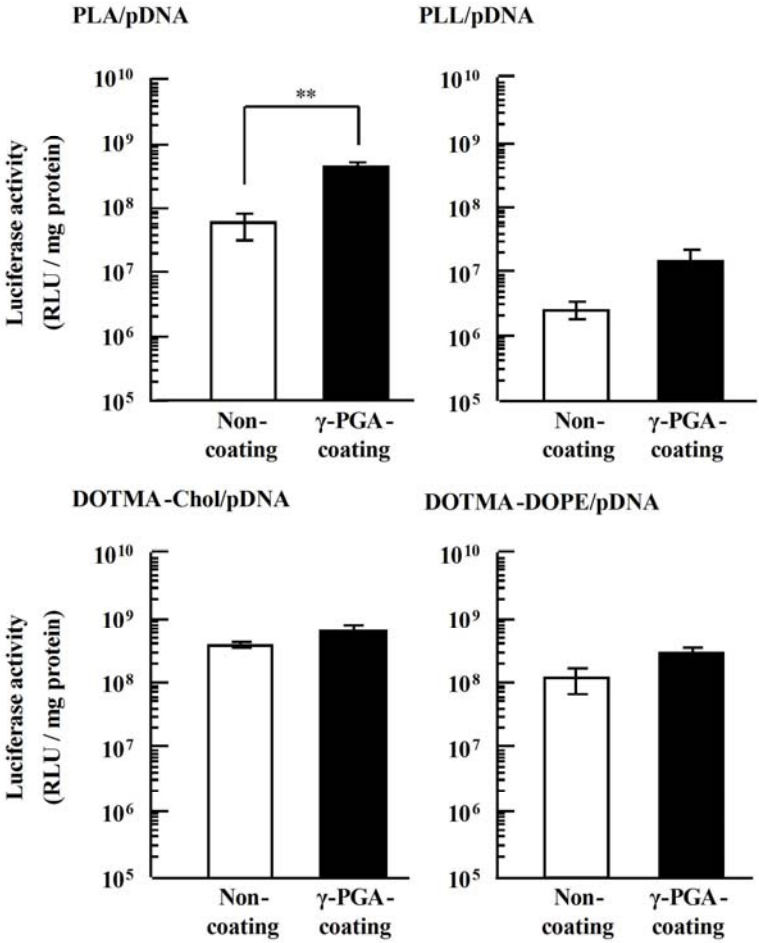


Fig. 4

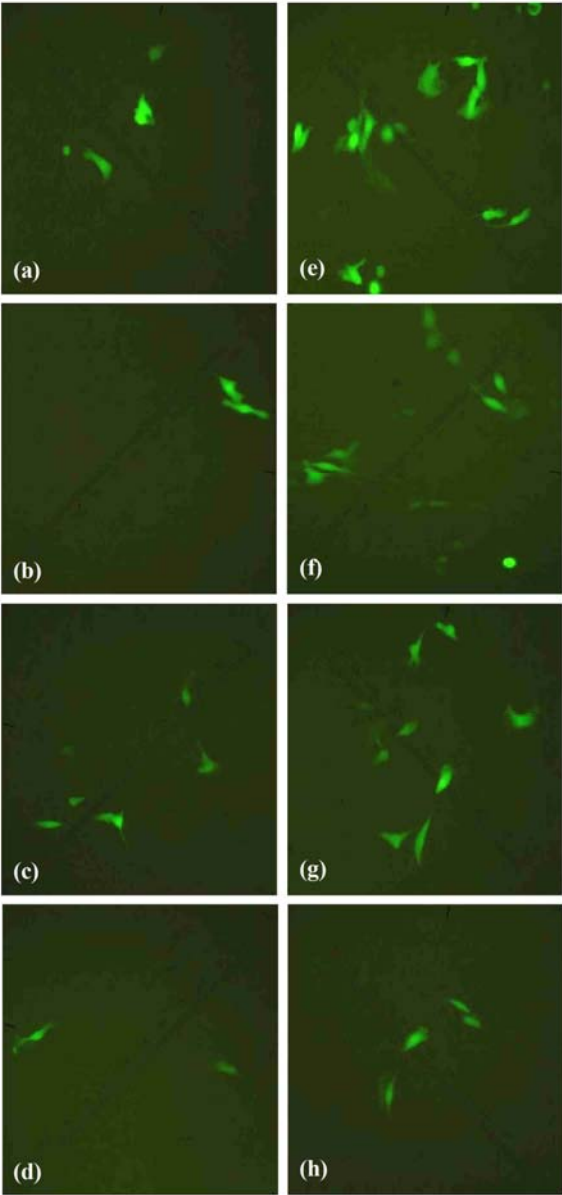


Fig. 5

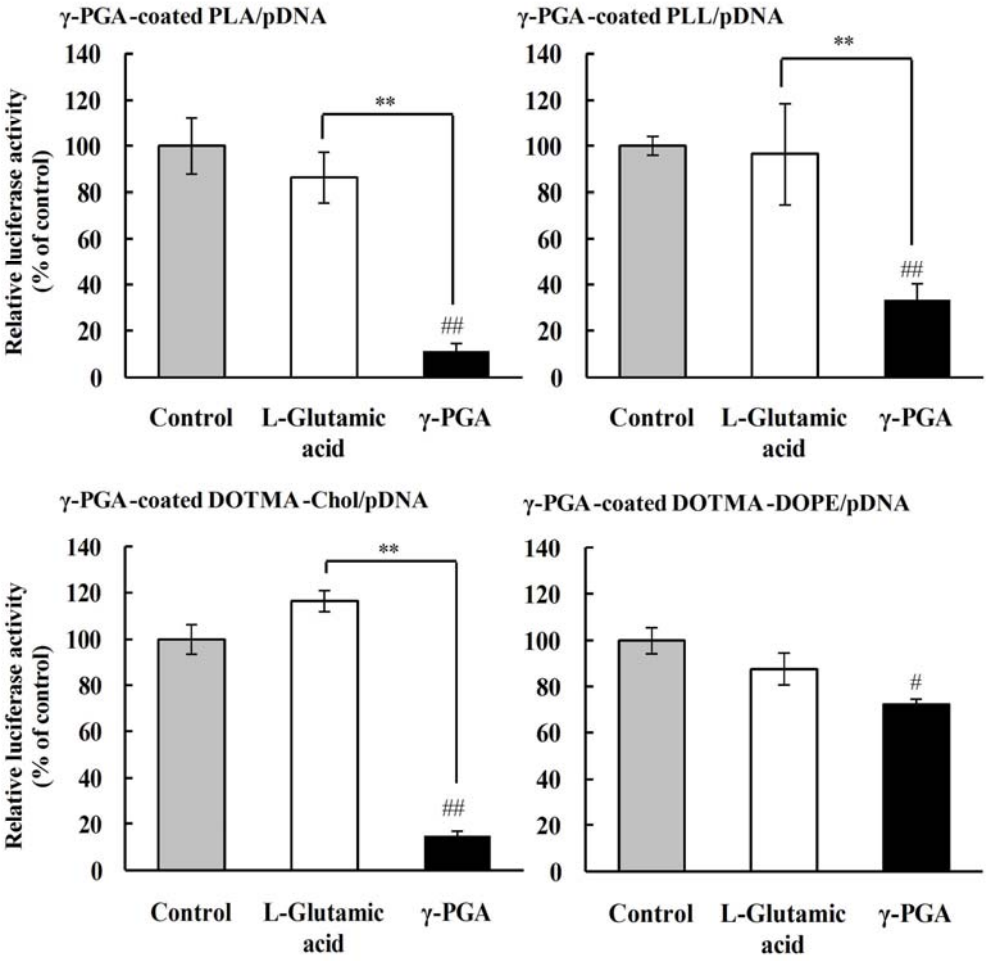


Fig. 6

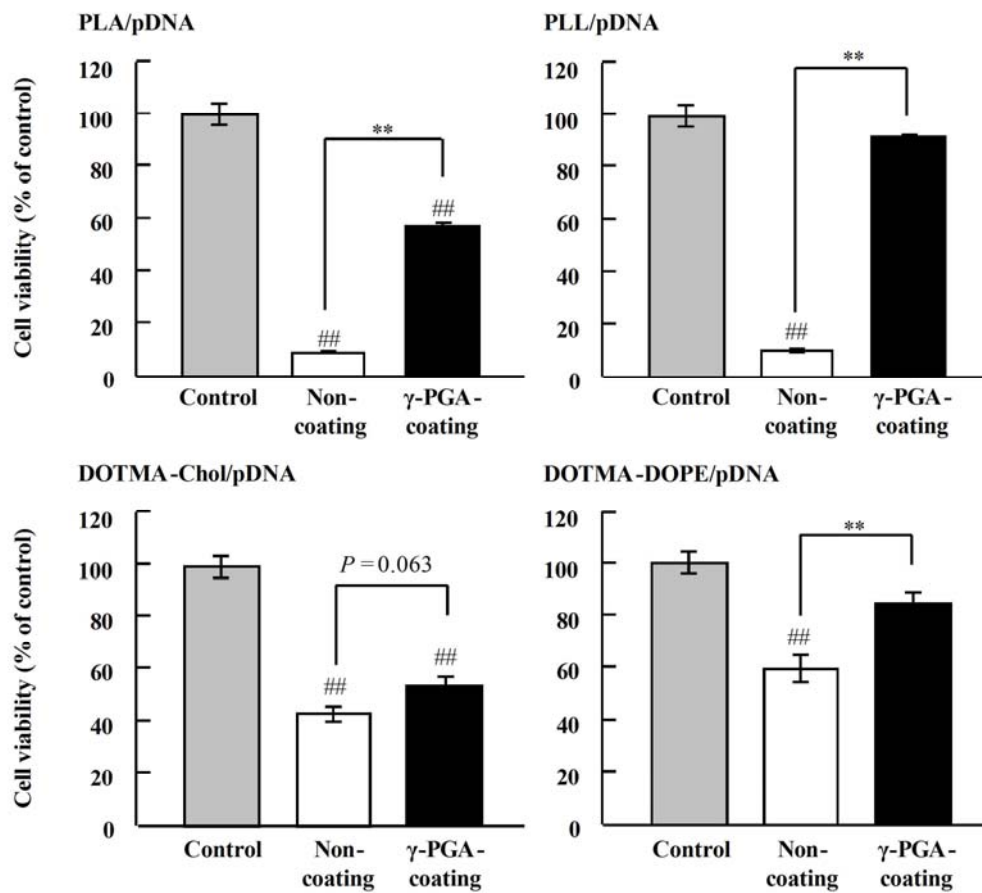


Fig. 7

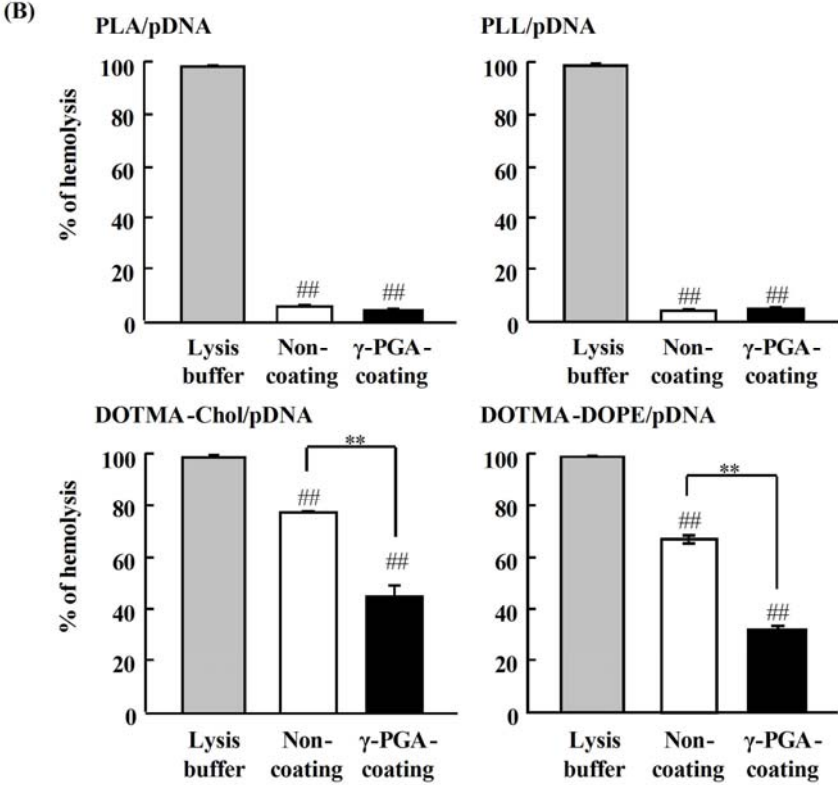
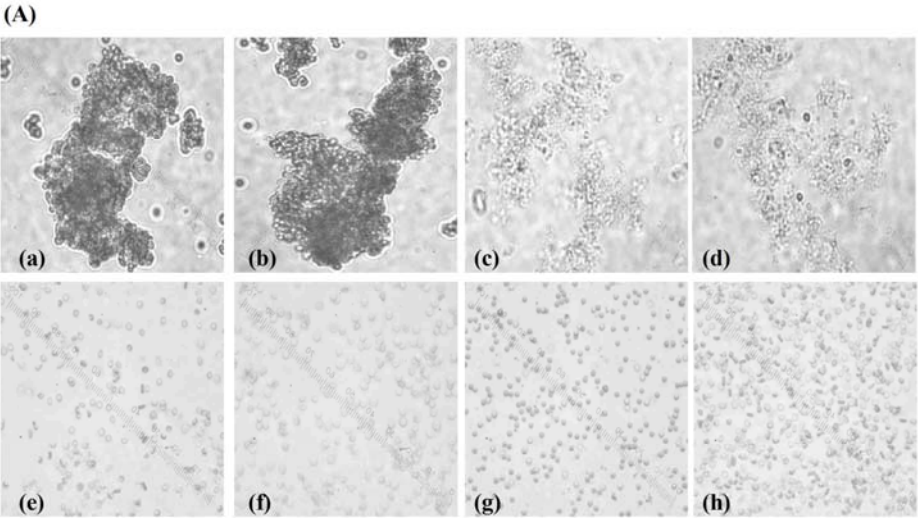


Fig. 8

