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Secure and effective gene delivery system of plasmid DNA coated by polynucleotide

Yukinobu Kodama\textsuperscript{a}, Chikako Ohkubo\textsuperscript{a}, Tomoaki Kurosaki\textsuperscript{a}, Kanoko Egashira\textsuperscript{a}, Kayoko Sato\textsuperscript{a}, Shintaro Fumoto\textsuperscript{b}, Koyo Nishida\textsuperscript{b}, Norihide Higuchi\textsuperscript{a}, Takashi Kitahara\textsuperscript{a}, Tadahiro Nakamura\textsuperscript{a}, Hitoshi Sasaki\textsuperscript{a,*}

\textsuperscript{a} Department of Hospital Pharmacy, Nagasaki University Hospital, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan; \textsuperscript{b} Department of Pharmaceutics, Graduate School of Biomedical Sciences, Nagasaki University, 1-14 Bunkyo-machi, Nagasaki 852-8521, Japan

*Corresponding author: Department of Hospital Pharmacy, Nagasaki University Hospital, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan

Tel.: +81-95-819-7245

Fax: +81-95-819-7251

E-mail: sasaki@nagasaki-u.ac.jp
Abstract

Polynucleotides are anionic macromolecules which are expected to transfer into the targeted cells through specific uptake mechanisms. So, we developed polynucleotides coating complexes of plasmid DNA (pDNA) and polyethylenimine (PEI) for a secure and efficient gene delivery system and evaluated their usefulness. Polyadenylic acid (polyA), polyuridylic acid (polyU), polycytidylic acid (polyC), and polyguanylic acid (polyG) were examined as the coating materials. pDNA/PEI/polyA, pDNA/PEI/polyU, and pDNA/PEI/polyC complexes formed nanoparticles with a negative surface charge although pDNA/PEI/polyG was aggregated. The pDNA/PEI/polyC complex showed high transgene efficiency in B16-F10 cells although there was little efficiency in pDNA/PEI/polyA and pDNA/PEI/polyU complexes. An inhibition study strongly indicated the specific uptake mechanism of pDNA/PEI/polyC complex. Polynucleotide coating complexes had lower cytotoxicity than pDNA/PEI complex. The pDNA/PEI/polyC complex showed high gene expression selectively in the spleen after intravenous injection into mice. The pDNA/PEI/polyC complex showed no agglutination with erythrocytes and no acute toxicity although these were observed in pDNA/PEI complex. Thus, we developed polynucleotide coating complexes as novel vectors for clinical gene therapy, and the pDNA/PEI/polyC complex as a useful
candidate for a gene delivery system.

**Keywords**: polynucleotide; gene delivery; polyethylenimine; pDNA; ternary complex
1. Introduction

Gene therapy is expected to be an effective method to treat cancer, infectious diseases, innate immunodeficiency, and cardiovascular diseases (Cavazzana-Calvo et al., 2012; Zarogoulidis et al., 2012; Su et al., 2012). Nucleic acids such as plasmid DNA (pDNA), antisense oligonucleotide, and small interfering RNA (siRNA) are used as therapeutic agents. The success of gene therapy highly depends on the development of an effective and secure gene delivery system, but there are many obstacles to the delivery of naked nucleic acids, such as rapid enzymatic degradation, rapid elimination via the kidney, and poor cellular uptake because of their large molecules and anionic charge, which govern biodistribution in the body (Nishikawa et al., 2005; Sato et al., 2007). In order to overcome these obstacles, a number of viral and non-viral vectors for nucleic acids have been developed for targeting cells and tissues through specific mechanisms (Nishikawa et al., 2005; Kurosaki et al., 2011; Corey, 2007; Tagalakis et al., 2011; Kawakami et al., 2000). Non-viral vectors for gene delivery have emerged as a promising alternative to viral vectors, because non-viral vectors have advantages such as much lower immunotoxicity, a clear structure, and easy modeling (Liu et al., 2004). Cationic compounds, such as cationic polymers and cationic liposomes, are mainly used as the non-viral vectors. On the other hand, Anionic polymers are little used as the
gene delivery vectors because they repulse the cellular membrane electrostatically and are not taken up by cells.

However, several anionic polymers have been reported to be selectively taken up by endothelial cells, smooth muscles, and macrophages (McCourt et al., 2004; McCourt et al., 1999; Qian et al., 2009; Martens et al., 2006; Li et al., 2009). Among them, polyguanylic acid was found to bind the scavenger receptor (SR) family (Class A, Class C, Class E, and Class F) (Gough et al., 2000). The SR was originally coined to describe a macrophage receptor that mediates endocytosis of a broad range of polyanionic molecules. Takakura et al. also demonstrated specific cellular uptake of pDNA in murine dendritic cells DC2.4 because the cellular association of pDNA was inhibited by pDNA itself, polyinosinic acid, and dextran sulfate (Yoshinaga et al.; 2002). Following intravenous injection, pDNA was rapidly eliminated from the circulation and taken up by liver sinusoidal endothelial cells (Hisazumi et al., 2004). These results suggest a potential of polynucleotides for an effective gene delivery system using a specific uptake mechanism. However, there are no reports on the development of nanoparticles using polynucleotides.

In the present study, we prepared ternary complexes using pDNA, polyethylenimine (PEI), and polynucleotide for a gene delivery system and evaluated their usefulness.
PEI is widely used as a polymeric vector. Polynucleotides with large molecular and relatively stable properties were used as coating materials, including polyadenylic acid (polyA), polyuridylic acid (polyU), polycytidylic acid (polyC), and polyguanylic acid (polyG). We newly found that ternary complexes coated by polyC were a useful candidate for the gene delivery system because of their high gene expression without toxicity.
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). Polyadenylic acid potassium salt (polyA), polyuridylic acid potassium salt (polyU), polycytidylic acid potassium salt (polyC), and polyguanylic acid potassium salt (polyG) were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from Biological Industries Ltd. (Kibbutz Beit Haemek, Israel). RPMI 1640, 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, USA). 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-1) and 1-methoxy-5-methylphenazinium methylsulfate (1-methoxy PMS) were purchased 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.
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 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 polynucleotide was mixed with pDNA/PEI complex by pipetting and left for another 15 min at room temperature. In this study, we constructed those complexes at a theoretical charge ratio: phosphate of pDNA: nitrogen of PEI: phosphate of polynucleotide = 1:8:0 (pDNA/PEI complex), 1:8:2 (pDNA/PEI/polyA2, pDNA/PEI/polyU2, pDNA/PEI/polyC2, or pDNA/PEI/polyG2 complexes), 1:8:4 (pDNA/PEI/polyA4, pDNA/PEI/polyU4, pDNA/PEI/polyC4, or pDNA/PEI/polyG4 complexes).
complexes), 1:8:6 (pDNA/PEI/polyA6, pDNA/PEI/polyU6, pDNA/PEI/polyC6, or pDNA/PEI/polyG6 complexes), 1:8:8 (pDNA/PEI/polyA8, pDNA/PEI/polyU8, pDNA/PEI/polyC8, or pDNA/PEI/polyG8 complexes), and 1:8:10 (pDNA/PEI/polyA10, pDNA/PEI/polyU10, pDNA/PEI/polyC10, or pDNA/PEI/polyG10 complexes).

2.3. Physicochemical property of ternary complexes

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

To determine complex formations, 10 µL aliquots of 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 50 V in running buffer solution (40 mM Tris/HCl, 40 mM acetic acid, and 1 mM ethylenediaminetetraacetic acid (EDTA)) for 60 min. The retardation of pDNA was visualized with ethidium bromide staining.

2.4. Transfection experiments

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. B16-F10 cells were plated on 24-well plates (Becton-Dickinson, Franklin Lakes, NJ, USA) at a density of 1.0 × 10⁴ 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 (FBS (-)) or culture medium (FBS (+)) 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, German). The protein content of the lysate was determined by a Bradford assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using BSA as a standard. Absorbance was measured using a microplate reader (Sunrise RC-R; Tecan Japan Co., Ltd., Kanagawa, Japan) at 595 nm. Luciferase activity was indicated as
relative light units (RLU) per mg protein.

2.5. WST-1 assay

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 (Millipore 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 $3.0 \times 10^3$ cells/well in the culture medium. The 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 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. Fluorescent microscopy
To visualize the uptake of the complexes and gene expressions, B16-F10 cells were transfected by various complexes constructed with pEGFP-C1, Rh-PEI, and polynucleotide. After 22 h incubation, the relative levels of Rh-PEI and GFP expression were characterized using fluorescent microscopy (200 × magnification; BZ-9000; KEYENCE, Osaka, Japan).

2.7. Inhibition study

For determination of the endocytotic pathway, after 23 h pre-incubation, the cells were treated with 0.014 mM chlorpromazine (CPZ) as an inhibitor of clathrin-mediated endocytosis, 0.2 mM genistein as an inhibitor of caveolae-mediated endocytosis, or 1 mM amiloride as an inhibitor of macropinocytosis for 1 h. After treatment, the pDNA/PEI/polyC6 complexes and pDNA/PEI complexes were added to the medium containing each inhibitor and incubated for 2 h. After 2 h transfection, the medium was replaced with culture medium, cells were cultured for a further 22 h at 37 °C, and then the luciferase activities were determined.

For the inhibition study, the cells were transfected as described above with pDNA/PEI/polyC6 complexes in transfection medium containing various concentrations of polyC. After transfection, the medium was replaced with culture
medium, cells were cultured for a further 22 h at 37 °C, and then luciferase activities were determined.

2.8. **Morphology of pDNA/PEI/polyC**

Transmission electron microscopy (JEM-1230; JEOL, Tokyo, Japan) was used to observe the configuration of pDNA/PEI/polyC6.

2.9. **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 day before experiments.

2.10. **Agglutination study**

Erythrocytes from mice were washed three times at 4 °C by centrifugation at 5000 rpm (Kubota 3500; Kubota, Tokyo, Japan) for 5 min and resuspended in PBS. A 2% (v/v) stock suspension was prepared. Various complexes were added to the
erythrocytes (complexes: stock suspension = 1:1). The suspensions were incubated for 15 min at room temperature. The 10 µL suspensions were placed on a glass plate and agglutination was observed by microscopy (200 × magnification).

2.11. In vivo study

Each complex containing 40 µg pCMV-Luc at a volume of 300 µL per mouse was injected intravenously into mice to examine the transgene efficacy of pDNA/PEI and pDNA/PEI/polyC6 complexes. At 6 h following injection, the mice were sacrificed, and the liver, kidney, spleen, heart, and lung were dissected. The tissues were washed twice with cold saline and homogenized with lysis buffer. The homogenates were centrifuged at 15,000 rpm (Kubota 3500; Kubota) for 5 min and the supernatants were used for luciferase assays. Luciferase activity was indicated as RLU per gram of tissue.

To evaluate the acute toxicity of pDNA/PEI and pDNA/PEI/polyC6 complexes, each complex containing 200 µg pCMV-Luc at a volume of 1.5 mL per mouse was injected intravenously into mice. Twenty-four hours after administration, surviving mice were counted.
2.12. *Statistical analysis*

Statistical significance between two groups was identified by Student’s $t$ test. Multiple comparisons among groups were made by Dunnett’s pairwise multiple comparison $t$ test.
3. **Results**

3.1. **Physicochemical characteristics and gel retardation assay**

The particle size and $\zeta$-potential of various complexes are shown in Table 1. The pDNA/PEI complexes had 46.6 ± 5.5 nm particle size and 47.0 ± 0.5 mV $\zeta$-potential. The pDNA/PEI/polyA, pDNA/PEI/polyU, and pDNA/PEI/polyC complexes with a charge ratio of polynucleotide to pDNA greater than 4 were less than 100 nm particle size, although pDNA/PEI/polyG complexes showed aggregation and particle size was not detected. Addition of polynucleotides decreased the $\zeta$-potential of pDNA/PEI complexes concentration-dependently. The pDNA/PEI/polyA, pDNA/PEI/polyU, and pDNA/PEI/polyC complexes with a charge ratio greater than 4 showed an anionic surface charge.

Complex formations were examined by a gel retardation assay (Fig. 1). Naked pDNA was detected as a band on agarose gel. The complexes showed no band of pDNA, which was released.

On the basis of the results of physicochemical properties, we performed further studies using the pDNA/PEI/polyA, pDNA/PEI/polyU, and pDNA/PEI/polyC complexes.
3.2. *In vitro transfection efficiency*

The complexes were incubated with B16-F10 cells and luciferase activity in the cells was determined. The *in vitro* transgene efficiencies of the complexes at various charge ratios are shown in Fig. 2. The pDNA/PEI complexes exceeded $10^{10}$ RLU/mg protein in transgene efficiency. Addition of polynucleotides to pDNA/PEI complexes decreased luciferase activities with an increase of the amount. The pDNA/PEI/polyC complexes retained high gene expression over $10^9$ RLU/mg protein, although pDNA/PEI/polyA complexes and pDNA/PEI/polyU complexes showed low gene expression of less than $10^9$ RLU/mg protein.

3.3. *Cellular toxicity*

Each complex was added to B16-F10 cells and cell viability was evaluated by WST-1 assay (Fig. 3). The pDNA/PEI complexes showed significantly higher cellular toxicity than the control ($P < 0.01$). On the other hand, no cellular toxicity was observed in the pDNA/PEI/polyA complexes. The pDNA/PEI/polyU complexes showed moderate cytotoxicity concentration-dependently. The addition of polyC decreased the cytotoxicity of pDNA/PEI complexes, and the pDNA/PEI/polyC complexes with a charge ratio greater than 6 did not affect the cell viability of B16-F10 cells.
3.4. **Fluorescent microscopy**

The cellular uptake and gene expression were visualized in the pDNA/PEI/polyA6, pDNA/PEI/polyU6, and pDNA/PEI/polyC6 complexes. B16-F10 cells were transfected with the complexes containing Rh-PEI and pEGFP-C1 (Fig. 4).

The pDNA/PEI complexes and pDNA/PEI/polyC6 complexes showed red dots of Rh-PEI in the cells. The bright green fluorescence of GFP was observed in the cells transfected with pDNA/PEI complexes and pDNA/PEI/polyC6 complexes, although no fluorescence was observed in pDNA/PEI/polyA6 complexes and pDNA/PEI/polyU6 complexes.

3.5. **Inhibition study**

The gene expression of pDNA/PEI/polyC6 complexes in medium containing various concentrations of polyC is shown in Fig. 5A. PolyC significantly inhibited the transgene efficiency of pDNA/PEI/polyC6 complexes concentration-dependently. Fig. 5B shows the influence of endocytotic inhibitors on the transgene efficiency of pDNA/PEI/polyC6 complexes and pDNA/PEI complexes. All endocytotic inhibitors decreased the transgene efficiency of pDNA/PEI/polyC6 complexes. Especially, the
inhibition of caveolae-mediated endocytosis with genistein significantly decreased the transgene efficiency of the pDNA/PEI/polyC6 complexes (P < 0.01), which was lower than 20%. On the other hand, the transgene efficacy of pDNA/PEI complexes was only suppressed by the addition of genistein. Chlorpromazine and amiloride had little effect on the transgene efficiency of the pDNA/PEI complexes.

3.6. The effect of serum on the transgene efficiency

The effect of serum on transgene efficiencies of the complexes was examined. pDNA/PEI/polyC complexes were unaffected by serum. On the other hand, the transfection effect of pDNA/PEI complexes was decreased by serum (Fig. 6).

3.7. Morphology of pDNA/PEI/polyC

pDNA/PEI/polyC6 complexes was observed as clumped nano-particles in the TEM (Fig. 7).

3.8. In vivo study

In vivo transgene efficiency of pDNA/PEI complexes and pDNA/PEI/polyC6 complexes, which showed high transgene efficiency in an in vitro experiment, was
examined in ddY male mice and is shown in Fig. 8. Luciferase activities in several tissues were determined 6 h after intravenous administration of the complexes. The pDNA/PEI complexes showed high gene expression in all tissues. On the other hand, pDNA/PEI/polyC6 complexes showed high gene expression selectively in the spleen.

3.9. In vivo acute toxicity and agglutination study

In vivo acute toxicity of pDNA/PEI and pDNA/PEI/polyC6 complexes was evaluated by the survival rate 24 h after intravenous infection of the complexes to ddY male mice. All mice survived the injection of pDNA/PEI/polyC6 complexes, although 4 of 9 mice died after injection of pDNA/PEI complexes (Fig. 9A).

Agglutination activities of pDNA/PEI/polyC6 complexes were compared to pDNA/PEI complexes in erythrocytes (Fig. 9B). The pDNA/PEI/polyC6 complexes showed no agglutination, although the pDNA/PEI complexes agglutinated many erythrocytes.
4. Discussion

Advances in the field of non-viral gene therapy have been the subject of intensive research because of the inherent safety issues associated with most viral vectors. Numerous non-viral vectors for increasing transfection efficiencies and decreasing toxicities have been developed. Among the non-viral vectors, cationic lipids and cationic polymers are the most probable alternatives to viral vectors and are increasingly being used in vitro and in vivo (Gao et al., 2007). A cationic polymer (at physiological pH) can be combined with DNA to form a particulate complex, polyplex, capable of gene transfer to the targeted cells (Pezzoli et al., 2012). The most obvious difference of cationic polymers from cationic lipids is that they do not contain a hydrophobic moiety and are completely soluble in water. Compared with cationic lipids, cationic polymers have the obvious advantage of compressing DNA molecules to a relatively small size. PEI is one of the most studied and employed non-viral gene carriers. Many factors affect the efficiency/cytotoxicity profile of PEI polyplexes, such as molecular weight, degree of branching, zeta potential and particle size (Kunath et al., 2003; Kircheis et al., 1999). PEI is known to cause severe adverse effects such as cytotoxicity and agglutination. The toxic effect is mainly determined by the cationic nature of PEI (Lv et al., 2006; Pack et al., 2005; Lugwitz et al., 2005).
Here, we hypothesized that anionic polymer-coating of complexes of pDNA and PEI could improve toxicity of PEI. Among anionic polymers, polynucleotides were expected to transfer the complexes into the targeted cells through a specific uptake mechanism. PolyA, polyU, polyC, and polyG were used as a coating material with large molecular weight and relatively stable properties.

We checked the physicochemical properties of the ternary complexes. Table 1 shows the particle size and ζ-potential of the complexes. The pDNA/PEI/polyA, pDNA/PEI/polyU, and pDNA/PEI/polyC complexes with a charge ratio greater than 4 showed less than 100 nm particle size and a negative surface charge; indicating the particle sizes of the ternary complexes were suitable for \textit{in vivo} gene delivery. The negative surface charge of the ternary complexes suggested that polynucleotides effectively coated the particles. Furthermore, the size and ζ-potential of pDNA/PEI/polyA, pDNA/PEI/polyU, and pDNA/PEI/polyC complexes were not changed in serum (data not shown); thus, stability of the ternary complexes was expected to be good enough for \textit{in vivo} applications. Some polyanions such as heparin are known to release pDNA from pDNA/PEI complexes (Moret et al., 2001). However, we found that the addition of polynucleotides did not push pDNA out of particles (Fig. 1). Furthermore, the presence of serum did not promote pDNA release from the ternary complexes.
complexes (data not shown). These results support that pDNA/PEI/polyA, pDNA/PEI/polyU, and pDNA/PEI/polyC complexes are stable as self-assembled nano-particles without de-complexation, even in blood.

The *in vitro* transgene efficiency was evaluated with B16-F10 cells by luciferase activity (Fig. 2). The pDNA/PEI complexes exceeded $10^{10}$ RLU/mg protein in transgene efficiency. Addition of polynucleotides to pDNA/PEI complexes decreased luciferase activities concentration-dependently. These results reflect the low interaction of the negative surface charge of complexes with the cellular surface (Chen et al., 2011; Fröhlich, 2012). In particular, the pDNA/PEI/polyA complexes and pDNA/PEI/polyU complexes showed low gene expression after the addition of polynucleotides. In fact, cellular uptake of Rh-PEI was not observed in the pDNA/PEI/polyA complexes and pDNA/PEI/polyU complexes at a charge ratio of 6 (Fig. 4). On the other hand, the pDNA/PEI/polyC complexes retained high gene expression over $10^9$ RLU/mg protein and showed cellular uptake of Rh-PEI and gene expression of GFP. These results suggest that the uptake mechanism of pDNA/PEI/polynucleotide complexes differs with the type of polynucleotide and that there is a specific uptake mechanism of pDNA/PEI/polyC complexes. pDNA/PEI/polyC complexes must release DNA so that it can be transported into the
nucleus, although the detailed mechanism is unclear.

We therefore performed an inhibition study with polyC and various endocytotic inhibitors. The gene transfection of pDNA/PEI/polyC6 complexes was inhibited by polyC concentration-dependently. These results strongly indicate the specific uptake mechanism of pDNA/PEI/polyC complexes. CPZ, genistein, and amiloride all suppressed the transgene expression efficiency of the pDNA/PEI/polyC6 complexes, suggesting that it was taken up by the cells via several mechanisms. Among inhibitors, genistein especially suppressed the gene expression of pDNA/PEI/polyC6 complexes. These results showed that the pDNA/PEI/polyC6 complexes were mainly taken up by caveolae-mediated endocytosis. It is generally believed that caveolar uptake does not lead to lysosomal degradation (Harris et al. 2002; Ferrari et al., 2003). Therefore, pDNA/PEI/polyC6 complexes seem to be advantageous in terms of DNA delivery. On the other hand, the transgene expression efficiency of the pDNA/PEI complexes was only suppressed by genistein. These results suggested that the uptake mechanism of pDNA/PEI/polyC complexes differed from that of pDNA/PEI complexes.

We confirmed that the particle size of pDNA/PEI/polyC6 complexes was suitable for \textit{in vivo} gene delivery using TEM (Fig. 7). Then, \textit{in vivo} transgene efficiency of pDNA/PEI/polyC6 complexes, which showed high transgene efficiency and low
cytotoxicity in vitro (Figs. 2 and 3), was examined in ddY male mice (Fig. 8). Naked pDNA did not show gene expression in any tissues (data not shown). The pDNA/PEI/polyC6 complexes showed high gene expression selectively in the spleen, although pDNA/PEI complexes showed gene expression non-selectively in all tissues. The positive charges of polyplexes are known to strongly interact with the negative surface of the cellular membrane and be taken up by endothelial cells through endocytosis. The strong positive charge of pDNA/PEI complexes may contribute to the high gene expression in all tissues. On the other hand, the high gene expression in the spleen of pDNA/PEI/polyC complexes may result in the low serum effect (Fig. 6). The spleen plays important roles in regard to erythrocytes and the immune system (Cesta, 2006; Zandvoort et al., 2002), acting primarily as a blood filter and having many macrophages and dendritic cells, which express many types of receptors that mediate the endocytosis of a broad range of polyanionic molecules. A specific uptake mechanism of pDNA/PEI/polyC complexes was strongly suggested in the present study; therefore, pDNA/PEI/polyC complexes may show specifically high transgene efficiency in the spleen. A splenic gene delivery system must be a promising approach for DNA vaccination. We have already demonstrated that a splenic gene delivery system markedly inhibited malaria infection in mice (Cherif et al., 2011).
There were mainly two types of cytotoxicity in the process of PEI-mediated cell transfection; immediate toxicity associated with free PEI and delayed toxicity associated with cellular processing of pDNA/PEI complexes (Godbey et al., 2001). The pDNA/PEI complexes showed significant cellular toxicity (Fig. 3). On the other hand, the ternary complexes reduced cytotoxicity. The negative surface charge of the polynucleotide-coating complexes may result in their safety because of low interaction with the cellular membrane. When administered into the circulatory system, the free PEIs interacted with negatively charged serum proteins and red blood cells, precipitated in huge clusters and adhered to the cellular surface (Lv et al. 2006). This effect could destabilize the plasma membrane and induce immediate toxicity. In fact, the pDNA/PEI complexes showed severe agglutination with erythrocytes. Four of 9 mice had died 24 h after injection of pDNA/PEI complexes at a 5-fold dose; however, pDNA/PEI/polyC6 complexes showed no agglutination with erythrocytes and acute toxicity (Fig. 9). These results indicate the high safety of pDNA/PEI/polyC complexes compared to pDNA/PEI complexes.
5. Conclusion

In this study, we developed polynucleotide-coating complexes as a novel vector for clinical gene therapy. This vector consisted of stable particles with apparently negative $\zeta$-potential. In particular, the pDNA/PEI/polyC6 complexes showed high in vitro gene expression without cytotoxicity. The inhibition study suggested the specific uptake mechanism of pDNA/PEI/polyC6 complexes. The pDNA/PEI/polyC6 complexes showed selective in vivo gene expression in the spleen, showing neither agglutination with erythrocytes nor acute toxicity. The pDNA/PEI/polyC6 complexes are a useful candidate for a gene delivery system. Further studies are necessary to reveal the detail uptake pathway of polyC.

Acknowledgements and Declaration of Interest

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Declaration of Interest statement

The authors report no declarations of interest.
References


sinusoidal endothelia, avascular tissues, and at solid/liquid interfaces,


Caveolae-mediated internalization of extracellular HIV-1 tat fusion proteins
visualized in real time. Mol Ther, 8, 284-94.


is next. *AAPS J*, 9, E92-104.

delivery affects endothelial cell function and viability. *Biomaterials*, 22,
471-80.


caveolin in immune cells: distribution and functions. *Trends Immunol*, 23,
158-64.


[28] Pezzoli D, Chiesa R, De Nardo L, Candiani G. 2012. We still have a long way to go to effectively deliver genes! *J Appl Biomater Function Mater*, 10, 82-91.


Figure captions

Fig. 1. Gel retardation assay of each polynucleotide coating complex. Each complex was loaded onto agarose gel, and electrophoresis was carried out. Retardation of pDNA was visualized using ethidium bromide. (A): pDNA/PEI/polyA complexes; (B): pDNA/PEI/polyU complexes; (C): pDNA/PEI/polyC complexes; (D): pDNA/PEI/polyG complexes.

Fig. 2. In vitro transgene efficiency in B16-F10 cells of pDNA/PEI/polyA complexes (A), pDNA/PEI/polyU complexes (B), and pDNA/PEI/polyC complexes (C). B16-F10 cells were transfected with each complex containing pCMV-Luc in Opti-MEM. Twenty-two hours after transfection, luciferase activity was evaluated. Each bar represents the mean ± S.E. of three experiments.

Fig. 3. Cytotoxicity of various complexes on B16-F10 cells. Viability of cells treated with pDNA/PEI/polyA complexes (A), pDNA/PEI/polyU complexes (B), and pDNA/PEI/polyC complexes was measured by WST-1 assay. Cells were incubated with each complex for 2 h and cell viability was measured 22 h after treatment. Data are the percentage to untreated cells. Each bar represents the mean ± S.E. of eight
experiments. *: $P < 0.05$, **: $P < 0.01$ vs control.

Fig. 4. Fluorescent microscopy images of B16-F10 cells transfected with each complex. 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/polyU; (d): pDNA/PEI/polyC.

Fig. 5. Influence of polyC (A) and endocytotic inhibitors (B) on transgene efficiency. (A) pDNA/PEI/polyC6 complexes were transfected in medium containing various concentrations of polyC. (B) pDNA/PEI/polyC6 complexes and pDNA/PEI complexes were transfected in medium with various endocytotic inhibitors. After 22 h transfection, luciferase activities were evaluated. pDNA/PEI/polyC6 complex (■), pDNA/PEI complex (□). Each bar represents the mean ± S. E. of three experiments. *: $P < 0.05$, **: $P < 0.01$ vs control.

Fig. 6. Influence of serum on transgene efficiency of the complexes. B16-F10 cells were transfected with each complex containing pCMV-Luc in culture medium.
Twenty-two hours after transfection, luciferase activity was evaluated. Each bar represents the mean ± S.E. of three experiments.

Fig. 7. Transmission Electron Microscopy (TEM) Image of pDNA/PEI/polyC6 complexes.

Fig. 8. *In vivo* transgene efficiencies of the pDNA/PEI (A) and pDNA/PEI/polyC6 (B) complexes in mice. The complexes were injected intravenously into mice (40 µg DNA per mouse). At 6 h after injection, mice were sacrificed and each organ was dissected to quantify luciferase activity. Each bar represents the mean ± S.E. (n = 3-6).

Fig. 9. Acute toxicity (A) and agglutination with erythrocytes (B) of the complexes. (A) Surviving mice were counted 24 h after injection of each complex. (B) Each complex was added to erythrocytes, and agglutination was observed by microscopy (200 × magnification).
Gel retardation assay of each polynucleotide coating complex.
52x76mm (300 x 300 DPI)
In vitro transgene efficiency in B16-F10 cells of pDNA/PEI/polyA complexes (A), pDNA/PEI/polyU complexes (B), and pDNA/PEI/polyC complexes (C).

52x76mm (300 x 300 DPI)
Fig. 3

Cytotoxicity of various complexes on B16-F10 cells.
52x76mm (300 x 300 DPI)
Fig. 4

Fluorescent microscopy images of B16-F10 cells transfected with each complex. 44x63mm (300 x 300 DPI)
Influence of polyC (A) and endocytotic inhibitors (B) on transgene efficiency.

52x76mm (300 x 300 DPI)
Influence of serum on transgene efficiency of the complexes.
52x76mm (300 x 300 DPI)
52x76mm (300 x 300 DPI)
In vivo transgene efficiencies of the pDNA/PEI (A) and pDNA/PEI/polyC6 (B) complexes in mice.
52x76mm (300 x 300 DPI)
Fig. 9

### Table A

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<th>pDNA/PEI</th>
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<tr>
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<tr>
<td>Alive</td>
<td>5</td>
<td>10</td>
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<tr>
<td>Total</td>
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### Table B

- pDNA/PEI
- pDNA/PEI/polyC6

Acute toxicity (A) and agglutination with erythrocytes (B) of the complexes.  
52x76mm (300 x 300 DPI)
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<tr>
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<th>Size (nm)</th>
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<td><strong>polyA-coated complexes</strong></td>
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<tr>
<td>pDNA/PEI</td>
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Each data are the mean ± S.E. (n = 3). n.d.: not detected.