Quaternary complexes modified from pDNA and poly-L-lysine complexes to enhance pH-buffering effect and suppress cytotoxicity

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ABSTRUCT

We developed a modified complex of pDNA and poly-L-lysine (PLL) by the addition of poly-L-histidine (PLH) and γ-polyglutamic acid (γ-PGA) to enhance its pH-buffering effect and suppress cytotoxicity. The binary and ternary complexes of pDNA with PLL or/and PLH showed particle sizes of approximately 52-76 nm with cationic surface charge. The ternary complexes showed much higher gene expression than the binary complexes with PLL. The mixed solution of PLL and PLH showed higher buffering capacity than PLL solution. The high gene expression of ternary complexes was reduced by bafilomycin A_1. These results indicated the addition of PLH to PLL complexes promoted endosomal escape by enhancing the pH-buffering effect. The binary and ternary complexes showed cytotoxicity and blood agglutination because of their cationic surface charge. We therefore developed quaternary complexes by the addition of anionic γ-PGA, which was reported to decrease the toxicity of cationic complexes. In fact, quaternary complexes showed no cytotoxicity and blood agglutination. Also, quaternary complexes showed higher gene expression than ternary complexes regardless of their anionic surface charge. Quaternary complexes showed selectively high gene expression in the spleen after their intravenous administration.

Thus, we successfully developed the quaternary complexes with high gene expression and no toxicity.

Keywords: Non-viral gene delivery; Plasmid DNA; Biocompatibility; Biodegradable polymers; Nanoparticles;

Abbreviations: PLL, poly-L-lysine; PLH, poly-L-histidine; γ-PGA, γ-polyglutamic acid; BSA, bovine serum albumin; FBS, fetal bovine serum; WST-1, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt; 1-methoxy PMS, 1-methoxy-5-methylphenazinium methyl sulfate; EDTA, ethylenediaminetetraacetic acid; RLU, relative light units; CPZ, chlorpromazine

INTRODUCTION

A wide range of non-viral gene vectors has been developed, such as cationic

polymers and cationic lipids, for successful gene therapy.[1,2]   Several advantages of

cationic polymers in the process of gene transfection have been reported: condensing

pDNA by electrostatic interaction, binding to the cell surface, take up by the endocytotic

pathway, and release of pDNA into the cytoplasm.[3,4]   Among cationic polymers,

poly-L-lysine (PLL) was one of the first polymers employed for gene transfer, which is

considered to have an advantage for clinical use because of its biodegradable peptide

structure.   At physiological pH, the amino groups of PLL were positively charged and

interact electrostatically with negatively charged DNA, which condensed the DNA in

the complexes and protected it.[5]   PLL, however, had a couple of disadvantages for

clinical use, such as low gene expression and agglutination of blood.[6]

The low gene transfection efficiency of PLL was reported to be due to its lack of an

efficient mechanism for endosomal escape.[7]   Generally, non-viral gene vectors that can

escape from endosomes express high gene transfection efficiency.   According to the

results provided by several groups, using a polymer with high buffering capacity, the

efficiency of endosomal escape could be increased by increasing the buffering

capacity.[8,9]   Gene vectors with buffering capacity lead to osmotic swelling and rupture

of endosomes, resulting in the release of vectors into the cytoplasm, a phenomenon

called the "pH-buffering effect".

Histidine has potential as a modification group for gene vectors because of its

biocompatibility and buffering capacity in the pH range of endosomes.[10-13]   The

accumulation of histidine residues inside acidic vesicles can induce the pH-buffering

effect, which could enhance the endosomal escape rate of the gene vector.   In previous

studies, there are several reports of histidine vectors such as histidylated polylysine,[14]

histidine-rich polymers,[15] and histidine-rich peptides.[16]   Then, we constructed ternary

complexes which incorporated poly-L-histidine (PLH) into PLL-based gene vectors

(binary complexes) to achieve more efficient gene transfection.

On the other hand, the cationic surface of pDNA complexes with cationic polymers

induced cytotoxicity and blood agglutination caused by their strong interaction with the

anionic surface of cells and erythrocytes.[17]   Agglutination of the complexes often led

to their rapid elimination and adverse effects, such as embolism and inflammatory

reactions.[18-20]   Recharging cationic complexes with an anionic compound was reported

to be a promising method for overcoming these toxicities.[21,22]   In our previous study,

we discovered anionic polymers such as γ-polyglutamic acid (γ-PGA),[23] chondroitin

sulfate,[24] and hyaluronic acid,[24] which decreased the toxicity of cationic complexes

while maintaining high transgene efficiency.   Among them, the addition of γ-PGA

showed the highest gene expression.

In the present study, we developed modified complexes of pDNA and PLL by the

addition of PLH and γ-PGA to enhance their pH-buffering effect and suppress

cytotoxicity.   We investigated the efficacy and safety of these quaternary complexes as

gene vectors *in vitro* and *in vivo*.

MATERIALS AND METHODS

Chemicals

Bovine serum albumin (BSA), PLL (mean molecular weight: 22,500), and PLH (molecular weight: ≥ 5,000) were obtained from Sigma-Aldrich Co. LLC. (St. Louis, MO, USA). The γ-PGA (mean molecular weight: 55,000) was provided by Yakult Pharmaceutical Industry Co., Ltd. (Tokyo, Japan). 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). YOYO-1 was purchased from Molecular Probes (Leiden, The Netherlands). All other chemicals were of reagent grade.

Construction of pDNA

pCMV-Luc was constructed by subcloning the Hind III/Xba I firefly luciferase cDNA fragment from the pGL3-control vector (Promega, Madison, WI, USA) into the

polylinker of the pcDNA3 vector (Invitrogen, Carlsbad, CA, USA).   The 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.

Preparation of binary, ternary, and quaternary complexes

PLL-PLH mixture was prepared by mixing PLL solution and PLH solution.   To

prepare binary and ternary complexes, pDNA solution and PLL-PLH mixture were

mixed by pipetting thoroughly.   The charge ratios of pDNA, PLL, and PLH were 1:8:0

(PLL8-PLH0 complexes), 1:6:2 (PLL6-PLH2 complexes), 1:4:4 (PLL4-PLH4

complexes), 1:2:6 (PLL2-PLH6 complexes), or 1:0:8 (PLL0-PLH8 complexes).   To

prepare quaternary complexes, γ-PGA solution was added to PLL6-PLH2 complexes at

various charge ratios of 1:6:2:4 (PLL6-PLH2-PGA4 complexes), 1:6:2:6

(PLL6-PLH2-PGA6 complexes), 1:6:2:8 (PLL6-PLH2-PGA8 complexes), and 1:6:2:10

(PLL6-PLH2-PGA10 complexes).

Physicochemical properties and gel retardation of complexes

The particle sizes and ζ-potentials of several 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, 20 µL aliquots of complex solutions containing 1

µg pDNA were mixed with 4 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.

Transfection and cellular uptake 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% CO_2 in air at 37 °C.   B16-F10 cells

were plated on 24-well plates (Becton-Dickinson and Company, Franklin Lakes, NJ,

USA) at a density of 1.0×10^4 cells/well and cultivated in 500 µl culture medium.   In

the transfection experiment, the medium was replaced with 500 µl Opti-MEM I medium

after 24 h pre-incubation and each complex containing 1 µg pCMV-Luc was added to

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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 under a humidified

atmosphere of 5% CO_2 in air 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 Co., Ltd., 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 the 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.

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

complexes containing of YOYO-1-labeled pCMV-Luc.　After 22 h incubation, the

relative levels of YOYO-1 in the cells were characterized using fluorescence

microscopy (200 × magnification).

pH titration

The pHs of PLL solution (3.9 µM) and mixed solution of PLL and PLH were

adjusted to 3.0 with 2 M HCL, and then the solutions were titrated with 0.01 M NaOH.

The change of pH was recorded using a pH meter (LAQUA F-72; Horiba Ltd., Kyoto,

Japan) and plotted for comparison of the buffering capacity between groups.   The

molar ratio of PLL to PLH in mixed solution was the same as that of PLL6-PLH2

complexes.

WST-1 assay

Cytotoxicity tests of various complexes on B16-F10 cells were carried out using a

WST-1 commercially available cell proliferation reagent.   The WST-1 reagent was

prepared (5 mM WST-1 and 0.2 mM 1-methoxy PMS in PBS) and filtered through a

0.22 µm filter (Millex-GP; Millipore Co., Bedford, MA, USA) just before the

experiments.   B16-F10 cells were plated on 96-well plates (Becton-Dickinson and

Company) at a density of 3.0×10^3 cells/well in the 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 µl culture

medium and incubated for another 22 h at 37 °C.   The medium was replaced with 100

µl culture medium and 10 µl 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 the percentage to untreated cells.

Inhibition study

  For determination of the endocytotic pathway, after 23 h pre-incubation, B16-F10 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.

  On the other hand, to research their release from endosomes, the cells were treated

with 100 nM bafiromycin A_1 as an ATPase proton pump inhibitor for 1 h.

  After treatment, PLL6-PLH2 complexes or PLL6-PLH2-PGA8 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 luciferase activities were determined.

Agglutination study

  Erythrocytes from mice were washed three times at 4 °C by centrifugation at 5,000

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.   Ten microliters of these suspensions were placed on a

glass plate and agglutination was observed by microscopy (400 × magnification).

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 weeks old) were

purchased from Japan SLC (Shizuoka, Japan).   After shipping, mice were acclimatized

to the environment for at least one day before the experiments.

In vivo study

   Each complex containing 40 µg pCMV-Luc at a volume of 250 µl per mouse was

injected intravenously into mice to examine the transgene efficacy of PLL-PLH

complexes and PLL-PLH-PGA complexes.   Six hours after the 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) for 5 min and the

supernatants were used for luciferase assays.   Luciferase activity is indicated as RLU

per gram of tissue.

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*.

RESULTS

Physicochemical properties and electrophoresis assay of binary and ternary

complexes

The particle sizes and ζ-potentials of the binary and ternary complexes are

summarized in Table 1.   The complexes showed a particle size of approximately 52-76

nm and a ζ-potential of approximately 40-48 mV.

Complex formations were examined using a gel retardation assay (Fig. 1).   Naked

pDNA was detected as bands on an agarose gel.   No band was detected in the lanes of

binary and ternary complexes.

In vitro transfection efficiency and cellular uptake of binary and ternary

complexes

B16-F10 cells were transfected with the binary and ternary complexes and luciferase

activity was evaluated in the cells.   The *in vitro* transgene efficiencies of the complexes

are shown in Fig. 2A.   The complexes of pDNA and PLL (PLL8-PLH0 complexes)

showed 1.34×10^6 RLU/mg protein in transgene efficiency.   On the other hand, the

gene expressions of ternary complexes were 42.0-fold (PLL6-PLH2 complexes) and

35.7-fold (PLL4-PLH4 complexes) higher than that of binary complexes (PLL8-PLH0

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complexes).   PLL2-PLH6 and PLL0-PLH8 complexes showed lower luciferase

activity than PLL8-PLH0 complexes.

    B16-F10 cells were transfected with PLL8-PLH0, PLL6-PLH2, or PLL0-PLH8

complexes containing YOYO-1-labeled pCMV-Luc to allow the uptake of the

complexes to be visualized (Fig. 2B).   As a result, many green dots representing

YOYO-1 were observed in most of the transfected cells for PLL8-PLH0 and

PLL6-PLH2 complexes.   On the other hand, PLL0-PLH8 showed low green dots.

pH titration

    With the addition of 0.01 M NaOH, the pHs of PLL solution and mixed solution of

PLL and PLH gradually increased.   The mixed solution of PLL and PLH had higher

buffering capacity between pH 5 and 7 than PLL solution (Fig. 2C).

Physicochemical properties and electrophoresis assay of quaternary complexes

    In the preliminary experiment, the ternary complexes of pDNA (PLL-PLH

complexes) showed cytotoxicity and agglutination.   Therefore, we prepared quaternary

complexes of pDNA (PLL-PLH-PGA complexes) by the addition of γ-PGA to

PLL6-PLH2 complexes.   The particle size and ζ-potential of these PLL-PLH-PGA

complexes are shown in Table 2.   While PLL6-PLH2-PGA4 complexes showed

agglutination, the complexes with charge ratios of 8 or 10 for γ-PGA to pDNA showed a

particle size of around 100 nm.   The addition of γ-PGA decreased the ζ-potential of

complexes in a concentration-dependent manner.   PLL-PLH-PGA complexes with

charge ratios of 8 or 10 for γ-PGA to pDNA had an anionic surface charge.   Gel

retardation assays were employed to examine the formation of the complexes (Fig. 3).

Naked pDNA was detected as bands on an agarose gel.   On the other hand,

PLL-PLH-PGA complexes showed no band of pDNA that was released.

In vitro transfection efficiency of quaternary complexes

   The quaternary complexes containing pCMV-Luc were incubated with B16-F10 cells

and the luciferase activity in the cells was determined.   The *in vitro* transgene

efficiencies of the complexes at various charge ratios are shown in Fig. 4A.   The

transgene efficiencies of PLL6-PLH2 complexes showed 5.5×10^7 RLU/mg protein.

The luciferase activity of PLL-PLH-PGA complexes was higher than that of

PLL6-PLH2 complexes.

Endocytotic pathway and release from endosome of ternary and quaternary

complexes

For assessment of the endocytotic pathway, we examined the effects of endocytotic inhibitors on the transgene efficiencies of PLL6-PLH2-PGA8 complexes (Fig. 4B). The transfection efficiency of PLL6-PLH2-PGA8 complexes in the presence of CPZ, genistein, and amilirode decreased to 79.7%, 56.0%, and 40.6% of the original efficiency, respectively.

The influence of 100 nM bafiromycin A_1 on transfection efficiency of PLL6-PLH2 complexes (Fig. 2D) or PLL6-PLH2-PGA8 complexes (Fig. 4C) was determined to examine endosomal escape.   Transgene efficiencies were significantly suppressed by bafiromycin A_1 ($P < 0.05$).

Cytotoxicity and agglutination study of the quaternary complexes

Each complex was added to B16-F10 cells and cell viability was determined by the WST-1 assay to evaluate cytotoxicity (Fig. 5A).   Both PLL8-PLH0 complexes and PLL6-PLH2 complexes showed significantly higher cytotoxicity than the control ($P < 0.05$).   On the other hand, PLL6-PLH2-PGA8 complexes had no cytotoxicity under the same condition.

Also, each complex was incubated with erythrocytes to evaluate agglutination (Fig.

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5B).   Severe agglutination was observed in both PLL8-PLH0 complexes and

PLL6-PLH2 complexes.   However, no agglutination was observed in

PLL6-PLH2-PGA8 complexes.

In vivo study

The *in vivo* transgene efficiencies of PLL8-PLL0 complexes, PLL6-PLH2 complexes,

and PLL6-PLH2-PGA8 complexes containing pCMV-Luc were examined in ddY male

mice (Fig. 6).   Luciferase activities in several tissues were determined 6 h after

intravenous administration of the complexes.   The transgene efficiencies of

PLL8-PLH0 complexes showed 1.1×10^5 RLU/mg protein in the lung.   On the other

hand, the gene expressions of PLL6-PLH2-PGA8 complexes were lower in the lung

(4.7×10^4 RLU/mg protein), and significantly higher in the spleen (3.4×10^5 RLU/mg

protein) than those of PLL8-PLH0 complexes and PLL6-PLH2 complexes.

DISCUSSION

Cationic polymers are one of the most popular non-viral gene vectors due to their excellent ability to compact anionic genes, synthetic controllability, better flexibility achieved simply by varying the chemical composition, molecular weight, and architecture (linear, randomly branched, dendrimer, block, and graft copolymer).[25] Espcially, polyethylenimine (PEI) and polyamidoamine (PAMAM) dendrimers show high gene expression *in vitro* and *in vivo*.[26,27] These vectors strongly interact with the cell surface, and are well taken up by cells because of their strong cationic surface. Moreover, these vectors achieve efficient escape from endosomes to cytoplasm via the pH-buffering effect and the gene released from the vector in the cytoplasm expresses protein in the nucleus.

The cationic polymer PLL was reported to show low gene expression because of its lack of pH-buffering effect, regardless its high uptake into the cells.[6,7,28] Therefore, we tried to develop ternary complexes of pDNA with PLL and PLH, which are polymers with pH-buffering capacity. Histidine of PLH, a non-essential amino acid, contains an imidazole ring which increases the buffering capacity in endosomes and lysosomes. Histidine-containing dipeptides, such as carosine, anserine, and balenine, are also known to act as endogenous buffers in skeletal muscle.[11,29]

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The binary complexes of pDNA with PLL or PLH showed 76.4 nm or 51.6 nm in particle size, and 48.4 mV or 39.9 mV in ζ-potential (Table 1). The ternary complexes of pDNA with PLL and PLH showed similar particle size and ζ-potential. The binary and ternary complexes can include pDNA stably (Fig. 1). Addition of PLH to pDNA and PLL did not greatly affect the manufacturing characteristics of the particles.

As projected, the gene expressions of ternary complexes which replaced PLL with PLH at rates of 25% and 50% (PLL6-PLH2 and PLL4-PLH4 complexes) were 42.0-fold and 35.7-fold higher than that of binary complexes of pDNA and PLL (PLL8-PLL0 complexes), respectively (Fig. 2A). On the other hand, binary complexes with PLH (PLL0-PLH8 complexes) and ternary complexes which replaced PLL with PLH at a rate of 75% (PLL2-PLH6 complexes) showed lower luciferase activities. PLL0-PLH8 complexes showed very low uptake by cells (Fig. 2B, iii). Therefore, the low gene expression of PLL0-PLH8 complexes was mainly due to the low uptake of PLL0-PLH8. Zhao et al. reported that synthetic histidinylated PLL based gene vector showed unstable and low gene expression by introducing many histidine group.[30] Introducing of histidine group to the PLL complexes reduced cationic characteristics of the complexes. The low uptake of PLL0-PLH8 may be caused by slight low cationic surface and peculiar structure of PLH.

The mixed solution of PLL and PLH showed higher buffering capacity between pH 5

and 7 than PLL solution (Fig. 2B).   PEI and histidylated polymers showed similar

titration profiles through neutral to weakly acidic conditions.[31-33]   The endosomal

escape mechanism of pDNA medicated by PEI was supposed to occur via the

pH-buffering effect.   The imidazole group of histidine had pKa 6.0 [34] and thus became

cationic in slightly acidic conditions.

Furthermore, the transfection efficiency of PLL6-PLH2 complexes was markedly

reduced when cells were incubated in the presence of bafilomycin A$_1$, a specific

inhibitor of the ATPase responsible for the accumulation of protons inside the endosome

lumen.[35,36]   On the other hand, strong cellular uptake of both the PLL8-PLH0 and

PLL6-PLH2 complexes was observed during experiments involving complexes

containing pDNA labelled with YOYO-1.   These results indicate that the addition of

PLH to PLL complexes induced endosomal escape by enhancing the pH-buffering

effect to increase the gene expression.

The strong interaction of cationic complexes to anionic cell membrane often cause to

strong cytotoxicity and blood agglutination.   In fact, cationic complexes such as binary

complexes with PLL and ternary complexes of PLL6-PLH2 complexes showed strong

cytotoxicity and blood agglutination (Fig. 5).   One promising approach to overcoming

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such cytotoxicity is the addition of anionic polymers to cationic complexes. For

example, we previously found that γ-PGA decreased the toxicity of cationic

compounds.[23] Therefore, we developed quaternary complexes of pDNA by the

addition of γ-PGA to PLL6-PLH2 complexes.

The addition of anionic polymers often caused the release of pDNA containing the

complexes. However, the addition of PGA did not release pDNA from quaternary

complexes (Fig. 3). Complexes with charge ratios of 8 or 10 for γ-PGA to pDNA

(PLL6-PLH2-PGA8 and PLL6-PLH2-PGA10 complexes) showed a particle size of

around 100 nm with anionic surface charge, suggesting stable anionic particles. The

anionic surface charge of complexes largely reduced cell surface interaction and blood

agglutination to decrease cytotoxicity and blood toxicity. In fact, the change in the

surface charge from positive to negative markedly decreased cytotoxicity and blood

agglutination (Fig. 5). The addition of alginic acid and anionic PEG derivatives to

cationic complexes was also demonstrated to reduce cytotoxicity and blood

agglutination by changing the surface charge.[37,38]

On the other hand, the anionic surface charge of particles also reduced gene

expression by decreasing cell surface interactions. We prepared various anionic

coating complexes and most could not be taken up well by cells and showed low gene

expression.[23]   Among these anionic polymers, however, we discovered that γ-PGA was

a rare constituent which was taken up by cells and showed high gene expression.[23]   In

fact, all quaternary complexes coated with γ-PGA showed high gene expression

comparable to ternary complexes (PLL6-PLH2 complexes) (Fig. 4A).   These

transfection efficiencies were as great as the commercially available transfection reagent,

lipofectin $(1.5 \times 10^8$ RLU/mg protein)[39], although their transgene efficacies were lower

than those of the binary complexes of pDNA with PAMAM $(1.4 \times 10^9$ RLU/mg protein)

or PEI $(2.2 \times 10^{10}$ RLU/mg protein).   However, PAMAM and PEI are not

biodegradable and exhibit strong toxicity [40,41], therefore they are not available yet for

clinical use. The present quaternary complexes are biodegradable and are constructed by

self-assembly.   They showed no cytotoxicity and blood toxicity.   The complexes may

be easily manufactured with sterilization and expected for clinical use.

   In the previous report, we confirmed that γ-PGA-coated complex was taken up by an

energy-dependent process and γ-PGA-specific receptor-mediated pathway using

B16F10 cells.[23]   Then, to clarify the mechanisms responsible for the uptake of the

complexes, we performed an inhibition study with various endocytotic inhibitors, such

as chlorpromazine (CPZ) for clathrin-mediated endocytosis, genistein for

caveolae-mediated endocytosis, and amiloride for macropinocytosis.[4]   The transgene

efficiency of PLL6-PLH2-PGA8 complexes was markedly decreased in the presence of

amiloride, suggesting that macropinocytosis must be the primary route of transfection

for PLL-PLH-PGA complexes (Fig. 4B).   Furthermore, the transfection efficiency of

PLL6-PLH2-PGA8 complexes was markedly inhibited by bafiromycin A1.   This result

indicated that quaternary complexes have the pH-buffering efficiency of PLH.

    For the clinical gene therapy, not only high transfection efficiency but also

biocompatibility for frequent dosing is indispensable.   PLL6-PLH2-PGA8 complexes

showed high gene transfection efficiency without cytotoxicity and agglutination,

indicating their utility as a clinical gene delivery system.   We therefore determined the

in vivo transgene efficiencies of PLL6-PLH2-PGA8 complexes.   PLL6-PLH2-PGA8

complexes showed high gene expression in the spleen (3.4×10^5 RLU/mg protein) after

intravenous injection (Fig. 6).   Sutherland et al. reported that poly-γ-D-glutamic acid

(γDPGA), which is a capsular component of Bacillus anthracis, was mainly

accumulated in the spleen and liver after intravenous injection into mice.[42,43]   In

particular, γDPGA was located in red pulp in the spleen and Kupffer cells and sinusoidal

endothelial cells in the liver. PLL6-PLH2-PGA8 complexes may be taken up by splenic

macrophage through a similar mechanism of γDPGA.   Various γ-PGA-coated

complexes were reported to be accumulated in the limbic system of the spleen and

showed gene expression after their intravenous injection into mice.[44]   It was reported

that the gene vector which showed high gene expression in the spleen was an effective

DNA vaccine for malaria.[45]

   Thus, we successfully modified binary complexes of pDNA with PLL by the addition

of PLH and γ-PGA to develop quaternary complexes with high gene expression and no

toxicity.

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REFERENCES

1. Canine BF, Hatefi A 2010. Development of recombinant cationic polymers for gene therapy research, Adv Drug Deliv Rev 62: 1524-1529.

2. Tros de Ilarduya C, Sun Y, Duzgunes N. 2010. Gene delivery by lipoplexes and Polyplexes. Eur J Pharm Sci 40: 159–170.

3. Boussif O, Lezoualc'h F, Zanta MA, Mergny MD, Scherman D, Demeneix B, Behr JP 1995. 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.

4. Khalil IA, Kogure K, Akita H, Harashima H 2006. Uptake pathways and subsequent intracellular trafficking in nonviral gene delivery. Pharmacol Rev 58: 32-45.

5. Wagner E, Cotton M, Foisner R, Birnstiel ML 1991. Transferrin-polycation-DNA complexes: the effect of polycations on the structure of the complex and DNA delivery to cells. Proc Natl Acad Sci U.S.A. 88: 4255–4259.

6. Farrell LL, Pepin J, Kucharski C, Lin X, Xu Z, Uludag H 2007. A comparison of the effectiveness of cationic polymers poly-l-lysine (PLL) and polyethylenimine (PEI) for non-viral delivery of plasmid DNA to bone marrow stromal cells (BMSC). Eur J Pharm Biopharm 65: 388–397.

7. Yamagata M, Kawano T, Shiba K, Mori T, Katayama Y, Niidome T 2007. Structural advantage of dendritic poly(L-lysine) for gene delivery into cells. Bioorg Med Chem

15: 526-532.

8.   Sonawane ND, Szoka FC, Verkman A 2003. Chloride accumulation and swelling in endosomes enhances DNA transfer by polyamine-DNA polyplexes. J Biol Chem 278: 44826–44831.

9.   Tseng SJ, Tang SC, Zeng YF, Cherng JY, Shih MF 2005. Structural characterization and buffering capacity in relation to the transfection efficiency of biodegradable polyurethane. Bioconjug Chem 16: 1375–1381.

10.   Shigeta K, Kawakami S, Higuchi Y, Okuda T, Yagi H, Yamashita F, Hashida M 2007. Novel histidine-conjugated galactosylated cationic liposomes for efficient hepatocyte-selective gene transfer in human hepatoma HepG2 cells. J Controll Release 118: 262–270.

11.   Abe H 2000. Role of histidine-related compounds as intracellular proton buffering constituents in vertebrate muscle. Biochemistry (Mosc.) 65: 757–765.

12.   Kichler A, Leborgne C, März J, Danos O, Bechinger B 2003. Histidine-rich amphipathic peptide antibiotics promote efficient delivery of DNA into mammalian cells. Proc Natl Acad Sci U.S.A. 100: 1564–1568.

13.   Pichon C, Gonçalves C, Midoux P 2001. Histidine-rich peptides and polymers for nucleic acids delivery. Adv Drug Delivery Rev 53: 75–94.

29

14.   Midoux P, Monsigny M 1999. Efficient gene transfer by histidylated

polylysine/pDNA complexes. Bioconjug Chem 10: 406–411.

15.   Swami A, Aggarwal A, Pathak A, Patnaik S, Kumar P, Singh Y, Gupta KC 2007.

Imidazolyl-PEI modified nanoparticles for enhanced gene delivery. Int J Pharm 335:

180–192.

16.   AokiY, Hosaka S, Kawa S, Kiyosawa K 2001. Potential tumor-targeting peptide

vector of histidylated oligolysine conjugated to a tumor-homing RGD motif. Cancer

Gene Ther 8: 783–787.

17.   Dekie L, Toncheva V, Dubruel P, Schacht EH, Barrett L, Seymour LW 2000.

Poly-L-glutamic acid derivatives as vectors for gene therapy. J Control Release 65:

187–202.

18.   D. Fischer, T. Bieber, Y. Li, H.P. Elsasser, T. Kissel 1999. A novel non-viral vector

for DNA delivery based on low molecular weight, branched polyethylenimine: effect of

molecular weight on transfection efficiency and cytotoxicity. Pharm Res 16:

1273–1279.

19.   Ogris M, Brunner S, Schuller S, Kircheis R, Wagner E 1999. PEGylated

DNA/transferrin–PEI complexes: reduced interaction with blood components, extended

circulation in blood and potential for systemic gene delivery. Gene Ther 6: 595–605.

20.   Ogris M, Wagner E 2002. Targeting tumors with non-viral gene delivery systems.

Drug Discov Today 7: 479–485.

21   Trubetskoy VS, Wong SC, Subbotin V, Budker VG, Loomis A, Hagstrom JE, Wolff JA 2003.

Recharging cationic DNA complexes with highly charged polyanions for in vitro and in vivo gene

delivery. Gene Ther 10: 261–271.

22.   Ito T, Iida-Tanaka N, Koyama Y 2008. Efficient in vivo gene transfection by stable

DNA/PEI complexes coated by hyaluronic acid. J Drug Target 16: 276–281.

23.   Kurosaki T, Kitahara T, Fumoto S, Nishida K, Nakamura J, Niidome T, Kodama Y,

Nakagawa H, To H, Sasaki H 2009. Ternary complexes of pDNA, polyethylenimine,

and gamma-polyglutamic acid for gene delivery systems. Biomaterials 30: 2846-2853.

24.   Kurosaki T, Kitahara T, Kawakami S, Nishida K, Nakamura J, Teshima M,

Nakagawa H, Kodama Y, To H, Sasaki H 2009. The development of a gene vector

electrostatically assembled with a polysaccharide capsule. Biomaterials 30: 4427-4434.

25.   Kabanov AV 1999. Taking polycation gene delivery systems from in vitro to in

vivo. Pharm Sci Technol Today 2: 365–372.

26.   Godbey WT, Wu KK, Mikos AG 1999. Poly(ethylenimine) and its role in gene

delivery. J Control Release 60: 149–160.

27.   M.X. Tang, F.C. Szoka 1997. The influence of polymer structure on the

interactions of cationic polymers with DNA and morphology of the resulting complexes.

Gene Ther 4: 823–832 .

28.   Cherng JY 2009. Investigation of DNA spectral conformational changes and

polymer buffering capacity in relation to transfection efficiency of DNA/polymer

complexes. J Pharm Pharm Sci 12: 346-356.

29.   Abe H, Dobson GP, Hoeger U, Parkhouse WS 1985. Role of histidine-related

compounds to intracellular buffering in fish skeletal muscle. Am J Physiol 249:

R449–454.

30.   Zhao GX, Tanaka H, Kim CW, Li K, Funamoto D, Nobori T, Nakamura Y,

Niidome T, Kishimura A, Mori T, Katayama Y 2014. Histidinylated poly-L-lysine-based

vectors for cancer-specific gene expression via enhancing the endosomal escape. J

Biomater Sci Polym Ed. 25: 519-534.

31.   Thomas M, Lu JJ, Ge Q, Zhang C, Chen J, Klibanov AM 2005. Full deacylation

of polyethylenimine dramatically boosts its gene delivery efficiency and specificity to

mouse lung. Proc Natl Acad Sci U. S. A. 102: 5679–5684.

32.   Benns JM, Choi JS, Mahato RI, Park JS, Kim SW 2000. pH-sensitive cationic

polymer gene delivery vehicle: N-Ac-poly(l-histidine)-graft-poly(l-lysine) comb shaped

polymer. Bioconjug Chem 11: 637–645.

33.   Leng Q, Mixson AJ 2005. Modified branched peptides with a histidine-rich tail enhance in vitro gene transfection. Nucleic Acids Res 33 e40.

34.   Patchornic A, Berger A, Katchalski E 1957. Poly-l-histidine. J Am Chem Soc 79: 5227–5236.

35.   Furuchi T, Aikawa K, Arai H, Inoue K 1993. Bafilomycin A1, a specific inhibitor of vacuolar-type H(+)-ATPase, blocks lysosomal cholesterol trafficking in macrophages. J Biol Chem 268: 27345–27348.

36.   Singh RS, Gonçalves C, Sandrin P, Pichon C, Midoux P, Chaudhuri A 2004. On the gene delivery efficacies of pH-sensitive cationic lipids via endosomal protonation: a chemical biology investigation. Chem Biol 11: 713–723.

37.   Jiang G, Min SH, Kim MN, Lee DC, Lim MJ, Yeom YI 2006. Alginate/PEI/DNA polyplexes: a new gene delivery system. Yao Xue Xue Bao 41: 439–445.

38.   Maruyama K, Iwasaki F, Takizawa T, Yanagie H, Niidome T, Yamada E, Ito T, Koyama Y 2004. Novel receptor-mediated gene delivery system comprising plasmid/protamine/sugar-containing polyanion ternary complex. Biomaterials 25: 3267–3273.

39. Kurosaki T, Morishita T, Kodama Y, Sato K, Nakagawa H, Higuchi N, Nakamura T, Hamamoto T, Sasaki H, Kitahara T. 2010. Nanoparticles electrostatically coated with

folic acid for effective gene therapy. Mol Pharm. 8: 913-919.

40.   Moghimi SM, Symonds P, Murray JC, Hunter AC, Debska G, Szewczyk AA 2005.

Two-stage poly(ethylenimine)-mediated cytotoxicity: implications for gene

transfer/therapy. Mol Ther 11: 990–995.

41.   Mukherjee SP, Lyng FM, Garcia A, Davoren M, Byrne HJ. 2010. Mechanistic

studies of in vitro cytotoxicity of poly(amidoamine) dendrimers in mammalian cells.

Toxicol Appl Pharmacol 248: 259–268.

42.   Sutherland MD, Thorkildson P, Parks SD, Kozel TR. 2008. In vivo fate and

distribution of poly-gamma-D-glutamic acid, the capsular antigen from Bacillus

anthracis. Infect Immun 76: 899–906.

43.   Sutherland MD, Kozel TR. 2009. Macrophage uptake, intracellular localization,

and degradation of poly-gamma-D-glutamic acid, the capsular antigen of Bacillus

anthracis. Infect Immun 77: 532–538.

44.   Kurosaki T, Kodama Y, Muro T, Higuchi N, Nakamura T, Kitahara T, Miyakoda M,

Yui K, Sasaki H 2013. Secure splenic delivery of plasmid DNA and its application to

DNA vaccine. Biol Pharm Bull 36: 1800-1806.

45.   Cherif MS, Shuaibu MN, Kurosaki T, Helegbe GK, Kikuchi M, Yanagi T, Tsuboi

T, Sasaki H, Hirayama K 2011. Immunogenicity of novel nanoparticle-coated MSP-1

C-terminus malaria DNA vaccine using different routes of administration. Vaccine 29:

9038-9050.

FIGURE LEGENDS

Figure 1.

Electrophoretic migration of pDNA in binary and ternary complexes through agarose gel. PLL-PLH complexes were loaded onto an agarose gel, and electrophoresis was carried out.   The retardation of pDNA was visualized using ethidium bromide.

Figure 2.

(A) *In vitro* transfection efficiencies of PLL-PLH complexes.   B16-F10 cells were transfected with various complexes containing pCMV-Luc.   Twenty-two hours after transfection, cells were lysed and luciferase activities were determined.   Each value is the mean ± S.E. ($n = 3$).   *P <0.05 vs. PLL8-PLH0 complex.

(B) Fluorescence microscopy images of transfected B16-F10 cells. B16-F10 cells were transfected with YOYO-1 labeled pCMV-Luc-containing complexes. Twenty-two hours after the cells had been transfected, the uptake of PLL8-PLH0 (i), PLL6–PLH2 (ii), and PLL0-PLH8 complexes (iii) was assessed (200 × magnification).

(C) Buffering capacity of PLL and PLL-PLH solution.   NaOH 0.01 M was used to titrate the PLL solution or mixed solution of PLL and PLH.   Molar ratio of PLL to PLH in mixed solution was the same as for PLL6-PLL2 complexes. ●: PLL, ○:

PLL-PLH

(D) PLL6-PLL2 complexes were transfected in medium with bafiromycin A_1.

Twenty-two hours after transfection, cells were lysed and luciferase activities were

determined.   Each value is the mean $\pm$ S.E. ($n = 3$).   *P $<$0.05 vs. control.

Figure 3.

Electrophoretic migration of pDNA in quaternary complexes through agarose gel.

PLL-PLH-PGA complexes were loaded onto an agarose gel, and electrophoresis was

carried out.   The retardation of pDNA was visualized using ethidium bromide.

Figure 4.

(A) *In vitro* transfection efficiencies of PLL-PLH-PGA complexes.   B16-F10 cells

were transfected with various complexes containing pCMV-Luc.   Twenty-two hours

after transfection, cells were lysed and luciferase activities were determined.   Each

value is the mean $\pm$ S.E. ($n = 3$).   *P $<$0.05 vs. PLL6-PLH2 complex.

(B) Influence of endocytotic inhibitors on the transfection efficiency of

PLL6-PLH2-PGA8 complexes.   PLL6-PLL2-PGA8 complexes were transfected in

medium with various endocytotic inhibitors.   Twenty-two hours after transfection, cells

were lysed and luciferase activities were determined.   Each value is the mean $\pm$ S.E. (n

$= 3$).

(C) Influence of endocytotic inhibitors bafiromycin A_1 on the transfection efficiency of

PLL6-PLH2-PGA8 complexes.   PLL6-PLL2-PGA8 complexes were transfected in

medium with bafiromycin A_1.   Twenty-two hours after transfection, cells were lysed

and luciferase activities were determined.   Each value is the mean $\pm$ S.E. ($n = 3$).   *P

<0.05 vs. control.

Figure 5.

(A) Cytotoxicity of the complexes on B16-F10 cells.   The viability of cells treated with

each complex was measured by the WST-1 assay.   Cell viability was measured 22 h

after cells had been incubated with the complexes for 2 h.   Data are the percentage to

untreated cells.   Each value is the mean $\pm$ S.E. ($n = 8$).   *P <0.05 vs. control.

(B) Agglutination of complexes with erythrocytes.   Each complex was added to

erythrocytes, and agglutination was observed by microscopy ($400 \times$ magnification).

(i) PBS, (ii) PLL8-PLL0 complexes, (iii) PLL6-PLH2 complexes, (IV)

PLL6-PLH2-PGA8 complexes.

Figure 6.

In vivo transgene efficiencies of the complexes in mice.   The complexes were injected

intravenously into mice (40 µg DNA per mouse).   Six hours after the injection, mice

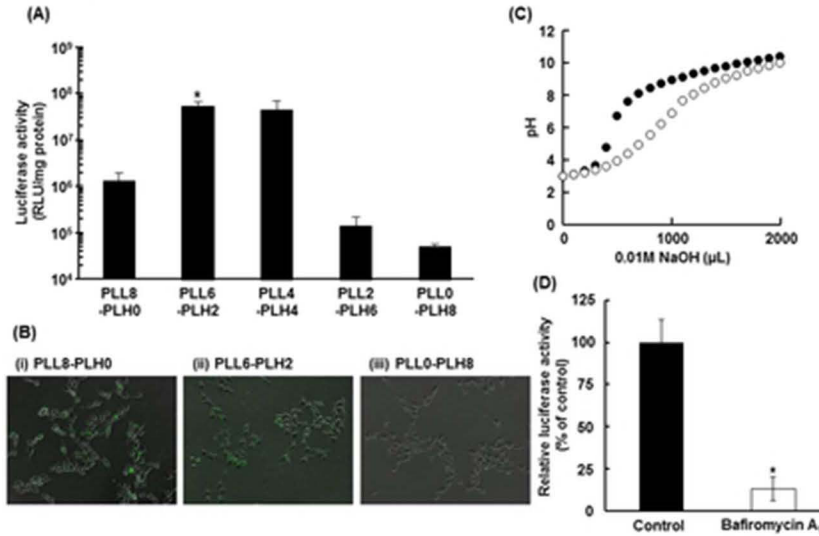were sacrificed and each organ was dissected to quantify luciferase activity.

PLL8-PLL0 complex (■), PLL6-PLH2 complex (▨) and PLL6-PLH2-PGA8 complex

(□).   Each value is the mean ± S.E. (n = 3).   *P <0.05, vs PLL complex, †P<0.05, vs

PLL-PLH complex.

Fig. 1
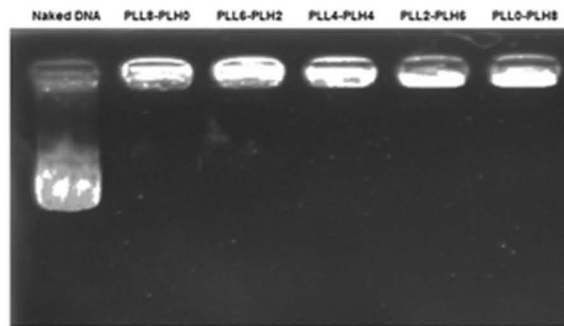
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Fig. 2

(A) In vitro transfection efficiencies of PLL-PLH complexes. B16-F10 cells were transfected with various complexes containing pCMV-Luc. Twenty-two hours after transfection, cells were lysed and luciferase activities were determined. Each value is the mean ± S.E. (n = 3). *P <0.05 vs. PLL8-PLH0 complex.

(B) Fluorescence microscopy images of transfected B16-F10 cells. B16-F10 cells were transfected with YOYO-1 labeled pCMV-Luc-containing complexes. Twenty-two hours after the cells had been transfected, the uptake of PLL8-PLH0 (i), PLL6–PLH2 (ii), and PLL0-PLH8 complexes (iii) was assessed (200 × magnification).

(C) Buffering capacity of PLL and PLL-PLH solution. NaOH 0.01 M was used to titrate the PLL solution or mixed solution of PLL and PLH. Molar ratio of PLL to PLH in mixed solution was the same as for PLL6-PLL2 complexes. ●: PLL, □○: PLL-PLH

(D) PLL6-PLL2 complexes were transfected in medium with bafiromycin A1. Twenty-two hours after transfection, cells were lysed and luciferase activities were determined. Each value is the mean ± S.E. (n = 3). *P <0.05 vs. control.
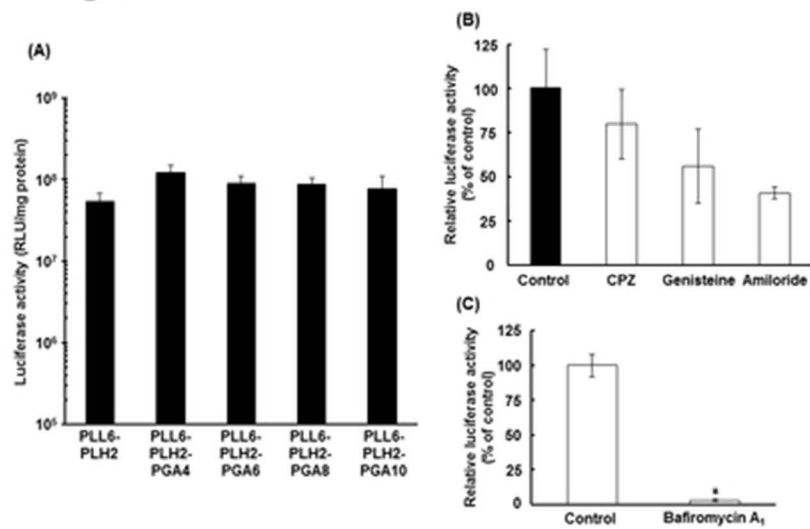
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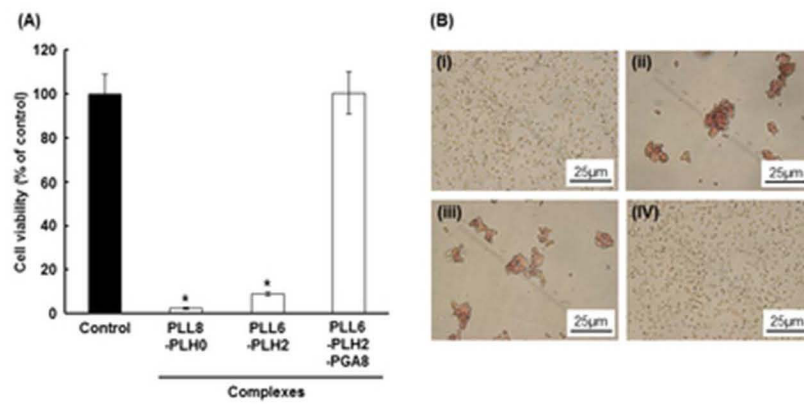
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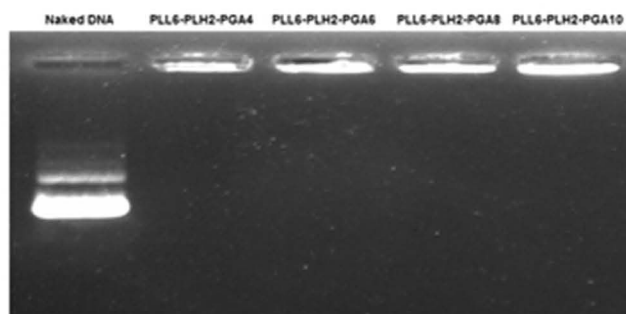
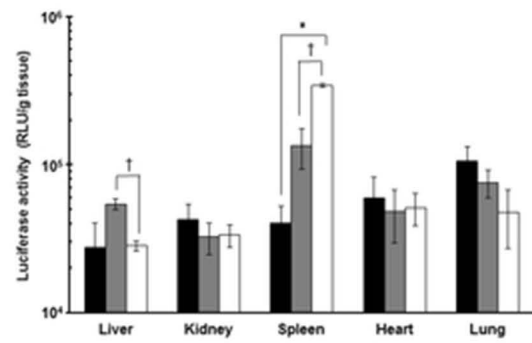36x27mm (300 x 300 DPI)

Fig. 4

36x27mm (300 x 300 DPI)

Fig. 5

36x27mm (300 x 300 DPI)

Fig. 6

36x27mm (300 x 300 DPI)

Tables

Table 1

Particle size and ζ-potential of binary and ternary complexes.

Complexes	ζ-potential (mV)	Size (nm)
PLL8-PLH0 complex	48.4 ± 0.6	76.4 ± 22.1
PLL6-PLH2 complex	42.3 ± 0.1	64.1 ± 8.3
PLL4-PLH4 complex	41.3 ± 0.8	70.1 ± 4.7
PLL2-PLH6 complex	40.3 ± 0.8	62.7 ± 4.0
PLL0-PLH8 complex	39.9 ± 0.5	51.6 ± 8.8

Each data is mean ± S.D. (n = 3)

1

Table 2

Particle size and ζ-potential of quaternary complexes.

Complexes	ζ-potential (mV)	Size (nm)
PLL6-PLH2-PGA4 complex	0.5 ± 0.3	undetectable
PLL6-PLH2-PGA6 complex	-7.4 ± 0.1	272.5 ± 1.6
PLL6-PLH2-PGA8 complex	-22.0 ± 0.5	106.9 ± 6.5
PLL6-PLH2-PGA10 complex	-28.6 ± 0.6	120.0 ± 9.0

Each data is mean ± S.D. (n =3)