Quaternary Complexes Modified from pDNA and Poly-L-Lysine Complexes to Enhance pH-Buffering Effect and Suppress Cytotoxicity

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ABSTRACT: 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

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 that 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

Abbreviations used: 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.

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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 Da), and PLH (molecular weight: ≥5,000 Da) were obtained from Sigma–Aldrich Company LLC. (St. Louis, Missouri). The γ-PGA (mean molecular weight: 55,000 Da) was provided by Yakult Pharmaceutical Industry Company, 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, New York). 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 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 *Hin*d III/*Xba*I firefly luciferase cDNA fragment from the pGL3-control vector (Promega, Madison, Wisconsin) into the polylinker of the pcDNA3 vector (Invitrogen, Carlsbad, California). 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

Poly-L-lysine–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 was 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, New Jersey) 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 preincubation 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 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 was mixed with 50 μL luciferase assay buffer (PicaGene; Toyo Ink Company, Ltd., 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 (Bio-Rad Laboratories, Inc., Hercules, California) using BSA as a standard. Absorbance was measured using a microplate reader (Sunrise RC-R; Tecan Japan Company, 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 pH 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 Company, Bedford, Massachusetts) 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

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