PEG grafted polylysine with fusogenic peptide for gene delivery: high transfection efficiency with low cytotoxicity

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Abstract

For efficient gene delivery into cells, a new formulation method based on using polyethylene glycol (PEG) grafted poly(l-lysine) (PLL) and a fusogenic peptide is presented in this study. First, PEG grafted PLL (PEG-g-PLL) was complexed with DNA by controlling the polymer/DNA ratio to form negatively charged nano-particulate complexes. A positively charged fusogenic peptide, KALA, was then coated by ionic interaction onto the surface of polymer/DNA complexes to make net positively charged KALA/polymer/DNA complexes. The use of PEG-g-PLL for KALA coating significantly suppressed the aggregation of complexes due to steric stabilization effect of PEG present on the surface, while the use of PLL alone induced severe aggregation of the complexes via KALA mediated inter-particulate cross-linking. For PEG-g-PLL/DNA complexes, enhanced transfection efficiency was observed with increasing amount of KALA. This suggests that maintaining the size of DNA/polymer complexes after KALA coating plays an important role in gene transfection. KALA/DNA/PEG-g-PLL complexes exhibited lower cytotoxicity compared with other polymer/DNA complexes. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

Efficient delivery of foreign DNA into cells is an extremely critical step in gene therapy. To avoid using viral vector systems that may elicit undesirable safety problems, non-viral vector systems are an attractive alternative method to deliver DNA into cells [1]. Increased attention has been given to non-viral delivery systems such as cationic liposome/DNA complexes and polymer/DNA complexes [2,3]. Among various cationic polymers, poly(l-lysine) (PLL) has been widely used as a gene carrier because of its excellent DNA condensing ability [4]. However, PLL itself is not an ideal transfection agent because of its cytotoxic effect and relatively low transfection efficiency in vitro and in vivo. PLL has been modified with hydrophilic and biocompatible polyethylene glycol (PEG) for increasing transfection efficiency in the presence of serum proteins and with targetable ligands for increasing tissue specificity [5–7]. PEG was conjugated to PLL to form either graft or block copolymer structures [8–10]. The PEG modified PLL/DNA complexes showed decreased interaction with plasma proteins,
reduced cell toxicity, and consequently increased transfection degree. Steric effect of surface exposed PEG strands reduced the extent of non-specific protein adsorption onto positively charged polymer/DNA complexes and this eventually contributed to the cellular uptake of the complexes within cells.

For efficient gene transfection, rapid escape of DNA from endosomal membrane to cytoplasm is required. A variety of membrane disruptive peptides and fusion peptides such as GALA and KALA are used for that purpose [11–14]. In most cases, the membrane active peptide elements have an amphipathic character [15–17]. Under appropriate conditions, they can interact with the endosomal membranes, perturb them, and thus enable DNA to be delivered into the cell cytoplasm. These membrane disruptive and fusogenic peptides have a tendency to undergo conformational change in a pH-dependent manner [14]. Simple non-covalent ionic interactions between positively charged membrane active peptides and negatively charged DNA molecules were utilized for transfecting cells, because they have the ability to condense DNA [18–23]. Previously, we reported that a di-block copolymer of poly(N,N'-dimethylaminoethyl methacrylate-co-N-vinylpyrrolidone)-block-PEG-galactose could be used as a gene delivery vector for hepatocyte targeting [24]. It was demonstrated that significantly enhanced level of gene expression could be achieved by simple coating of KALA onto the PEG modified surface of negatively charged DNA/polymer complexes. The study suggested that positively charged KALA coated DNA/polymer complexes had a higher tendency to be taken into cells by adsorptive endocytosis, along with an increased capacity for endosomal escape by means of co-delivering a fusogenic peptide into cells. Recently, we additionally reported that PEG-KALA conjugates were coated on the surface of PEI/DNA complexes to attain increased transfection efficiency while minimizing the cytotoxicity of PEI [25].

This study demonstrates a versatile method of formulating polymer/DNA complex using a fusogenic peptide, KALA, to obtain high transfection efficiency with low cytotoxicity. PEG-g-PLL was synthesized and used for the condensation of DNA/DNA/PEG-g-PLL and DNA/PLL complexes were formulated to produce negatively charged surfaces, onto which KALA was coated to form net positively charged KALA/DNA/polymer complexes. The size and zeta ($\zeta$) potential value of the complexes were evaluated with changing KALA/DNA weight ratios. The complexes were transfected against 293T cells using RSV luciferase plasmid DNA as a reporter gene. To demonstrate the steric repulsion effect of PEG, transfection experiments were also carried out in the presence of serum proteins. Cell cytotoxicities were evaluated for PLL and PEG-g-PLL and their complexes with DNA and KALA.

2. Materials and methods

2.1. Materials

Poly(ℓ-lysine) (MW 30,000) was purchased from Sigma (St. Louis, MO). Succinimidyl succinamide methoxy-poly(ethylene glycol) (SSA-PEG; MW 5000) was the product of Shearwater Polymers (Huntsville, AL). Dialysis membrane (MW cut-off, 10,000) was purchased from Spectrum (Houston, TX). Phosphate-buffered saline (PBS), Dulbecco’s Modified Eagle’s Medium (DMEM), fetal bovine serum (FBS), t-glutamine, penicillin, streptomycin, and trypsin were the products of Gibco-BRL (Grand Island, NY). 293T cells (transformed primary embryonic kidney, human) were supplied from the Korean Cell Line Bank (Seoul, South Korea). Microbicinechinonic acid (BCA) protein assay kit was purchased from Pierce (Rockford, IL). Plasmid RSV luciferase was extracted from transformed Escherichia coli by a standard alkaline lysis technique [26] and purified by a DNA-purification column which was purchased from QIAGEN (CA, USA). The purity and concentration of plasmid DNA was determined by measuring absorbance ratio at 260/280 nm using a spectrophotometer (Shimadzu UV-1601).

2.2. Synthesis of PEG-g-PLL

SSA-PEG (90 mg, 18 $\mu$mol) dissolved in 1 ml of anhydrous dimethylsulfoxide (DMSO) was slowly added to 2 ml of DMSO containing PLL (100 mg, 3.3 $\mu$mol). The stoichiometric molar ratio of SSA-PEG and PLL was adjusted to 6:1. The reaction
continued for 12 h at room temperature under nitrogen atmosphere and was terminated by adding 30 ml of de-ionized water. The reaction mixture was dialyzed to remove unreacted PEG and subsequently lyophilized. PEG-g-PLL was analyzed by $^1$H-NMR using D$_2$O as a solvent. $^1$H-NMR spectra were taken with a Bruker DRX 400 spectrometer operating at 400 MHz. Chemical shift (δ) was measured in ppm by using 3-(trimethyl-silyl) propionic-2,2,3,3-d$_4$ acid (TSP) sodium salt as an internal reference.

2.3. Preparation of KALA/DNA/polymer complexes

PEG-g-PLL (15 μg/ml) and DNA (100 μg/ml) stock solutions were prepared in HBS (10 mM HEPES, 30 mM NaCl, 1.5 mM sodium phosphate dibasic, pH 7.4). The PEG-g-PLL solution (1 ml) was added dropwise to the DNA solution (1 ml). The weight ratio of PEG-g-PLL and DNA in the solution mixture was 1:6.67 (N/P ratio=1:5). At this ratio, negatively charged complexes were formed. They were incubated for 15 min at room temperature. The DNA/PEG-g-PLL complex particles were coated with varying amounts of KALA by slowly adding the above solution into 1 ml of KALA solutions containing 0.25–1.5 mg of KALA. Then 60 μl of the solution containing KALA/DNA/PEG-g-PLL complex particles was added in each well for cell transfection experiments. For the preparation of KALA/DNA/PLL complexes, 8 μg/ml of PLL was used for DNA compaction by considering the grafted weight percentage of PEG chains onto PLL backbone.

2.4. Size and surface charge of KALA/DNA/polymer complexes

Size and surface zeta potential values of KALA/DNA/PLL and KALA/DNA/PEG-g-PLL complexes were measured as a function of KALA coating amount by a dynamic light scattering instrument (Zetaplus, Brookhaven, NY) equipped with a He-Ne laser at a wavelength of 632.2 nm. Selecting an appropriate buffer solution to measure the zeta potential value of the complexes was very critical. When the complexes were prepared in phosphate buffered saline (PBS) or DMEM, yellow-colored aggregates might be produced immediately due to the electro-chemical reaction. These aggregates were metallic phosphate aggregates which were formed at the platinum-plates of zeta potential analyzer. However, HEPES buffered saline (HBS) did not produce such metallic phosphate aggregates.

2.5. KALA/DNA/PLL and KALA/DNA/PEG-g-PLL complexes for cell transfection

293T cells were grown in Dulbecco’s Modified Eagles Medium (DMEM) supplemented with 10% fetal bovine serum, 10 U/ml penicillin-streptomycin, and 2 mM L-glutamine at 37°C in a humidified atmosphere under 5% CO$_2$. The cultured cells were plated in a six-well plate at an initial density of 100,000 cells/well. The suspended solution (60 μl) of KALA/DNA/PLL or KALA/DNA/PEG-g-PLL complex nano-particles was added into each well. Cell transfection experiments were performed in the absence or presence of serum proteins. The complexes in the serum environment were removed after 4 h. Transfected cells were incubated for 2 days before measuring the luciferase activity in each well. The extent of luciferase gene expression was determined by using a luciferase gene assay kit. Transfected cells were harvested with subsequent lysis, and the cell lysate was centrifuged at 14,000 rpm for 30 min. The supernatant (50 μl) was used for measuring luciferase activity and for determining protein concentration. Luminescence was recorded for 15 s in a Lumat LB 9501 luminometer (Berthold, Wildbach, Germany). Transfection efficiency was expressed as relative light unit (RLU) per milligram cell protein. All transfection experiments were performed in triplicate and two independent experiments were carried out to verify the reproducibility.

2.6. Cytotoxicity assay

293T cells were grown in 96-well plate at an initial density of 10,000 cells/well in 0.2 ml of growth medium and were incubated in environment of 5% CO$_2$ for 24 h. Growth media were replaced by new growth media containing KALA (15 μg/ml), PLL (15 μg/ml), and PEG-g-PLL (25 μg/ml). Various DNA complexes, and KALA/DNA, PLL/DNA, PEG-g-PLL/DNA and KALA/PEG-g-PLL/
DNA complexes were also tested. After 12-h incubation, the metabolic activity of 293T cells in each well was measured by using thiazolyl blue (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, MTT) assay [27].

3. Results and discussion

PEG grafted PLL has been widely used for non-viral gene delivery [6–8]. The introduction of PEG onto the PLL chain enhanced cell transfection efficiency despite the presence of serum proteins. The protein repelling capacity of PEG chains on the DNA/polymer complexes plays a critical role in suppressing protein adsorption, maintaining a positively charged surface, and facilitating the adsorptive endocytosis process. PEG grafted PLL was synthesized by using NHS-activated methoxy-PEG as described elsewhere [8]. From 1H-NMR spectra (data not shown) of the synthesized PEG-g-PLL, the numbers of PEG chains grafted onto a PLL backbone could be calculated from the relative integration ratio of a single –CH2– proton peak in PEG (s, 3.7–3.8 ppm) and multiple –CH– proton peaks in PLL (m, 4.3–4.4 ppm). It was found that 4.5 PEG chains were grafted onto a PLL backbone. The synthesized PEG-g-PLL was used for the compaction of plasmid DNA. To generate the negatively charged surface of DNA/PEG-g-PLL complexes, the weight ratio of PEG-g-PLL and DNA was adjusted to 1:6.67 (nitrogen/phosphate (N/P) ratio was 1:5). Fig. 1 shows surface zeta potential values of DNA/PEG-g-PLL and DNA/PLL complexes with increasing coating amount of cationic, pH-dependent fusogenic peptide, KALA. KALA undergoes a conformational change in response to pH change, exhibiting membrane disruptive activity in an acidified endosomal environment. KALA has an amino acid sequence of WEAK-LAKH-LAKH-LAKH-LAKH-LKAC-EA with a MW of 3.1 kDa. It has net five positive charges per molecule considering the total number of positively charged and negatively charged amino acids. The amount of KALA used for coating the DNA/polymer complexes is expressed as the relative weight ratio of KALA and DNA. It can be seen that without KALA coating, both DNA/PEG-g-PLL and DNA/PLL complexes had surface zeta potential values of ~23 mV, indicating that phosphate anions in DNA were not sufficiently masked by nitrogen cations of PEG-g-PLL or PLL. The size of the DNA/PEG-g-PLL and DNA/PLL complexes at this weight ratio was ~150 nm. The zeta potential value of both complexes increased with increasing amount of KALA due to the gradual and preferential coverage of positively charged KALA onto the surface. At the KALA/DNA weight ratio of 7.5, surface zeta potential value almost reached the saturation level, ~21 mV. DNA/DNA/PEG-g-PLL complexes had slightly higher zeta potential values than KALA/DNA/PLL complexes above the weight ratio of 5, probably because the KALA mediated aggregation of DNA/PLL complexes, as shown in Fig. 2, affected in measuring the zeta potential value. Fig. 2 shows the mean sizes of DNA/PEG-g-PLL and DNA/PLL complexes as a function of KALA coating amount. DNA/PLL complexes exhibit significant increment in size with KALA coating. DNA/PLL complexes were likely to aggregate upon increasing KALA amount, because positively charged KALA could form ionic bridges between the negatively charged complex particles. The aggregation tendency of the complex particles by KALA was more pronounced as the KALA amount to be coated was increased. The KALA coated DNA/PLL, as measured by a
complexes at this ratio because of interactions between the neutralized complexes. However, subsequent slight size reduction at the KALA/DNA ratio of 5.0 was observed with further coating of KALA molecules. This was due to the fact that the surface of the complexes became positively charged with concomitant enhancement of their colloidal stability. The observation can be attributed to the aggregation between the complexes having neutral surface charges at the KALA/DNA ratio of 2.5.

Fig. 3 shows transfection efficiencies of KALA/DNA/PEG-g-PLL and KALA/DNA/PLL complexes. As control experiments, positively charged KALA/DNA complexes (KALA/DNA weight ratio=10) and PEG-g-PLL/DNA complexes (N/P ratio=10) were also transfected, having luciferase activities of $3.1 \times 10^5$ and $1.1 \times 10^6$, respectively. For KALA/DNA/PLL, transfection efficiency was significantly increased with KALA coating up to the KALA/DNA ratio of 10, but decreased thereafter. This was clearly caused by the formation of KALA mediated inter-particle aggregates upon the addition of excessive KALA as shown in Fig. 2. The size of DNA/polymer complexes is very important in the cellular uptake process, i.e. endocytosis. On the other hand, the transfection efficiency of KALA/DNA/PEG-g-PLL complexes with KALA coating gradually increased without showing a decreasing tendency. The increasing transfection tendency of KALA/DNA/PEG-g-PLL complexes was presumably caused by the membrane disruptive action of co-delivered KALA for endosomal escape. Additionally, increasing positive charges on the surface by KALA coating, which resulted in the increased cellular uptake, might enhance transfection efficiency.

Fig. 4 shows the effect of serum proteins on the transfection efficiency of KALA/DNA/PEG-g-PLL and DNA/PLL complexes. The serum proteins were added during the transfection. Positively charged DNA/PLL (N/P ratio=10) complexes were used as a control. In the presence of 10% fetal bovine serum (FBS), PLL/DNA complexes had significantly reduced transfection efficiency compared to that without 10% FBS. This reduction can be attributed to the adsorption of serum proteins onto the complexes, which hampered the adsorptive endocytosis process of the complexes within cells. The KALA/DNA/PEG-g-PLL complexes showed slightly higher trans-
Fig. 3. Transfection efficiencies of KALA/DNA/PLL and KALA/DNA/PEG-g-PLL complexes against 293T cells. A 2-μg amount of of plasmid DNA was used per well. Transfection experiments were performed in triplicate and two independent experiments were carried out.

Fig. 4. Comparison with transfection efficiencies between KALA/DNA/PEG-g-PLL and DNA/PLL complexes in the presence of 10% fetal bovine serum (FBS). In the case of DNA/PLL complex, N/P ratio was fixed at 10. Transfection experiments were performed in triplicate. Two independent experiments were carried out.

Fig. 5. Effect of KALA and PEG-g-PLL on cell viability against 293T cells. PLL exhibits the lowest cell viability due to its charge density as reported in previous studies [8,28,29]. However, KALA and PEG-g-PLL had cell viabilities of ~80%.
Fig. 5. In vitro cytotoxicity of KALA/DNA/PEG-g-PLL complexes against 293T cells. The cytotoxicity of KALA, PLL, and PEG-g-PLL molecules was also evaluated after complexation with DNA or not. KALA/DNA weight ratio for KALA/DNA complex was 10, and KALA/DNA weight ratio for KALA/PEG-g-PLL/DNA complex was 15, with an N/P ratio of 10 for both PLL/DNA and PEG-g-PLL/DNA complexes. Control means no treatment on the cells.

for the cationic carriers alone and 90% for being complexed with DNA (N/P ratio = 10 for PLL and KALA/DNA weight ratio = 15). It is very encouraging that no noticeable cytotoxicity was observed in KALA/DNA/PEG-g-PLL complexes probably because of reduced charge density by complexation and the presence of surface exposed PEG chains. Fig. 6 describes a schematic sketch of the formulation presented in this study. The current formulation provides a capacity of high transfection efficiency with minimal cellular cytotoxicity. Furthermore, the formulation approach described here could be widely applied to various cationic polymers such as PLL, PEI and poly(N,N'-dimethylaminoethyl methacrylate) (PDMAEMA) [23,24].

4. Conclusions

In this study, it was demonstrated that PEG grafted PLL could be utilized to enhance gene transfection efficiency by formulation with a fusogenic peptide, KALA. Simple ionic binding of positively charged KALA onto negatively charged complexes of DNA/PEG-g-PLL resulted in improved transfection efficiency. The grafted PEG chains played an essential role in minimizing the inter-particle aggregation, which resulted in high transfection efficiency in the presence of serum due to surface exposed PEG molecules. This polymeric gene delivery system also exhibited very low cytotoxicity.
Fig. 6. A schematic diagram of the formulation of the DNA/PLL and DNA/PEG-g-PLL complexes with KALA coating.

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References


