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Enhancing Transfection Efficiency Using Polyethylene Glycol Grafted Polyethylenimine and Fusogenic Peptide

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Abstract This study presents a new formulation method for improving DNA transfection efficiency using a fusogenic peptide and polyethylene glycol grafted polyethylenimine. Succinimidyl succinate polyethylene glycol (PEG-SSA) was conjugated with polyethylenimine (PEI). PEI is well known for a good endosomal escaping and DNA condensing agent. The positively charged synthetic fusogenic peptide, KALA, was coated on the negatively charged PEG-g-PEI/DNA and PEI/DNA complexes. The KALA/PEI/DNA complexes exhibited aggregation behavior at higher KALA coating amounts with an effective diameter of around 1,000 nm. However, the KALA/PEG-g-PEI/DNA complexes were 100–300 nm in size with a surface zeta-potential (ζ) value of about +20 mV. The conjugated PEG molecules suppressed any KALA-mediated inter-particle aggregation, and thereby improved the transfection efficiency. Consequently, the transfection efficiency of the KALA/PEG-g-PEI/DNA complexes was obtained by utilizing both the fusogenic activity of KALA and the steric repulsion effect of PEG.

Keywords: KALA, polyethylene glycol (PEG), polyethylenimine (PEI), a DNA delivery system

INTRODUCTION

In the development of vectors for gene therapy, both viral and nonviral approaches have been extensively explored. Recently, nonviral gene delivery is a rapidly expanding field in gene therapy. The attractive features of nonviral systems are based on their easy manipulation, the dimensional flexibility of DNA, large-scale and economical production, and safety in *in vivo* application. Therefore, an increasing interest has been given to nonviral systems using cationic liposomes, cationic polymers, DNA binding peptides or naked DNA [1-5].

Cationic polymers, such as polylysine, poly(N,N'-dimethylaminoethyl methacrylate, (DMAEMA)), polyethylenimine, and polyamidoamine dendrimers have been used as efficient gene delivery vectors [6-11]. Among them, PEI has been demonstrated as one of the most efficient delivery polymers, since its introduction in 1995 [12]. Primary, secondary, and tertiary amine groups are positioned at every third nitrogen atom of PEI, thereby making it one of the most positively charged polymers. At physiological pH, PEI is partially protonated with a very large buffering capacity, leading to an excellent lysosomotropic effect for endosomal escape [13]. This is referred as the "proton sponge" effect.

To achieve efficient gene transfection, three major obstacles for foreign gene expression, that is, cellular uptake, endosome escape, and nuclear localization, must be considered in designing DNA carriers. To enhance cellular uptake, polyelectrolyte complex nanoparticles, formed between DNA and cationic polymers, are transported within cells by an endocytosis process. Also, many membrane active peptides have been used to increase gene delivery either by themselves or in association with other molecules [14-16]. These peptides enable the DNA/polymer complexes to escape from endosome to cytoplasm by destabilizing the endosomal membrane. Such peptides have been characterized in terms of their DNA binding, fusogenic activity, and cytotoxicity [17,18]. Arg and Lys residues are the most common amino acids binding to the negatively charged phosphate backbone of DNA. In particular, KALA (WEAK-LAKA-LAKA-LAKH-LAKA-LAKA-LKAC-EA) was synthesized and applied for enhancing transfection efficiency [18,19]. The cationic and α-helical conformation of this peptide was coated onto negatively charged polymer/DNA surfaces [20,21]. However, peptide mediated inter-particle aggregation is a major barrier in the formulation of KALA/polymer/DNA complexes.

Accordingly this paper presents a new formulation method for increasing transfection efficiency by using the fusogenic peptide, KALA, and polyethylene glycol (PEG). A PEG-SSA derivative was used to conjugate with PEI including primary amines in its backbone. The PEG-g-PEI conjugate was then characterized by ^{1}H-NMR. The positively charged KALA peptide was coated onto negatively charged PEI/DNA and PEG-g-PEI/DNA complexes to improve transfection efficiency. The sizes and surface charge values of the KALA/PEI/DNA and KALA/PEG-g-PEI/DNA complexes were characterized as a function of the KALA coating amount.

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MATERIALS AND METHODS

Materials

The poly(ethylenimine) (MW 25,000) was purchased from Aldrich (St. Louis, MO, USA). The PEG-SSA (MW 5,000) was the product of Shearwater Polymers (Huntsville, AL). The dialysis membrane (MW cutoff, 25,000) was purchased from Spectrum (Huston, Texas, USA). The phosphate-buffered saline (PBS), Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), L-glutamine, penicillin, streptomycin and trypsin-EDTA were the products of Gibco-BRL (Grand Island, NY, USA). The KALA peptide was provided by Peptron (Taejon, Korea) and its purity was checked with HPLC. 293T cells (transformed primary embryonic kidney, human) were supplied with the Korean Cell Line Bank (Seoul, Korea). The BCA protein assay reagent was the product of Pierce (Rockford, IL, USA). Plasmid RSV luciferase was extracted from transformed *Escherichia coli* using a standard alkaline lysis technique and purified by a DNA-purification column purchased from QIAGEN (CA, USA). The purity and concentration of the plasmid DNA were determined by the absorbance ratio at 260/280 nm using a spectrophotometer (Shimadzu UV-1601).

Methods

Synthesis and Characterization of PEG-g-PEI Conjugate

One gram of PEI (MW 25,000) (40 μmol) was dissolved in 25 mL of a sodium phosphate buffer (pH 8.0). Five hundred micrograms of PEG-SSA (100 μmol, MW 5,000) dissolved in 5 mL of dehydrated DMSO was added to the PEI solution in a drop-wise fashion. The reaction was left at room temperature for 4 h. The stoichiometry of the reaction was 1:2.5 (PEI:PEG-SSA). The reaction was stopped by the addition of 60 mL of deionized water followed by dialysis (MWCO 25,000). The conjugate was then freeze-dried and stored at −20 °C until use. The PEG-g-PEI was analyzed by ^{1}H-NMR using D_2O as a solvent. The ^{1}H-NMR spectra were taken using a Brucker DRX 300 spectrometer operating at 300 MHz. The chemical shift (δ) was measured in ppm using 3-(trimethyl-silyl) propionic-2,2,3,3-d_4 acid (TSP) sodium salt as an internal reference.

Formulation of KALA/PEG-g-PEI/DNA and KALA/PEI/DNA Complexes

Ten milliliters of PEI (5 μg/mL) and DNA (100 μg/mL) were separately prepared in an HBS (10 mM HEPES, 30 mM NaCl, 1.5 mM sodium phosphate, pH 7.4) solution. One mL of the PEI solution was added to 1 mL of the DNA solution in a drop-by-drop manner. The weight ratio of PEI and DNA in the solution mixture was 1:20 (nitrogen/phosphate ratio = 1:5), and the same amount of PEI was also formulated for making PEG-g-PEI/DNA conjugates. Negatively charged complexes were formed at this ratio. After incubation for 15 min, the PEI/DNA and PEG-g-PEI/DNA complex particles were coated with varying amounts of KALA and subsequently incubated for 15 min.

Size and Surface Charge of Polymer/DNA Complexes

The size and surface charge of the KALA/PEG-g-PEI/DNA and KALA/PEI/DNA complexes were measured using a dynamic light scattering instrument (Zetaplus, Brookhaven, NY) equipped with a He-Ne laser at a wavelength of 632.2 nm (Detection angle = 15°).

Formulation of KALA/PEG-g-PEI/DNA and KALA/PEI/DNA complexes for transfection

293T cells were grown in Dulbecco's Modified Eagles Medium (DMEM), supplemented with 10% fetal bovine serum, 100 units/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 6-well plate at an initial density of 100,000 cells/well. After 24 h incubation, sixty μL of two kinds of complexes containing 2 μg of DNA was transfected to each well. Cell transfection experiments were performed in the absence of serum proteins. The transfected cells were incubated for 48 h before measuring the luciferase activity in each well. The activities of luciferase gene expression were determined by using a luciferase gene assay kit (Promega, USA) with a Lumat LB 9501 luminometer (Berthold, Wildbach, Germany). Briefly, transfected cells were harvested and lysed using a lysis buffer (50 μL) as recommended by the manufacturer, and then the cell lysate was centrifuged at 14,000 rpm for 30 min. The supernatant (20 μL) was used for measuring the luciferase activity, then protein concentration was determined using a BCA protein assay kit. The luminescence was recorded for 15 sec in a luminometer. The transfection efficiency was expressed as the 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.

RESULTS AND DISCUSSION

Fig. 1 shows a schematic diagram of the PEG-g-PEI synthesis. PEG-SSA was reacted specifically with the primary amine groups of PEI. The ^{1}H-NMR spectra confirmed that the PEG molecules were successfully grafted to the PEI backbone (data not shown). From this result, the number of PEG chains grafted onto a PEI backbone was calculated based on the relative integration values between the single -CH_2- proton peak in PEG (s, 3.7-3.8 ppm) and the multiple –HC_2- proton peaks in PEI (m, 2.3-2.6 ppm). According to the ^{1}H-NMR results, the average number of conjugated PEG was 1.8 per PEI backbone. The surface charges of the KALA/PEI/DNA and KALA/PEG-g-PEI/DNA complexes were measured as a function of a KALA/DNA weight ratio (Fig. 2). Strongly negative surface charges, −42 ± 5.4 mV for KALA/PEG-g-PEI/DNA and −39 ± 4.2 mV for KALA/

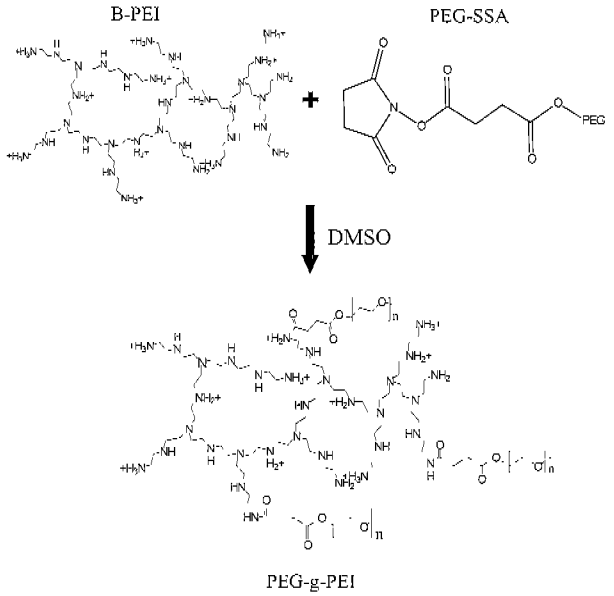

Fig. 1. Schematic sketch of the PEG-grafted PEI conjugates.

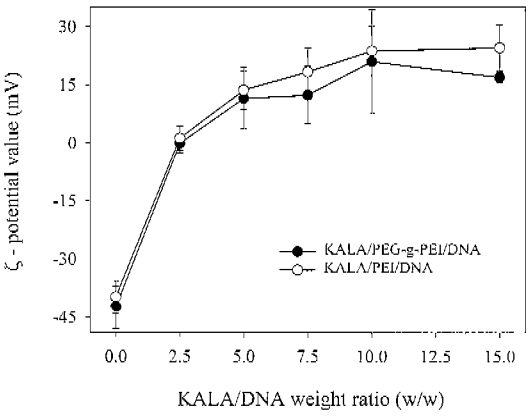

Fig. 2. Zeta-potential of KALA/PEI/DNA and KALA/PEG-g-PEI/DNA complexes as a function of KALA/DNA weight ratio with fixed amount of DNA.

PEI/DNA complexes, were measured at a KALA/DNA weight ratio of 0. Thereafter, the ζ-potential values increased with an increasing amount of KALA coated onto the surface of the complexes. At a KALA/DNA weight ratio of 2.5, both complexes were electrically neutral. Fig. 2 also shows saturation behavior at about +20 mV for the KALA/PEG-g-PEI/DNA complexes and nearly +25 mV for the KALA/PEI/DNA complexes with an increasing amount of KALA coating.

The effective diameters of the KALA/PEG-g-PEI/DNA and KALA/PEI/DNA complexes were evaluated using laser light scattering equipment (Fig. 3). The KALA/PEI/DNA complexes were aggregated above a KALA/DNA weight ratio of 10. This aggregation behavior resulted from KALA mediated inter-particle aggregation;

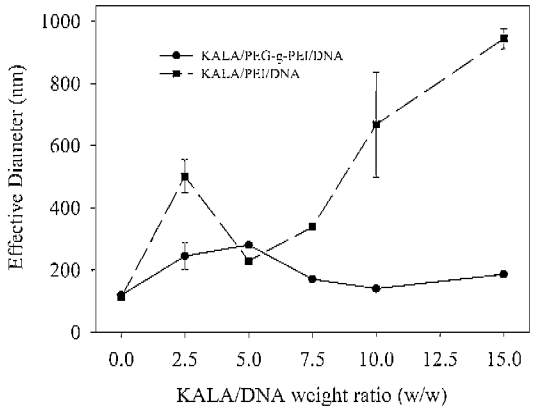

Fig. 3. Effective diameters (nm) of KALA/PEI/DNA and KALA/PEG-g-PEI/DNA complexes as a function of KALA/DNA weight ratio with fixed amount of DNA.

the effective diameter became about 1,000 nm at a KALA/DNA weight ratio of 15. However, the complexes containing PEG-g-PEI molecules exhibited no aggregation even above a KALA/DNA weight ratio of 10. This implies that highly mobile PEG molecules grafted to PEI suppressed any KALA mediated aggregation. It is also worth noting that at a KALA/DNA weight ratio of 2.5, the ζ-potential value approached zero (Fig. 2). The neutralized nanoparticles became more hydrophobic than the unneutralized ones. Therefore, the size of the KALA/PEI/DNA complexes slightly increased at this ratio (501 ± 51.2 nm), but no significant size increment was observed with the KALA/PEG-g-PEI/DNA complexes (245 ± 41.7 nm in effective diameter) (Fig. 3). Accordingly, the conjugated PEG molecules prevented, due to the steric stabilization effect of PEG, the KALA/PEG-g-PEI/DNA complexes from being aggregated. The transfection efficiency of these complexes was evaluated on 293T cells; the transfection efficiency was expressed as the relative light unit per mg cell protein. Fig. 4 shows the transfection efficiency of the KALA/PEI/DNA and KALA/PEG-g-PEI/DNA complexes with varying KALA/DNA weight ratios and a fixed DNA amount. In Fig. 4, a KALA/DNA weight ratio of zero means that negatively charged PEI/DNA complexes were formulated at an N/P ratio of 0.2. These were used as a KALA negative control. From a KALA/DNA weight ratio of 0 to 10, the transfection efficiency profile showed an increasing pattern and a decreasing trend thereafter. At a KALA/DNA weight ratio of 15, transfection efficiency was about 100-fold smaller than that of a KALA/DNA weight ratio of 10. The increased transfection efficiency within the KALA/DNA weight range of 0 to 10 was attributed to the co-delivered fusogenic activity of KALA for facilitated endosome escape and to becoming positive surface charge of the complexes to enhance cellular uptake. The decreasing transfection efficiency at higher KALA/DNA ratios was possibly caused by KALA-mediated aggregation that significantly reduced the cellular uptake of the KALA/PEI/

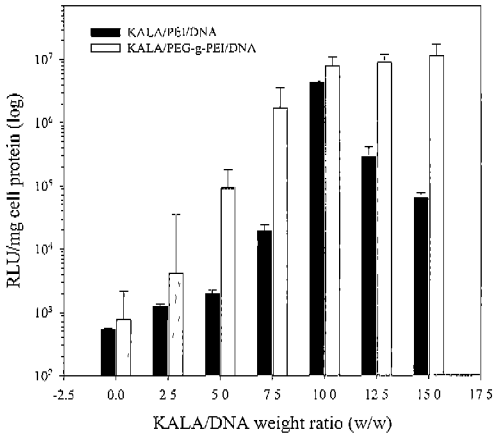

Fig. 4. Transfection efficiency of KALA/PEI/DNA and KALA/PEG-g-PEI/DNA complexes against 293T cells. The Transfection experiments were performed in triplicate with two independent experiments.

DNA complexes. This result indicates that the particle size is a dominant factor affecting transfection efficiency. However, the transfection efficiency of the KALA/PEG-g-PEI/DNA complexes exhibited an increasing transfection efficiency profile without a decreasing tendency observed in the formulation of the KALA/PEI/DNA complexes. It is conceivable that the surface-exposed PEG can enhance the transfection efficiency by preventing any inter-particle aggregation of the complexes in contrast to the KALA/PEI/DNA complexes. This result confirms that the maintenance of an appropriate particle size is an important factor for enhancing transfection efficiency. KALA coating on the surface of the PEG-g-PEI/DNA complexes not only changed the surface charge value of the complexes to be more positive, which is favorable for cellular uptake by an adsorptive endocytosis process, but also provided an opportunity to destabilize an endosomal membrane by co-delivering KALA to the endosome. Both effects had a direct influence on attaining high transfection efficiency. Further investigation is needed to determine which factor is more dominant in this system. A unique advantage of this delivery formulation is that the amount of PEI used for DNA condensation can be minimized to reduce the cytotoxic effect of PEI. The amounts of PEI required for the formulation of conventional PEI/DNA complexes are relatively very high, compared to that of the present formulation. The cytotoxic concern related to PEI has been well documented in previous literatures [22-26]. However, the current study obtained a similar transfection level to that of conventional DNA/PEI formulation while significantly reducing the cytotoxicity of PEI because only a minimal amount of PEI was required to condense the DNA [21]. The overall formulation strategy for the KALA/PEG-g-PEI/DNA and KALA/PEI/DNA complexes is described in Fig. 5.

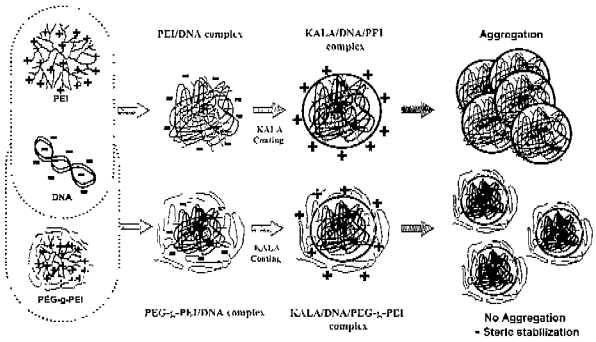

Fig. 5. Schematic diagram of formulation strategies of the KALA/PEI/DNA and KALA/PEG-g-PEI/DNA complexes.

CONCLUSION

This report demonstrated the comparative transfection efficiencies of the KALA/PEG-g-PEI/DNA and KALA/PEI/DNA complexes. Both PEI and PEG-g-PEI conjugates effectively condensed DNA with negatively charged surfaces, then positively charged KALA was coated on the surface of these complexes. The PEG corona in the KALA/PEG-g-PEI/DNA complexes suppressed any inter-particle aggregation mediated by KALA. The non-aggregated complexes of KALA/PEG-g-PEI/DNA increased the transfection efficiency with little affect on the cell viability.

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