Enhancement of the Transfection Efficiency of Poly(ethylenimine) by Guanidylation

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Chemists have not only developed new drugs but also some novel methodology for drug or gene delivery. Both the synthesis of new delivery carrier and the development of new formulation of existing materials could be a good target of chemists in these fields. Previously, gene delivery had also belonged to only biological field owing to the viral vector,[1] but it entered into the area of chemistry after the development of synthetic non-viral vectors.[2]

Several non-viral vectors composed of polymers,[3] lipids,[4] metals,[5] etc. have various merits over viral vectors including low toxicity, mass production, and the capacity for the gene even though they had relatively lower transfection efficiency than the viral vectors. Some polymeric vectors, such as poly(ethylenimine) (PEI)[6] and poly(amidoamine) (PAMAM)[7] showed the highest transfection efficiencies among non-viral gene delivery vectors, which were comparable to those of the viral vectors. It was suggested that the high transfection efficiency of PEI or PAMAM could be explained by the endosome buffering effect originated from its high charge density or membrane disruption.[8]

The transfection efficiency of these polymers is highly dependent upon their molecular weights. For example, whereas PEI with the molecular weight over 25 kDa showed the highest transfection efficiency among all non-viral gene delivery vectors, PEI with low molecular weight showed much less transfection efficiency.[9]

However, toxicity, which is another important factor for the *in vivo* gene delivery, increases sharply as the molecular weight increases. The PEI 2 kDa shows almost no toxicity, but PEI 25 kDa showed very high toxicity both in vitro and in vivo applications. Therefore, there have been many efforts to combine the merits of these two types of PEI-high transfection efficiency of high-molecular-weight PEI and low toxicity of low-molecular-weight PEI- for an ideal gene delivery vector. For example, several derivatives of low-molecular-weight PEI were linked with biodegradable bonds for enhancing transfection efficiency, with limiting success.[10]

Meanwhile, it has been reported that arginine residues in the TAT viral protein sequence could improve the delivery efficiency into the cell nucleus.[11] Other polymers conjugated with TAT sequence or arginine-rich sequence also showed high transfection efficiency. In our previous report, the PAMAM dendrimer showed significantly improved transfection efficiency after the modification of the surface with arginine.[12]

Because there is a specific guanidine residue other than primary amine in arginine, it is supposed that we can improve the transfection efficiency of PEI by the modification of its primary amine into guanidine. In this paper, we will report how to improve the transfection efficiency of low-molecular weight PEI as well as to maintain its low toxicity.

The modification of primary amines in PEI 2 kDa into guanidines is performed using pyrazole-1-carboxamidine (Figure 1). As a control, the amines in high-molecular-weight PEI 25 kDa was also converted to guanidines. PEI in 0.01 M potassium carbonate aqueous solution was added with 5 equivalents of pyrazole-1-carboxamidine per each primary amine residue. The reaction mixture was stirred at r.t. for 20 hr, dialyzed against distilled water, and lyophilized to get the final product, poly(ethylenimine guanidine) (PEGu).

^{1}H-NMR and elemental analysis were used for the measurement of amine-guanidine conversion ratios. 12% (25 kDa) and 19% (2 kDa) of total amine residues in PEI

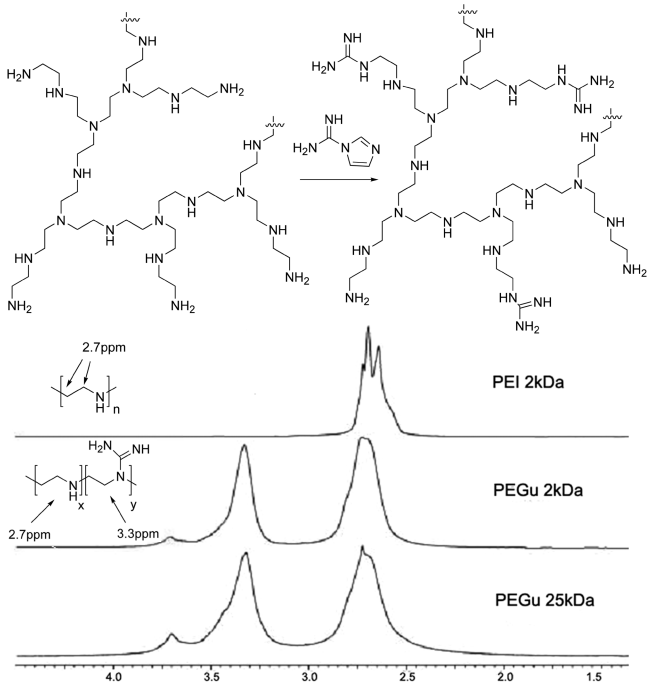

Figure 1. Synthetic scheme of PEGu synthesis and ^{1}H-NMR spectrums of PEI and PEGu.

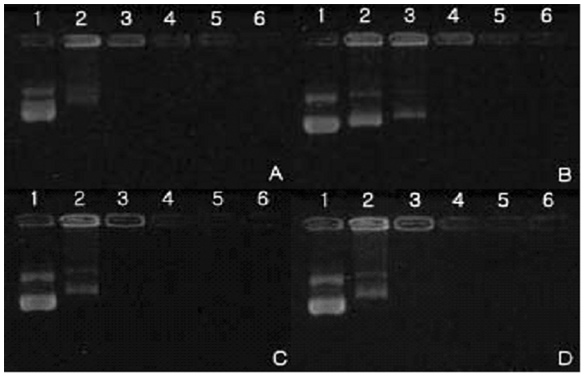

Figure 2. Agarose gel retardation assay of plasmid DNA and PEI 25 kDa (A), PEGu 25 kDa (B), PEI 2 kDa (C), PEGu 2 kDa (D). Plasmid DNA (1 μg) only (lane 1), N/P ratio of polymer/DNA = 1, 2, 4, 8 and 11 (lanes 2, 3, 4, 5, and 6 respectively).

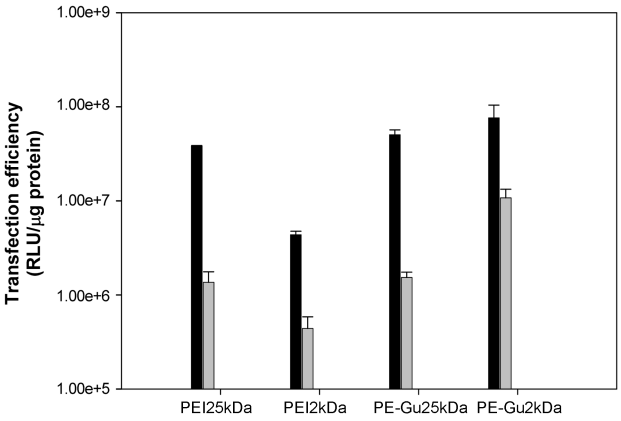

Figure 3. Transfection efficiency on C2C12 cell lines. Black bars represent FBS-free condition, and gray bars represent FBS condition. The numbers represent weight ratios between DNA and polymers. The error bars mean standard deviation of three experiments.

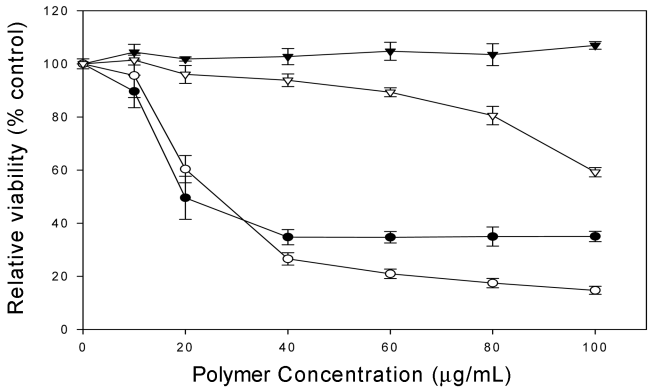

Figure 4. Cytotoxicity assay on C2C12 cells of PEI 25 kDa (●), PEGu 25 kDa (○), PEI 2 kDa (▼), and PEGu 2 kDa (▽). The error bars mean standard deviation of six experiments.

were converted into guanidine. Considering that the primary amine groups are 25% of total amine groups in PEI, the primary amine-guanidine conversion yields are 48% (25 kDa) and 76% (2 kDa). These conversion yields by pyrazole-1-carboxamidine are similar to that in the previous report.[13] The reason of the lower conversion yield of PEI 25 kDa was probably because the portion of the exposed primary amines in PEI 25 kDa, which could react with pyrazole-1-carboxamidine easily, was lower than that in PEI 2 kDa.

Because the electrostatic complex formation between cationic polymers and anionic DNA is very important for transfection, the complex formation ability of initial PEI and PEGu were compared each other using well-known agarose gel electrophoresis method (Figure 2). The numbers in the figure represent the ratios between moles of (amine (N) + guanidine (G)) residues in polymers and moles of phosphate (P) residues in DNA. As shown in the figure, the mobility of DNA with PEGu was retarded at (N + G)/P ratio around 3 in both molecular weights. Although the complex formation abilities of PEGus are slightly weaker than those of initial PEIs, the differences are not so significant. The polymer-DNA complex can be formed easily after the conversion from PEI to PEGu.

The transfection on the mouse myoblast C2C12 cell line was performed with the polymer-DNA complex (Figure 3). PEGu 25 kDa showed 30% higher transfection efficiency than unmodified PEI 25 kDa. The transfection increase by guanidylation is more remarkable in the transfection by low-molecular weight polymers. As mentioned above, the transfection efficiency of the unmodified PEI 2 kDa is only one tenth of that of PEI 25 kDa at their optimal conditions. However, after the guanidylation, PEGu 2 kDa showed 20 times higher efficiency than PEI 2 kDa and two times higher than PEI 25 kDa. Moreover, the transfection efficiency of PEGu 2 kDa was eight times higher than that of PEI 25 kDa in presence of 10% fetal bovine serum condition, which was more similar to the in vivo environment.

As mentioned above, the transfection efficiency of high-molecular-weight PEI is largely dependent upon the endosome buffering effect of its unprotonated amines. Whereas the guanidylation could facilitate the nucleus localization of the gene, the resulting decrease of unprotonated amine density could inhibit the endosome buffering effect somewhat. Therefore, the final transfection efficiency of the PEGu 25 kDa was not increased so much. Meanwhile, it is supposed that the transfection of low-molecular-weight PEI could not proceed by the endosome buffering effectively, so that the guanidylation could increase the transfection efficiency over 20 times.

Because our main objective was the development of the gene delivery carrier with low toxicity as well as high transfection efficiency, the cytotoxicity of PEGu was also analyzed in the C2C12 cell line (Figure 4). The cytotoxicities of the PEGus increased slightly comparing to those of their corresponding PEIs. However, IC_{50} value of PEGu 2 kDa is more than five times of that of PEI 25 kDa. Considering that the transfection efficiency of the PEGu 2 kDa was two times higher than PEI 25 kDa, the guanidylation of low-molecular-weight PEI could be a good way to obtain an efficient and safe gene delivery carrier. The concentration of the gene delivery carrier for the transfection is generally below 20

mg/mL, so that the toxicity of PEGu 2 kDa could be negligible.

In summary, we could improve the trasnfection efficiency of PEI 2 kDa 20 times by guanidylation as well as maintain its low cytotoxicity. The guanidylated polymer, PEGu, can be a potential gene delivery carrier, and the guanidylation of primary amine groups can be applied to increase the transfection efficiency of other cationic polymers for gene delivery.

Experimental Section

The synthesis of PEGu. PEI (0.010 mol amine residues/L) was dissolved in potassium carbonate aqueous solution. The concentration of potassium carbonate is 0.050 mol/L. The solution was added with pyrazole-1-carboxamidine (5 eq. per amine residue). The reaction mixture was stirred for 20 h and purified by dialysis (M.W.C.O = 1,000 Da; Hankook Spectrum Co.) against distilled water to obtain their corresponding PEGus. The ^{1}H-NMR spectrums of PEI and PEGu were shown in Figure 1. The conversion yield could be calculated from the elemental analysis data. The conversion yield is 12% and 19% for PEI 25 kDa and PEI 2 kDa, respectively.

Agarose gel electrophoresis. DNA-polymer complexes were formed at different charge ratios between the polymer and pCN-Luciferase (pCN-Luci) plasmid by incubating in HEPES buffer (25 mM, PH 7.4, 10 mM $MgCl_2$) at rt for 30 min. Each sample was then analyzed by electrophoresis on a 0.7% agarose gel and stained by incubation for 1 h in buffer containing ethidium bromide (0.5 μg/mL) at 37 °C.

Transfection assay in the C2C12 cell line. The C2C12 cells were seeded at a density of 5×10^4 cells/well in 24-well plates in 600 μL of media containing 10% Fatal Bovine Serum (FBS) and grown to 70-80% confluence. The cells were treated with polyplex solution containing 2 μg of plasmid DNA at different N/P ratios for 4 h at 37 °C. Following 4 h treatment of polyplexes, the medium was replaced by 600 μL of fresh medium containing 10% FBS. Cells were incubated further for 2 days before the luciferase assay. Then the growth medium was removed, and the cells were rinsed with phosphate buffered saline and lysed for 30 min at room temperature using 150 μL of Reporter lysis buffer (Promega). Luciferase activity was measured using a LB 9507 luminometer (Berthold, Germany) and the protein content was measured by using a Micro BCA assay reagent kit (Pierce, Rockford, IL).

Cytotoxicity assay. For the cytotoxicity assay, the colorimetric MTT assay was performed. Briefly, HepG2 cells were seeded at a density of 5×10^3 cells/well in a 96-well plate and ground in 90 μL of media for 1 day prior to the incubation with polymers. After treating cells with PEI 2 kDa, PEI 25 kDa, PEGu 2 kDa, and PEGu 25 kDa for 1 day, 25 μL of MTT stock solution (5 mg/mL) was added each well and incubate further 2 h. Then 100 μL extraction buffer (20% w/v of SDS is dissolved at 37 °C in a solution of 50% of each DMF and water, using magnetic stirring: pH is adjusted to 4.7 by adding 2.5% of an 80% acetic acid and 2.5% 1 N HCl) was added, and incubate overnight at 37 °C and the absorbance was measured at 570 nm using a microplate reader.

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