PAMAM Dendrimers Conjugated with 1-Arginine and γ-Aminobutyric Acid as Novel Polymeric Gene Delivery Carriers

Sang Jae Son,† Gwang Sig Yu,‡ Yun Hui Choe,† Youn-Joong Kim,†§ Eunji Lee,† Jong-Sang Park,§,* and Joon Sig Choi†,*

†Graduate School of Analytical Science and Technology (GRAST), Chungnam National University, Daejeon 305-764, Korea
‡E-mail: joonsig@cnu.ac.kr
§Department of Biochemistry, College of Natural Sciences, Chungnam National University, Daejeon 305-764, Korea
Korea Basic Science Institute (KBSI), Daejeon 305-333, Korea
School of Chemistry and Molecular Engineering, Seoul National University, Seoul 151-742, Korea
*E-mail: pfjspark@plaza.snu.ac.kr

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In this study, we synthesized functional dendrimer derivatives as nonviral gene delivery vectors. Poly(amidoamine) dendrimer (PAMAM, generation 4) was modified to possess functional amino acids to enhance gene transfection efficiency. PAMAM G4 derivatives conjugated with 1-arginine (Arg) and γ-aminobutyric acid (GABA) showed higher transfection efficiency and lower cytotoxicity compared to the native PAMAM G4 dendrimer. The polyplex of the PAMAM G4 derivative/pDNA was evaluated using an agarose gel retardation assay and Picogreen reagent assay. Additionally, the MTT assay was performed to examine the cytotoxicity of synthesized polymers. All PAMAM G4 derivatives showed lower cytotoxicity than PEI25kD. Particularly, PAMAM G4-GABA-Arg displayed enhanced transfection efficiency compared to the native PAMAM G4 dendrimer.

Key Words: PAMAM dendrimer, Gene delivery, 1-Arginine, γ-Aminobutyric acid (GABA)

Introduction

Effective gene therapy requires development of vectors containing therapeutic genes, which can deliver these genes safely and selectively to a target site.1,2 Particularly, the vector system should be able to deliver either DNA or RNA since delivery of the native forms of these molecules is difficult because of their negatively charged nature.

Vector systems can be divided into two categories: viral vector systems and non-viral vector systems. Viral vector systems such as adenovirus and retrovirus are efficient for gene transfection, but their cytotoxicity and the possibility of mutations in the host genome limits their clinical use.3 In contrast, non-viral vector systems show low cytotoxicity and transfection efficiency compared to viral vectors. To overcome the limitations of both viral and non-viral vector systems, various chemical alteration or conjugation methods have been developed for non-viral vector systems to enhance the transfection efficiency and biocompatibility by using cationic polymers, liposomes, and micelles.4,5

Cationic polymers such as poly(amidoamine) (PAMAM) dendrimer and polyethylenimine (PEI) have been commonly used as gene delivery agents.5,6 PAMAM dendrimers are also widely used for both drug and gene delivery.5,6 PEI is a highly efficient cationic polymer since it has numerous amine groups in its structure. However, it also shows strong cytotoxicity. Thus, PEI should be chemically modified to improve its suitability as a gene delivery agent.

PAMAM dendrimers can be synthesized using repeating Michael-addition and amidation methods with diverse core units such as ammonia and ethylenediamine (EDA). PAMAM dendrimers have a spherical structure; their surface primary amine groups can be chemically modified to possess various functional units. Moreover, the tertiary amine groups inside the repeating backbone structure allow them to act as a proton sponge during the endocytosis. Despite these advantages, PAMAM dendrimers require further modifications of their peripheral end groups since the intact structure shows low transfection efficiency. Therefore, PAMAM dendrimers have been subjected to chemical alteration with various amino acids.8

Oligo-arginine (Arg) is a cell-penetrating peptide (CPP) that is able to penetrate the cell membrane.10 Many studies have shown that modifying Arg in the conventional vector system increases cell-penetration efficiency, and increasing Arg concentration corresponds to enhanced transfection efficiency.11,12 Since GABA is composed of four carbons, it can be used as a linker molecule. In our previous study, we showed that polymers coupled with a 6-carbon linker between PAMAM and Arg show higher transfection efficiency compared to native PAMAM dendrimers.13 Therefore, we hypothesized that introducing a GABA linker between PAMAM G4 and its peripheral arginines would be also effective for enhancing the flexibility of arginine units, which interact with the plasma membrane for internalization into the cell. Moreover, conjugating GABA as a linker may be beneficial in terms of biocompatibility because GABA is a common neurotransmitter found in neuronal cells. Here, we report the synthesis, characterization, and in vitro evaluation of PAMAM G4-GABA-Arg as a novel cationic gene
carrier in the non-viral gene delivery systems.

Materials and Methods

Materials. N-Hydroxybenzotriazole (HOBt) and 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyloxonium (HBTU) were purchased from Anaspec (San Jose, CA). PAMAM G4 dendrimer, N,N-diisopropylethyl-amine (DIPEA), dimethyl sulfoxide (DMSO), trifluoroacetic acid (TFA), trisopropylsilane (TIS), and piperidine were purchased from Sigma-Aldrich (St. Louis, MO). Fmoc-Arg(pbf)-OH and Fmoc-γ-Abu-OH were purchased from Novabiochem (San Diego, CA). Fetal Bovine Serum (FBS), Dulbecco’s modified Eagle medium (DMEM), Dulbecco’s phosphate buffered saline (DPBS) and 100× antibiotic-antimycotic agent were purchased from Gibco (Gaithersburg, MD). The luciferase assay kit was purchased from Promega (Madison, WI).

Methods.

Synthesis of PAMAM G4 Derivatives: PAMAM G4 was allowed to react with 4 equivalents of Fmoc-Abu-OH (GABA), HOBt, HBTU, and 8 equivalents of DIPEA in anhydrous DMSO for 16 h. After the first reaction, PAMAM G4-GABA-Fmoc was precipitated using cold ether and dried using N₂ gas. Next, 30% (v/v, DMSO) piperidine was used to deprotect the Fmoc groups for 1 h in the dark. Next, PAMAM G4-GABA-NH₂ was further reacted with 4 equivalents of Fmoc-Arg(pbf)-OH, HOBt, HBTU, and 8 equivalents of DIPEA in anhydrous DMSO for 16 h. The resulting PAMAM G4-GABA-Arg(pbf)-Fmoc was precipitated, dried, and deprotected in the same manner described above. To deprotect the pbf groups on Arg units, the product was dissolved in deprotection solution (TFA:TIS:H₂O, 95%, 2.5%, 2.5%) and reacted for 6 h. After the deprotection reaction, the final products were precipitated and dried. To separate the final product from the un-reacted molecules, the final product was dialyzed against distilled water using a dialysis membrane (MWCO3500, Spectrum/por®, Spectrum Laboratories, CA, USA) for 2 days. After dialysis, the final product was lyophilized and collected in a microcentrifuge tube. The synthesis scheme is shown in Scheme 1. For structure identification, PAMAM G4 derivatives were analyzed using 400 MHz ¹H NMR spectroscopy (Bruker DRX 300).

Complex Formation Analysis: Polyplexes of PAMAM G4 derivatives with plasmid DNA were evaluated using agarose gel electrophoresis. Increasing volumes of polymer were reacted with a fixed volume of plasmid DNA in HEPES buffer (125 mM) for 30 min and analyzed on a 0.7% agarose gel containing ethidium bromide (0.5 mg/mL). Electrophoresis was performed in 1× Tris-acetate EDTA (TAE) buffer for 30 min at 100V. The hydrodynamic diameter of the polyplexes was measured using a dynamic light scattering ELS-Z2 system (Photol, Otsuka Electronics, Japan).

Picogreen Reagent Assay: The Picogreen reagent assay is more sensitive for detecting complex formation. Complexes

Scheme 1. Synthesis scheme of (a) PAMAM G4-Arg-GABA, and (b) PAMAM G4-GABA-Arg.
of plasmid DNA with PAMAM G4 derivatives were dissolved in HEPES buffer, adjusted to 200 µL, and incubated for 30 min at room temperature. Next, 200 µL of Picogreen reagent dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4) was added to the complex solution and incubated for at least 2 min at room temperature. The mixed solutions were adjusted to 2 mL with the TE buffer. Finally, the fluorescence of the complex solutions was measured with an excitation wavelength of 480 nm and an emission wavelength of 520 nm by using a spectrofluorometer (JASCO FP-750).

**Cell Culture:** Human cervical carcinoma HeLa and mouse neuroblastoma Neuro-2A cells were used and maintained in Dulbecco’s Modified Eagle Medium (DMEM, GIBCO) containing 10% fetal bovine serum (FBS, GIBCO) and 1% antibiotics (100× antibiotic-antimycotic, GIBCO) at 37 °C in a 5% humidified CO₂ incubator.

**Cytotoxicity Assay:** To measure cell viability, we performed WST-1 and MTT assays with the HeLa cells. Cells were seeded in a 96-well plate at 1.5 × 10⁴ cells/well and incubated in 100 µL of medium for 24 h. After incubation, the cells were treated with various concentrations of polymer samples (PEI 25 kD, PAMAM G4, PAMAM G4-Arg, PAMAM G4-Arg-GABA, PAMAM G4-GABA, and PAMAM G4-GABA-Arg) and further incubated for 24 h. After 24 h, 26 µL of the EZ-cytox reagent was added to each well for the WST-1 assay, and absorbance was measured at 450 nm in a VERSAmax microplate reader (Molecular Devices, Sunnyvale, CA). For the MTT assay, the MTT reagent (2 mg/mL solution, dissolved in DPBS) was prepared and added to each well, and the cells were incubated for 4 h. After removing the medium, 100 µL of DMSO was used to dissolve the MTT formazan crystals. Absorbance from each well was measured at 570 nm.

**Transfection Assay:** HeLa and Neuro-2A cells were plated in a 24-well plate at 2.0 × 10⁵ and 5 × 10⁴ cells/well, respectively. After cell seeding into the plate, cells were incubated for 24 h. Plasmid DNA was combined with various weight ratios of polymers, incubated for 30 min at room temperature, and used to treat the cells. Complexes composed of PEI25kD/pCN-Luc (w/w = 2:1) and PAMAM G4/pCN-Luc (w/w = 4:1) were used for comparison of transfection efficiency. After an additional 24 h of incubation, the medium was removed, and the cells were washed with DPBS and lysed using reporter lysis buffer for 30 min. Luciferase activity was detected using the Lumat LB 9507 instrument (Berthold Technology, Bad Wildbad, Germany) and the protein content was evaluated using the Micro BCA™ protein assay kit (Pierce, Rockford, IL).

**Results and Discussion**

**Characterization of PAMAM G4 Derivatives.** Based on the ¹H NMR results, conjugation yields of the polymers were evaluated by calculating the integral values of the respective peaks. The information about the molecular weights, synthesis yields are calculated from ¹H NMR and presented in Table 1.

### Table 1. Synthesis results of PAMAM G4 derivatives analyzed by ¹H NMR results

<table>
<thead>
<tr>
<th>Molecular weight</th>
<th>Arginine conjugation yield (%)</th>
<th>GABA conjugation yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAMAM G4</td>
<td>14214.17</td>
<td>-</td>
</tr>
<tr>
<td>PAMAM G4-R</td>
<td>24011.03</td>
<td>98</td>
</tr>
<tr>
<td>PAMAM G4-R-GABA</td>
<td>30584.44</td>
<td>99</td>
</tr>
<tr>
<td>PAMAM G4-GABA</td>
<td>18463.36</td>
<td>-</td>
</tr>
<tr>
<td>PAMAM G4-GABA-Arg</td>
<td>28632.01</td>
<td>93</td>
</tr>
</tbody>
</table>

**Figure 1.** ¹H NMR data. (a) PAMAM G4, (b) PAMAM G4-Arg, (c) PAMAM G4-Arg-GABA, and (d) PAMAM G4-GABA-Arg.
1.669 (\(-\text{HCCH}_2\text{CH}_2\text{NH}-\)), 3.537 (\(-\text{HCCH}_2\text{CH}_2\text{CH}_2\text{NH}-\)) of the Arg units, 2.147 (\(-\text{COCH}_2\text{CH}_2\text{CH}_2\text{NH}-\)) of the GABA and 2.306 (\(\text{NCH}_2\text{CH}_2\text{CO}^-\)) of the PAMAM G4 unit.

**Agarose Gel Retardation Assay and Picogreen Assay.**

To confirm polyplex formation of the synthesized PAMAM G4 derivatives with plasmid DNA, increasing amounts of polymers were combined with a fixed volume of pDNA in HEPES buffer and incubated for 30 min at room temperature. Combined polyplexes were run on a 0.7% agarose gel and imaged using a UV transilluminator (Fig. 2). Synthesized PAMAM G4 derivatives, including PAMAM G4-Arg, PAMAM G4-GABA, and PAMAM G4-GABA-Arg, completely retarded pDNA at a weight ratio of 2:1 (polymer/pDNA), whereas PAMAM G4-Arg-GABA showed complete retardation at 4:1. To evaluate complex formation precisely, we performed a Picogreen assay. As shown in Figure 3, the optimal weight ratio of synthesized PAMAM G4 derivatives revealed 4:1 or higher ratios. Interestingly, PAMAM G4-Arg-GABA showed a relatively increased weight ratio for complex formation compared to other polymers for both gel electrophoresis and Picogreen assay results. These differences in weight ratios for complex formation can be explained on the basis of the location of Arg units. PAMAM derivatives with arginine units at their peripheral ends may interact more effectively with negatively charged pDNA because of the presence of guanidyl groups. For PAMAM G4-Arg-GABA, since guanidyl groups on the arginine units are located in the inner space of the surface primary amines, they are less capable of forming complexes than other PAMAM G4 derivatives. However, these results indicate that all synthesized PAMAM G4 derivatives effectively interacted with pDNA at relatively low polymer concentrations.

**Dynamic Light Scattering (DLS) Study.**

According to the results from the agarose gel electrophoresis and Picogreen assays, the hydrodiameter of polyplexes at the optimal weight ratio were measured using dynamic light scattering (Fig. 4). As shown in Figure 4, reduced size distribution was observed when pDNA combined with synthesized PAMAM G4 derivatives. Naked pDNA revealed a nearly 2-μm size distribution. This pDNA cannot be internalized into cells because of its large hydrodynamic diameter and rapid clearance in the bloodstream by degradation enzymes such as DNase. Therefore, it is important to reduce the size of pDNA by introducing compacting agents that can electrostatically interact with negatively charged pDNA. Polyplex formation also helps to evade enzymatic degradation by burying the binding site, which is essential for docking to the active site. Synthesized PAMAM G4 derivatives formed nanometer-size complexes (around 100-200 nm) efficiently with pDNA.

**Transfection Efficiency and Cytotoxicity.** To evaluate the cytotoxicity of synthesized PAMAM G4 derivatives, human cervical carcinoma HeLa cells and mouse neuroblastoma Neuro-2A cells were incubated in the presence of various polymer concentrations. The relative viability of...

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**Figure 2.** Agarose gel retardation was performed to analyze the complex formation. Complexes were prepared at various weight ratios as indicated. (a) PAMAM G4-Arg, (b) PAMAM G4-GABA, (c) PAMAM G4-Arg-GABA, (d) PAMAM G4-GABA-Arg.

**Figure 3.** Picogreen assay was performed to evaluate complex formation between pDNA and PAMAM G4 derivatives.

**Figure 4.** Size distribution of naked plasmid DNA and polyplexes formed by PAMAM G4 derivatives.

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PAMAM displayed moderate cytotoxicity over a wide range of polymer concentrations in both cell lines. HeLa cells and Neuro-2A cells were incubated with synthesized PAMAM G4 derivatives complexed with pDNA at various weight ratios for 24 h. Luciferase activity was measured to evaluate transfection efficiency. As shown in Figure 6, GABA-terminated PAMAM G4 derivatives showed decreased transfection efficiency compared to native PAMAM G4 dendrimer even at high weight ratios. However, arginine-terminated PAMAM G4 derivatives showed enhanced transfection efficiency. Interestingly, PAMAM G4-GABA-Arg displayed significantly enhanced transfection efficiency compared to PAMAM G4 and PAMAM G4-Arg-GABA. So, it seems that the location of the arginine residues in the PAMAM G4 derivatives significantly affects complex formation and transfection efficiency. Furthermore, the chain length is related with hydrophobicity and will influence the physicochemical properties of the synthesized polymer and complexes with DNA. GABA, which is used as a linker compound in this study, possesses less hydrophobicity compared to Ahx. However, the transfection efficiency was not considerably affected by the different length of the spacer molecule. Although the transfection efficiency is not improved significantly compared to Ahx conjugation, introducing GABA may be beneficial in terms of cytotoxicity and biocompatibility because GABA is a common neurotransmitter in neuronal cells.

Conclusion

In summary, we synthesized novel cationic PAMAM G4 derivatives containing γ-aminobutyric acid (GABA) and l-arginine (Arg). Grafting GABA to the surface of PAMAM G4 reduced not only the complexation strength between pDNA and polymer but also the transfection efficiency. We also found that the location of Arg units in the polymer structure significantly affects complex formation and transfection efficiency. Furthermore, introducing GABA onto the PAMAM dendrimer improved the cytotoxicity of the polymer. Synthetic PAMAM G4 derivatives conjugated with GABA and Arg may be ideal gene delivery carriers because of their low cytotoxicity and high transfection efficiency.

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References and Notes