Polyplexes Assembled with Internally Quaternized PAMAM-OH Dendrimer and Plasmid DNA Have a Neutral Surface and Gene Delivery Potency

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INTRODUCTION

Numerous gene delivery systems based on viral (1–3) and nonviral (4, 5) vectors have been developed and tried so far. Recently, several recurring issues about safety of viral vectors have led to a careful reconsideration of the use of them in human clinical trials such as in Gelsinger’s case (6, 7). Moreover, they have significant limitation in large-scale production and available DNA size they can carry. In response to these problems, nonviral gene delivery systems such as cationic polymers or cationic lipids have attracted great attention to achieve a break-through in the development of an “ideal” gene carrier (8).

Several polymeric materials have been investigated as candidates for gene delivery; among them, cationic polymers with hydrophilic segments are gaining attention (9–14). It is because hydrophilic segments detoxify cationic polymers (11, 12), improve solubility (12, 13, 15) and prevent polycation/DNA complexes from aggregation in vivo.

Starburst PAMAM dendrimers are highly branched spherical polymers characterized by primary amine groups at the surface and tertiary amine groups in the interior. The polyplexes formed by PAMAM showed efficient transfection in which the primary amine groups participate in DNA binding and the tertiary amine groups exert endosome buffering effect (14, 16, 17). Biological behavior of dendrimers depends to a large extent on their surface groups. In one example, it was reported that amine-terminated PAMAM dendrimer interacted with bovine serum albumin more strongly than carboxyl-terminated one (18). Serum albumin has been considered to cause aggregation of nonviral carriers in vivo (19).

PAMAM-OH dendrimers are structurally identical to PAMAM except that surface amine functions have been replaced by hydroxyl groups. Absence of surface primary amine groups in PAMAM-OH makes this polymer nearly neutral which might be advantageous in terms of cytotoxicity and an aggregation problem. However, PAMAM-OH is nearly unable to form DNA polyplex because of the low pK_a of interior tertiary amines (20).

To overcome this hurdle, we introduced internal quaternary ammonium salt to the tertiary amine of PAMAM-OH dendrimers by methylation in order to provide binding sites for negatively charged plasmid DNA. Some polymers containing quaternary ammonium groups have been known to make polyplexes with DNA more efficiently than the polymers having primary or secondary amine groups. In addition, the polyplexes of quaternary amine-based polymers were relatively smaller compared to those prepared by cationic polymers containing primary and tertiary amine groups (21). Quaternary amine-based polymers are cationic at most pHs as a strong polyelectrolyte, while the charge density in the primary amine-based polymers depend on the pH of media (22).

From such a viewpoint, here we report the synthesis and characterization of internally quaternized PAMAM-OH. We expected that interior quaternary amine groups of QPAMAM-OH would interact negatively charged DNA while preserving a neutral polymer and/or a polyplex surface, which would act affirmatively with regard to cytotoxicity and an aggregation behavior of polyplexes.
EXPERIMENTAL PROCEDURES

Materials. PAMAM-OH G4, PAMAM G4 (Starburst), Methyl iodide, anhydrous N,N-dimethylformamide (DMF), and PEI (average molecular weight 25 kDa) were purchased from Aldrich (Milwaukee, WI). PGL3-control vector (plasmid DNA) was purchased from Promega (Madison, WI). Fetal bovine serum (FBS) and Dubecco's modified Eagle's medium (DMEM) were purchased from GIBCO (Gaithersburg, MD).

Synthesis of Quaternized PAMAM-OH (QPAMAM-OH). The solvent (methanol) was vacuum-evaporated and dried from manufacturer's PAMAM-OH solution prior to reaction. After redissolving PAMAM-OH (0.1 g, 7 μmol) in DMF (0.5 mL), methyl iodide of various molar ratios diluted in DMF (0.5 mL) was added. The mixture was stirred at each optimal reaction temperature (Table 1). After 24 h, the mixture was precipitated into diethyl ether and vacuum-dried and the residue obtained was redissolved in 1 mL of water. The solution was placed into a dialysis membrane (SpectraPore, MwCO 6000–8000) and dialyzed against 2 M NaCl and pure water in succession. Freeze-drying of water resulted in a white powder of QPAMAM-OH. 1H NMR (300 MHz, D2O) δ 2.47 (br m, CH3CO), 2.69 (br m, NCH2CH2NHCO), 2.88 (br m, NCH2CH2), 3.15 (s, CH3), 3.34 (br m, CH3CH2OH), 3.52 (br m, CH3N+), 3.66 (br m, CH2OH).

Ethidium Bromide Exclusion Assay. Ethidium bromide (1.0 μg) in 10 μL of water and plasmid DNA (1.0 μg) in 10 μL of water were mixed for 10 min at room temperature. After incubation, the plasmid DNA/ethidium bromide mixture was added to quaternized PAMAM-OH dendrimers with various charge ratios, ranging from 0.25 to 10 (+/-) and incubated further for 30 min. The charge ratio was calculated by relating the number of quaternary amine groups of QPAMAM-OH derivatives and the number of phosphate groups of DNA. The complexes were diluted to a total of 2 mL of Hepes buffered saline (HBS, 25 mM Hepes, 150 mM NaCl, pH 7.4) prior to measuring fluorescence intensity with a spectrofluorometer (Jasco FP-750). Excitation (λex) and emission (λem) wavelengths were 260 and 600 nm, respectively. The fluorescence of the DNA solution in HBS with ethidium bromide was calculated as 100%. The buffer containing ethidium bromide only without DNA was used as a blank control.

Dynamic Light Scattering (DLS) Measurement. The size of complexes was determined using a BI-200SM Goniometer (Brookhaven Instruments Corporation, Holtsville, NY) with a Lexel laser model 95 argon laser (100 mW output power at a wavelength of 514.5 nm). Correlator, PD2000 (Precision Detectors) was used and the scattering angle was 90°. Complexes were formed at a final concentration of 5 μg/mL plasmid DNA in water. DNA stock solution was added to QPAMAM-OH derivatives or PAMAM G4 prepared at various concentrations. DLS was performed in triplicate with the sampling time set to automatic.

Potential Measurement. The complexes of PAMAM or 0.97 QPAMAM-OH and plasmid DNA at various charge ratios were prepared in water. Surface charges were measured using a Zetasizer (Malvern Instrument Ltd, Malvern UK) equipped with a He–Ne laser at a wavelength of 680 nm.

RESULTS AND DISCUSSION

Synthesis of QPAMAM-OHs. QPAMAM-OHs with various degrees of internal quaternization were synthesized by partial or near complete methylation of interior tertiary amines groups of PAMAM-OH. The peak area of methylated N-methyl group was compared to the total proton numbers of the measured by 1H NMR (Figure 1). The area of methyl iodide mixture was added to quaternized PAMAM-OH dendrimers with various charge ratios, ranging from 0.25 to 10 (+/-) and incubated further for 30 min. The charge ratio was calculated by relating the number of quaternary amine groups of QPAMAM-OH derivatives and the number of phosphate groups of DNA. The complexes were diluted to a total of 2 mL of Hepes buffered saline (HBS, 25 mM Hepes, 150 mM NaCl, pH 7.4) prior to measuring fluorescence intensity with a spectrofluorometer (Jasco FP-750). Excitation (λex) and emission (λem) wavelengths were 260 and 600 nm, respectively. The fluorescence of the DNA solution in HBS with ethidium bromide was calculated as 100%. The buffer containing ethidium bromide only without DNA was used as a blank control.

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Atomic Force Microscopy (AFM). Atomic force microscopy (Nanoscope IIIa System, Digital Instruments, Inc., Santa Barbara, CA) was used for imaging the shape of complexes at 4 (N/P or ± ratio). Complexes were formed at a total of 1 μg/mL of plasmid DNA concentration in water. In the case of PAMAM-OH/plasmid mixture, 2.5 mM of MgCl2 was applied to the solution. Complexes containing 1 ng of plasmid DNA were applied to freshly cleaved mica and incubated on the mica for 5 min. After incubation, excess fluid was wicked off using filter paper. The solution was dried at room temperature prior to imaging. The image mode was set to tapping mode and average scan speed was 2 Hz.

Cytotoxicity Assay in Vitro. For the cytotoxicity assay, an MTT assay was performed (23). 293T cells were seeded at a density of 1 x 104 cells/well in a 96-well plate and grown in 95 μL of DMEM containing 10% FBS for 24 h, supplying 5% CO2 at 37 °C. After treating cells with PEI 25 kDa, PAMAM-G4, PAMAM-OH-G4, and QPAMAM-OHs for 1 day, 25 μL of MTT stock solution (5 mg/ml) was added to each well and incubated for 2 h. Then, 100 μL of extraction buffer (20% w/v of SDS in 50% DMF, pH 4.7) was added. Absorbance was measured at 570 nm after overnight incubation.

Transfection. 293T cells (5 x 104 cells/well) were seeded in 24-well plates and grown in 600 μL of DMEM containing 10% FBS for 1 day. Polyplexes of plasmid DNA and dendrimers were prepared by mixing 0.5 μL of plasmid DNA (4 μg/mL) and 0.5 μL of PAMAM or QPAMAM-OHs at various N/P or charge ratios, respectively, in FBS-free DMEM, and the mixtures were incubated for 30 min at room temperature. Following 4 h treatment of polyplexes, the medium was replaced by 1 mL of DMEM containing 10% FBS. Cells were incubated further for 2 days at 37 °C. After the growth medium was removed, cells were washed with PBS and lysed for 30 min at room temperature by 100 μL of Reporter lysis buffer (Promega, Madison, WI). The lysate was cleared by centrifugation. Luciferase activity was measured using a LB 9507 luminometer (Berthold, Germany), and the protein content was measured by Micro BCA assay reagents (Pierce, Rockford, IL).

Table 1. Quaternization of PAMAM-OH G4

<table>
<thead>
<tr>
<th>PAMAM-OH</th>
<th>0.27 Q</th>
<th>0.52 Q</th>
<th>0.78 Q</th>
<th>0.97 Q</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH3</td>
<td>0.5 equiv</td>
<td>0.75 equiv</td>
<td>0.9 equiv</td>
<td>4.16 equiv</td>
</tr>
<tr>
<td>temperature</td>
<td>25 °C</td>
<td>37.5 °C</td>
<td>37.5 °C</td>
<td>37.5 °C</td>
</tr>
</tbody>
</table>

a Eqv means the equivalent moles of methyl iodide relative to interior tertiary amines in PAMAM-OH G4.
QPAMAM-OH), and 97% (0.97 QPAMAM-OH) were obtained after dialysis and lyophilization.

The PAMAM-OH dendrimers were transformed to be functional by equipping quaternary amine groups for DNA condensation, the hydroxyl groups of their surface for low cytotoxicity, and internal positively charged amines by modifying tertiary amines to quaternary ammonium salts, which screened their charges due to the exterior hydroxyl groups leading to neutral surface charges.

Analysis of Complex Formation by Agarose Gel Electrophoresis and Ethidium Bromide Exclusion Assay. The PAMAM-OHs having various degrees of quaternization and plasmid DNA were mixed, and the mixtures were electrophoresed in agarose gel to see if polyplexes could be formed by interior positive charges (Figure 2). No indication of polyplex formation between PAMAM-OH and DNA was found (Figure 2A). Low pKₐ values of PAMAM-OH’s interior tertiary amines and ensuing low charge density should be the reason for this. Slowly migrating bands in Figure 2B might be the polyplex of 0.27 QPAMAM-OH and DNA; however, no complete retardation of DNA was observed in this polymer. It was observed that the number of charges per copolymer was an important factor in condensing DNA into small particles and in determining other physicochemical characteristics of a polymer/DNA complex (24). As the degree of quaternization increased, complete retardation of DNA was observed (Figure 2C, 2D, and 2E). More highly quaternized polymers were more efficient in polyplex formation. The polyplex formation in 0.97 QPAMAM-OH was nearly stoichiometric.

As shown in Figure 3, ethidium bromide exclusion assay was performed to quantify and confirm the complex formation ability of QPAMAM-OHs. Initial fluorescence of DNA and ethidium bromide complex decreased as dendrimers bound to DNA, releasing intercalated ethidium bromide. In 0.27 QPAMAM-OH, the relative fluorescence intensity decreased to 70% at a charge ratio of 5 and plateaued after that point. A low charge density of 0.27 QPAMAM-OH might be the reason and it is in line with the electrophoresis result (vide ante). DNA was completely retarded by 0.52 QPAMAM-OH above a charge ratio of 2 in the electrophoresis experiment; however, ethidium exclusion did not go beyond around 60% even at a charge ratio of 10. The unexpected phenomenon was revealed that interaction between 0.52 QPAMAM-OH and DNA is too weak to change DNA conformation sufficiently to exclude intercalated ethidium. 0.78 QPAMAM-OH and 0.97 QPAMAM-OH expelled ethidium from DNA, completely above a charge ratio of about 2. In summary, as the degrees of quaternization...
The formation of polyplexes was more efficient.

Characterization of QPAMAM-OHs/DNA Particles.

For analysis of particle size, we performed dynamic light scattering measurements. Briefly, in the presence of quaternary amine-based PAMAM-OH, relatively smaller, compared to primary amine-based PAMAM, particles were formed (Table 2). Interestingly, in the case of 0.52 QPAMAM-OH, very large particles (680.2 nm) were detected at a charge ratio of 1 due to the poor ability of complex formation with DNA. This is also seen from the neutralization between DNA and 0.52 QPAMAM-OH, in which cancelation of net charge (25) and minimization of charge-to-charge repulsion between complex particles might cause the large particles (26). However, increasing the charge ratio up to 2, the particle size became small (122.6 nm). The size distribution of the particles of more quaternized QPAMAM-OHs with DNA was below 157 nm at all charge ratios, which means these particles were small enough to be taken up by receptor-mediated endocytosis requiring a smaller size than 150 nm (27). It can be also noted that the diameter of complex particles gradually decreased as the degree of quaternization in QPAMAM-OH increased. This result indicates that the more compact polyplexes are produced at higher degrees of QPAMAM-OHs. We believe from these results that plasmid DNA having a extended structure can contact and bind to internal positive charges.

Figure 3. Ethidium bromide exclusion assay. N/P ratio of PAMAM-OH G4 and charge ratio (+/-) of QPAMAM-OH/plasmid DNA was 0.25, 0.5, 0.75, 1, 2, 5, and 10. Data are expressed as a mean relative fluorescence intensity (%; n = 3) at each ratio and the mean ± standard deviation are shown at each data point.

Table 2. The Complex Size of QPAMAM-OH/DNA Polyplexes and PAMAM/DNA Polyplexes As Determined by Dynamic Light Scattering

<table>
<thead>
<tr>
<th>(+/-)</th>
<th>PAMAM</th>
<th>0.52 Q</th>
<th>0.78 Q</th>
<th>0.97 Q</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>168.0</td>
<td>680.2</td>
<td>157.0</td>
<td>129.3</td>
</tr>
<tr>
<td>2</td>
<td>126.0</td>
<td>122.6</td>
<td>108.4</td>
<td>111.3</td>
</tr>
<tr>
<td>4</td>
<td>96.4</td>
<td>90.9</td>
<td>84.8</td>
<td>74.0</td>
</tr>
<tr>
<td>10</td>
<td>78.0</td>
<td>66.1</td>
<td>51.3</td>
<td>53.3</td>
</tr>
</tbody>
</table>

* Data are the mean diameter (nm) of each polyplex observed in water at 5 μg/mL of plasmid DNA concentration. Charge ratio in PAMAM was calculated on the assumption that only primary amines are protonated in near neutral pH (97% > 78% > 52% > 27%) in QPAMAM-OH increased, the formation of polyplexes was more efficient.

Figure 4. Surface charge of particles measured by ζ potential experiments. The polyplexes of 0.97 QPAMAM-OH (•) or PAMAM G4 (○) and plasmid DNA were formed at 0.5, 1, 4, and 10 of charge ratios (+/-). Results are shown as mean ± standard deviation (n = 3).
inner space (28) to accommodate large plasmid DNA molecules. This should be the reason hydroxyl groups exposed at their surface are able to screen internal positive charges even increasing their charge ratios. Thus, the condensates are considered to be electrostatically neutralized at their surface.

The morphology and size of the complexes depending on the degree of quaternization was investigated by atomic force microscopy (AFM) at a charge ratio of 4.0 (Figure 5). Only free plasmid DNA, or very large and loose condensates were observed for PAMAM-OH/DNA polyplexes and 0.27 QPAMAM-OH/DNA polyplexes. 0.52 QPAMAM-OH showed partially condensed structures of DNA. Theses results were reconfirmed by ethidium bromide exclusion assay results in which no sharp decrease of relative fluorescence was observed by 0.52 QPAMAM-OH/DNA polyplexes.
QPAMAM-OH. The images of polyplexes composed of 0.78 QPAMAM-OH and 0.97 QPAMAM-OH were observed to be small and spherical particles.

**Cytotoxicity Issue.** Cytotoxic effects of polycations are mainly mediated by interactions of the polymers with cell membranes and/or by cellular uptake of the polymers, and subsequent activation of intracellular signal transduction pathways (29). We examined the cytotoxicity of QPAMAM-OHs with various degrees of quaternization, comparing it with PAMAM G4 and branched PEI (25 kDa) (Figure 6). The cells exposed to PAMAM G4 were more viable than that treated with PEI. In general, the relative cell viability (RCV) of both PEI and PAMAM G4 decreased significantly as the concentration increased. However, quaternized PAMAM-OHs were less toxic than PAMAM G4 and PEI 25kDa at various concentrations. Relative cell viability (RCV, %) is expressed as a percentage of viable cells divided by untreated cells.

The degree of quaternization was observed: PAMAM-OH > 0.26 QPAMAM-OH > 0.52 QPAMAM-OH > 0.78 QPAMAM-OH > 0.97 QPAMAM-OH with cell cytotoxicity mirroring the degree of quaternization. The results suggest that the cytotoxicity is a function of the nature of the polycation charged moiety (primary, secondary, tertiary, or quaternary amine group) rather than its charge density (34). The quaternary amines were always charged and strongly hydrophilic; thus, the majority of quaternary amine-containing PAMAM-OHs appeared to be less toxic than tertiary amine-containing PAMAM-OH. Moreover, the higher density of quaternary amine-containing PAMAM-OH benefited from improved hydrophilicity and showed less cytotoxicity. Therefore, the transformation of the water-soluble polymers had previously resulted in a marked reduction in toxicity (33, 35).

Introducing quaternary amines to PAMAM-OH resulted not only in less cytotoxicity, but also the ability for condensation with plasmid DNA while the parent PAMAM-OH could not. These hydroxyl group-modified dendrimer-based delivery systems are supposed to be useful for reduction of the toxicity of PAMAM dendrimers.

**Transfection Efficiency in Vitro.** The gene delivery efficiency of QPAMAM-OH/DNA polyplexes as judged by luciferase gene expression was tested on 293T cell lines. The result (Figure 7) indicated that the transfection efficiency of QPAMAM-OH was lower than that of PAMAM G4 and PEI. The neutral surface charge of QPAMAM-OH/DNA polyplexes, as shown by measurement of the \( \zeta \) potential, might decrease chances of the polyplexes to bind electrostatically to cell—matrix and cell—cell anchoring proteins, such as heparan sulfate proteoglycans (36, 37).

In principle, cellular uptake of a polycation is a nonspecific process. A positively charged complex binds to a negatively charged cell membrane with electrostatic interaction. However, anionic proteoglycans are too ubiquitous for cationic particles to reach specific targeting organs in vivo. Therefore, the introduction of a system which decreases the surface charge of particles is necessary to avoid such a nonspecific uptake into cells. QPAMAM-OHs have such a low nonspecific interaction potential due to the neutral surface of particles; however,
they should be equipped with cell-surface targeting residues that could trigger their receptor-mediated endocytosis. Although the transfection efficiency of QPAMAM-OH derivatives was lower by 1 order of magnitude than PAMAM G4, our particles have the merit of much lower cytotoxicity and the chance to attach a ligand at the hydroxyl groups of their surface. These properties are useful for the strategy of receptor-mediated transfection by attaching a ligand as is being done in our further works.

CONCLUSION

We introduced quaternary ammonium salts that were placed in an internal location of starburst PAMAM-OH dendrimer (generation 4) by methylation of interior tertiary amines of PAMAM-OH. Improvements were made, not only in the ability to bind to DNA, but also in the cytotoxicity. The surface hydroxyl functionalities of QPAMAM-OHs could provide low cytotoxicity, and the polyplex of the polymer exhibited a neutral surface charge. Another major advantage of QPAMAM-OH dendrimers is that unreacted hydroxyl groups can be exploited for the conjugation of targeted ligands in order to provide cell entry by receptor-mediated endocytosis, while maintaining decreased nonspecific interaction with ubiquitous cellular proteins.

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LITERATURE CITED

influence of polymer structure on cell viability and hemolysis. Biomaterials 24, 1121–1131.


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