Quaternized amino poly(glycerol-methacrylate)s for enhanced pDNA delivery

Zhixiang Liang,a Xinshi Wu,a Ying-Wei Yang,b Cui Li,a Guolin Wu,c and Hui Gao*a

Linear and 8-arm amino poly(glycerol methacrylate)s (PGOHMAs) were synthesized from poly(glycidyl methacrylate)s (PGMAs) via ring opening reactions with methylethylamine (MEA), diethylamine (DEA) and dipropylamine (DPA), respectively. Further modification of tertiary amine groups of amino PGOHMAs by quaternization reaction with methyl iodide resulted in partially quaternized PGOHMAs, namely, QPGOHMAs. QPGOHMA/plasmid DNA (pDNA) complexes at different nitrogen-to-phosphate (N/P) ratios were then prepared. In addition, eight-arm star-shaped MEA-modified PGMA (SB-MEA) was also used to complex with pDNA as a positive control to compare with QPGOHMA/pDNA complexes. QPGOHMA/pDNA complexes were spherical in shape as observed by scanning electron microscopy (SEM), and smaller in size than SB-MEA/pDNA complexes as suggested by dynamic light scattering (DLS). Interestingly, gel electrophoresis and ethidium bromide displacement assay indicated that QPGOHMAs could condense pDNA at lower N/P ratios than that of SB-MEA. In vitro experiments on Huh-7 cells showed that QPGOHMAs displayed lower cytotoxicity and better transfection efficacy compared to SB-MEA. The cellular uptake analysis revealed that QPGOHMAs enhanced the cellular uptake level of pDNA. Overall, amino PGOHMAs modified by quaternization were proved to be effective gene delivery systems for gene therapy.

Introduction

Gene therapy, for either RNA or DNA, has been deemed as a useful curative method for both inherited and acquired genetic diseases.1,2 The main aim of gene therapy is the successful transfer of genetic materials in vivo to targeted tissues.3–5 However, naked curative genes are rapidly degraded by nucleases and show poor cellular uptake. For this reason, the development of safe and efficient gene carriers is one of the prerequisites for the success of gene therapy.5–7

Cationic lipids and polymers, the most important non-viral vectors, have many advantages over viral ones, such as non-immunogenicity, easy producibility and non-oncogenicity, holding the promise to replace viral vectors in clinical applications.5,8–10 In the past decade, cationic polymers have attracted more and more attention as nonviral gene delivery vectors,11 due to their flexible properties, facile synthesis, robustness, and proven gene delivery efficiency.1 Nevertheless, low transfection efficiency and undesirable cytotoxicity remain the most challenging aspects of these cationic polymers. To overcome the disadvantages, various modifications have been made to improve their gene delivery efficacy.12,13 Among them, partially quaternary ammonium salt-functionalized amino groups of cationic polymers displayed promising results. The structure of the remaining amino groups in polycations is an essential factor to determine the physicochemical characteristics of polycationic polymers and the transfection efficacy.14,15 In particular, quaternary ammonium polymers have several advantages for gene delivery, including the reduction of cytotoxicity and the improvement of binding affinity with DNA, and enhanced transfection efficiency compared to amino polymers.16–18 Quaternized star-shaped polymers have been used to form micelles in water from a series of amphiphilic quaternized poly[2-(dimethylamino)ethyl methacrylate]-block-poly(methyl methacrylate) copolymers, to aggregate into stimuli-responsive micelles from ABC triblock terpolymers,19 to form a body-centered-cubic structure near the overlap threshold from quaternized poly(ethylene oxide) (PEO) stars.20,21 However, quaternized star-shaped polymers for DNA delivery are still rare.

Some amino poly(glycerol methacrylate)s (PGOHMAs) have successfully been applied as cationic polymeric carriers of antisense oligonucleotides in our previous study.22,23 Excess positive charges are usually required for high transfection efficiency. However, these excess charges are usually associated with cellular toxicity. Our previous results have shown that N,N,N’-trimethylethylenediamine, methylethylamine (MEA),...
and 4-amino-1-butanol modified PGOHMA exhibited better transfection efficacy than PEI at a rather low nitrogen-to-phosphate (N/P) ratio (N/P ratio = 2). Hydrogen-bond from multi-hydroxyl PGOHMAs may be attributed to their enhanced complexation stability with DNA. To further enhance the transfection efficacy and lower the cytotoxicity of PGOHMA derivatives, quaternized amino PGOHMAs were designed and synthesized in the present study. Amino PGOHMAs were synthesized from poly(glycidyl methacrylate)s (PGMAs) via ring opening reactions with different fatty amines and further modified via a quaternization reaction with methyl iodide (MeI), to form partially quaternized PGOHMAs, namely, QPGOHMAs (Fig. 1). Their condensation capacity with pDNA, cell toxicity, transfection efficiency, as well as cellular uptake and endosomal escape ability, were evaluated and compared with S8-MEA.

Materials and methods

Materials

The plasmid pEGFP-N1 was purchased from Shanghai CPG Biotech Co., Ltd (Shanghai, China). Glycidyl methacrylate, 2-bromoisobutyryl bromide, bipyridyl, CuBr, and dipropylamine (DPA) were purchased from Shanghai Adams Reagent Co., Ltd (Shanghai, China). MEA and diethylamine (DEA) were obtained from Tianjin Guangfu Fine Chemical Research Institute (Tianjin, China). A plasmid vector coding for luciferase with a CAG promoter was used in the in vitro cell viability and transfection experiment (RIKEN, Japan). The luciferase assay kit was a product of Promega. Methyl iodide (MeI) was obtained from Josiah chemical Co., Ltd (Chengdu, China). Prior to use, tetrahydrofuran (THF) was dried by refluxing over sodium, in the presence of benzophenone as an indicator. All other reagents were obtained from Tianjin Chemical Reagent Co. (Tianjin, China).

Synthesis of amino PGOHMAs

2,3,4,6,1',2',3',6'-Octa-O-isobutyrylbromide cellobiose, as an eight-arm initiator, was synthesized from cellobiose according to our previous report. Linear (L-PGMA) and 8-arm PGMAs (S8-PGMA) were synthesized and modified with different amines according to previous reports. Briefly, PGMA was dissolved in acetonitrile at a concentration of 12 mg mL⁻¹. Then, MEA, DEA and DPA were added in excess. The mixture was refluxed at 90 °C overnight under argon. After the solution was cooled down, the products were dialyzed (Spectra/Por RC, cutoff 7000) against water and then freeze-dried. The obtained polymers were named as L-MEA, S8-MEA, L-DEA and S8-DEA, L-DPA, and S8-DPA (L represents linear polymers; S8 represents 8-arm polymers).

Synthesis and characterization of quaternary ammonium salts of amino PGOHMA (QPGOHMAs)

The quaternization reaction of the tertiary amino groups of amino PGOHMAs was carried out using MeI. Briefly, e.g., L-MEA (50 mg, 0.34 mmol of amino groups) was dissolved in methanol (15 mL), and excess of MeI (44 mL, 0.68 mmol, 2 molar eq. vs. the amino groups) was added. The mixture was stirred for 24 h at room temperature. Purification was carried out by dialyzing (molecular weight cut-off of 7000, Tianjin Unite Stars Biotech Co., Ltd) against distilled water for 4-5 days to obtain a QPGOHMA named QL-MEA. The polymers were then freeze-dried. Similarly, QS8-MEA, QL-DEA, and QS8-DEA, QL-DPA, and QS8-DPA were synthesized using the same procedure.

Characterization of the polymers

All 1H NMR spectra were recorded in CDCl₃ or D₂O using a 400 MHz Bruker Avance-400 spectrometer (400 MHz, Bruker, Freemont, CA). FTIR spectra were recorded on a Bio-Rad 6000 (Thermo Electron, USA) spectrometer using KBr pellets. Each
sample was ground along with 50 mg of KBr to afford a fine powder and compressed into a pellet. Gel permeation chromatography (GPC) measurements were performed in THF or AcOH aqueous solution (10 mM of AcOH, 500 mM of NaCl). Adequate molecular weight separation was achieved using three Waters Styragel columns (HT3, HT4 and HT5) in series at a flow rate of 1.0 mL min\(^{-1}\) at 35 °C. Calibration curves were obtained with nearly monodisperse polyethylene glycol. The molar content of amino groups per gram of polymer was measured with an elemental analysis instrument (Elementar Vario EL, GER). The degree of quaternization (DQ) of the QPGOHMA polymers were determined by X-ray photoelectron spectroscopy (XPS).

**pH titration**

Amino PGOHMA and QPGOHMA were dissolved in double distilled water. Aqueous HCl solution (0.1 mol L\(^{-1}\)) was used to adjust the pH to 2. The resulting polymer solution was stirred for 24 h to ensure complete dissolution, followed by titration with aqueous NaOH solution (0.05 mol L\(^{-1}\)) from pH 2.0 to 12.0 with a 3 min interval between two dosages for equilibrium. The ionization degree of the amine groups in the copolymer was calculated as 

\[
\alpha = \frac{[\text{basic}] - [\text{OH}^-] + [\text{H}^+]}{C_{\text{NaOH}}},
\]

where [basic], [OH\(^-\)] and [H\(^+\)] were the molarity of the added NaOH for titration, free hydroxide ions and hydrogen ions, respectively, and \(C_{\text{NaOH}}\) is the total molar concentration of the amino groups in molarity. There are two inflection points on the pH titration curve, [\text{OH}^-] and [H\(^+\)] were calculated from the titration curve, based on the volume of the first inflection point and the second inflection point. The pH at \(\alpha = 0.5\) is considered as the apparent dissociation constant (pK\(_a\)) of the PGMA derivatives.\(^{26}\)

**Preparation and characterization of DNA complexes**

UV absorption at 260 nm was used to determine the concentration of plasmid DNA (pDNA). Complexes were formed by slowly dropping the polymer solution into an equal volume of Tris buffer (50 mM, pH 7.4) of DNA (25 μg mL\(^{-1}\)) to obtain N/P ratios of 0.5–6. Both the polymer and DNA solutions were passed through 0.22 μm nylon filters, the passage rate of which was over 95%. Then they were mixed and vortexed for 10 seconds and incubated at room temperature for 45 min. S8-MEA/pDNA complexes were prepared using the same method as a positive control to compare with QPGOHMA/pDNA complexes. The mean hydrodynamic diameter and zeta potential of the polymer/pDNA complexes were investigated \((n = 3)\) in Tris buffer (pH 7.4) at 25 °C on a Zetasizer Nano ZS90 instrument (Malvern Instruments, Southborough, MA).

**Ethidium bromide (EB) displacement assay**

The stability of DNA condensation was evaluated as a function of the N/P ratio by a fluorescence method using EB.\(^{28}\) Fluorescence spectra were recorded with three readings at an excitation wavelength of 485 nm and an emission wavelength of 595 nm using a Hitachi F-4500 fluorescence spectrophotometer (Hitachi Scientific Instruments, Finchampstead, UK). Fluorescence (EtBr) was first recorded using a pure EB solution. The fluorescence was re-measured after the addition of pDNA (25 μg mL\(^{-1}\), 1 mL) to obtain fluorescence (DNA + EtBr). An aliquot of polymer (1 mL) was then added into the solution at a predetermined N/P ratio. Samples were gently mixed, and readings were taken after 15 min of incubation to obtain fluorescence (obs). The relative fluorescence was calculated as follows:\(^{31}\)

\[
\text{relative fluorescence} = \frac{\text{fluorescence (obs)} - \text{fluorescence (EtBr)}}{\text{fluorescence (DNA + EtBr) − fluorescence (EtBr)}} \times 100
\]

**Agarose gel electrophoresis**

The pDNA binding capacity of QPGOHMA was evaluated by agarose gel electrophoresis. Various formulations of polymer/pDNA complexes were prepared with N/P ratios ranging from 0.5 to 6. The complexes were electrophoresed on 1% agarose gel (stained with 1 μL of 10 mg mL\(^{-1}\) EB per 50 mL of agarose solution) in 0.5 × TBE buffer at 80 V for 60 min. The gel was then analyzed under a UV illuminator (Gel Documentation Systems, Bio-Rad, Hercules, CA) to reveal the relative position of the complexed DNA to the naked DNA.

**Scanning Electron Microscopy (SEM)**

SEM samples were prepared by depositing solutions of Q88-MEA/pDNA complexes (N/P = 3) on the glass slide. After evaporation of water, the samples were coated with a thin gold layer. The morphology of QPGOHMA/DNA complexes was observed with a scanning electron microscope (SEM) on a JSM-6700F type field emission scanning electron microscope (JEOL, South Korea).

**Cell viability assay**

The MTT assay was performed to evaluate cell viability after treatment with complexes.\(^{24}\) Huh-7 cells replicated continuously with a doubling time of approximately 50 h. Huh-7 cells were cultured at a cell density of 10 000 cells per well for 18 h in DMEM with 10% FBS supplemented in 24-well plates (at 70% confluency). The cells were then exposed to a serial concentration of the polypeptide solutions at 37 °C for 48 h. The medium was replaced with 100 μL of MTT solution (5 mg mL\(^{-1}\), PBS) and incubated for another 4 h. Cells incubated with media were tested for control. The culture medium was replaced with 100 μL of DMSO, and the resulting solution was measured using a microplate reader (Model 680, Bio-Rad). The cell viability in each well was calculated from the obtained values as a percentage of control wells. The results were presented as a mean and standard deviation obtained from three samples.

**In vitro transfection**

For transfection experiments, Cag-luc was used to evaluate the transfection efficiency of the three polymers against Huh-7 cells. A linear PEI-based commercial transfection reagent (ExGen 500) was used as a control. Huh-7 cells were seeded in...
24-well plates at a density of 10 000 cells per well and cultured with 400 µL of DMEM containing 10% FBS for 24 h at 37 °C in a humidified atmosphere containing 5% CO₂ until the cells reached 70% confluency. The QPGOHMA/pDNA complexes formed at different N/P ratios ranging from 1 to 5 were added into the culture plate (2.5 µg of pDNA mL⁻¹) and incubated with cells at 37 °C for 48 h, and then washed twice with PBS and harvested with reporter lysis buffer. Luciferase activity was measured by detecting the light emission from an aliquot of cell lysate incubated with 100 µL of a luciferin substrate (Promega) in a luminometer (Lumat LB9507, Berthold). The results were expressed as relative light units per milligram of cell protein (RLU per mg protein), and the protein concentration of each well was measured using protein assay kits (Pierce). The results were presented as a mean and standard deviation obtained from three samples.

Flow cytometric assay
Huh-7 cells were seeded into 24-well culture plates at a density of 100 000 cells per well in DMEM containing 10% FBS. Cy5-labeled DNA complexes were prepared with QPGOHMA and S8-MEA polymer. Then the medium was replaced with fresh medium and 150 µL of the complex solution (50 g pDNA mL⁻¹) was applied to each well (n = 3). After 24 h of incubation, the medium was removed and the cells were washed with 0.5 mL of PBS. The cells were treated with a trypsin–EDTA solution for 2 min and suspended in PBS, then analyzed using the flow cytometer (BD LSR II, BD, Franklin Lakes, NJ).

Confocal laser scanning microscopy (CLSM)
The intracellular distribution of polyplexes was investigated with CLSM. In short, pDNA was labeled with Cy5 using a Label IT Nucleic Acid Labeling Kit. Huh-7 cells were seeded on a 35 mm glass base dish (Iwaki, Tokyo, Japan) at a density of 50 000 cells per well and incubated overnight in 2 mL of DMEM containing 10% FBS. After the medium was exchanged, 150 µL of complex solutions (33.3 µg pDNA mL⁻¹) was applied to each sample. After 24 h of incubation, the old medium was removed and the cells were washed twice with PBS. The intracellular distribution of the complexes was observed by CLSM after staining acidic late endosomes/lysosomes with LysoTracker Green (Molecular Probes, Eugene, OR), and nuclei with Hoechst 33342 (Dojindo Laboratories, Kumamoto, Japan). CLSM observation was performed using an LSM510 (Carl Zeiss, Oberlochen, Germany) equipped with a 63× objective (C-Apochromat, Carl Zeiss) at the excitation wavelengths of 488 nm (Ar laser) for LysoTracker Green, 633 nm (He-Ne laser) for Cy5, and 710 nm (MaiTai laser, 2 photon excitation; Spectra-Physics, Mountain View, CA) for Hoechst 33342. To evaluate the endosomal escaping behavior of complexes, the rate of co-localization of Cy5-labeled pDNA with LysoTracker Green was quantified. Twenty five cells were counted to estimate the co-localization ratio.

Statistical analysis
Significant differences between two groups were evaluated by a Student’s t-test.

Results and discussion
Synthesis and characterization of the polymers
L-PGMA and S8-PGMA with an average molecular weight (Mₙ) of ca. 8000 were synthesized by atom transfer radical polymerization (ATRP) (Table 1). These two polymers were then functionalized with three different amines, i.e., MEA, DEA, and DPA, by ring-opening addition (Scheme 1) to obtain L-MEA and S8-MEA, L-DEA and S8-DEA, L-DPA and S8-DPA, respectively (Table 2). The amination conversion is over 88%, calculated from the ‘N’ percent determined by elemental analysis.

As shown in the FT-IR spectra (Fig. 2), aminolysis of L-PGMA to L-DEA caused identifiable changes. After aminolysis, the peak at around 991 cm⁻¹ attributed to the epoxide band disappeared. While a broad and strong peak emerged at 3400 cm⁻¹, corresponding to the presence of the hydrogen bond stretching.

Table 1 Molecular weights of PGMAs

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Mn/Da</th>
<th>PDIa</th>
<th>Degree of polymerizationb</th>
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<tr>
<td>L-PGMA</td>
<td>8400</td>
<td>1.21</td>
<td>58</td>
</tr>
<tr>
<td>S8-PGMA</td>
<td>8200</td>
<td>1.26</td>
<td>47</td>
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a PDI = polydispersity index. b The same as the epoxide groups per polymer.

Scheme 1 Synthetic route to amino PGOHMs and QPGOHMs ((a) represents the 8-arm ATRP initiator: 2,3,4,6-1',2',3',6'-octa-O-isobutyryl bromide cellulobiose and (b) represents the linear ATRP initiator: 2-bromoisobutyryl bromide).
vibration of hydroxyl and amino functionalities. In comparison with L-DEA, a noticeable change occurred in the spectra of QL-DEA. A strong peak at 1450 cm\(^{-1}\) of QL-DEA appeared, corresponding to the C–N stretching vibration of the quaternary ammonium group, confirming the occurrence of quaternization reaction.

Fig. 3 shows the \(^1\)H NMR spectra of L-DEA and QL-DEA. L-DEA features typical signals of the methylene groups linked to the amino group appearing at around 2.6–2.8 ppm. The characteristic peaks of QL-DEA at around 3.2 ppm indicates the presence of methyl and methylene groups in the quaternary ammonium group, and the peak attributed to methylene groups connected to the tertiary amine group at around 2.6 ppm remains, reflecting partial quaternization.

The \(pK_a\) values were calculated from pH titration curves. The amino PGMAs and QPGOHMA had an apparent \(pK_a\) comprised between 6.4 and 8.3, which was influenced by the chemical structure and the density of amino groups in the polymers (Table 2). Only one \(pK_a\) was obtained with QPGOHMAs bearing tertiary amine and quaternary ammonium groups, which is different from the case of PEI. PEI has \(-\text{NH}_2\) and \(\equiv\text{NH}\) groups which are almost completely charged at low pH values upon bonding of H\(^+\) cations,\(^{34}\) thus, it is not possible to obtain one, specific value of \(pK_a\) for PEI.

Characterization of polymer/DNA complexes

**Particle size and zeta-potential measurements.** Due to the poor solubility of L-MEA, L-DEA, L-DPA, S8-DEA and S8-DPA in buffer solutions, only S8-MEA with a good solubility was used as a positive control. It is generally believed that the particle size and surface charge of polyplexes are important factors in
adjusting their cellular uptake efficiency. Fig. 4a shows the hydrodynamic diameter of S8-MEA/pDNA and QPGOHMA/pDNA complexes, determined by DLS. For the S8-MEA/pDNA complexes, the particle size decreased with increasing N/P ratio of samples and reached a minimum value at around 400 nm. For the QPGOHMA/pDNA complexes, the particle sizes of all complexes tended to decrease sharply with an increase of the N/P ratio, similar results were observed by Lee et al. The smallest particle sizes of QPGOHMA complexes ranged from 130 to 180 nm, except for QS8-DPA/pDNA complexes. A minimum value of around 250 nm was obtained for QS8-DPA/pDNA complexes due to the steric hindrance effect, as well as the branching architecture of QS8-DPA. In addition, the decrease in particle size of QL-MEA/pDNA complexes is not as sharp as the other quaternized polymers, which may be caused by the relatively poor ability of complex formation with DNA as indicated by the relatively low zeta potentials at N/P ratios of 1.5–4.

The zeta-potentials of all complexes ranged from –49 to 45 mV (Fig. 4b). Negative values were observed at N/P ratios of 0.5–1 for all complexes, which is due to incomplete condensation between the pDNA and polymers. Specifically, the surface charges of QPGOHMA/pDNA complexes became positive when the N/P ratio reached 1.5. The transition of the surface charge of S8-MEA/pDNA complexes from negative to positive occurred at an N/P ratio of 2. After the quaternization reaction, the positive zeta potential of QPGOHMA/pDNA was much more than that of S8-MEA, and similar results were observed by Wang et al. Moreover, because the DQ of QL-MEA was the lowest among QPGOHMAs, the zeta potential of QL-MEA/pDNA was slightly lower than other QPGOHMA polyplexes. The surface charge of gene delivery vectors plays a pivotal role in determining the gene transfection efficiency. The presence of positive surface charge would allow an electrostatic interaction to occur between negatively charged cellular membranes and positively charged complexes.

EB displacement assay
The polymer/pDNA condensation can be characterized by the decrease of the fluorescence of EB due to the dissociation of the DNA–EB complex. When EB displacement is complete, the fluorescence reaches a plateau. As shown in Fig. 5, compared with S8-MEA/pDNA, the fluorescence of the QPGOHMA/pDNA complexes reached a plateau at lower N/P ratios. All of the QPGOHMAs quenched the fluorescence of the EB/pDNA complex to 25–35% of the initial fluorescence value, except for QS8-DPA (about 45% of initial fluorescence), which was probably due to the relatively loose complexes on account of its larger average particle size, making the interaction between QS8-DPA and pDNA too weak to change DNA construction adequately to displace the intercalated EB.

Morphology of complexes by SEM
Morphology of QS8-MEA/pDNA complexes at an N/P ratio of 3 was observed by SEM (Fig. 6). The compacted complex existed in the form of near spherical nanoparticles with an average size of 150 nm. The mean diameter determined by DLS is 244 nm, which was the hydrodynamic diameter of nanoparticles, and usually higher than that obtained by SEM. The other complexes showed similar morphology to QS8-MEA/pDNA complexes (data not shown).
Gel electrophoresis

To estimate the DNA binding stability of complexes, a gel retardation assay was performed. Fig. 7 shows the gel retardation results of QPGOHMA/pDNA complexes with increasing N/P ratios. For S8-MEA/pDNA, the complete retardation of pDNA was observed at the N/P ratio of 2 (data not shown). While for QS8-MEA, the complete retardation of pDNA was achieved at the N/P ratio of 1.5. It is clear that QPGOHMA could completely condense pDNA at a low N/P ratio, indicating that the increased cationic charges from quaternization could enhance the condensation capability.

Cytotoxicity of the complexes and transfection efficiency

The aim of designing a series of QPGOHMA was to compare the effect of structures on their properties. However, we found that the quaternized PGOHMAs showed similar properties upon being complexed with pDNA, for instance, similar hydrodynamic diameter, zeta potential, and stability as evaluated by the EtBr displacement experiment. Moreover, we want to compare the gene delivery properties of amino PGOHMAs and QPGOHMAs, only S8-MEA is well soluble in water. The other amino PGOHMAs are not water-soluble, and cannot be used as positive controls. Therefore, the cytotoxicity assay and luciferase gene expression study were performed for MEA series (Fig. 8). The Huh-7 cell line was selected as the target cell line due to its sensitivity to cytotoxicity and transfection. Compared with S8-MEA and PEI (ExGen 500, Cell viability was equal to 65% at N/P 5), both QS8-MEA and QL-MEA are less cytotoxic. Even though a few reports evidenced that introducing quaternary ammonium groups increased the cytotoxicity, our results were in accordance with many previous reports, which showed increased cell viability upon quaternization.

The in vitro gene transfection efficiencies of S8-MEA and QPGOHMA copolymers were measured in Huh-7 cells using Cag-luc plasmid as a reporter gene. The results are shown in Fig. 9. The transfection efficacy of S8-MEA and QPGOHMA was lower than that of PEI (ExGen 500) at N/P ratios of 3, 4 and 5. In comparison with S8-MEA/pDNA complexes at the same N/P ratio, QPGOHMA/pDNA complexes yield a substantial increase in transfection efficiencies ranging from 1 to 5, except that S8-MEA complexes showed better efficacy than those of QL-MEA of N/P = 3. The relatively small size, high zeta-potential, and enhanced condensation ability of QPGOHMA may be attributed to its better transfection efficiency as compared to its precursor.
amino PGOHMA. In addition, the cytotoxicity of amino PGOHMA samples can disrupt various cellular functions including the protein synthesis, which probably decreased gene delivery efficiency as compared with QPGOHMA. The best activity was observed at the N/P ratio of 4 for all of the QPGOHMA samples. QL-MEA/pDNA complexes were clearly less efficient than QS8-MEA. Because of the increased surface cationic charges, QS8-MEA was expected to strongly complex pDNA to obtain a higher transfection yield than S8-MEA.

Cellular uptake of complexes
Flow cytometric analysis was used to quantify the cellular uptake of complexes with respect to pDNA using Cy5-labeled pDNA. As shown in Fig. 10, QS8-MEA/pDNA complexes showed higher pDNA uptake than S8-MEA/pDNA complexes at the N/P ratio of 4, presumably due to the stronger and steady integration of QS8-MEA with DNA compared to S8-MEA. The enhanced cellular uptake of QS8-MEA/pDNA complexes may be a benefit for the effective transfection.

CLSM observation and evaluation of endosomal escape
The amount of Cy5-labeled pDNA observed in the cells was a little more for QS8-MEA, which was consistent with the flow cytometry results. The localization of Cy5-labeled pDNA (red) in the endosomes and lysosomes was observed by CLSM. Lyso-Tracker Green (green) and Hoechst 33342 (blue) were used to label late endosomes/lysosomes and nuclei, respectively. Co-localization of pDNA with the late endosomes/lysosomes was quantified from the average value of 25 cells. As can be seen in Fig. 11, at the N/P ratio of 4, about 27% of pDNA complexed with S8-MEA was localized in the late endosomes/lysosomes, while nearly 31% of that complexed with QS8-MEA. Their endosomal escape was similar, and S8-MEA internalized into the cells could achieve endosomal escape slightly effective than QS8-MEA, which was due to the protonation of the tertiary amine moieties under the acidic condition to enhance stability of the S8-MEA/pDNA complexes.

Conclusions
In the present study, a series of quaternary ammonium salt-functionalized amino PGOHMAS have been successfully synthesized and their physicochemical properties have been evaluated. Even though the transfection efficacy of QPGOHMA was lower than that of PEI, their cytotoxicity was lower than that of PEI. Quaternionization improved the water solubility of amino PGOHMA. QPGOHMA polymers exhibited good ability to complex pDNA as well as suitable particle size and zeta potential for gene transfection. Besides, QPGOHMA samples showed enhanced cellular uptake, much lower cytotoxicity as well as better gene transfection efficiency than unmodified amino PGOHMAS, indicating that amino PGOHMA modification by quaternionization can be helpful for the design of non-viral gene vectors for potential application in gene therapy.

Abbreviations

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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>PGMA</td>
<td>Poly(glycidyl methacrylate)s</td>
</tr>
<tr>
<td>PGOHMAS</td>
<td>Poly(glycerol methacrylate)s</td>
</tr>
<tr>
<td>MEA</td>
<td>Methylmethyleamine</td>
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DEA
DPA
QPGOHA
MeI
N/P
S8-MEA
L-MEA and S8-MEA, L-DEA and S8-DEA, L-DPA and S8-DPA
QL-MEA, QSS-MEA, QL-DEA, QSS-DEA, QL-DPA and QSS-DPA
THF
GPC
DQ
EB
SEM
DLS
Diethylamine
Dipropylamine
Quaternized PGGOHA
Methyl iodide
Nitrogen-to-phosphate
Eight-arm star-shaped MEA-modified PGMA
L represents linear polymers;
S8 represents 8-arm polymers
Quaternized L-MEA, S8-MEA, L-DEA, S8-DEA, L-DPA and S8-DPA
Tetrahydrofuran
Gel permeation chromatography
Degree of quaternization
Ethidium bromide
Scanning electron microscopy
Dynamic light scattering

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Notes and references