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Cationization of *Ganoderma lucidum* polysaccharides in concentrated alkaline solutions as gene carriers

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ABSTRACT

Quaternized *Ganoderma lucidum* glucans (QGs) were synthesized using 3-chloro-2-hydroxypropyltrimethylammonium chloride (CHPTAC) as the cationic reagent in 1 M NaOH aqueous solution at 60 °C. The chemical structure and solution properties of QGs were characterized by using elemental analysis, FTIR, ^{13}C NMR, laser light scattering, ζ-potential measurement and viscometry. The QG solutions in pure water exhibited a typical polyelectrolyte behavior, and normal viscosity behavior in 0.9% NaCl. Moreover, two QG samples with relatively high degree of substitution (DS = 0.43 and 0.51) were selected and studied as gene carriers. The results of gel retardation assay suggested that QGs could condense DNA efficiently. QGs displayed relatively lower cytotoxicity as compared with PEI, and QG/DNA complexes exhibited effective transfection compared to the naked DNA in 293T cells. The quaternized glucan derivatives prepared in NaOH aqueous solutions could be considered as promising non-viral gene carriers.

1. Introduction

Cationic polysaccharides (cationic celluloses, cationic guar, amino-modified starches, chitosan, chitosan derivatives and dextran derivatives) are large-scale commercial products that have many useful characteristics such as hydrophilicity, biodegradability and antibacterial properties (Azzam, Raskin, Makovitzki, Brem, & Vierling, 2002; Dong et al., 2009; Kabanov, 1999; Khai-Woon, Chen, Lo, Huang, & Wang, 2011; Rachel & Rinaudo, 2003). Cationic polysaccharides can be prepared by the reaction of the native polymer with various reagents possessing positively charged groups such as amino, imino, ammonium, sulfonium or phosphonium groups. Among these reagents, 2,3-epoxypropyltrimethylammonium chloride and 3-chloro-2-hydroxypropyltrimethylammonium chloride (CHPTAC) have been used extensively (Ghimici & Nichifor, 2006; Slita et al., 2007). Such derivatives can be reacted easily with the hydroxyl groups of polysaccharides in alkaline medium leading to the corresponding cationic ether.

Gene therapy has been progressively developed with the hope that it will be an integral part of medical modalities (Thomsen, Lichota, Kim, & Moos, 2011). The ultimate goal of gene therapy is to cure both inherited and acquired disorders in a straightforward manner by removing their cause, that is, by adding, correcting, or replacing gene (Eliyahu, Siani, Azzam, Dom, & Barenholz, 2006; Nagasaki et al., 2004; Souguir, Roudesli, & Picton, 2007). Gene-delivery vectors are classified routinely as viral and non-viral, and the advantages and disadvantages of each category are well-documented. The viral vectors usually show high transfect efficiency with high cell toxicity, while the non-viral vectors mostly have low transfect efficiency with low toxicity (Tony et al., 2002). The most important problem for scientists is to synthesis new vector which has high transfect efficiency with low toxicity. Based on the biodegradable and biocompatible natural products, cationic polysaccharides can provide a new way for the study of gene-delivery vectors.

In this work, we choose water-insoluble linear (1 → 3)-β-D-glucan extract from the fruit body of *Ganoderma lucidum* as the native material, and synthesized the quaternized glucan using CHPTAC as etherifying agent to prepare the cationic polysaccharides with good water-solubility. Furthermore, the structure and properties of the quaternized glucan were studied. Cytotoxicity and transfect efficiency of the cationic glucan/DNA complexes were also evaluated.

2. Experimental

2.1. Materials

Polysaccharides (GL-IV-I) were extracted from the fruit body of *G. lucidum*. Characterization and extraction of polysaccharides have been reported in our paper before (Wang & Zhang, 2009).

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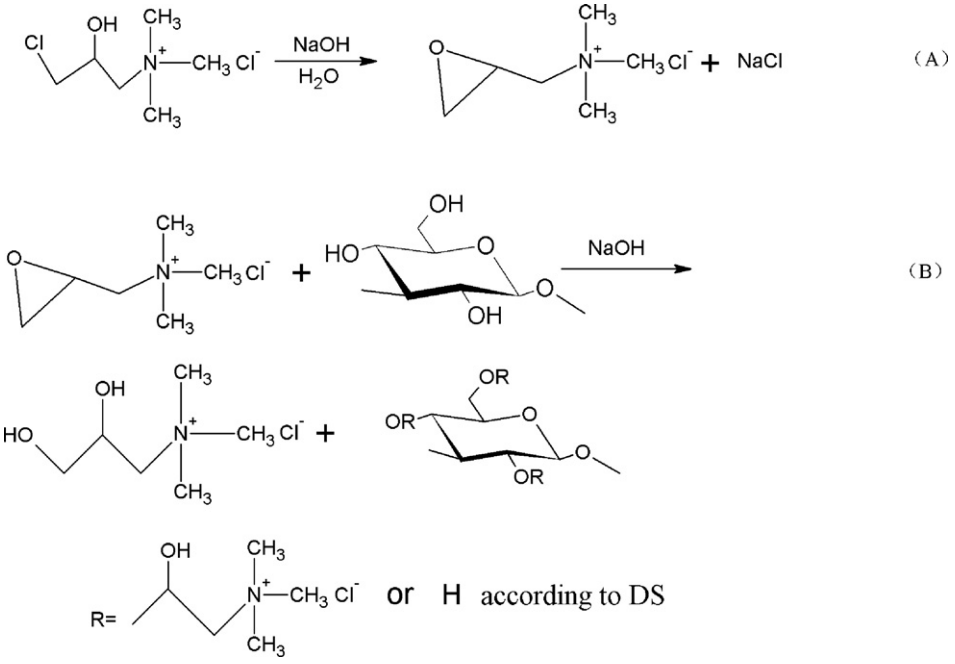

Scheme 1. Quaternization of GL-IV-I with 3-chloro-2-hydroxypropyl-trimethylammonium chloride (CHPTAC) in 1 M NaOH aqueous solutions at 60 °C.

The molecular weight (M_w) of the linear (1 → 3)-β-D-glucan is 13.3×10^4. CHPTAC was purchased from Guofeng Fine Chemical Co. Ltd., Shandong, China, and was used as etherifying reagent without further purification. Branched polyethylenimine, with a molecular weight of 25 kDa, was purchased from Sigma–Aldrich. Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), penicillin–streptomycin, trypsin, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), and Dulbecco's phosphatebuffered saline (PBS) were purchased from Invitrogen Corp. All other reagents were of analytical grade and were used without further purification. The binding capability of polycations to pDNA is a prerequisite as the gene vectors. The polycations can condense pDNA into compact structures and reduces the electrostatic repulsion between DNA and cell surface by neutralizing the negative charge, which facilitates the uptake of the complex to negatively charged cell membrane constituents and, therefore, to a higher rate of uptake (Farber & Domb, 2007; Merdan, Kopecek, & Kissel, 2002). When pDNA is condensed by polycations, it also can be better protected against enzymatic degradation by nucleases in serum and extracellular fluids (Xu et al., 2007).

2.2. Quaternization of GL-IV-I

In a 100 mL beaker, 0.25 g GL-IV-I was well-dispersed in 1 M NaOH solution under magnetic stirring for 1 h at 60 °C. In a typical reaction procedure, a certain amount of CHPTAC aqueous solution was added drop-wise into the solution obtained previously, and the mixture was stirred at 60 °C for 18 h. The molar ratios of GL-IV-I to CHPTAC were 1:6, 1:9, 1:12, 1:18 and 1:24. The reaction product was neutralized with 1 M HCl aqueous solution and dialyzed with regenerated cellulose tubes (M_w cutoff 8000, USA) against distilled water for 7 days, then centrifuged to give a supernatant. The supernatant was finally freeze-dried with lyophilizer (Christ Alpha 1-2, Osterode am Harz, Germany) to obtain the purified GL-IV-I derivatives (white powder), coded as QG-1–QG-6 (Scheme 1).

2.3. Characterization of QG-1–QG-6

FTIR spectra of QG samples and GL-IV-I were performed with a Nicolet 170SX Fourier transform infrared spectrometer. The test specimens were prepared by the KBr-disk method. Nitrogen contents (N%) of QGs were measured with an elemental analyzer (CHN-O-Rapid, Foss Hera us GmbH, Hanau, Germany). The degree of substitution determines the positive charge density of QGs, which affect the chain conformation and transfection efficiency of QGs. DS value of QG was determined by nitrogen content and calculated according to the following equation (Song, Sun, Zhang, Zhou, & Zhang, 2008):

$$\mathrm{DS} = \frac{162 \times N\%}{14 - 151.5 \times N\%} \quad (1)$$

^{13}C NMR measurements of the samples in D_2O at 25 °C were carried out on a Varian INOVA-600 spectrometer in the proton noise-decoupling mode with a standard 5 mm probe at ambient temperature, and the sample concentration was about 100 mg/mL. The chemical shifts were referenced to the signals of tetramethylsilane (TMS).

All the QG samples showed good water-solubility lowed than 100 mg/mL in distilled water at 25 °C. ζ-Potential of the QGs in distilled water (c = 1 mg/mL) was measured by Nano-ZS ZEN3600 (Malven Instruments, UK) at 25 °C. The viscosity of the samples in pure water and in 0.1 mol/L NaCl aqueous solutions was measured at 25 ± 0.1 °C with an Ubbelodhe capillary viscometer. The kinetic energy correction was always negligible. Huggins and Kraemer equations were used to estimate the [η] value by extrapolation to infinite dilution as follows (Huggins, 1942; Kraemer, 1938):

$$\frac{\eta_{sp}}{c} = [\eta] + k[\eta]^2 c \quad (2)$$

$$\frac{\ln \eta_r}{c} = [\eta] - k'[\eta]^2 c \quad (3)$$

where k and k' are constants for a given polymer under certain conditions in a given solvent, η_{sp}/c is the reduced specific viscosity, and $(\ln \eta_r)/c$ is the inherent viscosity.

The M_w values were measured by ALV/DLS/SLS-5000E laser light scattering (ALV/CGS-8F, ALV, Germany) equipped with ALV-5000/E multiple-τ digital time correlator. Vertical polarization light of 532 nm wavelength was selected as the incident light source. Toluene was used as a standard material for calibration of light scattering instrument. The Rayleigh factor (R_{ref}) of toluene against

532 nm vertical polarization light at 25 °C is 2.74×10^{-5} cm^{-1}. The scattering light intensity of QGs (I_θ) was measured and the Rayleigh factor of QGs can be calculated by the following equation:

$$R_\theta = \frac{\langle I_{\text{solution}}(q)\rangle - \langle I_{\text{solvent}}(q)\rangle}{\langle I_{\text{ref}}(q)\rangle} R_{\text{ref}} \left(\frac{n}{n_{\text{ref}}}\right)^2 \quad (4)$$

where q is the absolute value of scattering vector ($\vec{q}$), q can be calculated as follows:

$$q = |\vec{q}| = \frac{4\pi n_0}{\lambda_0} \sin\left(\frac{\theta}{2}\right) \quad (5)$$

where n_0 is the refractive index of the solvent, λ_0 is the wavelength of the incident light in vacuum. θ is the angle of incented light. $\langle I_{\text{solution}}(q)\rangle$, $\langle I_{\text{solvent}}(q)\rangle$ and $\langle I_{\text{ref}}(q)\rangle$ are the average scattering intensity of QG solution, solvent and the standard sample (toluene), n and n_{ref} are the refractive index of QG solution and toluene. The relationship of M_w and scattering light density at θ for polymers is as follows (Meunier, Nicolai, & Durand, 2003; Ngai, Wu, & Chen, 2004):

$$\frac{K_c}{R_\theta} = \frac{1}{M_{\text{app}}(c)}(1 + q^2\xi^2_{\text{static}}) \quad (6)$$

$$K = \frac{4\pi^2 n^2 (dn/dc)^2}{N_A \lambda_0^4} \quad (7)$$

where K is an optical constant, N_A is Avogadro's number; dn/dc is the refractive index increment of solvent. ξ_{static} is the static correlation length, which is related to the size of polymer in solution. $M_{\text{app}}(c)$ is the apparent molecular weight of polymer in solution. The relationship between $M_{\text{app}}(c)$ and M_w is as follows (Burchard, 2001):

$$\frac{1}{M_{\text{app}}(c)} \equiv \frac{1}{M_w}(1 + 2A_2 M_w c + 3A_3 M_w c^2 + 4A_4 M_w c^3 + \cdots) \quad (8)$$

A_i (i = 2, 3, 4, ...) is virial coefficient, describing the interaction between i polymer chains. The A_2 value of polymer is positive in good solvent, corresponding to the interaction of two polymer chains. Polymer molecular weight of polymer can be obtained by extrapolation to zero concentration. dn/dc was measured by differential refractometer (Optilab, DAWN® DSP, Wyatt Technology Co., Santa Barbara, CA, USA, λ = 633 nm) at 25 °C, and the dn/dc values of QG-5 and QG-6 in 0.9% NaCl aqueous solution were 0.140 and 0.142 mL/g, separately.

3. Gene carrier assay

3.1. Cell culture

Human embryonic kidney transformed (293T) cells were incubated in DMEM containing 10% FBS and 1% antibiotics (penicillin–streptomycin, 10,000 U/mL) at 37 °C in a humidified atmosphere containing 5% CO_2.

3.2. Amplification and purification of plasmids

In this study, pGL-3 plasmid used as the luciferase report gene was transformed in *Escherichia coli* JM109. pGL-3 plasmid was amplified in broth media at 37 °C overnight at 300 rpm. Then the purified plasmid was diluted by TE buffer solution and stored at −20 °C. The integrity of plasmid was confirmed by agarose gel electrophoresis. The purity and concentration of plasmid were determined by ultraviolet (UV) absorbance at 260 and 280 nm.

3.3. Agarose gel electrophoresis assay

Agarose gel electrophoresis experiments were performed to monitor the complexation of QG to DNA. Two series of QG/DNA complexes (QG-5 and QG-6) at different N/P ratios (the quaternary ammonium salt groups on cellulose backbone to phosphate groups of DNA) ranging from 0.5 to 6 were prepared by adding appropriate volume of QG (in 0.15 M NaCl solution) to 96 ng (0.8 μL) of pGL-3 DNA (120 ng/μL in 40 mM Tris–HCl buffer solution). The complexes were diluted by 0.15 M NaCl solution to 6 μL, and then the complexes were incubated at 37 °C for 30 min. After that, the complexes were subjected to electrophoresis on the 0.7% (w/v) agarose gel containing GelRed and with Tris–acetate (TAE) running buffer at 80 V for 80 min. DNA was visualized with a UV lamp using a Vilber Lourmat imaging system.

3.4. Particle size and ζ-potential measurements

Particle size and ζ-potential of QG/DNA complexes were measured by Nano-ZS ZEN3600 (Malven Instruments, UK) at 25 °C. The complexes at various N/P ratios ranging from 5 to 30 were prepared by adding appropriate volume of QG (QG-5 and QG-6) solution (in 0.15 M NaCl solution) to 1 μg of pGL-3 DNA solution (in 40 mM Tris–HCl buffer solution). The complexes were incubated at 37 °C for 30 min. After that the complexes were diluted by 0.15 M NaCl solution to 1 mL prior to measurement.

3.5. Cytotoxicity assay

The cytotoxicity of QGs (QG-5 and QG-6) and 25 kDa PEI was examined by MTT assay. The 293T cells were seeded in a 96-well plate at 5000 cells/well and cultured for 1 day in 200 μL of DMEM containing 10% FBS. After the polymers were added for 2 days, the medium was replaced with 200 μL of fresh medium. Then 20 μL of MTT solutions was added for 4 h. After that, the medium was removed and 150 μL of DMSO was added and mixed. The absorbance was measured at 570 nm using a microplate reader (BIORAD, Model 550, USA). The relative cell viability (Φ) was calculated as

$$\Phi\ (\%) = \frac{OD_{(\text{samples})}}{OD_{(\text{control})}} \times 100 \quad (9)$$

where $OD_{(\text{control})}$ was obtained in the absence of polymers and $OD_{(\text{samples})}$ was obtained in the presence of polymers.

3.6. In vitro transfection (luciferase assay)

For the in vitro transfection studies, the 25 kDa PEI/DNA at N/P ratio of 20 and naked pGL-3 DNA (1 μg) were respectively used as the positive and negative controls. The 293T cells were seeded at a density of 6×10^4 cells/well in 24-well plate with 1 mL DMEM containing 10% FBS and incubated at 37 °C for 24 h. The QG/DNA complexes at N/P ratios ranging from 5 to 30 were prepared by adding an appropriate volume of QG to 1 μg of pGL-3 DNA solution and then diluted by 0.15 M NaCl solution to 100 μL and incubated at 37 °C for 30 min. Before transfection, the cells were washed by PBS, and then the QG/DNA complexes, PEI/DNA complexes, and naked DNA were added with serum-free DMEM for 4 h at 37 °C. After that the serum-free DMEM was replaced by fresh DMEM containing 10% FBS, and the cells were further incubated for 2 days. The luciferase assay was performed according to manufacturer's protocols. Relative light units (RLUs) were measured with chemiluminometer (Lumat LB9507, EG&G Berthold, Germany). The total protein was measured according to a BCA protein assay kit (Pierce). Luciferase activity was expressed as RLU/mg protein. Data were shown as

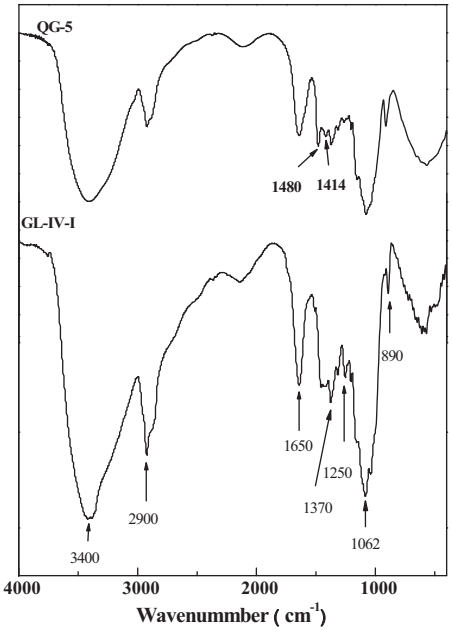

Fig. 1. IR spectra of GL-IV-I and QG-5.

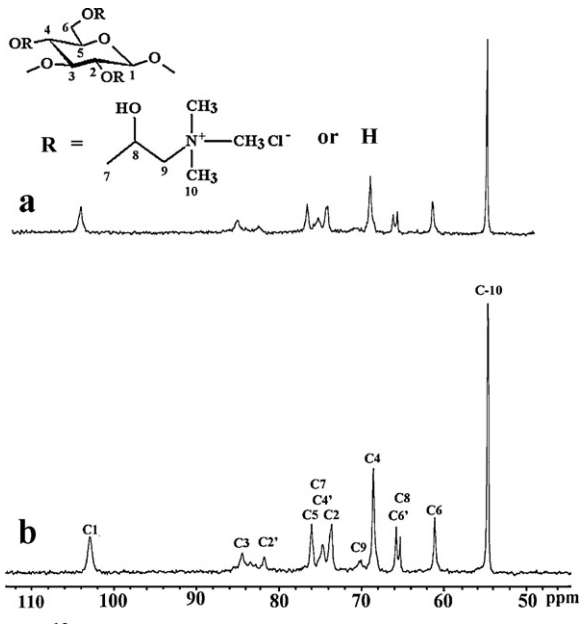

Fig. 2. ^{13}C NMR spectra of QG-5 and QG-6.

mean (standard deviation (SD)) based on three independent measurements.

4. Results and discussion

4.1. Chemical structures of GL-IV-I and QGs

The FTIR spectra of the native GL-IV-I and QG-5 are shown in Fig. 1. GL-IV-I exhibited the characteristic IR absorption of the polysaccharide at 1250 and 1650 cm^{-1}. The native GL-IV-I also exhibited absorption peaks at 890 cm^{-1}, which is the absorption peak of a β-glucan (Wang, Zhang, Li, Hou, & Zeng, 2004). The absorption peak at about 3400 cm^{-1} assigned to stretching vibration modes of O—H groups. The bands at 2900, 1370, and 1062 cm^{-1} were assigned to stretching vibration of —CH_2— groups (Kacurakova, Ebringerova, Hirsch, & Hromadkova, 1994; Loubaki, Ourevitch, & Sicsic, 1991). Compared to the native GL-IV-I, the most striking spectra of QG-5 at 1480 cm^{-1} are corresponding to the methyl groups of ammonium. Moreover, the peak of QG-5 positioned at 1414 cm^{-1} was referenced as the C—N stretching vibration (Pal, Mal, & Singh, 2005). FTIR spectra have given an evidence of the introduction of the quaternary ammonium salt group on the glucan backbone.

The ^{13}C NMR spectra of GL-IV-I had been reported in our paper before. Fig. 2 shows the ^{13}C NMR spectra of QG-5 and QG-6. The peak at the lower field around 103.7 ppm corresponded to C-l; peaks around 86.9, 76.0, 73.5, 69.1 and 61.6 ppm were assigned to C-3, C-5, C-2, C-4 and C-6. Peaks of substituted C-2′, C-4′ and C-6′ were around 81.7, 74.7 and 66.0 ppm. Signals at 74.6, 65.2, and 70.1 ppm were assigned for the C7, C8, and C9 atoms, respectively (Haack, Heinze, Oelmeyer, & Kulicke, 2002; Heinze, Haack, & Rensing, 2004). The typical signal of the $(CH_3)_3N^+$ moiety appeared at 54.5 ppm (Yu, Huang, Ying, & Xiao, 2007). Therefore, the results of NMR further proved the successful synthesis of quaternized glucan in concentrated alkaline solutions. From the ^{13}C NMR spectra of QGs, we can get the conclusion that quaternization of GL-IV-I is non-selective substitution, and the active hydroxyl groups at C-2, C-4, and C-6 exhibited different reactivities. Hydroxyl groups at C-6 exhibit highest reactivity because of the least steric hindrance.

4.2. Viscosity behavior

Fig. 3 shows the relationship between reduced viscosity (η_{sp}/c) and concentration (c) for QGs in 0.9% NaCl and pure water, separately. We can see the electrostatic repulsion effect of polyelectrolyte for QGs. This is due to the introduction of quaternary ammonium ion with a positive charge which makes the molecular chain in repulsion with thinning the concentration of the solution (Zhou et al., 2001). QGs exhibit normal viscosity character in 0.9% NaCl aqueous solution as we can see in Fig. 3.

The molecular weight, ζ-potential, DS, η and particle size of QG-5 and QG-6 are summarized in Table 1. Fig. 4 shows the M_w of QG-5 and QG-6 in 0.9% NaCl aqueous solution. As we can see, M_w of QGs is larger than that of the native glucan because the introduction of the new quaternary ammonium group. Increased degree of substitution makes more extended chain conformation and larger M_w value and larger particle size of QGs. The ζ-potential of QG-5 and QG-6 is positive, +34.2 and +41.5 mV, which provides the possibility of the successful gene transfection ability.

4.3. Interactions of QGs with pDNA

In this work, the binding capability of QG-5 and QG-6 to pDNA was studied by agarose gel electrophoresis, and plasmid DNA (96 ng) was mixed with QG samples at different N/P ratios. The gel electrophoresis measurements in Fig. 5 clearly show that both QG-5 and QG-6 bind the pDNA completely. The charge and particle size of the polymer/DNA complexes are very important for polycations used as gene vectors and play an important role in the entering of complex into nucleolus by cellular uptake. In this study, the particle sizes and ζ-potential of QG-5/DNA and QG-6/DNA complexes at various N/P ratios ranging from 5 to 30 were measured by adding QG solution to 1 μg DNA at physiological ionic strength condition (in 0.15 M NaCl solution). The particle size of QG/DNA complexes decreased sharply as a result of the net positive electrostatic repulsion between complexes.

Table 1
Molecular mass, DS, intrinsic viscosity, and ζ-potential of QGs.

	$M_w \times 10^{-4}$	$[\eta]$[a] (mL/g)	ζ-Potential[b] (mV)	Nitrogen (%)	DS	Particle size (nm)
GL-IV-I	12.4	27.8				
QG-5	27.3	37.5	34.2	2.84	0.43	186
QG-6	40.1	39.8	41.5	2.98	0.51	224

[a] Measured in 0.9% NaCl aqueous solution.
[b] Determined by the 1 mg/mL aqueous solutions at 25 °C.

4.4. In vitro cytotoxicity and transfection

One of the major requirements of the polymeric vectors for use in gene therapy is the low cytotoxicity. In our work, the cytotoxicity of QG-5 and QG-6 was evaluated in 293T cells by MTT assay, and the 25 kDa PEI was used as the control. As shown in Fig. 6, the cytotoxicity of QGs increased gradually with increasing of the concentrations and DS values due to the presence of a higher

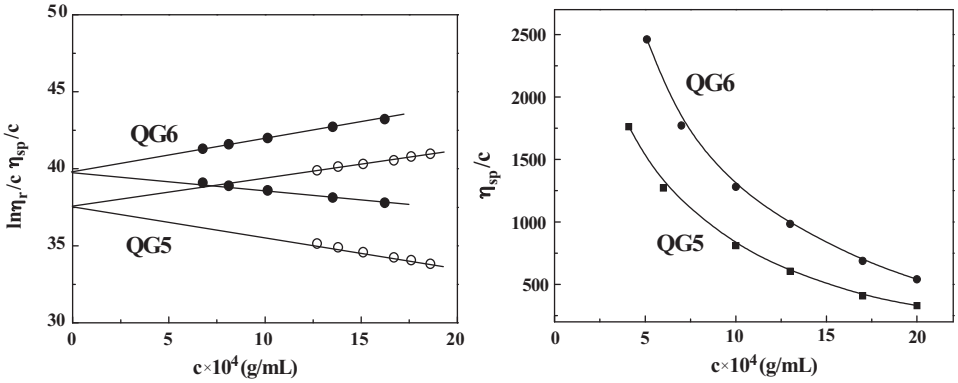

Fig. 3. Plots of η_{sp}/c–c and ln η_r/c–c in 0.9% aqueous NaCl solution (left) and reduced viscosity (η_{sp}/c) against concentration (c) of QG-5 and QG-6 in pure water (right) at 25 °C.

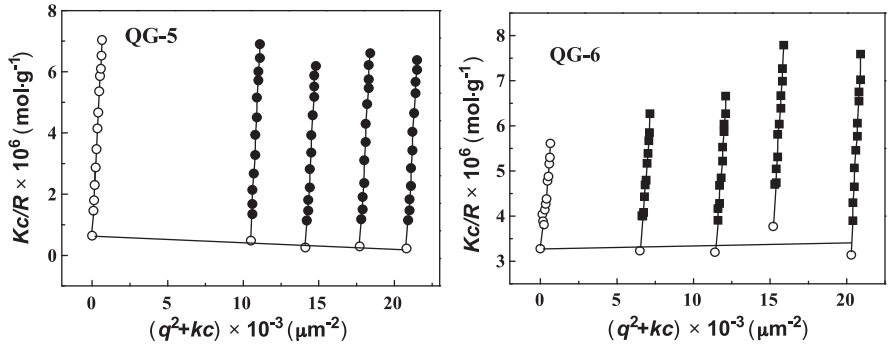

Fig. 4. Zimm plots of QG-5 and QG-6.

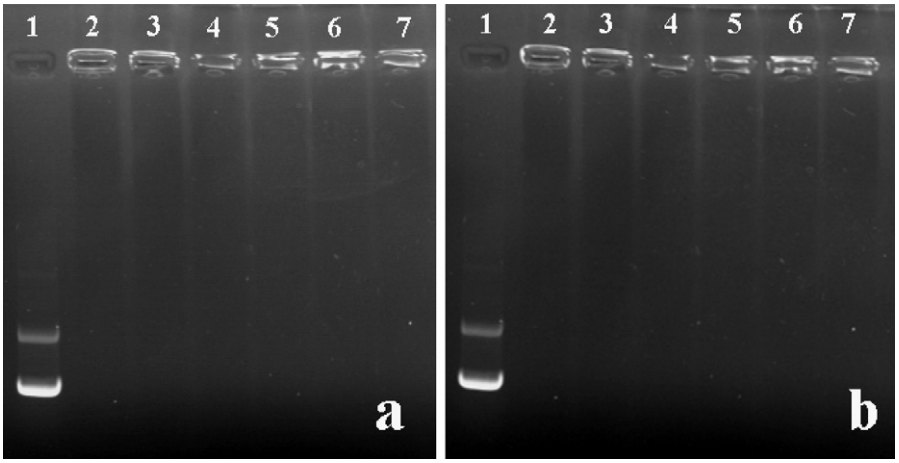

Fig. 5. Agarose gel electrophoresis retardation assay of (a) QG-5/DNA complexes and (b) QG-6/DNA complexes. Plasmid DNA (96 ng) was mixed with QGs at different N/P ratios: 1 (0), 2 (5), 3 (10), 4 (15), 5 (20), 6 (25), 7 (30).

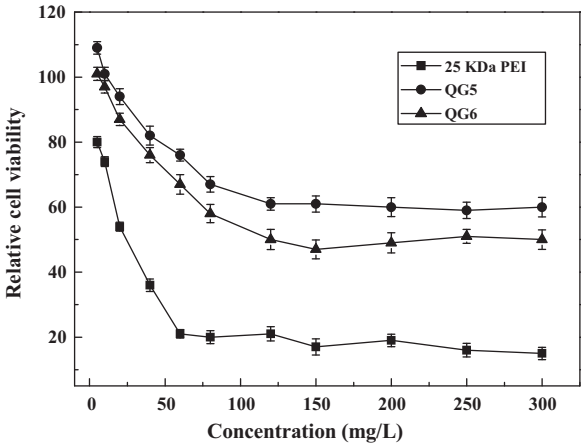

Fig. 6. Cell viabilities of 293T cells in the presence of QG-5, QG-6, and 25 kDa PEI.

amount of cationic glucan damaging the cellular membranes. However, the QGs exhibited much lower cytotoxicity when compared with PEI, even at the highest concentration (200–300 mg/L), the cell viability of the QGs groups is still higher than 50%, while cell viability in the same concentration of the PEI group is lower than 20%. The fact that QGs seemed to be well tolerated by cells was encouraging for further transfection experiments.

The transfection efficiency of QG/DNA complexes was evaluated in 293T cells at N/P ratios ranging from 5 to 30, using naked DNA and 25 kDa PEI/DNA complexes at its optimal ratio (N/P, 20) as the controls. The plasmid pGL-3 DNA was used as the luciferase reporter gene. On the basis of the results of low cytotoxicity, the N/P ratios ranging from 5 to 30 are fit for the transfection experiments. Fig. 7 shows the transfection efficiency of QG/DNA complexes, PEI/DNA complexes, and naked DNA. It indicated that the trends of transfection efficiency for QG-5/DNA and QG-6/DNA complexes were similar, and the transfection efficiency depended on the DS value of QGs. As shown in Fig. 7, although the transfection efficiency was relatively lower than that of PEI, QGs increased the transfection efficiency markedly compared with the naked DNA values (up to 1000-fold). Considering the much lower cytotoxicity compared with PEI, QGs could be considered as promising nonviral gene carriers in vitro.

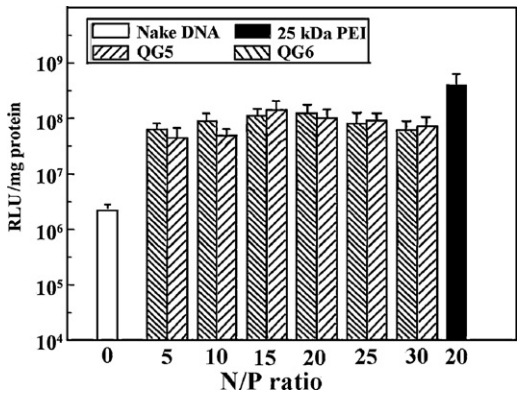

Fig. 7. Luciferase expression in 293T cells transfected by QG/DNA complexes at different N/P ratios. Transfection efficiency of the naked DNA and 25 kDa PEI/DNA complexes at N/P ratio of 20 are shown as control. Data are shown as mean ± SD ($n = 3$).

5. Conclusion

Quaternized *G. lucidum* polysaccharides were synthesized successfully in concentrated NaOH aqueous solution. Water-soluble QGs with different DS values could be obtained by adjusting the molar ratio of CHPTAC to glucan from 6 to 24, and the reaction time is no less than 18 h. Aqueous solutions of QGs displayed a typical polyelectrolyte behavior, QGs with relatively high DS could condense DNA efficiently. The cytotoxicity of QGs was evaluated in 293T cells and was found to be relatively low compared with PEI. The transfection efficiency of the QG/DNA complexes was measured by the luciferase gene in 293T cells, and the cells had been transfected effectively. The results indicated that the quaternized glucan derivatives obtained in the concentrated NaOH are promising vectors to be used as gene carriers.

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