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Synthesis and characterization of methoxy poly(ethylene glycol)-*O*-chitosan-polyethylenimine for gene delivery

Yuan Yuan Yu[a,1], Zheng Wang[a], Lin Cai[b], Gang Wang[c,1], Xiao Yang[b], Xiao Pin Wan[b], Xin Hua Xu[a], Ying Li[a,*], Rong Gao[b,**]

[a] College of Chemistry, Sichuan University, No. 29 Wangjiang Road, Chengdu, Sichuan 610064, PR China
[b] College of Life Science, Sichuan University, No. 29 Wangjiang Road, Chengdu, Sichuan 610064, PR China
[c] Biomaterial Engineering Center of Sichuan University, Chengdu 610064, PR China

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ABSTRACT

A novel water-soluble chitosan (CS) derivative methoxy poly(ethylene glycol)-*O*-chitosan-polyethylenimine (mPEG-*O*-CS-PEI), was synthesized. The synthesized intermediates and final product were characterized and confirmed by ^{1}H NMR and FT-IR spectra. The particle size and zeta potential of mPEG-*O*-CS-PEI/DNA complexes were 65 nm and +28.5 mV at the mass ratio of 20:1, respectively. Agarose gel electrophoresis study showed strong DNA binding ability of mPEG-*O*-CS-PEI. The transfection of L-02 cells proved that mPEG-*O*-CS-PEI/plasmid was significantly less toxic than PEI 35 kDa and Lipofectin. The result of real-time quantitative PCR and GFP expression imaging showed that the transfection efficiency of mPEG-*O*-CS-PEI was significantly higher than PEI 35 kDa and Lipofectin in L-02 cells ($P < 0.05$). Therefore, mPEG-*O*-CS-PEI copolymer may be attractive cationic polymers for nonviral gene therapy.

1. Introduction

Gene therapy is regarded as the most efficient method to treat many inheritable or acquired diseases (Ferber, 2001). However, the major problem for progress in human gene therapy is lack of effective vectors (Kim et al., 2007). Basically, gene delivery vectors are generally classified into two categories: viral vectors and nonviral vectors. Although viral vectors have higher transfection efficiency, they have fatal drawback such as mutagenesis, carcinogenesis, immune response and time consuming (Liu & Yao, 2002). Compared with viral vectors, nonviral vectors have several advantages including safer, easy to produce, the ability to transfer large DNA moleculars, and have been considered as potential alternatives (Strand, Issa, Christensen, Vårum, & Artursson, 2008). Among the wide number of nonviral vectors, chitosan, a cationic polymer, is one of the most potential candidates due to its outstanding biologic properties including biodegradability, biocompatibility and bioactivity (Borchard, 2001). However, the low transfection efficiently restrict it used as gene carrier (Kim et al., 2007). To overcome this problem, several ligands such as galactose ligand (Gao et al., 2003; Kim, Park, Nah, Choi, & Cho, 2004; Murata, Ohya, & Ouchi, 1997), transferrin ligand (Zhang, Mardyani, Chan, & Kumacheva, 2006), folate ligand (Lee, Lockey, & Mohapatra, 2006; Mansouri et al., 2006), mannose ligand (Kim, Nah, Cho, Park, & Cho, 2006) were introduced into chitosan chain. Polyethylenimine (PEI) is another type of cationic polymer and has been widely utilized for gene delivery due to its superior transfection in many different types of cells. Transfection efficiency and cytotoxicity of PEI depend on its molecular weight. A lower molecular weight PEI shows lower cytotoxicity and transfection activity (Fischer, Bieber, Li, Elsässer, & Kissel, 1999; Kunath et al., 2003). One rational way was suggested that low molecular weight PEIs could be combined together using degradable linkages to form combined PEI with suitable high molecular weights.

In this study, a new water-soluble chitosan derivative, mPEG-*O*-CS-PEI, was synthesized. Introduction of mPEG into chitosan chain not only increase its solubility but also reduce the particle sizes of the complexes (Park et al., 2001). Low molecular PEI was conjugated to chitosan to increase the transfection efficiency. The synthesized intermediates and final product were characterized by ^{1}H NMR and FT-IR spectra. The physicochemical properties of the copolymer/DNA complexes, particle size, and zeta potential were analyzed. The in vitro cytotoxicity and in vitro transfection efficiency were investigated.

* Corresponding author. Tel.: +86 28 85418495; fax: +86 28 85413601.
** Corresponding author. Tel.: +86 28 85416856; fax: +86 28 85416856.
E-mail addresses: profliying@sina.com (Y. Li), lake96@qq.com (R. Gao).
[1] These two authors are equally contributed to this research.

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2. Materials and methods

2.1. Materials

Chitosan (CS, Mn 48 kDa, deacetylation degree 90%) was provided by Nanjing Weikang Biotechnology Co. Ltd. (China). Methoxy poly(ethylene glycol) (mPEG, Mn 2 kDa) was obtained from Hannong Chemicals Inc. (Korea). Polyethylenimine (PEI, Mn 2 kDa) was purchased from Wuhan Qianglong New Chemical Materials Co. Ltd. (China). Dialysis tubing (MWCO 8–14 kDa) was purchased from Beijing Solarbio Technology Co. Ltd (China). L-02 cell: human liver cell line (provided by Shanghai Life Science Academy, China). Other chemicals were analytical-grade and used without further purification.

2.2. Preparation of mPEGylation chitosan (mPEG-O-CS, **5**)

The mPEG-*O*-CS copolymer was synthesized in two steps. In the first step, mPEG (20 g, 10 mmol) was dissolved in 25 ml toluene, and the solvent was distilled to azeotropically remove water. Triphenyl phosphite (8.0 ml, 50 mmol) and methyl iodide (1.9 ml, 50 mmol) were then added and the mixture was stirred at 120 °C for 5 h in darkness. The reaction mixture was cooled and dissolved in 20 ml toluene, followed by precipitating from ether. The precipitate was filtered and dried under vacuum to obtained pale yellow mPEG iodide (**2**) (Gorochovceva & Makuška, 2004). In the second step, chitosan (4.0 g, 24 mmol as monosaccharide residue) was added into 40 g 50% NaOH solution and put into refrigerator at −18 °C for 48 h for alkalization. The mPEG iodide (10.0 g, 5 mmol) was then added into the alkali chitosan (**4**), the resultant mixture was heated to 90 °C and stirred for 6 h. After cooling, the pH of the solution was adjusted to 7.0 with HCl aqueous (10%, v/v). The solution was dialyzed against distilled water, and then lyophilized.

2.3. Synthesis of mPEG-O-CS-PEI (**6**)

mPEG-*O*-CS (**5**) (6.0 g, 10 mmol as monosaccharide residue containing 8.9 mmol amino group) and triethylamine (0.9 g, 8.9 mmol) were dissolved in CH_2Cl_2 (20 ml), acryloyl chloride (0.81 g, 8.9 mmol) was then dropped slowly into the reaction mixture and stirred for 1 h with an ice bath then for 2 h at room temperature. The reaction mixture was dialyzed and dissolved in 50 ml of H_2O, PEI (3.0 g, 1.5 mmol) was then added to the solution, and the reaction was conducted for 48 h at 50 °C under argon atmosphere. The resultant solution was concentrated and then dialyzed against distilled water, and the solution was subsequently freeze-dried.

2.4. Characterization of copolymer

FT-IR spectra were recorded on a Perkin Elmer spectrometer. All samples were prepared as potassium bromicle pellets. ^{1}H NMR spectra were recorded on a Bruker AV400 spectrometer. Chitosan was dissolved in the mixed solvent D_2O/CF_3COOD. PEI, mPEG-*O*-CS and mPEG-*O*-CS-PEI were dissolved in D_2O according to their solubility.

2.5. Determination of mPEG and PEI contents

The content of mPEG units in the copolymer was determined by a modified colorimetric method based on the partitioning of a chromophore present in ammonium ferrothiocyanate regent from the aqueous to a chloroform phase in the presence of mPEG (Nag, Mitra, & Ghosh, 1996). The DS of PEI moiety could be calculated by comparing the ratio of the number of $-CH_2CH_2O-$ protons of mPEG and $-CH_2CH_2NH-$ protons of PEI (Park et al., 2005).

2.6. DNA condensation

Complex formation was confirmed by agarose gel electrophoresis. The nanoparticle solution of plasmid DNA with mPEG-*O*-CS-PEI copolymer was loaded into undivided wells of 0.7% agarose gel, electrophoresed at 100 V for 45 min and stained with 0.5 mg/ml ethidium bromide. The resulting DNA migration pattern was revealed under UV irradiation.

2.7. Size and zeta potential of the complexes

The average diameter and zeta potential of the polymeric micelles were measured by Zetasizer 3000 HS/IHPL instrument (Malvern Instruments Ltd., Malvern, UK).

2.8. Transmission electron microscopy (TEM)

TEM was used to observe the morphology of the mPEG-*O*-CS-PEI/DNA nanoparticles. The specimens were prepared by dropping the sample solution onto a copper grid covered with nitrocellulose, then a drop of 2% phosphotungstic acid was added to the sample to give a negative stain, which was air-dried at room temperature then the observation was performed with the electron microscopy.

2.9. Preparation of polymer/DNA nanoparticles

The nanoparticles of CS and mPEG-*O*-CS-PEI complexes were prepared using the method of ionic cross-linking (Bodmeier, Chen, & Paeratakul, 1989). CS and mPEG-*O*-CS-PEI were dissolved separately in 1% (v/v, pH 5.5) acetic acid solution, and heated at 65 °C for 10 min. Then the polycation solution and plasmid VRMFat-1 solution were mixed together to form complexes with different weight ratios of copolymer/DNA. The resultant mixture was left for 5 min to obtain the nanoparticle solution.

2.10. Cytotoxicity assay

Cytotoxicity of different reagents was measured using the MTT dye reduction assay. Cells were seeded in 96-well plates at an initial density of 3.0×10^5 cell/well in 0.1 ml of growth medium and incubated overnight. Then the cells were incubated in 100 μl serum-free medium containing various amounts of polymers (pH 7.4). After an additional incubation for 16 h, the medium was removed and the cells were rinsed twice with PBS. The wells were refilled with complete medium and cells were cultured for another 24 h. Then 10 μl of MTT (5 mg/ml) solution was added into each well after removal of the transfection solution. The solution was removed after 4 h of incubation, and replaced with 150 μl of DMSO to dissolve the formed crystals. Absorbance at 490 nm was measured with an ELISA plate reader (BioRad, Microplate Reader 3550).

2.11. Transfection efficiency in vitro

2.11.1. Expression of green fluorescence protein (GFP) in cells

L-02 cells were seed in 12-well plates at an initial density of 3×10^5 cells/well with 1 ml of DMEM, and incubated at 37 °C for 24 h in 5% CO_2. The cells were washed with DMEM and incubated with the polymer/DNA complexes in serum-free culture medium (pH 7.4) for 4 h at 37 °C. Then the medium was replaced with fresh medium containing serum and incubated 48 h. The luciferase assay was carried out according to the manufacture's instruction (Promega, USA).

2.11.2. Real-time quantitative RT-PCR

To detect the transcription of EGFP gene in transfected cell lines, L-02 cells cultured in 12-well plates were washed with ice-

cold PBS, lysed in 1 ml TRIZOL (Invitrogen), and RNA was isolated according to the manufacturer's protocol. cDNA was synthesized from 1 μg RNA in a 20 μl reaction volume containing 4 μl of 5× reaction buffer, 1 μl dNTP mix (10 mM each), 1 μl random hexamers (100 μM), 2 μl 0.1 M DTT, 0.5 μl RNaseOUT™ Recombinant Ribonuclease Inhibitor (40 U/μl, Invitrogen), 0.5 μl SuperScript II Reverse Transcriptase (200 U/μl, Invitrogen), and H_2O.

A portion of cDNAs was diluted with H_2O to be 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}. Standard curve assays for each gene were performed using the original and diluted cDNAs covering the range of all template concentrations in the BioRad iCycler system (BioRad). PCR efficiencies (Eff.) of each gene were calculated from the given slopes in the standard curves according to the equation: $E = 10^{-1}/\text{slope}$.

Real-time quantitative PCR was performed with 1 μl of cDNA using 2× TaKaRa SYBR Green Premix (TaKaRa) in a BioRad iCycler. All reactions were done in a 20 μl reaction volume in triplicate. The specificity of amplification was verified by melt curve analysis and agarose gel electrophoresis. EGFP forward and reverse primers were 5′-CTTCAAGGACGACGGCAACTACAAG-3′ and 5′-CGTTCTTCTGCTTGTCGGCCATGAT-3′. β-Actin forward and reverse primers were 5′-CTCCTCCCTGGAGAAGAGCTA-3′ and 5′-CCTTCTGCATCCTGTCGGCAA-3′, respectively. PCR parameters consisted of 1 min at 95 °C, 40 cycles of amplification at 95 °C for 10 s, 64 °C for 30 s, and 72 °C for 20 s, followed by a melt curve detection: 95 °C 1 min, 55 °C 1 min, 55 °C 10 s for 80 cycles with 0.5 °C increasing in each cycle. Ct (Cycle threshold) values of each reaction were determined using BioRad iQ5 software. The relative amount of EGFP mRNA was normalized to β-actin reference gene and calculated according to the formula as below.

$$\text{Rel. quantity} = \frac{\text{GOI}_{\text{sample}}/\text{GOI}_{\text{control}}}{\text{Norm}_{\text{sample}}/\text{Norm}_{\text{control}}} = \frac{(1+\text{Eff})_{\text{GOI}}^{Ct_{\text{control}}-Ct_{\text{sample}}}}{(1+\text{Eff})_{\text{Norm}}^{Ct_{\text{control}}-Ct_{\text{sample}}}}$$

Rel. quantity: relative quantity, relative gene expression level in sample compared to that in control; GOI: gene of interest; Norm: normalizer, reference gene, house keeping gene; Eff: efficiency of PCR amplification; Ct: cycle threshold, the point at which the fluorescence rises above the background.

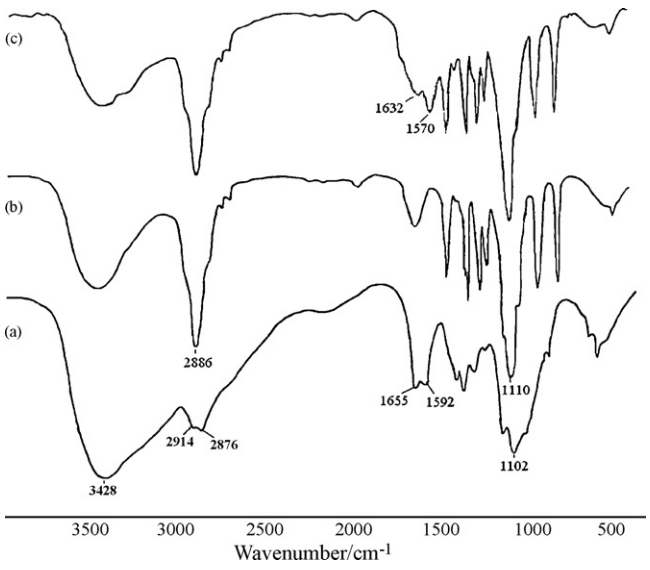

Fig. 2. The FT-IR spectra of CS (a), mPEG-*O*-CS (b), and mPEG-*O*-CS-PEI (c).

3. Results and discussion

3.1. Synthesis and structure analysis

The general synthetic route of mPEG-*O*-CS-PEI is shown in Fig. 1. mPEG iodide was obtained by the reaction between mPEG and methyl iodide. Then the alkali chitosan reacted with mPEG iodide to obtain a water-soluble mPEG-*O*-CS and acryloyl chloride as a linking reagent grafted onto CS to get the activated CS derivative. Finally, the product was obtained by the reaction between amino group of PEI and double bound of CS derivative. The structure of the copolymer was conformed by FT-IR spectra and ^{1}H NMR spectra. As shown in Fig. 2, compared with the FT-IR spectra of chitosan, the peak at 2886 cm^{-1} (C–H stretching) and 1112 cm^{-1} (–C–O–C stretching) observably increased, due to the introduction of mPEG chain. FT-IR spectra of mPEG-*O*-CS-PEI are very similar to that of mPEG-*O*-CS. More information about the graft copolymer was obtained by ^{1}H NMR spectra. As shown in Fig. 3, the ^{1}H

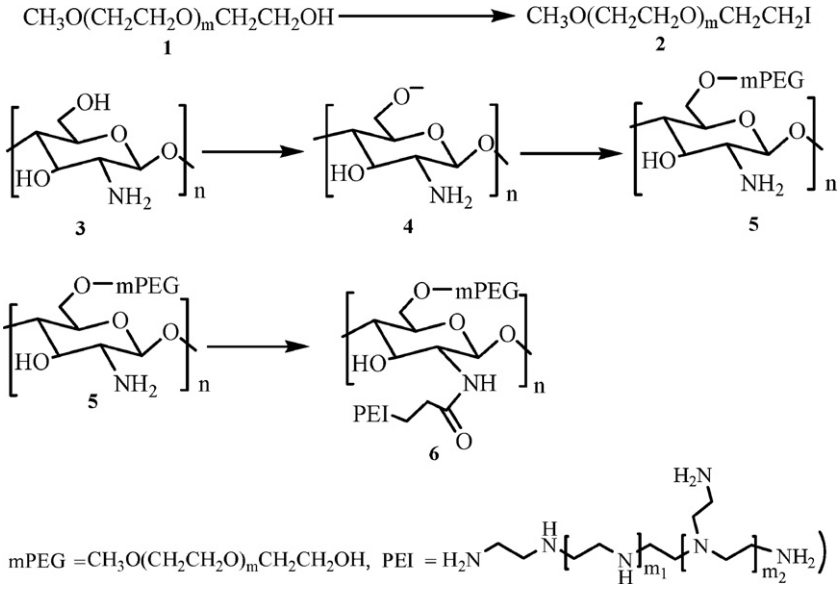

Fig. 1. Synthesis of mPEG-*O*-CS-PEI.

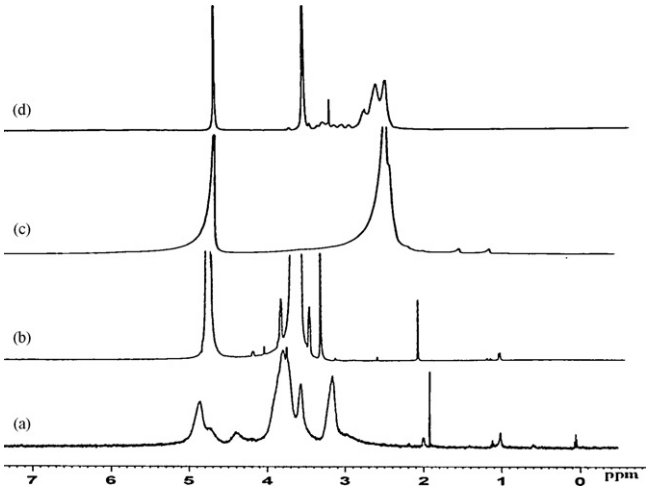

Fig. 3. The ^{1}H NMR spectra of CS (a), mPEG-*O*-CS (b), and PEI (c), mPEG-*O*-CS-PEI (d).

NMR data of chitosan were: δ 4.72 ppm (H-1 of glucosamine ring), δ 3.12 ppm (H-2 of glucosamine ring), δ 3.43–3.81 ppm (H-3, H-4, H-5, H-6 of glucosamine ring), δ 1.96 ppm (–$NHCOCH_3$ of acetyl group). After peglated reaction, the peaks at δ 3.38 ppm (–OCH_3 of mPEG) revealed the existence of mPEG group, the strong broad signals of the mPEG at δ 3.55–4.07 ppm partially covered over the signal of the glucosamine ring of chitosan on the ^{1}H NMR of mPEG-*O*-CS. Compared with the mPEG-*O*-CS, there exhibits the characteristic chemical shifts of PEI (δ 2.57–2.86 ppm) on the ^{1}H NMR of mPEG-*O*-CS-PEI, indicating that PEI was grafted to the chitosan chain (Petersen et al., 2002). All the information mentioned above indicated that PEI and mPEG were grafted onto CS. The DS of chitosan by mPEG was calculated by formula (1) and the DS of chitosan by PEI was calculated by formula (2)

$$DS_{\mathrm{mPEG}}\ (\%) = \frac{m_{\mathrm{mPEG}}/M_{\mathrm{mPEG}}}{m - m_{\mathrm{mPEG}}} \times 100 \quad (1)$$

$$DS_{\mathrm{PEI}}\,(\%) = \frac{I_{\mathrm{PEI}}}{I_{\mathrm{mPEG}} \times 1.32} \times DS_{\mathrm{mPEG}} \quad (2)$$

where m_{mPEG} represents the measured mPEG weight (g); M_{mPEG} represents the molecular weight of mPEG (2000 g/mol); m represents the sample weight (g); M_{CS} represents the molecular weight of chitosan monomer (165 g/mol); I_{PEI} represents the number of –CH_2CH_2NH– protons of PEI; I_{mPEG} represents the number of –CH_2CH_2O– protons of mPEG.

The DS of chitosan by mPEG and PEI were calculated as 33% and 28%.

3.2. DNA bonding ability

The combination ability of mPEG-*O*-CS-PEI with DNA was evaluated by measuring the fluorescence emitted when adding the ethidium bromide into the complexes. As shown in Fig. 4, the migration of DNA was suppressed completely when the mass ratio higher than 10:1. The agarose gel electrophoresis results indicated the good DNA condensation capability of mPEG-*O*-CS-PEI.

3.3. Particle size and zeta potential

The particle size and zeta potential of the nanoparticles were shown in Table 1. It can be concluded that mPEG-*O*-CS-PEI/DNA complexes showed higher zeta potential and smaller particle size compared with CS/DNA complexes, which contributed to the grafting of polycationic PEI and mPEG. The particle size of mPEG-

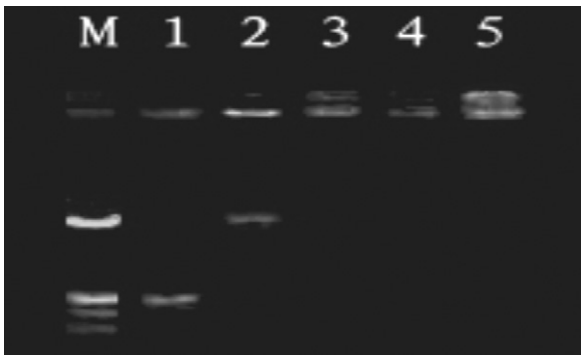

Fig. 4. Gel retardation assay (0.7% agarose gel) of mPEG-*O*-CS-PEI/DNA complexes. M: marker; Lane 1: VRfat-1; Lane 2–5: mPEG-*O*-CS-PEI/VRfat-1, mass ratios of 5:1, 10:1, 20:1, 30:1, respectively.

Table 1
The average diameter and zeta potential of CS and mPEG-*O*-CS-PEI.

Sample	T (°C)	w/w	Z-Ave (d.nm)	Zeta potential (mV)
CS/DNA	25	10:1	380	−1.59
	25	20:1	398	0.60
	25	30:1	381	11.1
mPEG-*O*-CS-PEI/DNA	25	10:1	154	14.2
	25	20:1	65	28.5
	25	30:1	78	30.3

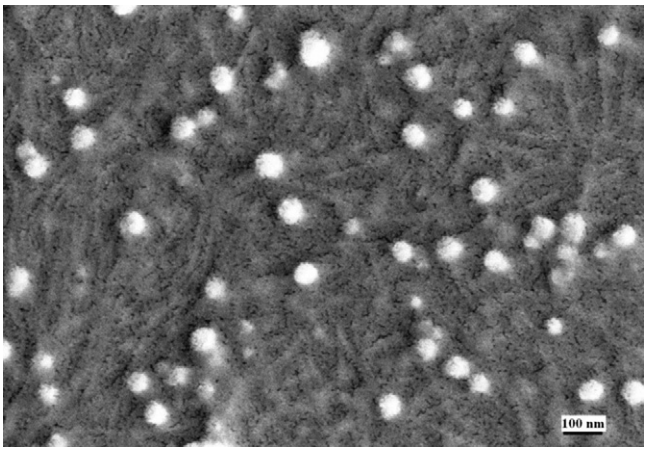

Fig. 5. TEM photograph of mPEG-*O*-CS-PEI nanoparticles (100,000×).

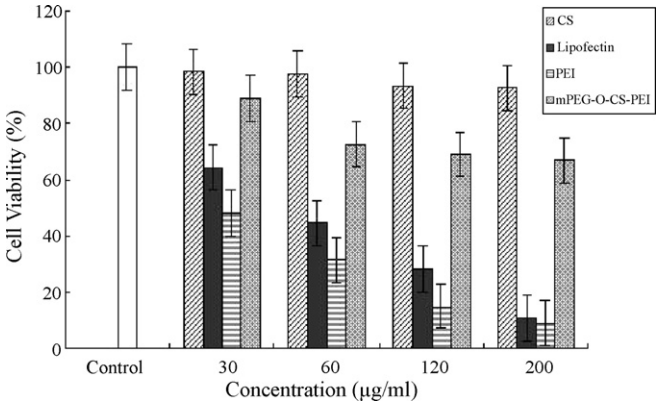

Fig. 6. Cell viability of CS, Lipofectin, PEI (35 kDa) and mPEG-*O*-CS-PEI in the concentration range 30–200 μg/ml in L-02 cell line, determined by the MTT assy.

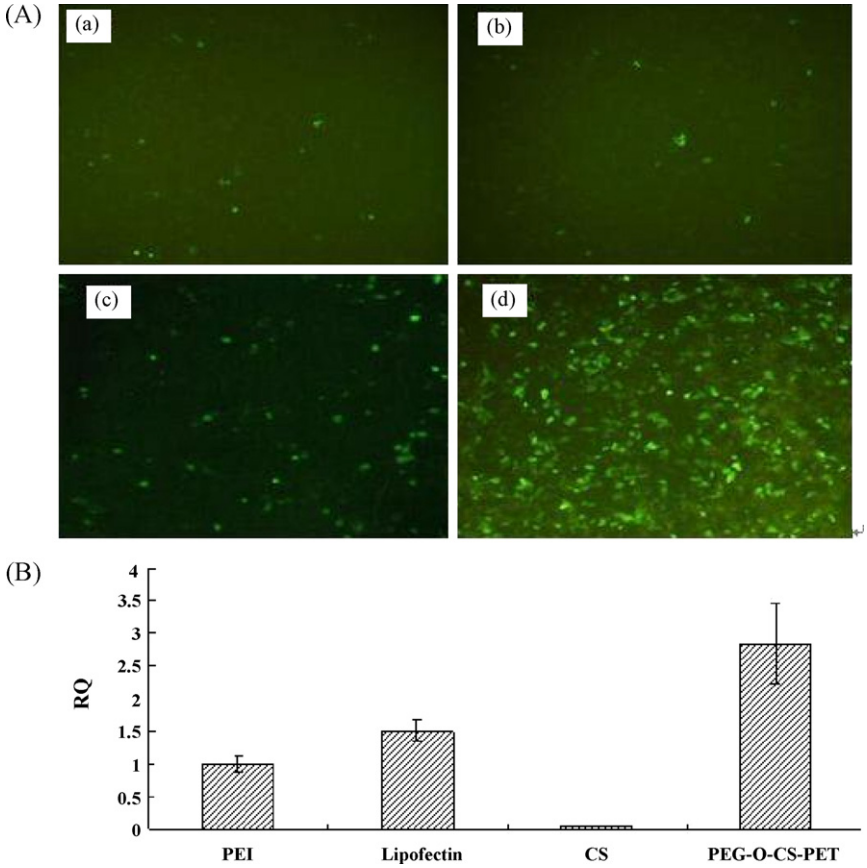

Fig. 7. (A) Fluorescence images of L-02 cells transfected with (a) CS (mass ratio 30:1), (b) PEI (35 kDa, mass ratio 1:1), (c) Lipofectin, and (d) PEG-*O*-CS-PEI (mass ratio 20:1) (magnification 100×). (B) In vitro transfection efficiency of the complexes of mPEG-*O*-CS-PEI/DNA (mass ratio 20:1) in comparison with that of CS (mass ratio 30:1), PEI (35 kDa, mass ratio 1:1) and Lipofectin.

O-CS-PEI/DNA complexes decreased with increasing mass ratio of mPEG-O-CS-PEI to DNA and had a minimum value at 65 nm at the mass ratio of 20:1. The zeta potential of mPEG-*O*-CS-PEI/DNA complexes rapidly increased to positive values with increasing mass ratio. A slight positive surface charge and small particle size are necessary to allow for endocytosis into cells (Jiang et al., 2007; Nomura et al., 1997). The shape of mPEG-*O*-CS-PEI/DNA nanoparticles showed spherical and polydisperse nature as shown in Fig. 5. Therefore, mPEG-*O*-CS-PEI/DNA complexes at the mass ratio of 20:1 thought to be profitable for the gene transfer cells.

3.4. Cell viability

The cytotoxicity of mPEG-*O*-CS-PEI copolymer on L-02 cells was evaluated by MTT assay at various concentration of the copolymer. As shown in Fig. 6, mPEG-*O*-CS-PEI/DNA complexes exhibited much lower cytotoxicity compared with that of 35 kDa PEI and Lipofectin. The cytotoxicity of mPEG-*O*-CS-PEI/DNA complexes increased gradually with increasing concentration. However, the cytotoxicity of PEI and Lipofectin increased drastically as concentration increased and the cell viability were below 30% when the concentration reached 120 μg/ml. The results showed that less decrease in viability was found for L-02 cells treated with mPEG-*O*-CS-PEI/DNA compared to CS/DNA nanoparticles. It was reported that covalently linking PEI to PEG is an effective method to minimized toxicity of PEI (Ahn, Chae, Bae, & Kim, 2002). In addition, the low molecular weight PEI was lower toxicity than high molecular weight PEI. The lower cytotoxicity of mPEG-*O*-CS-PEI copolymer may be due to the PEG group and low molecular weight of PEI.

3.5. Transfection efficiency in vitro

DNA transfection efficiency of mPEG-*O*-CS-PEI/DNA complexes was evaluated in L-02 cells. As shown in Fig. 7, transfection efficiency of mPEG-*O*-CS-PEI/DNA complexes into L-02 cells was assessed at mass ratio of 20:1. mPEG-*O*-CS-PEI showed much higher transfection efficiency than PEI and Lipofectin ($P < 0.05$), and furthermore, it manifested obviously lower cell toxicity to L-02 cells in comparison with those of PEI and Lipofectin ($P < 0.05$). The higher transfection efficiency of mPEG-*O*-CS-PEI should be attributed to higher amine content of complexes and small particle size at mass ratio of 20:1. The small particle sizes of complexes make the complexes endocytized by cells easily (Illum, Jabbal-Gill, Hinchcliffe, Fisher, & Davis, 2001). In addition, the higher amine content makes the complexes escape endosome easily due to the higher buffer capacity.

4. Conclusion

A water-soluble cationic copolymer of mPEG-*O*-CS-PEI was synthesized via a facile route. The structure was confirmed by FT-IR and ^{1}H NMR. The mPEG-*O*-CS-PEI/DNA nanoparticles had an appropriate particle size of 65 nm and positive surface charge of 28.5 mV, which showed an enhanced DNA condensation capability compared to CS. The cell viability results showed that the nanoparticles had weak toxicity on the growth of cells, and the image of the expression of GFP and real-time quantitative PCR showed that mPEG-*O*-CS-PEI can significantly enhance the transfection and expression efficiency of genes in L-02 cells in vitro in comparison with chitosan, PEI and Lipofectin. These results suggest that

the mPEG-*O*-CS-PEI is a promising and effective nonviral vector for gene therapy.

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