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Mannosylated chitosan-graft-polyethylenimine as a gene carrier for Raw 264.7 cell targeting

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ABSTRACT

Gene transfer using non-viral vectors is a promising approach for the safe delivery of therapeutic genes. Among non-viral vectors, chitosans have been proposed as alternative, biocompatible cationic polymers for non-viral gene delivery. However, the low transfection efficiency and low specificity of chitosan needs to be addressed prior to clinical application. In this study, mannosylated chitosan-graft-polyethylenimine (Man-CHI-g-PEI) copolymer was prepared by thiourea reaction between the isothiocyanate group of mannopyranosylphenylisothiocyanate and the amine groups of chitosan-graft-PEI (CHI-g-PEI) for targeting into antigen presenting cells (APCs) having mannose receptors. The composition and molecular weight were characterized using ^{1}H NMR and GPC, respectively. The copolymer was complexed with plasmid DNA in various copolymer/DNA (N/P) charge ratios, and the complexes were characterized. Man-CHI-g-PEI showed good DNA binding ability and high protection of DNA from nuclease attack and had low cytotoxicity compared with PEI 25K. The transfection efficiency of Man-CHI-g-PEI/DNA complexes into the Raw 264.7 macrophage cell line, which has mannose receptors, was higher than CHI-g-PEI itself as well as PEI 25K, indicating Man-CHI-g-PEI can be used as an APCs' targeting gene delivery carrier.

1. Introduction

Gene therapy is the transfer of genetic material to specific cells in order to have a therapeutic effect. While most gene therapy protocols currently in clinical trials employ recombinant viral vectors due to their high transfection efficiency, safety concerns have been raised because of their highly toxic nature. The use of non-viral vectors has attracted great interest not only because of their safety of use but also because the characteristics of non-viral vectors can be modified easily (Arthur et al., 1997; Raper et al., 2003; Lu et al., 2007).

Among non-viral vectors, chitosans have been proposed as alternative, biocompatible cationic polymers suitable for non-viral gene delivery (Köping-Höggård et al., 2004). However, this system is significantly limited by its low transfection efficiency and low cell specificity (Mansouri et al., 2006). To address this problem, several ligands-conjugated chitosans have been designed for receptor-mediated endocytosis gene delivery because receptor-mediated endocytosis offers the potential to target specific cells and enhances their uptake (Mao et al., 2001; Chan et al., 2007; Park et al., 2001; Jiang et al., 2007a, 2008; Kim et al., 2006). Among the ligand-conjugated chitosans, mannosylated chitosan (MC) has been reported as antigen presenting cells (APCs)-targeting gene carriers due to the specific ligand-receptor interactions between mannose-moieties and mannose receptors (MR) (Kim et al., 2006). The MR is predominantly present on macrophages and dendritic cells, which play a central role in innate and adaptive immune responses (Taylor et al., 1990). MC was expected to have significant potential as a safe, APCs-targeting gene carrier; however, the transfection efficiency of MC was not high enough for further clinical applications (Kim et al., 2006).

In a previous study, chitosan-graft-PEI (CHI-g-PEI) was prepared as a gene carrier (Jiang et al., 2007b). The CHI-g-PEI showed low cell toxicity and high transfection efficiency, although this system also had limited cell specificity. Therefore, in this study, mannosylated chitosan-graft-PEI (Man-CHI-g-PEI) was prepared to obtain better APC specificity. Physiochemical properties of Man-CHI-g-PEI/DNA complexes were analyzed. The cytotoxicity,

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macrophage cell specificity, and transfection efficiency were also investigated.

2. Materials and methods

2.1. Materials

Chitosan (molecular weight 100K; deacetylation degree 87%) was supplied by Jakwang (Ansung, Korea). Mannopyranosylphenylisothiocyanate, branched PEI 25K, and potassium periodate were obtained from Sigma–Aldrich (St. Louis, MO, USA). Branched PEI 1800 Da was purchased from Wako (Osaka, Japan).

2.2. Preparation of Man-CHI-g-PEI

Man-CHI-g-PEI copolymer was synthesized in two steps. In the first step, CHI-g-PEI was synthesized according to a previously reported method (Jiang et al., 2007b). In the second step, mannose was introduced to CHI-g-PEI copolymer in order to produce a thiourea reaction (Kim et al., 2006). Briefly, CHI-g-PEI (240 mg) dissolved in 10 mL of carbonate buffer solution (pH 9.0) was mixed with 4 mL of mannopyranosylphenylisothiocyanate solution (12.5 mg/mL DMSO) and then stirred for 24 h at room temperature. The Man-CHI-g-PEI copolymer was dialyzed (Spectra/Por® membrane: MWCO = 12,000–14,000) against deionized water. The synthetic scheme of Man-CHI-g-PEI is shown in Fig. 1.

2.3. Characterization of copolymer and copolymer/DNA complexes

The composition of the prepared Man-CHI-g-PEI copolymer was estimated by measuring ^{1}H NMR (Avance™ 600, Bruker, Germany). The molecular weight of the Man-CHI-g-PEI copolymer was measured using a gel permeation chromatography column (Dawn Eos, Wyatt, Santa Barbara, CA, USA) wavelength of 690 nm.

The DNA condensation ability of the copolymer was confirmed by electrophoresis. DNA retardation was observed by irradiation with UV light and assayed with Cam2com software. The morphology of Man-CHI-g-PEI/DNA (pGL3) complexes was observed using EF-TEM (LIBRA 120, Carl Zeiss, Germany) (Jiang et al., 2007b). The size and surface charge of the Man-CHI-g-PEI/DNA complexes were measured at 25 °C using an electrophoretic light scattering spectrophotometer. Protection and release of DNA in complexes were measured using electrophoresis (Jiang et al., 2007b).

2.4. Cell lines, cell culture, and cell viability assays

Raw 264.7 (murine macrophage cells) and HeLa (human cervix epithelial carcinoma cells) were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco BRL, Paris, France) and NCTC 3749 (murine macrophage cells) were incubated in RPMI 1640 medium (HyClone, Utah, USA) supplemented with 10% fetal bovine serum (FBS, HyClone, Utah, USA), streptomycin at 100 μg/mL, and penicillin at 100 U/mL. Cells were incubated at 37 °C in a humidified 5% CO_2 atmosphere. Cells were split using Trypsin/EDTA medium when almost confluent. *In vitro* cytotoxicity test was evaluated according to a previously reported method (Jiang et al., 2007b).

2.5. Transfection efficiency in vitro

The *in vitro* transfection efficiency of Man-CHI-g-PEI was evaluated in Raw 264.7 and HeLa cells. Cells were seeded in 24-well plates in 1 mL of growth medium at an initial density of 2×10^5 cells/well and 1×10^5 cells/well for the Raw 264.7 and HeLa cells, respectively. The luciferase assay was done as previously described (Jiang et al., 2007b).

For flow cytometry, cells transfected with polymer/pEGFP-N2 complexes. After transfection, cells were washed once with PBS and detached with 0.25% trypsin/EDTA. Transfection efficiency was evaluated by scoring the percentage of cells expressing GFP using a FACS Calibur System from Becton-Dickinson (San Jose, CA). Fluorescence parameters from 10,000 cells were acquired. In the case of the murine IL-12 (mIL-12) gene, Raw 264.7 cells were treated with polymer/pmIL-12 complexes and the culture supernatants were assayed for measurement of mIL-12 p70 using ELISA kit (R&D Systems, Minneapolis), as suggested by the manufacturer.

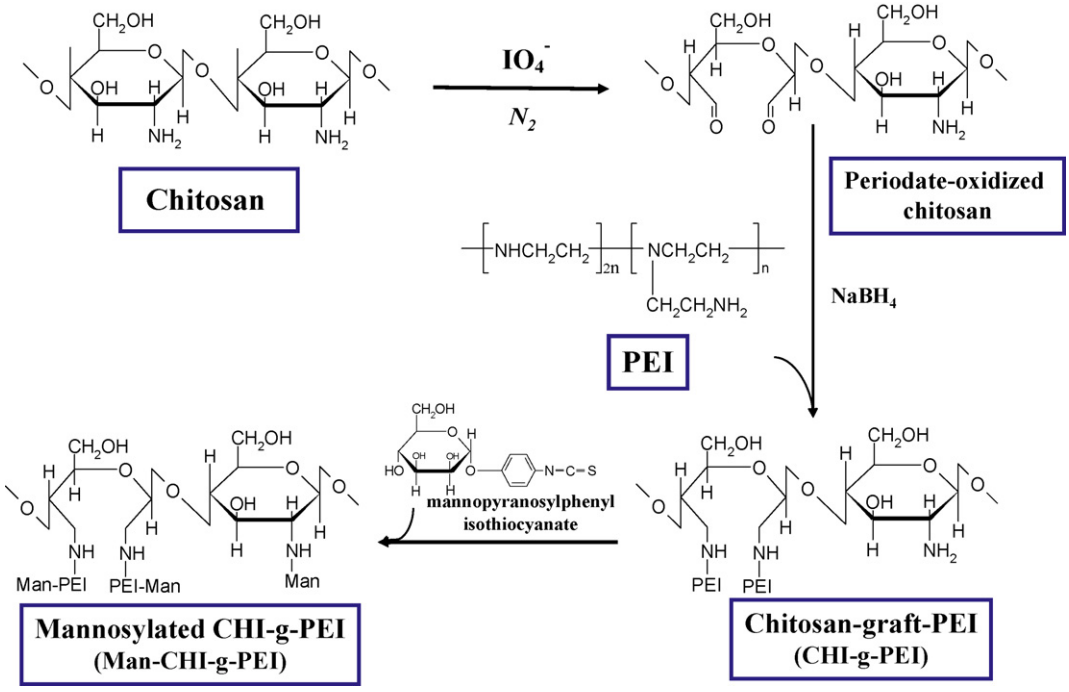

Fig. 1. Proposed reaction scheme for synthesis of Man-CHI-g-PEI.

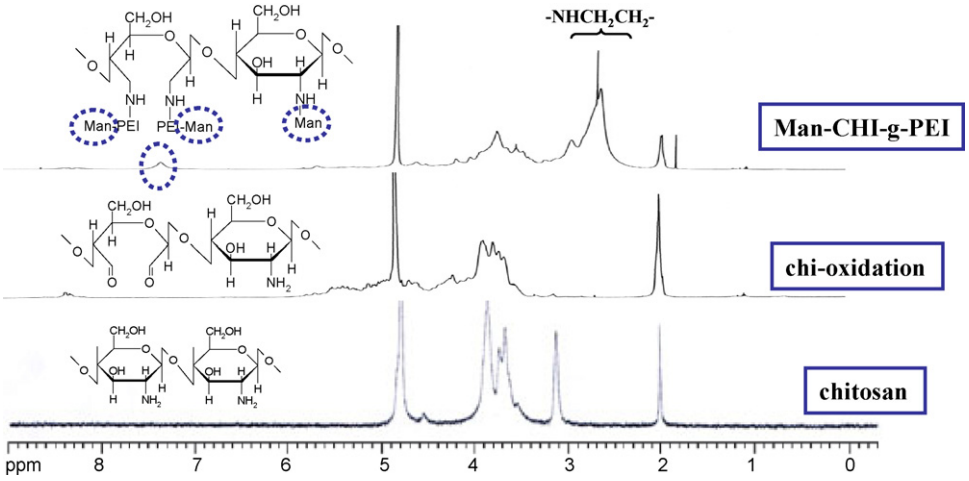

Fig. 2. Representative ^{1}H NMR spectra of Man-CHI-g-PEI chi-oxidation and chitosan in D_2O: δ = 3.2–3.1 (–CH_3–, 2-carbon of chitosan), 3.3–2.5 (–$NHCH_2CH_2$–, PEI ethylene) and 7.0–7.4 (–CH–, mannopyranosylphenyl isothiocyanate).

2.6. Statistical analysis

Statistical analysis was performed using Student's *t*-test (GraphPad Software, San Diego, CA). Data were expressed as mean ± S.D. Statistical significance was represented by $^*P < 0.05$ and $^{**}P < 0.01$.

3. Results

3.1. Synthesis and characterization of copolymer

The Man-CHI-g-PEI copolymer was synthesized by the thiourea reaction between the isothiocyanate group of mannopyranosylphenylisothiocyanate and the amine groups of CHI-g-PEI [Fig. 1]. The composition of the synthesized copolymer was analyzed by ^{1}H NMR [Fig. 2]. The degree of oxidized chitosan, determined from integral values of the 2-carbon proton peak, was 47 mol% [Table 1]. The assignment of chemical shifts of PEI was determined by a previously reported method (Park et al., 2005). Results showed 16.4 mol% of the PEI was grafted to the oxidized chitosan chain. The chemical composition of the mannose groups in Man-CHI-g-PEI was determined to be 15.4 mol% by assigning the protons of the acetyl group of chitosan and the protons of phenylisothiocyanate in mannopyranosylphenylisothiocyanate (shown by the dotted line).

3.2. Characterization of Man-CHI-g-PEI/DNA complexes

The condensation capability of Man-CHI-g-PEI with DNA was evaluated using agarose gel electrophoresis. The migration of DNA was completely retarded when the N/P ratio of the Man-CHI-g-PEI/DNA complexes was approximately 1 [Fig. 3A]. Fig. 3B shows representative morphologies of Man-CHI-g-PEI/DNA complexes, which had a well-formed, spherical shape and compact structure. All complexes were less than 95 nm, and particle sizes tended to decrease with an increase in the N/P ratio [Fig. 3C]. The relatively homogenous size distribution of the complexes measured by dynamic light scattering was unimodal [Fig. 3D]. The zeta potentials of Man-CHI-g-PEI/DNA complexes at various N/P ratios are shown in Fig. 3C. At an N/P ratio of 0.1, where complexes could not form completely, the zeta potential of the copolymer/DNA complexes was negative (−25.7 ± 3.7 mV). With increasing N/P ratios, the zeta potential rapidly increased to positive values (20.9 ± 4.5 mV, at N/P ratio 14). As shown in Fig. 3E, DNA in the complexes was protected from DNase I, in contrast with naked plasmid DNA as a control.

Table 1
Characteristics of prepared Man-CHI-g-PEI copolymer.

Degree of oxidation (mol%)	MW of periodate-oxidized chitosan (kDa)	MW of mannosylated CHI-g-PEI (kDa)	Degree of grafted PEI (1800) (mol%)	Degree of mannosylation (mol%)
47	12.6	26.3	16.4	15.4

3.3. Cytotoxicity of Man-CHI-g-PEI

The Man-CHI-g-PEI copolymer showed low cytotoxicity as compared with both PEI 25K and CHI-g-PEI in 3 different cell lines [Fig. 4]. Man-CHI-g-PEI copolymer-treated cells exhibited good cell viability (86.0, 68.9 and 65.6% in Raw 264.7, NCTC 3749 and HeLa cell lines, respectively) even at a high concentration (100 μg/mL), whereas the cell viability of PEI 25K-treated cells was drastically decreased with increasing concentration.

3.4. In vitro transfection efficiency

Raw 264.7 macrophage cells expressing moderate mannose receptors (Kim et al., 2006) were used to determine whether gene delivery with Man-CHI-g-PEI is mannose receptor-mediated. To determine the optimal N/P ratio of Man-CHI-g-PEI/DNA complexes for transfection, Raw 264.7 cells were transfected with complexes prepared at different N/P ratios [Fig. 5A]. The transfection efficiency of the copolymer increased with increasing N/P ratio, whereas the transfection efficiency of PEI 25K alone decreased due to its cytotoxicity. The maximum transfection efficiency of Man-CHI-g-PEI/DNA was observed at an N/P ratio of 14 and tended to decrease with further increases in the N/P ratio. Fig. 5B shows the transfection efficiencies of DNA, CHI-g-PEI/DNA, Man-CHI-g-PEI/DNA, and PEI/DNA complexes at functional N/P ratios. The transfection efficiency of Man-CHI-g-PEI/DNA complexes was found to be 10948.3-, 9.4- and 16.6-fold higher than those of DNA, CHI-g-PEI/DNA, and PEI/DNA complexes, respectively.

The transfection efficiency of Man-CHI-g-PEI/DNA complexes in Raw 264.7 and HeLa cells was also compared in order to demonstrate the effect of mannose on receptor-mediated gene transfer [Fig. 5C]. Man-CHI-g-PEI/DNA complexes showed higher transfection efficiency as compared with CHI-g-PEI/DNA complexes in Raw 264.7 cells, but not in HeLa cells, which have no mannose receptors on the cell surface, indicating that the mannose ligand on Man-CHI-

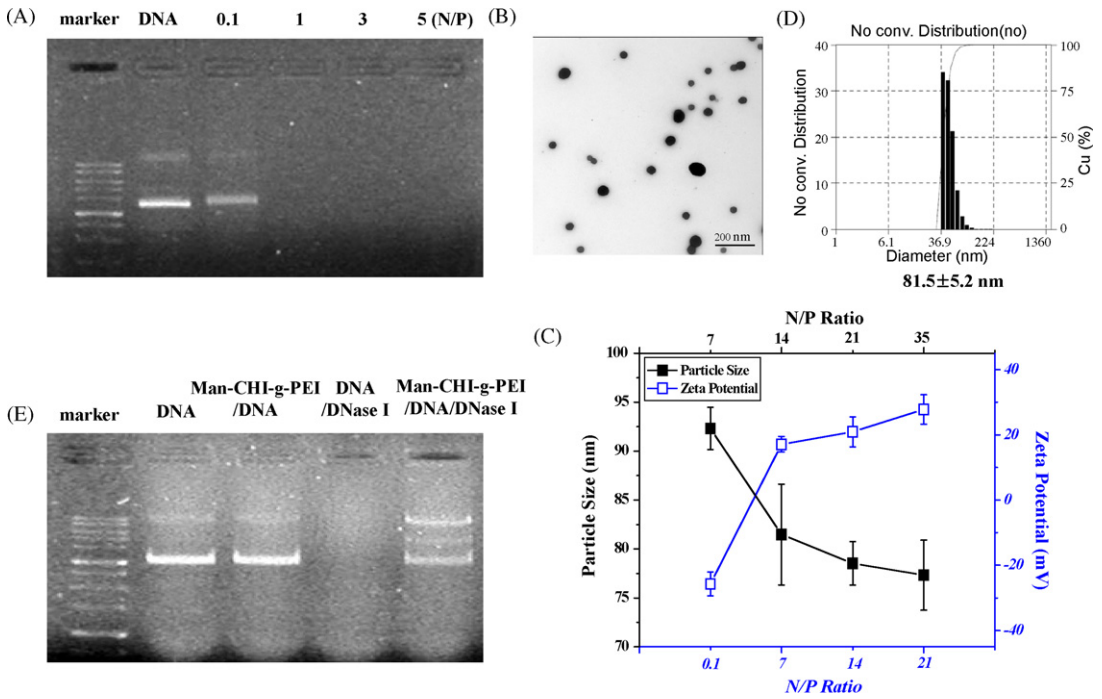

Fig. 3. (A) Agarose gel electrophoresis of Man-CHI-g-PEI/DNA complexes at various N/P ratios; (B) EF-TEM images of Man-CHI-g-PEI/DNA complexes at N/P ratio 14, scale bar: 200 nm; (C) particle sizes and zeta potentials of Man-CHI-g-PEI/DNA complexes at various N/P ratios ($n = 3$, error bars represent standard deviation); (D) size distribution of Man-CHI-g-PEI/DNA complexes prepared at N/P ratio 14 and (E) DNA protection and release assay.

g-PEI played a significant role in mannose receptors recognition and enhanced transfection efficiency in Raw 264.7 cells. The transfection efficiency of Man-CHI-g-PEI/DNA complexes was greatly reduced (14.9-fold) in the presence of an excess of free mannose (50 mM), indicating Man-CHI-g-PEI/DNA complexes are absorbed via receptor-mediated endocytosis [Fig. 5D].

To further elucidate the mechanism of transfection, the buffering capacity of the Man-CHI-g-PEI copolymer was analyzed. HeLa cells

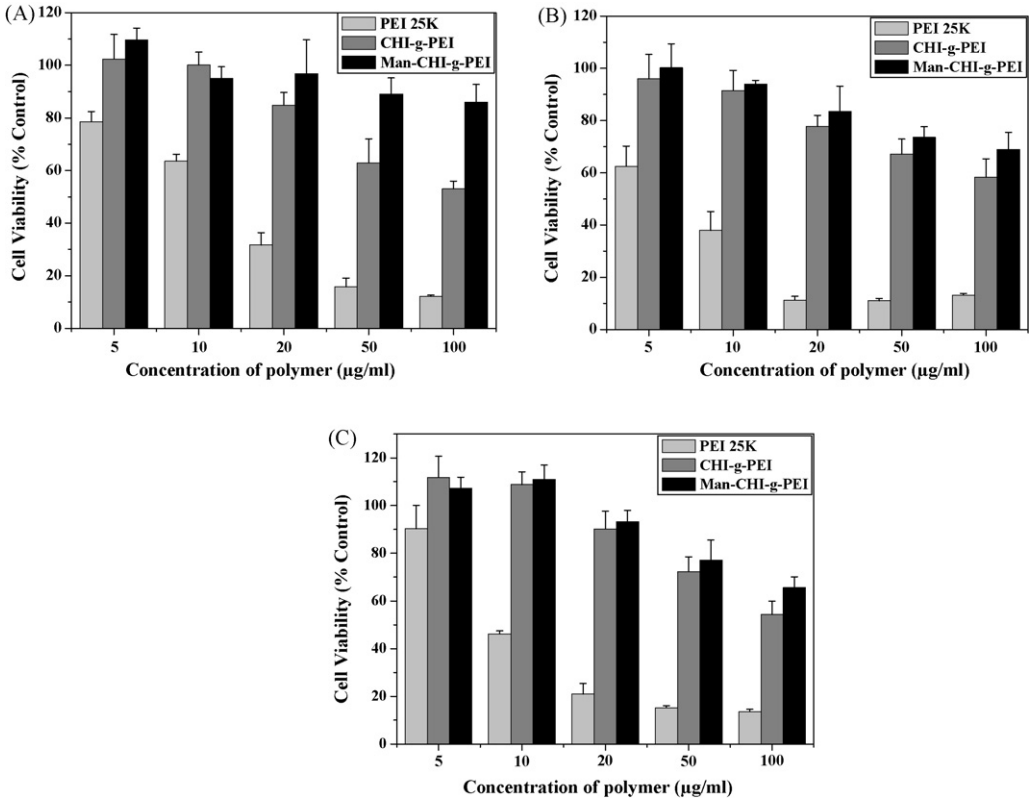

Fig. 4. Cytotoxicity of Man-CHI-g-PEI copolymer at various concentrations in different cell lines (A) Raw 264.7; (B) NCTC 3749 and (C) HeLa ($n = 3$, error bars represent standard deviation).

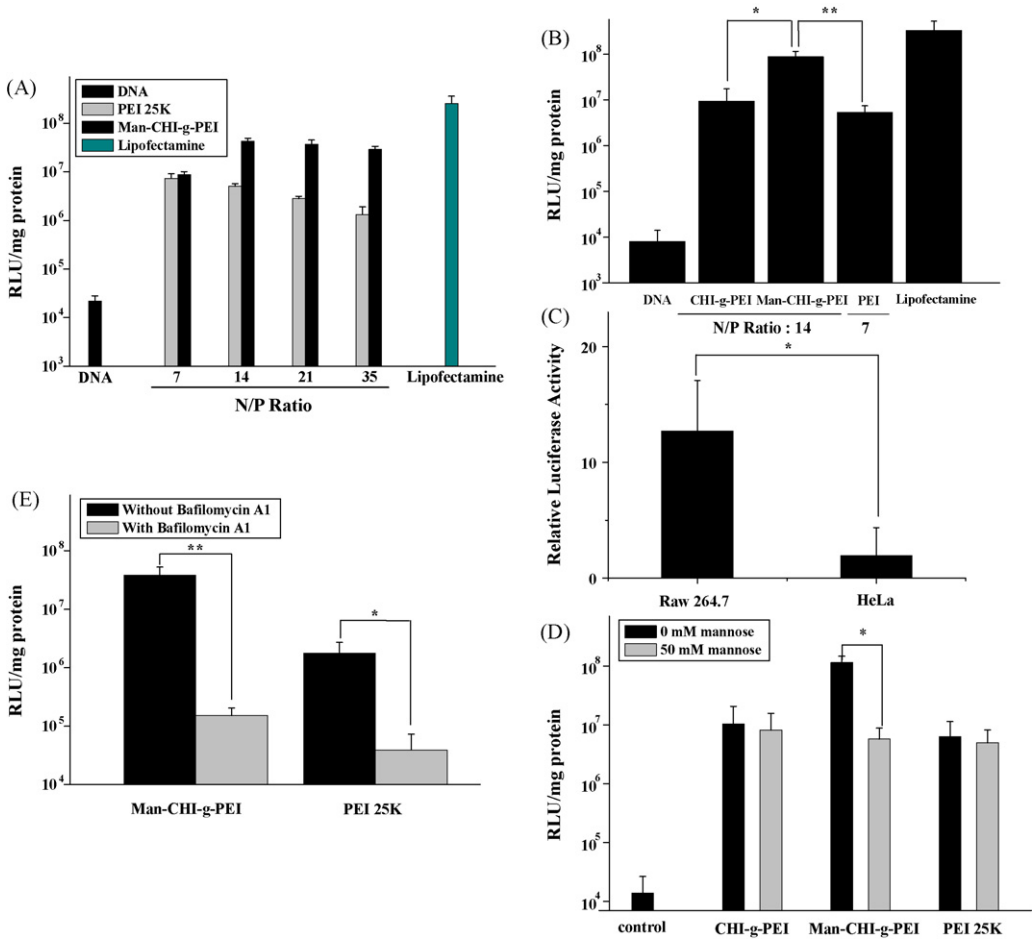

Fig. 5. (A) Transfection efficiency of Raw 264.7 cells by Man-CHI-g-PEI/DNA (pGL3-control) complexes at various N/P ratios ($n = 3$, error bars represent standard deviation); (B) transfection efficiency of polymer/DNA Man-CHI-g-PEI/DNA (pGL3-control) complexes at functional N/P ratios in Raw 264.7 cells; (C) luciferase activity from Man-CHI-g-PEI/DNA complexes at a charge ratio of 14 normalized by that from CHI-g-PEI/DNA complexes on Raw 264.7 and HeLa cells; (D) competition assay of PEI/DNA, CHI-g-PEI/DNA and Man-CHI-g-PEI/DNA complexes by adding free mannose (50 mM) as a competitor of the mannose and (E) effect of bafilomycin A1 on gene transfection. Values are means ± S.D. $^{*}P < 0.05$ and $^{**}P < 0.01$.

were treated during transfection with bafilomycin A1, a specific inhibitor of vacuolar type H^+ ATPase. Transfection of Man-CHI-g-PEI/DNA complexes was drastically decreased (241.2-fold) after bafilomycin treatment [Fig. 5E], suggesting the involvement of the proton sponge effect in PEI-mediated transfection. These results demonstrate that Man-CHI-g-PEI has enhanced gene transfer efficiency and cell specificity *in vitro*.

A greater GFP expression was observed with the Man-CHI-g-PEI/DNA complexes compared with the PEI 25K/DNA and CHI-g-PEI/DNA complexes (data not shown). In addition, using FACS to view GFP expression, the transfection efficiency was evaluated [Fig. 6A]. Likewise, the transfection efficiency of Man-CHI-g-PEI was higher than that of PEI 25K and CHI-g-PEI. Furthermore, IL-12 cytokine gene was delivered to Raw 264.7 cells as a therapeutic gene and the efficiency of Man-CHI-g-PEI-mediated cytokine production was evaluated. As shown in Fig. 6B, Man-CHI-g-PEI/pmIL-12 complexes as compared with PEI/pmIL-12 and CHI-g-PEI/pmIL-12 complexes significantly increased mIL-12 p70.

4. Discussion

An important step in the advancement of gene therapy is the development of an efficient, targeted gene delivery system. Receptor-mediated gene transfer is a promising gene delivery technique. In a previous study, CHI-g-PEI was prepared as a gene carrier (Jiang et al., 2007b). CHI-g-PEI showed good cell viability and high transfection efficiency but poor cell specificity. Therefore, to obtain both gene transfer ability and macrophage cell specificity and to reduce toxicity compared to PEI alone, Man-CHI-g-PEI was prepared using mannopyranosylphenyl isothiocyanate and chitosan-graft low molecular weight PEI.

The Man-CHI-g-PEI copolymer was successfully synthesized. As shown in Fig. 2, the proton peaks of PEI ($-NHCH_2CH_2-$) appeared at 3.3–2.5 ppm and the proton peak of the phenylisothiocyanate in mannopyranosylphenylisothiocyanate (the portion shown within the dotted line) appeared at 7.4–7.2 ppm, indicating PEI was grafted to the chitosan chain and mannopyranosylphenylisothiocyanate coupled with CHI-g-PEI.

As shown in Fig. 3A, Man-CHI-g-PEI exhibited good DNA binding ability. Previous research reported that for effective gene expression, the DNA in the gene vehicle should be protected from enzyme degradation (Park et al., 2005). In contrast with control naked plasmid DNA, DNA in the complexes was protected from DNase I [Fig. 3E]. This result suggests that more intact DNA could transfer to cells. Particle size is a particularly important factor that influences the access and passage of complexes through the targeted site. All complexes were less than 95 nm and particle sizes tended to decrease with an increase in the N/P ratio [Fig. 3C]. The hydrophilic groups of the mannose residue are thought to prevent the aggregation of complexes. Additionally, since the polymer complexes with DNA through ionic interactions at high N/P ratios, net electrostatic repulsive forces prevent aggregation. Upon self-assembly of DNA

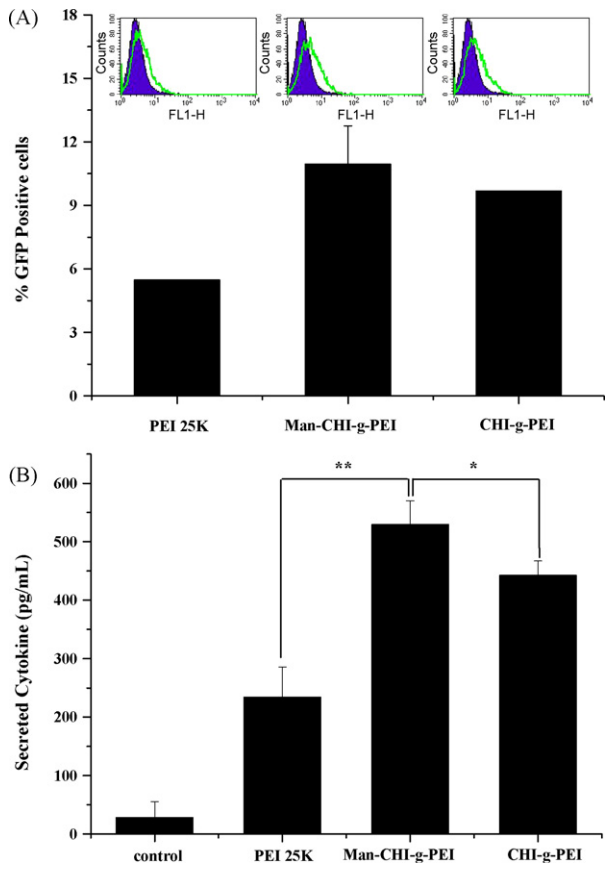

Fig. 6. GFP expressed in a Raw 264.7 cell line transfected with Man-CHI-g-PEI/DNA complexes (A) FACS analysis and (B) secreted cytokine analysis. Values are means ± S.D. $^{*}P < 0.05$ and $^{**}P < 0.01$.

and Man-CHI-g-PEI copolymer, the highly negative charge of the DNA is rapidly neutralized until the surface charge of the complexes becomes positive at higher N/P ratios. A positive surface charge was previously reported as necessary for binding to anionic cell surfaces, which facilitates uptake by the cell (Kunath et al., 2003).

The cytotoxicity of cationic polymers is likely due to polymer aggregation on cell surfaces, impairing important membrane functions. Hong et al. reported PEI induced more LDH leakage as compared with the other polycationic polymers, such as poly-L-lysine (PLL) and dendrimer (G5-NH_2) in KB and Rat2 cell lines because PEI possesses a much greater charge/monomer ratio (Hong et al., 2006). Conversely, chitosan has been reported to have a low toxicity (Moghimi et al., 2005), although a few investigators have observed a dose-dependent toxicity of chitosan at high doses *in vitro* (Illum, 1998). In a previous study, significantly higher cell viability of chitosan-treated cells was found compared with PEI 25K-treated cells in 3 different cell lines (293T, HeLa and HepG2) (Jiang et al., 2007b). In the present study, the cell viability was further increased after the mannosylation. The cell viability of Man-CHI-g-PEI (86.0% of control) was higher than that of CHI-g-PEI (53.1% of control) at a concentration of 100 μg/mL in the Raw 264.7 cell line. As previously reported, although cationic polymers with high charge density had strong cell lytic and toxic properties, a reduction of charge density resulted in less cell toxicity (Carreno-Gomez and Duncan, 1997). A greater number of low zeta potentials were found from Man-CHI-g-PEI/DNA complexes (+20.9 ± 4.5 mV, N/P = 14) than from PEI 25K/DNA complexes (+35.3 ± 3.6 mV, N/P = 7). Therefore, most likely, Man-CHI-g-PEI had lower toxicity than either PEI 25K or low-molecular-weight PEI 1800 Da due to the properties of biocompatible chitosan and the shielding of the primary amines of PEI 1800 Da after reaction with periodate-oxidized chitosan.

In comparison with previous studies, the Man-CHI-g-PEI copolymer from this study showed reduced cytotoxicity due to biocompatible chitosan and low molecular weight PEI, significantly enhanced transfection efficiency by the application of mannose for macrophage targeting, and high buffering capacity of PEI *in vitro*. In a previous study, mannosylated chitosan was developed as a receptor-mediated gene delivery carrier (Kim et al., 2006). MC/DNA complexes showed low cytotoxicity, though the transfection efficiency was very low. Mannosylated PEI (ManPEI) as alternative gene carriers have been introduced, owing to PEI-mediated high transfection efficiency, but ManPEI/DNA complexes also showed strong cytotoxicity (Diebold et al., 1999). Hashida group also developed a mannosylated cholesterol derivative (Man-C4-Chol) and demonstrated mannose-receptor molecules would be more effective for gene expression in macrophages than the nonspecific pathway (Lu et al., 2007; Hattori et al., 2005). In the same manner, Man-CHI-g-PEI/DNA complexes were more efficient for transferring reporter genes (luciferase, FACS and IL-12 cytokine assays) into macrophages in comparison with CHI-g-PEI/DNA complexes.

5. Conclusion

In this study, a novel Man-CHI-g-PEI copolymer as a new gene carrier for APCs targeting was successfully prepared and evaluated. Man-CHI-g-PEI demonstrated superior ability to form complexes with DNA and had suitable physicochemical properties for a gene delivery system. This copolymer had low cytotoxicity and exhibited enhanced gene transfer efficiency and specificity when compared with PEI 25K. Therefore, Man-CHI-g-PEI has the potential to be a safe and efficient APC-targeting gene carrier.

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