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Research paper

Chitosan-graft-spermine as a gene carrier *in vitro* and *in vivo*

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ABSTRACT

Chitosan has been proposed as a non-viral gene carrier because of its biodegradable and biocompatible cationic polymeric properties. However, the transfection efficiency of chitosan-DNA complexes is still too low for clinical trials. To improve transfection efficiency, we prepared a chitosan-graft-spermine (CHI-g-SPE) copolymer by an imine reaction between periodate-oxidized chitosan and spermine. The CHI-g-SPE copolymer was complexed with plasmid DNA in various copolymer-DNA weight ratios, and the complexes were characterized. The CHI-g-SPE copolymer showed good DNA binding ability and high protection of DNA from nuclease attack. The CHI-g-SPE/DNA complexes had well-formed spherical shapes and a nanoscale size with homogenous size distribution. The CHI-g-SPE copolymer had low cytotoxicity and CHI-g-SPE/DNA complexes showed transfection efficiency that was enhanced over that of chitosan-DNA. Furthermore, aerosol delivery of CHI-g-SPE/GFP complexes showed higher GFP expression compared with chitosan/GFP complexes, without toxicity. Our results indicate that the CHI-g-SPE copolymer has potential as a gene carrier.

1. Introduction

Gene therapy has received much attention in the fields of medicine, pharmaceutical sciences, and biotechnology because of its potential to cure genetic disorders and chronic diseases [1]. The field of gene therapy has advanced rapidly in the last decade. However, since naked therapeutic genes are rapidly degraded by nucleases and show poor cellular uptake, a major limiting factor for gene therapy remains the lack of suitable vectors [2,3]. Therefore, the development of safe and efficient gene carriers is a prerequisite for gene therapy success. Two different approaches, viral vectors and non-viral delivery systems, have primarily been used to deliver genes [4]. The vast majority of approved vectors for current clinical protocols are viral vectors because of their high transfection efficiency. However, they possess immunogenic properties and the potential ability to cause mutational infection and toxic side reactions that could limit their doses and frequency of treatment [5,6]. Non-viral vectors can be classified into lipoplex- and polyplex-forming carriers [7,8], and these have several advantages over viral vectors, including safety, lower immunogenicity, and the ability to transfer larger DNA molecules [4,7].

Chitosan is a family of linear binary polysaccharides comprised of beta (1-4) linked 2-amino-2-deoxy-beta-D-glucose (GlcN; D-unit) and the N-acetylated analogue (GlcNAc; A-unit) that has been investigated as a non-viral vector. Chitosan offers several advantages such as biodegradability, biocompatibility, and low toxicity with high cationic potential [9,10]. However, this system has a significant limitation in its low transfection efficiency [11–13]. This is primarily attributed to its minimal solubility and low buffering capacity at physiological pH leading to poor endosomal escape of the gene carrier [14]. To improve transfection efficiency, several derivatives of chitosan have been designed based on reactions with free amino groups. Kim et al. reported chitosan coupled to urocanic acid, which bears an imidazole ring that can enhance endosomal rupture through a proton sponge mechanism [11]. Wong et al. synthesized PEI-graft-chitosan by cationic polymerization of aziridine to water-soluble chitosan [15]. PEI-graft-chitosan had a lower cytotoxicity and higher transfection efficiency than PEI 25K due to the endosomal buffering effect of PEI both *in vitro* and *in vivo*.

Spermine is a tetra-amine with two primary and two secondary amino groups that is involved in cellular metabolism and is present

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in all eukaryotic cells [16]. It reversibly condenses DNA and is routinely used in DNA isolation [17]. Recently, the utility of hydrophobized dextran–spermine polyplexes as gene carriers was tested by Domb et al. [18–21]. Spermine was conjugated to the polymer via one of its primary amino groups, which became a secondary amine, resulting in a high buffering effect. Therefore, in this study, we designed the gene carrier chitosan-graft-spermine (CHI-g-SPE), a biocompatible copolymer, through an imine reaction between periodate-oxidized chitosan and spermine. The physicochemical properties of CHI-g-SPE/DNA complexes were analyzed. Cytotoxicity and transfection efficiency of the CHI-g-SPE/DNA complexes were investigated *in vitro* and *in vivo*. The toxicity study results *in vivo* was confirmed by histopathological examination.

2. Materials and methods

2.1. Materials

Chitosan (low molecular weight, degree of deacetylation: 85%), potassium periodate, and spermine were from Sigma–Aldrich (St. Louis, MO, USA). A pGL3 expression vector (5.3 kb, Promega, Madison, USA) contained a luciferase gene, driven by an SV40 promoter and enhancer. Plasmid pcDNA3.1/CT-green fluorescent protein (GFP) (6.1 kb), was from Invitrogen (CA, USA). Plasmids were propagated in *Escherichia coli*, extracted by the alkali lysis technique, and purified by a QIAGENR kit (Chatsworth, CA, USA). All other chemicals were reagent grade.

2.2. Preparation and characterization of copolymer and copolymer/DNA complexes

CHI-g-SPE copolymer was synthesized by an imine reaction between periodate-oxidized chitosan and spermine. First, periodate-oxidized chitosan was prepared by a modified method of Vold et al. [22]. Briefly, chitosan (25 mM) and potassium periodate (50 mM) were dissolved in sodium acetate buffer (pH 4.5). The potassium periodate solution was slowly added to the chitosan solution with magnetic stirring for 2 days at 4 °C before dialyzing (Spectra/Por membrane: MWCO = 3500) against NaCl (0.2 M, pH 4.5) three times and against deionized water (pH 4.5) five times. Spermine was grafted to periodate-oxidized chitosan by a modified method of Jiang et al. [12], with 50 mmol of spermine reacted with 5 mmol of periodate-oxidized chitosan solution with magnetic stirring for 2 days at 4 °C. Subsequently, the solution was treated with sodium borohydride (1 g $NaBH_4$/g chitosan), and thoroughly dialyzed (MWCO = 3500) against NaCl (0.2 M), and deionized water was added at 4 °C to remove unreacted spermine. The copolymer was lyophilized after dialysis. The composition of the prepared CHI-g-SPE copolymer was estimated by ^{1}H nuclear magnetic resonance (^{1}H NMR) (Avance 600, Bruker, Germany). The molecular weight of the CHI-g-SPE copolymer was measured by a gel-permeation chromatography column (GPC-MALS, Dawn Eos, Wyatt, USA).

All CHI-g-SPE/DNA complexes were freshly prepared before use and characterized by the method of Jiang et al. [12]. The DNA condensation ability of the CHI-g-SPE copolymer was confirmed by electrophoresis. DNA retardation was checked under ultraviolet illumination. The morphology of CHI-g-SPE/DNA complexes was observed by EF-TEM (LIBRA 120, Carl Zeiss, Germany). Dynamic light scattering spectrophotometer (ELS8000, Otsuka Electronics, Osaka, Japan) at 90° and 20° scattering angles was used to measure the particle size and surface charge of polyplexes at various *N*/*P* ratios.

DNA protection and release of DNA in complexes were measured using electrophoresis by the method of Jiang et al. [12]. Briefly, 1 μl of DNase I (2 units) or phosphate-buffered saline in DNase/Mg^{2+} digestion buffer (50 mM Tris–Cl, pH 7.6 and 10 mM $MgCl_2$) was added to 4 μl of polyplex solution (0.1 μg of plasmid DNA with 0.5 μg of polymer) or to naked plasmid DNA and incubated at 37 °C with shaking at 100 rpm for 30 min. For DNase inactivation, all samples were treated with 4 μl of EDTA (250 mM) for 10 min and mixed with 1% sodium dodecyl sulfate (SDS, pH 7.2) at a final volume of 15 μl. Final samples were incubated for 2 h, and electrophoresis was performed in 1% agarose gels with Tris–acetate–EDTA running buffer for 1 h at 50 V.

2.3. Cell lines, cell viability assays, and transfection efficiency studies in vitro

A549 (adenocarcinomic human alveolar basal epithelial cells) and WI-38 (human lung fibroblast cells) were cultured in RPMI 1640 (Gibco BRL, Paris, France) and HepG2 (human hepatoblastoma cells) were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco BRL). All media were supplemented with 10% fetal bovine serum (FBS, HyClone, Logan, UT, USA), streptomycin at 100 μg/mL, and penicillin at 100 U/mL. All cells were incubated at 37 °C in humidified 5% CO_2 atmosphere.

In vitro cytotoxicity tests were evaluated as previously reported [12]. Briefly, A549, WI-38, and HepG2 cells were seeded in 96-well plates at an initial density of 1×10^4 cells/well in 0.2 mL of growth medium and incubated for 18 h prior to medium replacement with serum-free medium containing various amounts of filtered polymers. After 24 h, medium was changed to growth media containing 20 μL of Cell Titer 96 A_{queous} One Solution Reagent. After incubation for 3 h, absorbance was measured at 590 nm, using an ELISA plate reader (GLR 1000, Genelabs Diagnostics, Singapore) to measure the metabolic activity of the cells.

In vitro transfection efficiency studies were evaluated as previously reported [12]. Briefly, A549 cells were seeded in 24-well plates at an initial density of 1.5×10^5 cells/well. After 18-h incubation, the medium was replaced with serum-free or media with polymer/pGL3 (1 μg) complexes and incubated for 4 h. The medium was replaced and incubation continued for 24 h. Luciferase assays were performed according to the manufacturer's protocols.

To assay transfection, bafilomycin A1 (200 nM) diluted in DMSO was put into the wells. After a 10-min incubation period, transfection efficiency studies were performed as described earlier.

2.4. In vivo aerosol delivery studies

Male C57Bl/6 mice, 17 weeks old, were housed in our laboratory animal facility and maintained at 23 ± 2 °C with relative humidity 50 ± 20%, under a 12-h light–dark cycle. To evaluate the efficiency of CHI-g-SPE delivery via aerosol, a nose-only exposure chamber (NOEC; Dusturbo, Seoul, Korea) was used, and 20 mice were randomly divided into five groups (four mice/group): untreated controls, GFP control (without polymer), CHI-g-SPE only (without GFP), CHI-g-SPE (with GFP), and CHI (with GFP). Each group received 1 mg DNA with GFP (alone or complexed with polymer) and aerosol delivery was performed as previously described [23]. At 48 h after inhalation studies, the mice were sacrificed and organs collected for analysis. For GFP analysis, lungs were fixed in 4% paraformaldehyde for 12 h and preserved in 30% sucrose for 48 h at 4 °C. Lungs were embedded with OCT compound (Sakura, Torrance, CA, USA) at <−20 °C. Microtome sections (10 μm) of lungs were evaluated for GFP signal using a Zeiss LSM510 confocal microscope (Carl Zeiss). For toxicity screening, organs were fixed in 10% neutral formalin, processed for paraffin section (3 μm), and evaluated for toxicity after H&E staining. All experimental procedures in this study were approved by the Animal Care and Use Committee at Seoul National University (SNU-100415-1).

3. Results and discussion

3.1. Synthesis and characterization of copolymer

We synthesized a CHI-g-SPE copolymer through an imine reaction between periodate-oxidized chitosan and the amine groups of spermine as shown in Fig. 1A. The molecular weight of CHI-g-SPE copolymer was 15 kDa. The composition of the synthesized copolymer was analyzed by ^{1}H NMR (Fig. 1B). Peaks for methylene protons at δ = 2.6–2.7 ppm [–NH–CH_2–] and for spermine at δ = 1.6–1.7 ppm [–CH_2–] in the copolymer appeared after the imine reaction. The peaks for chitosan protons, δ = 3.5–4.1 ppm [–CH_2–], were also detectable by ^{1}H NMR of the polymer. Although chitosan is soluble only in acidic conditions [24], after grafting with spermine, CHI-g-SPE was completely water soluble at physiological pH because of the hydrophilic nature of the added spermine.

3.2. Characterization of CHI-g-SPE/DNA complexes

The ability of polycations to interact with plasmid DNA is an important requirement for an effective gene delivery system. Therefore, we assessed the capability of CHI-g-SPE to condense with DNA using agarose gel electrophoresis. As shown in Fig. 2A, free plasmid DNA migrated through the gel and resolved into various distinct bands corresponding to super coiled and nicked circular forms of the plasmid. For the CHI-g-SPE copolymer, the migration of DNA was completely inhibited when the weight ratio of CHI-g-SPE to DNA was five. The formation of CHI-g-SPE/DNA complexes was also monitored by observation of the morphology and particle size distribution of the complexes at a functional weight ratio. Fig. 2B shows that the CHI-g-SPE/DNA complexes had well-formed spherical shapes and compacted structure. Surface quantification of properties such as particle size and surface charge of the complex is necessary to assure its uptake by cells. In particular, the particle size of polymer complexes is an important factor that influences the access and passage of complexes through the targeting site. Polycation/DNA complexes mostly enter the cell by endocytosis or pinocytosis and thus have a size requirement below 100 nm for maximum endocytosis by non-specialized cells [25]. As shown in Fig. 2C, complexes were 55.7 nm (PDI: 2.272e-001) and the relatively homogenous size distribution was measured by dynamic light scattering. A positive surface charge of polyplexes is necessary for binding to anionic cell surfaces, which facilitates cell uptake [26,27]. Fig. 2D shows the zeta potentials of complexes at various weight ratios. At weight ratio 0.1, where complexes could not form completely, the zeta potential of the CHI-g-SPE/DNA complexes was negative. With increasing weight ratio, the zeta potential of the complexes rapidly increased to positive values. For effective gene expression, the DNA in the gene vehicle must be protected from enzymatic degradation [28]. As shown in Fig. 2E, in contrast to the control naked plasmid DNA, DNA in the complexes was protected from DNase I. These

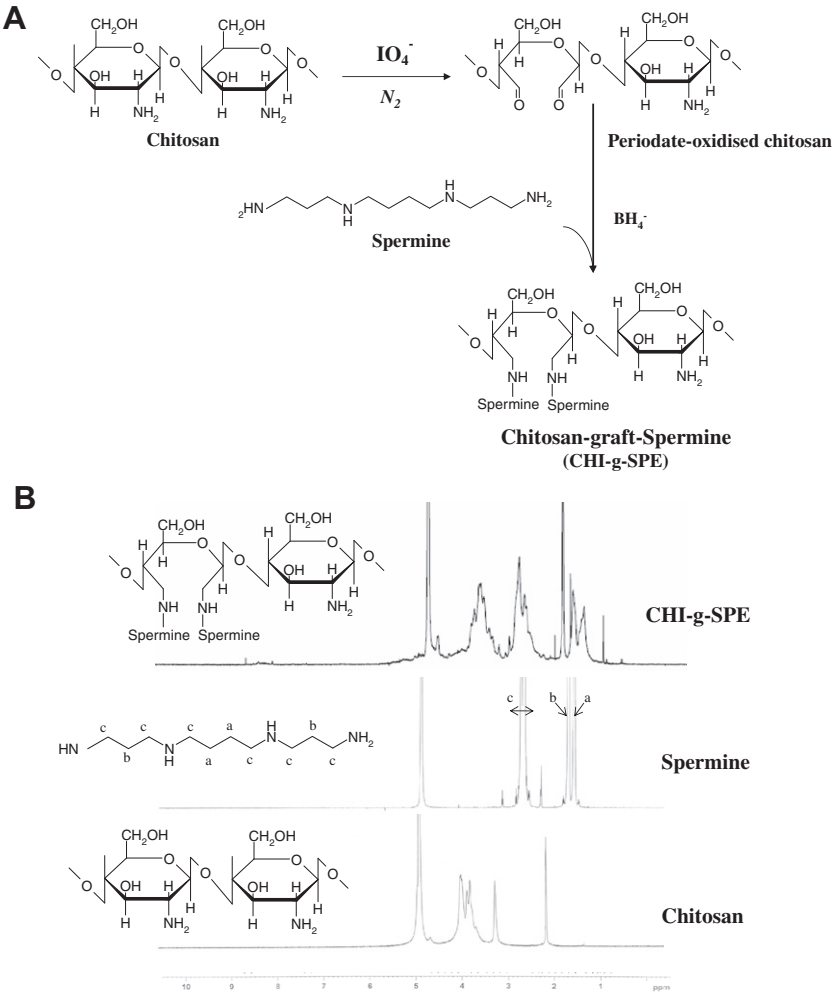

Fig. 1. (A) Proposed reaction scheme for synthesis of CHI-g-SPE. (B) Representative ^{1}H NMR spectrum of CHI-g-SPE in D_2O: δ = 2.6–2.7 ppm [–NH–CH_2–, spermine protons] and δ = 1.6–1.7 ppm [–CH_2–, spermine protons], δ = 3.5–4.1 ppm [–CH_2–, chitosan protons].

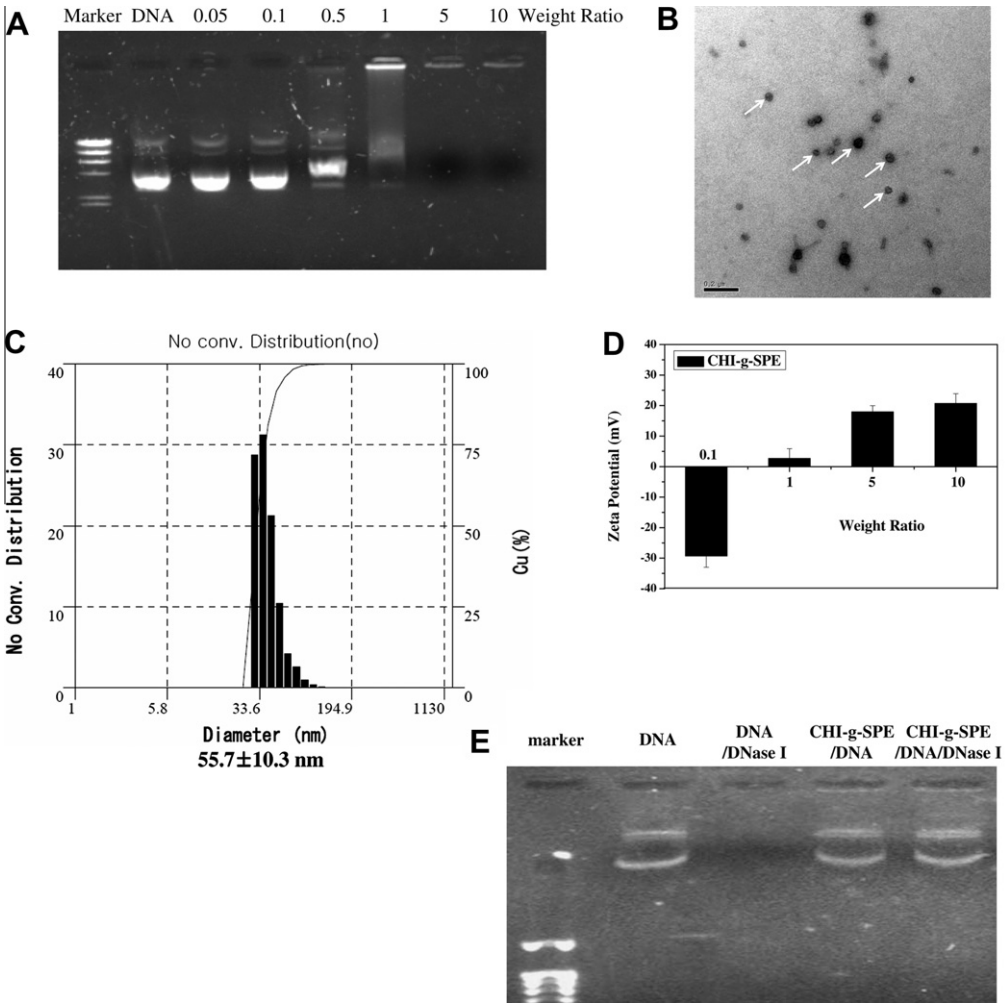

Fig. 2. Characterization of CHI-g-SPE/DNA complexes. (A) Agarose gel electrophoresis of CHI-g-SPE/DNA complexes at various weight ratios. (B) EF-TEM images of CHI-g-SPE/DNA complexes at weight ratio 10. (C) Particle size distribution of complexes prepared at weight ratio 10. (D) Surface charges of CHI-g-SPE/DNA complexes at various weight ratios (mean ± SD, $n = 3$). (E) Protection and release assay of DNA. DNA was released by adding 1% SDS to the CHI-g-SPE/DNA complexes at weight ratio 5.

results suggested that the polymer would allow more integral DNA to be transferred to cells without degradation.

3.3. Cytotoxicity of CHI-g-SPE copolymer

To investigate the cytotoxicity of the CHI-g-SPE copolymer, cell viability was determined by the Cell Titer96 AQ_{ueous} One Solution Cell Proliferation Assay at various concentrations of copolymer. Both chitosan and CHI-g-SPE copolymer-treated cells exhibited good cell viability (88.1%, 99.8% in A549 cells, 86.6%, 83.0% in WI-38 cells, and 85.1%, 90.9% in HepG2 cells) even at a high concentration (50 μg/mL), whereas the cell viability of PEI 25K-treated cells drastically decreased with increasing concentration (Fig. 3). As a polymeric gene carrier, PEI is commonly used in gene transfer because of its high transfection efficiency. However, PEI toxicity is a significant concern. The mechanism of cytotoxicity in PEI is poorly understood, but one hypothesis is that PEI aggregates on the cell surface and impairs important membrane functions. Another possibility is that PEI, free or in complexes with DNA, interferes with critical intracellular processes [29–31]. In contrast, chitosan has been reported to have low toxicity and appears to be a biocompatible cationic polymer for non-viral gene delivery [32–34]. Also, as a biogenic tetra-amine, spermine is safe and naturally present in body tissues. Therefore, we assumed that CHI-g-SPE had lower toxicity because of the properties of biocompatible chitosan and spermine. Although cationic polymers with high charge densities have strong cell lytic and toxic properties, a reduction in charge density is reported to result in lower cell toxicity [35]. We found a greater number of low zeta potentials in CHI-g-SPE/DNA complexes (20.7 ± 3.2 mV, at weight ratio 10) than in PEI 25K/DNA complexes (+40.5 ± 3.8 mV, at N/P ratio 10).

3.4. Cell transfection of CHI-g-SPE copolymer

To investigate transfection efficiency of the CHI-g-SPE copolymer, we performed luciferase activity assays *in vitro* as shown in Fig. 4A. Transfection efficiency of the CHI-g-SPE copolymer increased with increasing weight ratio and 4.6-fold higher transfection efficiency was observed for the CHI-g-SPE copolymer (weight ratio 10) than for chitosan (weight ratio 5). The higher transfection efficiency of copolymer has been reported and attributed to higher amine content in the complexes [15]. The higher amine content and the amine composition in the copolymer that is similar to PEI give the complexes a higher buffering capacity that allows them to escape the endosome easily. To further elucidate the mechanism of CHI-g-SPE copolymer transfection, we determined the buffering capacity of the copolymer. A549 cells were treated during transfection with bafilomycin A1, a specific inhibitor of vacuolar type H^+ ATPase. The transfection efficiency of CHI-g-SPE/DNA complexes was drastically decreased after bafilomycin

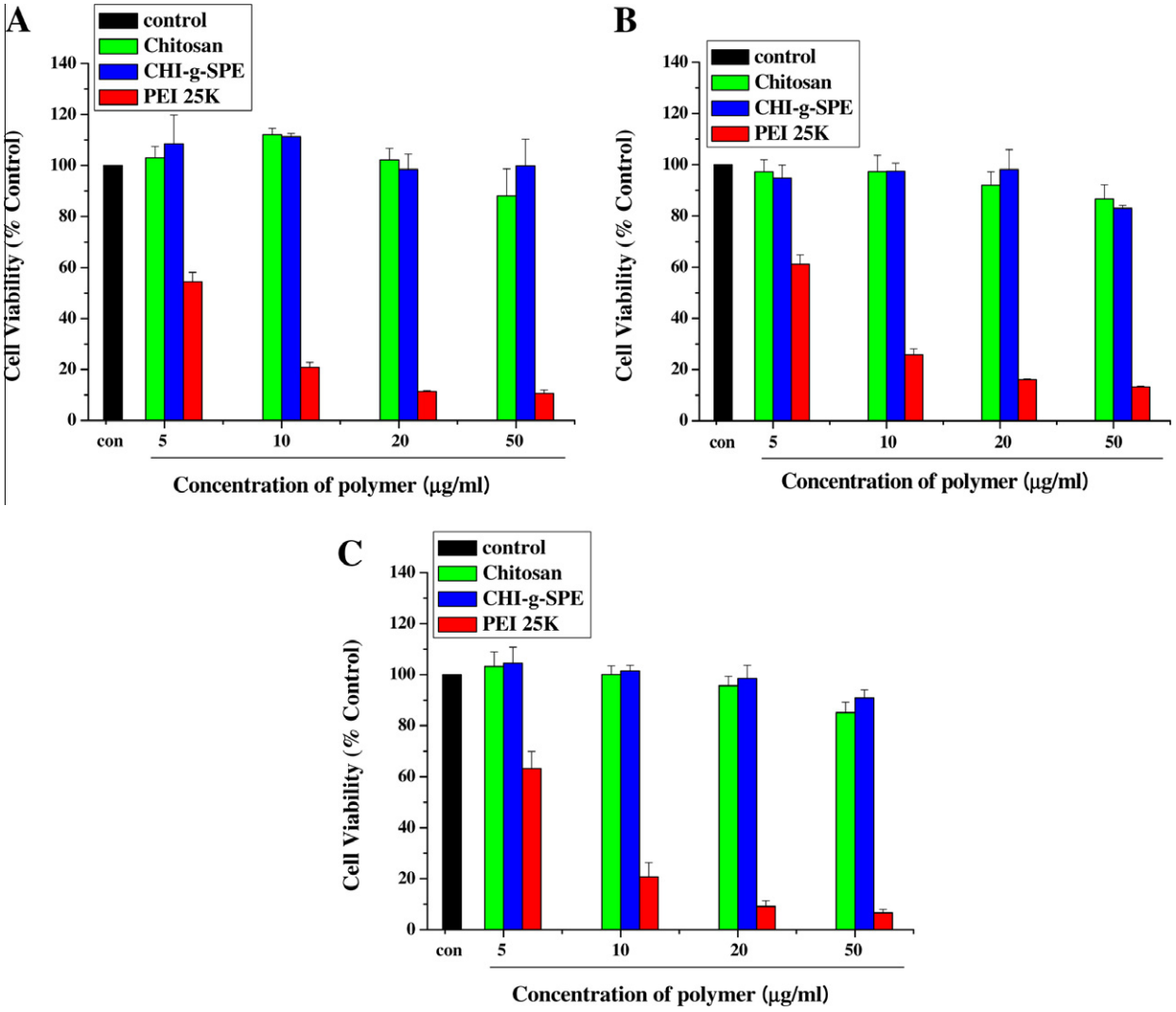

Fig. 3. Cytotoxicity of CHI-g-SPE at various concentrations. (A) A549. (B) WI-38. (C) HepG2 cell lines (mean ± SD, $n = 3$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

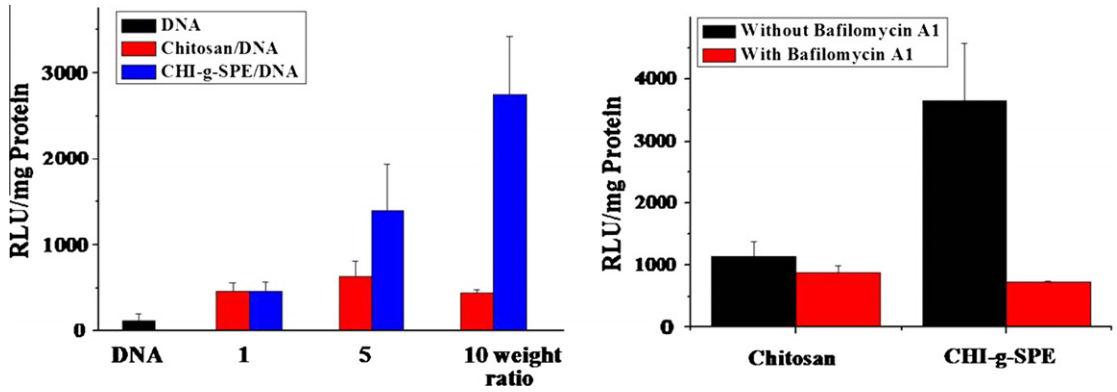

Fig. 4. (A) Transfection efficiency of CHI-g-SPE/DNA complexes at various weight ratios in A549 cells. (B) Effect of bafilomycin A1 on gene transfection. CHI-g-SPE/DNA complexes at weight ratio 10 in A549 cells. Bafilomycin A1 (200 nM) diluted in DMSO was added to the wells. After a 10-min incubation, transfection solutions were added into the wells for 4 h. Cells were incubated in growth medium for 24 h (mean ± SD, $n = 3$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

treatment, as shown in Fig. 4B. The decline in transfection efficiency of CHI-g-SPE copolymer in the presence of bafilomycin suggested that the CHI-g-SPE copolymer had a high buffering capacity. As CHI-g-SPE copolymer has a high buffering capacity, it can escape endosome by a proton sponge effect into the cell cytosol. However, when we assayed the effect of bafilomycin on

the transfection of the chitosan/DNA complexes into the A549 cell line, efficiency was not significantly decreased in the presence of bafilomycin.

3.5. In vivo aerosol delivery of CHI-g-SPE copolymer

Lung cancer is the leading cause of cancer death worldwide, and its mortality and morbidity figures are increasing steadily [36]. Inhalation is one of the most non-invasive approaches to drug delivery. The aerosol delivery of genes provides promises for the treatment of a broad spectrum of pulmonary disorders and offers numerous advantages over more invasive modes of delivery. Therefore, aerosol delivery is a possible lung cancer gene therapy because the anatomical structure and location of the lungs make instant access and a non-invasive approach possible, with a high delivery efficiency that does not affect other organs [37,38]. Chitosan is reported to have mucoadhesive properties [39], and CHI-*g*-SPE showed high transfection efficiency *in vitro*, so CHI-*g*-SPE could be as a novel gene therapeutic polymer.

GFP signal was evaluated to verify the delivery efficiency of CHI-*g*-SPE *in vivo*. As shown in Fig. 5, the GFP signal was evident in the group that inhaled CHI-g-SPE/GFP complexes compared to the other groups (GFP control and CHI/GFP group). Furthermore, histopathology of lungs (Fig. 6) showed one or two layers of pneumocyte type I and less than 10% of pneumocyte type II in the alveolar walls throughout the entire section. No necrosis, degeneration, metaplasia, anaplasia in pneumocytes, atelectasis, or emphysema were detected. Capillary vessels within the alveolar wall were not enlarged and damaged endothelial cells were rarely observed. Neither congestion nor hemorrhage was noticeable. Only a few alveolar macrophages in alveolar wall were observed, which appeared to be normal, and neither infiltration of inflammatory cells nor exudates were detected. In complete blood cells (data not shown) and other major organs, including brain, heart, lung, liver, kidney, spleen, and testis, no noticeable abnormal features were detected (see S1).

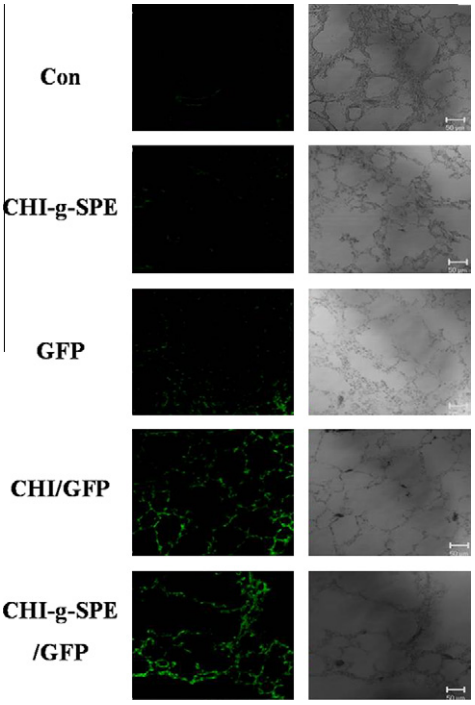

Fig. 5. *In vivo* GFP expression analysis after aerosol administration to lungs. Con, Control; CHI-g-SPE, Chitosan-graft-spermine without GFP; GFP, GFP only; CHI/GFP, Chitosan with GFP; CHI-g-SPE/GFP, Chitosan-graft-spermine with GFP. Magnification: 200×, Scale bar represents 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

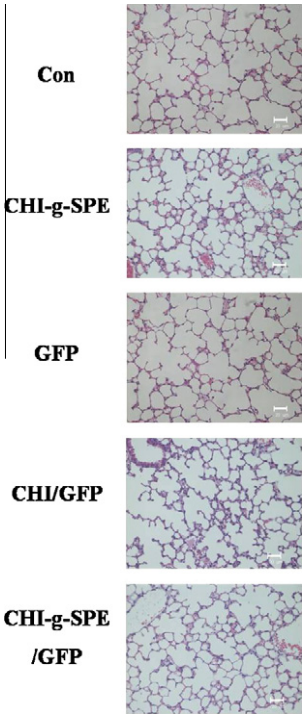

Fig. 6. Lung histopathology. Con, Control; CHI-g-SPE, Chitosan-graft-spermine without GFP; GFP, GFP only; CHI/GFP, Chitosan with GFP; CHI-g-SPE/GFP, Chitosan-graft-spermine with GFP. Staining: hematoxylin & eosin; Magnification: 200×, Scale bar represents 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Conclusions

We have successfully prepared and evaluated a new CHI-g-SPE copolymer as a new gene carrier. The CHI-g-SPE copolymer showed strong ability to form complexes with DNA and suitable physicochemical properties as a gene delivery system. This copolymer had low cytotoxicity and exhibited enhanced gene transfer efficiency *in vitro* and *in vivo*. Therefore, CHI-g-SPE has the potential to be a safe and efficient gene carrier. We are currently focusing on more comprehensive studies to characterize CHI-g-SPE, especially in lung cancer therapy via aerosol administration.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejpb.2010.09.014.

References

[1] T.G. Park, J.H. Jeong, S.W. Kim, Current status of polymeric gene delivery systems, Adv. Drug Deliv. Rev. 58 (2006) 467–486.
[2] C. Louise, Nonviral vectors, Methods Mol. Biol. 333 (2006) 201–226.
[3] A. Rolland, Gene medicines: the end of the beginning?, Adv Drug Deliv. Rev. 57 (2005) 669–673.
[4] T. Merdan, J. Kopecek, T. Kissel, Prospects for cationic polymers in gene and oligonucleotide therapy against cancer, Adv. Drug Deliv. Rev. 54 (2002) 715–758.
[5] M. L Edelstein, M.R. Abedi, J. Wixon, R.M. Edelstein, Gene therapy clinical trials worldwide 1989–2004 – an overview, J. Gene Med. 6 (2004) 597–602.
[6] I.M. Verma, N. Somia, Gene therapy – promises, problems and prospects, Nature 389 (1997) 239–242.
[7] W.F. Anderson, Human gene therapy, Nature 392 (1998) 25–30.
[8] M.D. Brown, A.G. Schatzlein, I.F. Uchegbu, Gene delivery with synthetic (non viral) carriers, Int. J. Pharm. 229 (2001) 1–21.
[9] M. Köping-Höggård, I. Tubulekas, H. Guan, K. Edwards, M. Nilsson, K.M. Varum, P. Artursson, Chitosan as a nonviral gene delivery system. Structure–property relationships and characteristics compared with polyethylenimine *in vitro* and after lung administration *in vivo*, Gene Ther. 8 (2001) 1108–1121.
[10] M. Köping-Höggård, K.M. Varum, M. Issa, S. Danielsen, B.E. Christensen, B.T. Stokke, P. Artursson, Improved chitosan-mediated gene delivery based on easily dissociated chitosan polyplexes of highly defined chitosan oligomers, Gene Ther. 11 (2004) 1441–1452.
[11] T.H. Kim, J.E. Ihm, Y.J. Choi, J.W. Nah, C.S. Cho, Efficient gene delivery by urocanic acid-modified chitosan, J. Control. Release 93 (2003) 389–402.
[12] H.L. Jiang, Y.K. Kim, R. Arote, J.W. Nah, M.H. Cho, Y.J. Choi, T. Akaike, C. S Cho, Chitosan-graft-polyethylenimine as a gene carrier, J. Control. Release 117 (2007) 273–280.
[13] H.L. Jiang, J.T. Kwon, Y.K. Kim, E.M. Kim, R. Arote, H.J. Jeong, J.W. Nah, Y.J. Choi, T. Akaike, M.H. Cho, C.S. Cho, Galactosylated chitosan-graft-polyethylenimine as a gene carrier for hepatocyte targeting, Gene Ther. 14 (2007) 1389–1398.
[14] B. Ghosn, S.P. Kasturi, K. Roy, Enhancing polysaccharide-mediated delivery of nucleic acids through functionalization with secondary and tertiary amines, Curr. Top. Med. Chem. 8 (2008) 331–340.
[15] K. Wong, G. Sun, X. Zhang, H. Dai, Y. Liu, C. He, K.W. Leong, PEI-g-chitosan a novel gene delivery system with transfection efficiency comparable to polyethylenimine in vitro and after liver administration in vivo, Bioconjug. Chem. 17 (2006) 152–158.
[16] J.C. Allen, Biochemistry of the polyamines, Cell. Biochem. Funct. 1 (1983) 131–140.
[17] D. Jere, J.E. Kim, R. Arote, H.L. Jiang, Y.K. Kim, Y.J. Choi, C.H. Yun, M.H. Cho, C.S. Cho, Akt1 silencing efficiencies in lung cancer cells by sh/si/ssiRNA transfection using a reductable polyspermine carrier, Biomaterials 30 (2009) 1635–1647.
[18] T. Azzam, H. Eliyabu, A. Makovitzki, M. Linial, A.J. Domb, Hydrophobized dextran-spermine conjugate as potential vector for in vitro gene transfection, J. Control. Release 96 (2004) 309–323.
[19] T. Azzam, H. Eliyahu, L. Shapira, M. Linial, Y. Barenholz, A.J. Domb, Polysaccharide-oligoamine based conjugates for gene delivery, J. Med. Chem. 45 (2002) 1817–1824.
[20] H. Eliyahu, A. Makovitzki, T. Azzam, A. Zlotkin, A. Joseph, D. Gazit, Y. Barenholz, A.J. Domb, Novel dextran-spermine conjugates as transfecting agents: comparing water-soluble and micellar polymers, Gene Ther. 12 (2005) 494–503.
[21] H. Hosseinkhani, T. Azzam, Y. Tabata, A.J. Domb, Dextran-spermine polycation: an efficient nonviral vector for in vitro and in vivo gene transfection, Gene Ther. 11 (2004) 194–203.
[22] I.M. Vold, B.E. Christensen, Periodate oxidation of chitosans with different chemical compositions, Carbohydr. Res. 340 (2005) 679–684.
[23] H.L. Jiang, C.X. Xu, Y.K. Kim, R. Arote, D. Jere, H.T. Lim, M.H. Cho, C.S. Cho, The suppression of lung tumorigenesis by aerosol-delivered folate-chitosan-graft-polyethylenimine/Akt1 shRNA complexes through the Akt signaling pathway, Biomaterials 30 (2009) 5844–5852.
[24] N. Kubota, N. Tatsumoto, T. Sano, K. Toya, A simple preparation of half N-acetylated chitosan highly soluble in water and aqueous organic solvents, Carbohydr. Res. 324 (2000) 268–274.
[25] P. Erbacher, S. Zou, T. Bettinger, A.M. Steffan, J.S. Remy, Chitosan-based vector/DNA complexes for gene delivery: biophysical characteristics and transfection ability, Pharm. Res. 15 (1998) 1332–1339.
[26] K. Kunath, A. von Harpe, D. Fischer, H. Petersen, U. Bickel, K. Voigt, T. Kissel, Low-molecular-weight polyethylenimine as a non-viral vector for DNA delivery: comparison of physicochemical properties, transfection efficiency and in vivo distribution with high-molecular-weight polyethylenimine, J. Control. Release 89 (2003) 113–125.
[27] H. Petersen, P.M. Fechner, A.L. Martin, K. Kunath, S. Stolnik, C.J. Roberts, D. Fischer, M.C. Davies, T. Kissel, Polyethylenimine-graft-poly(ethylene glycol) copolymers: influence of copolymer block structure on DNA complexation and biological activities as gene delivery system, Bioconjug. Chem. 13 (2002) 845–854.
[28] M.R. Park, K.O. Han, I.K. Han, M.H. Cho, J.W. Nah, Y.J. Choi, C.S. Cho, Degradable polyethylenimine-alt-poly(ethylene glycol) copolymers as novel gene carriers, J. Control. Release 105 (2005) 367–380.
[29] D. Fischer, T. Bieber, Y. Li, H.P. Elsasser, T. Kissel, A novel non-viral vector for DNA delivery based on low molecular weight, branched polyethylenimine: effect of molecular weight on transfection efficiency and cytotoxicity, Pharm. Res. 16 (1999) 1273–1279.
[30] S. Li, W. Dong, Y. Zong, W. Yin, G. Jin, Q. Hu, X. Huang, W. Jiang, Z.C. Hua, Polyethylenimine-complexed plasmid particles targeting focal adhesion kinase function as melanoma tumor therapeutics, Mol. Ther. 15 (2007) 515–523.
[31] S.M. Moghimi, P. Symonds, J.C. Murray, A.C. Hunter, G. Debska, A. Szewczyk, A two-stage poly(ethylenimine)-mediated cytotoxicity: implications for gene transfer/therapy, Mol. Ther. 11 (2005) 990–995.
[32] H.L. Jiang, Y.K. Kim, R. Arote, D. Jere, J.S. Quan, J.H. Yu, Y.J. Choi, J.W. Nah, M.H. Cho, C.S. Cho, Mannosylated chitosan-graft-polyethylenimine as a gene carrier for Raw 264.7 cell targeting, Int. J. Pharm. 375 (2009) 133–139.
[33] H.L. Jiang, J.T. Kwon, E.M. Kim, Y.K. Kim, R. Arote, D. Jere, H.J. Jeong, M.K. Jang, J.W. Nah, C.X. Xu, I.K. Park, M.H. Cho, C.S. Cho, Galactosylated poly(ethylene glycol)-chitosan-graft-polyethylenimine as a gene carrier for hepatocyte-targeting, J. Control. Release 131 (2008) 150–157.
[34] S.T. Kim, C.K. Kim, Water-soluble chitosan-based antisense oligodeoxynucleotide of interleukin-5 for treatment of allergic rhinitis, Biomaterials 28 (2007) 3360–3368.
[35] N.G. Schipper, K.M. Varum, P. Artursson, Chitosans as absorption enhancers for poorly absorbable drugs. 1: Influence of molecular weight and degree of acetylation on drug transport across human intestinal epithelial (Caco-2) cells, Pharm. Res. 13 (1996) 1686–1692.
[36] V. Donati, L. Boldrini, M. Dell'Omodarme, M.C. Prati, P. Faviana, T. Camacci, M. Lucchi, A. Mussi, M. Santoro, F. Basolo, G. Fontanini, Osteopontin expression and prognostic significance in non-small cell lung cancer, Clin. Cancer Res. 11 (2005) 6459–6465.
[37] A. Gautam, J.C. Waldrep, C.L. Densmore, Aerosol gene therapy, Mol. Biotechnol. 23 (2003) 51–60.
[38] C.L. Densmore, Advances in noninvasive pulmonary gene therapy, Curr. Drug Deliv. 3 (2006) 55–63.
[39] H.O. Alpar, S. Somavarapu, K.N. Atuah, V.W. Bramwell, Biodegradable mucoadhesive particulates for nasal and pulmonary antigen and DNA delivery, Adv. Drug Deliv. Rev. 57 (2005) 411–430.