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Preparation and characterization of nonaarginine-modified chitosan nanoparticles for siRNA delivery

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ABSTRACT

Chitosan-based nanoparticles have been widely used as a carrier for gene delivery due to their low toxicity and the positively charged amino groups in chitosan. In this study, we hypothesized that introduction of nonaarginine to chitosan could improve its ability to form a complex with siRNA, as well as enhance the cellular uptake and transfection efficiency of chitosan-based nanoparticles. To this end, a peptide with nine repeating arginine residues was chemically coupled to the chitosan backbone, and various characteristics of nonaarginine–chitosan/siRNA nanoparticles were investigated. The mean diameter and zeta potential of the nonaarginine–chitosan/siRNA nanoparticles were dependent on the amount of nonaarginine conjugated to chitosan. Nonaarginine-modified chitosan/siRNA nanoparticles demonstrated enhanced cellular association and transfection efficiency *in vitro*, while maintaining a low level of cytotoxicity. In conclusion, nonaarginine-modified chitosan should be considered a potential carrier for various gene delivery applications.

1. Introduction

RNA interference (RNAi) has gained a great deal of attention in recent years, largely due to its potential as a new class of therapeutics for the treatment of various diseases (Pecot, Calin, Coleman, Lopez-Berestein, & Sood, 2011; Shankar, Manjunath, & Lieberman, 2005). During RNAi processing, double stranded small interfering RNA (siRNA) degrades target mRNA with the help of RNA-induced silencing complex (RISC), subsequently inhibiting protein synthesis of the associated target gene (Plasterk, 2002). RNAi has prospective applications in the treatment of a variety of diseases including cancer, viral infection, and genetic disorders (Fougerolles, Vornlocher, Maraganore, & Lieberman, 2007; Kumar et al., 2008; Santel et al., 2006). However, the delivery of siRNA into the body has raised several issues, including rapid degradation, low intracellular uptake, and limited stability in the blood stream. To overcome these limitations, it remains essential to identify a suitable delivery vehicle that increases the stability of siRNA in the presence of nucleases and enhances intracellular uptake (Schaffert & Wagner, 2008).

Typical gene delivery systems include both viral and non-viral vectors. Viral vectors have a high transfection efficiency and are used extensively in numerous gene delivery applications. However, they have many safety concerns compared with non-viral vectors, such as the induction of inflammatory responses, undesirable immune responses, and oncogenic effects (Lundstrom, 2003). Thus, non-viral vectors have been increasingly explored as an alternative to viral vectors, due to their ease of synthesis and chemical modification, low immune response, and unrestricted plasmid size (Li & Huang, 2007; Park, Jeong, & Kim, 2006).

Chitosan, the linear and partly acetylated (1–4)-2-amino-2-deoxy-β-D-glucan, is obtained from chitin, the second most abundant natural polymer (Muzzarelli et al., 2012). Chitosan has been widely used in many drug delivery applications, especially in gene delivery applications (Jayakumar, Menon, Manzoor, Nair, & Tamura, 2010; Sashiwa & Aiba, 2004), owing to its positively charged amino groups, which allow for electrostatic interactions with negatively charged nucleic acids to form stable complexes (Lee, 2007; Mao, Sun, & Kissel, 2010). Indeed, chitosan/DNA nanoparticles have been prepared and their transfection efficiency has been tested in different cell types, including mesenchymal stem cells (Corsi, Chellat, Yahia, & Fernandes, 2003; Leong et al., 1998). Many studies have also shown effective silencing of siRNA in the presence of chitosan *in vitro* and *in vivo* (Howard et al., 2006; Ji et al., 2009; Katas & Alpar, 2006; Lee et al., 2009; Nielsen et al., 2010; Noh et al., 2010).

In this study, we hypothesized that introduction of nonaarginine residues to chitosan could improve its ability to form a complex with negatively charged siRNA, thereby enhancing the cellular association and gene silencing efficiency of chitosan/siRNA nanoparticles. Although oligoarginine is often used as a model non-viral gene delivery carrier (Kim et al., 2006, 2010), the use of highly

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charged cationic peptides may cause cytotoxicity. We anticipated that the use of nonaarginine-modified chitosan would enhance the stability of the chitosan/siRNA nanoparticles due to an increase in positive charge and enhance cellular uptake and transfection efficiency by action of the oligopeptide, which have been widely utilized as a cell-penetrating peptide to enhance the efficiency of intracellular delivery (Futaki et al., 2001; Liu et al., 2010; Saw, Ko, & Jon, 2010; Zhang et al., 2011). Thus, a peptide with nine repeating arginine (R_9) units was chemically coupled to the chitosan backbone and various characteristics of nonaarginine–chitosan/siRNA nanoparticles were investigated *in vitro*.

2. Materials and methods

2.1. Materials

Chitosan glutamate (MW = 470,000 g/mol) was purchased from FMC Biopolymer and used without further purification. Nonaarginine (R_9) was purchased from GenScript. 2-(N-morpholino) ethanesulfonic acid (MES) and 1-ethyl-3-(dimethylaminopropyl) carbodiimide (EDC) was purchased from Sigma–Aldrich. N-hydroxysulfosuccinimide (sulfo-NHS) was purchased from Pierce. Dulbecco's modified Eagle's medium (DMEM) was supplied from Gibco. Diethylpyrocarbonate (DEPC)-treated water was obtained from Samchulli Pharmaceutical and siRNA was purchased from Bioneer. Dimethyl sulfoxide (DMSO), potassium bromide (KBr), acetic acid-d_4 (CD_3COOD), deuterium oxide (D_2O), and 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Sigma–Aldrich and used without further purification.

2.2. Synthesis of nonaarginine–chitosan

Nonaarginine-modified chitosan was synthesized by covalent conjugation between the carboxylate of nonaarginine and the amino groups of the chitosan backbone using carbodiimide chemistry. In brief, chitosan glutamate was dissolved in an MES buffer solution (pH = 5.5), and nonaarginine (R_9) was added to the chitosan solution in the presence of EDC and sulfo-NHS (EDC:sulfo-NHS = 1:2, mole ratio). The degree of substitution was varied by changing the mole ratio between nonaarginine and chitosan. The conjugation reaction was carried out for 24 h at room temperature. The resulting nonaarginine-modified chitosan was purified by extensive dialysis against deionized water using dialysis membranes (molecular weight cut-off = 3500, Spectra/Por) for four days at room temperature to remove unreacted chemicals and byproducts, followed by activated charcoal treatment, sterilization using a 0.22-μm filter, and lyophilization (yield = approximately 80%).

2.3. Characterization of nonaarginine–chitosan

Chemical conjugation between nonaarginine and chitosan was confirmed by FT-IR spectroscopy (MAGNa-IR 760 E.S.P.). Briefly, chitosan samples were mixed with dry potassium bromide, and the mixture was ground into a fine powder using an agate mortar before being compressed into discs. Each disc was scanned at a resolution of 4 cm^{-1} with a rate of 4 mm/s over a wavenumber region of 800–4000 cm^{-1}. ^1H NMR spectra were recorded using a Varian Mercury Plus 300 MHz spectrometer at 70 °C. Samples were dissolved in 1% CD_3COOD/D_2O ([sample] = 10 mg/ml). The degree of substitution of nonaarginine conjugated to the chitosan backbone was calculated from elemental analysis (Thermo Finnigan Flash EA 1112).

2.4. Preparation of nonaarginine–chitosan/siRNA nanoparticles

Nonaarginine–chitosan was dissolved in sodium acetate buffer (0.1 M sodium acetate, pH 5.5) and added to a solution of siRNA in DEPC-treated water containing polyguluronate, which was used to prepare stable nanoparticles as previously described (Lee et al., 2009). The weight ratio of chitosan to siRNA ranged from 20 to 100. The nanoparticles were incubated at room temperature for 30 min before use or further analysis.

2.5. Gel retardation assays

Binding of siRNAs to nonaarginine–chitosan was confirmed by 3% agarose gel electrophoresis. Briefly, samples were loaded onto gels, and electrophoresis was carried out at 100 V for 30 min with TBE buffer (4.45 mM Tris–base, 1 mM sodium EDTA, 4.45 mM boric acid, pH 8.3). The siRNA bands were visualized with ethidium bromide using a UV transilluminator at a wavelength of 365 nm.

2.6. Measurements of particle size and zeta potential

The mean diameter and zeta potential of nanoparticles were determined at 25 °C using a Nano ZS Zetasizer (Malvern Instruments). Each experiment was carried out in triplicate and the average results were reported. The morphology of nanoparticles was observed by atomic force microscopy (AFM). Samples were loaded on a mica surface and purged with nitrogen. The microscopic images were obtained using a PSIA XE-100 AFM system in a non-contact mode.

2.7. Stability of nonaarginine–chitosan/siRNA nanoparticles in serum

Naked siRNA and chitosan nanoparticles loaded with siRNA were incubated at 37 °C in medium containing 50% fetal bovine serum (FBS). At predetermined time points (range: 0–24 h), the nanoparticles were collected from the medium and stored at −20 °C until gel electrophoresis was performed. A phenol/chloroform extraction method was used to detach the siRNA from the chitosan nanoparticles prior to gel electrophoresis. The integrity of the siRNA was subsequently analyzed by 3% agarose gel electrophoresis.

2.8. Cytotoxicity assay

In order to test the toxicity of nonaarginine–chitosan nanoparticles, HeLa cells were plated in 96-well tissue culture plates at a density of 5×10^3 cells/well in DMEM. After incubation with nanoparticles at 37 °C for 24 h, 10 μl of MTT was added to each well and subsequently incubated for 4 h to allow the formation of formazan crystals, which are insoluble in cell culture media. Unreduced MTT and medium were then removed, and 100 μl of DMSO was added to each well to dissolve the MTT formazan crystals. Lastly, MTT assay plates were incubated at room temperature for 30 min, followed by measurement of the absorbance of formazan products at 540 nm using a spectrophotometer (Molecular Devices).

2.9. Cellular association

The cellular association of nonaarginine–chitosan/siRNA nanoparticles was investigated using HeLa cells. Cells were plated on 24-well tissue culture plates (5×10^4 cells/well) in DMEM containing 10% FBS and 1% penicillin. Cells were then treated with 50 μl of chitosan nanoparticles containing 200 pmol FITC-conjugated siRNA dispersed in medium without serum. After 4 h of transfection, the medium was removed and replaced with fresh medium containing serum. The cells were then incubated for an

additional 20 h at 37 °C under a 5% CO_2 atmosphere, fixed with 2% formaldehyde, and analyzed by flow cytometry (Becton Dickinson FACS Caliber).

2.10. Gene silencing

To assess gene silencing by the investigated nonaarginine–chitosan/siRNA nanoparticles, HeLa cells were seeded in 12-well tissue culture plates at a density of 1×10^5 cells/well in DMEM containing 10% FBS and 1% penicillin 24 h prior to transfection. On the day of transfection, the medium was removed and replaced with 2 ml fresh media without serum. The cells were then treated with 100 μl of nanoparticles containing 200 pmol siRNA. Four hours after transfection, the medium was replaced with DMEM containing serum, after which cells were incubated at 37 °C under a 5% CO_2 atmosphere for 44 h. The efficiency of gene silencing was evaluated by real-time PCR analysis. Briefly, RNA was isolated from cells using an RNAiso kit (Takara). The mRNA expression of cyclophilin B (CypB) was quantitatively evaluated by applying real-time SYBR Green PCR technology with an ABI PRISM 7500 Real-Time PCR System (Applied Biosystems). The relative level of gene expression was determined by comparison with that of the reference gene GAPDH. The sequences of the primers used were as follows: mouse cyclophilin B, 5′-TGGAGAGCACCAAGACAGACA-3′ and 5′-GTCGACAATGATGACATCCTTCA-3′; mouse GAPDH, 5′-GGCAAATTCAACGGCACAGT-3′ and 5′-GGGTCTCGCTCCTG-GAAGAT-3′.

2.11. Statistical analysis

All data are presented as means ± standard deviations. Statistical analyses were performed using Student's *t*-test. *P*-values < 0.05 were considered statistically significant.

3. Results and discussion

3.1. Characterization of nonaarginine-modified chitosan

Nonaarginine (R_9)-modified chitosan was prepared by covalent conjugation between the carboxylate of nonaarginine and the amine of chitosan using carbodiimide chemistry. An EDC-mediated reaction can be enhanced through the formation of sulfo-NHS ester intermediate, as it maintains stability in aqueous solution against hydrolysis longer than the active ester formed from the reaction of EDC alone (Hermanson, 1996). The degree of substitution (DS), defined as the molar ratio between nonaarginine (substituent) and 100 glucosamines (repeating units in chitosan), was determined from elemental analysis (DS 0.06 and DS 0.6), and non-modified chitosan was used as a control (DS 0). Covalent conjugation of nonaarginine and chitosan was confirmed by FT-IR and NMR. Chitosan exhibited characteristic bands of the NH_2 scissoring vibration at 1633 cm^{-1}, carbonyl asymmetric stretching vibration at 1550 cm^{-1}, and C—O stretching vibrations of the pyranose ring at 1037–1076 cm^{-1}. C—C—N asymmetric bending was also observed at 1153 cm^{-1} (Fig. 1). A peak corresponding to the amide bond at 1535 cm^{-1} was clearly observed for nonaarginine-modified chitosan, indicating the successful formation of a covalent linkage between the carboxylate of nonaarginine units and the amino groups of chitosan (Liu et al., 2004; Xiao, Wan, Zhao, Liu, & Zhang, 2011). Non-modified chitosan showed the chemical shifts at $\delta = 5.23$ (H1), $\delta = 3.54$ (H2), $\delta = 3.70$–4.50 (H3, H4, H5, H6), and $\delta = 2.40$ ppm ($NHCOCH_3$) in ^1H NMR spectra (Fig. 2). After conjugation, the new peaks at $\delta = 1.47$–1.55 ppm were observed in nonaarginine-modified chitosan, indicating that nonaarginine was successfully coupled to the chitosan chain.

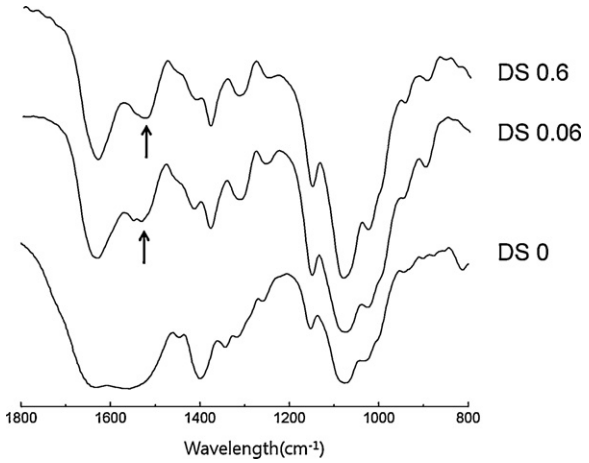

Fig. 1. FT-IR spectra of nonaarginine–chitosan with various degrees of substitution (DS). The arrows indicate newly formed amide bonds (1535 cm^{-1}) in nonaarginine–chitosan.

3.2. Interactions between siRNA and nonaarginine–chitosan

The formation of nonaarginine–chitosan/siRNA complexes was confirmed by gel electrophoresis. Nonaarginine–chitosan/siRNA nanoparticles were prepared at a weight ratio of 20–100. Movement of siRNA was substantially retarded when either chitosan or nonaarginine–chitosan was used for complex formation compared with control siRNA. However, use of nonaarginine–chitosan was much more effective in complex formation with siRNA at weight ratios greater than 40 (Fig. 3). Indeed, as the degree of substitution with nonaarginine became higher, more efficient complex formation was observed, which was likely due to the increased positive charges of the nonaarginine-modified chitosan.

3.3. Size and surface charge of nonaarginine–chitosan/siRNA nanoparticles

The mean diameter and zeta potential of the nanoparticles were determined using a zetasizer. The mean diameter of the nonaarginine–chitosan/siRNA nanoparticles ranged from 210 to

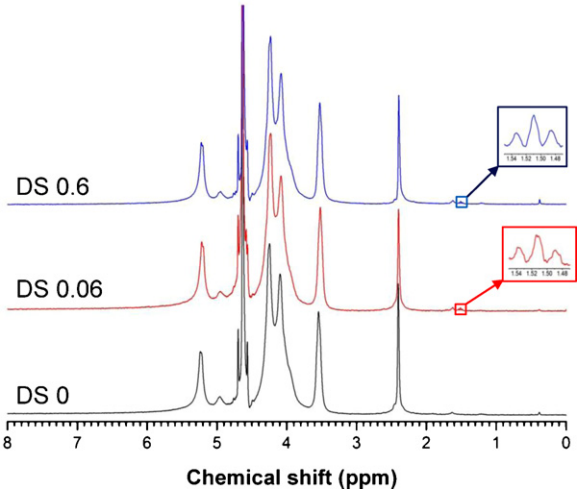

Fig. 2. ^1H NMR spectra of nonaarginine–chitosan with various degrees of substitution (DS).

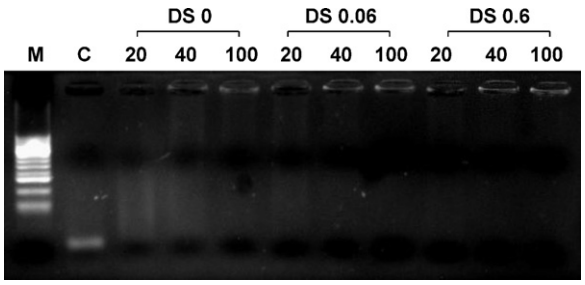

Fig. 3. Gel retardation assay of nonaarginine–chitosan/siRNA nanoparticles. C and M represent naked siRNA and DNA markers, respectively. Lanes marked with 20, 40, and 100 represent the weight ratio of nonaarginine–chitosan to siRNA, respectively.

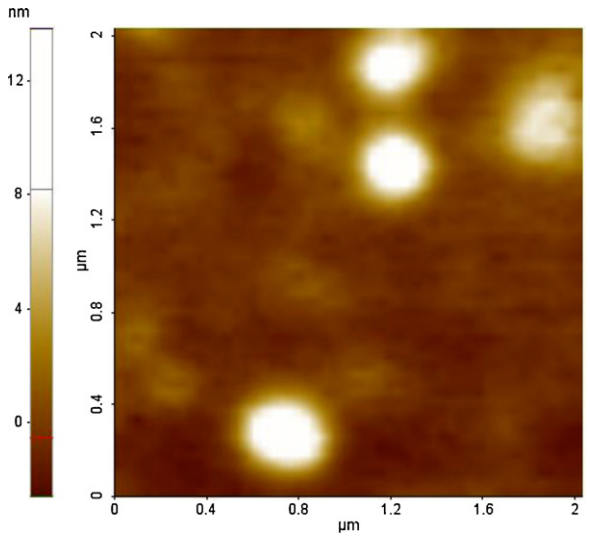

Fig. 4. Atomic force microscopic image of nonaarginine–chitosan/siRNA nanoparticles (DS = 0.06).

600 nm, depending on the DS of nonaarginine and weight ratio between chitosan and siRNA (Table 1). The mean diameter of the nanoparticles was increased by increasing either the DS of nonaarginine or the weight ratio of chitosan to siRNA. The zeta potential of the nanoparticles varied from +7.5 to +25 mV, which was dependent on the DS of nonaarginine and the chitosan/siRNA weight ratio. Zeta potentials were substantially improved by increasing either the DS of nonaarginine or the chitosan/siRNA weight ratio. The size and surface charges of nanoparticles are critical parameters for cellular uptake. Furthermore, positively charged surfaces facilitate adhesion of the nanoparticles to negatively charged cellular membranes, which may improve their potential as drug delivery carriers (He, Hu, Yin, Tang, & Yin, 2010). Based on the results described above, nanoparticles prepared at a weight ratio of 40

Table 1
Size and zeta potential of nonaarginine–chitosan/siRNA nanoparticles ($n = 3$).

DS	Weight ratio (chitosan/siRNA)	Size (nm)	PDI	Zeta potential (mV)
0	20	213 ± 17	0.104	7.5 ± 1.1
	40	249 ± 10	0.285	13.3 ± 0.4
	100	445 ± 22	0.231	18.0 ± 1.8
0.06	20	242 ± 10	0.176	9.4 ± 1.6
	40	329 ± 18	0.214	14.5 ± 0.6
	100	519 ± 20	0.206	22.8 ± 0.8
0.6	20	309 ± 5	0.18	13.9 ± 0.9
	40	338 ± 8	0.223	17.5 ± 1.9
	100	603 ± 12	0.262	25.4 ± 1.8

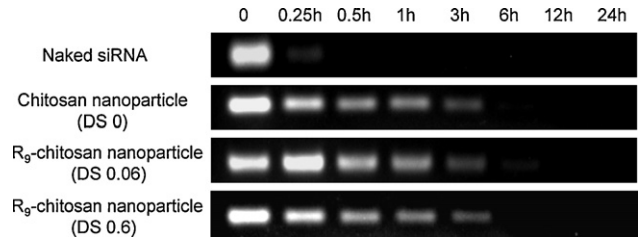

Fig. 5. Serum stability of nonaarginine–chitosan/siRNA nanoparticles incubated in 50% serum-containing medium at 37 °C.

were used to further investigate cellular association and gene silencing. The representative image of nanoparticles was obtained by AFM, suggesting a round shape of nonaarginine–chitosan/siRNA nanoparticles (Fig. 4).

3.4. Stability of nonaarginine–chitosan/siRNA nanoparticles in serum

We next tested the stability of nonaarginine–chitosan nanoparticles under physiological conditions. Briefly, the serum stability of naked siRNA and nonaarginine–chitosan/siRNA nanoparticles was tested in 50% serum-containing medium for 24 h. Rapid degradation of naked siRNA was observed after 15 min of incubation (Fig. 5). On the other hand, the degradation of siRNA protected by nonaarginine–chitosan proceeded much more slowly in the presence of serum, suggesting the potential of these nanoparticles for improving the gene transfection efficiency in the body.

3.5. Cytotoxicity

The viability of cells treated with chitosan-based nanoparticles was evaluated by the MTT assay using HeLa cells and normalized to non-treated cells as a control. The nonaarginine–chitosan/siRNA nanoparticles exhibited excellent cell viability compared with cells treated with liposome and nonaarginine. This may have been due to the inherently lower toxicity of chitosan and low DS of nonaarginine in the chitosan. However, Lipofectamine and nonaarginine resulted in a substantial loss of cell viability (Fig. 6). Consistent with this observation, although Lipofectamine is widely used as a non-viral gene delivery carrier, significant cytotoxicity limits its potential for *in vivo* applications (Lv, Zhang, Wang, Cui, & Yan, 2006).

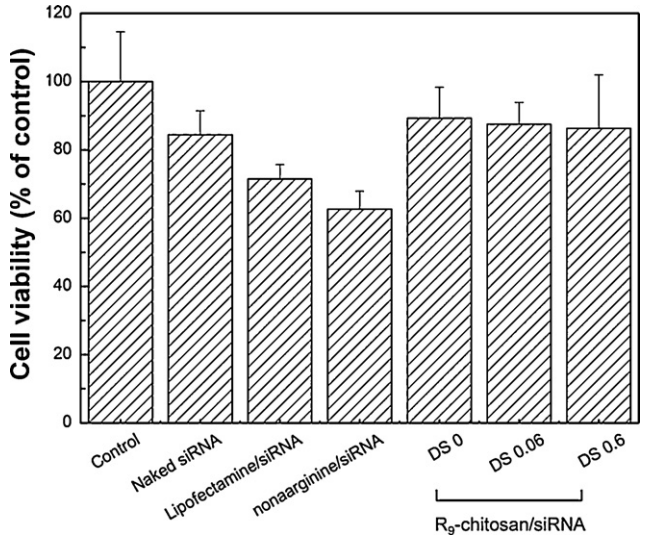

Fig. 6. Cytotoxicity of nanoparticles tested with HeLa cells ($n = 6$).

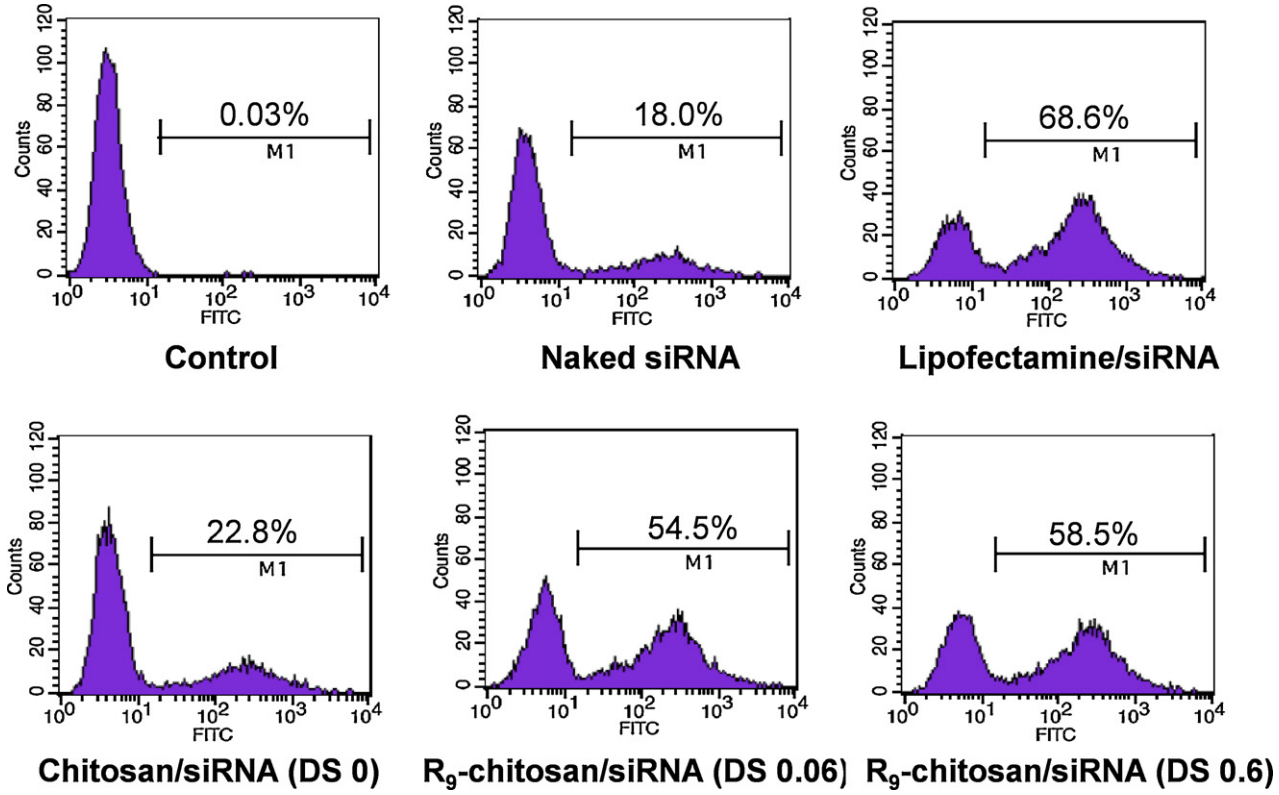

Fig. 7. Cellular association of nonaarginine–chitosan/siRNA nanoparticles determined by flow cytometry ($n = 3$).

3.6. Cellular association

The cellular association of nonaarginine–chitosan/siRNA nanoparticles was tested using HeLa cells. The cellular uptake efficiency of chitosan/siRNA nanoparticles was increased when nonaarginine–chitosan was used (Fig. 7). Likewise, the enhanced positive surface charge as well as increased stability of nanoparticles with introduction of nonaarginine in chitosan may have resulted in improved cellular association of the nanoparticles. Generally, naked siRNA is not substantially taken up by cells due to the electrostatic repulsion induced by interaction with the negatively charged cell membrane. However, cationic complexes significantly enhance the cellular uptake and nonaarginine–chitosan greatly improves the cellular association of nanoparticles compared with non-modified chitosan.

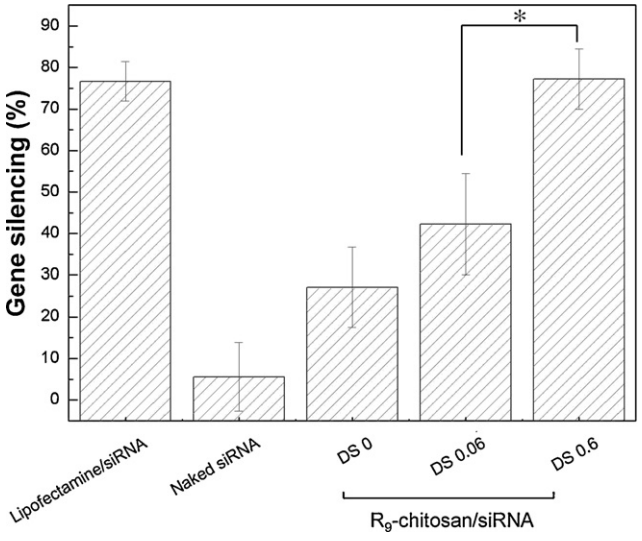

Fig. 8. Quantitative analysis of the gene silencing efficiency of nonaarginine–chitosan/siRNA nanoparticles determined by real-time PCR ($n = 5$). HeLa cells were treated with nonaarginine–chitosan/siCypB nanoparticles and the gene silencing efficiency was normalized to GAPDH expression ($^*P < 0.05$).

3.7. Gene silencing

Quantitative analysis of gene silencing was carried out with HeLa cells after treatment with nanoparticles containing siCypB using real-time PCR (Fig. 8). The gene silencing efficiency was increased in parallel with an increase in DS. Furthermore, the gene silencing efficiency of nonaarginine–chitosan/siRNA nanoparticles (DS 0.6) was comparable to that of Lipofectamine, which was used as a positive control (approximately 80%). This may have been due to the increased positive charge of the nonaarginine–chitosan/siRNA nanoparticles as well as the effect of nonaarginine as a cell-penetrating peptide. A negligible gene silencing effect was observed for naked siRNA.

4. Conclusions

In the present study, we demonstrated that oligoarginine-modified chitosan/siRNA nanoparticles are useful for delivering siRNA *in vitro*. Oligoarginine-modified chitosan was synthesized by chemical conjugation of nonaarginine to the chitosan backbone and used to prepare nanoparticles by charge complex formation with siRNA. The characteristics of the nanoparticles were regulated by changing either the degree of substitution of nonaarginine or the amount of nonaarginine–chitosan added to form the nanoparticles. The cellular association and gene silencing efficiency of nonaarginine–chitosan/siRNA nanoparticles were remarkably improved *in vitro* compared with non-modified chitosan while maintaining a low level of cytotoxicity. This approach may serve

as the basis for developing novel polysaccharide-based carriers for gene therapy.

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