Hyaluronic Acid–Polyethyleneimine Conjugate for Target Specific Intracellular Delivery of siRNA

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INTRODUCTION

A small interfering RNA (siRNA) is a short double-stranded RNA that contains 21–23 nucleic acids with 19 duplexed region.1,2 It shows a specific and effective gene silencing activity by the sequence-specific down-regulation of a complementary mes-
senger RNA. There have been many reports to use siRNA as a potential therapeutic agent for the treatment of numerous diseases including cancer and other diseases from genetic disorder or viral infection. However, the efficiency of gene silencing by siRNA is known to be very low because of its poor intracellular uptake and rapid enzymatic degradation in the body. Accordingly, siRNA is often complexed with cationic polymers facilitating the intracellular delivery as well as enhancing the stability from enzymatic attack. A variety of cationic polymers, which can electrostatically bind to siRNA forming a condensed complex nanoparticle, have been explored as siRNA delivery carriers. Among the cationic polymers, polyethylenimine (PEI) has been widely used to condense DNA plasmid, siRNA, and anti-sense oligodeoxynucleotide (ODN). The siRNA complexed with PEI can be readily delivered into cells through the endocytosis avoiding enzymatic degradation by nuclease. However, the inherent cytotoxicity of PEI limited its applications for in vivo gene therapy. Furthermore, siRNA/PEI complex was reported to cause the activation of complements, the coagulation of blood cells, and the self-aggregation with serum proteins, because of the positively charged surface characteristics. To alleviate the problems, various kinds of polyanions have been used to coat siRNA/PEI complex without disrupting its structure.

Recently, hyaluronic acid (HA) has been used as a novel drug carrier for various biopharmaceuticals. HA is a natural linear polysaccharide composed of alternating disaccharide units of β-1,4-interglycosidic linkage. It is the only nonsulfated glycosaminoglycan (GAG) with a molecular weight range from 1000 to 10,000,000 Da. More than 50% of HA in the body is present in skin, lung, intestine, and extracellular matrix (ECM). HA is also abundant in synovial fluid, umbilical cord, and blood. Currently, HA is commercially produced from animal tissues such as cock’s comb and from fermented microbial sources. The fact, that HA molecules from different sources have the same primary structure, explains the molecular basis for its natural biocompatibility. The biological function of HA has been widely investigated for the regulation of cell behaviors. A strong correlation between the presence of HA, and cell migration and proliferation has been demonstrated.

In this work, we developed a novel target specific siRNA delivery system using PEI-HA conjugates. Anti-PGL3-Luc siRNA was used as a model system suppressing the PGL3-Luc gene expression. PEI-HA conjugate was formed via amide bond formation between the carboxyl groups of HA and the amine groups of branched PEI (bPEI). PEI-HA conjugate was analyzed with 1H nuclear magnetic resonance (NMR) and gel permeation chromatography (GPC), and the siRNA/PEI-HA complex was characterized by atomic force microscopy (AFM) and ζ-potential analysis. The cytotoxicity of siRNA/PEI-HA complex was evaluated by MTT assay and compared with that of siRNA/PEI complex. The LYVE-1 receptor mediated endocytosis of siRNA/PEI-HA complex was clearly visualized by confocal microscopy of B16F1 and HEK-293 cells after treatment with fluorescein isothiocyanate (FITC) labeled siRNA/PEI-HA complex. In addition, the target specific gene silencing efficiency of anti-PGL3-Luc siRNA/PEI-HA complex was investigated by measuring the luciferase activity in the B16F1 cells pretransfected by PGL3-Luc vectors with and without free HA in the transfection medium.

**MATERIALS AND METHODS**

**Materials**

Hyaluronic acid (HA) with a molecular weight (M_w) of 130,000 was purchased from Lifecore Co. (Chaska, MN). Branched polyethyleneimine (bPEI) with a M_w of 25,000, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC hydrochloride), phosphate buffered saline (PBS) tablet, phosphate buffer (pH 6.6 at 25°C), methanol and paraformaldehyde were purchased from Sigma-Aldrich (St. Louis, MO). Dimethyl sulfoxide (dimethylsulfoxide (DMSO)) was obtained from Junsei Chemical Co. (Tokyo, Japan), and hydrochloric acid (HCl) and sodium hydroxide (NaOH) from Wako Pure Chemical Industries (Osaka, Japan). 1-Hydroxybenzotriazole monohydrate (HOBr) was purchased from Daejung Chemicals & Metals Co. (Gyeonggi-Do, Korea). B16F1 cells of murine melanoma and HEK-293 cells of human kidney were obtained from Korean Cell Line Bank (Seoul, Korea). Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), and antibiotics (penicillin) were purchased from JBI Co. (Seoul, Korea). MTT assay kit, PGL3-Luc (PGL3 Luciferase) plasmid, lysis solution, and luciferase assay reagents were obtained from Promega (Madison, WI), and Lipofectamine reagent from Invitrogen (Carlsbad, CA). All reagents were used without further purification.

**Small Interfering RNA**

Anti-PGL3-Luc small interfering RNA (siRNA) and FITC labeled siRNA (FITC-siRNA) were purchased from Bioneer Co. (Daejeon, Korea). The sequences of anti-PGL3-Luc siRNA are 5′-UUG UUU
HA–PEI Conjugate for Target Specific siRNA Delivery

**Synthesis and Characterization of PEI-HA Conjugate**

PEI-HA conjugate was synthesized via amide bond formation between the amine groups of branched PEI and the carboxyl groups of HA. The weight ratios of HA to PEI in the reaction solutions were 1:2, 1:5, and 1:20, respectively. The following are detailed description for the synthesis of PEI-HA conjugate with a feeding weight ratio of HA to PEI of 1:20. HA (Mw = 130 K, 20 mg) was dissolved in 10 ml of water. To the HA solution was added 10 ml of branched PEI (Mw = 25 K) aqueous solution at a concentration of 40 mg/ml. Then, the pH of the reaction mixture was adjusted to 6.5 by the addition of 1M HCl. EDC hydrochloride (40.4 mg) and HOBT (28.5 mg) were dissolved in the mixed solvent of 500 μl water and 500 μl DMSO, and then added to the solution containing HA and PEI. After mixing for 24 h, the reaction was stopped by raising the pH of the solution to 7.0 with 1M NaOH. The resulting solution was poured into the prewashed dialysis membrane tube (MWCO of 10 kDa) and dialyzed against a large excess amount of 100 mM NaCl solution for 2 days, 25% ethanol for 1 day and pure water for 1 day. The PEI-HA conjugate was analyzed and fractionated by gel permeation chromatography (GPC) using the following system: Waters 1525 binary HPLC pump, Waters 2487 dual absorbance detector, Waters 717 plus autosampler, Ultrahydrogel™ 1000 column (7.8 mm × 30 cm) (Milford, MA). The eluant was 34 mM phosphate buffer (pH 6.6) plus autosampler, UltrahydrogelTM 1000 column (7.8 mm × 30 cm) (Milford, MA). The eluant was 34 mM phosphate buffer (pH 6.6) with 20 vol% methanol and the flow rate was 1.0 ml/min. The detection wavelength was 210 nm. The degree of PEI conjugation to HA was determined by 1H NMR analysis (DPX300, Bruker, Germany).

**Preparation and Characterization of siRNA/PEI-HA Complex**

The siRNA/PEI-HA complex was prepared by mixing 1 μl of FITC-siRNA (50 pmol/μl) or anti-PGL3-Luc siRNA (0.75 μg/μl) with the specified amount of PEI-HA solution (2.6 μg/μl) followed by incubation at room temperature for 15 min. The weight ratio of PEI-HA to siRNA varied from 1.7 to 6.9. To the resulting complex solution, NaCl was added to adjust the final concentration to be 150 mM. A control, siRNA/PEI complex containing the same amount of PEI was also prepared as described above. After dilution of the complex solution with 750 μl of water, the ζ-potential of siRNA/PEI-HA complex (a weight ratio of 1:5.2) was measured with the same particle analyzer. In addition, the particle size of siRNA/PEI-HA complex was measured with atomic force microscope (AFM, Multimode 3100, VEECO Instrument Co., NY). The siRNA/PEI-HA complex solution (50 μl) was placed on a silicon wafer and then air-dried. AFM system was in a tapping mode with a scanning area of 8 × 8 μm.

**Cell Culture and Cytotoxicity Test**

B16F1 cells were cultured at 37°C in a humidified incubator containing 5% CO₂. DMEM was supplemented with 10 vol% FBS and 10 IU/ml of antibiotics (penicillin). The cytotoxicity was evaluated by the MTT assay.42 B16F1 cells were aliquoted into 5 × 10³ cells/well in a 96-well plate and incubated at 37°C for 24 h. The prepared siRNA/PEI complex and siRNA/PEI-HA complex (a weight ratio of 1.7 to 6.9) solutions (20 μl each) were incubated at room temperature for 20 min and added to the cells in the absence of FBS. HA solution without siRNA was used as a control. At the end of the transfection step, 20 μl of 2 mg/ml MTT solution in PBS was added to the plate and incubated at 37°C for additional 4 h. The medium containing MTT was removed and 300 μl of DMSO was added to dissolve the formazan crystal formed by live cells. The optical density was measured at 540 nm with UV spectrophotometer (Biotrak II plate reader, Biochrom, Cambridge, UK). Cell viability (%) was calculated using the following equation: Cell viability (%) = [OD₅₄₀ (sample)/OD₅₄₀ (control)] × 100, where OD₅₄₀ (sample) represents the optical density from the wells treated with siRNA/PEI complex or siRNA/PEI-HA complex and OD₅₄₀ (control) represents that from the wells treated with PBS.

**Intracellular Delivery of FITC Labeled siRNA/PEI-HA Complex**

B16F1 and HEK-293 cells were plated in culture slides (Bedford, MA, US) at a density of 5.0 × 10³ cells/plate and incubated at 37°C for 24 h, respectively. Then, the culture medium was replaced with 0.2 ml of serum free medium containing FITC-siRNA/PEI-HA complex with a weight ratio of 1:5.2. The culture slide was incubated at 37°C and retrieved in 2 h. Cells were fixed with 1 wt% paraformaldehyde and observed with a con-focal laser scanning microscope (LSM 510, Carl-Zeiss, Thornwood, NY) at an excitation wavelength of 543 nm. The magnification was ×20.

**PGL3-Luc Gene transfection and the gene silencing**

B16F1 and HEK-293 cells were aliquoted into 5 × 10³ cells/well in a 24-well plate and incubated at 37°C for 24 h. Anti-PGL3-Luc siRNA/PEI-HA complex was cotransfected along with PGL3-Luc plasmid/lipofectamine complex as described elsewhere.19 Briefly, cells were pretransfected with 0.5 ml of DMEM containing 2 μg of PGL3-Luc vector using a lipofectamine reagent (2 mg/ml). After incubation for 3 h and the subsequent washing, anti-PGL3-Luc siRNA/PEI-HA (a weight ratio of 5.2) complexes were transfected for the gene silencing in the serum-free medium. Anti-PGL3-Luc siRNA/lipofectamine complex was used as a positive control. After incubation for 24 h, the transfected cells were lysed with a lysis buffer (1% Triton X-100) and 5 μl of the lysed solution was mixed with 25 μl of luciferase assay solution. The luciferase activity was measured with a luminometer (Luminoskan Ascent, Lab systems, Germany). To confirm the LYVE-1 HA receptor mediated endocytosis of siRNA/PEI-HA complex to B16F1 cells, the effect of HA (Mw = 130 K, 10 or 20 μg/ml) in the transfection medium on PGL3-Luc gene silencing was investigated using a flow cytometry.

**RESULTS AND DISCUSSION**

**Synthesis and Characterization of PEI-HA Conjugate**

A novel target specific siRNA delivery system was successfully developed using PEI-HA conjugate. Figure 1 shows a sche-

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UGG AGC GAA AdTdT-3′ (sense) and 5'-UUU CCU UCC AAA ACA AdTdT-3′ (antisense). FITC-siRNA has the sequences of 5'-CCU ACG CCA AUU UCG UdTdT-3′ (sense) and 5'-AGG AAA UUG GUG GCG UAG GdTdT (antisense). The siRNA was labeled with FITC at the 5'-end of its sense strand.

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matic representation for the conjugation of HA with branched PEI through the formation of amide linkage. The carboxyl groups of HA were activated with EDC and then conjugated with amine groups of PEI. The weight ratios of HA to PEI in the reaction solutions were 1:2, 1:5, and 1:20, respectively. GPC is widely used as a tool for the analysis of molecular weight and molecular weight distribution. Figure 2 shows GPC chromatograms of HA and PEI-HA conjugates. The elution times of HA and PEI-HA conjugates were 5.48 and 9.97 min, respectively. Considering that large molecules pass through a GPC column faster than small ones with a short elution time on the chromatogram, the hydrodynamic volume of PEI-HA conjugate appeared smaller than that of HA. The shrinkage of HA chain after conjugation with PEI was likely because of the electrostatic neutralization. The repulsion force between the negatively charged carboxyl groups of HA was thought to be compensated by the positive charge of PEI. PEI-HA conjugate was fractionated by GPC for further applications.

To more clearly investigate the successful conjugation of PEI to the activated HA, the fractionated products have been analyzed by $^1$H NMR (see Figure 3). Compared with the $^1$H NMR spectra of HA (Figure 3A) and PEI (Figure 3B), that of

![Figure 1](image1.png)

**FIGURE 1** Schematic representation for the conjugation of hyaluronic acid (HA) with branch-type polyethyleneimine (bPEI) using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC).

![Figure 2](image2.png)

**FIGURE 2** Gel permeation chromatograms (GPC) for hyaluronic acid (HA) at left peak and polyethyleneimine (PEI)–HA conjugate at right peak detected at 210 nm.

![Figure 3](image3.png)

**FIGURE 3** $^1$H nuclear magnetic resonance (NMR) spectra of (A) hyaluronic acid (HA), (B) branch-type polyethyleneimine (bPEI), (C) a mixture of bPEI and HA, and (D) PEI–HA conjugate synthesized with a feeding weight ratio of HA to PEI of 1:2.
PEI–HA conjugate (Figure 3D) has both a methyl peak of acetoamide group of HA at 1.9 ppm and a PEI peak at 2.5 ppm shifted from 2.5 ppm as reported elsewhere. With the distinct HA peak, however, there was no shift of PEI peak on the 1H NMR spectrum of the mixed solution of HA and PEI (Figure 3C). The results confirmed that the fractionated products were not a mixture of PEI and HA but PEI-HA conjugates. From the integral values of 1H NMR spectra, it was determined that 5.7, 11.4, and 24.2 mol% of the carboxyl groups of HA were modified with PEI for three reaction conditions, respectively. The ζ-potential of PEI-HA conjugates increased from +3.6 to +13.2 and +24.9 mV with increasing degree of HA modification.

Preparation and Characterization of siRNA/PEI-HA Complex
Among three PEI-HA conjugates with 5.7, 11.4, and 24.2 mol% PEI contents, the last one appeared to retain a sufficient positive charge to make a complex with negatively charged siRNA. When the weight ratio of PEI-HA to siRNA varied from 1.7, 3.4, 5.2, to 6.9, the ζ-potential of siRNA/PEI-HA complex increased from −21.5, −19.93, −17.07, to −15.33 mV, respectively. The results reflected the core-shell structure of siRNA/PEI-HA complex formed by the electrostatic interaction between siRNA and PEI part of PEI-HA conjugate. Figure 4 shows the AFM image of siRNA/PEI-HA complex with a weight ratio of siRNA to PEI-HA of 1:5.2. The complex nanoparticles with an average size of ca. 21 nm were well dispersed on the silicon wafer.

Before the gene silencing experiment, a comparative cytotoxicity test for PEI and PEI-HA conjugate was performed in B16F1 cells (see Figure 5). Despite of the wide use of PEI for nonviral gene delivery, its serious cytotoxicity has been the obstacle to in vivo applications. In contrast, PEI-HA showed less cytotoxicity than PEI likely because of the electrostatic neutralization of the positively charged PEI by the negatively charged HA. In addition, when siRNA is complexed with PEI-HA conjugate, the biocompatible HA on the shell of siRNA/PEI-HA complex would greatly contribute for the reduction of cytotoxicity. The control of HA showed no cytotoxicity at all (see Figure 5).

Intracellular Uptake of FITC-siRNA/PEI-HA Complex
FITC labeled siRNA was used to visualize the receptor mediated endocytosis of siRNA/PEI-HA complex. As mentioned earlier, cluster determinant 44 (CD44), receptor for hyaluronate-mediated motility (RHAMM), and lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1) have been identified as HA receptors. B16F1 cells are reported to overexpress LYVE-1 at which HA is taken up to the cells through the receptor-mediated endocytosis. Intracellular uptake of FITC-siRNA/PEI-HA complex to B16F1 cells was compared with that to HEK-293 cells without HA receptors. Figure 6A shows the confocal microscopic images of FITC-siRNA/PEI-HA complex after incubation for 2 h. Green fluorescence was observed throughout the whole cytoplasms indicating that FITC-siRNA/PEI-HA complex had escaped from the endosome and translocated into the cytosol. After the endosomal escape, FITC-siRNA/PEI-HA complex was likely to degrade on the exposure to the reductive cytoplasmic environment being distributed throughout the cytosol. In contrast, FITC-siRNA/PEI-HA complex was not readily up-taken to HEK-
293 cells without HA receptors (Figure 6B). From the results, we could clearly observe the HA receptor mediated endocytosis of FITC-siRNA/PEI-HA complex to LYVE-1 over-expressing B16F1 cells. The specific interaction of HA with LYVE-1 receptor expressed on the tumor cells, such as B16F1 cells, has been well described elsewhere.36

**Gene Silencing of siRNA/PEI-HA Complex**

The PGL3-Luc gene silencing of anti-PGL3-Luc siRNA/PEI-HA complex in B16F1 cells was compared with that in HEK-293 cells. After the pretransfection of PGL3-Luc gene with Lipofectamine, the cells were transfected with anti-PGL3-Luc siRNA/PEI-HA complex for the subsequent gene silencing. PGL3-Luc gene expression was reduced for both LYVE-1 over-expressing B16F1 cells and LYVE-1 negative HEK-293 cells (Figure 7A). However, as shown in Figure 7B, the PGL3-Luc gene silencing efficiency of siRNA/PEI-HA complex was much higher in B16F1 cells than in the HEK-293 cells. The down-regulation in B16F1 cells was about 92%, whereas that in HEK-293 was only 38%. The results suggested that the anti-PGL3-Luc siRNA/PEI-HA complex with the outer-layer of HA was more efficiently delivered to LYVE-1 over-expressing B16F1 cells by the HA receptor mediated endocytosis than to HEK-293 cells without HA receptors. Figure 7C shows the effect of HA in the transfection medium on PGL3-Luc gene silencing in B16F1 cells by siRNA/PEI-HA complex. As the amount of HA increased in the medium, the PGL3-Luc gene silencing efficiency by anti-PGL3-Luc siRNA/PEI-HA complex in B16F1 cells was reduced by the competitive binding of free HA onto the LYVE-1, HA receptor. All these results confirmed that the HA receptor mediated endocytosis facilitated the intracellular delivery of anti-PGL3-Luc siRNA/PEI-HA complex into B16F1 cells with LYVE-1. The novel PEI-HA conjugate, which can deliver siRNA specifically to the cells containing HA receptor, will be investigated further for in vivo applications.

**CONCLUSIONS**

A novel target specific intracellular gene delivery system was successfully developed using PEI-HA conjugate. 1H NMR analysis confirmed the conjugation of HA with PEI through the formation of amide linkage. The negative $\zeta$-potential of siRNA/PEI-HA complex reflected the core/shell structure formed by the electrostatic interaction between the negatively charged siRNA and the positively charged PEI moiety of PEI-HA conjugate. AFM image of siRNA/PEI-HA complex showed well-dispersed spherical nanoparticles with an average size of ca. 21 nm on the silicon wafer. PEI-HA appeared
less cytotoxic than PEI because of the electrostatic neutralization of PEI by the negatively charged HA. FITC-siRNA/PEI-HA complex could be well up-taken to B16F1 cells through the LYVE-1 HA receptor mediated endocytosis. In addition, the PGL3-Luc gene silencing of anti-PGL3-Luc siRNA/PEI-HA complex was more efficient in the LYVE-1 over-expressing B16F1 cells than in HEK-293 cells without HA receptors. The gene silencing effect was reduced in the presence of free HA in the transfection medium, reflecting that anti-PGL3-Luc siRNA/PEI-HA complex was selectively taken up to B16F1 cells via the receptor mediated endocytosis. The novel PEI-HA conjugate, which can deliver siRNA specifically to the cells containing HA receptor, will be investigated further for in vivo applications.

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