Antisense Oligodeoxynucleotide-Conjugated Hyaluronic Acid/Protamine Nanocomplexes for Intracellular Gene Inhibition

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Received April 3, 2007; Revised Manuscript Received May 17, 2007

Green fluorescent protein (GFP) antisense oligodeoxynucleotide (ODN) was covalently conjugated to hyaluronic acid (HA) via a reducible disulfide linkage, and the HA–ODN conjugate was complexed with protamine to increase the extent of cellular uptake and enhance the gene inhibition efficiency of GFP expression. The HA–ODN conjugate formed more stable polyelectrolyte complexes with protamine as compared to naked ODN, probably because of its increased charge density. The higher cellular uptake of protamine/HA–ODN complexes than that of protamine/naked ODN complexes was attributed to the formation of more compact nanosized complexes (~200 nm in diameter) in aqueous solution. Protamine/HA–ODN complexes also showed a comparable level of GFP gene inhibition to that of cytotoxic polyethylenimine (PEI)/ODN complexes. Since both HA and protamine are naturally occurring biocompatible materials, the current formulation based on a cleavable conjugation strategy of ODN to HA could be potentially applied as safe and effective nonviral carriers for ODN and siRNA nucleic acid therapeutics.

INTRODUCTION

Recently, efficient and specific down-regulation of therapeutically important genes has received special attention to treat various genetic and infectious diseases, including cancer (1–3). Nucleic acid-based drugs, such as antisense oligodeoxynucleotide (ODN), ribozyme, and small interfering RNA (siRNA), have been shown to inhibit expression of target proteins at post-transcriptional level. While they show highly sequence-specific gene silencing behaviors, critical delivery problems such as poor intracellular uptake and severe enzymatic degradation still remain to be solved for successful in vivo therapeutic application (4–6). To improve their intracellular uptake and nuclease-resistant stability, a variety of cationic agents including polymers, polypeptides, and lipids were complexed with anionic nucleic acid drugs to produce nanosized polyelectrolyte and lipoplexes. The nanoscale complexes can readily be transported into cells via an endocytic pathway while protecting the genetic drugs from degradation by nuclease. Among the cationic synthetic polymers, poly(ethylenimine) (PEI) and poly(L-lysine) (PLL) have been widely used to form nanosized polyelectrolyte complexes to deliver plasmid DNA, ODN, and siRNA (7–12). It is generally known that high molecular weight (MW) PEI and PLL produce more stable polypelexes with plasmid DNA than low MW polypelexes, generally resulting in increased gene transfection efficiency (13). This is because the charge density (the number of charges per polymer chain) of cationic polymers plays an utmost important role in maintaining the colloidal structural stability of polyelectrolyte nanocomplexes, especially in the transfection medium containing high salt concentration. The charge density effect on complex stability is closely related to inter- and intramolecular charge entanglement and mixing degree of two oppositely charged polyelectrolytes upon complexation. However, using high MW PEI and PLL as nonviral gene carriers has often elicited harmful cytotoxic effects, adversely influencing gene transfection efficiency. For producing stable and compact polypelexes, two oppositely charged polyelectrolytes (cationic polymer and DNA) must have sufficient charge densities for complexation. To form stable and non-cytotoxic polypelexes with ODN, reducible or self-degradable cationic polymers have been used to increase charge density for improved complex stability without compromising the cytotoxicity problem (14–17). The immediate degradation of reducible or self-degradable cationic polymers after cellular uptake produced less cytotoxic low MW fragments tolerable to the cells.

Antisense ODN is a single strand DNA composed of about 20 nucleotides with MW of around 5000, which has very low charge density compared to plasmid DNA with MW of a few million. Accordingly, when ODN was complexed with cationic polymers, the resultant ODN polypelexes often exhibited much reduced colloidal stability than that of the plasmid DNA polypelexes, mainly because of insufficient charge density of ODN.

Protamine is a nontoxic, cationic, and naturally occurring polypeptide which is known to provide unique membrane-translocating and nuclear-localizing activities because of its arginine-rich sequence (18–22). Although protamine has been used as a condensing agent for ODN, the transfection efficiency of protamine/ODN polyelectrolyte complexes was far less than that of high MW PEI or PLL (23, 24). This was probably because both protamine and ODN did not have sufficient MW and charge density to form stable colloidal complexes, in addition to the presence of weak basic guanidine groups in protamine.

In this study, antisense GFP ODN was conjugated to anionic and biocompatible HA via a cleavable disulfide linkage to increase the charge density of ODN. HA, composed of N-acetyl-d-glucosamine and d-glucuronic acid, was expected to provide enough charge density to the conjugated ODN. The HA–ODN conjugate was complexed with clinically acceptable low MW protamine to improve the stability of HA–ODN/protamine polyelectrolyte complexes and increase gene inhibition efficiency. Anti-sense GFP ODN was used for conjugation to HA. HA–ODN/protamine complexes were physically characterized,...
and their inhibition extent of GFP expression was evaluated using HEK293 cells.

MATERIALS AND METHODS

Materials. Hyaluronic acid (sodium salt form, MW 17 000) was obtained from Lifecore Biomedical (Chaska, MN). Dialysis membrane (MW cutoff, 50 000) was a product from Spectrum (Houston, TX). GFP Antisense ODN (GAGCTGCACGCTGC-CGTC, 5’ amine modification, MW 5656) was purchased from Bioneer (Daejeon, Korea). Plasmid DNA (pEGFP-C1) was extracted from transformed Escherichia coli by a standard alkaline lysis technique and purified by a DNA-purification column which was obtained from QIAGEN (Valencia, CA) according to the manufacturer’s instruction. The purity and concentration of plasmid DNA was determined by measuring the absorbance ratio at 260/280 nm using a spectrophotometer (UV-1601, Shimadzu, Japan). N-Succinimidyld-3-(2-pyridyldithio)-propionate (SPDP) was purchased from Pierce (Rockford, IL). Dulbecco’s phosphate-buffered saline (PBS), fetal bovine serum (FBS), and DMEM were purchased from Gibco BRL (Grand Island, NY). A pEGFP-C1 vector was obtained from BD Bioscience Clontech (Palo Alto, CA). Protamine, [1-ethyl-3- (dimethylamino)propyl]carbodiimide hydrochloride (EDC), PEI (branched, MW 25 K), cystamine, and diithiothreitol (DTT) were purchased from Sigma (St. Louis, MO). Cell counting kit-8 (CCK-8) was obtained from Dojindo Laboratories (Kumamoto, Japan). All other chemicals and reagents were of analytical grade.

Methods. Synthesis and Characterization of ODN-Conjugated Hyaluronic Acid. For preparation of thiol-modified hyaluronic acid (HA, MW 17 000), HA (200 mg) dissolved in PBS solution (pH 7.0) was added into 4 mL of dimethyl sulfoxide (DMSO)/deionized water mixture (50/50 (v/v)) containing EDC (194 mg) and HOBt (138 mg). To the solution was added cystamine (229 mg) for conjugation overnight. After treating with DTT (DTT: cystamine molar ratio = 4:1), the whole solution was dialyzed against deionized water using a membrane (MW cutoff: 3500) for 2 days and lyophilized. 1H NMR spectra were taken with a Bruker DRX 300 NMR operating at 300.13 MHz by using D2O to determine the degree of cystamine modification. The degree of thiol substitution in HA was calculated based on the molar ratio of thiol groups in thiolated HA to the total carboxylic acid groups in unmodified HA. The amount of thiol groups per HA chain was also quantified by the Ellman’s assay.

To prepare the HA−SS−ODN conjugate (HA−ODN), 2-pyridyl disulfide-activated ODN was first synthesized by adding SPDP (275 μg) in DMSO to the PBS solution (pH 7.4) containing 5’-amine-modified antisense ODN (500 μg). After reaction for 5 h at room temperature, the unreacted SPDP was removed by using a desalting column (D-Salt dextran desalting column, Pierce, Rockford, IL). The activated ODN (340 μg) was reacted with 2.4 mg of thiol-modified HA in PBS solution at room temperature for 2 days. The unreacted antisense ODN was removed by dialysis against deionized water (MWCO 10 000). The resulting product, HA−ODN conjugate, was analyzed by polyacrylamide gel (20%) electrophoresis. The electrophoresis was carried out at a voltage of 120 V for 1 h in TAE buffer solution (40 nM Tris-HCl, 1% (v/v) acetic acid, 1 mM EDTA). Antisense ODN was visualized by ethidium bromide (EtBr) staining. HA−ODN conjugate was also analyzed by gel permeation chromatography (GPC) using a Waters 510 HPLC pump system (Waters 486, Waters, Milford, MA) equipped with a refractive index detector (Shodex RI-71, Kawasaki, Japan). GPC analysis was performed on a column (Ultrahydrogel 250, 7.8 × 300 mm, Waters) using distilled water as an eluant with a flow rate of 1 mL/min. To evaluate cleavability of the disulfide linkage for HA−ODN conjugate, the sample was incubated in 50 mM of glutathione solution for 1 h or 2 h and injected to the GPC system to separate the fraction of cleaved ODN from the HA−ODN conjugate.

Preparation and Characterization of Protamine/HA−ODN Complexes. Naked ODN or HA−ODN conjugate (4 μg) dissolved in 100 μL of PBS (pH 7.4) solution was mixed with various amounts of desalted protamine (0.4, 2, 4, and 64 μg) dissolved in 100 μL of PBS solution. After incubation for 15 min at room temperature, 2.8 mL of PBS solution was added to the mixture. To determine the hydrodynamic diameter of nanocomplexes formed from ODN or HA−ODN with protamine, the resulting complex solutions were analyzed by a dynamic light scattering instrument (Zeta-Plus, Brookhaven, NY). To evaluate stability of the protamine/HA−ODN complexes, a polyanion competition assay was done as previously reported (25). After preparation of nanocomplexes from ODN or HA−ODN (nucleotide 1 μg) with protamine (16 μg) by incubation in 20 μL of PBS solution for 15 min, different amounts of heparin (0, 4, 10, 50, and 100 μg) were added to the solution and incubated for 15 min. The mixture solution was subjected to 1% agarose gel electrophoresis running at 100 V for 20 min. The fraction of decomplexed ODN by heparin was visualized in the gel by staining with EtBr.

Intracellular Uptake Assay. HEK293 cells were maintained in DMEM medium supplemented with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere of 5% CO2. Cells were regularly passaged at subconfluence and plated on a four-well chamber slide at 24 h before transfection. On the next day, the cells were transfected with protamine/ODN or protamine/HA−ODN complexes formulated at a protamine/ODN weight ratio of 7.5. To visualize antisense ODN, a FITC-labeled sense ODN having a complementary sequence to antisense GFP ODN (FITC-sense ODN) was hybridized with either naked ODN or HA−ODN conjugate. After 5 h incubation, cells were washed three times with cold PBS solution and fixed with 1% (w/v) paraformaldehyde solution for 30 min at 4 °C. The nucleus was stained with DAPI (4′,6-diamidino-2-phenylindole) dye, a double strand DNA specific marker. Uptake of antisense ODN within the HEK293 cells was visualized by confocal microscopy (Carl Zeiss, Germany).

Cell Culture and Transfection. For GFP gene silencing experiments, GFP plasmid DNA was cotransfected with protamine/ODN or protamine/HA−ODN complexes to HeLa cells (human cervical cancer cell line), according the previously published method with minor modifications (26). Briefly, the cells were cotransfected in serum free medium with pEGFP-C1 plasmid (1 μg) using Lipofectamine 2000 reagent (2 mg/mL, Invitrogen, Carlsbad, CA) and protamine/ODN or protamine/HA−ODN complexes formulated at a protamine/ODN weight ratio of 1.5 and 6 (ODN amount: 1 μg). Polyelectrolyte complexes formed from PEI and naked ODN (1 μg) formulated at an N/P ratio of 8 were used as a positive control. After 5 h incubation, medium was replaced with new DMEM medium containing 10% FBS. After 48 h, transfected cells were harvested by treating with a cell lysis solution (1% Triton X-100 in PBS solution) and centrifuged to remove cell debris. The supernatant was analyzed to determine the amount of GFP protein expression using a spectrofluorophotometer (SLM-AMINCO 8100, SLM Instruments Inc., Rochester, NY) with an excitation and emission wavelength at 488 and 507 nm, respectively. All values were normalized by the cellular protein amount which was determined by the Micro-BCA protein assay (Pierce, Rockford, IL). Relative GFP expression level was then calculated as relative percentage using that of pEGFP-C1 transfected HeLa cells as 100% (26).
The cytotoxicity of the two cationic polymers, protamine and PEI, was evaluated by the CCK-8 cell viability assay which depends on the mitochondrial dehydrogenase activity of the cells. HEK 293 cells were seeded in a 96-well plate at a density of $1 \times 10^4$ cells per well and grown in DMEM medium supplemented with 10% (v/v) fetal bovine serum for 24 h at 37 °C. On the next day, protamine or PEI was added to the culture medium to give a final concentration of 0 to 160 µg/mL. After further incubation for 24 h, the number of viable cells was determined by the CCK-8 cell viability assay according to the manufacturer’s protocol.

RESULTS AND DISCUSSION

Synthetic schemes of thiol-modified hyaluronic acid (HA) and HA−ODN conjugate are shown in Figure 1. The degree of cystamine substitution in the total carboxylic acid group in HA was 27.4 ± 3.4% as quantified by $^1$H NMR (data not shown). The conjugation extent of cystamine was determined by relating the relative peak area of NHCH$_2$CH$_2$SH (2.55 ppm) protons of cystamine to that of COCH$_3$ (1.86 ppm) protons of HA. The degree thiol substitution in HA was 20.8 ± 9.2%, and the number of thiol group in HA after DTT treatment was 8.8 ± 3.9 per HA chain, as quantified by Ellman’s assay.

The primary amine group at the 5′-end of antisense ODN was first reacted with an active NHS group of a heterobifunctional cross-linker, SPDP (N-succinimidyl 3-(2-pyridyldithio)propionate), to produce 2-pyridyl disulfide-activated ODN. Thiol-modified HA was then added to the 2-pyridyl disulfide-activated ODN to obtain the HA−SS−ODN conjugate (HA−ODN) via a sulfhydryl exchange reaction. The HA−ODN conjugate was purified by using a gel elution method and analyzed by gel electrophoresis. Figure 2a shows the 20% polyacrylamide gel electrophoresis result, indicating that HA−ODN conjugate was successfully synthesized.

To calculate the number of ODN molecules conjugated onto the HA backbone, naked ODN and HA−ODN conjugate were...
analyzed by GPC, using a series of dextran molecules with different molecular weights as standards (Figure 2b). The HA–ODN conjugate eluted earlier at a retention time corresponding to MW 22,700, while the naked ODN was eluted later at a peak position of MW 5,656. From the GPC results, the number of ODN per HA chain was 0.83 (0.15, which was simply calculated based on the difference in elution times between HA–ODN conjugate and naked ODN. The calculation for the number of ODN molecules on the HA chain based on the MW difference, however, was somewhat misleading, because highly charged HA–ODN conjugate and ODN had different hydrodynamic volumes from neutral dextran molecules that were used as standards in the GPC analysis. It should be noted that the HA–ODN conjugate was eluted as doublet peaks in the chromatogram, implying that roughly one or two ODN chains were conjugated onto the HA backbone. To verify that the disulfide linkage of HA–ODN conjugate could be cleaved in reducing condition, the HA–ODN conjugate was incubated in a solution containing 50 mM glutathione (GSH). Figure 2c shows that free ODN is liberated from the conjugate as a result of cleavage of the disulfide linkage between HA and ODN. The doublet peak for the HA–ODN conjugate disappeared progressively, and a new peak for the released free ODN fraction appeared gradually with increasing incubating time. The sharply increasing signal observed in the late elution time was due to the nonspecific effect of GSH present in the sample. After 2 h incubation in reductive condition, a free form of ODN was completely regenerated from the conjugated form. The main reason for the introduction of a cleavable disulfide linkage in the HA–ODN conjugate was to release an intact form of antisense ODN into the cytosol. It is known that the cytoplasmic space maintains a reductive microenvironment mainly by the presence of high GSH concentrations of up to 10 mM (27). It was postulated that the free form of ODN might have a better gene silencing activity than the conjugated form of HA–ODN because the steric hindrance effect of adjacent HA on binding to a target mRNA sequence was likely to be minimized (28).

Dynamic light scattering (DLS) analysis revealed that the HA–ODN conjugate could form much smaller complexes than the unconjugated ODN (Figure 3a). The hydrodynamic sizes of protamine complexes with ODN and HA–ODN conjugate were 653.5 ± 5.1 nm and 166.0 ± 13.9 nm at a protamine/ODN weight ratio of 0.5, respectively. This suggests that HA–ODN conjugate could produce more stable and compact complexes with protamine primarily because of the increased charge density. The conjugated HA provided additional anionic charges for the formation of complexes between protamine and ODN. When increasing the protamine/ODN weight ratio, the diameters of protamine/HA–ODN conjugate complexes were still around 200 nm, while those of protamine/ODN complexes were about 800 nm. Gel retardation assay was also performed.

Figure 2. (a) Migration profiles of unconjugated ODN and HA–ODN conjugate in 20% polyacrylamide gel. (b) GPC result of unmodified ODN and HA–ODN conjugate. (c) Time-dependent cleavage of disulfide linkage in the HA–ODN conjugate when incubated in the medium containing 50 mM glutathione.

Figure 3. (a) Effective diameter of protamine/ODN and protamine/HA–ODN polyelectrolyte complexes as determined by dynamic light scattering. (b) Stability of polyelectrolyte complexes in the presence of competing polyanion, heparin. Protamine/ODN and protamine/HA–ODN complexes were incubated with various amount of heparin prior to loading on 1% agarose gel.
to confirm that ODN and HA-ODN conjugate could form nanoscale complexes with protamine by electrostatic interactions. Complete gel retardation of ODN and HA-ODN conjugate complexes was observed at a protamine/ODN weight ratio of 1 (data not shown).

To evaluate the relative structural stability of protamine/ODN and protamine/HA-ODN complexes, a competing polyanion, heparin, at various concentrations was added to induce decomplexation. After incubation with heparin, the fraction of decomplexed ODN was visualized in the gel (Figure 3b). Protamine/ODN and protamine/HA-ODN complexes were completely decomplexed at heparin concentrations of 10 μg and 50 μg, respectively. A 5-fold higher heparin concentration was required to destabilize the protamine/HA-ODN complexes than for the protamine/ODN complexes. It should be noted in Figure 3b that both ODN and HA-ODN conjugate bands appeared at the same migration position in the gel. This was because of the fact that 1% agarose gel electrophoresis did not provide sufficient resolution to separate the two species having a MW difference of around 20 000–30 000. From the above results, it is reasonable to say that HA-ODN conjugate formed more stable polyelectrolyte complexes than naked ODN. The stability and size of DNA polyplexes play an important role in promoting cellular uptake and enhancing gene transfection efficiency. It was reported that the size of polyplexes should be less than or around 200 nm for facile intracellular transport by an endocytic pathway (29). The DLS and heparin-induced decomplexation assay clearly indicate that more stable and compact polyplexes can be produced by conjugating ODN molecules to a highly charged HA chain backbone by increasing the charge density of HA-ODN conjugate for electrostatic interactions with protamine.

Figure 4a represents confocal images of HEK 293 cells incubated with protamine/ODN and protamine/HA-ODN complexes, respectively. The nuclei of cells were stained with the DAPI dye, which appeared blue. To visualize intracellular uptake of ODN and HA-ODN, an FITC-labeled sense ODN strand was used to hybridize an antisense ODN strand. The annealed FITC-labeled double strand ODN was used for complex formulation. Protamine/HA-ODN complexes appeared
scattered as green fluorescent dots within the cytosolic space of cells (Figure 4a right panel). They were likely to be preferentially located within the endosomal and lysosomal vesicles after cellular uptake. On the other hand, cells treated with PBS (left panel), protamine/ODN complexes (middle panel), and sense-FITC ODN only (data not shown) showed no measurable green fluorescent intensities. The confocal images evidently suggest that the conjugation of ODN to HA could greatly enhance the extent of intracellular uptake by producing more stable nanosized polyelectrolyte complexes suitable for intracellular endocytosis.

Protamine/HA–ODN and protamine/ODN complexes (formulated with a single strand GFP antisense ODN) were co-transfected with GFP plasmid formulated with Lipofectamine to HeLa cells. Their GFP gene inhibition degrees were comparatively evaluated by fluorescence microscopy as shown in Figure 4b. In the fluorescent images, it can be seen that protamine/HA–ODN complexes formulated at a protamine/ODN weight ratio of 1.5 inhibit expression of GFP to a greater extent (right panel), compared to protamine/ODN complexes (middle panel). The extent of GFP inhibition was quantitatively analyzed by measuring intracellular GFP fluorescence intensity after cell lysis. Protamine/HA–ODN conjugate complexes prepared at a protamine/ODN weight ratio of 1.5 and 6 showed more significant inhibition of GFP gene expression than the protamine/ODN complexes (Figure 4c). It is also noticeable that increasing the weight ratio of protamine/ODN enhances the extent of GFP expression. High molecular weight PEI (25 k) was used as a control in this study. PEI/ODN complexes exhibited 19.7 ± 14.5% of GFP gene expression at an N/P ratio of 8, which is known to be an optimal condition for gene transfection (30). Protamine/ODN and protamine/HA–ODN complexes prepared at a protamine/ODN weight ratio of 1.5 showed 46.9 ± 19.5% and 17.5 ± 13.7% of GFP expression levels, respectively. The results imply that the greater extent of gene silencing effect observed for protamine/HA–ODN complexes was mostly because of enhanced complex stability and compact complex size without causing any cytotoxic effects. Furthermore, the reductive cleavage of the disulfide linkage between HA and ODN released out unconjugated and intact ODN species into the cytoplasm, which additionally contribute to the effective inhibition of GFP expression. Although protamine/HA–ODN and PEI complexes showed similar gene silencing levels, PEI was very cytotoxic in contrast to protamine at the same weight ratio, as measured by the CCK8 assay (Figure 4d). Thus, the more biocompatible protamine compared to any other cationic synthetic polymers such as PEI and PLL could be used as an effective condensing agent for the HA–ODN conjugate.

Last, it is worthwhile to note that low MW HA fraction is known to be internalized into cancer cells overexpressing HA receptors such as CD44 via receptor-mediated endocytosis. It was recently reported that HA conjugated chemical drugs including paclitaxel showed higher intracellular uptake and better therapeutic efficiency compared to unconjugated ones (31–33). Various water insoluble drugs could be conjugated in a cleavable or noncleavable manner to HA. HA–conjugated drugs could be solubilized in aqueous solution as self-assembling micelles, enabling intravenous administration. Moreover, HA–based prodrugs were expected to show target-specific cellular uptake against HA receptor-positive cells. In this study, we introduced the HA–ODN conjugation approach to increase the stability of nanosized complexes with protamine. It cannot be ruled out that protamine/HA–ODN conjugate polypelexes could possibly be internalized within cells via HA–receptor mediated endocytosis to some extent, in addition to the nonspecific endocytic mechanism. Quite recently, we reported that siRNA-encapsulated HA nanogels could be delivered into cells by HA receptor-mediated endocytosis (34). Thus, more detailed studies will be necessary to elucidate the cellular uptake mechanism.

In conclusion, antisense ODN was conjugated to HA, a negatively charged natural polysaccharide, to increase the charge density of the resultant conjugate for facile complexation with another naturally occurring polypeptide, protamine. Stable and non-cytotoxic protamine/HA–ODN complexes were formed, which exhibited a comparable gene silencing level to a cytotoxic PEI-based ODN formulation. The current novel ODN formulations prepared only from naturally occurring and clinically safe materials would have potential applications for siRNA delivery in the future.

ACKNOWLEDGMENT

This study was supported by the grants from the National Research Laboratory project from the Ministry of Science and Technology and the National Cancer Center, Republic of Korea.

LITERATURE CITED


BC0701110