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Enhanced splicing correction effect by an oligo-aspartic acid–PNA conjugate and cationic carrier complexes

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ABSTRACT

Peptide nucleic acids (PNAs) are synthetic structural analogues of DNA and RNA. They recognize specific cellular nucleic acid sequences and form stable complexes with complementary DNA or RNA. Here, we designed an oligo-aspartic acid–PNA conjugate and showed its enhanced delivery into cells with high gene correction efficiency using conventional cationic carriers, such as polyethylenimine (PEI) and Lipofectamine 2000. The negatively charged oligo-aspartic acid–PNA ($Asp_{(n)}$–PNA) formed complexes with PEI and Lipofectamine, and the resulting $Asp_{(n)}$–PNA/PEI and $Asp_{(n)}$–PNA/Lipofectamine complexes were introduced into cells. We observed significantly enhanced cellular uptake of $Asp_{(n)}$–PNA by cationic carriers and detected an active splicing correction effect even at nanomolar concentrations. We found that the splicing correction efficiency of the complex depended on the kind of the cationic carriers and on the number of repeating aspartic acid units. By enhancing the cellular uptake efficiency of PNAs, these results may provide a novel platform technology of PNAs as bioactive substances for their biological and therapeutic applications.

1. Introduction

Peptide nucleic acids (PNAs), oligonucleotide mimics, bind to complementary DNA or RNA with high affinity according to Watson–Crick base-pairing rules [1–3]. In PNAs, the sugar-phosphate backbone of DNA/RNA is replaced with an achiral polyamide backbone, consisting of *N*-(2-aminoethyl)-glycine units that interact with the nucleobases via methylene carbonyl linkers [2,4,5]. By strict chemical alteration, PNAs obtain an uncharged atypical backbone, which confers increased affinity in the context of hybrid duplexes with complementary DNA/RNA sequences. Moreover, PNAs are much more stable in biological fluids compared with that of DNA and RNA because nucleases and proteases do not recognize or digest the altered backbones [6,7].

PNAs are regarded as antigene or antisense agents, because they can regulate protein expression by inhibiting mRNA translation or transcription. As such, PNAs are potential therapeutic agents in cases where modulation of gene expression may be beneficial [8,9]. However, poor cellular uptake of PNAs has limited their application in cell culture and *in vivo* [10–12]. The uptake issue has been approached by modulating a target sequence and length [13,14], or by structural modification of PNAs and treatment with chemical reagents. PNA modification by conjugation to various moieties, including DNA oligomers [15], cell-penetrating peptides (such as Tat, penetratin, or oligo-arginines) [16–18], or nuclear localization signal (NLS) peptides [19], enhances cellular internalization. Incorporation of positively charged oligopeptides, in particular, improves cellular uptake [20]. Because cell membranes have a negative charge, positively charged particles can be introduced inside cells after their adherence to the surface of cell membranes [18]. Chemical reagents can also be used to improve PNA delivery. For example, transfection reagents such as Lipofectin and Lipofectamine can encapsulate PNAs in the liposome interior or bind them at the liposome surface [14,21]. Lipid-mediated methods usually follow the endocytosis pathway to effectively deliver PNAs into the cytoplasm. Moreover, adding calcium ions, chloroquine, or, sucrose to the cell culture medium facilitates the release of PNAs from endosomes, and robustly increases PNA activity [22]. Another class of chemical reagents includes the cationic polymers, such as poly-L-lysine [23], PAMAM dendrimer [24], or, polyethylenimine (PEI) [25,26], which are also widely used as efficient non-viral vectors in drug and gene delivery systems. Although these chemical reagents may be helpful for improving the cellular uptake of PNAs, this approach is not applicable to native PNAs due to their uncharged neutral polyamide backbone. Thus, assigning negative

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charges directly to PNAs may be a good choice to improve their ability to make complexes with various typical delivery reagents without using unnecessary complementary DNA oligonucleotides.

Negative charges can be introduced to PNAs via conjugation of phosphate moieties, and this strategy combined with complex formation with cationic lipids significantly enhances delivery to cells [27]. The cationic lipoplexes also show antisense activity in the nanomolar range. Herein, we describe the use of oligo-aspartic acid conjugation to derive novel negatively charged PNA derivatives. The oligo-aspartic acid–PNA conjugates could be complexed with conventional transfection reagents (PEI polymer or Lipofectamine 2000) and displayed much enhanced gene correction effect. We report the transfection efficiency and cytotoxicity of oligo-aspartic acid–PNA relative to unmodified PNAs. Furthermore, we proved that oligo-aspartic acid–PNA delivered by PEI or Lipofectamine localized to the perinuclear and nuclear areas inside cells.

2. Materials and methods

2.1. Materials

Polyethylenimine (branched, 25 kDa and 2 kDa) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (Seoul, Korea). Lipofectamine 2000 transfection reagent, TRIzol reagent and Hoechst 33342 were purchased from Invitrogen (Carlsbad, CA, USA). The Luciferase 1000 Assay kit and reporter lysis buffer were purchased from Promega (Madison, WI, USA). *Taq* polymerase chain reaction (PCR) Master Mix kit and Omniscript reverse transcriptase were purchased from Qiagen (Valencia, CA, USA). Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were purchased from GIBCO (Gaithersburg, MD, USA). PNA and oligo-aspartic acid–PNA ($Asp_{(4)}$–PNA, $Asp_{(8)}$–PNA, $Asp_{(12)}$–PNA, $Asp_{(8)}$–PNA–M, $Asp_{(12)}$–PNA–M, PNA–Fam, and $Asp_{(12)}$–PNA–Fam) were synthesized by the Panagene Co. (Daejeon, South Korea).

2.2. Melting temperature analysis

The melting temperature values of PNA/DNA duplexes were obtained using the CFX-96 real-time PCR machine from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). The PNA/DNA duplex samples were prepared in 10 mM Tris–EDTA buffer (pH 8.5) at 1 μM. Samples were annealed at 95 ºC for 10 min, cooled at 30 ºC for 5 min, and then scanned from 40 to 90 ºC at a scan rate 0.1 ºC/s. This procedure was repeated 3 times.

2.3. Cells and cell culture

HeLa pLuc 705 cells were kindly provided by Prof. Ryszard Kole (Department of Pharmacology and Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, NC, USA). Cells were cultured in DMEM supplemented with 10% FBS. The cells were maintained at 37 °C in an incubator with a humidified atmosphere containing 5% CO_2.

2.4. PNA transfection

HeLa pLuc 705 cells were seeded at a density of 1.2 or 2.0×10^4 cells/well in 96 well plates for luciferase assay, and 1.0×10^6 cells/well in 6 well plates for RT-PCR experiments 24 h before transfection. PNAs and PNAs/PEI or Lipofectamine complexes were prepared at various molar ratios and incubated for 30 min at room temperature. The cells were treated with media containing PNAs, PNAs/PEI, or Lipofectamine complexes at the desired concentrations and incubated for 1 day at 37 °C in a 5% CO_2 incubator before they were assayed.

2.5. Luciferase assay

At 24 h post-transfection, the cells in 96 well plates were washed with phosphate-buffered saline (PBS), and reporter lysis buffer was added to each well. After 30 min incubation at room temperature, the cells were harvested and transferred to microcentrifuge tubes. After 15 s of vortexing, the cells were centrifuged at 1200 rpm for 20 min. Luciferase activity was measured in terms of relative light units (RLU) per μg protein by using a Lumat LB 9057 luminometer (Berthold, Bad Wildbad, Germany). The protein concentration of the extract was determined using a BCA protein assay kit (Pierce, Iselin, NJ, USA).

2.6. RT-PCR

The sequences of the primers used were as follows: luciferase sense, 5′-TTGATATGTGGATTTCGAGTCGTC-3′; antisense, 5′-AGCCACGCTTTC ATACTGCT-3′. Total RNA was isolated with TRIzol reagent (Invitrogen), according to the manufacturer's protocol. Total RNA concentration and purity were calculated by absorbance at 260 and 280 nm. First-strand cDNA was synthesized with 2 μg of total RNA and 1 μM of oligo-dT_{18} primers using Omniscript reverse transcriptase. Quantitative PCR amplification was performed using a Peltier thermal cycler (MJ Research, Watertown, MA, USA) and *Taq* PCR Master Mix kit, using first-strand cDNA diluted at 1:25 with 20 pmol of primers, according to the manufacturer's protocol. The PCR reaction consisted of an initial denaturation step at 55 °C for 35 min, 95 °C for 15 min, and three-step cycling (30 cycles) at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 5 min. PCR products were analyzed on a 2% agarose gel with 1× TBE buffer and visualized by ethidium bromide staining. Gel images were captured and analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

2.7. Fluorescence microscopy imaging

HeLa pLuc 705 cells were replated at a density of 5×10^3 cells/well in 96 well plates. The next day, the cells were transfected with 1 μM Fam–PNA or Fam–$Asp_{(12)}$–PNA and with 1 μg PEI or Lipofectamine in the medium. The cells were incubated for 6, 12, and 24 h, washed twice with PBS, and fixed with 10% formalin for 5 min. After the cells were washed with PBS, the nuclei were stained with 10 μg/mL Hoechst 33342 for 5 min in the dark. Stained cell images were captured using a fluorescent microscope (IX51) with a DP controller (Olympus Optical, Tokyo, Japan).

2.8. Confocal microscopy imaging

Exponentially growing cells were plated at a density of 5×10^3 cells/well in an 8 well μ-slides (ibidi, Munich, Germany) on the day before transfection. The cells were incubated with 200 μL/well of media, including 1 μM Fam–PNA or Fam–$Asp_{(12)}$–PNA and 1 μg PEI or Lipofectamine for 24 h. The cells were washed twice with PBS, fixed with 10% formalin for 5 min, and the nuclei were stained with 10 μg/mL Hoechst 33342 for 5 min. Stained cell images were analyzed using a Deltavision RT confocal system equipped with an argon laser (excitation and absorbance wavelengths, 528 nm and UV, respectively; Applied Precision, Issaquah, WA, USA) connected to an Olympus IX71 microscope (oil immersion 60 × 1.4 NA objective; Olympus). The Imaris software package (Applied Precision) was used for image acquisition and processing.

2.9. Cell viability assays

We used the MTT assay to assess cell viability. HeLa pLuc 705 cells were seeded at a density of 1.2×10^4 cells/well in 96 well plates and incubated for 1 day. After 24 h, PNAs or the PNAs/PEI complex, and PEI or Lipofectamine were added at 100 μL/well in medium and incubated for 1 day at 37 °C. After the incubation, 2 mg/mL MTT solution