Supporting Information

Utilizing the Bioorthogonal Base-Pairing System of L-DNA to Design Ideal DNA Nanocarriers for Enhanced Delivery of Nucleic Acid Cargos

Kyoung-Ran Kim, Taemin Lee, Byeong-Su Kim, and Dae-Ro Ahn*

MATERIALS AND METHODS

Synthesis and purification of oligonucleotides. The D-DNA oligonucleotides were synthesized by IDT DNA technologies (USA). The chemical synthesis of all the L-DNA oligonucleotides was performed on the 1.0 μmol scale on a Mermaid-4 DNA/RNA synthesizer by using standard phosphoramidite chemistry. The phosphoramidite building blocks of the natural nucleosides from Proligo and those of L-nucleosides were from ChemGenes, respectively. 3’-Fluorescein-modifier and 3’-amine-modifier CPG solid supports were purchased from Glen Research. Solvents and reagents used for the synthesis were purchased from Proligo and prepared according to the manufacturer’s indications. The oligonucleotides were cleaved from the resin and deprotected in 33% NH$_3$ solution at 55°C overnight (16 h). After evaporation the crude samples were filtered, taken up in water and purified by 10% denaturing gel electrophoresis followed by ethanol precipitation as described in the literature [1]. Concentrations of the oligonucleotide solutions were determined by UV absorption at 260 nm. The extinction coefficients of the L-oligonucleotides were supposed to be identical to the natural oligonucleotides and were calculated using the OligoAnalyzer 3.1 (www.idtdna.com). The concentrations of fluorescein-labeled S1 strands were estimated by UV absorption at 490 nm.

Preparation of DNA tetrahedral (Tds). The DNA Tds with or without AS1411 aptamer
were assembled by mixing four strands (250 nM each strand) in TM buffer (10 mM Tris-HCl, 5 mM MgCl₂, pH 8); S1, S2 (or S3-Ap), S3 (or S3 -Ap), S4 (or S4 -Ap). The mixture was denatured by heating to 95°C and annealed by cooling to 4°C using a PCR machine (Applied Biosystems, USA).

**Gel electrophoresis.** Non-denaturing polyacrylamide gels (6%) were run in TBE buffer with 100V at 4°C for 40 min. After electrophoresis, the images were visualized using a fluorescence scanner (Typhoon9400, GE healthcare, USA)

**Dynamic light scattering (DLS).** The hydrodynamic sizes of the DNA Tds were measured with Zetasizer (Malvern, UK) by following a literature procedure [2]. The concentration of samples used for the DLS analysis was 250 nM.

**AFM imaging.** The 100 μL of each DNA samples with specific concentration in buffer solution were spin-coated on a freshly cleaned Si wafer (10 mm × 10 mm) at 2000 rpm for 60 s. DNA Td samples were imaged in a tapping mode on Dimension AFM instrument (Dimension D3100, Veeco, USA) in air, using 0.01 – 0.025 Ohm·cm antimony (n) doped Si probes having a resonance frequency in the range of 332 – 376 kHz (Veeco-TESP). AFM data were processed with NanoScope 7.20 software.

**Transfection of DNA nanoconstructs into HeLa and NIH3T3 cells.** HeLa cells and NIH3T3 cells were plated in glass bottomed 35 mm dishes with DMEM medium (Gibco, USA) containing 10% heat inactivated fetal bovine serum, 1% penicillin and streptomycin. After 25000 cells were seeded in each dish, the dishes were incubated at 37°C, in humidified atmosphere containing 5% CO₂. The growth medium was removed from each cell sample,
and the cells were washed twice with PBS (Gibco, USA). Each transfection mixture was made using Tds (250 nM) in the fresh medium (250 μL) without serum and antibiotics. The final concentration of DNA samples was 10 nM.

**Microscopic imaging.** The nuclei were stained using Hoechst 34580 (3 μg/mL, invitrogen, USA) and the cells were washed with PBS (200 μL) twice. The cell culture medium (200 μL) was then added. Live cells were imaged using a fluorescence microscopy (LSM 700, Carl Zeiss Microscopy, Germany). Excitation/emission filters used for fluorescein and Hoechst 34580 were 480-500/509-547 nm, and 340-380/432-482 nm, respectively.

**Flow cytometry analysis.** HeLa cells and NIH-3T3 cells were seeded on 24-well culture plates at a density of 10⁵ cells/mL and cultured for 24 h and then washed twice with PBS. They were incubated with the fluorescently labeled DNA molecules by using the same method adopted for the transfection experiment, harvested, and washed three times with PBS. Then, 200 μL of trypsin replacement (TrypLE™, Gibco, USA) was added to each sample, and the samples were incubated for 5 min at 37°C. Then 1 mL of the medium was added to each sample, and the resulting cell suspensions were transferred to conical tubes (Falcon™ tubes, BD Biosciences, USA) and centrifuged for 2 min at 1200 rpm. Supernatant was removed, and the cell pellets were resuspended in 1 mL of PBS. Fluorescence intensity of the cells was estimated by flow cytometry (FC500, Beckman coulter, USA). Samples of at least 1000 cells were analyzed in triplicate.

**Nuclease resistance.** For the stability test, 10% mouse serum (10 μL, Sigma Aldrich, USA) were added to the DNA solutions (90 μL, 900 nM), and the mixture were incubated at 37°C. At each point, the solutions were quenched by adding the stop solution composed of 98%
deionized formamide, 10 mM EDTA, 0.5 mg/ml bromophenol blue and xylene cyanole, and then analyzed on a denaturing 12% PAGE (7M urea). The amount of undamaged DNA structures was estimated by visualization of fluorescein-labeled S1 on a fluorescence scanner (Typhoon9400, GE Healthcare, USA).

**Endocytosis assays.** HeLa cells (2×10^5 cells/well) were seeded with DMEM media (2 mL) on a 6-well plate and incubated overnight at 37°C in humidified atmosphere containing 5% CO₂. For the treatment of inhibitors, the media were replaced with the fresh medium (2 mL) containing amiloride (2 mM, an inhibitor for macropinocytosis), chlorpromazine (10 μM, an inhibitor for clathrin-mediated endocytosis), or genistein (200 μM, an inhibitor for caveolae-mediated endocytosis). After 30 min, Td (10 nM) was added into the cell media and incubated at 37°C for 2 h. After washing with PBS, the cells were trypsinized, centrifuged and lysed in RIPA buffer, and fluorescence intensity of fluorescein labeled on Td was measured using an fluorescence microplate reader (Applikan™, Thermo Fisher Scientific, USA) for quantitation of intracellularly delivered Td via endocytosis. The amount of Td in cell lysates was normalized to the total cellular protein content of cells, which was determined by protein assay kit (BIO-RAD, USA). For the K⁺ depletion experiment, cells were depleted of potassium essentially as described by Larkin et al. [3]. The cells were incubated for 5 min at 37°C in hypotonic medium (culture medium/water, 1:1, v/v), followed by incubation in isotonic K⁺-free buffer (140 mM NaCl, 20 mM Hepes, pH 7.4, 1 mM CaCl₂, 1 mM MgCl₂, and 1 mg/mL glucose).

**Intracellular FRET analysis.** HeLa cells were plated in glass-bottomed 35 mm dishes with DMEM media (Gibco, USA) containing 10% heat inactivated fetal bovine serum, 1% penicillin and streptomycin. After 25000 cells were seeded in each dish, the dishes were
incubated overnight at 37°C in humidified atmosphere containing 5% CO₂. The growth medium was removed from each cell sample, and the cells were washed twice with PBS (Gibco, USA). D-Td and L-Td labeled with dual fluorophores (FAM-Td-TMR) were assembled by mixing S1, S2, S3 and S4-TMR. The mixtures were denatured by heating to 95°C and annealed by cooling to 4°C using a PCR machine. The transfection was performed by using the same manner adopted for the uptake experiment. Final concentration of DNA samples was 100 nM. The cells were washed twice by PBS, and incubated with fresh cell culture medium (200 μL) at 37°C, and imaged at different time points (0, 4, 8, 24 and 48h) using excitation/emission filters such as donor/donor (FAM/FAM), donor/acceptor (FAM/TMR), and acceptor/acceptor (TMR/TMR) in a fluorescence microscopy (Delta Vision). The FRET efficiencies were calculated by using ImageJ as described in the literature [4].

Subcellular localization of Tds. Subcellular localization of Tds was studied by staining selected cellular organelles such as lysosome, mitochondria, and nuclei with Lysotracker Deep Red (100 nM, Invitrogen, USA), MitoTracker Orange CMTMRos (100 nM, Invitrogen, USA), and Hoechest34580 (3 μg/mL, Invitrogen, USA), respectively. After transfection with Tds, the NIH-3T3 cells were treated with the staining reagents and examined by a confocal fluorescence microscopy (LSM 700, Carl Zeiss Microscopy, Germany).

Cell cycle analysis. NIH-3T3 cells were harvested, washed with PBS, re-suspended in 1.5 mL cold PBS, and permeabilized with 3.5 mL cold ethanol at -20°C for overnight. Fixed cells were stained with propidium iodide solution (0.1 mg/mL, PBS containing 0.06% Triton-X) at 37°C for 45 min before analyses on a flow cytometer (guava easyCyte, Millipore).
Cell viability assays with DNA Tds. Cytotoxicity of Tds was estimated using the MTT assay. Briefly, $8 \times 10^3$ HeLa cells and NIH-3T3 cells were seeded with media (100 μL) in 96-well plates and cultured overnight to reach ~80% confluency. The cells were then incubated in the fresh media containing 10% FBS, 1% antibiotics, and DNA molecules at 37°C for 6 h in the CO$_2$ chamber. To examine the multivalent effect, the monomeric aptamer (AS1411) was also incubated at same conditions. Then, we washed the cells twice using DPBS, and incubated them in the freshly replaced media for 48 h at 37°C, in humidified atmosphere containing 5% CO$_2$. Thiazolyl blue tetrazolium bromide (MTT, TACS, Germany) solution (10 μL) was added to each well, followed by 4 h incubation at 37°C. Next, cells were lysed with 200 μL of dimethyl sulfoxide (DMSO, Sigma-Aldrich, USA). After overnight incubation at room temperature, the absorbance was measured at 580 nm using a microplate reader (SpectraMax™ Plus, Molecular Devices, USA).

Table S1. DNA sequences used in this study. L-DNA sequences are underlined and the linker parts are expressed with the italic style

<table>
<thead>
<tr>
<th>DNA</th>
<th>Sequence</th>
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<tr>
<td>S1 (D)</td>
<td>ACATTCTAAGTCTGAAAACATTACAGCTTGGCTACACGAGAAGGCCCATAGTA-fluorescin</td>
</tr>
<tr>
<td>S2 (D)</td>
<td>${\text{TATCCCGCGCGACGGAGGTGAATGCGAGGGCAGGCGCTCTTC-NH}_2}$</td>
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| S3 (D) | $\{\text{TCAAGGTTGAGAATGTCTCTGGTTATGGACAGGCTTACATCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTY
S3 (D)- Ap GGTGGTGTTGGTTGGTGGTGGTGGTTTTTTCAACTGCCTGGTGATAAAACGACACTACGTGGG
S4 (D)- Ap GGTGGTGTTGGTTGGTGGTGGTGGTGGTTTTTTTCAGACTTAGGAATGTGCTTCCCACGTAGTGTCGTTTGTATTGGACCCCTCGCAT-NH₂
S2 (L)-Ap GGTGGTGTTGGTTGGTGGTGGTGGTGGTTTTTTATCACCAGGCAGTTGACAGTGTAGCAAGCTGTAATAGATGGCAAGGCTCCAATACTT-NH₂
S3 (L)-Ap GGTGGTGTTGGTTGGTGGTGGTGGTGGTTTTTTCAACTGCCTGGTGATAAAACGACACTACGTGGG
S4 (L)-Ap GGTGGTGTTGGTTGGTGGTGGTGGTGGTTTTTTTCAGACTTAGGAATGTGCTTCCCACGTAGTGTCGTTTGTATTGGACCCCTCGCAT-NH₂

Figure S1. AFM images and the size distribution of (A) D- and (B) L-Td.
Figure S2. Analysis for intracellular distribution of Tds into (A) mitochondria, (B) nucleus, and (C) lysosome. (D) Cell cycle analysis after uptake of Tds.
**Figure S3.** Gel electrophoretic analysis for the stability of (A) D- and (B) L-Td in 10% mouse serum.
Figure S4. Intracellular FRET. HeLa cells were incubated for 0, 4, 8, 24, and 48 h after treatment with (A) D-Td or (B) L-Td dually labelled with fluorescein (donor) and tetramethylrhodamine (acceptor). (C) The normalized FRET efficiencies expressed with numbers in the microscopic images are displayed with blue bars (D-Td) and red bars (L-Td).
in the chart.

**Figure S3.** Gel electrophoretic analysis of D- and L-Td assemblies (200 nM) constructed by annealing in the presence or absence of the free AS1411 aptamer (200 nM).

**Figure S6.** DLS data of (A) Ap3-D-Td and (B) Ap3-L-Td.
Figure S7. The cytotoxicity of free AS1411 against (A) HeLa cells and (B) NIH-3T3 cells. The assay was performed by using the same manner adopted in the cell viability assays for the Td-treated cells.
Figure S8. (A) The correctly assembled portion (lane 1) was extracted from the gel and reloaded to examine whether the assembly state was retained (lane 2) (B) Flow cytometry analysis for the cellular uptake of DNA constructs including the refined portion of Ap3-D-Td into HeLa cells (top) or NIH-3T3 cells (bottom). The green and blue traces represent the cells treated with the unpurified structures and the refined portion of Ap3-D-Td, respectively. The cells treated with the Td without the aptamer attached are shown with red (D-Td) and magenta (L-Td) traces. The orange traces represent the cells treated with Ap3-L-Td. The control untreated cells are shown with black traces. The concentration of DNA constructs was 10 nM. (C) Cytotoxicity of D-Td (yellow up-triangles), unpurified Ap3-D-Td (blue squares), purified Ap3-D-Td (magenta squares), L-Td (red circles), Ap3-L-Td (green down-triangles), and the AS1411 aptamer (black circles).

