Supporting Information

Multivalent Comb-Type Aptamer-siRNA Conjugates for Efficient and Selective Intracellular Delivery

*Hyundong Yoo†, Hyosook Jung†, Seung An Kim, and Hyejung Mok**

Department of Bioscience and Biotechnology, Konkuk University, 120 Neungdong-ro, Gwangjin-gu, Seoul 143-701, Republic of Korea

Experimental details

1. Materials

MUC1 aptamer[1] (5′-dGdCdAdGdTdTdGdAdTdCdCdTdTdTdGdGdAdTdAdCdCdCdTdGdG-3′), Bcl-2 siRNA[2] (Sense, 5′-GCUGCACCUGACGCCCUUCdTdT-3′; Antisense, 5′-GAAGGGCGUCAGGUGCAGCdTdT-3′), MUC1 aptamer Bcl-2 siRNA (Sense, 5′-dGdCdAdGdTdTdGdAdTdCdCdTdTdTdGdGdAdTdAdCdCdCdTdGdG -GCUGCACCUGACGCCCUUCdTdT-3′; Antisense, 5′-GAAGGGCGUCAGGUGCAGCdTdT-3′), GFP siRNA[3] (Sense, 5′-GCAAGCUGACCCUGAAGUUdTdT-3′; Antisense, 5′-AACUUCAGGGUCAGCUUGCdTdT-3′), and MUC1 aptamer GFP siRNA (Sense, 5′-dGdCdAdGdTdTdGdAdTdCdCdTdTdTdGdGdAdTdAdCdCdCdTdGdG - GCAAGCUGACCCUGAAGUUdTdT -3′; Antisense, 5′-AACUUCAGGGUCAGCUUGCdTdT-3′) were purchased from Bioneer (Daejeon, Korea). LPEI (molecular weight, 2.5 kDa) was obtained from Polysciences, Inc (Warrington, PA, USA). Streptavidin-coated gold nanoparticles with a core diameter of 5 nm were purchased from Cytodiagnostics (Ontario, Canada). POPO-3, Roswell Park Memorial Institute (RPMI) 1640 medium, and penicillin/streptomycin (P/S) were obtained from Invitrogen (Carlsbad, CA, USA). Fetal bovine serum (FBS) was purchased from Gibco BRL (Grand Island, NY, USA). Cell counting kit-8 (CCK-8) was obtained from Dojindo Laboratories (Kumamoto, Japan). CaspACE colorimetric assay kit and Micro-BCA protein assay kit were purchased from Promega (Madison, WI, USA) and Pierce (Rockford, IL, USA), respectively.

2. Preparation of Comb-Apt-siRNA

To de-protect thiol groups, two types of thiol-functionalized antisense (AS) siRNAs (20 nmol) at both the 3′- /5′- ends and only at the 3′-ends were incubated with a dithiothreitol (DTT, 1M) reducing agent overnight at pH 8.0, respectively. The reactants were then purified via a desalting column (MWCO 7k) to remove the DTT. The resulting two types of AS siRNAs with de-protected thiol groups in 20 μL of high salt solution (2.7 M NaCl, 54 mM KCl, 162 mM Na_2HPO_4, 29.4 mM KH_2PO_4) were reacted with dithiobismaleimidoethane (DTME, 20 nmol) overnight, respectively. The prepared multimeric and dimeric AS siRNA was loaded onto 15% polyacrylamide gels, and gel electrophoresis was performed for 45 min at 180 V. The siRNA within the gels was stained with ethidium bromide and visualized using a UV-trans-illuminator.

To prepare the common aptamer-siRNA conjugates (Apt-siR), dimeric aptamer-siRNA conjugates (Di-Apt-siR), and multivalent comb-type aptamer-siRNA conjugates (Comb-Apt-siR), monomeric, dimeric, and multimeric AS siRNA in phosphate-buffered saline (PBS) solution at an RNA concentration of 1 μg/μL was annealed with aptamer-sense siRNA by heating at 95°C for 5 minutes and sequential cooling to 25 °C for 2 h in an Biometra thermocycler machine. For the preparation of monomeric siRNA (Mono-siR) and multimeric siRNA (Multi-siR), monomeric and multimeric AS siRNA in phosphate-buffered saline (PBS) solution at an RNA concentration of 1 μg/μL was also annealed with a sense strand of siRNA. The Apt-siR and Comb-Apt-siR were loaded on 15% polyacrylamide gel (45 min, 180 V), stained with ethidium bromide, and visualized using a UV-trans-illuminator.

3. Characterization of Comb-Apt-siRNA

For transmission electron microscopy (TEM) analysis, monomeric and multimeric antisense siRNAs were annealed with a biotin-labeled sense strand in PBS solution at 37°C for 1 h. After column purification to remove salts, the resulting solution was then incubated with streptavidin-coated gold nanoparticles at a biotin: gold nanoparticle molar ratio of 1:5 at room temperature for 30 min. To prevent binding of several biotin-labeled siRNAs per single streptavidin-coated gold nanoparticle, 5-fold molar excess of gold nanoparticles were incubated with RNAs. The resulting solution was loaded onto a carbon-coated grid (300 mesh, Ted Pella Inc, Redding, CA, USA) and then washed with diethylpyrocarbonate (DEPC)-treated water. Gold nanoparticles tethered on monomeric and multimeric siRNAs were visualized under a 200-kV field-emission transmission electron microscope (FE-TEM, JEOL ltd., Japan) in bright field mode.

To investigate the structure of multimeric antisense/sense-biotin siRNAs in the presence of LPEI (2.5k), gold-tethered multimeric siRNA were mixed with LPEI at a weight ratio of 1 for 20 min. The resulting solution was loaded onto a carbon-coated grid and then washed with DEPC-treated water. Gold-tethered multimeric siRNA/LPEI mixtures were visualized under a 200-kV FE-TEM in bright field mode.

4. Cell culture and intracellular uptake

MCF-7 cells (human breast cancer), HepG2 cells (human hepatocellular liver carcinoma), and A549 cells (human lung adenocarcinoma epithelial) were maintained in RPMI supplemented with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37°C in a humidified atmosphere of 5% CO_2. MCF-7 cells and HepG2 cells were plated on 4-well chamber slides at a density of 2×10^5 cells/well for 24 h prior to transfection. To visualize nucleotides, Apt-siR, Di-Apt-siR, and Comb-Apt-siR were stained with POPO-3 dye at room temperature according to the manufacturer's instructions. Briefly, 20 μg of siRNA was added to 20 μl of 100 μM POPO-3 dye and incubated for 1 h at room temperature. To remove free POPO-3 dye, POPO-3-labeled siRNAs were purified by conventional ethanol precipitation. Three kinds of POPO-3-labeled siRNAs (Apt-siR, Di-Apt-siR, and Comb-Apt-siR) were incubated with MCF-7 cells and HepG2 cells at a final RNA concentration of 0.18 μM for 4 h. After incubation, cells were washed twice with cold PBS and then fixed with 4% paraformaldehyde in PBS solution. Next, each sample was observed by confocal microscopy (FV-1000 spectral; Olympus, Japan).

To examine the concentration-dependent intracellular uptake, POPO-3-labeled Apt-siR and Comb-Apt-siR were administered to cells by using different concentrations of RNA (0.07, 0.15, 0.3, 0.6, and 1.2 μM) for 4 h. Next, the media were removed and cells were washed three times with a PBS solution, followed by cell lysis with 1% Triton X-100 in PBS solution. The cell lysates were centrifuged at 13,000 rpm for 5 min. Fluorescence intensity in the supernatant of each sample was measured using a spectrofluorophotometer (Molecular Devices, Sunnyvale, CA, USA) at excitation/emission wavelengths of 534/570 nm, respectively.

To examine time-dependent intracellular uptake, POPO-3-labeled Apt-siR and Comb-Apt-siR at an RNA concentration of 0.3 μM were administered to cells and incubated for different time periods (0, 0.5, 1, 4 and 6 h). After washing and lysing the cells, the fluorescence intensity in the supernatant of each sample was measured using a spectrofluorophotometer (Molecular Devices) at excitation/emission wavelengths of 534/570 nm, respectively.

For inhibition of intracellular uptake, POPO-3-labeled Comb-Apt-siR (0.3 μM) was administered to cells in solutions containing different types of inhibitors, including wortmannin (100 nM), nystatin (50 μg/mL), and chlorpromazine (30 μM), for 4 h. POPO-3 dye (0.3 μM) was also treated to cells in solutions containing each inhibitor for 4 h as a control. After washing and lysing the cells, intracellular fluorescence intensity was measured at excitation/emission wavelengths of 534/570 nm, respectively. POPO-3-labeled Comb-Apt-siRs without LPEI and those with LPEI at a weight ratio of 1 were treated to cells for 4 h at different RNA concentrations (0, 0.3, and 0.6 μM), respectively. After washing and lysing the cells, intracellular fluorescence intensity was measured at excitation/emission wavelengths of 534/570 nm, respectively.

5. Transfection and gene suppression

Green fluorescence protein (GFP)-expressing A549 cells (A549-GFP cells) were plated on a 24-well plate at a density of 5×10^4 cells/well for 24 h prior to transfection. Common GFP siRNA without aptamer (Mono-siR), multimerized GFP siRNA without aptamer (Multi-siR), Apt-siR, and Comb-Apt-siR were administered to cells at an RNA concentration of 144.6 nM after mixing with LPEI 2.5k at a weight ratio of 1 in the presence of serum-containing media for 24 h. Three types of GFP RNA conjugates (Apt-siR, Di-Apt-siR, and Comb-Apt-siR) were also treated to A549-GFP cells in the absence of LPEI 2.5k at an RNA concentration of 144.6 nM in serum condition. After further incubation for 24 h, the extent of GFP gene silencing was quantitatively analyzed using a spectrofluorophotometer (Molecular Devices) with excitation and emission wavelengths of 488

and 507 nm, respectively. The relative GFP expression level in cells transfected with each sample was then calculated as a relative percentage of GFP gene expression compared to non-transfected A549-GFP cells (control): %GFP expression = (GFP intensity in sample/GFP intensity in control) × 100.

6. Cell viability assay

MCF-7 cells and HepG2 cells were plated on a 96-well plate at a density of 5×10^3 cells/well for 24 h prior to transfection. Mono-Bcl-2 siRNA (Mono-siR), multi-Bcl-2 siRNA (Multi-siR), mono-apt-Bcl-2 siRNA (Apt-siR), and multi-apt-Bcl-2 siRNA (Comb-Apt-siR) were administered to cells at an RNA concentration of 250 nM after mixing with LPEI 2.5K at a weight ratio of 1 in the absence of serum for 5 h. After incubation, the medium was changed and replaced with fresh RPMI medium supplemented with 10% FBS, and cells were further incubated for 3 days. Cell viability was determined using the CCK-8 assay, according to the manufacturer's protocol. In addition, four types of control GFP siRNAs including Mono-siR, Multi-siR, Apt-siR, and Comb-Apt-siR were also administered to cells at the same RNA concentration of 250 nM after mixing with LPEI 2.5K at a weight ratio of 1 in the absence of serum for 5 h. Free aptamer was also treated to cells at the concentration of 250 nM. Cell viability was assessed by CCK-8 assay.

7. Caspase-3 activity

MCF-7 cells were plated on a 6-well plate at a density of 2.5×10^5 cells/well for 24 h prior to transfection. Monomeric Bcl-2 siRNA (Mono-siR), multimuric Bcl-2 siRNA (Multi-siR), monomeric aptamer-Bcl-2 siRNA (Apt-siR), and multimeric aptamer-Bcl-2 siRNA (Comb-Apt-siR) were administered to cells at an RNA concentration of 250 nM after mixing with LPEI 2.5K at a weight ratio of 1 in the presence of serum-containing media for 24 h. Next, cells were further incubated in fresh serum-containing media for 24 h. For the caspase-3 assay, cells were collected, washed with ice-cold PBS, and lysed with cell lysis buffer (CaspACE colorimetric assay kit, Promega). Complete cell lysis was ensured by freezing and thawing three times and incubation on ice for 15 min. To precipitate cell debris, samples were spun by centrifugation at 15,000 × *g* for 20 min at 4°C. The caspase-3 activity in the supernatant was measured using a spectrophotometer at 405 nm with DEVD-*p*-nitroanilide (DEVD-pNA) as a substrate, according to the manufacturer's instructions. The amount of total protein in cell lysates was measured with a BCA protein assay kit (Pierce). Liberated pNA was normalized to the amount of total protein in cell lysates.

References

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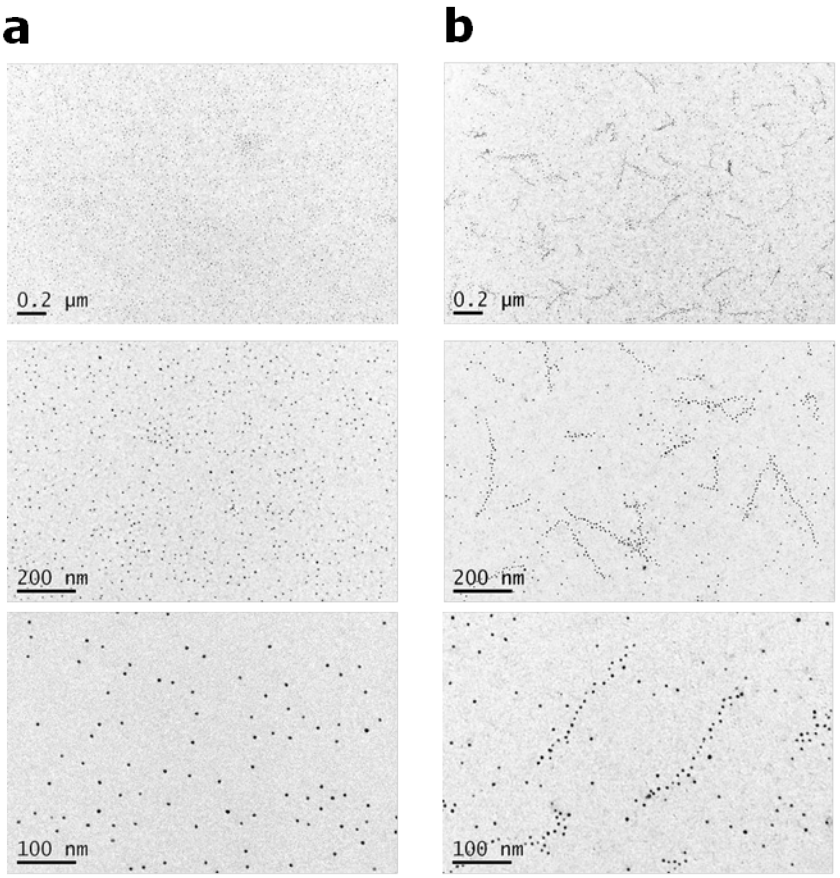
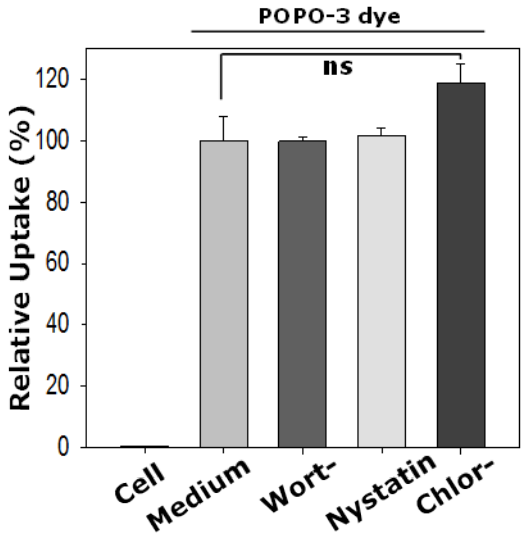
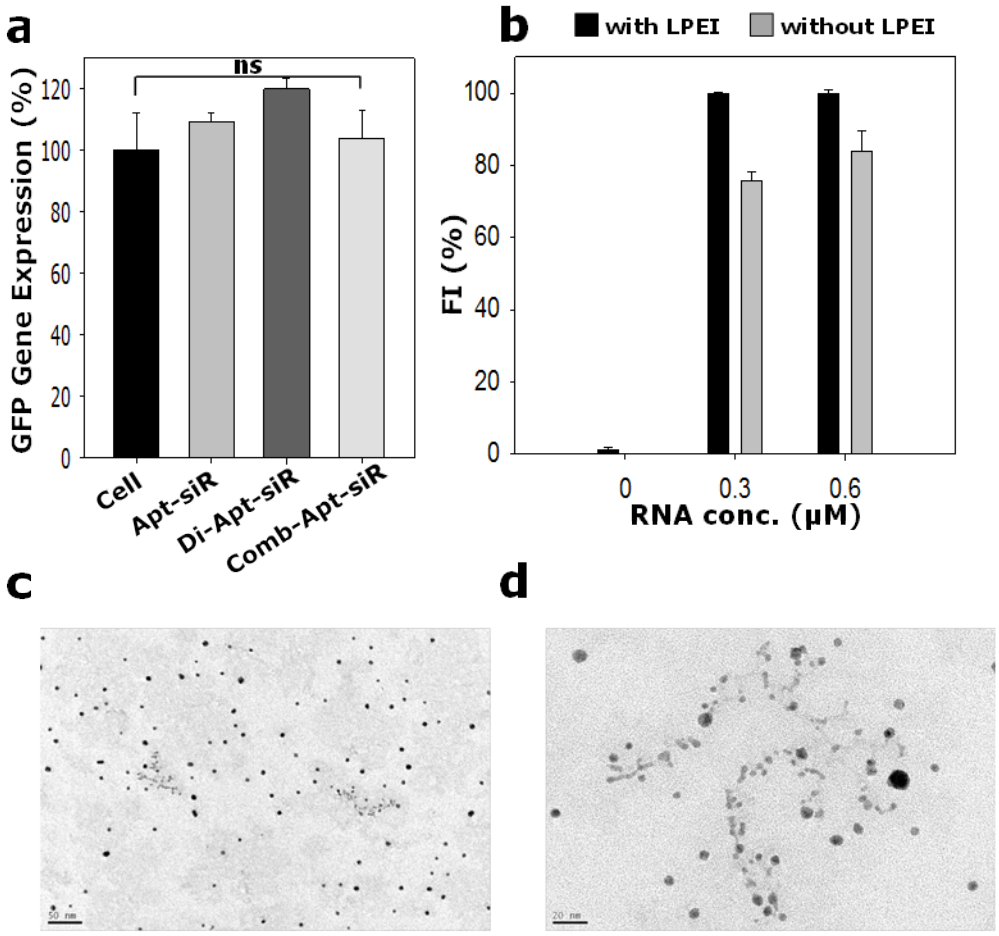
(3) Mok, H.; Lee, S. H.; Park, J. W.; Park, T. G. *Nat Mater* **2010**, *9*, 272.



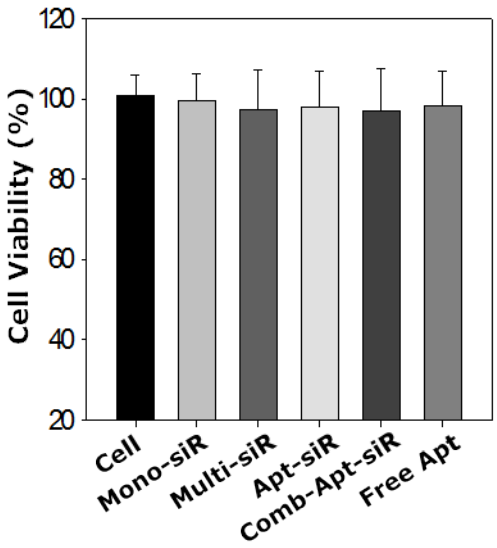
Supplementary Figure 1. TEM images of (a) monomeric AS/biotin-S and (b) multimeric AS/biotin-S double strand siRNAs coated with streptavidin-gold nanoparticles.



Supplementary Figure 2. Inhibition assay of cellular uptake by chemical inhibitors after treatment of POPO-3 dye alone to MCF-7 cells (ns=not significant).



Supplementary Figure 3. (a) The extent of GFP gene expression for A549 cells after transfection with three types of apt-siRNA conjugates in the absence of LPEI 2.5K (ns=not significant). (b) Fluorescence intensities (FI) (%) in MCF-7 cells after incubation with POPO-3-labeled Comb-Apt-siR with or without LPEI (2.5K) at different RNA concentrations. (c-d) TEM images of multimeric AS/biotin-S double strand siRNAs coated with streptavidin-gold nanoparticles with LPEI (2.5K).



Supplementary Figure 4. Cell viability after transfection with four types of GFP siRNA conjugates with LPEI 2.5k at a weight ratio of 1. Free MUC1 aptamer was also used as a control.