Catalytic Self-assembly of DNA Dendritic Complex for Efficient Gene Silencing

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Supporting Information

Reagents

The sequences of oligonucleotides used in this paper are listed in Table S1. DNA synthesis reagents were purchased from Glen Research (Sterling, VA). A solution of 0.1 M triethylamine acetate (pH 6.5) was used as HPLC buffer A, and HPLC-grade acetonitrile from Oceanpak (Sweden) was used as HPLC buffer B. 1×TAE/Mg^{2+} buffer (40 mM Tris-acetic acid, 1 mM EDTA and 12.5 mM magnesium acetate, balanced to pH 7.2) was used for all self-assembled reactions. Stainsall was obtained from Sigma Aldrich (Shanghai, China). Lipofectamine™ RNAiMAX was obtained from Invitrogen. Complete medium (RPMI 1640 and DMEM) with 10% fetal bovine serum and penicillin (100 U/ml) -streptomycin (100ug/ml) were used for cell culture. Washing buffer contained glucose (4.5 g L^{-1}) and $MgCl_2$ (5 mM) in Dulbecco's phosphate buffered saline (D-PBS; Gibco). CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega) was used for cytotoxicity study. All other chemicals were obtained from Dingguo Changsheng Biotechnology Co. Ltd. (Beijing, China) and used without further purification. Milli-Q water (resistance >18 MΩ cm) was used to prepare all solutions.

Equipment

HPLC (Agilent, 1260) equipped with C-18 reversed-phase column (5μm, 4.6×250mm, 100Å; GL Science, Inertsil ODS-3) was used, in addition to a Nano Drop 2000 spectrophotometer (Thermo Scientific), Bio-Rad molecular imager (ChemiDoc XRS+ with lab imaging software), confocal laser-scanning microscope (Olympus, FV1000), atomic force microscope (Veeco, Nanoscope IIIa), flow cytometer (BD FACSVerse), TC 10 automated cell counter (Bio-Rad), and microplate reader (BioTek).

DNA synthesis

DNA sequences were synthesized on the PolyGen DNA synthesizer. The synthesis protocol was set up according to the requirements specified by the reagents' manufacturers. Following on-machine synthesis, the DNA products were deprotected and cleaved from CPG by incubating with 2 mL of AMA (Ammonium Hydroxide and 40% methylamine, 1:1) for 30min at 65 °C in water bath. The cleaved DNA product was transferred into a 15 mL centrifuge tube and mixed with 200 μL of 3.0 M NaCl and 5.0 mL of ethanol, after which the sample was placed into a freezer at −20 °C for ethanol precipitation. Afterwards, the DNA product was spun at 4000 rpm under 4 °C for 30 min. The supernatant was removed, and the precipitated DNA product was dissolved in 400 μL of 0.1 M triethylamine acetate (TEAA) for HPLC purification. HPLC purification was performed with a cleaned C18 column on an Agilent 1260 HPLC machine. The collected DNA product was dried and processed for detritylation by dissolving and incubating in 200 μL of 80% acetic acid for 20 min. The detritylated DNA product was mixed with 20 μL of 3.0 M NaCl and 500 μL of ethanol and placed into a freezer at −20 °C for 30 min. Afterwards, the DNA product was spun at 14000 rpm under 4 °C for 5 min. The DNA product was dried by a vacuum dryer and resolved with ultrapure water, followed by desalting with desalting columns. The DNA products were quantified and stored in ultrapure water for subsequent experiments. The detailed sequences are given in Table S1.

Agarose gel electrophoresis

A 1% agarose gel was prepared with ethidium bromide. Reaction mixtures were eventually quantified in a volume of 8 μL to give a desired concentration. Then, to each sample, 1.6 uL of 6x loading buffer were added directly for electrophoresis experiments. Electrophoresis was carried out in 1× Tris-borate-EDTA (TBE) buffer (90 mM Tris, 90 mM boric acid, and 10 mM EDTA, pH 8.0) at 100 V for 50 min at room temperature. After stopping electrophoresis, gel was removed, and DNA bands were imaged and analyzed using a Bio-Rad molecular imager with imaging software under UV light.

AFM imaging of DNA dendrimer complex

10 µL of dendrimer complex and 5 µL of 30 mM Ni^{2+} were mixed on the surface of freshly cleaved mica. After the solution dried at room temperature, the mica was rinsed with Milli-Q water three times and dried in ambient air. Atomic force microscopy of samples was observed on a Multimode 8 (Bruker/USA) using ScanAsyst mode.

Cell culture

All cells were obtained from ATCC (American Type Culture Collection, Manassas, VA, USA). Cell lines CCRF-CEM (Human T-cell ALL), Ramos (Human B-cell Burkitt's lymphoma) and A549 cells (human lung adenocarcinoma epithelial cell line) were cultured in RPMI 1640 or DMEM medium supplemented with 10% fetal bovine serum (FBS, heat inactivated) and penicillin (100 U/ml) -streptomycin (100 µg/ml) in a cell culture incubator at 37 °C with 5% CO_2. Cell density was determined by using a hemocytometer. For adherent A549 cell lines, short-term (30 s–1 min) trypsin treatment was adopted to dissociate cells from culture flask or dish.

Selective binding assay

Selectivity of DNA dendrimer was studied using flow cytometry. Each sample used for binding assay contained 2×10^5 cells. Cells were incubated with 50 ul of 150 nM DNA dendrimer in a centrifuge tube at 37 °C for 2 h or 8 h and then washed and resuspended with DPBS. Cells were treated with trypsin and washed with DPBS to remove the membrane binding before fluorescent microscopy imaging or flow cytometric analysis.

Silencing assay using flow cytometer

A549 cells were plated in a 30 mm cell culture dish and grown to around 80-90% confluence for 24 h before experiments. After removing the culture medium and washing twice with DPBS, cells were incubated with 100 µl of 150 nM DNA dendrimer in DMEM medium without FBS for 8 h at 37 °C in 5% CO_2. Cells were then washed twice with 1 ml of washing buffer and incubated in DMEM medium with 10% FBS. After incubation for another 2 days, cells were washed with DPBS and detached with 400 µl of trypsin. Finally, the cells were washed and resuspended in 400 µl of DPBS for flow cytometric analysis on a BD FACSVerse™ flow cytometer.

Silencing assay using confocal laser-scanning microscopy imaging

A549 cells were plated in a 35 mm confocal dish and incubated for 24 h. After washing twice with DPBS, A549 cells were incubated with 100 μl of 150 nM DNA dendrimer in FBS-free DMEM medium for 8 h at 37 °C in 5% CO_2. Cells were then washed twice with 1 ml of washing buffer and incubated in DMEM medium with 10% FBS. After incubation for another 2 days, cells were washed with DPBS and subjected to confocal microscope imaging using a FV1000 confocal microscope (Olympus).

Western blot analysis

A549 cells were grown for 24 hours on 35 mm culture plates until about 80% confluence before further treatment. Cells were then treated with 100 ul of 150 nM DNA dendrimer in FBS-free culture medium. After 8-hour incubation, the culture medium was replaced by another 2 ml of fresh culture medium with 10% FBS for another two days. The process of total cellular protein harvesting was performed at 4°C by lysing cells for 20 min in 60 μl of lysis buffer (50 mM HEPES, pH 7.6, 250 mM NaCl, 0.1% NP-40, 5 mM EDTA, 1 mM PMSF, 10 mM N-ethylmaleimide, and 2 μg each of aprotinin, bestatin, and leupeptin/ml). The lysates were clarified by microcentrifugation for 20 min at 14000 g. Protein concentrations were determined with a spectrophotometer (NanoDrop 2000, Thermo Scientific). Total cellular proteins were separated on 5-10% SDS-PAGE with 1× running buffer and then electrotransferred to nitrocellulose membranes with transfer buffer at 400 mA for 90 min. The membranes were blocked with 5% nonfat dry milk in PBS buffer containing 0.2% Tween 20 (PBST) for at least 1 h at room temperature. The membranes were incubated overnight with GFP-Tag (7G9) mouse mAb in PBST containing 5% nonfat dry milk at 4 °C. After washing three times with PBST for 10 min, the membranes were incubated with peroxidase-conjugated goat anti-mouse IgG antibody in PBST containing 5% nonfat dry milk for 2 h. After washing with PBST and treating with imaging reagents, protein expressions were determined using a Bio-Rad molecular imager with imaging software.

Cytotoxicity

For cytotoxicity experiments, the number of A549 cells was counted with a hemocytometer. 5×10^3 cells were used for one sample. Cells were seeded in a 96-well plate in FBS-free medium and incubated at 37 °C for 24 h to ensure good adherence. Cells were incubated with concentration gradient of dendritic complex and cationic lipofectamine for 2 h in a cell culture incubator at 37 °C. Supernatant was removed from medium directly with a pipette, and 200 μl of fresh medium (with 10% FBS) were added into each well and incubated for 48 h in a cell culture incubator at 37 °C for further cell growth. The medium was directly removed. Each 20 μl of CellTiter reagent was diluted and added to 100 μl of fresh FBS-free medium. Then 100 μl of the diluted reagent were added to each well and incubated for 1-2 h in a cell culture incubator at 37 °C. A microplate reader was used to record the absorbance at 490 nm. Cell viability was determined according to the manufacturer's description.

Table S1. Sequences used in this experiment (5'-3').

I	AA-TGGCAGA-GATAGAT-AA-GAGTTTG
IF	AA-TGGCAGA-GATAGAT-AA-GAGTTTG-FAM
A1	CAAACTC-TT-ATCTATC-TCTGCCA-TT-TT-AA-TGCAATG-TCACGGT-AA-TGGCAGA-GATAGAT-AA-TGCAATG-TCACGGT-AA
A2	TCTGCCA-TT-ACCGTGA-CATTGCA-TT-TT-AA-GCTACAG-GACTACG-AA-TGCAATG-TCACGGT-AA-GCTACAG-GACTACG-AA
A3	CATTGCA-TT-CGTAGTC-CTGTAGC-TT-TT-AA-GTATCAG-ATCGCCG-AA-GCTACAG-GACTACG-AA-GTATCAG-ATCGCCG-AA
A4	CTGTAGC-TT-CGGCGAT-CTGATAC-TT-TT-AA-TGACCAA-ACCACCT-AA-GTATCAG-ATCGCCG-AA-TGACCAA-ACCACCT-AA
A5	CTGATAC-TT-AGGTGGT-TTGGTCA-TT-TT-AA-CTCCACT-CCTACTC-AA-TGACCAA-ACCACCT-AA
Sgc8c	ATCTAACTGCTGCGCCGCCGGGAAAATACTGTACGGTTAGA
cA5-Sgc8c	ATCTAACTGCTGCGCCGCCGGGAAAATACTGTACGGTTAGA-TT-AGGTGGT-TTGGTCA-TT-GAGTAGG

S11e	ATGCGAACAGGTGGGTGGGTTGGGTGGATTGTTCGGCTTCTTGAT
cA5-s11e	ATGCGAACAGGTGGGTGGGTTGGGTGGATTGTTCGGCTTCTTGAT--TT-AGGTGGT-TTGGTCA-TT-GAGTAGG
B2	AA-GCTACAG-GACTACG-AA-TGCAATG-TCACGGT-AA-GCTACAG-GACTACG-AA-TT-TT-ACCGTGA-CATTGCA-TT-ATCTATC
B3	AA-GTATCAG-ATCGCCG-AA-GCTACAG-GACTACG-AA-GTATCAG-ATCGCCG-AA-TT-TT-CGTAGTC-CTGTAGC-TT-ACCGTGA
B4	AA-TGACCAA-ACCACCT-AA-GTATCAG-ATCGCCG-AA-TGACCAA-ACCACCT-AA-TT-TT-CGGCGAT-CTGATAC-TT-CGTAGTC
B5	AA-TGACCAA-ACCACCT-AA-CTCCACT-CCTACTC-AA-TT-TT-AGGTGGT-TTGGTCA-TT-CGGCGAT
cB5-sense	AGGTGGTTTGGTCATT-AcAuGAAGcAGcACGACuU dTdT
Antisense	AAGUCGUGCUGCUUCAUGUdTdT

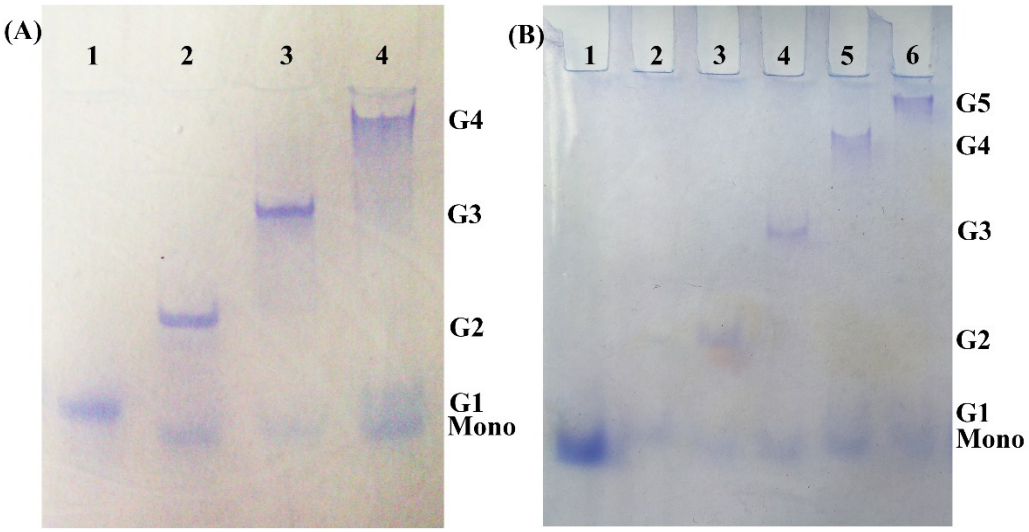

Figure S1. Polyacrylamide gel electrophoresis results of self-assembly of G4 and G5 DNA dendrimer. (A) 3.5% polyacrylamide gel, running at 70 V for 30 min. (B) 2% polyacrylamide gel, running at 70 V for 30 min. DNA bands were stained by Stainsall.

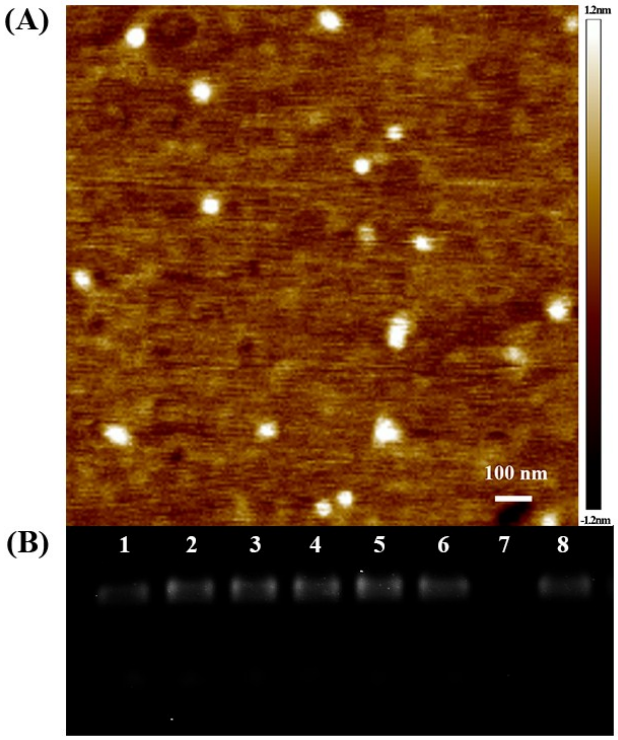

Figure S2. (A) AFM characterization of DNA dendrimers with high monodispersity: AFM image of G5 dendrimer with a wide visual field. (B) Stability of G5 DNA dendrimer (lane1). Lane 2: 1 mM GSH, lane 3: 2 mM GSH, lane 4: pH 6.0; lane 5: pH 7.0; lane 6: pH 8.0; lane 7: A3 monomer in DNase I; lane 8: G5 dendrimer in 0.2U DNase I.

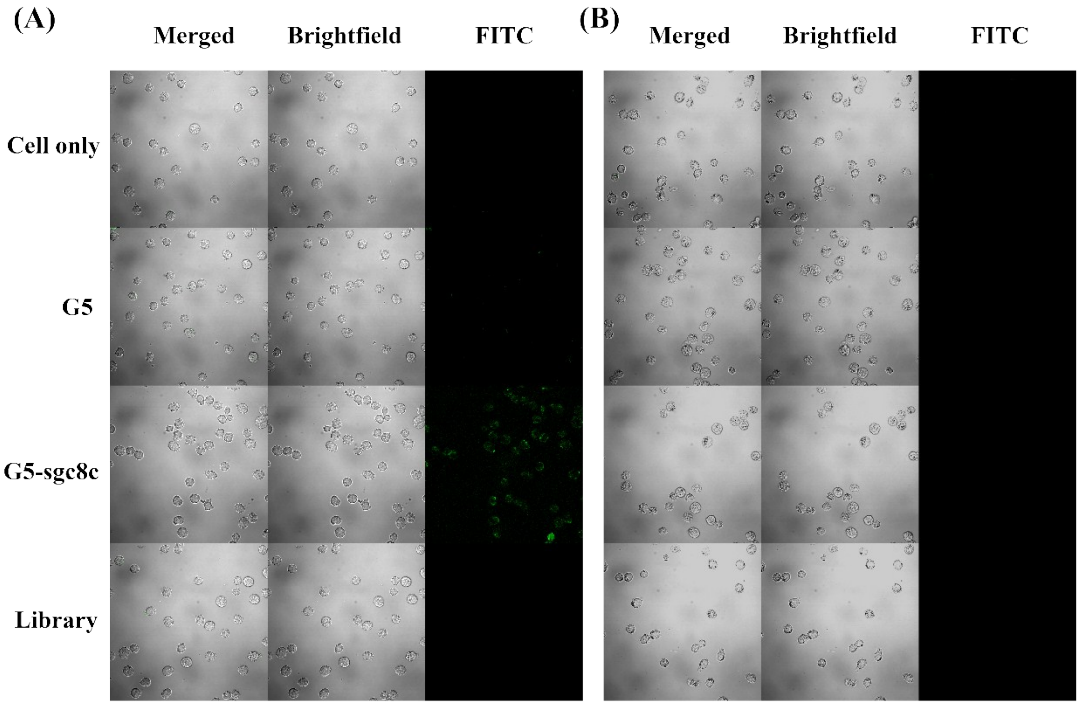

Figure S3. Confocal fluorescent imaging of selective cell targeting of DNA dendrimers. Benefiting from the high selectivity of sgc8c, G5-sgc8c complex could bind to target CEM (A), but not nontarget Ramos (B), cells, giving obvious fluorescent signal. In contrast, G5 complex without aptamer couldn't been uptaken by cells, hence no fluorescence was observed.

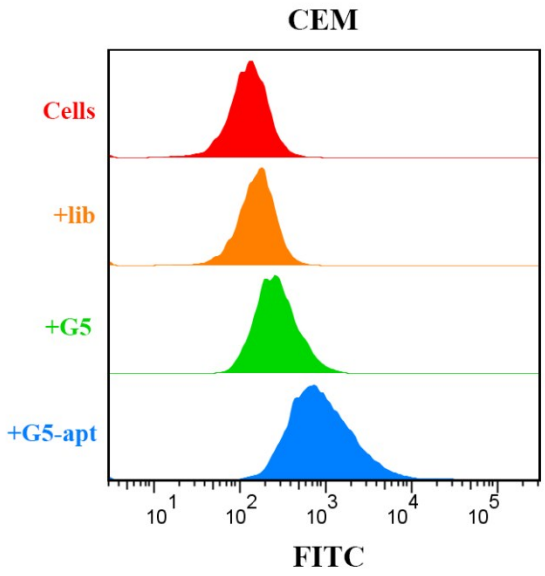

Figure S4. Flow cytometric analysis of G5-sgc8c complex incubated with CEM cells for 8 hours. A large shift was observed after incubating CEM cells with G5-sgc8c complex for 8 hours. In contrast, only a very small shift was observed after incubating CEM cells with G5 without aptamer, which demonstrated the high cell targeting ability of G5-aptamer complex.

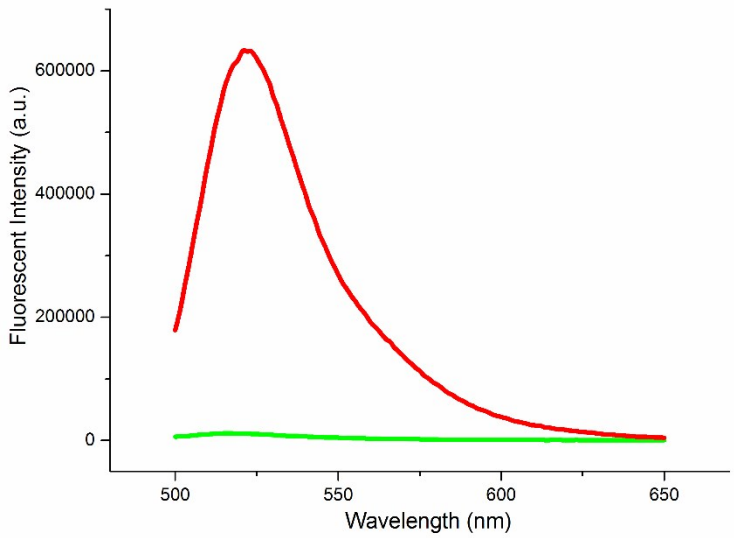

Figure S5. Endocytosis efficiency determined by lysing the cells and quantifying fluorescence

signal. The red line indicates the fluorescent intensity of FITC-labelled dendrimer complex, marked as $I_{dendrimer}$. The green line indicates the fluorescent intensity of CEM lysate solution in the same volume after incubating with dendrimer, trypsin treatment and cell lysing, marked as I_{cell}. The efficiency was estimated to be 1.8±0.1% by three independent assays using a formulae: Efficiency= $I_{cell}/I_{dendrimer}$.

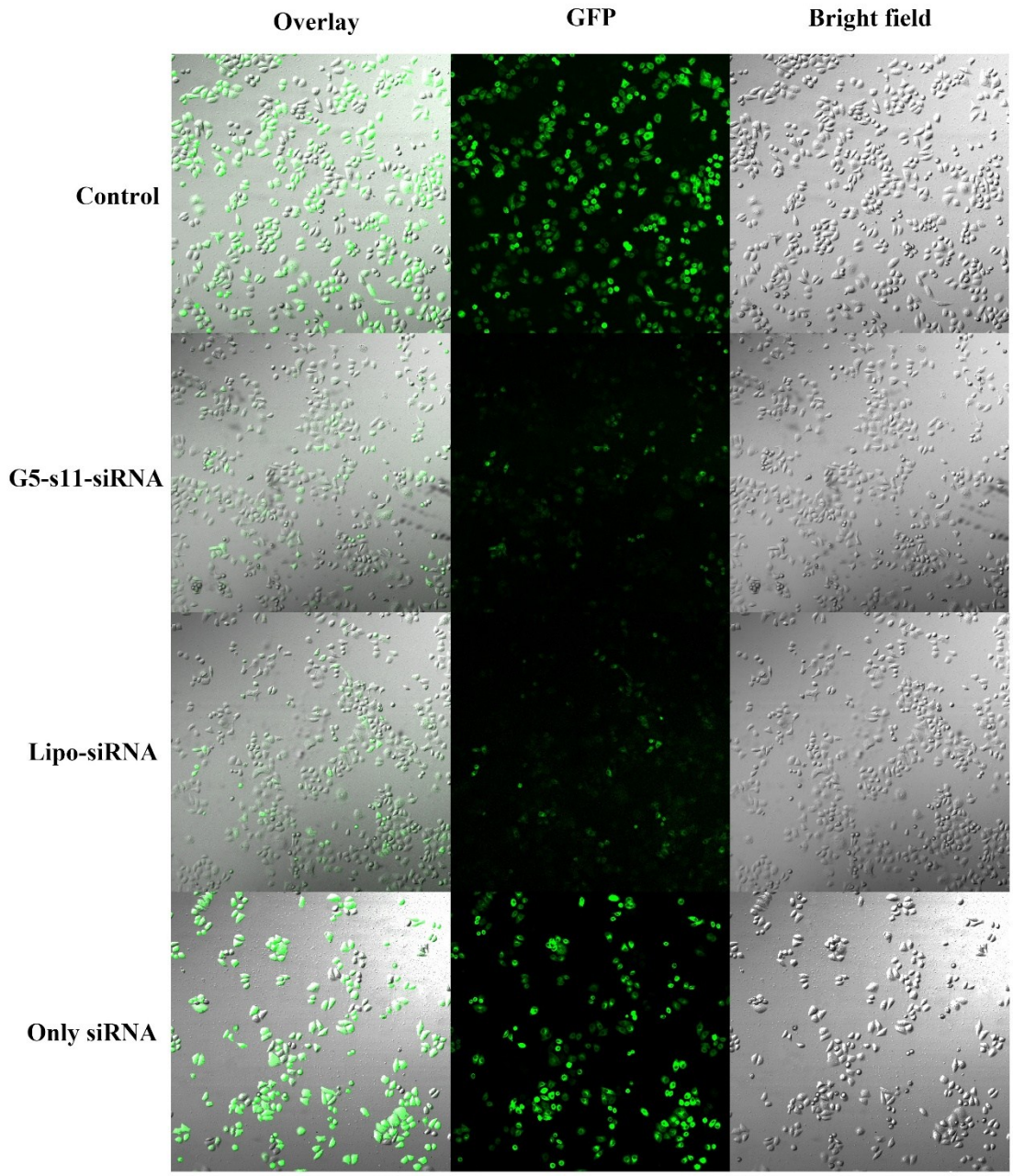

Figure S6. DNA dendrimer was used for efficient gene silencing. A549G cells showed strong GFP fluorescent signal without dendrimer treatment. After treatment with G5-s11e-siRNA

complex or Lipofectamine-siRNA complex and incubation for 2 days, siRNA-induced gene silencing led to decreased fluorescent intensity resulting from significantly reduced GFP expression. Because of the negative backbone, siRNA without carrier can't traverse the cell membrane or block the expression of GFP.

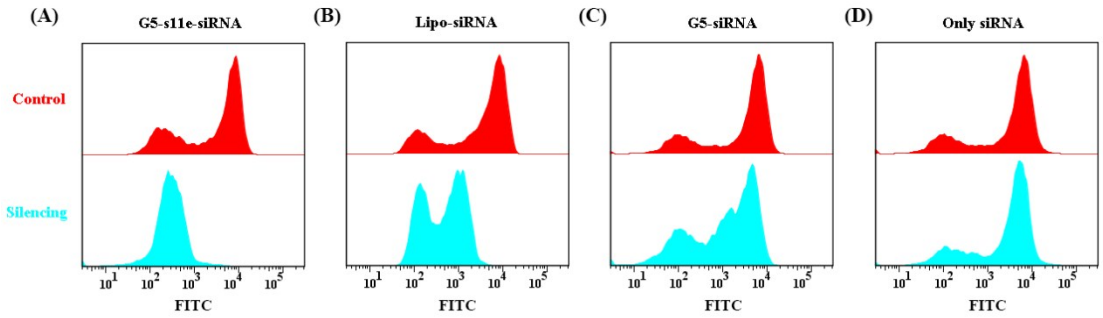

Figure S7. Flow cytometric results of siRNA-based gene silencing. (A) DNA dendrimer with aptamer as siRNA carrier for gene silencing. (B) Lipofectamine™ RNAiMAX as siRNA carrier for gene silencing. (C) DNA dendrimer without aptamer as siRNA carrier for gene silencing. Without the help of aptamer, G5-siRNA complex shows a poor silencing efficiency. (D) SiRNAs without carriers were incubated with cells. Because of the negative backbone, siRNA can't traverse the cell membrane or block the expression of GFP.

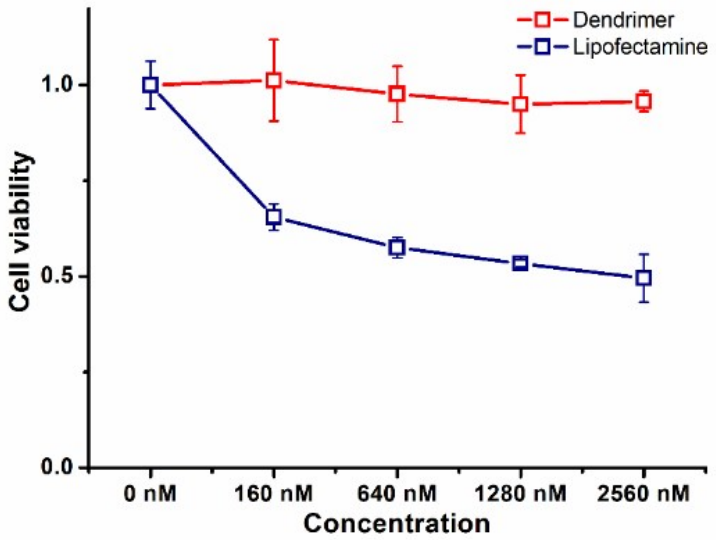

Figure S8. Cytotoxicity results of DNA dendrimer and lipofectamine when delivering different concentrations of siRNA. Red line: DNA dendrimer as siRNA carrier for gene silencing. Blue line: Lipofectamine™ RNAiMAX as siRNA carrier for gene silencing. For a reasonable comparison,

DNA dendrimers and lipofectamines carrying the same amount of siRNA were used for each group of samples.