Electronic Supplementary Information (ESI)

DNA nanoclew templated spherical nucleic acids for siRNA delivery

Weimin Ruan[a], Meng Zheng*[a], Yang An[b], Yuanyuan Liu[a], David Lovejoy[c], Mingcong Hao[a], Yan Zou[a], Albert Lee[c], Shu Yang[c], Yiqing Lu[a], Marco Marsh[c], Roger Chung[c] and Bingyang Shi*[a]

a. International Joint Center for Biomedical Innovation, School of Life Sciences, Henan University, Kaifeng, Henan, 475004, P. R. China.

b. School of Basic Medical Sciences, Henan University, Kaifeng, Henan 475004, P.R. China.

c. Motor Neuron Disease Research Centre, Department of Biomedical Sciences, Faculty of Medicine and Health Science, Macquarie University, NSW, 2119, Australia

* Corresponding Author: mzheng@henu.edu.cn and bs@henu.edu.cn

Experimental procedures Section:

1. Preparation of DNA nanoclew (DC)

DNA oligos were synthesized by GENEWIZ Inc. (Beijing, China). T4 DNA ligase and ϕ29 DNA polymerase were purchased from New England BioLabs Inc. (Ipswich, MA, USA). siRNA duplexes labeled at the 5' end of the sense strand with either Cy3 or Cy5 dyes were obtained from Genepharma Inc. (Shanghai, China). DNA rolling-circle amplification were performed using a modified protocol.[1] Briefly, 5' phosphate-ssDNA template was hybridized with primer in 1×T4 ligase buffer, heated at 95°C for 4 min, followed by keeping at room temperature for at least 4 h. T4 ligase was added into the mixture to ligase the click. The reaction mixtures were precipitated (phenol-chloroform extraction followed by 75% ethanol washing) and dissolved in ddH_2O. Template at final concentration of 500 nM under ϕ29 DNA polymerase is used in performing RCA for 12 h, followed by heat inactivation at 70 °C for 15 min. Then the reaction mixture was washed with ddH_2O, and separated by centrifuge at 16000×*g*. The wash-centrifuge procedure was repeated until the OD260/280 reached above 1.80. DC concentration and purity was measured by Nanodrop 2000 spectrometer (Thermo Scientific).

DC Template: 5'- Phosphate -CGCCACTGATTTCACCGCTTCAAGCTAGATGC ATCTAGCAATGGGCACGGGCAGCTTGCCGGTGGAAGCTAGATGCATCTAG CAAGCGC

DC primer: ATCAGTGGCGGCGCTTG

2. Synthesis of DC-siRNA

DNA nanoclew (DC) particle and 20 nt overhang tailored siRNA were mixed together at different weight ratio (15:1, 10:1, 5:1, 2.5:1, 1:1, 1:2.5, 1:5) in 1× TM buffer containing 10 mM Tris and 5 mM Mg^{2+}. The solution was heated to 95 °C for 5 min and rapidly cooled on ice to generate DC-siRNA SNA nanoparticles. All siRNAs were chemically modified with site-specific 2′-OMe to improve nuclease resistance. The optimum ratio of DC to siRNA was determined by native PAGE electrophoresis of

SNA nanoparticles using 8% PAGE gel (Bio-Rad) with 0.5× TBE buffer followed by silver staining. Additional overhang sequences was attached on the 3' end of anti-sense strand, while the sequence is complementary to RCA template oligonucleotides. The siRNA sequences (GL-3 siRNA) against luciferase and control sequences (Scramble siRNA) as follows:

GL-3 Luc：Sense: 5' -(Cy3 or Cy5)- AAcGcuGGGcGuuAAucAAdTdT - 3'
Antisense: 5'- UUGAUuAACGCCcAGCGUUdTdT -overhang-3'
(overhang, GGGCACGGGCAGCTTGCCGG)

Scrambled siRNA (Negative Control):
Sense: 5' -UUcUCCGAAcGuGUCAcGUdTdT-3'
Antisense: 5'-AcGuGACACGuuCGGAGAAdTdT-overhang-3'
(overhang, GGGCACGGGCAGCTTGCCGG)

3. Characterization of DC-siRNA

To examine the morphology of DC and DC-siRNA, the DC and DC-siRNA solutions were adjusted to 10 ng/μl and deposited on silicone matrices, then dried in oven at 70 °C and coated with Au, followed by observation on an S-7100 scanning electron microscope (HITACHI, Japan). We also measured the hydrodynamic size of DC and DC-siRNA by a Zetasizer Nano ZS (Malvern, UK). The fluorescent images of DC-siRNA were shot by confocal microscopy imaging (Leica TCS SP8), followed by treating with HyVolution.

4. Cell Culture

HeLa or HeLa cells expressed with luciferase (HeLa-Luc cells) were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% (v/v) FBS and 1% Pen/Strep, in a 37 °C incubator (Thermo Scientific) under 5% CO_2.

5. Cellular Uptake

The take up ability of HeLa cells to DC-siRNA nanoparticles was measured by confocal imaging and flow cytometry assay, respectively. Free siRNA was used as a control in both measurements. For confocal imaging, HeLa cells were seeded at a density of 1×10^4 cells/well in 8-units-chamber-slide (Nunc, Thermo-fisher). The next day, DC-siRNA nanoparticles (siRNA was labeled by Cy5) were added into serum-containing medium at a final siRNA concentration of 200 nM. After 12 hours' incubation, the cells were washed with PBS plus 0.01% Tween20 and replaced with fresh growth medium. Images were taken at 12 h after transfection by fluorescence confocal microscope (Zeiss, 710). For flow cytometry assay, HeLa cells were incubated with DC-siRNA nanoparticles at a siRNA concentration of 200 nM in growth medium for 12 h, followed by being washed twice with PBS plus 0.01% Tween20. Then, cells were trypsinized and suspended in PBS prior to analysis by flow cytometer (BD Aria II). For each sample, 10000 events were monitored and evaluated. A 633 nm (He-Ne) laser was used to excite the Cy5 fluorophore, and its emission was observed with a 660/20 nm filter through APC channel. Flow cytometer data were analyzed by the FlowJo software.

6. FRET Assay

Firstly, siRNA was labeled with Cy3 and Cy5 respectively, and subsequently Cy3-siRNA and Cy5-siRNA mixed at an amount ratio of 1:1. Then, the yielded siRNA mixture was hybridized with DC as mentioned above. To test the digestion of DC-siRNA against Dicer, the two fluorophore labeled DC-siRNA solution treated with or without 1.5 unit Dicer enzyme (Genlantis, San Diego, CA, USA) was incubated at 37 °C. After 12 h and 24 h incubation, the nanoparticles were excited at 543 nm and the fluorescence intensity was read at 570 and 665 nm, respectively, using a microplate reader (Molecular Device, i3X). FRET efficiency is defined as the ratio of emission fluorescence intensities at 665 nm to 570 nm (Em665/Em570). To study the intracellular release of siRNA from DC-siRNA nanoparticles, Cy3 and Cy5 labeled DC-siRNA nanoparticles were transfected into HeLa cells in 8-units-chamber-slide (Thermo Fisher). After 1 h and 12 h incubation, HeLa cells were washed with PBS/0.01% Tween20 and replaced with fresh growth medium before imaging. The

fluorescence signals were measured by inverted confocal microscope (Zeiss, 710) at an excitation of 543 nm, and collected at Cy3 and Cy5 channel, respectively. As a control, image of Cy5 at excitation of 643 nm was also shot.

7. Cell Viability Assay

HeLa cells were seeded in 96-well plate (5×10^3 cells/well). After 24 h, cells were added with DC-siRNA nanoparticles at different siRNA concentrations. After 24 h incubation, 10 μl MTS solution (5 mg/ml, Promega) was added to cultured medium in each well and incubated for 1.5 h. The absorbance at a wavelength of 490 nm of each well was measured using a microplate reader (Molecular Device, i3X). The relative cell viability was determined by comparing the absorbance at 490 nm with control wells which treated with PBS. Data are presented as average ± SD (n = 4).

8. Gene Silencing by DC-siRNA

To evaluate gene-silencing ability by DC-siRNA, HeLa-Luc cells were seeded in 24-well plate at a density of 1×10^5 cells/well. After 24 h, the medium was replaced and DC-siRNA nanoparticles were added at a siRNA concentration ranging from 50 nM to 500 nM. All transfection experiments were carried out in triplicates.

Quantitative real time-polymerase chain reaction (qRT-PCR) assay. HeLa-Luc cells were collected after 48 h transfection. Total RNA was isolated from cell samples using Trizon reagent (CWbiotech, Beijing) following the manufacturer's instruction. qRT-PCR was performed with standard procedure (Lightcycler480, Roche). The PCR primers to detect luciferase genes were purchased from GENEWIZ Inc. using the following sequences:

Forward：5'- GGAACCGCTGGAGAGCAACT -3'

Reverse: 5'- GTCCCTATCGAAGGACTCTGGCA -3'

GAPDH was used as a housekeeping gene to be normalized.

Forward：5'- CAGGGCTGCTTTTAACTCTG -3'

Reverse: 5'- GATGATCTTGAGGCTGTTGTC -3'

Luciferase knockdown assay. After 48 h transfection, HeLa-Luc cells were lysed and the luciferase activities were measured by a luminometer (Molecular Device, i3X) using luciferase reporter gene assay kit (Beyotime, China). Each experiment was performed in triplicates, and the data represent means ± SD of three independent experiments after normalized to Hela-Luc control.

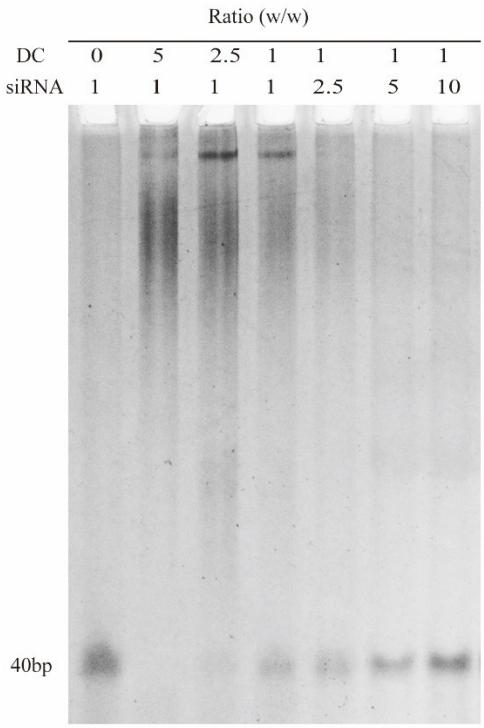

Figure S1. Native PAGE electrophoresis of DC-siRNA.

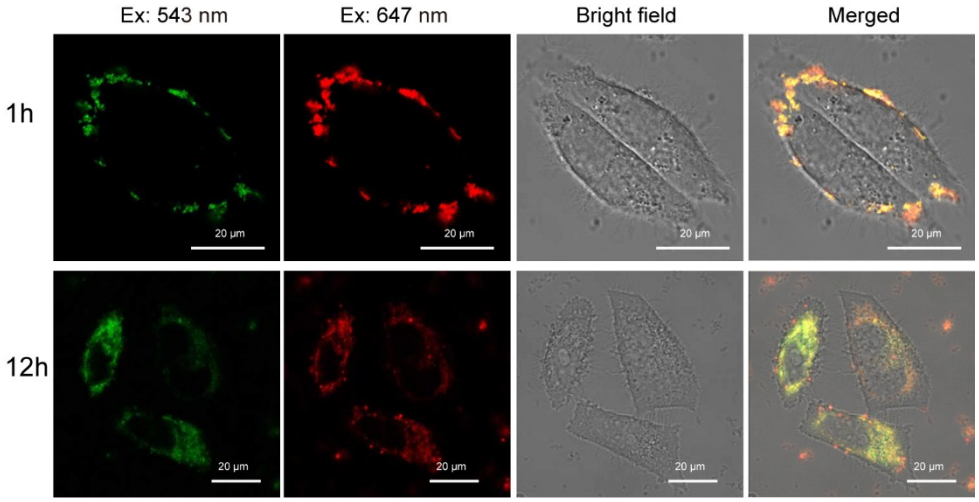

Figure S2. Confocal images of HeLa cells transfected at 1 h and 12 h by DC-siRNA nanoparticles with Cy3 and Cy5 fluorophore.

Reference

1. Lv, Y.; Hu, R.; Zhu, G.; Zhang, X.; Mei, L.; Liu, Q.; Qiu, L.; Wu, C.; Tan, W., Preparation and biomedical applications of programmable and multifunctional DNA nanoflowers. *Nat Protoc* **2015,** *10* (10), 1508-24.