Supporting Information

Protecting microRNAs from RNase degradation with steric DNA nanostructures

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Experimental methods

1.1 Synthesis and characterization of DNA Shuriken

The DNA sequences of the DNA Shuriken were designed using a computer program SEQUIN[1]. All the DNA strands (PAGE purified) were purchased from Sigma and used as received. Strand L: 5'-AGG CAC CAT CGT AGG TTA TTT AAT CTT GCC AGG CAC CAT CGT AGG TTA TTT AAT CTT GCC AGG CAC CAT CGT AGG TTA TTT AAT CTT GCC-3'; M: 5'- AAC GAC TAG CAA CCT GCC TGG CAA GCC TAC GAT GGA CAC GGT AA-3'; S: 5'-TCC TGG GAA AAC TGG ACA AAC CGT GTG GTT GCT AGT CGT T-3'; miR-145: 5'-GUC CAG UUU UCC CAG GAA UCC CU-3'; Fluorophore modified miR-145: 5'-Cy3-GUC CAG UUU UCC CAG GAA UCC CU-3'. The component DNA strands L, M and S were mixed together at a molar ratio of 1:3:3 in Tris-Acetic-EDTA-Mg^{2+} (TAE/Mg^{2+}) buffer (pH 8.0), and then subjected to an annealing process as follows: 95°C/5 min., 65°C/30 mins, 50°C/30 mins, 37°C/30 mins, and 22°C /30 mins. After the annealing, the DNA strands form a 3-point-star shaped motif that carrying 3 overhangs which are supposed to hybridize the miR-145 strands. The 3-point-star motif was then mixed with miR-145 or fluorophore modified miR-145 at a molar ratio of L:M:S:miR-145=1:3:3:3 to form the DNA Shuriken at room temperature for 30 mins.

5% native polyacrylamide (diluted from 40% 19:1 acrylamide/bis acrylamide) gel (PAGE) was run on a Hoefer SE 600 Chroma Vertical Electrophoresis System at room temperature for 1 hour under constant voltage of 250 V. 1× TAE/Mg^{2+} was used as running buffer, and the gel was stained in Stains-all solution (0.01%).

The DNA Shuriken solution was diluted to ~90 nM and measured by DLS (Brookhaven, USA) in 1× TAE/Mg^{2+} buffer. Pure 1× TAE/Mg^{2+} was used as blank.

1.2 Cellular uptake of DNA Shuriken

The internalization of DNA Shuriken was observed with a confocal fluorescence microscope, microplate reader and RT-PCR. For live imaging, human colorectal adenocarcinoma cell line DLD-1(ATCC, USA) was cultured in a 8 well glass bottom chamber slide (Lab-Tek Ⅱ chamber Slide Nunc) overnight with a seeding density of 1×10^5 cells/cm^2 in Dulbecco's Modified Eagle's Media (DMEM, Gibco) under standard culture conditions (37 °C, 5% CO_2). The DMEM medium was supplemented with 10% FBS (Thermo Scientific) and Penicillin/streptomycin solution (1%, PAA Laboratories Inc., USA). Then the cells were treated with fluorescently labeled DNA Shuriken at a final concentration of 200 nM (miR-145 cargo concentration is 600 nM) for 2 hours under 37 °C. The volume ration between DNA sample and DMEM was 1:8. After the treatment, the cells were washed 3 times with PBS buffer and then stained with Hoechst 33342 DNA dye (Final concentration: 1μg/mL) and cell mask (Deep Red, Invitrogen, 2 μg/mL) for the nucleus and cytoplasm membrane. The live imaging of intracellular DNA Shuriken was conducted under Olympus FV100 confocal microscope using 60 × oil immersed objective lens.

The comparative uptake study was determined with Synergy H4 Hybrid Multi-Mode Microplate Reader (Bio-Tek). Briefly, DLD-1 and human breast adenocarcinoma (MCF-7, ATCC) cells were seeded at seeding density of 1×10^5 cells/cm^2 while primary human microvascular endothelial cells (HMVEC passage 16, Life Technologies, USA) were seeded at seeding density of 4×10^4 cells/cm^2. The cells were cultured overnight in a 96-well plate (Costar, black) prior the different time point treatments (0.5, 1, 2 h). The comparative internalization study was conducted by exposing the cells with 600 nM of Cy3 labeled miR-145 in different delivery packaging, namely naked miR-145 (miRNA), miR-145 polyplex (miRNA+Lipofectamine) and DNA Shuriken which carries miR-145 (DNA Shuriken). Opti-MEM reduced serum medium (ThermoScientific, USA) was

used as diluent and was also introduced to the untreated control group. The miRNA lipoplex was formed by utilizing 320 nL of Lipofectamine 2000 (Thermoscientific, USA) for every 411 ng of Cy3 labelled miR-145, as previously reported [2]. Following the treatment, the cells were washed twice with PBS, and counterstain with Cell Tracker Green (Life Technologies, USA) following the supplier's protocol. Thereafter, the cells were washed with PBS before microplate reader measurement. Internalization was determined by normalizing the Cy3 signals (Ex/Em: 540/566) with the signal from the Cell tracker green (Ex/Em: 492/517).

RT-PCR was utilized to quantify intracellular miR-145 level after 24 hours. The cells were cultured exactly as previously described except that they were cultured in a 24-well plated and incubated with DNA Shuriken for 24 hours. After washing thrice with PBS, the cells were harvested using trypsin and lysed (RLT buffer, Qiagen). Total miRNAs were collected using a RNeasy mini kit (Qiagen). RT-qPCR was performed using MiRXES miRNA qPCR Assay kit (MIRXES PTE. LTD., Singapore) following the manufacture's instruction. Each sample was triplicated, and let-7a was used as internal control.

Extracellular and intracellular stability of DNA Shuriken was evaluated by native PAGE gel. DNA Shuriken was incubated with enzymes (1 μg/mL RNase A) or cell culture medium for different time points at 37 °C and then run on a Hoefer SE 600 Chroma Vertical Electrophoresis System. Gel concentration was 8%; running time and voltage were 1 hour and 250 V, respectively.

1.3 Cell proliferation experiment

DLD-1 cells were cultured in a 24-well plate overnight with a seeding density of 1×10^5 cells/cm^2. The cells were then treated with DNA Shuriken (200 nM), naked miR-145 (600 nM) and DNA star motif (200 nM, DNA Shuriken without miR-145) separately for another 24 hours. Thereafter, cells were washed thrice with PBS and collected using trypsin. Muse Ki67 cell proliferation kit (EMD Millipore, Hayward, CA) was used to evaluate cell proliferation on the Muse cell analyzer.

1.4 3D spheroid tumor model study

DLD-1 cell spheroid was prepared as described in previous reports.[3,4] Briefly, the cells were cultured on 2% agarose (First Base, Singapore) micro-molds for 24 hours to form cell spheroids. The seeding density of cell was maintained at 1.2×10^5 cells for each micro-mold. For treatment, DNA Shuriken solution was mixed with DMEM to a final concentration of 200 nM, and then added to the cell spheroid. The control group was cultured with DMEM supplemented with 1× TAE/Mg^{2+} buffer. The treatment solution was changed every 12 h during the experiment. The bright field image of the cell spheroid was captured by inverted microscope Olympus-CX41 (Olympus, Japan) every 24 h during the treatment.

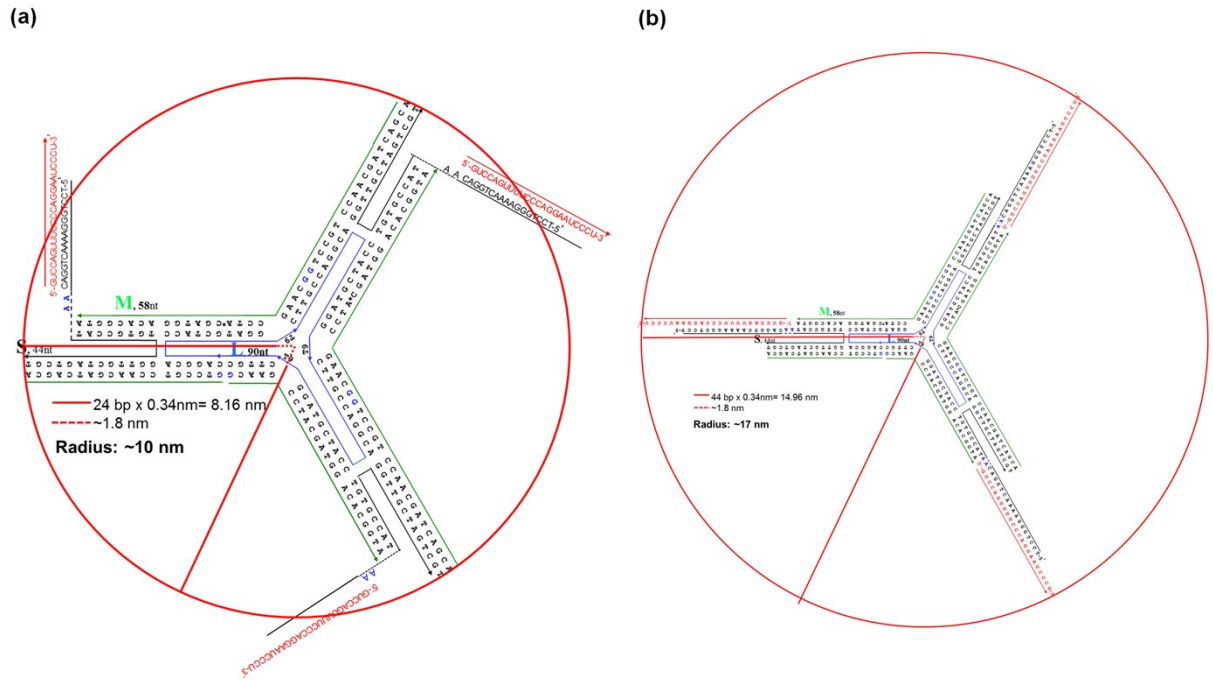

Fig. S1 Two typical conformations of DNA shuriken. In case (a), the DNA shuriken is not fully extended and has a radius of about 10 nm; while for case (b), the three duplex overhangs align with the three arms and represent the biggest size of DNA shuriken (~17 nm in radius and ~34 nm in diameter). Note that the three arms and overhangs are highly flexible and not necessarily in the same plane. The DNA shuriken might be much smaller in diameter if all the three arms bent to the same direction. Calculations were based on the assumptions that the width of DNA duplex is 2 nm, and each base pair is 0.34 nm along the helical direction.

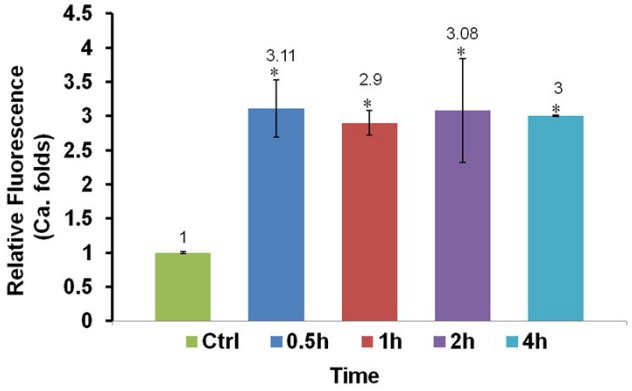

Fig. S2 Quantification of DNA Shuriken uptake with different incubation time. The relative fluorescence intensities of DLD1 cells reach to a maxima of around 3 fold higher compared to control within 30 mins. The fluorescence signal was also normalized to cell numbers by using cell tracker green. Data represent mean ± SD, n = 3. Student's t-test, $p < 0.05$, *significantly different from control group (ctrl).

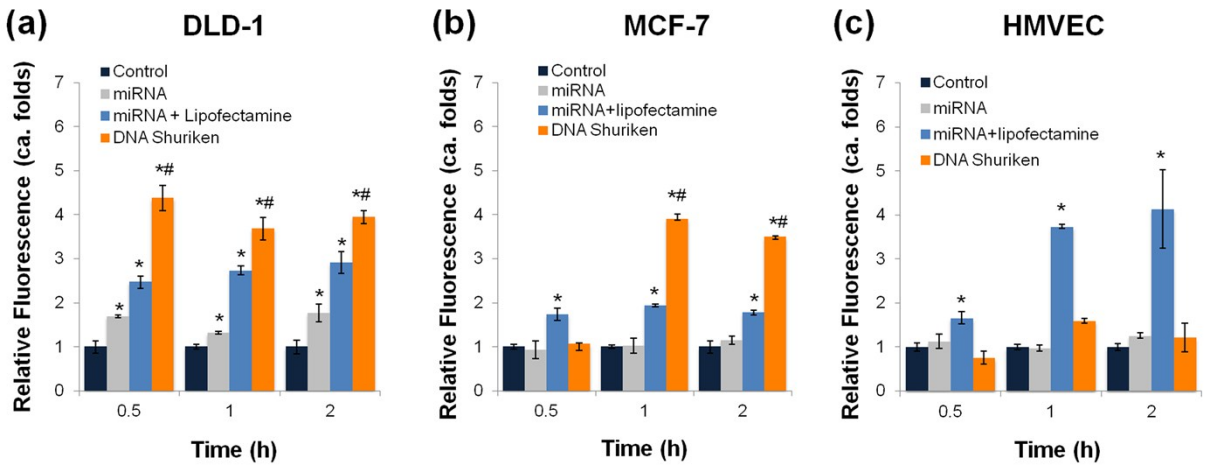

Fig. S3 Comparative study of different miRNA-145 formulation uptake into different cell lines. (a) DLD-1, (b) MCF-7 and (c) HMVEC was incubated with the naked miRNA-145 (miRNA), miRNA-145 lipoplex (miRNA + Lipofectamine) DNA Shuriken carrying miRNA-145 (DNA shuriken) at different time points (0.5, 1, 2 hours). Exposure dose was maintained at same miRNA concentration of 600 nM (which is equivalent to 200 nM DNA Shuriken). Internalization was determined by measuring the Cy3 signals that originated from the Cy3 labelled miRNA and normalizing the readout with the cell numbers and untreated control. Data represent mean ± SD, n = 3. Student's t-test, $p < 0.05$, *significantly different from control group (ctrl), #significantly different from the Lipofectamine treated group.

Reference

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4. C. Y. Tay, M. S. Muthu, S. L. Chia, K. T. Nguyen, S. S. Feng, D. T. Leong, *Adv. Funct. Mater.*, 2016, **26**, 4046-4065.