Electronic Supplementary Information

Delivery and releasing of microRNA-34a into MCF-7 breast cancer cell by using spherical nucleic acid nanocarriers

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EXPERIMENTAL SECTIONS

Preparation of Sterile, DNase/RNase-Free Solution. 0.2M Phosphate (NaH_2PO_4/Na_2HPO_4) buffer, 4M NaCl solution, 1XPBS buffer (137mM NaCl, 10mM Phosphate, 2.7mM KCl, pH 7.4, Hyclone), Nanopure water (18.2 MΩ) and Au nanoparticles colloidal were treated by 1‰ diethylprocarbonate (DEPC; Sigma-Aldrich) at room temperature (RT) overnight, and then autoclaved. The tips and tubes also were sterile, and DNase/RNase-free.

Synthesis of Spherical Nucleic Acid Nanoconjugates. The 13±1nm citrate-capped AuNPs were prepared as previously described[1]. Thiol-modified DNA oligonucleotides were reduced by Tris (2-carboxyethyl) phosphine hydrochlorides (TCEP, Sigma-Aldrich) for 1h at RT. Then, they were added to gold nanoparticle colloids at a concentration of 3μM of oligonucleotide in 10nM of colloids solution. After 6h, sodium dodecylsulfate (SDS) and phosphate buffer were added to the colloid solution to achieve the final concentrations of 0.1% and 10mM, respectively. After another 12h incubation period, the mixture was brought to 0.05M of NaCl by addition of 4M concentrated solution and this addition was repeated in 30 min intervals until a final concentration of 0.3 M NaCl. Then, the mixture was allowed to gently shake for 24h at RT. To purify the SNA conjugates, the mixture was centrifuged (13,500 rpm, 25 min) and resuspended in 1XPBS buffer three times. The concentration of SNA was determined by measuring the extinction at 520 nm ($\varepsilon=2.7\times10^8$ L mol^{-1} cm^{-1}). Subsequently, the fluorophore labeled strand was added to the ~ 30 nM DNA-AuNP solutions to a concentration 3.3μM, and the mixture was heated to 75 °C for 30 min, slowly cooled to RT, and stored in dark for 12 hours. Then the excess fluorophore-labeled strands are removed by centrifugation three times. Before cellular uptake, the SNAs were filter sterilized by using a 0.2 μm acetate syringe filter.

Extracellular Fluorescence Measurements. The extracellular fluorescence measurements were performed on Jobin Yvon Fluorolog FL3-22. Cy5 and Cy3 dye were excited at 633 and 533 nm, respectively, and their emissions were recorded in ranges of 650-750 nm, and 550-620 nm, respectively in 1 nm increments.

Oligonucleotide Loading Quantification. The DNA-AuNP colloid (400μL, 1 nM) was treated with KCN solution (0.1 M) to dissolve the gold core and release the oligonucleotides. After centrifugation, the fluorescence intensity of supernatant was measured and compared to

a standard curve.

Cell Culture. MCF-7 cell lines were obtained from Cell Bank of the Committee on Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China), and grown in Dulbecco's modified Eagles medium (DMEM, Hyclone) with 10% heat inactivated fetal bovine serum (FBS, Hyclone) and 1% Penicillin/Streptomycin (Gibco) in 5% CO_2 at 37 °C.

Cell Confocal Fluorescence Imaging. Cells were seed on coverglass bottom dish (diameter 35 mm, SPL Lifescience Co., Ltd.). After 1 day, the cells were treated with SNAs in Opti-MEM (Gibco). After a 4h incubation period, cells were washed with PBS buffer and cultured in fresh DMEM for another 44 hours. Then, cells were imaged under Lecia SP5 confocal microscope with HeNe laser excitation at 633 nm (Cy5) and 543 m (Cy3).

Flow Cytometry Analysis. Cells were prepared as described above. Then cells were trypsinized, collected, and suspended in PBS buffer. Flow cytometry was performed with BD Analyses were performed on BD FACSCanto II system with BD FACSDiva software.

Cellular Uptake of SNAs. Cells were co-incubated with 1nM SNAs, and then trypsinized, collected and counted. Cell pellets were dissolved in aqua regia (HCl: HNO_3 = 3:1) overnight at 37 °C. Then each sample was diluted to 5mL in solution consisting of 3% HNO3, 5 ppb Indium (internal standard) and Nanopure™ water. Cellular uptake of SNAs was measured by ICP-MS (Thermo-Fisher) and Au content incorporated by single cell or cell population was calculated according to the published protocol[2].

Cellular TEM Imaging. The preparation of cell sample is tedious. Briefly, after a 4h treatment period with SNAs (5 nM), cells were trypsinized and collected. Subsequently, cells were fixed in 3% glutaraldehyde containing 2% paraformaldehyde overnight at 4 °C. After PBS buffer wash, cells were further fixed with 1% osmic acid for 1.5 h at RT. Then, cells were thoroughly rinsed by PBS and dehydrated by using a series of concentration gradients of ethanol (30%, 50%, 70%, 80%, 95%, and 100%). A mixture of resin and ethanol (1:1) was added for 1h, and then, mixture was replaced by pure resin to infiltrate into cell pellet overnight (~12h). The cell pellet embedded in resin was stored at 70 °C for 48 hours to finish resin polymerization. Finally, the ultrathin section samples (80 nm-thick) were deposited on copper grids and stained with 2% uranyl acetate and lead nitrate. TEM imagings were obtained with FEI TECNAI G2 SPIRIT TEM SYSTEM, 120KV.

GSH Assay. For GSH effect study, the SNAs were diluted to 2.5 nM in PBS, and then the solution was degassed by repeated freeze-thaw cycles to prevent the oxidation of GSH. SNAs were incubated with 1470 μM GSH and 30 μM GSSH which is in accordance with the literature values[3] overnight at 37 °C. Then, the loading of oligonucleotides was measured.

Total RNA Extraction and Quantitative RT-PCR. Cells were cultured with R-F/control SNA (5 nM) and miRNA SNA (2 nM) for 6 hours, followed by PBS wash. In this section, all SNAs did not carry any fluorophore. After a final 72h culture period, cells were harvested and total RNA was extracted by using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. For miR-34a expression analysis, quantitative RT-PCR was performed with the miRcute miRNA First-Stand cDNA Synthesis Kit and miRcute miRNA qPCR Detection Kit (SYBR Green; Tiangen). U6 was used as a control. The primers for miR-34a mimics and U6 were purchased from Gene Pharma. For survivin mRNA expression analysis, total RNA was isolated from cells and cDNA was prepared by using the transcriptor First-Stand cDNA Synthesis Kit, following the indicated protocol. Quantitative RT-PCR was performed with LightCycler 480 SYBR Green I Master on a LightCycler 480 system (Roche). The relative expression of survivin mRNA was normalized to the internal control of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and compared to the untreated cells. All relative expression levels were calculated by the following equation: $Relative\ gene\ expression = 2^{-(\Delta Ct\ sample - \Delta Ct\ control)}$. Finally, primers sequences were listed in Table S1.

Cell Viability Assay. Cell viability assay was measured by using Cell Counting Kit (CCK-8). Cells were seeded in a 96-well plate at 3000 cells per well. Cells were treated by R-F/control SNA (5 nM) and miRNA SNA (2 nM) for 6 hours, followed by PBS wash. In this section, all SNAs did not carry any fluorophore. After a final 96h incubation period, 10 μL CCK-8 solution was added to each well and incubated for 2 hours. The absorbance of each well was recorded at 450 nm.

Cell Apoptosis Measurement. MCF-7 cells were treated by R-F/control SNA (5 nM) and miR-34a SNA (2 nM) for 6 hours, followed by PBS wash. In this section, all SNAs did not carry any fluorophore. After a final 72h incubation period, apoptosis was measured by treating cells with FITC-Annexin V and PI Cell Apoptosis Detection Kit (Invitrogen), according to the manufacturer's protocol. Analyses were performed on BD FACSCanto II system with BD FACSDiva software.

Table S1 DNA/RNA/primers sequence.

Name	Type	Sequence (5'→3')
R-F strand	DNA	TGTCAGTGGCAGTG(T-Cy5)CAAGGAGCTGG
Thiol-strand of R-F SNA	DNA	CCCAGCCTTCCAGCTCCTTGA-$(T)_9$-HS
miR-34a mimics	RNA	UGGCAGUGUCUUAGCUGGUUGU
Thiol-strand of miR-34a SNA	DNA	SH-$(T)_9$-GCTAAGACACTGCCACTGACT
Thiol-strand of control SNA	DNA	TCTCCCCAGCCAGCTCCTTGA-$(T)_9$-HS
miR-34a forward	DNA	AAGGCCACGGATAGGTCCATA
miR-34a reverse	DNA	CGCTTTGGTGGTTCTGAAAGG
U6 forward	DNA	AAGGCCACGGATAGGTCCATA
U6 reverse	DNA	CGCTTTGGTGGTTCTGAAAGG
Survivin forward	DNA	ATGGGTGCCCCGACGTTG
Survivin reverse	DNA	AGAGGCCTCAATCCATGG
GAPDH forward	DNA	TGCACCACCAACTGTTTAGC
GAPDH reverse	DNA	GGCATGGACTGTGGTCATGAG

Additional Supporting Data

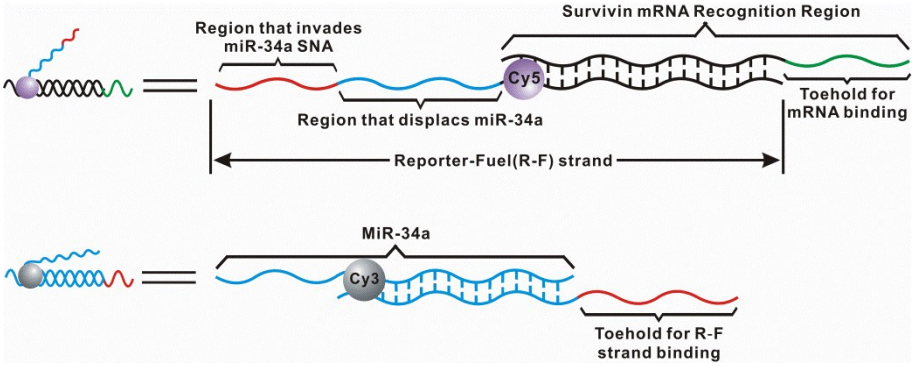

Fig. S1 The functions of each domain of oligonucleotides are detailed.

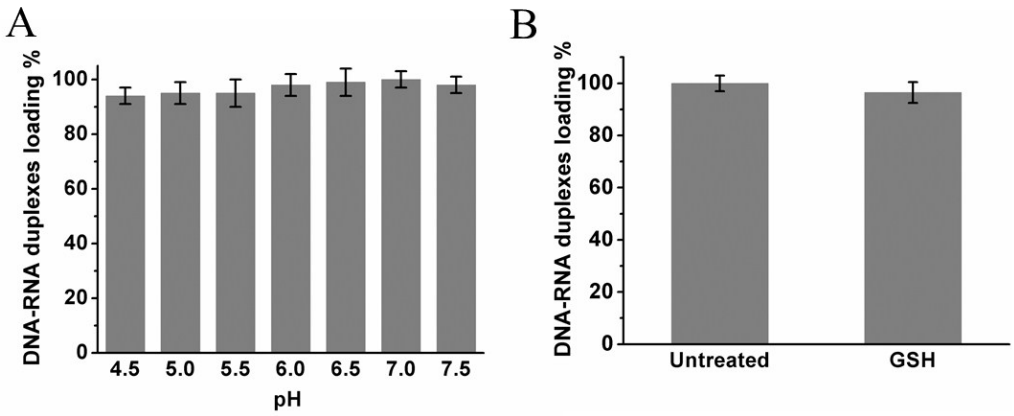

Fig. S2 (A) Effect of pH on oligonucleotide loading of miR-34a SNA (1 nM). (B) Effect of GSH on oligonucleotide loading of miR-34a SNA (1 nM). We used 1470 μM GSH and 30 μM GSSG in accordance with the literature values[3]. The error bars are the standard deviations from three repetitive experiments.

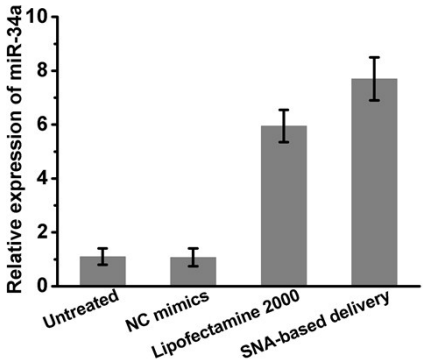

Fig. S3 Compared to the transfection regent, cells treated with SNA-based delivery system shows a higher miR-34a expression level, indicative of a higher efficiency of SNA nanocarrier.

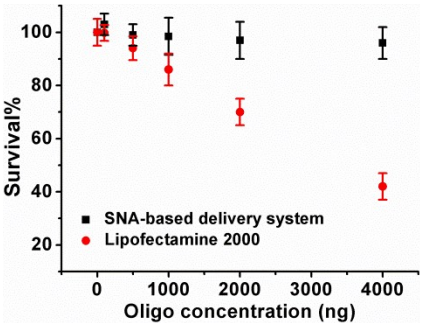

Fig. S4 Cytocoxicity assay of SNA-based delivery and transfection regent.

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2. D. A. Giljohann, D. S. Seferos, P. C. Patel, J. E. Millstone, N. L. Rosi and C. A. Mirkin, *Nano Lett.*, 2007, **7**, 3818-3821.
3. C. Hwang, A. Sinskey and H. Lodish, *Science*, 1992, **257**, 1496-1502.