Electronic Supplementary Material (ESI) for Biomaterials Science.

Supplementary Information

Kissing loop-mediated fabrication of RNA nanoparticles and their potential as cellular and *in vivo* siRNA delivery platforms

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Table S1. Sequences of oligonucleotides used in this study. Orange: T7 promoter. Blue: kissing loop. Green: siGFP

	Sequence (5' to 3')
S1-KL	Phosphate-GCT GAC CCT GAA GTT CAT CTG CAC CTT TGC GCG CTT TTG GTG CAG ATG AAC TTC AGG
S2-KL	Phosphate-GTC AGC TTC CCT ATA GTG AGT CGT ATT ATT TGC GCG CTT TTT AAT ACG ACT CAC TAT AGG GAA
S1-NL	Phosphate-GCT GAC CCT GAA GTT CAT CTG CAC CTT TTT TTT TTT TTG GTG CAG ATG AAC TTC AGG
S2-NL	Phosphate-GTC AGC TTC CCT ATA GTG AGT CAT ATT ATT TTT TTT TTT TTT AAT ACG ACT CAC TAT AGG GAA
Sense (siGFP)	r(GCUGACCCUGAAGUUCAUC)TT
Antisense (siGFP)	r(GAUGAACUUCAGGGUCAGC)TT

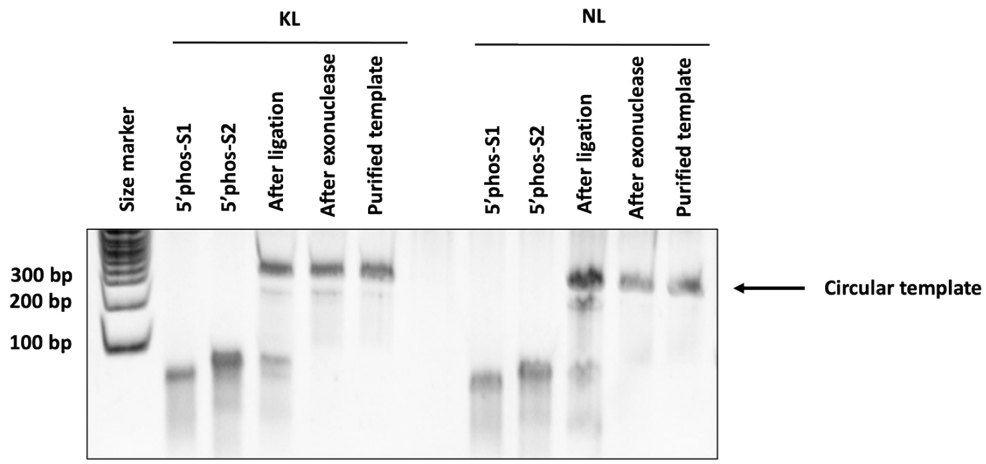

Figure S1. Preparation of circular templates (20% denaturing PAGE).

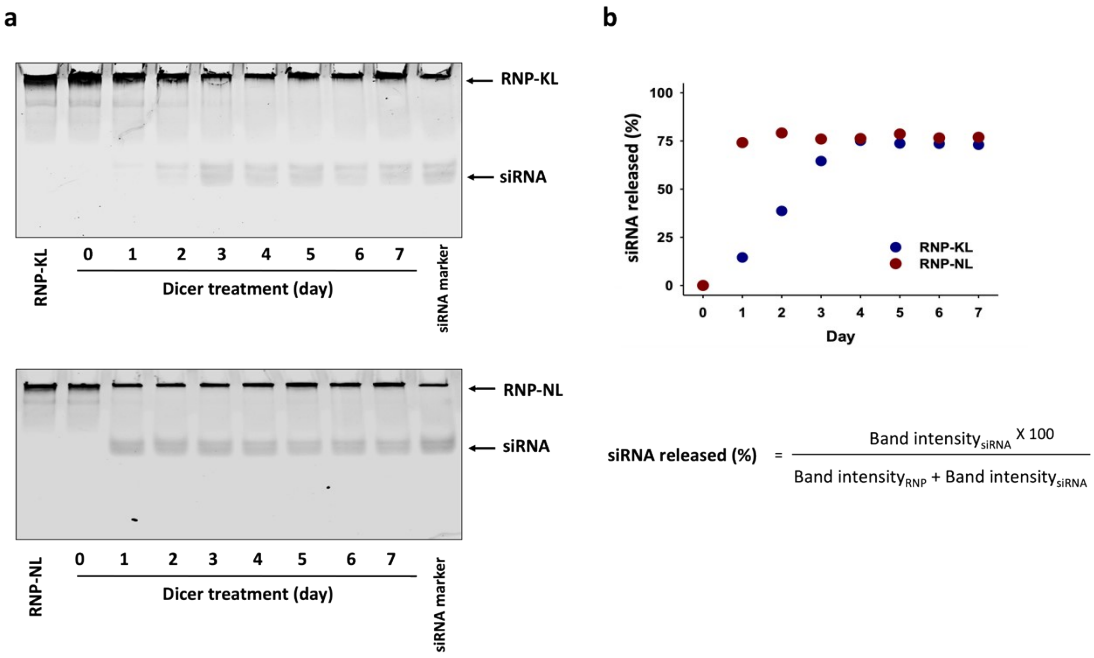

Figure S2. (a) Gel electrophoresis results of RNPs after reaction with Dicer. (b) The relative amount of siRNA (%) released from RNPs by Dicer treatment. The band intensity of RNP used for the calculation was obtained after subtraction of the non-specific band intensity by the staining agent (SYBR gold) in the well of the siRNA marker lane.

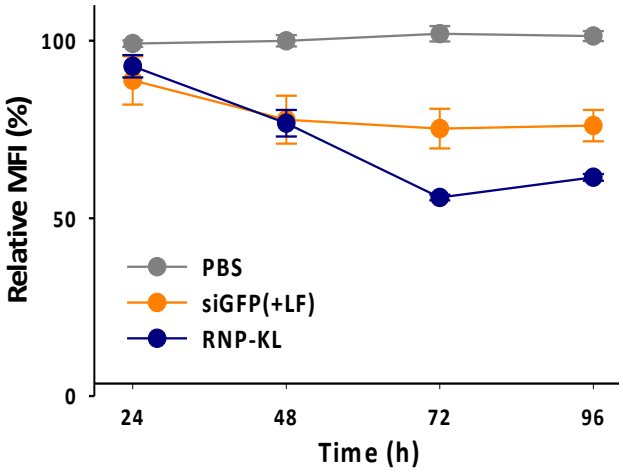

Figure S3. Time-dependent change in relative mean fluorescence intensity (MFI) of HeLa/GFP cells after treatment with PBS, siGFP in the presence of LF, and RNP-KL.

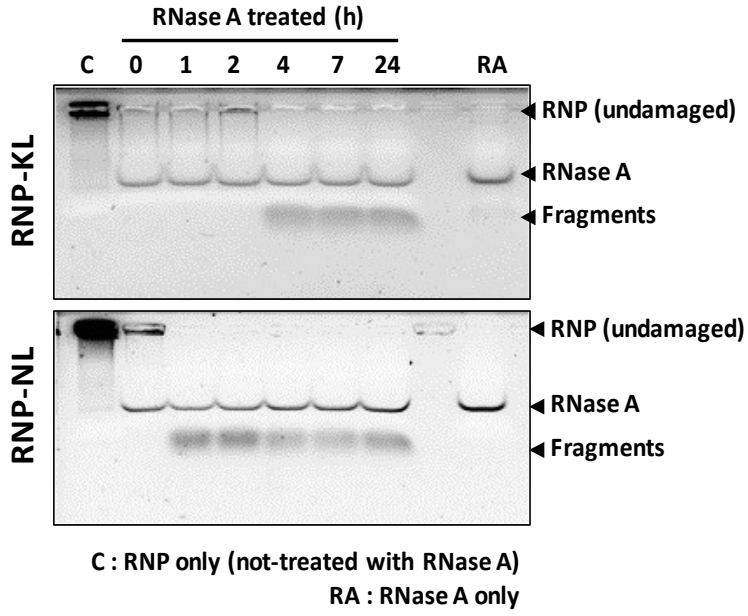

Figure S4. Gel electrophoresis results of RNPs after treatment with RNase A.

Experimental Section

Statistical analysis

Data were represented as mean ± standard deviation. Statistical analysis was performed with one-way ANOVA (Tukey's multiple comparison test) or unpaired t-test using the Prism software; the data with $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ were considered statistically significant.

Materials

5'-phosphate-oligonucleotides, primers for quantitative RT-PCR, and RNase A were synthesized and obtained by Bioneer (Daejeon, Korea). T4 DNA ligase, exonucleases (I and III), T7 RNA polymerase, rNTP mix, and HiScribe™ T7 High Yield RNA Synthesis Kit were purchased from New England Biolabs (MA, USA). SYBR® gold nucleic acid gel stain, Super

signal™ west pico chemiluminescent substrate, quantitative Elisa kits (Mouse TNF-α and IFN-γ), SYBR green master mix, and lipofectamine (RNAi MAX) were acquired from Thermo Fisher Scientific (CA, USA). The Amicon Ultra-15 Centrifugal Filter Unit Ultra-4 (MWCO 100 kDa) was obtained from Merck Millipore (MO, USA). Antibodies (anti-GFP Rabbit mAb and anti-β-actin Rabbit mAb) and 10X RIPA buffer were purchased from Cell Signaling Technologies (USA). RNeasy Mini kit and cDNA synthesis kit were purchased from QIAGEN (Hilden, Germany) and Enzynomics (Daejeon, Korea) respectively. DMEM, RPMI, FBS (heat-inactivated), and antibiotics (1% penicillin and 1% streptomycin) were obtained from Welgene (Gyeongsan, Korea). Other chemical reagents, organic solvents, and buffers were acquired from Sigma Aldrich (MO, USA), Daejung chemicals (Seoul, Korea), and Biosesang (Seongnam, Korea), respectively.

Preparation of RNPs (Circularization of linear DNA and RCT reaction)

For preparation of two types of RNP (kissing loop and no loop), at first, linear oligonucleotides were circularized. 5'-phosphate-S1 and S2 (single stranded-DNA, **Table S1**) were circularized by T4 DNA ligase reaction. Reaction mixtures were incubated at 16℃ for overnight and heat inactivated at 65℃ for 10 min. Uncircularized oligonucleotides were removed by exonuclease I and III treatment (37℃, 1 h). Circularized DNA was separated on 20% denaturing PAGE (7M urea) and purified, followed by ethanol precipitation. The purified circular DNA was quantified by measuring UV absorbance at 260 nm. Then, circular DNAs (final concentration, 0.7 μM) were mixed with T7 RNA polymerase (5 U, New English Biolabs, USA), rNTP mix (2 mM each), and reaction buffer (1X). Reaction solutions were then incubated for 1, 4, 7, and 24 h at 37℃ and purified by Amicon Centrifugal Filter (MWCO 100 kDa). After removal of unreacted precursors, RNPs were quantified by Nanodrop (Thermo Fisher Scientific, CA, USA).

Characterization of RNPs

RNPs were verified on SYBR gold containing agarose gel (1%). Gel was run in 0.5X TBE buffer for 30 min at 100V, 4℃. After electrophoresis, gel was visualized by iBright FL1000 imaging system (Thermo Fisher Scientific, CA, USA). The hydrodynamic size and surface charge of RNPs were measured using Zetasizer™ (Malvern Instruments, Worcestershire, UK). SYBR gold pre-stained RNPs were visualized using confocal fluorescence microscopy (LSM700, Carl Zeiss, Oberkochen, Germany). Images were analyzed using ZEN software. For analyzing the morphology and size of RNPs with scanning electron microscopy (SEM), they

were deposited onto Si-wafers and coated with Pt after being dried. Nova Nano SEM 200 (FEI, OR, USA) was used to acquired high resolution images of samples at an accelerating voltage of 5 kV. For observing the time-dependent size change in dried state of RNPs, atomic force microscopy (AFM, Park systems, XE-100, Suwon, Korea) was used. The samples were dropped and dried onto a mica coated with $NiCl_2$, and dried. Images were obtained using a non-contact cantilever (PPP-NCHR, Park systems, Korea) and analyzed using XEI software.

Cell culture

GFP expressed cell lines (HeLa-GFP cells and SCC7-GFP cells) were cultured in DMEM and RPMI (Welgene, Gyeongsan, Korea) containing 10% FBS (heat-inactivated) and antibiotics (1% penicillin and 1% streptomycin) in a CO_2 incubator (37 °C, 5% CO_2), respectively.

Cellular uptake

RNPs were fluorescently labeled by exchanging 5% of UTP with Cyanine 5-Aminoallyluridine-5'-Triphosphate (Cy5-UTP) when RCT reaction was performed. HeLa cells (1×10^5) were treated with Cy5-labeled RNPs (100 ng/mL) in serum-free media and incubated at 37°C for 6 h. After washing two times with ice-cold PBS, the uptake level of RNPs was measured using a flow cytometer (Guava, Millipore, MA, USA). 10,000 events were recorded for each sample and experiments were triplicated. For confocal microscopic imaging, cells were seeded onto 35 mm^2 glass-bottomed dish (2×10^4), treated with RNPs (100 ng/mL) for 6 h and imaged by confocal microscopy (LSM700) after staining with DAPI solutions.

Cell viability assay

The cytotoxicity of the RNPs was estimated by Cell Counting Kit-8 (CCK-8, Dojindo, Japan) assays. Briefly, cells (1×10^4) were seeded on 96-well plates containing the media (100 μL) and cultured overnight to reach ~70% confluency. The cells were then incubated with the fresh media containing RNP (100 ng/mL to 500 ng/mL; 8.33 nM to 41.7 nM of siRNA). The cells were incubated at 37°C for 24 h in a CO_2 incubator (37 °C, 5% CO_2). Cell Counting Kit-8 (CCK-8, Dojindo, Japan) solution (10 μL) was added to each well and incubated for 4 h at 37 °C. The absorbance at 450 nm indicating the cell viability was measured using a microplate reader (SpectraMax 340, Molecular Devices, CA, USA).

Immunogenicity analysis

RAW264.7 cells (5×10^4) cultured in 24-well plate (500 μL) were treated with RNPs (100 ng/mL; 8.33 nM of siRNA). The concentration of siRNA in RNPs, which was estimated as follows: [siRNA] = [RNP]*(the number of bases for siRNA)/(the total number of bases in the circular template). After 24 h, the supernatant was collected and analyzed using mouse TNF-α and IFN-γ quantitative cytokine ELISA kits. The absorbance was measured at 450 nm using a microplate reader. The amount of cytokines was quantified based on the standard curved obtained from ELISA at various concentrations of TNF-α and IFN- γ (15.6, 31.3, 62.5, 125, 250 and 500 pg/mL). The cytokine levels of the cells treated with lipopolysaccharide (LPS) were also determined as the positive control for immunogenicity.

***In vitro* gene knockdown analysis**

HeLa/GFP cells in confocal dishes (2×10^4) were treated with RNPs (100 ng/mL; 8.33 nM of siRNA) and incubated in a 5% CO_2 incubator. After 72 h post-transfection, cells were washed with DPBS (1 mL, 3 times), stained with Hoechst 34,580 (3 μg/mL) in DPBS (0.2 mL) for 15 min and washed with DPBS (1 mL, 3 times). Fluorescence images of the cells were obtained using confocal microscopy (LSM 700).

For quantitative analysis of the GFP expression by flow cytometer, cells in 12-well cell culture plate were treated with RNPs (100 ng/mL; 8.33 nM of siRNA) for 24, 48, 72 and 96 h. siGFP complexed with lipofectamine was used as the positive control. At each time points, cells were harvested and analyzed using flow cytometer (Guava).

To assess relative mRNA expression level, HeLa/GFP cells were treated with RNPs (100 ng/mL; 8.33 nM of siRNA). GFP siRNA (10 nM)/lipofectamine RNAi MAX complex (Thermo Fisher Scientific, USA) was treated, according to manufacturer's protocl, for positive control groups. After 72 h post-transfection, cells were washed with DPBS 3 times, harvested and extracted their total RNA using RNeasy Mini kit (QIAGEN, UK). The amount of RNA was measured by NanoDrop 2000 (Thermo Fisher Scientific, USA), and cDNA was synthesized from 2 μg of RNA using cDNA synthesis kit (Enzynomics, Daejeon, Korea) according to the manufacturer's protocol. Then, the cDNAs were amplified using 2X SYBR green master mix, and they were normalized by amplifying the housekeeping gene (GAPDH). The $2^{-\Delta\Delta Ct}$ method was used to analyze the relative changes in gene expression. Primer sequences for qRT-PCR were as follows; GFP (Forward) 5'-AAGCTGACCCTGAAGTTCATCTGC-3'; (Reverse) 5'-CTTGTAGTTGCCGT

CGTCCTTGAA-3' and GAPDH (forward) 5'-AGAGCTACGAGCTGCCTGAC-3'; (Reverse) 5'-AGCACTGT GTTGGCGTACAG-3'.

Animal experiments

All experiments with live animals were performed in compliance with the relevant laws and institutional guidelines of KIST, and the Institutional Animal Care and Use Committee (IACUC) of the Korea Institute of Science and Technology (KIST) (the committee chair: Dr. Heh-In Im, the head of Research Animal Resource Center, KIST) have approved the experiments (2020-182).

Biodistribution of RNP in xenografted mouse model

SCC7/GFP cells (suspension, 1×10^6) were injected subcutaneously into the thigh of the BALB/c nude mice. When the tumor volume reach to ca. 100 mm^3, tumor-bearing mice were divided randomly to 3 groups (N=3 for each group). PBS, Cy5-labeled RNP-KL and RNP-NL (2.4 μg of RNA; 1 μM of siRNA) were injected *via* tail vein. At 2 h post-injection, mice were sacrificed and major organs were collected for *ex vivo* imaging. Images were obtained by IVIS Living Imaging System (Filter set: excitation at 620 nm and emission at 670 nm; PerkinElmer, MA, USA) and analyzed using IVIS Imaging 3.0 software.

***In vivo* gene knockdown analysis**

To evaluate *in vivo* gene silencing effect, PBS and RNP-KL were intravenously injected to the tumor-bearing mice (120 μg/kg per injection, 3 injections, 2-day interval). *In vivo* fluorescence emission from GFP in the tumor was monitored from day 1 to 7 days using IVIS imaging system. (Filter set: Excitation at 490 nm and Emission at 520 nm). At 2 days post-3rd injection of RNP-KL, the mice were sacrificed, and the tumors were harvested for further analysis.

qRT-PCR and western blot of tumor lysates

To examine GFP mRNA expression levels in the tumor of RNP-KL-treated mice, total RNA was isolated from homogenized tumor tissues using RNeasy Mini Kit (QIAGEN), according to the manufacturer's recommendation and qRT-PCR analysis was performed as the same method described above (*in vitro* gene knockdown analysis section).

For estimation of GFP protein expression level, homogenized tumor tissues were lysed in RIPA buffer containing protease inhibitor cocktail. The mixture was centrifuged at 12,000 rpm for 20 min at 4 ˚C. Proteins (20 μg) in the supernatant were separated by 5-12% SDS-

PAGE and transferred to PVDF membrane (4˚C, 100 min, 350 mA). The relative GFP protein expression levels were analyzed by same procedures for western blot as described in the *in vitro* gene knockdown analysis section.

Tumor lysate analysis

The excised tumors were homogenized under liquid nitrogen and lysed in RIPA buffer. The lysed tumor tissues were centrifuged (12,000 rpm, 10 min, 4 °C), and the supernatant of each sample was analyzed using a fluorescence spectrophotometer (F7000, Hitachi, Tokyo, Japan). After excitation at 480 nm, the maximum emission intensity was acquired at 510 nm in the profile measured at 490–600 nm.

Tumor section analysis

The tumors were embedded with optimal cutting temperature (OCT) compound (Leica Biosystems, Germany) and frozen in deep freezer. The frozen tissue blocks were cut in 15 μm by Lab Core Incorporation (Seoul, Korea). The section slides were washed with PBS, mounted with DAPI containing-mounting solution (Abcam, UK) and imaged using confocal microscopy LSM 700.

Nuclease stability assays

RNPs were incubated with RNase A (80 μg) for 1, 2, 4, 7, and 24 h at 37℃. At the end of each time points, in order to degrade proteins and observe fragmented pattern, 80 μg of proteinase K, 50 μm EDTA, 0.1% SDS were added to the solution and incubated for 1 h at 37℃. The samples were analyzed on 2% agarose containing 1X SYBR gold. After electrophoresis, gels were visualized using iBright FL1000.

Dicer-mediated release of siRNAs

For assessment of siRNA generation from RNPs, the nanoparticles were incubated with 0.1 U/μL of DICER (recombinant, human DICER enzyme) in reaction buffer (1 mM ATP, 2.5 mM $MgCl_2$). The cleaved products were analyzed by 10% native PAGE run in 0.5X TBE buffer at 100 V for 40 min. Then, gels were stained with SYBR gold solutions and analyzed by iBright imaging system. The band intensity was quantified by ImageJ software.