Electronic Supplementary Material (ESI) for Chemical Communications

Supporting Information Available

Branched RNA nanostructures for RNA interference

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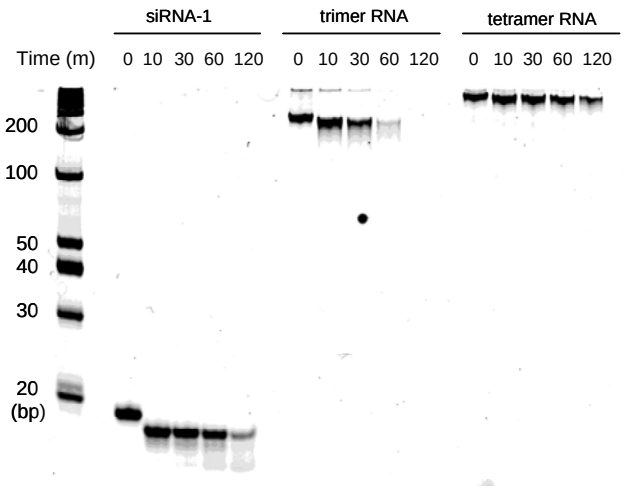

Figure S1. Stability of trimer RNA, tetramer RNA, and siRNA (siRNA-1). The RNAs were incubated with SVPD at 37 °C, and aliquots were taken after 10, 30, 60, and 120 min. The reaction mixture was analyzed using 15% native PAGE and visualized using SYBR Green I staining.

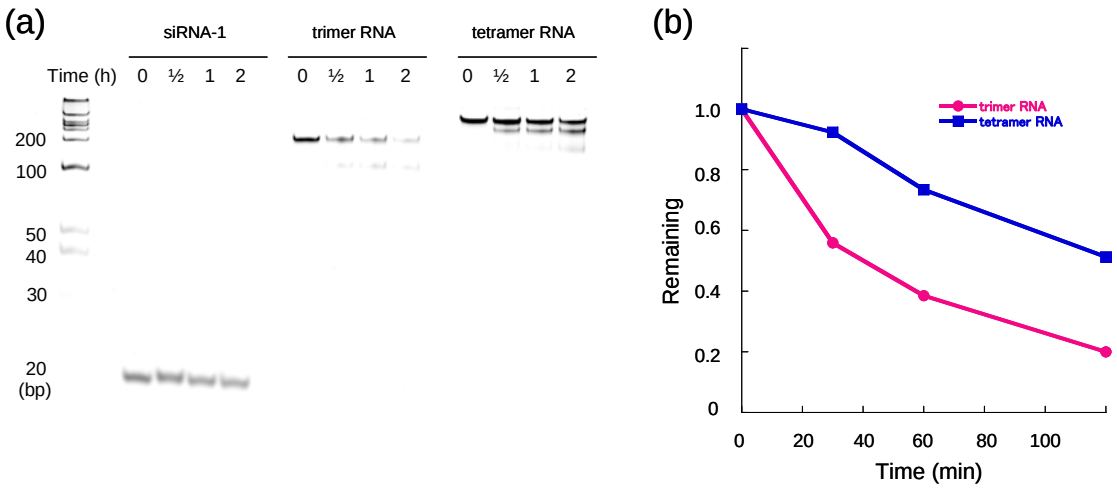

Figure S2. Analysis of the Dicer cleavage reaction of trimer RNA and tetramer RNA. (a) The RNAs were incubated with Dicer enzyme at 37 °C, and aliquots were taken after 0.5, 1, and 2 h. The reaction mixture was analyzed using 15% native PAGE and visualized using SYBR Green I staining. (b) Quantification of the longest sequences of trimer RNA (circles) and tetramer RNA (squares) shown in Figure S1a.

Experimental section

Synthesis of trimer RNA and tetramer RNA.

RNA oligonucleotides were synthesized using 2′-*O*-TOM protected β-cyanoethyl phosphoramidites (Glen Research, USA). Deprotected oligonucleotides were purified by 15% denature PAGE, isolated by the crush and soak method (elution buffer: 0.2 M NaCl, 10 mM EDTA, pH 8.0). Desalted RNAs were precipitated with sodium acetate (pH 5.2) and 2-propanol.

siRNA targeting GL3 luciferase (siRNA-1,-2, -3, -4) corresponded to the coding region nucleotides 849-869, 153-173, 196-216 and 498-518, respectively. Trimer RNA and tetramer RNA targeted the same regions.

Trimer RNA was constructed by mixing three oligonucleotide components (Sequence 1 + 2 + 3; 1:1:1 molar ratio) in annealing buffer (100 mM potassium acetate, 30 mM HEPES-KOH at pH 7.4, 2 mM magnesium acetate) with final concentration of 5 μM. Hybridizations were performed by heating at 90 °C for 3 min, then cooled slowly to room temperature. The solutions were further incubated at 4 °C. the annealed products were stored at -20 °C. Tetramer RNA was constructed in the same manner as for trimer RNA with Sequence 2 + 3 + 4 + 5.

Stability of RNAs against snake venom phosphodiesterase.

Annealed siRNA (6 μM), trimer RNA (2 μM) or tetramer RNA (1.5 μM) were incubated with 2×10^{-4} units/ μL Phosphodiesterase I from *Crotalus adamanteus* venom (Worthington Biochemial, USA) in 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 15 mM $MgCl_2$ at 37 °C. After 0.5, 1 and 2 h, aliquots (3 μL) were taken from the mixture and the reaction stopped by mixing with 5.3 μL of 0.5 M EDTA (pH 8.0) and frozen. They

were analyzed by 15% native PAGE at 3 °C. The total amount of RNA loaded was adjusted according to the mass of RNA to obtain a uniform intensity for the bands. siRNA Ladder marker (TaKaRa Bio, Japan) was used as the size marker for dsRNA. The gel was stained with SYBR Green I (Cambrex, USA), and visualized by scanning on a BioRad Molecular Imager FX (BioRad, USA).

Dicer cleavage reaction of trimer RNA and tetramer RNA.

Annealed siRNA (6 μM), trimer RNA (2 μM) or tetramer RNA (1.5 μM) were incubated with 0.1 units/ μL Rcombinant Human Turbo Dicer Enzyme (Genlantis, USA) in 1× Dicer Reaction Buffer, 1 mM ATP, 0.5× BSA and 5 mM $MgCl_2$ at 37 °C. After 1, 5 h and 20 h, aliquots (3.3 μL) were taken from the mixture and the reaction stopped by mixing with 0.6 μL of Dicer Stop Solution and frozen. They were analyzed by 15% native PAGE at 3 °C. The total amount of RNA laoded was adjusted according to the mass of RNA to obtain a uniform intensity for the bands. siRNA Ladder marker (TaKaRa Bio, Japan) was used as the size marker for dsRNA. The gel was stained with SYBR Green I (Cambrex, USA), and visualized by scanning on a BioRad Molecular Imager FX (BioRad, USA).

Measurements of RNAi activity.

All RNAs used were annealed at 5 μM concentrations in 1× annealing buffer by heating at 90 °C for 3 min, then cooled slowly to room temperature. The solutions were further incubated at 4 °C overnight.

HeLa cells (RIKEN Cell Bank, Japan) were grown at 37 °C under 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, Wako, Japan) supplemented with 10%

FBS. Cells were regularly passaged to maintain exponential growth. One day before transfection at 50% confluency, cells were plated in 12 well plates (5×10^4 cells/mL per well). Cotransfection of reporter plasmids and RNAs was carried out with GeneSilencer (Genlantis, USA) as described by the manufacturer for adherent cell lines. Just before cotransfection, the culture medium was replaced with 921 μL of DMEM without FBS, and 2 μg of GL3-Control (Promega, USA), 2 μg of pRL-TK (Promega), 25 pmol of RNA formulated into liposomes (79 μL) were added to each well. Four hours after the transfection, transfection medium was replaced with 1 mL of 10% FBS in DMEM. Luciferase expression was subsequently monitored with the Dual-Luciferase Reporter Assay System (Promega) on a Wallac ARVO SX (PerkinElmer, USA).