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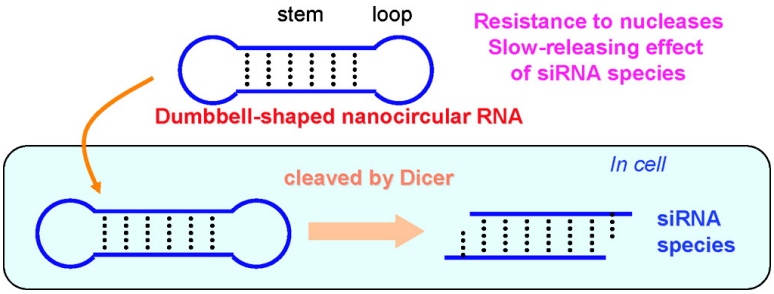


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# Dumbbell-Shaped Nanocircular RNAs for RNA Interference

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RNA interference (RNAi) is a potent and highly specific gene-silencing phenomenon that is initiated or triggered by double-stranded RNAs (dsRNAs).[1a] Shortly after the development of RNAi, small interfering RNAs (siRNAs), which are 21 nucleotides in length with a 3′ nucleotide overhang, were shown to be very effective in mammalian cells.[1b] Much effort has been dedicated to the application of siRNAs, both as biological tools and as therapeutic agents.[1c−e] These approaches can be divided into two main classes. In the first, DNA vectors encoding the RNA polymerase II or III promoter are used to transcribe siRNAs or short hairpin RNAs (shRNAs) in mammalian cells.[1c−e] In the second, chemically synthesized siRNA is introduced directly into cells.[1c−e] Although the vector system is suitable for biological experiments, there are safety problems in clinical applications.[1c−e] Currently, synthetic siRNA would be the method of choice for clinical purposes.[1e] However, natural RNA strands are quickly degraded in biological fluids.[2a] Chemically synthesized unnatural nucleotides have been developed and introduced into the siRNA strand.[1d] For example, modification of the ribose moiety with a 2′-deoxy, 2′-*O*-methyl, or 2′-fluoro group or modification of the phosphate backbone has been examined.[1d] Although these modifications improve the stability of siRNA in serum, they often cause a decrease in RNAi activity.[1d] There is also concern that unnatural RNA derivatives are toxic in the human body. A method to stabilize nontoxic natural RNA strands should be very useful for applications in RNAi technology.

DNA dumbbells, which consist of a double-helical stem closed by two hairpin loops, have been synthesized historically as physical models that can be used to analyze local structures in DNA.[3a,b] They have more recently been applied successfully to transcriptional regulation, where they act as decoy systems because they exhibit increased stability against exonucleases and biological fluids without the need to introduce unnatural nucleotides.[3c−f] A dumbbell-shaped RNA aptamer that contains two deoxynucleotides[4a] and DNA/RNA chimera dumbbells for antisense applications[4b,c] has been reported. Because 3′-exonuclease is a major enzyme involved in the degradation of nucleic acids in vivo,[2b] RNA dumbbells are expected to be more stable than dsRNA. However, to our knowledge, this design has never been tested as an RNAi strategy. A key point to be determined is whether an RNA dumbbell is cleaved by the Dicer enzyme[5] to generate dsRNA, as shRNA is,[5c] to achieve an RNAi effect in cells (Figure 1).

To test this idea, we designed and synthesized circular RNA dumbbells that contain a stem sequence encoding the firefly luciferase gene[1b] and two 9-mer loops (Figure 2a). The loop sequence is used widely in shRNA expression systems.[6] The stem sequences were designed according to a previous report, which showed that the expressed shRNA sequence worked well.[6b] We added one more loop sequence on the other end of this shRNA to form dumbbell structures. To identify the appropriate length of the stem, four dumbbells of differing stem lengths, ranging from 15 to

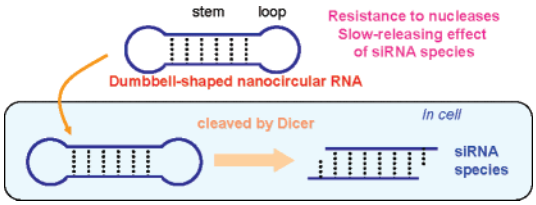


***Figure 1.*** Proposed mechanism of the RNAi effect induced by a dumbbell-shaped circular RNA.

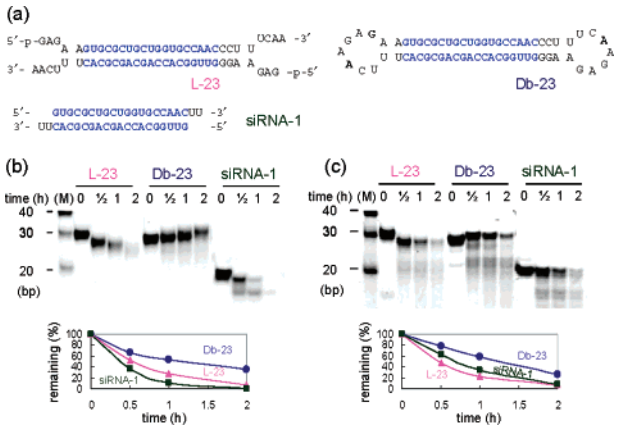


***Figure 2.*** Stability of an RNA dumbbell (Db-23), its linear counterpart (L-23), and an siRNA (siRNA-1). (a) Sequence and structure of RNAs. (b, upper panel) Digestion of RNAs with SVPD. (c, upper panel) Digestion of RNAs with 20% human serum. (b and c, lower panels) Native PAGE analysis of the reaction. Quantification of the longest sequences of Db-23 (circles), L-23 (triangles), and siRNA-1 (squares) in the upper panel.

27 base pairs (bp), were designed and synthesized (Figures 2a and S1). To construct the circular RNA dumbbell Db-23, dsRNA L-23 was closed at both ends with the loop sequences using T4 RNA ligase (Figure S2). A newly formed band that migrated more slowly than the starting single-stranded 32-mer appeared in polyacrylamide gel electrophoresis (PAGE) analysis (Figure S2, lane 6). The new band was inferred to be a circular RNA by comparing it with a synthetic hairpin-shaped RNA (Figure S3). These observations confirmed that RNA dumbbells can be prepared with a good yield from two RNA strands by treatment with T4 RNA ligase. All other dumbbells were prepared by the same method.

The circular dichroism (CD) spectrum of Db-23 was measured and compared with that of its linear counterpart (L-23) to investigate the effect of cyclization on its structure (see Figure 2a for their sequences). Their spectra were almost the same and corresponded to the A-form structure of RNA (Figure S4).[7] Therefore, cyclization had little effect on the helical structure. However, Db-23 showed higher thermal stability in melting experiments. The melting temperatures ($T_m$) of Db-23 and L-23 at a concentration of 0.5 $\mu$M in 10 mM sodium phosphate (pH 7.0) and 1 mM EDTA were about 90 and 71 °C, respectively (Figure S5b).



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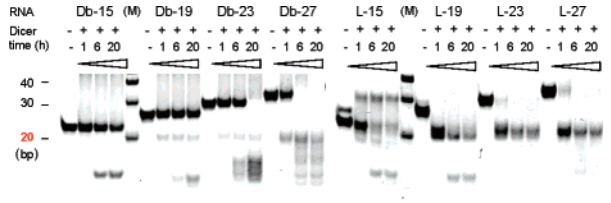


***Figure 3.*** Analysis of the Dicer cleavage reaction of RNA dumbbells. RNAs were incubated with Dicer enzyme at 37 °C, and aliquots were taken after 1, 6, and 20 h. The reaction mixtures were analyzed by 15% native PAGE and visualized with SYBR Green I staining.

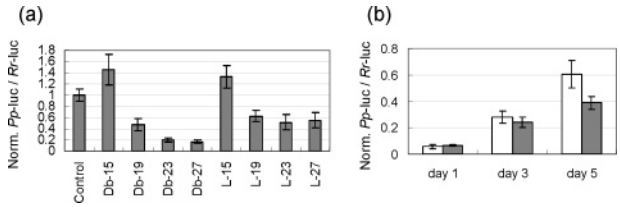


***Figure 4.*** RNA interference with RNA dumbbells. NIH/3T3 cells were cotransfected with 25 nM RNA and the vectors. The expression of the two luciferase genes was assayed after (a) 24 h or (b) 1, 3, or 5 days. Ratios of target to control luciferase were normalized to a control (no RNA). (a) Comparison of the activities with those of their linear counterparts. The plotted data are the means ± standard deviation of six independent experiments. (b) Persistence of the silencing effect of an RNA dumbbell (Db-23, gray) and siRNA (siRNA-1, white) normalized to a control. The plotted data are the means ± standard deviation of three independent experiments.

To determine its biological stability, the RNA dumbbell Db-23 was treated with snake venom phosphodiesterase (SVPD), a 3′-exonuclease. Db-23 showed higher stability compared with that of its linear counterpart, L-23 or an siRNA control, siRNA-1 (Figure 2a,b). After 2 h incubation, 36% of Db-23 retained its sequence in contrast to 6% of L-23. In this condition, no siRNA-1 was detected after 2 h. This improved stability of Db-23 can be attributed to its endless structure, as also reported for DNA dumbbells.[3e,f,4b] Next, Db-23, L-23, and siRNA-1 were incubated in 20% human serum at 37 °C (Figure 2c). In human serum, the differences in stability were not as clear as those observed with SVPD. However, Db-23 was still the most resistant to the degradation among the three tested. As shown in Figure 2c, 26% of Db-23 retained its sequence after 2 h, in contrast to 6% of L-23 and 8% of siRNA-1. We conclude that RNA dumbbells can be stabilized by their structures in biological environments.

Next, we tested whether the RNA dumbbells can be cleaved by the Dicer enzyme to form dsRNA, which can activate RNAi pathways. Recombinant human Dicer enzyme was used to treat four synthetic dumbbells, whose stem lengths differed (Db-15, Db-19, Db-23, and Db-27). Corresponding linear dsRNAs (L-15, L-19, L-23, and L-27, respectively) were tested as a control. As shown in Figure 3, dsRNAs of about 20 bp were produced from all dumbbells except Db-15, although RNAs shorter than 20 bp were observed as the major products. Under the same reaction conditions, linear dsRNAs were digested almost completely to shorter sequences of about 20 bp within 1 h (Figure 3). In contrast, the RNA dumbbells were digested much more slowly. This slow processing could be explained by structural evidence that Dicer recognizes the helical end of the RNA before digestion.[5] As the stem length increased, the rate of digestion of the dumbbells increased. Db-27 was digested almost completely after 6 h. In contrast, Db-15 and Db-19 were digested only partially even after 20 h.

To measure the RNAi activity of the RNA dumbbells, all dumbbells (Db-15 to Db-27) were analyzed. RNAs (25 nM) and the two vectors were cotransfected into NIH/3T3 cells, and the expression levels of the two luciferase genes were measured.[1b] The RNAi activity of the dumbbells and their linear counterparts (L-15 to L-27) was measured to study the effect of cyclization 24 h after transfection (Figure 4a). Db-15 and L-15 showed no interference activity because they were too short.[1b−e] Maximum suppression was induced by Db-23 and Db-27, and this suppression was about threefold more potent than that induced by L-23 and L-27. Db-19 showed moderate suppression activity compared with Db-23 or Db-27 probably because its insufficient stem length caused poor digestion by the Dicer enzyme (Figure 3). Therefore, we conclude that dumbbells with stem lengths longer than 23 bp offer strong RNAi effects and that cyclization has a positive effect on the interference activity.

Finally, the interference activities of Db-23 and the siRNA sequence (siRNA-1, Figure 2a) that targets the same region of the gene were compared over a longer period of 5 days (Figure 4b). Both RNAs showed similar suppression levels on days 1 and 3. However, on day 5, the suppression activity of Db-23 was 1.5-fold more potent than that of siRNA-1. These results confirm that an RNA dumbbell has prolonged RNAi activity. This effect could be induced by the slow release of the siRNA species from the dumbbell.

In conclusion, we have demonstrated that, despite their natural RNA strands, dumbbell-shaped RNAs withstand enzymatic degradation and offer prolonged RNAi activity because of the shape of the molecule, an endless structure. Our ongoing work is aimed at improving this loop structure.

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**Supporting Information Available:** Experimental details and figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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