RNA Nanostructures Hot Paper

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Crystal-Structure-Guided Design of Self-Assembling RNA Nanotriangles

*Mark A. Boerneke, Sergey M. Dibrov, and Thomas Hermann**

Abstract: *RNA nanotechnology uses RNA structural motifs to build nanosized architectures that assemble through selective base-pair interactions. Herein, we report the crystal-structure-guided design of highly stable RNA nanotriangles that self-assemble cooperatively from short oligonucleotides. The crystal structure of an 81 nucleotide nanotriangle determined at 2.6 Å resolution reveals the so-far smallest circularly closed nanoobject made entirely of double-stranded RNA. The assembly of the nanotriangle architecture involved RNA corner motifs that were derived from ligand-responsive RNA switches, which offer the opportunity to control self-assembly and dissociation.*

Nucleic acids have been used extensively to build nanosized objects by controlling assembly through designed base-pair interactions. Complex nanoobjects have been obtained by recursive folding of long nucleic acid sequences through inclusion of alternating double- and single-stranded regions, junctions, and helper oligonucleotides. The design of nanoobjects has exploited structural motifs observed in crystal structures.[1–14] We previously used short oligonucleotides to construct a self-assembling RNA nanosquare of 100 nucleotides.[12] Here we describe the crystal-structure-guided design of RNA nanotriangles that self-assemble in a cooperative process from multiple copies of short oligonucleotides. Crystal-structure analysis of an RNA triangle containing 81 nucleotides reveals the so-far smallest circularly closed nanoobject made entirely of double-stranded RNA. The self-assembly and dissociation of nanotriangles is sequence-dependent and may be modulated by ligands which bind recognition motifs incorporated in the RNA architectures.

The simplest known ligand-responsive RNA switches were recently discovered in the internal ribosome entry sites (IRES) of positive-strand RNA viruses of the *Flavi-* and *Picornaviridae* families. These RNA switch motifs are located in subdomain IIa of IRES elements and regulate viral protein synthesis through a ligand-dependent conformational transition.[15,16] Unlike traditional riboswitches, viral RNA switches do not undergo changes in secondary structure, but rather undergo a purely mechanical switching between two distinct and stable conformations. The viral RNA motifs adopt a bent conformation in the absence of a ligand, while an elongated conformation is captured by a bound ligand. Structures of ligand-free IIa switches from the IRES elements of hepatitis C virus (HCV) and Seneca Valley virus (SVV) as well as the ligand-bound switch from HCV were previously determined by X-ray crystallography.[16–18] The ligand-free IIa switch motif from HCV has previously been used as a corner building block to design and construct a self-assembling nanosquare and a nanoprism.[12,19] We concluded that other RNA nanoobjects may be obtained from viral IIa switch motifs by rational design, including variation of the length of the helices flanking each corner unit and adjusting the orientation of the corners relative to a common plane.

Crystal-structure analysis of the IIa corner motif from the SVV IRES provided design directions to construct triangular nanoobjects (Figure 1). The three-dimensional structure of the SVV motif was obtained from crystals of a short and long RNA construct, both of which showed identical corner structures.[16] The crystal packing of both constructs revealed circularly closed triangles involving pseudocontinuous stacking and intermolecular base-pair formation between a 3′-terminal overhanging nucleotide on both ends of each of three identical corner units (Figure 1b; Figures S1–S3 in the Supporting Information). Self-assembling nanotriangle constructs were designed guided by the triangular arrangement seen in the SVV IIa RNA crystal packing. To promote self-assembly of the corner motifs into a triangle, constructs were designed with the same total number of nucleotides and base identity, but various 3′-terminal overhang lengths. Secondary-structure models of exemplary self-assembling constructs containing four nucleotide overhangs are shown in Figure 1c. A small triangle is constructed from two oligonucleotides, with inner and outer strands of 11 and 16 residues, respectively (Figure 1c, top). Three copies of each of the inner and outer strands are designed to self-assemble as a single small triangle of 81 nucleotides. Similarly, a large triangle is constructed from strands of 20 and 26 residues, which form a nanoobject consisting of 138 nucleotides (Figure 1c, bottom).

Short and long SVV IIa corner motif constructs (Figure 1a) which contain single 3′-nucleotide overhangs were analyzed by native polyacrylamide gel electrophoresis (PAGE) and found to migrate as single bands consistent with their respective size (Figure 2a, 1 nucleotide overhang both; see the Supporting Information for all experimental details). In contrast, nanotriangle RNA constructs carrying four overhanging nucleotides (Figure 1c) to promote self-

[*] M. A. Boerneke, S. M. Dibrov, T. Hermann
Department of Chemistry and Biochemistry
University of California, San Diego
9500 Gilman Drive, La Jolla, CA 92093 (USA)
E-mail: tch@ucsd.edu
T. Hermann
Center for Drug Discovery Innovation
University of California, San Diego
9500 Gilman Drive, La Jolla, CA 92093 (USA)

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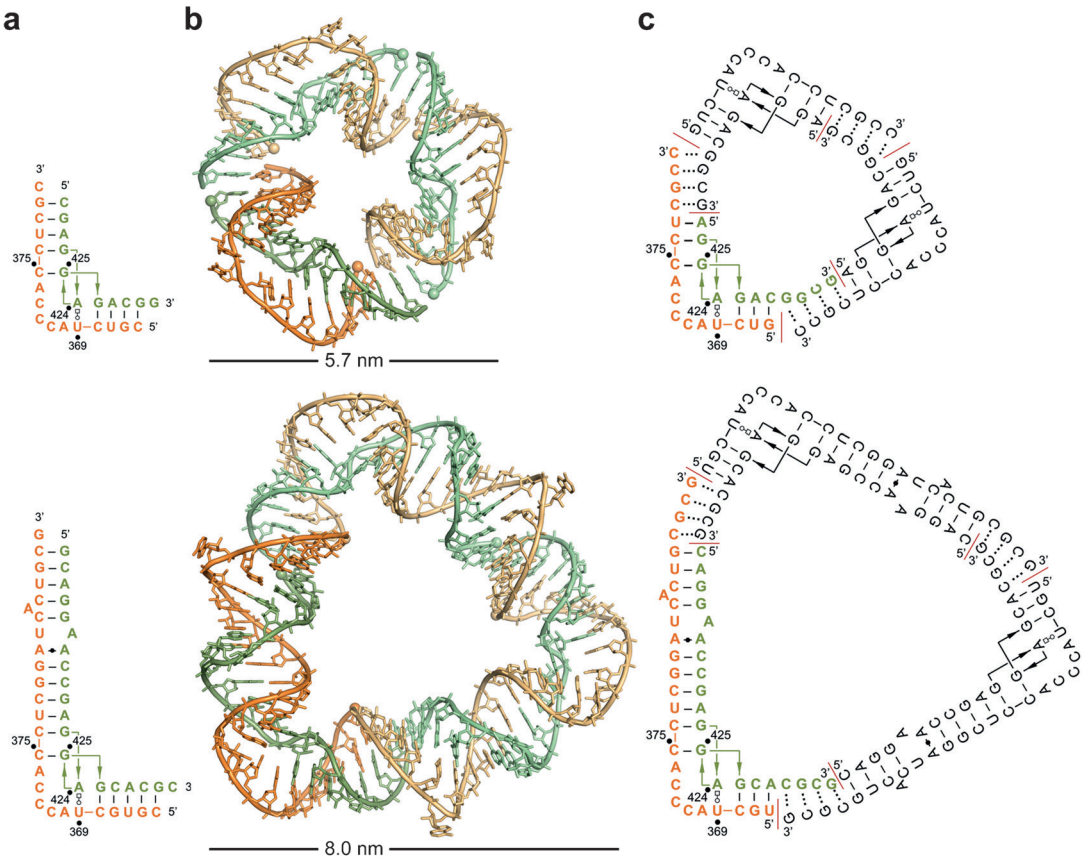

Figure 1. Design of self-assembling RNA nanotriangles. a) Secondary structures of short and long oligonucleotide constructs representing the subdomain IIa motif from the SVV IRES. Single-nucleotide overhangs aided crystallization by facilitating RNA packing. Residue numbering refers to the SVV genome. b) Circularly closed triangles seen in the packing of both short and long IIa RNA crystal structures (PDB ID: 4P97 and 4PHY). c) Secondary structure models of self-assembling RNA triangles containing four-nucleotide overhangs and designed using the crystal packing of short and long IIa constructs. Red lines indicate oligonucleotide termini.

assembly were found to migrate slower and consistent with the size of symmetrical triangles comprised of three identical corners (Figure 2a; 4 nucleotide overhang both). The small triangle construct migrated as a single band, while the large triangle construct gave rise to a faster moving major band and a slower moving minor band, the latter consistent with the size of a dimer of triangles (see Figure S4 for discussion). The faster moving major band was confirmed as a single triangle of three corners by comparative analysis of an identically sized programmable triangle that contains three distinct corners (A, B, C), each with a unique single-stranded overhang sequence that allows exclusive formation of the designed triangle with an A-B-C configuration but not other assemblies (Figure 2c,d). Both RNA nanotriangles were evaluated for their chemical and thermostability, and shown to resist boiling in water as well as incubation with 8 M urea (Figure 2b, see the Supporting Information for discussion).

Preliminary studies were performed to determine the effects of switch-binding ligands on the assembly and dissociation of the RNA nanotriangles. Native PAGE analysis revealed a decrease in the assembly efficiency of the large triangle in the presence of 500 μM ligand[20] (Figure 3a,c). As a consequence of the ligand capture of elongated IIa RNA switches in the self-assembling constructs, end-to-end association of straightened corner units of the large triangle led to the formation of multimers. This was not observed when ligand was added to the small triangle, perhaps due to the lability of the construct, which has only seven base pairs flanking an internal loop of five unpaired bases and forms by an all-or-nothing assembly from single strands. Dissociation of already assembled large triangles, which are less compact and stabilized by 15 base pairs flanking the internal loop, was observed upon incubation with ligand in the case of the symmetrical (Figure 3b) as well as the programmable construct (Figure S6). These findings suggest that the RNA corner units retain their function as ligand-responsive switches when incorporated into nanotriangles. The ligand-triggered dissociation of nanotriangles and assembly of alternate structures opens potential avenues for the construction of RNA nanoobjects that respond to environmental signals.

To investigate the three-dimensional structure of the small and large RNA nanotriangles, we crystallized both constructs. Well-diffracting crystals were obtained for the small triangle. Structure determination by X-ray diffraction at 2.6 Å resolution (Figure 4, see also Figures S7–S10 and Table S1) revealed a circularly closed and continuously double-stranded RNA which exhibited an architecture similar to the pseudo-

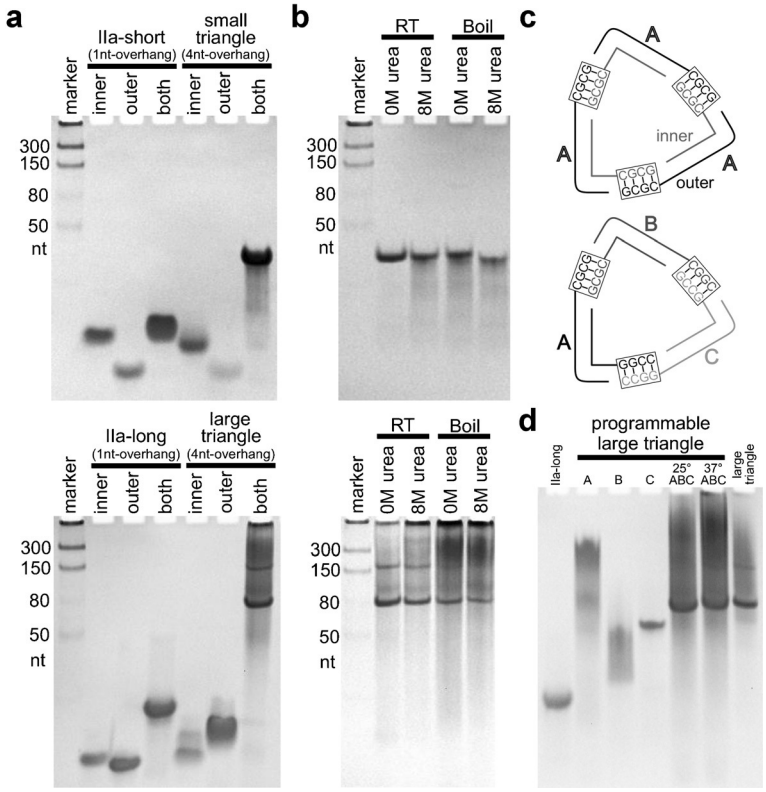

Figure 2. Assembly and stability of the nanotriangles. The structures were analyzed by native PAGE in the presence of 2.5 mM $MgCl_2$. a) Top gel: Assembly of a subdomain IIa short crystal construct containing single-nucleotide (nt) overhangs (secondary structure shown in Figure 1a, top) and self-assembling small triangle construct containing four-nucleotide overhangs (secondary structure shown in Figure 1c, top). Bottom: Assembly of subdomain IIa long crystal construct containing single-nucleotide overhangs (secondary structure shown in Figure 1a, bottom) and a self-assembling large triangle construct containing four-nucleotide overhangs (panel (c), top; secondary structure shown in Figure 1c, bottom). b) Stability of the small triangle (top) and large triangle (bottom) when treated with or without 8 M urea at room temperature (RT) or in boiling water. c) Diagram of the large triangle (AAA, top) and programmable large triangle (ABC, bottom). d) Assembly of the programmable large triangle (ABC) from corners A, B, and C.

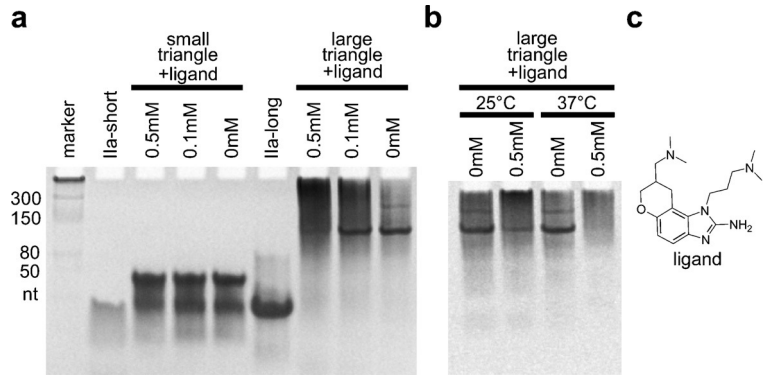

Figure 3. Self-assembly efficiency and dissociation of the nanotriangle in the presence of ligand. Ligand binding of the IIa switch captures an elongated conformation of the RNA.[16] a) Assembly of the small triangle is not affected by the presence of a switch-binding ligand. Assembly of the large triangle is partially prevented in the presence of a switch-binding ligandbut not affected by control compounds, which lack target-specific binding (Figure S5). The single corner units of the large triangle construct, captured by the ligand in an elongated conformation, prevent triangle formation and promote end-to-end multimerization into longer species. b) Dissociation of large triangles was observed when incubated post-assembly with a binding ligand. c) Structure of the benzimidazole ligand which binds to the IIa RNA switch element of the nanotriangles.

continuously closed small triangle seen in the crystal packing of the short IIa construct (Figures 1b and 4). As designed, the nanotriangle comprises three identical and symmetrical corner units, each forming from an inner and outer RNA strand with four overhanging nucleotides that hybridize with neighboring corner strands. The sides of the triangle are composed of 11 base pairs and measure about 5 nm in length, while the corners contain the IIa internal loop of 5 bases. The overall structure appears hexagonal at first glance, as it is not planar but has distorted sides that twist to accommodate three 90° corner motifs in a closed triangular architecture. The resulting structure is more compact than a planar triangle with a comparable side length. The 12 termini of the 6 single strands constituting the RNA triangle are located on the same face of the nanostructure (Figure 4 and Figure S7a). This feature offers an opportunity to build more complex structures and to functionalize the nanotriangle for sensor and materials applications.

The design of nanoscale objects that self-assemble from short oligonucleotides remains a key challenge in the emerging field of RNA nanotechnology. Although larger nanoarchitectures have previously been constructed from structurally complex RNA motifs or long nucleic acid sequences in conjunction with helper oligonucleotides, we aimed to create minimalist RNA nanoobjects by the efficient assembly of short sequences which by themselves do not adopt stable structures. We exploited detailed insight from X-ray crystallography to design and construct two different RNA nanotriangles that self-assembled from six oligonucleotides in solution and were crystallized for structural studies. The nanotriangles display remarkable stability towards denaturation and their composition offers unique structural features that promise applications in medicine, nanomaterials engineering, and as tools to test nanoscale phenomena. As a consequence of the hierarchical self-assembly from six short oligonucleotides, the RNA nanotriangles can be readily modified by conjugation at any of the 12 component strand termini to introduce additional functionality. Since the corner units used in the construction of the nanotriangles are also ligand-dependent conformational switches, the association and dissociation of the resulting RNA architectures is tunable, which will enable the design of nanodevices sensitive to environmental or cellular milieus.

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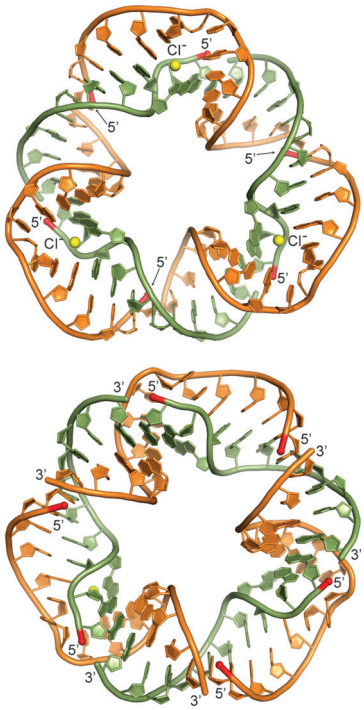

Figure 4. Crystal structure of the self-assembling RNA nanotriangle. Views from both sides of the triangle plane are shown. The back view (top) reveals three Cl^- ions (yellow spheres) bound at the Watson–Crick edge of nucleotides A374 and C375. The terminal residues of all the constituting oligonucleotides reside on one face of the triangle (front view, bottom). The 5′-termini are highlighted in red. Atomic coordinates and structure factors have been deposited in the Protein Data Bank (PDB ID: 5CNR).

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