Self-assembling RNA square

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The three-dimensional structures of noncoding RNA molecules reveal recurring architectural motifs that have been exploited for the design of artificial RNA nanomaterials. Programmed assembly of RNA nanoobjects from autonomously folding tetraloop–receptor complexes as well as junction motifs has been achieved previously through sequence-directed hybridization of complex sets of long oligonucleotides. Due to size and complexity, structural characterization of artificial RNA nanoobjects has been limited to low-resolution microscopy studies. Here we present the design, construction, and crystal structure determination at 2.2 Å of the smallest yet square-shaped nanoscale platform. We demonstrate the programmed self-assembly of RNA squares from complex mixtures of corner units and establish a concept to exploit the RNA square as a combinatorial nanoscale platform.

Noncoding RNA sequences can adopt intricate three-dimensional architectures whose complexity rivals those of proteins. The folding of RNA is governed by recurring structural motifs (1), the most common of which is the double helix that involves consecutively stacked pairs of complementary nucleobases interacting via hydrogen bonds. Structural motifs in RNA often form locally without the involvement of long-range tertiary interactions (2). The synthetic combination of RNA motifs has been exploited in the design of functional and architectural RNA structures (3–5), including artificial ribosensors and RNA “Lego” (6–8). Similar to well-established methods for the design and construction of DNA “origami” and nanomaterials (9, 10), approaches toward artificial RNA architectures have relied on the ability of RNA strands to hybridize via complementary base sequences (11, 12). Long oligonucleotides of considerable sequence complexity have been used to build complex RNA Lego as well as square- and cube-shaped objects (7, 13, 14). Here, we describe the design and crystal structure analysis of an RNA nanoobject that self-assembles from multiple copies of two short oligonucleotides which give rise to the smallest possible square structure that may be built from double-stranded RNA. We demonstrate sequence-dependent programmed self-assembly of the RNA square which might be exploited as a nanoscale platform for the directed combination of up to four molecular entities.

Results and Discussion

Previously, we had determined the three-dimensional structure of the domain IIA bulge in the internal ribosome entry site (IRES) of the hepatitis C virus (HCV) RNA genome (15) (Fig. 1 A and B). The domain IIA, which is a potential target of antiviral drugs that block HCV protein synthesis (16), adopts a sharply bent fold that is required for the correct spatial positioning of the HCV IRES during recruitment of host cell ribosomes (17, 18). Structural analysis of the unique 90° bend in the IIA domain led us to conclude that this viral RNA motif may constitute the most compact L-shaped object, or nanocorner, that can be built from contiguous double-stranded RNA. Molecular modeling based on the IIA crystal structure suggested that combination of four such nanocorners would allow the assembly of a double-stranded RNA square (Fig. 1 C) in which the internal loop motifs are separated by 10 base pairs, corresponding to a full turn in an A-form RNA double helix. Introduction of appropriate termini in the modeled RNA square allow a design that required only two distinct oligonucleotides (“inner” strand of 10 nucleotides and “outer” strand of 15 nucleotides) that would self-assemble via complementary overlapping sequences of four bases. The resulting RNA square is comprised of four identical copies of each, inner and

Fig. 1. Design of the self-assembling RNA square. (A) Secondary structure of the IIA-1 RNA representing the subdomain IIA of the IRES from HCV. Numbering was adopted from the HCV genome. (B) Three-dimensional structure of the IIA-1 RNA (15). Positions of magnesium ions are indicated by spheres. (C) Secondary structure of the RNA square. The four copies of the IIA-1 core are highlighted in different colors. Lines indicate boundaries of oligonucleotides. (D) Native polyacrylamide gel electrophoresis of IIA-1 and square RNA. Inner and outer oligonucleotides were loaded at higher concentrations compared to assembled IIA and square RNA.
outer strand, totaling 100 nucleotides. The design of the RNA square was tested by annealing of inner and outer strand oligonucleotides which produced a single RNA species that migrated in a native polyacrylamide gel at a size compatible with the square architecture (Fig. 1D and SI Appendix, Fig. S1).

The three-dimensional structure of the 100-nucleotide RNA square was investigated by X-ray crystallography. Single wavelength anomalous diffraction data collected from crystals of inner strand oligonucleotide annealed to a 5-bromo uridine-labeled (5BrU59) outer strand was used to determine the structure of the RNA square at 2.2 Å resolution (Fig. 2 and SI Appendix, Figs. S2–S6 and Tables S1 and S2). The crystal structure revealed a fully double-stranded RNA architecture that corresponded to the original design of the square, containing four outer strand oligonucleotides (designated A, C, E, and G) as well as four inner strands (designated B, D, F, and H) which assemble via overlapping complementary sequences and fold into the 90° bent corners of the HCV IRES domain IIa. The sides of the square measure approximately 60 Å. The distance across the interior space is about 18 Å. The termini of the outer strands are located at the interior of the square, whereas those of the inner strands are oriented at the outer circumference.

In agreement with the migration behavior in native polyacrylamide gel electrophoresis, the RNA square adopts a highly compact architecture in which 96 of the 100 nucleotides have their bases stacked with at least one neighboring residue. The bases of C55 and U56 in strand C are rotated out from the RNA fold and participate in stacking with a neighboring square. Nucleotides U56 and A57 in strand G were disordered in the electron density map. The overall shape of the RNA square in the crystal revealed unexpected asymmetry, attested by the fact that the four corners all adopt slightly different structures (Fig. 3). Corners formed from strands designated as A/B and G/H (Fig. 2) are most similar to each other and to the structure of the core in the HCV IRES domain IIa (SI Appendix, Table S3). The corner comprised of strands E/F is most dissimilar to all others, attested by the presence of a unique non-Watson-Crick A57−C111 base pair as well as a neighboring G110−C58/C55 base triple (Fig. 4), which are both absent in the original HCV IRES domain IIa. Participation of C111 in the A−C pair is at the expense of the canonical G52−C111 pair which is absent in the corner formed by strands E/F. As a consequence, the helix connecting corners E/F and G/H contains only nine base pairs, whereas the helix between corners C/D and E/F has 11 pairs. The other sides of the square, the helices between corners A/B and C/D as well as G/H and A/B, are each comprised of 10 base pairs as was designed originally.

The asymmetry observed in the four corners extends to the intermolecular contacts involved in packing of squares in the crystal (SI Appendix, Table S4). Within the plane, squares line up along diagonals with corners A/B in one square tightly packing against corners E/F in the adjacent molecule (SI Appendix, Fig. S3), forming numerous intermolecular hydrogen bonds (SI Appendix, Table S4). Corners C/D and G/H are pointing toward the cleavage formed between sides between corners G/H and A/B as well as E/F and G/H in neighboring squares. The tight interactions between A/B and E/F corners of adjacent squares

Fig. 2. Structure of the RNA square. (A) Minor groove side of the helical regions (2Fo−Fc electron density map contoured at 1σ). (B) View from the major groove side. (C) Secondary structure revised according to the crystal structure. Dashes at nucleotides in the corner loops indicate continuous stacking of bases on an adjacent helix; arrows depict rotated-out residues that do not stack on neighboring bases. In the EF corner, A57−C111 form a cis-Watson-Crick pair and C55 participates in a base triple while docking at the major groove edge of C58−G110. Binding sites of Mg2+ and Co3+ are indicated.

Fig. 3. Structure of the four corners within the RNA square. To facilitate comparison, the corners were rotated to show the same orientation in each case.
is likely the cause for distortions in the secondary structure of the E/F strand pair as was discussed above. The distinct involvement of corners in the crystal packing is reflected in less surface exposure and lower thermal factors for the A/B and E/F corners as opposed to the pairs of C/D and G/H strands which show larger exposed surfaces as well as higher B factors (SI Appendix, Fig. S4 and Table S5).

Crystallization of the RNA square was critically dependent on the presence of metal ions, as was expected from the important contribution of magnesium cations in the structure of the HCV IRES domain IIa (15). In the crystallized RNA square, 10 hexamminecobalt(III) and 2 magnesium cations are associated with the RNA (Fig. 2C and SI Appendix, Table S6). The four double-helical regions that connect the corners and contain the overlapping termini of the outer and inner strands are each stabilized by two hexamminecobalt(III) ions. One such cation is bound at the major groove edges of guanines in both strands (G60, G106, G107), while another bridges the major groove edges of two guanines in the outer strands only (G48, G49). These two metal positions are identical to magnesium ion binding sites in the crystal structure of the HCV IRES domain IIa. The four internal loops that form the corners of the RNA square have additional metal ions associated, albeit each at a different position. The metals in three of the corners have magnesium counterparts in the domain IIa (one Mg\(^{2+}\) each, bridging C55 and A109 in strands A/B as well as a Co\(^{3+}\) at G52 in strand G; one Mg\(^{2+}\) bridging G51 and G52 in strand E). A Co\(^{3+}\), associated with both A53 and A54 of strand C, is unique to the square structure.

After gel shift analysis and crystal structure determination confirmed the design concept of the RNA square, proof of principle experiments were performed to establish the utility of the RNA square, to determine overlapping sequences for assembly via strand hybridization. In the crystallography construct, all four recognition sequences were identical (A), resulting in corners designated as A^A. Permutations were generated (B, C, D) for the programmed assembly of fluorescently labeled corners with nonidentical recognition sequences (X^Y), which are unable to self-associate. (β) Fluorescence of dye-labeled corners A^B-cy3 and C^D-cy5 in complementation experiments (○, cy3; □, cy5; ⊗, quencher). A + symbol indicates the presence of a corner unit. Species that contribute to the fluorescence in each experiment are depicted.

or mismatched corners (B ^ A or D ^ C, or an equimolar mixture of B ^ C and D ^ A) and recorded the fluorescence signal of the cyanine dyes (Fig. 5, 1–12). We hypothesized that when a dye-labeled corner unit is incorporated in an RNA square, the cyanine residue at the 5’ end of the outer strand is placed inside the square where fluorescence is reduced relative to an unincorporated corner in which the dye points freely into the solvent. The corner units used in the complementation experiments were designed such that, upon square formation, two dye labels would be confined in close proximity inside the 18-Å cavity of the square, which would result in self-quenching. The complementation experiments supported these hypotheses.

Fig. 5. Programmed self-assembly of fluorescently labeled RNA squares. (A) Four 5’-terminal bases in the oligonucleotides of the corner units constitute overlapping sequences for assembly via strand hybridization. In the crystallography construct, all four recognition sequences were identical (A), resulting in corners designated as A^A. Permutations were generated (B, C, D) for the programmed assembly of fluorescently labeled corners with nonidentical recognition sequences (X^Y), which are unable to self-associate. (B) Fluorescence of dye-labeled corners A^B-cy3 and C^D-cy5 in complementation experiments (○, cy3; □, cy5; ⊗, quencher). A + symbol indicates the presence of a corner unit. Species that contribute to the fluorescence in each experiment are depicted.

As a control, a unit carrying a high-efficiency quencher was added at 10-fold excess to a mixture of the dye-labeled corners, along with the B^- C and D^+ A units, which led to reduction of fluorescence in both dyes comparable to self-quenching (experiment 12).

These experiments demonstrate that the selective association of distinct RNA squares from complex mixtures of corner units can be controlled by addition of sequence-complementary units. The programmed self-assembly described here establishes a concept to exploit the RNA square as a nanoscale platform for the directed combination of up to four molecular entities that are linked to the corner units. Simple modifications of the square architecture promise to provide unique RNA architectures as well as to exploit the RNA square as a nanoscale platform for the construction of nanostructures. For example, elongation of the square sides by one base pair (to 11 pairs; see SI Appendix, Fig. S7) would force the adjoining corners to rotate out of plane, which would prevent the closure of the square and perhaps allow the association of more than four corner units to give spiral-like structures. Preliminary experiments suggest that populations of such spirals of varying number of constituting corners might indeed form as a consequence of the unhindered recognition sequences of the terminal units (SI Appendix, Fig. S7).

Materials and Methods

RNA Preparation. RNA was annealed from stoichiometric amounts of HPLC-purified oligonucleotides in 10 mM sodium cacodylate buffer, pH 6.5, 5 mM MgCl$_2$ (SI Appendix, Table S1).

Gel Electrophoresis. RNA was analyzed on 13% native polyacrylamide gel in 40 mM MOPS buffer and 2.5 mM MgCl$_2$. Visualization was performed under UV after ethidium bromide staining.

Crystalization, Data Collection, and Structure Determination. Square RNA at 0.2-mM concentration was mixed with an equal volume of precipitating solution containing 50 mM sodium cacodylate, pH 6.5, 150 mM MgCl$_2$, 8-10 mM (Co(NH$_3$)$_6$)$_3$Cl$_2$, 50 mM KCl, and 15–20% PEG4000. Crystals grew at 16 °C by hanging drop vapor diffusion after equilibration against 17% PEG6000 in 20 mM sodium cacodylate, 50 mM KCl, at pH 6.4. Diffraction data were collected on beamlines 17-ID-B at the Advanced Photon Source, Argonne National Laboratory. Data were processed with HKL2000 (19). Examination of diffraction data by the program Xtriage (20) revealed a twinning component with a fraction of 0.498 and following the twin law -h, -k, l. Initial phases were calculated in PHENIX (20) by the single wavelength anomalous diffraction method using the anomalous scattering from a bromine incorporated at US9. An initial model was automatically built in PHENIX followed by iterative rounds of manual building and refinement, alternating between Refmac (21) using twin refinement within CCP4 (22) and manual rebuilding in Coot (23) based on the obtained $2F_o - F_c$ and $F_o - F_c$ maps. Metal ions were assigned based on electron density and geometry of coordinating ligands. Final refinement was carried out in PHENIX with combined translation libration screw motion, individual isotypic atomic displacement parameters, and water picking.

Fluorescence Experiments. Fluorescence experiments were performed at 100-nM RNA concentration in black 96-well plates on a Spectra Max Gemini monochromator plate reader (Molecular Devices) at 25 °C. Excitation was at 540 (Cy3) or 640 nm (Cy5). Emission was read at 570 (Cy3) or 670 nm (Cy5).

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Supporting Information for

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Fig. S1. Denaturing polyacrylamide gel electrophoresis analysis of IIa-1 and square RNA. The same samples as shown in Figure 1D were analyzed. Marker oligonucleotides are single stranded. Individual inner and outer oligonucleotides were loaded at higher concentrations compared to assembled IIa-1 and square RNA (“both”).
Fig. S2. Stereo views of the self-assembling RNA square overlaid with a 2Fo-Fc electron density map contoured at 1σ. (A) Front view. (B) Back view. The color scheme of the strands corresponds to the colors in Fig. 1C.
Fig. S3. Crystal packing of the self-assembling RNA square. View of next neighbors within a plane of squares. The color scheme of the strands corresponds to the colors in Figure 1C.
**Fig. S4.** Crystal packing of the self-assembling RNA square. View of next neighbors within a plane of squares. Strands are colored according to B factors (blue = low, red = high B factors).
**Fig. S5.** Stereo view of the crystal packing of the self-assembling RNA square. View of next neighbors in adjacent planes of squares. The color scheme of the strands corresponds to the colors in Fig. 1C.
Fig. S6. Stereo view of the crystal packing of the self-assembling RNA square. View of next neighbors in adjacent planes of squares. Strands are colored according to B factors (blue = low, red = high B factors).
Fig. S7. Native polyacrylamide gel electrophoresis analysis of IIa-1, square RNA and an RNA that contained one additional base pair in each side of the square (square +1bp). Marker oligonucleotides are single stranded.
Table S1. Oligonucleotides used for experiments.

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gel electrophoresis:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IIA1-inner strand</td>
<td>IIA1I</td>
<td>5’ GCG UGU CUG GCA GCC UCC G</td>
</tr>
<tr>
<td>IIA1-outer strand</td>
<td>IIA1O</td>
<td>5’ CGG AGG AAC UAC UGU CUU CAC GCC</td>
</tr>
<tr>
<td>square inner strand</td>
<td>AAI</td>
<td>5’ CCG GCA GCC U</td>
</tr>
<tr>
<td>square outer strand</td>
<td>AAO</td>
<td>5’ CCG GAG GAA CUA CUG</td>
</tr>
<tr>
<td><strong>Crystallization (square):</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>inner strand</td>
<td>AAI</td>
<td>5’ CCG GCA GCC U</td>
</tr>
<tr>
<td>outer strand</td>
<td>AAO5Br</td>
<td>5’ CCG GAG GAA CUA C^5BrUG^1</td>
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<td><strong>Fluorescence Experiments:</strong></td>
<td></td>
<td></td>
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<tr>
<td>inner strand for A^B-cy3</td>
<td>ABI</td>
<td>5’ GGC CCA GCC U</td>
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<tr>
<td>outer strand for A^B-cy3</td>
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<td>5’ GCG CCA GCC U</td>
</tr>
<tr>
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<tr>
<td>outer strand for C^D-Q</td>
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<td>5’ GGC CAG GAA CUA CUG</td>
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<td>outer strand for D^C</td>
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<tr>
<td>outer strand for D^A</td>
<td>DCO</td>
<td>5’ GCG CAG GAA CUA CUG</td>
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^1^5BrU = 5-bromo uridine  
^2^cy3, cy5 = fluorescent cyanine dyes attached to the 5’ O atom via a (CH2)_4- linker.  
^3^IAbRQ = Iowa Black RQ Dark quencher attached to the 5’ O atom via a (CH2)_4- linker.
Table S2. Crystallographic data collection and refinement statistics for the square RNA.

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<th>Native</th>
<th>Crystal 1</th>
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<tr>
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<tr>
<td>(a, b, c (\text{Å}))</td>
<td>62.38</td>
<td>62.38</td>
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<tr>
<td></td>
<td>62.38</td>
<td>126.27</td>
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<tr>
<td>(\alpha, \beta, \gamma (\degree))</td>
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<td>120</td>
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<tr>
<td></td>
<td>90</td>
<td>90</td>
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<td>(R_{\text{sym}}) or (R_{\text{merge}})</td>
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<td>(I/\sigma I)</td>
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<td>Completeness (%)</td>
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<td>(R_{\text{work}})/(R_{\text{free}})</td>
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<tr>
<td>Ligand/ion</td>
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<td>Bond angles (°)</td>
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*Highest resolution shell is shown in parenthesis.*
Table S3. Root mean square deviation [Å] within strands of the square RNA and compared to IIa-1 RNA.

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<td>GH</td>
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*Nucleotides 107-113 of the inner strand and 50-55/58-60 of the outer strand were used for superposition.*
Table S4. Potential hydrogen bonds and stacking interactions in crystal contacts of the square RNA.

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<td>A</td>
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<td>O4</td>
<td>F'</td>
<td>G110</td>
<td>N2</td>
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<td>F'</td>
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*Primed strand designations indicate a neighboring square RNA.
Table S5. Exposed surface area and B-factors in the crystal structure of the square RNA.

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*Hexamminecobalt(III) ions are listed as "Co", hydrated magnesium ions as "Mg".*