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Composing RNA Nanostructures from a Syntax of RNA Structural Modules

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Abstract

Natural stable RNAs fold and assemble into complex three-dimensional architectures by relying on the hierarchical formation of intricate, recurrent networks of noncovalent tertiary interactions. These sequence-dependent networks specify RNA structural modules enabling orientational and topological control of helical struts to form larger self-folding domains. Borrowing concepts from linguistics, we defined an extended structural syntax of RNA modules for programming RNA strands to assemble into complex, responsive nanostructures under both thermodynamic and kinetic control. Based on this syntax, various RNA building blocks promote the multimolecular assembly of objects with well-defined three-dimensional shapes as well as the isothermal folding of long RNAs into complex single-stranded nanostructures during transcription. This work offers a glimpse of the limitless potential of RNA as an informational medium for designing programmable and functional nanomaterials useful for synthetic biology, nanomedicine, and nanotechnology.

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L.J. and C.G. conceived and designed experiments; C.G., A.C., and E.V. performed biochemical experiments; A.C. and C.G. performed AFM; N.V. performed cryo-EM; L.J. wrote the paper with the help of C.G. and A.C.

Notes

The authors declare no competing financial interest.

ASSOCIATED CONTENT

Supporting Information

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Additional details on materials and methods. Tables showing RNA and tectoRNA sequences and the composition of studied nanostructures and G3-heart sequences. Figures showing the syntax of RNA structural modules, the design and construction process of RNA self-assembling architectures, a diagram describing molecule preparation sequences, experimental material characterizations, AFM images, secondary structure diagrams and assembly rules, RNA self-assembly, native PAGE results, three-dimensional organization of RNA units and modularity, G1 and G2 hearts self-assembly, visualization of G1 hearts by AFM, characterization of G2 and G3 hearts, analysis and assembly of G2 and G3 hearts, design criteria. (PDF)

Graphical Abstract

Keywords

RNA nanotechnology; RNA self-assembly; RNA folding; nanostructures; nanoparticles; tectoRNAs; RNA architectonics

The rational design and engineering of complex three-dimensional (3D) nanostructures of RNA remain a great challenge for nanotechnology, nanochemistry, synthetic biology, and nanomedicine.^{1–8} In nature, functional RNAs, like riboswitches and ribozymes, fold into 3D shapes through the sequence-dependent formation of exquisite 3D networks of noncovalent interactions. While the RNA secondary (2D) structure is mostly driven by the formation of local RNA helices, the RNA 3D structure is induced by recurrent tertiary modules^{9–11} that constitute the lexicon enabling control of the RNA folding and assembly into larger self-folding domains¹² through the orientation, stacking, and bending of adjacent helical struts and the formation of long-range interactions (Figures 1 and S1). We previously developed the RNA architectonics approach,^{5,13} a method that aids designing and building self-assembling nanoarchitectures from the structural information on natural RNAs.^{14–20} So far, RNA nanodesign has relied on the repetitive use of a very limited repertoire of structural modules, short RNA sequences promoting self-assembly through Watson–Crick base pairs,^{6,15,17–19} and strategies that seldom take advantage of the ability of RNA to fold and assemble during transcription in isothermal conditions.^{17,21,22} Borrowing concepts from linguistics, we hypothesized that we could describe and generate complex RNA structures from an extended syntax: RNA modules act as “words” that are arranged with respect to one another to define larger self-assembling domains (or tectoRNA units), thereby expressing nanostructures with complex and well-defined 3D shapes specified by the context of the constituent modules, reminiscent of the way that words make up sentences that express meanings. We demonstrate herein that such a syntax of RNA modules offers a limitless potential of expression for building complex, responsive 3D nanostructures under thermodynamic and kinetic control (Figures 1, S1, and S2).

RNA structural modules (Figure 1, left) define a wide range of possible geometries that can be used to join helical struts. The UA_handle three-way junction (UAh_3WJ) and class II tRNA (tRNA-5WJ) are 90° bend modules suitable for the construction of tetrameric assemblies, also called tectos-quares.^{10,18,23} While both modules can flex, the UAh_3WJ module is more planar, and one of its helical stems preferentially flexes in one direction with respect to the two others, leading to straight, obtuse, and acute angles.²⁴ Obtuse and acute

units were both programmed with kissing loops (KLs) to either assemble into tectosquares or tectotriangles (Figures 1 and S3). Tectotriangles TT with acute units are more thermostable than those with obtuse units (TTo). The opposite trend is observed for tectosquares (Figures 2A and S4). Tectotriangles TT are nonetheless more thermostable than tectosquares TS with obtuse units. By varying of the length of the arms bearing complementary tail–tail ends, tectosquares TS and tectotriangles TT can be controlled to assemble into regular nanoarrays, ladders, and nanogrids (Figures 1, 2B, and S5A–C and Table S1AB). Because of the preferential, directional flexing of the UA_h-3WJ module, obtuse units designed for tectotriangles TTo also assemble into tectohexagons (Figures 2 and S4). When programmed with tail–tail interactions, they form mixed TTo-2TTo “sponge-like” networks (Figures 2B and S5A). These results demonstrate that UA_h-3WJ units can serve different geometrical purposes in a programmable and context-dependent fashion.

The A-minor-3WJ junction module (Figure S1) controls the preferential stacking of adjacent RNA helices according to its thermodynamic stability.¹⁹ With magnesium and increasing RNA concentrations, A-minor-3WJ units TF1 assemble end-to-end through kissing loops into nanorings and nanofibers of various sizes, whereas units TF2, with a thermostable GAAA/11nt-3WJ junction, form long nanofibers¹⁹ (Figures 1, S6, and S7 and Table S1C). AMP-TF1 and AMP-TF2 were derived from these tectoRNAs by incorporating the AMP aptamer^{25–27} into their design (Figures 1, S1, and S6). The AMP aptamer offers structural modulation of the flexibility of RNA bending: upon recognition of AMP (adenosine or ATP),^{25,26} AMP-TF units form a ~120° bend by induced-fit folding. Consequently, AMP-TF1 and AMP-TF2 nanostructures can be controlled in response to ligand binding. Native polyacrylamide gel electrophoresis (PAGE) analysis indicates that AMP-TF1 and AMP-TF2 assemble into mixtures of tetramers, hexamers, and nanofibers in the presence of AMP, whereas they mostly form dimers in the absence of the cognate ligand (Figures 2D and S7). AMP-TF1 and AMP-TF2 variants, which inactivate the AMP-aptamer, behave as in the absence of AMP (Figures 2D and S7). Atomic force microscopy (AFM) imaging reveals that AMP-TF1 units generate long dynamical nanofibers with numerous kinks that suggest a spiraling curvature induced by AMP (Figure S7F).

To expand the versatility of these RNA modules, new tectoRNA units were derived from those above to create responsive nanostructures with distinctive shapes. Heart shapes were chosen because they require a complex collection of different bends and coaxial stacks; they are planar and easily identifiable by microscopy. As for their universal meaning, there is no greater message than “LOVE” to give to the world. We first designed tectoRNA units able to self-assemble into G1 and G2 nanohearts (Figures 1 and 3). In a second step, these units acting as structural domains were encoded into single-stranded RNA to fold into G3 nanohearts (Figure 4).

The design of the G1 heart minimizes the number of different RNA units necessary to build a symmetrical six-unit object assembling through four distinct kissing loops. Units HX and HY, which are, respectively, derived from TF and AMP-TF units, are used twice to form the two sides of the heart. They are joined by HW and HZ, a pair of 90° angle units derived from tRNA-5WJ units that were previously used in tectosquares and polyhedrons^{18,23} (Figures 1, S6, and S8A and Table S1D). Consequently, partial control of the positioning of

HW with respect of HZ results in two distinct cis and trans assembly products (Figure S8A). Unit HX forms the straight edges of the heart: alongside an A-minor-3WJ module, it incorporates a small A-bulge module that induces a slight 160° curvature to the sides of the object and also relieves the heart shape structure from possible structural stress (Figures 1 and S1). Unit HY, which forms the two lobes of the heart, merges features from AMP-TF1 and AMP-TF2 units. Its strong curvature is induced by two consecutive AMP aptamers connected through an A-minor-3WJ module. The G1 heart assembles with ~70% yields on native PAGE (Figure S9). Structural Pb(II) probing of unit HY, alone or within the context of G1 hearts, indicates that its AMP-aptamer modules fold into compact, rigid bends in both contexts upon addition of AMP (Figures 3 and S10). Several discrete particles can unambiguously be identified as nanohearts by AFM (Figures 3 and S11). However, the protruding tRNA-5WJ modules within the heterogeneous mix of cis and trans G1 hearts do not favor RNA adhesion on the mica surface under solution, rendering AFM visualization challenging at high resolution (Figures 3 and S11).

The G2 heart, derived from the G1 heart, is designed to resemble a “pierced” heart. As an asymmetric and addressable object, its six RNA units are easily identifiable by microscopy (Figures 1, S6, and S8B and Table S1D). Units HA and HD, which replace units HZ and HW from the G1 heart, are derived from TT and TS units, respectively (Figures 1 and S6). Their constituent AUh_3WJ module confers an increased flatness compared to other 90° modules.^{15,16,23,28} Units HC and HE are structurally identical to unit HY but programmed with different kissing loops. Units HB and HF are both derived from HX: instead of an A-minor-3WJ, HB has a UA_h_3WJ module, whereas HF contains the GAAA/1Int-3WJ of TF2 (Figures 1 and S6). HB forms the G2-heart crossbar by assembling with unit HD through a tail–tail triple interaction (Figure S6C). The G2-heart design presents the UA_h_3WJ module in three different orientations, leading to ~65°, 115°, and 180° angles. In the presence of AMP, the G2 heart assembles with a yield of ~85%, as determined by native PAGE and cryo-electron-microscopy (cryo-EM) (Figures S9 and S13). Approximately ~60% of the objects visualized by AFM are single G2 hearts, out of which 95% present a well-defined crossbar (Figures 3 and S12). About ~75% of them present the crossbar on the right, suggesting that the overall 3D shape of G2 hearts leads to preferential orientation on the mica surface (Figure S12). This bias is not observed by cryo-EM (Figures 3 and S13D). In high-resolution AFM images, the heart shape and dimension closely match the 3D model, with regions of increased height corresponding to the locations of tertiary modules within the assembly (Figures 3 and S13C). Likewise, a particle average of 2456 particles visualized by cryo-EM shows increased density only in areas corresponding to tertiary modules and corroborates their orientational and topological properties (Figure S13D–F). Notably, the three different orientations of the UA_h_3WJ module in units HA, HB, and HD are validated by AFM and cryoEM data (Figure S13). The ability to control precise helical-stacking through a single tail–tail interaction of seven base pairs was also tested for G2 hearts. A pair of sets of hearts were designed and assembled through a two-step assembly protocol into two different G2-heart dimers (HA1-A1 and HA2-A2) with distinctive patterns of 12 units (Figures 1, 3, and S8 and Table S1D). AFM reveals that both samples exhibit a greater surface coverage than single G1 and G2-heart constructs (Figures 3 and S14). It also

demonstrates that single tail–tail interactions connecting two UAh_3WJ modules stack strongly enough to specify the directionality and orientation between two G2 hearts.

We hypothesized that G2-heart units could be encoded within a single-stranded RNA to generate the G3 heart, a ~715 nucleotide long molecule anticipated to fold during transcription into a heart shape. Encoded units should act as thermodynamic and kinetic control in isothermal conditions.²⁹ Depending on the way these domains are encoded within the RNA sequence, the location of kissing loops, and choice of 5′–3′ ends, it is theoretically possible to design multiple constructs with distinct folding pathways (Figures 4A and S15). Therefore, G3-heart constructs with efficient co-transcriptional folding are supposed to favor local formation of modular RNA subdomains while minimizing the length of transient single-stranded regions occurring during transcription. In the G2 heart, units HC, HE, and HF, which contain the A-minor junction and AMP aptamer modules, are structurally more complex than units HA, HB, and HD, which only relies on the UAh_3WJ module. Among a total of 14 different constructs with their 5′–3′ ends localized in domain dA, we chose the construct KL1–6, which promotes heart closure through KL1 and KL6 (Figures 4 and S15 and Table S1e). This construct maximizes the local modular formation of domains dC, dE, and dF while also minimizing the length of transient single-stranded regions during transcription. G3-heart co-transcriptional folding was investigated by AFM under solution²² (Figures 4 and S16). Approximately 70% of the objects on the mica surface correspond to G3-heart single particles. Multiple AFM scans of the same regions on the mica surface suggest that most G3 hearts are well-formed with structural characteristics similar to G2 hearts (Figures 4C–F and S16). G3 hearts have the same preferential orientation as G2 hearts when lying on the mica surface. The degrees of structural flexibility observed for few particles likely result from interacting with the scanning probe during AFM imaging under solution.

Drawing an analogy between the structural organization of RNA architectures and the way that words are put together to form phrases, clauses or sentences in linguistics, we have demonstrated herein that a syntax of RNA 3D modules can be used to program RNA strands to fold into complex, responsive nanostructures with well-defined 3D shapes. While these structural modules can control the multimeric assembly of distinct RNA units, they can also be encoded within long unique RNAs to promote the isothermal folding and assembly of single-stranded RNA nanoarchitectures during transcription. The latter approach has great potential for the low-copy-number expression of responsive RNA scaffolds inside the cell.^{30,31} Our RNA syntax was used herein to express a symbol of hope and love, but it can also be applied to the design of denser, solid 3D nanostructures conceivably as complex as the ribosome. In summary, this work emphasizes the remarkable amenability of RNA to rational design and the limitless potential of expression that RNA offers for engineering complex 3D nanostructures for synthetic biology, nano-medicine, and nanotechnology.^{1,3–6,26} By testing the orientational and topological behavior of RNA tertiary modules, our approach can significantly contribute to solving the sequence to 3D structure relationship in RNA folding. Finally, our “heart” study suggests that stable RNAs, like the ribosome, might have increased in size and complexity during evolution by the recombination and accretion of modular self-assembling domains at kissing-loop recombination sites.^{32,33}

RNA 3D Modeling and Sequence Design.

RNA design and nanoconstruction based on RNA structural modules is described in Figures S1 and S2. The heart-shaped atomic models were manually constructed in silico with the software SwissPdbviewer (<http://spdbv.vital-it.ch/>) by combining RNA fragments extracted from known X-ray crystallographic structures (<http://www.rcsb.org>). The merged RNA fragments were then refined to fix the backbone stereochemistry at the locations of the splices with Assemble 2.0 (<http://bioinformatics.org/assemble/>) and UCSF Chimera (<http://www.cgl.ucsf.edu/chimera/>). RNA sequences folding into a unique 2D structure were optimized to minimize alternative folds using NUPACK and the Vienna 2.0 package.

RNA Synthesis.

All RNA molecules were prepared by in vitro T7 RNA polymerase runoff transcription from PCR generated templates as previously described.² For native PAGE, temperature-gradient gel electrophoresis (TGGE), and structural probing, RNA assembly was typically monitored by PAGE using ³²P- α -ATP-labeled RNA or 3'-[³²P]pCp-labeled RNA as previously described² (see also the Supporting Information).

RNA Self-Assembly, Native PAGE, and TGGE assays.

Folding Protocol 1.

RNAs were prepared by mixing different tectoRNAs (200 nM each, unless otherwise specified) in water. RNA samples were subjected to a denaturation–renaturing step by heating the samples to 90 °C for 3 min, cooling on ice 3 min, incubation at 30 °C for 3 min, followed by the addition of 5× concentrated buffer to reach 10 mM Tris–borate at pH 8.2 (TB), 50 mM KCl, and 0.2 mM Mg(OAc)₂, prior to further incubation at 30 °C for 30 min.

Folding Protocol 2.

In addition to protocol 1, RNA nanostructures were further stabilized for native PAGE and AFM studies by raising the magnesium concentration to 15 mM Mg(OAc)₂ followed by heating at 60 °C for 10 min and slow-cooling to 10 °C over 30 min. Native PAGE and TGGE experiments were performed essentially as described in refs 18 and 23 (see the Supporting Information for details).

Structural Probing.

RNAs were assembled via protocol 1. Chemical probing was performed at various concentrations of Pb(II) according to established protocols¹⁸ (see the Supporting Information for details). The RNA fragments were separated on denaturing polyacrylamide gels (15% acrylamide 29:1, 8 M urea) before quantitation using a phosphorimager.

AFM Characterization of Multimeric Nanostructures.

RNA architectures were assembled in solution, deposited on a mica surface in 15 mM Mg²⁺ buffer and visualized either under solution or dried under nitrogen before AFM imaging in

air (3WJ-based heart, G2) (see the Supporting Information for details). Multimode AFM imaging was performed at room temperature in tapping mode using a Nanoscope IIIa controller (Veeco, Santa Barbara, CA).

G3 Heart Co-Transcriptional Folding and AFM Visualization.

RNAs were co-transcriptionally folded at 37.0 °C for 20 min in one-pot reactions (50 μ L) containing the template DNA (4 ng/ μ L final), NTPs (0.5 mM each final), dithiothreitol (1 mM final), 6 mM Mg(OAc)₂, 40 mM Na-OAc, 40 mM KCl, and 50 mM Tris-OAc (pH 7.8) in the presence of T7 RNA polymerase (~0.2 U/50 μ L). For AFM imaging, 5 μ L of the transcription mix was directly mixed with AFM buffer (12.5 mM Mg(OAc)₂, 40 mM KCl, 40 mM NaCl, and TB at pH7.8) on a freshly cleaved mica surface and preheated to 37.0 °C. After 1 min, the fluid was removed from the surface and fresh AFM buffer (40 μ L) and 4 μ L of 60 mM NiCl₂ were added. Multimode imaging was performed with 9.7kHz “solution imaging” tips under these AFM conditions.

Cryo-EM for Imaging G2 Hearts.

RNA heart samples were prepared according to protocol 2. Micrographs were acquired using a Tecnai F20 Twin transmission electron microscope operating at 120 kV, a nominal magnification of 80 000 $\times$, a pixel size of 0.105 nm at the specimen level, and a dose of ~30 e-/Å². Images were automatically collected by the Leginon system³⁴ and recorded with a Tietz F415 4K $\times$ 4K pixel CCD camera. Experimental data were processed using the Appion software package.^{35,36} A total of 4467 particles were automatically selected from the micrographs using the FindEM, template-based particle picker.³⁷ The particle class averages were created using the Xmipp MLalign2D program³⁸ for reference-free alignment and classification using the fast algorithm.³⁹ A resolution of 20.8 Å for the class average based on 2456 particles was determined by converting the spectral signal-to-noise ratio into a Fourier ring correlation taken at a cutoff of 0.5.⁴⁰

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

RNA structural syntax behind the rational design of 3D RNA nanostructures. (left) RNA 3D modules defining angles ranging from 60° to 180°. (middle) A set of different RNA domains, or tectoRNA units, derived from the 3D modules. Arrows indicate the structural relationship between tectoRNAs. (right) Examples of RNA shapes described in the text. The color coding for modules is the same for all panels. For more details, see also Figures S1 and S2.

Figure 2.

RNA module control of the assembly of tectoRNA units into repetitious nanostructures. (A) Assembly principle of "obtuse" or "acute" UA_h3WJ RNA units into tectosquares (TS and TSa), tectotriangles (TT and TTo), and hexameric nanorings (2TTo). Tectosquares formed of four units (A–D) assemble through four different kissing loops. Tectotriangles and hexamers are formed of three units (A, B, and T). Apparent melting temperatures (T_{ms}) obtained by TGGE at 0.2 mM Mg(OAc)₂ are indicated for each assembly (see details on the materials and methods in the text). (B) TS, TT, and TTo constructs can assemble further through complementary 3' tails to form programmable nanoarrays and nanogrids (Figure S3). Except for TT5 + TT6 + TT7 imaged in air, atomic force microscopy (AFM) visualization was performed under solution with 15 mM Mg(OAc)₂, as described in the text and in Figure S5. All scale bars are 50 nm. (C) Principle of assembly of AMP-TF units responsive to AMP into nanorings and fibers of different size and length. (D) Self-assembly of AMP-TF2 units in the absence or the presence of AMP at the indicated concentration is monitored by native polyacrylamide gel electrophoresis at 1 mM Mg(OAc)₂ and 10 °C. AMP-TF2 variant is shown in Figure S6. (E) AFM images of AMP-TF1 nanofibers (25 nM) acquired under solution in the presence of 2 mM ATP and 15 mM Mg(OAc)₂ at 20 °C.

Figure 3.

G1 and G2 nanohearts and their characteristic heart shapes, which are responsive to AMP. (A) G1 heart unit HY rigidifies upon the addition of 1 mM AMP. Pb(II) (42 mM) cleavage profiles of HY within G1 hearts, with (red) or without (blue) AMP, compared to those of HY alone, with (green) or without (yellow) AMP (Figure S10). Boxed 3D model of HY: regions in yellow and blue have reduced Pb(II) cleavage with AMP and units H-WXZ; regions in red retain significant cleavage. (B) AFM images of G1 hearts obtained under magnesium solution (Figure S11). (C,D) AFM images of G2 hearts acquired in air. (E) Single G2-heart images. (F) High-resolution AFM image contour-plot of a G2 heart. Each contour represents a 0.1 nm change in height. (G) The same image compared to the G2-heart 3D model. Tall points fit to the tertiary modules. (H) Cryo-EM image of G2 hearts on a thin layer of carbon. (I) Combined left and right reference-based alignments of 2456 single G2-heart particles obtained from cryo-EM images. For panels E–I, see also Figures S12 and S13. (J) G2 hearts

with two different orientations of unit HA (HA1 or HA2) assemble into two distinctive dimers when linked through one tail–tail interaction. AFM images were acquired in air (Figures S8 and S14).

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Figure 4.

Single-stranded G3 hearts, with structural modularity similar to G2 hearts, are produced under kinetic control in isothermal conditions during transcription. (A) Design criteria for the G3-heart KL1–6 (see the text and Figure S15). (B) Co-transcriptional folding and visualization procedure for G3 hearts. (C) Typical AFM images of G3 hearts acquired in solution according to procedure in panel B. A portion of the mica surface displayed on the right was scanned twice by AFM. Most hearts do not change much in shape (circled in blue), but a few can be disrupted by the AFM tip (circled in red). (D) Yield of well-formed G3 hearts vs malformed or partial transcripts. (E) Examples of well-resolved single G3 hearts from 0.5 μm AFM scans. (F) Examples of well-resolved single G3 hearts from 1 μm AFM scans. For panels C–F, see also Figure S16.