

Organization of Intracellular Reactions with Rationally Designed RNA Assemblies

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The rules of nucleic acid base-pairing have been used to construct nanoscale architectures and organize biomolecules, but little has been done to apply this technology in vivo. Here we designed and assembled multi-dimensional RNA structures and used them as scaffolds for the spatial organization of bacterial metabolism. Engineered RNA modules were assembled into discrete, one- and two- dimensional scaffolds with distinct protein docking sites, and used to control the spatial organization of a hydrogen-producing pathway. We increased hydrogen output as a function of scaffold architecture. Rationally designed RNA assemblies can thus be used to construct functional architectures in vivo.

In cells, multi-enzymatic pathways are often physically and spatially organized onto scaffolds, clusters or into micro-compartments (1). Spatial organization helps substrates flow between interacting proteins, limits cross-talk between signaling pathways, and increases yields of sequential metabolic reactions (1, 2). The ability to organize protein complexes and biological pathways spatially presents a strategy to engineer cells (3, 4).

The spatial organization of biomolecules has been the focus of DNA nanotechnology (5–8). This approach utilizes DNA's base-pairing to generate one-, two- and three-dimensional assemblies. DNA structures have largely remained limited to in vitro applications (9). RNA provides a compatible material for in vivo nucleic acid based construction (10). It can be produced via the transcription machinery, and forms stable interactions. RNA has been used to build higher-order assemblies in vitro (11, 12) and can potentially be used in vivo to engineer the intracellular environment.

Here, we engineered synthetic RNA modules that assemble into functional discrete, one-, and two-dimensional scaffolds in vivo, and used them to control the spatial organization of bound proteins (Fig. 1A). Scaffold **D0** was constructed from a single RNA module **d0**, which folded into

a duplex with PP7 and MS2 aptamer domains that bind PP7 and MS2 fusion proteins (Fig. 1B) (13).

We developed an approach for the in vivo isothermal assembly of extended RNA scaffolds by constructing sequence-symmetric RNA building blocks (Fig. 1, C and D) inspired by two-dimensional DNA analogs (13, 14). These RNA strands possess dimerization (DD) and polymerization domains (PD). To prevent the formation of ill-defined networks, it was necessary to disfavor the collapse of the palindromic regions (15) and control assembly-order by insuring tile formation before polymerization. We achieved this by designing PDs that fold intramolecularly into kinetically protected hairpin structures (Fig. 1D, step i). The stem of these hairpins is an overlapping shared domain with DD that discourages collapse (Fig. 1D, red segments), allowing DD to “activate” PD upon self-binding (Fig. 1D, step iii). We further destabilized the collapsed-state by incorporating wobble-pairs and mispairs (figs. S1 to S4).

The one-dimensional RNA assembly **D1** was derived from a single RNA **d1** with PP7 and MS2 binding domains (Fig. 1E). **d1** assembled into **d1-1** (step i), which self-assembled into **d1-2** (step iii). The torsion in **d1-2** induced folding into an RNA nanotube capable of growing into the one-dimensional scaffold **D1** (step iv). The two-dimensional RNA assembly **D2** was formed from **d2'** and **d2''**, each carrying a distinct PP7 and MS2 aptamer (Fig. 1F). The dormant tile **d2'** spontaneously generated the pro-tile **d2-1** (step i), which interacted with **d2''** to generate tile **d2-2** (step ii). **d2-2** then self-assembled into the two-dimensional RNA scaffold **D2** with PP7 and MS2 binding domains (step iv).

In vitro transcribed RNA modules **d1** and **d2'/d2''** were characterized using atomic force microscopy (AFM). **d1** formed one-dimensional RNA fibers **D1**, while **d2'/d2''** assembled into two-dimensional extended RNA fibers **D2** (Fig. 2A). The width of **D1** (~5 nm, a few tiles wide) is smaller than that of its DNA analog (14), and might thus also correspond to one-dimensional ribbons **D1₂** constructed from

a continuous line of single-tiles (Fig. 1E, step ii). Given that **D2** preferentially grows in a single direction when compared to its DNA analog (13), it might also correspond to RNA nanotubes **D2₂** that are relatively wider than **D1** (Fig. 1D, step iii). To confirm the validity of our assemblies, we used analogs of **d1** and **d2'** with a poly-T stretch in place of the DD incapable of assembling; **d1_T** and **d2'_T** did not generate extended assemblies (Fig. 2B).

We developed a DNA-based precipitation (DP) method to purify our RNA assemblies from cells. Streptavidin-coated magnetic beads with a biotinylated DNA capture probe (**DP_C**) were added to cell lysates. The capture domain (CD) of **DP_C** binds the T7 terminator in our RNA molecules (Fig. 2C, step i). The RNA assemblies were released upon addition of **DP_R** that bound the release domain (RD) of **DP_C** (Fig. 2C, step ii). We were able to capture and release RNA (Fig. 2D).

In vivo synthesized **D1** and **D2** revealed extended one- and two-dimensional assemblies (Fig. 2E and fig. S5). Cross-sectional height analysis showed **D1** to have two populations of distinct height (3 and 6 nm), which is characteristic of open versus closed nanotubes. In vivo **D2** assembled into two-dimensional structures smaller and somewhat different than their in vitro counterparts, suggesting that the assembly process in cells is of lower fidelity. To confirm that the assemblies formed in vivo, we engineered a set of inhibitory strands (IS) that bound the trigger-domains of **d1'** and **d2'**. The inhibition by these strands was confirmed in vitro (Fig. 2F). The purification of **D1/D2** in the presence of excess IS did not eliminate the observed one- and two-dimensional assemblies (Fig. 2G), confirming the formation of **D1** and **D2** in cells pre-lysis.

Transmission electron microscopy (TEM) analysis of whole bacterial cells expressing **D1** or **D2** confirmed their assembly in cells. The RNA assemblies were tagged with gold-binding metallothionin-PP7 fusion proteins **P_{Au}** that form clusters (Fig. 2H) (16). Cells co-expressing **P_{Au}** and **D1** formed thin filaments 200 to 300 nm long, while cells co-expressing **D2** formed compact sphere-like structures ~100 nm in diameter. **D0**, **D1** or **D2** do not affect cell growth (fig. S12). Cells carrying the **D1** and **D2** scaffolds had higher RNA levels relative to cells expressing mutated poly-T RNA analogs (Fig. 2I), consistent with the formation of degradation resistant assemblies. Thus, **d1** and **d2'/d2''** assembled in vivo into **D1** and **D2**.

We used fluorescence complementation to detect protein assembly on our RNA scaffolds (Fig. 3). Green fluorescent protein (GFP) split into two halves **F_A** and **F_B**, fused to the PP7 or MS2 aptamer binding proteins, was used (Fig. 3A). Cells expressing **F_A** and **F_B** alone (Fig. 3B) or **D0**, **D1** or **D2** without the split GFPs displayed little fluorescence. However, the co-expression of **D0**, **D1** or **D2** with the split GFPs showed increased fluorescence (Fig. 3C). Thus our RNA

scaffolds served as docking sites to promote protein-protein interactions in cells.

Biological hydrogen production has both fundamental and practical implications. Co-expression of [FeFe]-hydrogenase and ferredoxin catalyzes the reduction of protons to hydrogen through electron transfer (17). We used this system to assess the potential of our RNA scaffolds to constrain flux through spatial organization. We fused the hydrogenase to a single copy of PP7 (**H_P**), and ferredoxin to a dimer of MS2 (**F_M**). Electrophoretic gel-shift analysis of the binding of **F_M** and **H_P** to **D0** was conducted (Fig. 4A). Addition of **H_P** to **D0** resulted in a single product termed **D0_H**. The addition of **F_P** to **D0** resulted in the formation of **D0_F**. The addition of **H_P** and **F_M** to **D0** resulted in a single product assigned to the protein-RNA assembly **D0_{FH}**. **H_P** and **F_M** assembled onto **D0** in cells to form **D0_{FH}** (Fig. 4B).

To determine whether our RNA scaffolds increased hydrogen biosynthesis, cells expressing the hydrogen-producing pathway along with the different RNA assemblies were analyzed using gas chromatography (fig. S11). The relative levels of **F_M** and **H_P** expression in **D0**, **D1** and **D2** cells were comparable (fig. S8). **D0**, **D1** and **D2** assembled **F_M** and **H_P** into **D0_{FH}**, **D1_{FH}**, and **D2_{FH}** (Fig. 1). **D0** resulted in a 4.0 ± 1.3 fold increase in hydrogen production compared to unscaffolded **H_P** and **F_M** (Fig. 4C). Hydrogen output with the extended assemblies **D1** and **D2** resulted in a 11 ± 2.8 and 48 ± 1.5 fold increase in hydrogen production (Fig. 4C). When normalized against the amount of RNA in cells (Fig. 2I and fig. S7), **D0**, **D1** and **D2** resulted in a 4.0-, 6.2-, and 24-fold increase. The increase with **D2** is consistent with its assembly in vivo into “organelle-like” structures effective at concentrating proteins and their products (Fig. 2H). Mutating the PP7 and MS2 binding sites prevented protein scaffolding (Fig. 4, D to G). Thus, RNA can be used to organize enzymatic pathways in vivo to increase output as a function of architecture.

Here we controlled the spatial organization of proteins in cells using RNA molecules that are sequence-programmed to isothermally assemble into pre-defined discrete, one- and two-dimensional structures in vivo. These assemblies scaffolded proteins, and were used to organize a hydrogen-producing biosynthetic pathway. Hydrogen production was optimized as a function of scaffold architecture. Unlike protein-based approaches (3, 4, 17), RNA-based scaffolds allow for the formation of complex multi-dimensional architectures with nanometer precision. In vivo RNA assemblies can thus be used to engineer biological pathways through spatial constraints (18, 19).

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Acknowledgments: Support was from Enerbio-Tuck foundation and IFP Energies Nouvelles to C.J.D., ANR France, INSERM-INRIA projet d'envergure and Axa

Foundation Chair on Longevity to A.B.L., NSERC Canada to F.A.A., and the Wyss Institute for Biologically Inspired Engineering. We thank HMS EM Center, and the reviewers for their insight.

Supporting Online Material

www.sciencemag.org/cgi/content/full/science.1206938/DC1

Materials and Methods

Figs. S1 to S13

References (20-35)

13 April 2011; accepted 6 June 2011

Published online 23 June 2011; 10.1126/science.1206938

Fig. 1. Design of RNA modules to organize proteins. **(A)** Proteins A and B scaffolded onto discrete, one- and two-dimensional RNA assemblies. **(B)** **D0** is a RNA strand that folds into a duplex with PP7 and MS2 sites. Ferredoxin/MS2 (**F_M**) and hydrogenase/PP7 (**H_P**) bind **D0** to generate **D0_{FH}**. **(C and D)** RNA with dimerization and polymerization domains (DD and PD) initiates the formation of extended assemblies. Capping the palindromic sequences in DD with PD prevents its collapse (i), and allows for self-assembly (ii) into functioning tiles (iii). **(E)** **D1** is constructed from a RNA strand **d1** bearing PP7 and MS2, and assembles into tile **d1-1** (i). **d1-1** assembles into a ribbon **D1₂** (ii), or into a nanotube **d1-2** (iii) that grows into **D1** (iv). **D1** organizes **F_M** and **H_P** into **D1_{FH}** (v). **(F)** **D2** is constructed from **d2'** and **d2''** bearing PP7 and MS2 respectively. **d2'** assembles into the pro-tile **d2-1** (i) and interacts with **d2''** to generate **d2-2** (ii). **d2-2** self-assembles into a nanotube **D2₂** (iii) or the two-dimensional **D2** (iv). **D2** organizes **F_M** and **H_P** into **D2_{FH}** (v).

Fig. 2. Characterization of RNA assemblies. **(A)** In vitro transcribed **d1** and **d2'/d2''** assemble into **D1** and **D2** (AFM; phase images; bars 0.25 μ m). **(B)** In vitro transcribed mutated RNA **d1_T** and **d2'_T/d2''** do not assemble. **(C)** DNA-based precipitation of in vivo RNA assemblies uses **DP_C** (i), and a release probe (**DP_R**) for recovery (ii). **(D)** Capture and release of substrate **DP_S** (left gel = beads, right gel = solution). Lane 1, conjugation of **DP_C** to streptavidin-coated magnetic beads. Lane 2, capture of **DP_S**. Lane 3, release of **DP_S** using **DP_R**. **(E)** AFM analysis of purified assemblies. **(F)** Inhibitory strands (IS) bind the DD of **d1** and **d2'** to prevent their assembly into **D1** or **D2** (circular structures are drying artifacts). **(G)** When used during the purification of **d1** and **d2'/d2''**, **D1** and **D2** assemblies are still observed. **(H)** TEM analysis revealed the formation of one-dimensional assemblies for **D1** and two-dimensional aggregates for **D2** (bars 100 nm). **(I)** qRT-PCR analysis of in vivo RNA production levels. Error bars represent the SEM.

Fig. 3. Fluorescence protein complementation in vivo. **(A)** GFP split into two halves, each of which is fused to PP7 or

MS2 (**F_A** and **F_B**). **F_A** and **F_B** bind their respective aptamers (i), and reconstruct functional fluorescent GFP (ii). **(B)** Fluorescence microscopy imaging of cells expressing **F_A** and **F_B** revealed little to no fluorescence (bars 10 μ m). **(C)** Cells co-expressing **F_A** and **F_B** with **D0**, **D1** or **D2** reveals an increase in fluorescence indicating that **D0**, **D1**, and **D2** scaffold PP7 and MS2 protein chimeras. Gray line in flow cytometry separates OFF and ON cells.

Fig. 4. Scaffolding hydrogen production. **(A)** In vitro gel-shift of **H_P** (lane 1) binds **D0** to form **D0_H** (lane 2). **F_M** (lane 3) binds **D0** to form **D0_F** (lane 4). **H_P** and **F_M** bind **D0** to form **D0_{FH}** (lane 5). **(B)** In vivo gel-shift of **H_P** and **F_M** (lane 1), and **H_P** and **F_M** in the presence of **D0** (lane 2). **(C)** Hydrogen biosynthesis as a function of scaffold, normalized to unscaffolded cells expressing **H_P** and **F_M**. **(D)** Mutating aptamer binding sites **(E)** does not affect self-assembly, **(F)** but prevents protein binding (bars 10 μ m) and **(G)** hydrogen production. Error bars represent the SEM.







