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RNA nanotechnology in synthetic biology

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We review recent advances in the design and expression of synthetic RNA sequences inside cells, to regulate gene expression and to achieve spatial localization of components. We focus on approaches that exploit the programmability of the secondary and tertiary structure of RNA to build scalable and modular devices that fold spontaneously and have the capacity to respond to environmental inputs.

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Introduction

RNA is an attractive molecule for synthetic biology for multiple reasons: first, RNA is involved in the control of gene expression at the level of transcription and translation, and is an essential component within the CRISPR/Cas gene editing system; second, the relationship between RNA sequence and secondary or tertiary structure is well characterized, making it possible to program the interactions of individual or multiple RNA strands to achieve a target structure or a desired sequence of binding events; third, RNA molecules can be designed to bind to a variety of ligands (small molecules, proteins, and RNA), enabling their use as sensors and actuators. Finally, because RNA is naturally present in all living organisms, synthetic RNA molecules can be easily ported to different hosts.

This review discusses recent advances in the use of engineered RNA in synthetic biology applications. We focus in particular on different methods to control gene expression with synthetic RNA, and on approaches to build RNA scaffolds for organizing molecular components inside cells

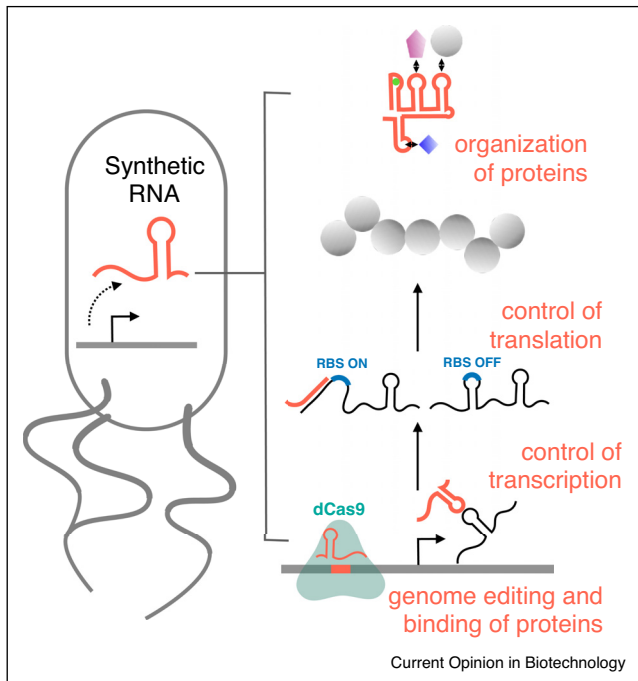
(Figure 1). A common thread of the methods reviewed here is their focus on rational design of secondary or tertiary structure, which determines the function of the engineered RNA.

Synthetic RNA for transcription regulation

Although protein regulators have played a key role in the early development of synthetic biological circuitry, remarkable progress has been made in creating libraries of synthetic RNA genetic regulators. RNA-mediated control of gene expression often involves specific structural motifs within mRNA, such as the intrinsic terminator structure that prevents transcription elongation in bacteria. Building on the natural pT181 transcriptional attenuator system, Lucks *et al.* showed that orthogonal variants of transcriptional attenuators can be obtained by strategic changes in the attenuator hairpin loop and stem sequences [1]. The orthogonal attenuator and antisense sequences could be used as tandem attenuators in transcriptional cascades. Takahashi and Lucks further expanded the set of transcription attenuators by chimeric fusion of pT181 attenuator and natural antisense RNA translational regulators [2]. The chimeric fusion and mutation strategy allowed them to construct 7 orthogonal transcriptional attenuators.

The same group later developed sRNA-based transcriptional activators termed Small Transcription Activating RNAs (STARs) [3]. *Trans*-acting STAR RNAs activate the transcription by preventing the formation of terminator hairpins (Figure 2a). A number of orthogonal, composable STAR variants were demonstrated and their performance was improved by a combination of tuning promoter strengths and engineering the RNA molecules [4]. A modest improvement of fold activation was achieved by increasing the expression level of the STAR antisense relative to its target sense RNA (to decrease OFF-state leakage) and by adding a 5' stability hairpin and a 3' sRNA scaffold (to improve RNA stability of STAR antisense). More significant improvements in fold regulation (repression from 85% to 98%, or activation from 10-fold to over 900-fold) were achieved by combining transcriptional termination and ribosome binding site (RBS) sequestration, a strategy that takes inspiration from a natural pT181 dual transcription/translation repression mechanism [5]. More recently, *de novo* computational design of STAR library identified high-dynamic range devices with near background level leakage and fold-activation of thousands [6*]. These STAR elements were demonstrated to function with existing regulatory elements as modular devices and were used to control multi-gene metabolic pathway expression.

Figure 1



Synthetic RNA molecules expressed within cells can be optimized for a variety of tasks that include control of gene expression and organization of small molecules and proteins. Here, we review recent research advances that exploit RNA structure for synthetic biology applications.

Engineering RNA to direct the CRISPR/Cas system

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) loci are naturally present in prokaryotes. CRISPR and the CRISPR-associated proteins (Cas) function as a microbial analog to the acquired immune system present in higher organisms [7]. The diversity, modularity, and efficacy of CRISPR-Cas systems are driving a biotechnological revolution including genome editing, posttranscriptional engineering, imaging, and diagnostics. One of the most commonly used systems *Streptococcus pyogenes* Cas9, for instance, can take a single guide RNA species to determine the complementary DNA sequence that it targets. Earlier works showed that a catalytically dead Cas9 lacking endonuclease activity, when co-expressed with a guide RNA, can specifically interfere with transcriptional elongation, RNA polymerase binding, or transcription factor binding. This system, CRISPR interference (CRISPRi), can efficiently repress expression of targeted genes in *Escherichia coli*, with no detectable off-target effects [8]. Engineering Cas proteins, for instance, as split-Cas9 and split-dCas9 variants responsive to small molecule inducers, could provide genetic strategies for specifically

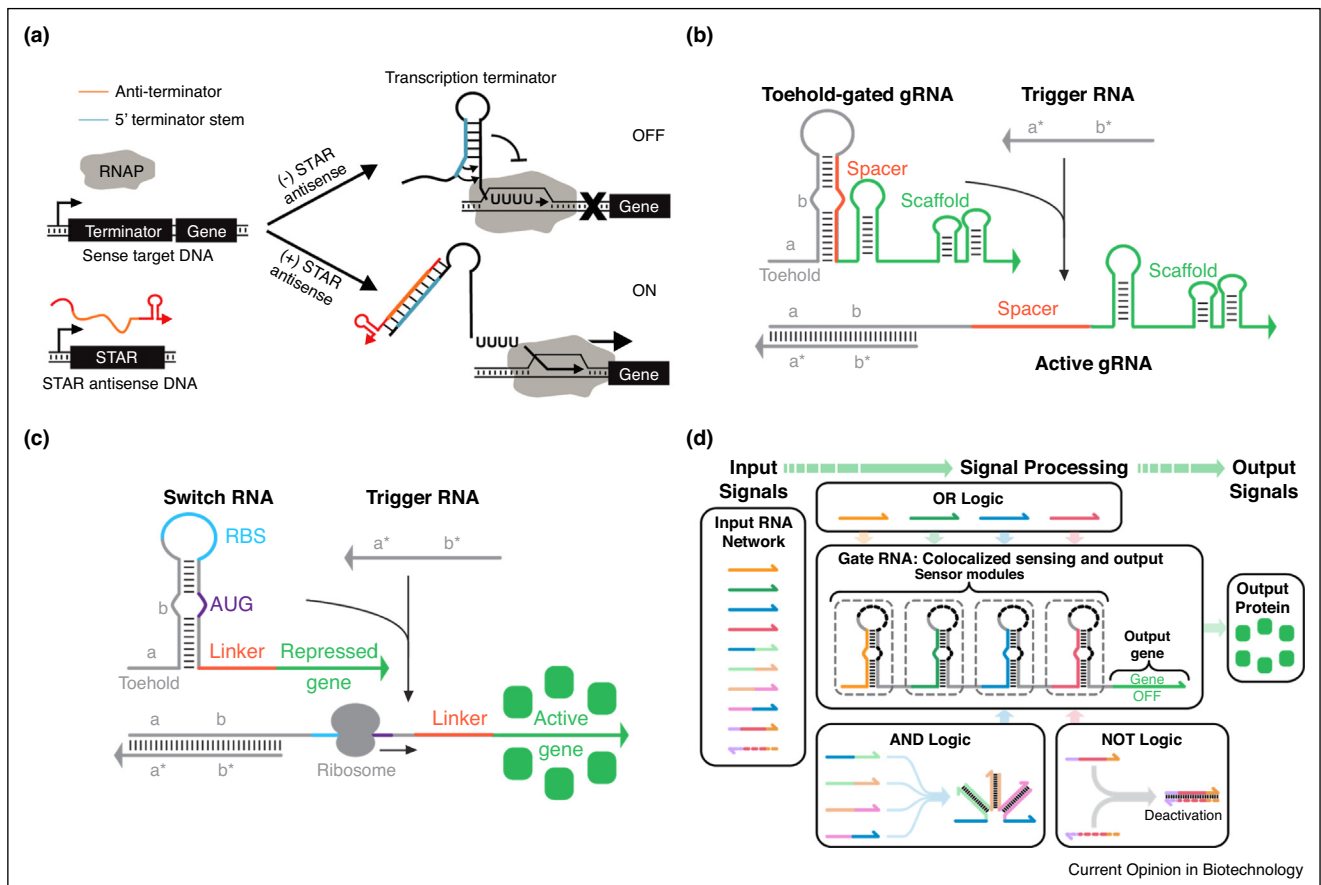
controlling Cas9 activity and constructing synthetic transcriptional networks [9].

Engineering the structure of guide RNA (gRNA) species for conditional control of Cas9 activity presents an efficient alternative to engineering Cas9 protein itself. RNA design using an RNA nanotechnology toolbox could lead to efficient control of complex synthetic biological circuitry using CRISPR-Cas system, and this is feasible because Cas9 activity is tolerant to significant modifications to the standard gRNA structure [10] that involve auxiliary domains to control gRNA activity by small-molecules [11,12], and nucleases [13]. Antisense RNAs were shown to be effective to repress the active form of gRNA [14,15]. The regulatory potential of gRNA could be further expanded by programmable RNA structure switching through RNA nanotechnology and several recent works have demonstrated the success and utility of switchable or conditional gRNAs. Cell-free transcription assays to monitor the activity of a number of distinct Cas nucleases could be combined with toehold-mediated strand displacement strategy to demonstrate conditional activation of Cas nuclease activity [16]. Jin *et al.* demonstrated engineered gRNA designs that function as logic gates in *in vitro* settings [17]. Computational design of conditional gRNAs for activation and repression was demonstrated for control of gene expression in *E. coli* [18^{*}]. Oesinghaus and Simmel showed that an analogous design strategy can be used for conditional and logic-gated activation of Cas12a system in *E. coli* [19]. Siu and Chen demonstrated engineered gRNA designs that possess toehold riboswitch structures (see next section) to respond specifically to cognate RNAs for multiplex control as well as to endogenous small RNA in *E. coli* [20^{*}] (Figure 2b). The gRNA engineering could be a useful tool in mammalian system as well. The conditional RNA design of Hanewich-Hollatz *et al.* was shown to be functional in HEK293T cells [18^{*}]. In another recent work, gRNA designs activated by miRNA-mediated processing were demonstrated, and these in turn could be used to monitor differentiation state of stem cells [21^{*}]. These results point to the potential of CRISPR-Cas gRNA engineering strategy as the tool of choice to allow control of synthetic gene circuits in bacteria and mammalian cells.

Synthetic RNA for control of translation

Synthetic RNA translational activators called 'riboregulators' are one of the first synthetic regulatory RNA devices [22]. Like natural sRNA regulators, the molecular structure of synthetic riboregulators prevents the binding of ribosomes to the RBS, thus inhibiting translation initiation. A *trans*-acting RNA can be designed to unwind the hairpin structure, free up the RBS, and allow ribosomal access and translation to proceed. In this seminal work, simple base-pairing design rules were used to create a family of two

Figure 2



Synthetic RNA regulators. **(a)** STAR design as a synthetic transcriptional regulator. Figure adapted from Ref. [3]. **(b)** Toehold-gated gRNA design as a synthetic controller of CRISPR activity. Figure adapted from Ref. [20]. **(c)** Toehold switch design as a synthetic translational regulator. Figure adapted from Ref. [23]. **(d)** Ribocomputing device integrating multiple toehold switches to evaluate complex logic expression in bacteria. Figure adapted from Ref. [24].

orthogonal devices. Recently, the library of riboregulators have been dramatically expanded by Green *et al.* through the adoption of toehold-mediated strand displacement, a tool widely used in DNA nanotechnology [23]. This new set of riboregulators, called ‘toehold switches,’ utilizes an exposed linear toehold domain to interact with target trigger sequence and cause the release of the RBS allowing protein translation to proceed (Figure 2c). The main advantage of toehold switches is the lack of sequence constraints, which facilitated the development of a large library of toehold switches that allow sRNA and mRNA detection as well as simultaneous control of up to 12 genes in *E. coli*. The modularity, orthogonality, and high-dynamic range provided by toehold switches make it possible to compose multiple toehold switches in a single RNA transcript for ‘ribocomputing’, a strategy that was exemplified by building complex AND/OR/NOT logic processing of 12 RNA inputs in *E. coli* [24] (Figure 2d). This

12-input logic circuit in bacteria provided evidence that the composition of RNA regulators could help in scaling up the synthetic biological circuits.

The design principles of toehold switches were recently used also to develop libraries of high-performance RNA-based repressors. Building on the copy-number control element from the insertion sequence IS10, wherein an antisense RNA inhibits transposase expression, Mutalik *et al.* demonstrated a family of orthogonal RNA-based translation repressors [25]. This RNA repressor system successfully reduced GFP expression by ~10-fold and formed an orthogonal library of six devices with under 20% crosstalk. By using toehold switch design principles to inactivate translation via trigger RNAs, called toehold repressor and three-way junction (3WJ) repressor, it was possible to strongly repress translation decreasing gene expression in excess of 100-fold with nearly arbitrary sequences, an efficiency comparable to protein repressors

[26]. These repressor modules could be concatenated to enable a more complex and Versatile logic with universal NAND and NOR gates using ribocomputing strategy. An independent work explored analogous design for RNA repressors, termed looped antisense oligonucleotide, which targets sequences around the exposed RBS to suppress translation [27]. These designs exhibited slightly weaker repression than toehold repressors, but they enabled straightforward targeting of endogenous mRNA transcripts.

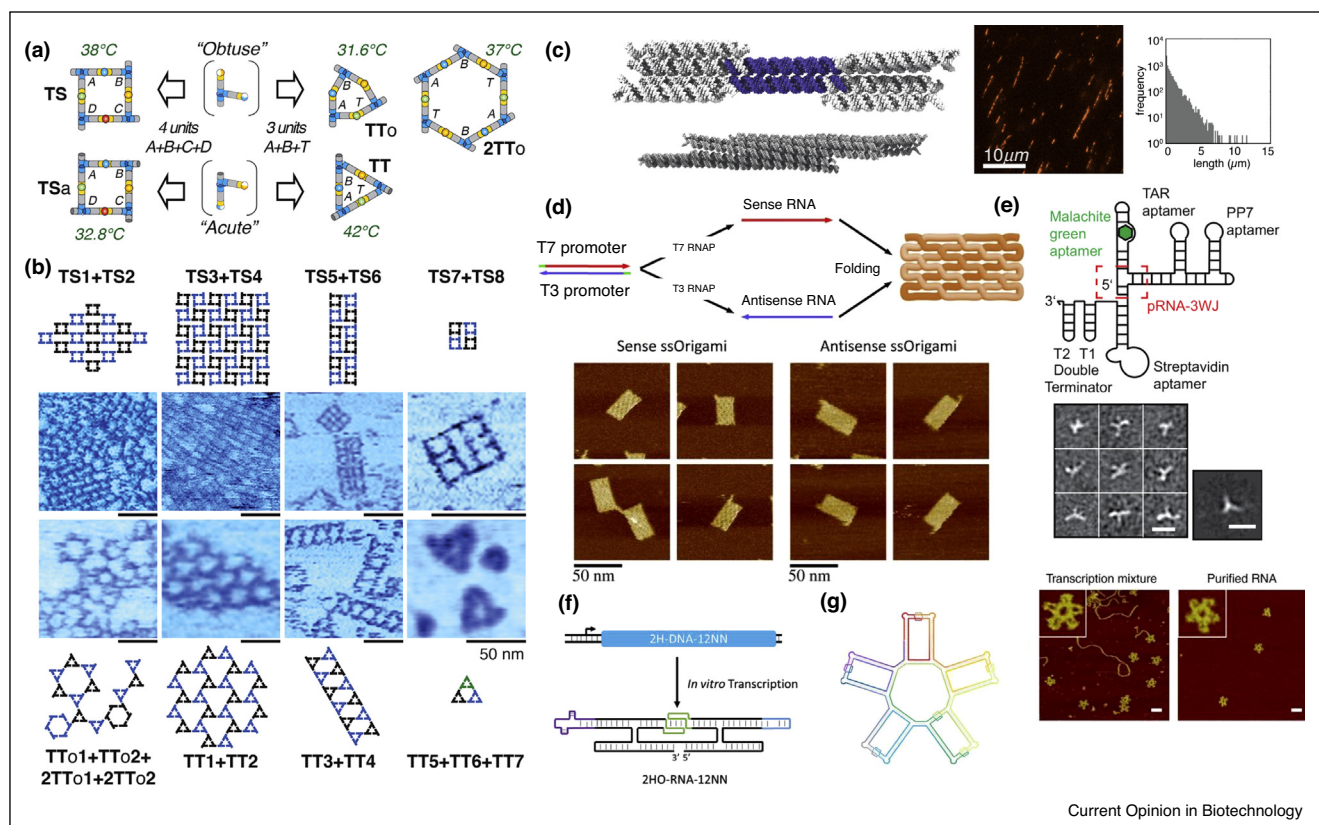
Organizing molecular components with RNA

Because RNA base-pairing can be predicted using a variety of algorithms, RNA has emerged as versatile polymer to build self-assembling structures at the nano-scale. These nanostructures include natural and engineered structural motifs, that is, sequences that locally fold into known secondary or tertiary structures. These motifs can be modularly composed to obtain molecular

scaffolds with desired shape, and include aptamers to spatially organize target ligands on the scaffold.

Many sophisticated RNA nanostructures have been developed for use as biomolecular materials in non-biological context, or for operation as drug delivery vectors. Some of the first RNA self-assembly demonstrations were based on ‘tecto RNA’ motifs, short conserved sequences with known tertiary structure found in nature [28] (Figure 3a). Interactions between tecto RNA motifs can be programmed via duplex formation or via loop–loop interactions with prescribed three dimensional shape, making it possible to build filaments, lattices, and polyhedra [29] (Figure 3b). Some of these structures have proved useful as nanoparticle carriers [30], in particular those based on the pRNA three-way junction [31]. The recent success of DNA nanotechnology has also spurred the development of RNA nanostructures that fold based on duplex-forming domains in which base pairs are designed with optimization algorithms, as opposed to conserved tecto RNA motifs. These methods have

Figure 3



Synthetic RNA nanostructures. (a) and (b): Tecto RNA modules with different geometries (a) interact via loop-loop bonds to form lattices with programmable features (b). Figure adapted from Ref. [29]. (c) RNA tiles assembling into nanotubes that reach micrometers in length. Figure adapted from Refs. [32,33]. (d) RNA origami squares folding from a single, long RNA strand. Figure adapted from Ref. [37]. (e) A multi-domain RNA for simultaneous localization of proteins and reporters, adapted from Ref. [40]. (f) Single-stranded RNA tile for localization of two ligands; the tile folds co-transcriptionally; adapted from Ref. [41]. (g) Three dimensional RNA objects produced and folded inside cells, adapted from Ref. [42].

produced micron-scale lattices and nanotubes assembling from multi-stranded tiles, that may find application as nanoparticle delivery systems [32,33] (Figure 3c). RNA origami designs that take inspiration from the well-known DNA origami method have been tailored for therapeutic applications [34–36]. This approach has also produced complex topologies that include knotted structures, folding from a single long strand of RNA, in which multiple double helical domains are held together by parallel crossovers [37]; these long RNA molecules can be transcribed *in vitro* or inside cells, and later folded via thermal annealing [38] (Figure 3d).

Because RNA is naturally produced inside cells, it is desirable to develop RNA structures that can fold and assemble as they are being transcribed in cytoplasmic conditions, both to produce scaffolds inside cells as well as to lower the cost of RNA device synthesis. For this purpose, it is convenient to build assemblies from a single-strand of RNA, avoiding stoichiometric imbalances and diffusion limits that may affect assembly of multiple strands. Multi-domain, single-stranded RNA structures based on the pRNA three-way junction have been optimized for rapid, automated nanoparticle production [39], and for simultaneous localization of small molecules and different fluorescent proteins in cell-free extracts and *in vivo* [40] (Figure 3e). When working with long RNA molecules, proximal domains interact and fold rapidly as the molecule is transcribed, and interactions between distal domains can be promoted once local interactions have occurred. On the basis of this idea, single stranded RNA origami has been demonstrated by substituting Holliday crossovers with kissing-loop motifs (borrowed from tecto RNA motifs). These structures do not need thermal annealing, and their modular design makes it possible to include aptamers to recruit and organize small molecules [41] (Figure 3f). Recent efforts have been successful at co-transcriptionally producing three dimensional objects with single-stranded RNA, some of which can be produced, folded, and harvested from biological cells [42] (Figure 3g).

Conclusions and outlook

The field of synthetic biology has seen a growing interest in the use of RNA for temporal and spatial control of molecular components. In parallel, RNA nanotechnology is rapidly developing methods to build structural RNA scaffolds of unprecedented complexity and size. As regulatory function and structure of RNA molecules are tightly connected, cross-pollination between these fields is essential to achieve the goal of building functional RNA devices based on predictive models of sequence interactions.

As new RNA tools for control of gene expression are rapidly expanding, design rules need to be established to open up the possibility for *de novo* design to leverage the large libraries of natural and synthetic regulators. *De novo*

design methods are desirable for transcriptional regulators [6] as well as for RNA devices and circuits in mammalian cells, as the repertoire of regulatory motifs is often still limited by the use of few well-characterized aptamers and ribozymes [43,44]. Because it is possible to deliver nucleic acid logic gates in mammalian cells and then use endogenous signals to trigger them [45–47], it is likely feasible to design and express large, composable RNA logic gates operating via strand displacement. Interestingly, recent work demonstrated that a toehold switch architecture was functional in mammalian cells to respond to miRNA as inputs [48]. These works illustrate the potential of RNA devices that recognize intracellular RNAs through base pairing and strand displacement in mammalian cells much as those devices reported for bacterial systems.

RNA regulators are also emerging as a useful tool for point of care diagnostics, an application that is rapidly expanding. For example, a recent report demonstrated that plant pathogens can be detected by engineered STARS, inducing production of enzymes for a visible change of sample color [49]. Toehold switches have been repurposed as sensors expressed within paper-based devices, to detect viruses such as Ebola [50–52], as monitoring systems for microbiome composition with detection limit in the femtomolar range [53]. The paper devices are infused with freeze-dried cell-free components that can be activated simply by adding water [50]. Further improvements in sensitivity and reduction of sample pre-processing requirements may lead to the adoption of these RNA sensing devices in the field and secure commercial success.

The potential usefulness of RNA as a scaffolding molecule inside cells has only been partially explored. The participation of RNA to a number of very large and complex natural structures like the ribosome and ribonucleoprotein condensates suggests that synthetic RNA may be engineered to have a structural role in artificial assemblies of comparable complexity. Recent work supports the hypothesis that engineered RNA scaffolds can assemble in the cytoplasm [42], and localize target proteins to control cell fate [54]. Yet, these structures include few RNA species and do not exceed 100 nanometers in size (The assembly of larger RNA tubular structures that could spatially organize specific enzymes in bacteria was demonstrated only indirectly by verifying improvements in the yield of the targeted metabolic pathway [55]). Plausible routes to building scalable RNA assemblies may be that of designing simple structural monomers that then interact to build larger structures, or molecules that form multi-strand networks with random connections but controllable density [56]. Taking inspiration from the architecture of RNA regulators, the structural features of these assemblies may be dynamically controlled using other RNA inputs to induce conformational changes of key components.

Despite still unsolved constraints, the studies we have reviewed herein clearly demonstrated the merits of utilizing novel programmable RNA-based devices in diverse areas of synthetic biology. As we gain more insight into RNA design principles that directly translates to functional applications alongside recent progress in computational tools, next-generation synthetic biology devices that mainly capitalize on RNA and RNA-processing modules could become a reality. We envision that future endeavors aimed at interfacing programmable RNA systems inside and outside cells with diverse platforms including electronic sensors and point-of-care devices could further expand the horizon of RNA-device-driven synthetic biology applications.

Conflict of interest statement

Nothing declared.

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References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest

- Lucks JB, Qi L, Mutalik VK, Wang D, Arkin AP: **Versatile RNA-sensing transcriptional regulators for engineering genetic networks.** *Proc Natl Acad Sci U S A* 2011, **108**:8617-8622.
 - Takahashi MK, Lucks JB: **A modular strategy for engineering orthogonal chimeric RNA transcription regulators.** *Nucleic Acids Res* 2013, **41**:7577-7588.
 - Chappell J, Takahashi MK, Lucks JB: **Creating small transcription activating RNAs.** *Nat Chem Biol* 2015, **11**:214-220.
 - Meyer S, Chappell J, Sankar S, Chew R, Lucks JB: **Improving fold activation of small transcription activating RNAs (STARs) with rational RNA engineering strategies.** *Biotechnol Bioeng* 2016, **113**:216-225.
 - Westbrook ?am?, Lucks JB: **Achieving large dynamic range control of gene expression with a compact RNA transcription-translation regulator.** *Nucleic Acids Res* 2017, **45**:5614-5624.
 - Chappell J, Westbrook A, Verosloff M, Lucks JB: **Computational design of small transcription activating RNAs for versatile and dynamic gene regulation.** *Nat Commun* 2017, **8**:1051.
- In this study, computational design of small transcription activating RNAs substantially increased performance and orthogonal library size of synthetic transcriptional regulators.
- Knott GJ, Doudna JA: **CRISPR-Cas guides the future of genetic engineering.** *Science* 2018, **361**:866-869.
 - Qi LS, Larson MH, Gilbert LA, Doudna JA, Weissman JS, Arkin AP *et al.*: **Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression.** *Cell* 2013, **152**:1173-1183.
 - Zetsche B, Volz SE, Zhang F: **A split-Cas9 architecture for inducible genome editing and transcription modulation.** *Nat Biotechnol* 2015, **33**:139-142.
 - Briner AE, Donohoue PD, Gomaa AA, Selle K, Slorach EM, Nye CH *et al.*: **Guide RNA functional modules direct Cas9 activity and orthogonality.** *Mol Cell* 2014, **56**:333-339.
 - Kundert K, Lucas JE, Watters KE, Fellmann C, Ng AH, Heineke BM *et al.*: **Controlling CRISPR-Cas9 with ligand-activated and ligand-deactivated sgRNAs.** *Nat Commun* 2019, **10**:2127.
 - Tang W, Hu JH, Liu DR: **Aptazyme-embedded guide RNAs enable ligand-responsive genome editing and transcriptional activation.** *Nat Commun* 2017, **8**:15939.
 - Ferry QRV, Lyutova R, Fulga TA: **Rational design of inducible CRISPR guide RNAs for de novo assembly of transcriptional programs.** *Nat Commun* 2017, **8**:14633.
 - Lee YJ, Hoynes-O'Connor A, Leong MC, Moon TS: **Programmable control of bacterial gene expression with the combined CRISPR and antisense RNA system.** *Nucleic Acids Res* 2016, **44**:2462-2473.
 - Mücl A, Schwarz-Schilling M, Fischer K, Simmel FC: **Filamentation and restoration of normal growth in *Escherichia coli* using a combined CRISPRi sgRNA/antisense RNA approach.** *PLoS One* 2018, **13**:e0198058.
 - Cox KJ, Subramanian HKK, Samaniego CC, Franco E, Choudhary A: **A universal method for sensitive and cell-free detection of CRISPR-associated nucleases.** *Chem Sci* 2019, **10**:2653-2662.
 - Jin M, Garreau de Loubresse N, Kim Y, Kim J, Yin P: **Programmable CRISPR-Cas repression, activation, and computation with sequence-independent targets and triggers.** *ACS Synth Biol* 2019, **8**:1583-1589.
 - Hanewich-Hollatz MH, Chen Z, Hochrein LM, Huang J, Pierce NA: **Conditional guide RNAs: programmable conditional regulation of CRISPR/Cas function in bacterial and mammalian cells via dynamic RNA nanotechnology.** *ACS Central Sci* 2019, **5**:1241-1249.
- Computational design of engineered conditional guide RNAs was demonstrated in *E. coli* and HEK 293T cells.
- Oesinghaus L, Simmel FC: **Switching the activity of Cas12a using guide RNA strand displacement circuits.** *Nat Commun* 2019, **10**:2092.
 - Siu K-H, Chen W: **Riboregulated toehold-gated gRNA for programmable CRISPR-Cas9 function.** *Nat Chem Biol* 2019, **15**:217-220.
- Toehold-gated guide RNAs showed multiplexed regulation and response to endogenous RNA signals in *E. coli*.
- Wang X-W, Hu L-F, Hao J, Liao L-Q, Chiu Y-T, Shi M *et al.*: **A microRNA-inducible CRISPR-Cas9 platform serves as a microRNA sensor and cell-type-specific genome regulation tool.** *Nat Cell Biol* 2019, **21**:522-530.
- microRNA-mediated single guide RNA-releasing strategy showed microRNA sensing capability in mouse embryonic stem cells.
- Isaacs FJ, Dwyer DJ, Ding C, Pervouchine DD, Cantor CR, Collins JJ: **Engineered riboregulators enable post-transcriptional control of gene expression.** *Nat Biotechnol* 2004, **22**:841-847.
 - Green AA, Silver PA, Collins JJ, Yin P: **Toehold switches: de-novo-designed regulators of gene expression.** *Cell* 2014, **159**:925-939.
 - Green AA, Kim J, Ma D, Silver PA, Collins JJ, Yin P: **Complex cellular logic computation using ribocomputing devices.** *Nature* 2017, **548**:117-121.
- In this paper, several synthetic riboregulators were co-localized on a long single-stranded RNA to scale up the complexity of logic evaluation in *E. coli*.
- Mutalik VK, Qi L, Guimaraes JC, Lucks JB, Arkin AP: **Rationally designed families of orthogonal RNA regulators of translation.** *Nat Chem Biol* 2012, **8**:447-454.
 - Kim J, Zhou Y, Carlson PD, Teichmann M, Chaudhary S, Simmel FC *et al.*: **De novo-designed translation-repressing riboregulators for multi-input cellular logic.** *Nat Chem Biol* 2019, **15**:1173-1182.
 - Carlson PD, Glasscock CJ, Lucks JB: **De novo design of translational RNA repressors.** *BioRxiv*. DOI: <https://doi.org/10.1101/501767>.

28. Chworos A, Severcan I, Koyfman AY, Weinkam P, Oroudjev E, Hansma HG *et al.*: **Building programmable jigsaw puzzles with RNA**. *Science* 2004, **306**:2068-2072.
29. Geary C, Chworos A, Verzemnieks E, Voss NR, Jaeger L: **Composing RNA Nanostructures from a syntax of RNA structural modules**. *Nano Lett* 2017, **17**:7095-7101.
30. Jedrzejczyk D, Chworos A: **Self-assembling RNA nanoparticle for gene expression regulation in a model system**. *ACS Synth Biol* 2019, **8**:491-497.
31. Pi F, Binzel DW, Lee TJ, Li Z, Sun M, Rychahou P *et al.*: **Nanoparticle orientation to control RNA loading and ligand display on extracellular vesicles for cancer regression**. *Nat Nanotechnol* 2018, **13**:82-89.
32. Stewart JM, Viard M, Subramanian HKK, Roark BK, Afonin KA, Franco E: **Programmable RNA microstructures for coordinated delivery of siRNAs**. *Nanoscale* 2016, **8**:17542-17550.
33. Stewart JM, Geary C, Franco E: **Design and characterization of RNA nanotubes**. *ACS Nano* 2019, **13**:5214-5221.
34. Hoiberg HC, Sparvath SM, Andersen VL, Kjems J, Andersen ES: **An RNA origami octahedron with intrinsic siRNAs for potent gene knockdown**. *Biotechnol J* 2019, **14**:e1700634.
35. Kozyra J, Ceccarelli A, Torelli E, Lopiccio A, Gu J-Y, Fellermann H *et al.*: **Designing uniquely addressable bio-orthogonal synthetic scaffolds for DNA and RNA origami**. *ACS Synth Biol* 2017, **6**:1140-1149.
36. Krissanaprasit A, Key C, Fergione M, Froehlich K, Pontula S, Hart M *et al.*: **Genetically encoded, functional single-strand RNA origami: anticoagulant**. *Adv Mater* 2019, **31** 1808262.
37. Han D, Qi X, Myhrvold C, Wang B, Dai M, Jiang S *et al.*: **Single-stranded DNA and RNA origami**. *Science* 2017, **358**:eaao2648. This paper presents new methods to build RNA origami folding from a single strand.
38. Qi X, Zhang F, Su Z, Jiang S, Han D, Ding B *et al.*: **Programming molecular topologies from single-stranded nucleic acids**. *Nat Commun* 2018, **9**:4579. This paper describes the production and *in vivo* amplification of RNA molecules that yield single-stranded two-dimensional and three-dimensional shapes exploiting knotted architectures.
39. Jasinski DL, Binzel DW, Guo P: **One-pot production of RNA nanoparticles via automated processing and self-assembly**. *ACS Nano* 2019, **13**:4603-4612.
40. Schwarz-Schilling M, Dupin A, Chizzolini F, Krishnan S, Mansy SS, Simmel FC: **Optimized assembly of a multifunctional RNA-protein nanostructure in a cell-free gene expression system**. *Nano Lett* 2018, **18**:2650-2657.
41. Jepsen MDE, Sparvath SM, Nielsen TB, Langvad AH, Grossi G, Gothelf KV *et al.*: **Development of a genetically encodable FRET system using fluorescent RNA aptamers**. *Nat Commun* 2018, **9**:18.
42. Li M, Zheng M, Wu S, Tian C, Liu D, Weizmann Y *et al.*: **In vivo production of RNA nanostructures via programmed folding of single-stranded RNAs**. *Nat Commun* 2018, **9**:2196.
- RNA nanostructures are synthesized and assembled inside cells from single strands of RNA.
43. Win MN, Smolke CD: **Higher-order cellular information processing with synthetic RNA devices**. *Science* 2008, **322**:456-460.
44. Ausländer S, Stücheli P, Rehm C, Ausländer D, Hartig JS, Fussenegger M: **A general design strategy for protein-responsive riboswitches in mammalian cells**. *Nat Methods* 2014, **11**:1154-1160.
45. Hemphill J, Deiters A: **DNA computation in mammalian cells: MicroRNA logic operations**. *J Am Chem Soc* 2013, **135**:10512-10518.
46. Groves B, Chen Y-J, Zurla C, Pochekailov S, Kirschman JL, Santangelo PJ *et al.*: **Computing in mammalian cells with nucleic acid strand exchange**. *Nat Nanotechnol* 2016, **11**:287-294.
47. Chatterjee G, Chen Y-J, Seelig G: **Nucleic acid strand displacement with synthetic mRNA inputs in living mammalian cells**. *ACS Synth Biol* 2018, **7**:2737-2741.
48. Wang S, Emery NJ, Liu AP: **A novel synthetic toehold switch for MicroRNA detection in mammalian cells**. *ACS Synth Biol* 2019, **8**:1079-1088.
49. Verosloff M, Chappell J, Perry KL, Thompson JR, Lucks JB: **PLANT-Dx: a molecular diagnostic for point-of-use detection of plant pathogens**. *ACS Synth Biol* 2019, **8**:902-905.
50. Pardee K, Green AA, Ferrante T, Cameron DE, DaleyKeyser A, Yin P *et al.*: **Paper-based synthetic gene networks**. *Cell* 2014, **159**:940-954.
51. Pardee K, Green AA, Takahashi MK, Braff D, Lambert G, Lee JW *et al.*: **Rapid, low-cost detection of Zika virus using programmable biomolecular components**. *Cell* 2016, **165**:1255-1266.
52. Ma D, Shen L, Wu K, Diehnelt CW, Green AA: **Low-cost detection of norovirus using paper-based cell-free systems and synbody-based viral enrichment**. *Synth Biol* 2018, **3**:ysy018.
53. Takahashi MK, Tan X, Dy AJ, Braff D, Akana RT, Furuta Y *et al.*: **A low-cost paper-based synthetic biology platform for analyzing gut microbiota and host biomarkers**. *Nat Commun* 2018, **9**:3347.
54. Shibata T, Fujita Y, Ohno H, Suzuki Y, Hayashi K, Komatsu KR *et al.*: **Protein-driven RNA nanostructured devices that function in vitro and control mammalian cell fate**. *Nat Commun* 2017, **8**:540. Synthetic RNA scaffolds are designed to control the assembly and oligomerization of proteins in mammalian cells.
55. Sachdeva G, Garg A, Godding D, Way JC, Silver PA: **In vivo co-localization of enzymes on RNA scaffolds increases metabolic production in a geometrically dependent manner**. *Nucleic Acids Res* 2014, **42**:9493-9503.
56. Jain A, Vale RD: **RNA gelation in repeat expansion disorders**. *Nature* 2017, **546**:243-247. Synthetic single strands of RNA characterized by repeated motifs assemble into liquid droplets in mammalian cells.