SURVEY AND SUMMARY

Opportunities in the design and application of RNA for gene expression control

Maureen McKeague, Remus S. Wong and Christina D. Smolke*

Department of Bioengineering, Stanford University, Stanford, CA 94305, USA

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ABSTRACT

The past decade of synthetic biology research has witnessed numerous advances in the development of tools and frameworks for the design and characterization of biological systems. Researchers have focused on the use of RNA for gene expression control due to its versatility in sensing molecular ligands and the relative ease by which RNA can be modeled and designed compared to proteins. We review the recent progress in the field with respect to RNA-based genetic devices that are controlled through small molecule and protein interactions. We discuss new approaches for generating and characterizing these devices and their underlying components. We also highlight immediate challenges, future directions and recent applications of synthetic RNA devices in engineered biological systems.

INTRODUCTION

The emerging field of synthetic biology focuses on the development of tools and frameworks for the design, construction and characterization of biological systems (1–3). A primary focus in this field has been the engineering and refinement of biological parts and devices to advance the probing and programming of biological systems (4). In particular, ‘sensing-actuation’ devices represent an important class of genetic devices that can detect, report on and act on environmental and intracellular signals, including small molecules and proteins, to study and control cellular function (5).

Researchers have discovered examples of natural functional RNAs that exhibit activity as regulatory RNAs, structural scaffolds or biological catalysts (6). Inspired by the diverse activities that RNA has evolved in nature, researchers have created synthetic, RNA-based, genetic control devices (7). RNA devices are typically composed of two domains: a small sensory RNA (an aptamer) (8) and an RNA-based regulatory element that controls gene expression through a variety of mechanisms, including transcription, translation or stability (9,10). The sensing and regulatory activities are coupled within the device, sometimes through a communication module, such that ligand binding alters the activity of the gene-regulatory element. Allosteric control over the gene-regulatory activity can be achieved through various molecular mechanisms, but generally, ligand binding at the sensor domain causes or stabilizes a conformational change in the regulatory domain that affects its activity. RNA-based control strategies offer many useful features, including the ability to tailor ligand-sensing and gene-regulatory activities and quantitative tuning of the system’s response (11). RNA-based controllers also offer unique advantages for translation into clinical systems, such as adaptability to diverse input molecules and regulatory targets and a compact RNA-only platform that is non-immunogenic (in contrast to platforms based on heterologous proteins) (12).

Here, we review and discuss recent progress in the field and challenges that must be addressed to realize the full potential of RNA-based genetic devices. We begin by discussing new strategies for generating the underlying components (sensor, gene-regulatory element and communication module) that comprise the RNA device and discuss challenges in the field with regard to generalizing, scaling and accelerating the design process. We further discuss measurement and characterization practices and highlight opportunities where the introduction of standards and rigorous characterization methods will fuel advances in design. Finally, we highlight recent applications of synthetic RNA devices in advancing foundational and applied biological research. While RNA devices have been designed to respond to diverse molecular inputs, for the purposes of this perspective we will focus on protein and small molecule-based inputs, as devices responsive to RNA-based inputs have been recently reviewed (6,11).
RNA DESIGN AND ENGINEERING

For the design of new RNA devices, individual sensor and regulatory RNA components are often identified, evolved de novo, or tuned independently and then assembled into device platforms. Research efforts have thus been directed to the identification and development of individual device components, which are generated in isolation or within the context of the device platform. Current efforts are focused on accelerating the design and discovery of new RNA components and developing strategies to create integrated RNA devices that enable novel gene-regulatory capabilities.

Generating new RNA device components

There is a general lack of available RNA devices that respond to diverse molecular inputs. The biggest bottleneck for new RNA device engineering continues to be the limited diversity in available sensor components. Overall, the strategies for generating new sensing functions are relatively limited in terms of throughput and efficiency, which can deter researchers from pursuing efforts to generate novel sensing activities de novo. As a result, major advances in the field have focused on improving our ability to generate new sensor components.

Reengineering of natural riboswitches. One source for sensor components is from natural riboswitches. Riboswitches are natural ligand-responsive regulatory elements that are composed of an evolutionarily conserved sensor (aptamer) domain and a variable gene-regulatory element (13). Currently, there are riboswitches identified to ∼20 different ligands. For example, the aptamer domain from the ribD riboswitch (14), which binds flavin mononucleotide (FMN), has been employed in several RNA device designs (15,16). More recently, the aptamer for guanine (17,18) has similarly been incorporated. With the discovery of each new ligand-responsive RNA, the pool of potential sensors increases (19). In particular, deep-sequencing technologies (e.g. RNA-seq) and genome-wide identification of transcription start sites (dT-RNA-seq) have been used to more rapidly identify new potential riboswitches (20). One particular advantage of natural aptamers is that they have been evolved to function under in vivo conditions and thus may be more readily incorporated into RNA device platforms for in vivo applications. On the other hand, the majority of riboswitches recognize cellular metabolites such as coenzymes, purines, amino acids and metal cations (21); applying these natural metabolites exogenously to cells may compromise their tightly-regulated intracellular concentrations and potentially impact normal cellular function (22). Therefore, these aptamers typically cannot be used to detect orthogonal or synthetic molecules (23).

To mitigate the limited orthogonality of natural aptamers, new sensor components can be obtained by altering the specificity of existing aptamers. For example, researchers performed structure-guided mutations on the natural adenine-binding addA aptamer and screened the variants against a large library of orthogonal ligands. They subsequently identified two additional ligands that bound to the aptamer with high affinity, resulting in improved in vivo gene-induction properties and reduced cellular toxicity (22). Although this strategy is low throughput and not broadly implemented, it can harness the existing in vivo capabilities of natural aptamers while avoiding inputs that may affect cellular function. We expect that with further development and application, this approach may be used to generate RNA devices responsive to new ligands.

De novo selection of RNA binding elements via SELEX. The largest potential pool for new sensor components is from aptamers generated de novo against new ligands via an in vitro selection procedure known as Systematic Evolution of Ligands by Exponential enrichment (SELEX) (24–26). While hundreds of aptamers have been generated against protein ligands, only ∼60 have been reported for small molecules (27,28). One prominent example is the theophylline RNA aptamer (29), which has been incorporated into ∼60% of in vivo engineered RNA devices (9,30). A major challenge associated with the standard in vitro SELEX method is that ligand binding does not occur in the context of co-transcriptional folding and the majority of aptamers require conditions that are unsuitable in vivo (e.g. non-physiological pH, high-magnesium concentrations) (31,32). Therefore, the field has shifted toward selecting aptamers under conditions that more closely mimic those intended for their application. For example, aptamers recently selected (31) and characterized (23) using low, physiologically-relevant magnesium levels (e.g. 0.5 mM) resulted in RNA devices with predictable activity. Additionally, methods such as in vivo SELEX (33) have been described and should be employed when generating parts for gene expression regulation. However, the major bottleneck is most likely attributed to several technical challenges associated with the SELEX method, including the necessity for ligand immobilization, a high-failure rate and a low-throughput (34). Other ongoing efforts in the field are directed toward new enhancements to the SELEX method (35–37), such as improving partitioning efficiency (e.g. Hi-Fi SELEX (38)) and reducing the number of rounds to reduce labor and cost (e.g. single round SELEX (39)).

In particular, the introduction of high-throughput sequencing (HTS), or next-generation sequencing (NGS), into the SELEX process has revolutionized the selection of new aptamers (Figure 1). A major drawback of conventional SELEX experiments is the lack of obtainable information regarding sequence enrichment during the selection rounds. Consequently, many selection experiments fail in identifying high-affinity aptamers. The introduction of HTS into the SELEX process enables comprehensive characterization of obtained aptamers, identification of functional and rare motifs, comparison of functional motifs in oligonucleotide populations and quantification of their abundance. Following its first application in 2002 (40), SELEX with HTS has become the standard for identifying aptamers in large selection experiments over the last 5 years. Furthermore, several groups have coupled the data-rich information obtained with HTS to many novel selection approaches including microfluidic SELEX (41), semi-automated SELEX (42), CE-SELEX (43–45), capture-SELEX (46) and branched SELEX (47). As a result, the number of reports describing new aptamers have increased in the past 5 years (48).
To support the data-rich approaches for aptamer selection, there has been substantial work in developing tools to leverage rich datasets through new bioinformatic programs and computational search methods. For example, FASTAptamer is a bioinformatic, open-access toolkit that allows researchers to count, normalize and rank sequencing reads. It enables facile comparison of populations for sequence distribution, generation of sequence family clusters and calculation of sequence enrichment throughout the course of a selection (49). Additionally, AptaTools is a set of computational tools developed for data processing, tracking changes in aptamer families throughout selection cycles and identifying sequence-structure motifs. These tools have already been successfully employed in the selection of new aptamers to protein ligands (e.g. Interleukin 10 receptor α chain (50)), and we expect that they will facilitate aptamer selection to small molecule targets and become important tools for all aptamer-related research.

In silico selection of RNA binding elements. A relatively unexplored approach to generating new, small-molecule-based sensor components is in silico design and selection. To our knowledge, there are currently no cases of de novo aptamer design for small molecule ligands using a computational approach (51). However, the most recent and exciting example of in silico design involved designing aptamers to recognize the cytochrome P450 51A1 using docking and molecular dynamics simulation. Importantly, 15 unique sequences were uncovered without using any in vitro screening or selection. Subsequent characterization indicated that these aptamers bound to the P450 protein with dissociation constant (K_D) values in the low μM range (52). This initial success will pave the way for improving our ability to successfully generate aptamers using in silico approaches. However, one limitation of this method is that the spatial structure of the ligand of interest must be known.

As an alternative to de novo, in silico aptamer selection, computational approaches may be used in combination with in vitro selection. For instance, computational methods may be used to design a starting oligonucleotide library (53) or a high affinity template to reduce the sequence space and improve the success of an in vitro selection experiment. In one study, researchers used an entropic fragment-based
in silico approach (54) to design aptamer templates that bound to phosphatidylserine (PS). When tested experimentally, the designed sequences bound to PS with a $K_D$ of 300 $\mu$M (55), thus providing a rich library for performing selections against this ligand.

Taken together, these recent findings highlight the promise of incorporating HTS and computational methods into aptamer selection processes. We expect that a combination of computational methods with in vitro selection will continue to be broadly explored and leveraged in the field, ultimately enhancing the selection process and reducing the cost and time to the final product.

**Integrating components into RNA device frameworks**

Typically, RNA devices are designed by integrating existing underlying components (sensor, communication module, regulatory RNA) into a functional device framework. Alternatively, a single component in the context of the device framework may be screened to identify a sequence that functionally couples with the other components in the device. The most common design strategies focus on functionally connecting the sensor and regulatory components (e.g. through the design of an appropriate communication module) using either rational design, screening or selection strategies. However, there exists one case where both the sensing and regulatory RNA were simultaneously engineered de novo (56).

**Rational design of RNA devices.** In early work, rational design was frequently employed in the design of the communication module for coupling the sensor and regulatory components (9,57). Many reviews have described the utility of this approach (10,11,58). However, the main drawback of rational design is inefficiency, as it often requires several iterations through the design-build-test cycle to achieve the desired in vivo gene-regulatory response. A second challenge is that this method typically relies on secondary structure prediction programs that implicitly focus on modeling global RNA structures (59). It is known that RNA begins to form structures in vivo during transcription, and this co-transcriptional folding defines the functional RNA molecule. This process is difficult to mimic in vitro and capture with global secondary structure prediction. To address these challenges, a few recent developments have drawn particular interest. First, there has been excellent progress in the development of design methods that use stochastic kinetic folding simulations (60–62). In addition, several reports have described new strategies directly comparing in vitro and in silico prediction methods to in vivo gene-regulatory activities through massively parallel assays (63,64). Finally, a recent study described the development of a statistical thermodynamic model to explain complex sequence-structure-activity relationships of RNA devices that function via translational regulation. Specifically, researchers demonstrate how aptamer structure, ligand affinity and switching free energy collectively control RNA device activity (65). More studies of this kind will allow researchers to thoroughly examine and identify complex sequence-structure-activity relationships of RNA devices. We expect that with advancements in kinetic simulation, increased usage of data-rich comparison strategies and a better understanding of the quantitative rules that govern device function, RNA device design may become more systematic and predictive.

**In vitro selection of RNA devices.** As with aptamer selection, HTS and selection strategies have become increasingly popular and allow researchers to go through the design-build-test cycle more efficiently. In particular, a recent strategy for developing RNA devices linked the desired sensor and regulatory RNA components via a randomized communication module and performed in vitro selection to isolate functional devices. Specifically, the cyclic diguanylosyl-5’-monophosphate (c-di-GMP) aptamer was linked to stem II of a hammerhead ribozyme (HHRz) through a library of randomized bridge sequences. After seven rounds of parallel selections, functional devices that inhibited or activated gene expression were isolated (66). The broader applicability of this strategy was also demonstrated in selecting tetracycline-inducible RNA devices (67). A selection-based approach offers the advantages to enrich large libraries and isolate variants that display a range of device activities within a given threshold. However, devices isolated in vitro do not always retain activities under in vivo conditions (68); therefore, a secondary screen must be performed on a larger, in vitro-validated set of devices to identify particular sequences that retain activity under in vivo conditions.

**In vivo screening of RNA devices.** An increasingly popular method of generating RNA devices is to apply high-throughput in vivo screens to identify functional sequences from large device libraries. These screens use gene expression activity as a measure of device performance and to-date they have incorporated a variety of functional readouts: colorimetric enzyme assays (e.g. $\beta$-galactosidase activity) (69), growth-based selection assays (e.g. antibiotic resistance, auxotrophy) (70,71), cell motility assays based on regulated expression of the chemotactic gene cheZ (72) and fluorescent reporter assays (73,74). Importantly, direct screening of device activity in the organism of interest ensures functionality and mitigates any challenges due to species-specific mechanisms that may be difficult to predict and study (e.g. co-transcriptional folding) (12,73,75).

Recently, high-throughput in vivo screens have been combined with strategies that allow for the simultaneous measurement of many distinct genetic devices. For example, FACS-Seq is a massively parallel assay that provides activity data on all members of a library by coupling fluorescence activated cell sorting (FACS) with NGS. This recent transition toward data-rich screening approaches will permit a more streamlined and expedited process for developing new RNA devices (64).

**RNA devices based on higher-ordered structural interactions.** Recent work in RNA device design highlighted a new mechanism for achieving allostery based on the alteration of higher-ordered structural interactions. The mechanism is hypothesized to utilize ligand binding to block long-range tertiary interactions and/or the formation of structural motifs that can act to prevent RNA recognition by protein cofactors or directly inhibit the regulatory activity of the RNA.
element (Figure 2). Importantly, such mechanisms have been demonstrated to be modular, thereby allowing new sensors to be incorporated into an RNA platform with minimal redesign. In one study, a non-coding RNA (ncRNA) that regulated transcription in *Escherichia coli* was independently engineered to respond to different ligands (theophylline and MS2 coat protein) (76). The aptamer element was fused to the 5′ end of the pT181 ncRNA regulatory element such that the aptamer loop nucleotides formed a pseudoknot interaction with the ncRNA loop nucleotides to disrupt its regulatory function. The binding of ligand to the aptamer region then disrupted the pseudoknot interaction, thereby liberating the ncRNA molecule to regulate transcription. This work highlights the ability of using modular platforms in engineering RNA devices to respond to different ligand inputs. Further design of RNA devices leveraging this mechanism may expedite the combinatorial implementation of RNA regulators to achieve multi-input logical processing.

The modularity and ease of design associated with the alteration of higher-ordered structural interactions were also demonstrated in the development of RNA interference (RNAi)-based devices. In this study, researchers controlled RNAi-based gene silencing with ligand-responsive microRNAs (miRNAs) by integrating an aptamer into the miRNA basal segments to impart control over miRNA processing (77). Aptamer sequences responsive to various small molecule and protein ligands (78) have been incorporated into this design, thus highlighting the modularity of the mechanism.

In another study, researchers developed protein-responsive, HHRz-based devices by modulating the tertiary stem–loop interactions of the HHRz to control gene expression in mammalian cells (12). The natural HHRz was screened for complementary inter-loop tertiary interactions to a natural ribonucleoprotein complex and the U1Ap protein. These engineered RNA devices exhibited cleavage activity in both *E. coli* and mammalian cells, thus suggesting that the ribozyme cleavage mechanism is maintained in different cell types. This work has recently been extended to small molecules by selecting for alternate HHRz loop sequences that interacted with small-molecule aptamer sequences. A general method was described in which one of the HHRz loops was replaced with an aptamer for an arbitrary ligand (e.g. theophylline, neomycin, tetracycline) and the opposite HHRz loop was randomized. The resulting library was subsequently screened to identify specific loop sequences that restored cleavage activity to the synthetic HHRz in the absence of ligand (64).

We expect that further application of this RNA device design approach will create new capabilities in gene expression control, expand the regulatory properties of current synthetic RNA devices, improve modularity and expedite the design process, thereby ultimately solving key challenges in the field.

### ROBUST CHARACTERIZATION AND BEST VALIDATION PRACTICES FOR RNA DEVICES

RNA engineering relies on our ability to alter RNA function through design. Thus, robust, quantitative characterization is critical for enabling the development of more effective components, improving their assembly into functioning architectures and ultimately generating highly functional RNA devices. Here, we discuss important parameters of RNA design and function and the corresponding in vitro and in vivo methods for measuring these parameters. We also highlight more recent high-throughput measurement strategies that are rapidly advancing our design capabilities.

#### Quantitative device performance parameters and characterization strategies

Device performance can be evaluated both in vitro and/or in vivo using a variety of specific quantitative and qualitative parameters. First, the individual components comprising the device can be characterized. For example, aptamers can be characterized for affinities, binding kinetics, selectivities, buffer dependence and structure (28). Similarly, regulatory RNAs can be characterized for mechanism of action, kinetics, ion dependence, nucleotide composition and structure (79). Thorough component characterization can inform which components may be most compatible and useful for a given device architecture or application. However, the properties of the individual components do not necessarily dictate performance within the context of the device (68). Thus, thorough quantitative characterization of parameters related to the entire RNA device is needed. These metrics will improve our understanding between RNA device design and function and provide important insight into the underlying molecular mechanisms by which the device acts to regulate gene expression.

#### Probing secondary structure of RNA devices

The link between sequence, structure and function is central to informed or directed RNA device design. Classically, in-line probing assays have been used extensively to elucidate the secondary structure and binding capabilities of natural riboswitches. This technique makes use of the natural instability of RNA, resulting in differential degradation patterns based on structure (80). Detailed information on three-dimensional structures are typically obtained through x-ray crystallography (81).

More recent methods such as selective 2′-hydroxyl acylation analyzed by primer extension (SHAPE) chemistry (82), single-molecule fluorescence resonance energy transfer (smFRET) imaging (83) and 2-aminopurine fluorescence (84), have been used to characterize nucleotide structure and RNA device dynamics (85,86). Importantly, these methods are amenable to parallelization and automation, thus permitting simultaneous and rapid measurements. As one example, in-cell SHAPE-Seq has emerged as an important methodology for RNA characterization. This method combines information about RNA structure within the cell by measuring regions of RNA flexibility using an in-cell chemical probe, reverse transcription, NGS and bioinformatics. By coupling this information to gene expression activities,
Figure 2. Strategies for constructing RNA devices based on higher-ordered structural interactions. (A) The aptamer (red sequence) is coupled to a non-coding RNA (ncRNA). In the absence of ligand (red pentagon), the structural interaction between the aptamer and the ncRNA (blue sequence) inactivates the ncRNA regulatory function. Ligand binding to the aptamer abolishes such structural interactions and activates the ncRNA for translational inhibition. (B) The aptamer (blue sequence) is integrated into the basal segment domain of a microRNA (miRNA). Processing of the miRNA results in incorporation of the targeting strand (red sequence) into the RNA-induced silencing complex (RISC), which silences target gene expression. Ligand (blue hexagon) binding to the aptamer inhibits proper processing of the miRNA, thereby reducing RNAi-mediated gene silencing and increasing target gene expression. (C) The aptamer (purple sequence) is integrated onto one of the loops of a HHRz, which is encoded into the 3′ UTR of a gene. Self-cleavage (at red arrow) destabilizes the transcript for degradation by ribonucleases, hence decreasing gene expression. Ligand (purple circle) binding to the aptamer disrupts tertiary interactions required for self-cleavage, thereby permitting translation of the target gene.
researches may rapidly obtain quantitative information regarding cellular RNA structure-function relationships (87). SHAPE-Seq has been used to study the structure-function relationships of two RNA regulatory systems in E. coli—the synthetic RNA riboregulator translational activator system and the natural IS10 translational repressor system (88)—and we expect this method to be applied in the future for studying other RNA devices.

**Quantification of device sensitivity to ligand.** Ligand sensitivity of a device, or the half maximal effective concentration (EC50), is a key parameter in understanding the interaction between the RNA device and its cognate ligand. Typical strategies for measuring EC50 values in vitro rely on gel-based assays that assess ligand binding via RNA conformational changes (16,80,89,90); however, these conventional analytical methods are inefficient and labor intensive. More recently, methods that reduce assay time and parallelize sample measurements have been developed. A kinetic isothermal titration calorimetry (kinITC) method was described to measure thermodynamic and kinetic data of RNA-ligand interactions for the natural thiamine pyrophosphate (TPP) riboswitch (91). Specifically, the titration curve obtained through the kinITC method provides enthalpic, affinity, stoichiometric and kinetic information about the binding reaction (91). A second high-throughput method involving a microfluidic mobility shift assay was used to characterize five computationally predicted SAM-I riboswitches (92). This microfluidic platform measures ligand binding and RNA conformational changes and reduces assay time to 0.3% of that required for similar gel-based mobility shift assays. A third method was developed for characterizing ribozyme-based devices via real-time, continuous monitoring of ribozyme cleavage using surface plasmon resonance (SPR) (93). The assay was applied to a panel of theophylline-responsive ribozyme-based devices and the SPR-measured dissociation rate constants correlated well with rates obtained via traditional gel-based cleavage assays.

**Quantification of gene-regulatory activity of RNA devices.** The two main parameters that are often reported when describing RNA device gene-regulatory performance are activation ratio and dynamic range, calculated as the ratio and difference between gene expression levels in the presence and absence of ligand, respectively. Quantitative gene-regulatory activities can be matched with the activities of the regulated proteins to ultimately produce the desired performance for an application of interest (94). To measure gene-regulatory activities, devices are typically cloned into a characterization construct in which a reporter protein is used as the signal output.

A number of reporter proteins and corresponding assays have been used to measure gene-regulatory activities of RNA devices. One common class of reporter proteins is enzymes, which can convert a substrate into a colorimetric or fluorescent product. For example, β-galactosidase (69,95), firefly luciferase (96) and alkaline phosphatase (18) are all frequently used enzyme reporters because of their high sensitivity to small changes in gene expression levels, thus permitting the measurement of small differences in device performance. However, these assays do not allow for single-cell quantification of gene expression (i.e. provide bulk measurements across a cell population) and are indirect (i.e. may not linearly correlate to changes in reporter protein levels).

Fluorescent proteins are by far the most commonly used reporter for characterizing device performance. The emitted fluorescence signal is a direct measurement of the protein and corresponding mRNA levels (97) and thus gene expression. Numerous variants of fluorescent proteins exhibiting different spectral properties are available (98); however, the majority of studies to-date utilize GFP and measure fluorescence through flow cytometry, a fluorescent plate reader or microscopy assays. Researchers often utilize fluorescent proteins coupled with flow cytometry assays to simultaneously quantify RNA device performance in single cells and across a cell population (99,100). More recently, assays that couple FACS with NGS (i.e. FACS-Seq) are being used as massively-parallel measurement strategies to simultaneously quantify the activities of up to millions of different RNA devices in a single experiment (101). In addition to improving our ability to generate new RNA devices, these data-rich strategies increase our understanding of RNA sequence-function-activity landscapes and may provide new opportunities for rational design.

However, one challenge in quantifying in vivo device performance via reporter proteins is the significant variation in absolute gene expression levels that may be observed due to differences in characterization methods, instrumentation and other environmental factors. Relative measures of gene expression have thus been proposed to improve the consistency of reported device performance. For instance, researchers have incorporated a normalizing expression construct into the RNA device characterization system and reported relative device activity (calculated as the ratio of gene expression levels measured from the RNA device construct to that from the normalizing construct) (102). The normalizing reporter (e.g. a fluorescent protein) served as an independent signal to provide a measure of noise in gene expression across the cell population (103).

**Qualitative features of RNA device architecture**

There are also qualitative features of device architecture and design that are important when implementing new device candidates into various systems. These 'qualitative' features relate to a synthetic biology-based design approach and consist of species portability and genetic modularity. Portability refers to the ability of a device to function in a species other than the one for which it was initially designed. Cross-species portability is dependent on the regulatory mechanism of the device and not on cellular machinery specific to a host. For example, the HHRz exhibits self-cleavage activity in various organisms, and RNA devices that incorporate ribozyme-based regulators have been demonstrated in organisms such as yeast and mammalian cells. Other regulatory mechanisms have also exhibited functionality across different species. One study demonstrated the activity of theophylline-responsive ribosome binding sequence-based RNA devices in four species of cyanobacteria (104), and another study used theophylline riboswitches from bacteria for translational regulation in chloroplasts (105). As re-
researchers begin to implement these genetic controllers in non-model systems, we expect a diversity of RNA devices to be characterized in more complex organisms, including plants.

Genetic modularity is a second qualitative attribute that refers to the ability of an RNA device to function independently of the target gene (i.e. result in the same dynamic range and activation ratio). Researchers have assessed genetic modularity by using different reporters to quantify the gene-regulatory activity. For example, researchers found the activity of a theophylline-responsive riboswitch to be similar for two different types of reporter genes (enzymatic and fluorescent) in mycobacteria (95). Ongoing work in the field may consider developing standards and benchmarks to measure qualitative features such as portability and genetic modularity.

Standards in RNA device characterization

Currently, there are no standards in place for reporting RNA device activities. Furthermore, the individual components themselves are poorly characterized (106) despite improvements for the sensor component (8,23,106). In contrast, quantitative descriptions of devices including comprehensive datasheets are widely used in other engineering disciplines (107). Recently, synthetic biologists have introduced the concept of standards in device characterization to more readily support the sharing and benchmarking of measured activities across laboratories. For instance, researchers proposed standardized descriptions regarding the compatibility of genetic devices with different genetic backgrounds, growth conditions or other genetic devices (107). The introduction of such measurement and reporting standards into RNA device design will facilitate comparison and implementation into new biological systems. In particular, having this information readily available can assist researchers in determining whether a particular device will meet the requirements of a system or application. However, as RNA devices are incorporated into increasingly complex genetic circuits, other parameters such as noise propagation, temporal response, evolutionary robustness, and device crosstalk will need to be examined, measured and potentially modified (5).

APPLICATIONS

As the examples of synthetic RNA devices continue to increase, so do the studies describing their roles as genetic controllers and sensors within different biological systems. The applications explored to-date for RNA devices are varied and here we highlight recent progress in the areas of molecular biosensors, metabolic pathway optimization and monitoring and programmed cellular behavior (Figure 3).

Quantification of in vivo metabolites and proteins

The most common application of RNA devices demonstrated to-date applies to analyte quantification. In general, numerous methods have been developed and implemented for measuring intracellular metabolites and proteins (108), and they have laid the foundation in our understanding of metabolic and signaling pathways. However, real-time and non-invasive methods that allow quantification at a single-cell level are required to obtain a better understanding of cellular processes. Researchers have been exploring the use of RNA devices as metabolite and protein sensors due to their relative simplicity and ability to translate molecular recognition to a change in protein expression.

Studies have implemented natural riboswitches for the non-invasive quantification of intracellular metabolites, including cyclic-di-GMP (66,109) and cyclic-di-AMP (110). These endeavors highlight the possibility of using synthetic RNA devices as genetically encoded sensors to probe biological transport and intracellular metabolite levels within living cells. In a recent example, protein-responsive synthetic miRNA devices regulating GFP were used to detect endogenous protein levels in mammalian cells. The RNA devices served as protein concentration detectors by non-invasively measuring changes in the nuclear concentration of β-catenin due to changes in the Wnt signaling pathway (78).

A recent breakthrough in RNA-based molecular quantification was highlighted by the development of the Spinach aptamer, which emits a green fluorescence upon formation of the RNA–fluorophore complex (termed Spinach) (111). Researchers have coupled the fluorescent properties of the Spinach aptamer to small-molecule RNA-based sensing elements to create standalone RNA devices that do not require control over fluorescent reporter gene expression. Spinach-based biosensors have been applied to image and dynamically monitor the binding of TPP, guanine and adenine in E. coli (112). Furthermore, newer iterations of the Spinach aptamer with improved and/or novel spectral properties have been developed (113–115), and they are expected to enhance current capabilities in RNA-based metabolite imaging and detection.

The studies to-date have focused on applying RNA devices to non-invasively quantify in vivo metabolites and proteins through the incorporation of natural RNA sensing elements. However, as improvements are made in implementing synthetic aptamers into RNA devices, our ability to sense a broader diversity of ligands within complex biological systems will continue to expand.

Tools for advancing metabolic pathway engineering

Recent advances in metabolic engineering have led to the production of a wide range of chemicals, and these processes typically require highly engineered metabolic pathways or organisms. Directed evolution techniques have been used to enhance pathway activities and product yields, but such approaches are generally limited by the throughput of the assays for the desired metabolite(s) or enzyme activities. By linking intracellular metabolite levels to changes in gene expression, RNA devices enable the development of high-throughput screening tools for library-based approaches to enzyme and pathway design.

Numerous metabolite-responsive RNA devices have now been applied to develop high-throughput screens or selections for metabolic pathway engineering. In an early study, researchers used a synthetic theophylline-responsive biosensor and a FACS-based screening approach to evolve
an enzyme capable of demethylating caffeine to theophylline in yeast. Large libraries of the caffeine demethylase were quantitatively screened for theophylline production within single cells, which enabled iterative identification of beneficial mutations that ultimately increased enzyme activity in vivo by 33 fold and product selectivity by 22 fold (116). More recently, a similar strategy was applied in Bacillus subtilis with a FMN-responsive RNA device to rapidly screen a genome library for strains displaying an elevated production efficiency of vitamin B2 (117).

Metabolite-responsive RNA devices can similarly be applied to develop high-throughput selections for enzyme activity. For example, the natural lysine riboswitch was used in a growth-based selection assay to enrich pathway-optimized E. coli strains to up to 75% of the total population (118). In another study, the natural glmS ribozyme that senses glucosamine 6-phosphate (GlcN6P) was incorporated into a synthetic suicide RNA construct in yeast to isolate a high producer strain for N-acetyl glucosamine (GlcNAc), a downstream product of GlcN6P (119).

A relatively untapped but exciting potential for RNA devices in metabolic pathway engineering is their application in dynamically controlling flux through a biosynthetic pathway. In a recent study, researchers used the native lysine riboswitch to control competing (but essential) metabolic by-pathways of lysine biosynthesis in response to changing L-lysine levels. Specifically, the lysine-responsive riboswitch was used to control the expression of citrate synthase (gltA)
and thus metabolic flux into the tricarboxylic acid cycle. This dynamic control strategy resulted in higher metabolic flux into the lysine synthesis pathway (120). The work to-date in the field highlights the flexibility and utility of RNA devices as generalizable tools for improving our capabilities in high-throughput screening applications (118). The bottleneck for this technology lies with the limited sensing diversity of our current toolset. Future work may focus on the development of aptamers to key metabolites associated with specific biosynthetic pathways of interest.

**Programming cellular behavior**

Advances in RNA synthetic biology are also improving our ability to design sophisticated genetic circuits capable of directing complex cellular behaviors in mammalian cells. To-date, RNA devices have been employed to program cell fate decisions, therapeutic activities and viral replication. In one study, ribozyme-based RNA devices were used to control cell signaling networks in yeast to conditionally direct cell fate (121). Researchers built RNA-based transducers incorporating theophylline- and tetracycline-responsive riboswitches to control the expression of negative and positive pathway regulator proteins, respectively. When integrated simultaneously within a genetic circuit, the RNA transducers conditionally routed cells to one of three fates—wild-type, mating or non-mating—in response to distinct environmental signals.

Researchers have also recently explored the use of synthetic RNA devices as programmable controllers in translational applications in health and medicine (122). Due to its small genetic footprint and non-immunogenic nature, RNA provides distinct advantages over protein counterparts in clinical applications. Synthetic RNA devices allow user-defined control and programming of therapeutic activity as an output in response to specific levels of an input marker. For example, a theophylline-responsive RNA device that was previously optimized in mammalian cells (123) was used to regulate the expression of genes transferred by adenovirus vectors and oncolytic adenoviruses (124). The insertion of an RNA device into viral vectors enabled dose-dependent and dynamic regulation of the transferred transgenes in several mammalian cell lines. In another study, theophylline-responsive ribozyme-based devices were engineered into an alphavirus-based replicon, and they mediated drug-responsive control of antigen expression and modulation of the type I interferon response (125). The system also enabled control over replication of the parental vaccine virus, hence demonstrating the feasibility of RNA-based controllers as viral vaccine safety-switches. These recent examples highlight the role of RNA devices in providing robust and titratable control over transgene expression and therapeutic activities, thereby permitting safer and more effective strategies for gene therapy.

**CONCLUSION**

The ongoing development of RNA synthetic biology has greatly improved our capacity to probe, interface with and control biology. There is significant potential and interest in using RNA devices as genetically-encoded sensors and controllers in diverse biological systems. However, current applications are limited by the availability of sensing components. Thus, the broader implementation of these genetic devices requires new strategies to support efficient generation of functional RNA-based sensing components. Given the rapid progress in high-throughput methods, data-rich assays and in silico design, we anticipate that the toolbox will continue to expand. One particularly exciting direction is the rapidly expanding integration of NGS into novel high-throughput measurement and screening strategies (49,64,88,114,126). These data-rich methods will permit rapid assessment of sequence-structure-function landscapes, thus improving our ability to construct new RNA devices with improved gene-regulatory activities. These methods will also provide the community with rich data sources to help increase our understanding of the relationships between RNA sequence and function. Finally, the adoption of measurement standards in RNA device characterization will have a profound impact to the larger research community, facilitating comparison, device implementation into diverse biological systems and the engineering of sophisticated applications.

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