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RNA modularity for synthetic biology

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Abstract

RNA molecules are highly modular components that can be used in a variety of contexts for building new metabolic, regulatory and genetic circuits in cells. The majority of synthetic RNA systems to date predominately rely on two-dimensional modularity. However, a better understanding and integration of three-dimensional RNA modularity at structural and functional levels is critical to the development of more complex, functional bio-systems and molecular machines for synthetic biology applications.

Introduction

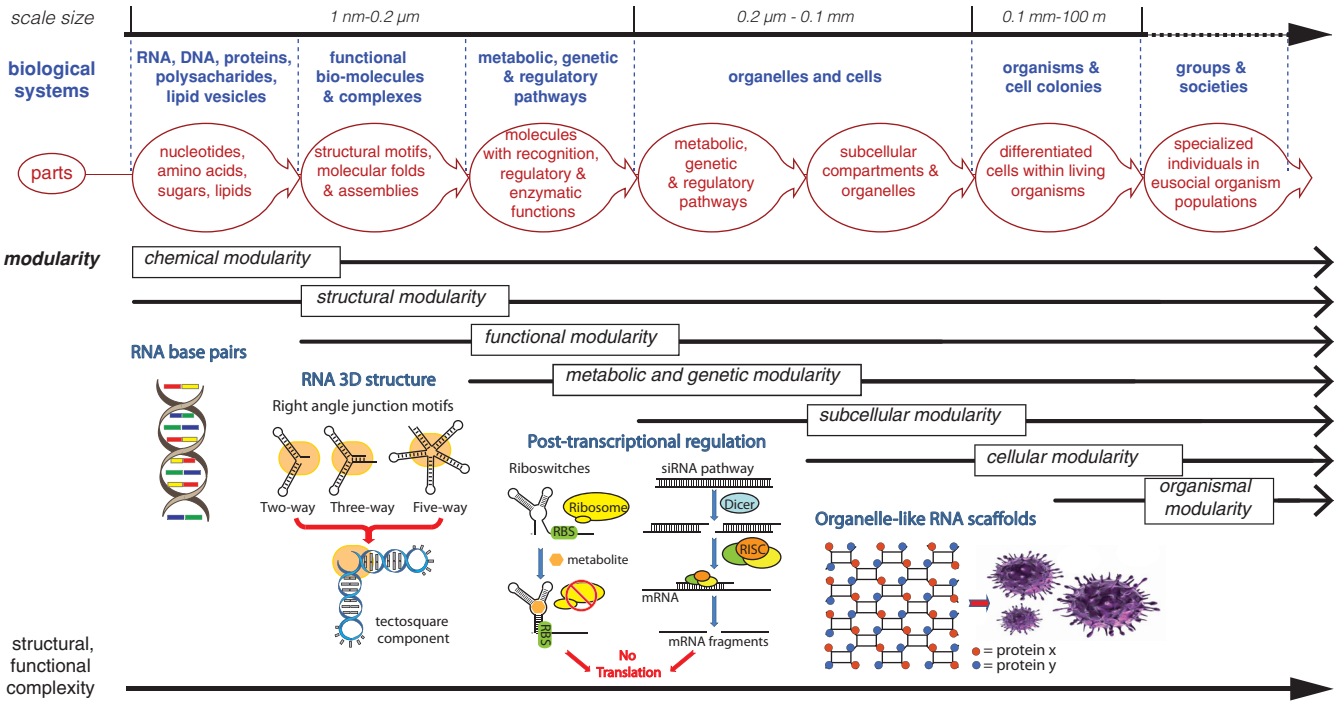
In the broadest sense, synthetic biology attempts to understand and mimic biological systems in order to provide novel biologically inspired solutions for a variety of challenges, such as medicine, energy production and product manufacturing. RNAs, such as short interfering RNAs (siRNAs), aptamers, riboswitches and ribozymes, hold significant promise as modular components for developing regulatory genetic circuits and other biological tools for many synthetic biology applications [1-10]. As exemplified by complex cellular machineries, like the ribosome [11-13], RNase P RNAs [14,15], group I and group II introns [16-19] and the spliceosome [20,21], RNA is a material of choice for building complex, functional nano-architectures [22,23]. The number of reviews highlighting the remarkable progress achieved in RNA synthetic biology over the past few years points to this [24-28]. However, when compared to the variety, and structural and functional complexity of natural systems, RNA synthetic biology still has a tremendous way to go.

A key structural attribute of RNA relates to its inherent ability to form diverse tertiary interactions through non-canonical base-pairings [29]. In this regard, the biologically relevant or active structure of an RNA molecule most often has three-dimensional implications. While significant gains have been made in RNA synthetic biology using nothing more than the knowledge of an RNA secondary structure as the primary determinant of activity *in vitro* [30-34], transition to the vastly more complex cellular context can still present unforeseen challenges for these same RNA moieties [35,36]. Some of this difficulty may stem from the construction of large artificial RNA-based systems and devices that lack a critical degree of structural and functional robustness. Thus, in our view, further developments in RNA synthetic biology complexity—including genetic regulatory elements, signaling devices, and molecular architectures—will depend on more focused efforts to understand and incorporate RNA structural principles at the tertiary level. Operating under this supposition, the following report is limited to more recent developments inspired by RNA nano-technology that offer opportunities to construct RNA devices or bio-systems containing significant increases in structural and functional complexity for use in RNA synthetic biology.

Structural and functional parts from naturally occurring RNAs

Similar to the other fundamental biomolecules, RNA is a hierarchical molecule containing multiple levels of modularity [37,38] (see Figure 1). The first and most basic layer of modularity at the chemical level concerns the four nucleotide building blocks themselves, which may be mixed and matched in any arrangement to form primary sequences. At the next level, the formation of regular

Figure 1. The multiple degrees of modularity in biological systems



As an example, RNAs are chemically, structurally and functionally modular. They can be integrated at the level of multiple metabolic, genetic and regulatory pathways that are themselves parts of subcellular components or cellular units. At a higher level of integration, RNA regulatory circuits are involved in the cellular modularity of multicellular organisms and in the developmental mechanisms leading to the specialization of individual organisms. Individual organisms can themselves be parts within colonies of eusocial species.

Watson-Crick helices that define RNA secondary (2D) structures constitutes the most basic form of structural modularity. The utility of 2D structure modularity has been demonstrated through the fusion of various types of RNA aptamers to other functional RNA elements—providing the regulatory RNAs (i.e. siRNAs and micro-RNAs [miRNAs]) with the ability to selectively target specific cell types or allosterically respond to environmental metabolites [39-41]. This type of modularity gives RNA synthetic biology a "plug-and-play" quality that facilitates the design of modular composite devices. This important capability allows different sequence modules to be swapped in and out to tailor the specific activities encoded in a particular device without needing to redesign the linkages between the modules of the device each time to maintain functional activity. Although presently less utilized in RNA synthetic biology, the same type of modularity is found at the tertiary level of RNA structures. It is at this highest level that structural modularity involves the three-dimensional (3D) nature of RNAs and their tertiary motifs.

Tertiary RNA motifs consist of highly conserved canonical and non-canonical hydrogen bonding patterns between semi-conserved nucleotides. The majority of tertiary motif information to date has come from the structural data of large naturally occurring RNAs, like the ribosome, group I intron, and RNase P. Characterization of recurrent hydrogen bonding patterns has led to the identification of a variety of recurrent structural motifs, including small submotifs (e.g. the U-turn [42], A-minor and GA-minor motifs [37,43-44], the UA_handle [38] and the ribose zipper [45]), terminal and internal loops [38,46-51], turns and junctions [37,38,52-59], long-range interactions [60-65] and pseudoknots [38,66-67]. A unifying characteristic of tertiary RNA motifs relates to their ability to also operate in a "plug-and-play" type fashion. Properly understood, RNA motifs can be swapped in and out of different sequence contexts and maintain their structural 3D identities [38,68].

Until recently, the use of tertiary RNA motifs (at least as far as it relates to synthetic biology) has been generally limited to the design of artificial RNA assemblies for the construction of RNA nano-structures, nano-particles (NPs) and/or scaffolds [5,6,43,68-73]. Because folded RNAs can be decomposed into smaller tertiary motifs, these structural building blocks can be used for

engineering artificial molecular units (tectoRNAs) able to self-assemble into large nano-structures [5,22,23,68]. This foundational approach, called RNA architectonics, was employed to generate *in vitro* (in the test tube) self-assembling RNA filaments (1D), RNA planar arrays (2D) and RNA polyhedral NPs (3D) with precise control and positioning of functional components in 3D space (e.g. [5,10,23,68,72-77]). Such work has demonstrated that tertiary motifs, forming thermodynamically stable and well-defined topologies, can be isolated and inserted into a variety of artificial structural contexts without compromise. By unraveling the sequence-structure relationship for RNA tertiary folds (e.g. [38,43,59]), the toolkit for rationally designing and constructing more complex and larger modular RNA assemblies is beginning to be established [78-80].

With respect to the more complex naturally occurring RNAs (i.e. the ribosome and RNase P), tertiary motifs are the core structural elements that facilitate folding and assembly as well as molecular recognition, and enzymatic and/or regulatory functions. In the case of smaller, less complex RNAs, the relationship between RNA structure and RNA function remains unchanged, in that structural modularity leads to functional modularity. For example, RNA takes advantage of a variety of structural contexts to regulate the expression of genes—ranging from riboswitches to small anti-sense RNA regulators (Figure 1) [27,28]. This ability is greatly facilitated by functional modularity. While different RNA folds can have different functions, structurally different RNAs can share identical or similar regulatory functions. The usage of different structural modules with identical functions as well as the mixing and matching of different RNA functions is a key component of natural RNA biology. This same principle will certainly facilitate the design of novel metabolic and genetic regulatory pathways by customizing them to particular structural, genomic contexts and organisms (Figure 1). Additionally, the interchangeability of different functional RNA parts could also be used to unravel and discover new principles of functional equivalence between apparently distinct cellular operations [81,82]. Therefore, rather than being limited to generating new divergent synthetic pathways in cells, we anticipate that synthetic biology will also contribute to the functional convergence and modularity of molecular circuits and metabolic pathways at the origin of the buildup of cells and organisms [81,82].

RNA parts from directed selection and evolution

The ability to generate novel RNAs with virtually any specific predetermined phenotype, using directed evolution and *in vitro* selection, has been instrumental in RNA nano-technology and RNA synthetic biology [83,84]. In this regard, SELEX (systematic evolution of ligands by exponential enrichment) has become the strategy of choice for the directed evolution and selection of RNA having novel binding and/or catalytic properties [85-89]. From a structural perspective, *in vitro* selection offers the possibility of creating new structural motifs, not selected for in natural systems, including novel long-range interactions (e.g. [65,77,90-91]). The possibility of selecting new RNA interactions and shapes significantly increases our ability to create more complex nano-structures and nano-machines. One consequence of its success relates to the relative ease associated with generating new phenotypes compared to the time and effort it takes to thoroughly ascertain the structural characteristics of each new aptamer selected [92,93]. The ultimate goal of directed evolution strategies is often, at times, more concerned with generating a specific desired phenotype from a pool of sequences of rather short sizes than it is with characterizing the resulting RNA's unique structural features.

Recently, Wittmann and Seuss [36] pointed out that, despite the number and diversity of RNA aptamers isolated to date [94,95], only a limited number of artificially selected aptamers have been successfully incorporated into useful riboswitch applications. They suggest that a majority of aptamers selected for *in vitro* lack the structural complexity necessary to function reliably *in vivo*. For example, when placed in the context of a regulatory element like a natural riboswitch, neomycin-binding aptamers screened *in vivo* have greater functional activity than those initially isolated *in vitro* [96,97]. Aptamers selected for *in vivo* regulation, like the tetracycline aptamer [36,98], tend to have increased structural complexity—allowing for larger conformational changes, higher binding-affinity with fast ligand binding and slow release, and greater thermal stability upon ligand binding [36]. It is possible that increased structural complexity may generate sequences that can support more interactions with the target ligand. This in turn could lead to higher binding affinity and/or increased binding specificity, which could explain the greater activity *in vivo* where ligand concentrations are likely to be more limited. Without knowing the precise cause, such findings, nevertheless, suggest that the selective pressures present *in vivo* give rise to aptamers with greater structural complexity and overall increased robustness, which may in turn allow them to work more effectively in these same conditions.

Structural studies on a variety of natural riboswitches suggest that long-range tertiary interactions are fundamental to their functional activity [99-103]. The important contributions that long-range interactions make in riboswitches (with respect to functional activity) are

reminiscent of past research involving the minimal hammerhead ribozyme. Over a decade of research based on the minimal hammerhead sequence provided inconclusive information on its active structure until the full-length ribozyme structure was discovered and its atomic structure solved, revealing a long-range interaction [104]. At first sight, isolation by *in vivo* SELEX of structurally complex aptamers might appear challenging but it is sure to offer greater potential for synthetic biology applications in comparison to many minimalist aptamers primarily selected for binding affinity *in vitro*. Furthermore, because evolution and selection often lead to the isolation of more than one RNA fold able to carry the same function, conducting selection experiments *in vivo* may increase the potential to produce multiple solution sets having the desired functional activity *in vivo*. Isolating multiple RNA solutions (each having functional modularity between the structurally distinct and unrelated 3D structures) promises to enhance the potential for building up more complex RNA molecules by providing additional structural choices among a specific type of function.

The inherently modular nature of RNA has spawned the study of RNA structures generated from completely random nucleotide sequences devoid of any selection pressures. These "never born RNAs" investigate the sequence/structure space associated with random RNA sequences not tied to selection pressures (whether natural or unnatural) [105,106]. In addition to highlighting how a particular structure can arise from many unrelated and different RNA sequences, "never born RNAs" could provide important insight into the ways in which the emergence of RNA structures are influenced by selection pressures—or the lack thereof. The use of structured "never born RNAs" as scaffolds could hold the promise of generating new devices with new or novel functionalities, but will most importantly contribute to unraveling the underlying sequence and structural constraints that are important in the selection and design of novel RNA functions for biological applications.

RNA parts with enhanced biomolecular interoperability for greater complexity

Future advancements in RNA synthetic biology will require greater interoperability between RNA and other types of materials. With respect to DNA and proteins, RNA offers distinct advantages. RNA is highly compatible with DNA in that it can form predictable base-pairings. In this regard, RNA can be used to form complex 2D circuits with itself and/or with DNA [107-109]. Furthermore, it can regulate and be naturally transcribed from DNA templates *in vivo*. Besides coding for proteins, RNA can co-operate with proteins to form ribonucleo-protein (RNP) complexes for regulation (i.e. in transcription and translation) and for building complex functional cellular machineries (i.e. the ribosome and RNase P).

As the technologies and methods for the selection of artificial RNA aptamers targeting proteins continue to advance [110], so do their applications. RNA aptamers targeting specific proteins have been used for applications including controlled localization of RNA [111], visualization of cellular RNA [112], and directing metabolic pathways through the use of engineered RNA scaffolds [7]. On the other hand, several RNP complexes have been designed to develop responsive genetic switches and reprogram cellular behavior [113-118]. The elucidation of the binding rules between RNA and Pumilio and FBF homology protein (PUF) [119-121] and pentatricopeptide repeats [122] offers interesting possibilities for the rational design of novel RNP constructs that can work in conjunction with one another [123].

Some natural non-coding RNAs, like DsrA, have the potential to form large RNA architectures within bacterial cells [124-126]. In the same manner, it was demonstrated that rationally designed RNA self-assembling nanostructures could promote the organization of intracellular reactions to produce an artificial hydrogen-producing pathway in bacteria (e.g. [7]). In view of these results and the potential of RNP complexes to generate self-assemblies [127], the development of novel intracellular RNP functions associated with subcellular self-assembling structures seems limitless for synthetic biology.

Chemically modified RNA parts and synthetic ligands

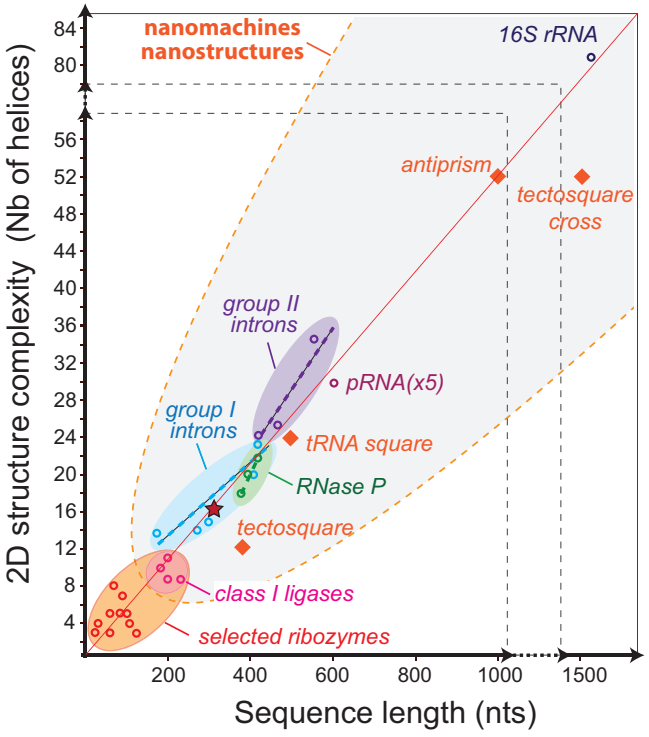
One of the areas where RNA synthetic biology has truly embraced its "synthetic" side is in the area of synthetic chemistry. Chemically modified RNA nucleotides and hybrids containing non-natural nucleic acids offer distinct possibilities in addition to their ability to increase an RNA's chemical and/or enzymatic stability. Non-natural nucleic acids (also referred to as XNA) have the potential to expand RNA's functional diversity as well as the ability to offer orthogonal systems capable of replication, heredity, and evolution [128-130]. As *in vitro* evolution and selection of novel functional XNAs has been recently demonstrated [129-131], XNAs open the door to the engineering of truly artificial living systems based on polymers other than DNA, RNA and proteins. Biocompatible XNAs containing reactive groups capable of undergoing enzyme-free ligation reactions [132] and nucleic acids containing photo-responsive moieties [133] are other examples of expanded functionality. Additionally, unmodified RNAs have been selected for their ability to bind synthetic small molecules or ligands in order to induce fluorescence signals [8,134,135].

As areas of RNA synthetic biology become more and more synthetic, structural and functional considerations of new synthetic parts will need to be continually re-investigated. For example, their effects on molecular recognition and assembly will determine the ways in which they can ensure seamless biocompatibility with existing cellular processes, such as self-assembly, regulation, and transcription processes.

The rise of nano-machines and RNA synthetic biology

The engineering of complex molecular nano-machines offers us the prospect of being able to modify, repair and/or control cellular operations for various therapeutic purposes. Their development may also increase the present toolkit of molecular biology and biochemistry for circularizing, modifying or synthesizing RNA and novel informational polymers. Recently, DNA self-assembly was used for engineering several mechanical devices, artificial nano-machines and assembly lines (e.g. [136-140]). However, these DNA nano-machines are still far from the remarkable efficiency and complexity of natural cellular machines, which essentially rely on RNA and proteins. Presently, one strategy for building nano-machines consists of deriving new functionalities from existing RNA machines like the ribosome. The creation of orthogonal ribosomes, operated by an expanded genetic code and using four codons per amino acid represents one of these seminal achievements for synthetic biology [141-143]. Other strategies take advantage of directed evolution and *in vitro* selection to isolate new complex artificial ribozymes from random RNA libraries (e.g. [144-146]), or modular RNA libraries that consist of a pre-existing functional domain to which random loops are appended (e.g. [85,147-151]). While a great deal of novel functional RNAs have been isolated by directed evolution and *in vitro* selection from combinatorial libraries of 30 nts to 200 nts (e.g. [85-87]), their structural and functional complexity are still far from that observed in nature (Figure 2). Presently, one of the most advanced nano-machines selected is a 200 nt RNA polymerase ribozyme (tC19z) with enhanced polymerase activity and fidelity with respect to previous class I ribozymes from which it was derived [150,152,153] (Figure 2). However, the resulting tC19z ribozyme is still partially template sequence-dependent and relatively slow (RNA polymerization reactions occur over several days [152]), in contrast to those catalyzed by RNA polymerase proteins that require only minutes to copy much longer templates. The question is whether nano-machines with the functional and structural complexity of large natural ribozymes can be developed in the laboratory. If they can, it will be a significant milestone paving the way to complex nano-factories with great potential for synthetic biology.

Figure 2. Structural complexity of natural and artificial ribozymes and RNA nano-structures in function of sequence length



A reasonable estimate of the two-dimensional (2D) structural complexity of a folded RNA is its number of constituent Watson-Crick helices. Note that most natural ribozymes and the 16S ribosomal RNA (rRNA) are aligned. *In vitro* selected ribozymes are circled in orange. Class I ligases are the most complex ribozymes originating from purely random sequences [146,150,152,153]. The red star indicates the most complex RNA ligase isolated by SELEX from a partially random library based on a natural structural scaffold [147,151]. Diamonds indicate modular nano-structures (reported in [68,72-73]).

As the functional complexity of natural molecular machines is proportional to their structural complexity (Figure 2), we hypothesize that the current knowledge in RNA nano-structure design will provide a foundation for building larger artificial nano-machines that approach the functional complexity of natural ones. Because of technical limitations associated with synthesizing large random RNA pools and RNA precipitation, functional parts isolated from purely random pools is limited to less than 200 nts regions (e.g. [144-146]). With the RNA architectonics approach, it is possible to engineer much larger RNA nano-structures or modular parts that can be used as scaffolds for pre-orienting and positioning random loops in 3D space, thereby allowing isolation of functional modules within large structural contexts that are not accessible from purely random pools (e.g. [147-150]. Thus, we anticipate that combining RNA

architectonics with directed evolution and *in vitro* selection would allow new modular ribozymes with structural complexity comparable to those of large ribozymes (i.e. group I and group II introns, RNase P) to be isolated. Additionally, selection pressure could be applied to select directional moving parts with functional modules to work in a concerted fashion to achieve a particular functional task. At the present time, the *de novo* development of complex nano-machines that operate in cells is exciting but essentially uncharted territory in synthetic biology.

Prospects and questions

Recent advances in RNA synthetic biology present fascinating possibilities but also raise a number of intriguing questions. For example, to what degree is structural complexity required for implementing complex cellular behaviors? It is true that rather simple molecules like miRNAs, siRNAs, or antisense RNA—having limited complexity at the part level—offer enough control to enable a great variety of different cellular behaviors. At the same time it is also clear, from the vantage point of both artificial and natural RNAs, that increased structural complexity has its advantages in certain cases. This seems to be particularly true when it comes to creating RNAs with interesting chemistry (as in the case of the ribosome) or selective RNAs having multiple functionalities (as in the case of riboswitches that require specific binding and allosteric regulatory properties).

The identification and characterization of large noncoding RNAs (lncRNAs) presents a new and emerging frontier in which to explore the correlation between structural and functional complexity further. Recent profiling of the secondary structure of some lncRNAs, on the order of several hundred nucleotides, suggests that they fold into complex highly ordered conformations [154-157]. While the degree to which lncRNAs form RNP complexes remains to be seen, at least three possible structural scenarios regarding their possible interactions have been put forth [158]. It would seem that whether lncRNAs exist as compact cores with largely peripheral protein binding sites, as relatively unstructured RNAs with loosely organized protein binding domains, or as RNAs lacking a central core but with ordered protein binding sites would be the result of very different and distinct folding and structural principles. For instance, compact 3D RNAs would most likely need to rely on and be optimized for long-range tertiary interactions while the other two would be optimized to avoid long-range tertiary interactions. Uncovering some of these fundamental characteristics regarding these types of structures could provide further insights into the design of synthetic RNP particles [71,123] and/or reveal unknown functionalities. Ultimately, such findings have the potential to advance our understanding of modern biology as well as provide new tools and strategies for RNA synthetic biology.

Abbreviations

lncRNA, large noncoding RNA; PUF, Pumilio and FBF homology protein; RNP, ribonucleo-protein; SELEX, systematic evolution of ligands by exponential enrichment; XNA, non-natural nucleic acid.

Disclosures

None.

Acknowledgments

Luc Jaeger wishes to dedicate this paper to Saint Thomas the Apostle, patron saint of architects and great missionary, and to Our Lady of Expectation.

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