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PERSPECTIVE

Twins, quadruplexes, and more: functional aspects of native and engineered RNA self-assembly *in vivo*

Richard A. Lease[a,*†], Véronique Arluison[b,c,d,e,*†] and Christophe Lavelle[e,f,g,h*]

[a]*Department of Chemical and Biomolecular Engineering, The Ohio State University, Columbus, Ohio, USA;* [b]*Université Paris Diderot-Paris 7, Sorbonne Paris Cité, Paris, France;* [c]*Laboratoire Jean Perrin CNRS FRE 3231, Paris, France;* [d]*Laboratoire Léon Brillouin, Commissariat à l'Energie Atomique, CNRS UMR 12, Gif-sur-Yvette, France;* [e]*CNRS GDR 3536, Paris, France;* [f]*National Museum of Natural History, 43 rue Cuvier, Paris 75005, France;* [g]*CNRS UMR 7196, Paris, France;* [h]*INSERM U 565, Paris, France*

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The primacy and power of RNA in governing many processes of life has begun to be more fully appreciated in both the discovery and inventive sciences. A variety of RNA interactions regulate gene expression, and structural self-assembly underlies many of these processes. The understanding sparked by these discoveries has inspired and informed the engineering of novel RNA structures, control elements, and genetic circuits in cells. Many of these engineered systems are built up fundamentally from RNA–RNA interactions, often combining modular, rational design with functional selection and screening. It is therefore useful to review the particular class of RNA-based regulatory mechanisms that rely on RNA self-assembly either through homomeric (self–self) or heteromeric (self–nonself) RNA–RNA interactions. Structures and sequence elements within individual RNAs create a basis for the pairing interactions, and in some instances can even lead to the formation of RNA polymers. Example systems of dimers, multimers, and polymers are reviewed in this article in the context of natural systems, wherein the function and impact of self-assemblies are understood. Following this, a brief overview is presented of specific engineered RNA self-assembly systems implemented *in vivo*, with lessons learned from both discovery and engineering approaches to RNA–RNA self-assembly.

Keywords: RNA structure; dimeric and oligomeric RNA; self-assembly; RNA engineering; noncoding RNA

Introduction

RNA self-assembly interactions constitute a vast array of interesting and useful phenomena in biology. Aside from questions of self-assembly by intramolecular RNA folding, wherein a given sequence forms its secondary and tertiary structure in *cis* (recently reviewed in Woodson (2010)), self-assembly broadly means intermolecular RNA–RNA interactions, i.e. two or more molecules of RNA bind to one another in *trans*. Here, binding is intrinsic and no catalyst is required, although crowding agents or molecular chaperones may facilitate these RNA–RNA interactions (Rajkowitsch et al. 2007; Kilburn et al. 2010). Self-assembly can involve homomeric interactions (self–self), i.e. formation of dimers or multimers of a single RNA, or heteromeric interactions (self–nonself), which create heterodimers and assemblies of disparate RNA molecules. One example of a heteromeric RNA–RNA assembly involves 'antisense' regulatory noncoding RNA (ncRNA), where an RNA sequence at least partially complementary to another RNA (e.g. sRNAs in bacteria or miRNAs in eukaryotes) governs mRNA stability, translation efficiency, or transcription attenuation processes by base-pairing with a corresponding mRNA 'target' (Figure 1).

Two RNAs that can form dimers or multimers might undergo formation of a stable pair or multimer if the resulting complex resides at lower free energy and if barriers for RNA folding and steric rearrangements are either absent or can be mediated by chaperones. That these RNAs may dimerize suggests that the entropy of the momomer interactions, which favors higher disorder (e.g. monomers over multimers and stem–loop self-annealing over complex formation), is outweighed by a favorable enthalpy of formation, i.e. that dimerization is enthalpy-driven. In addition, it may also be that some structures of self-assembly could be 'kinetic products', resulting from a lower energy of activation, rather than the thermodynamically most stable structure.

Within the category of homomeric intermolecular interactions, a variety of structural interactions may participate in the formation of bonds between two identical RNA molecules. A principal, though not exclusive, mean of RNA–RNA association is via complementary base-pairing.

*Corresponding authors. Email: lease.22@osu.edu; veronique.arluison@univ-paris-diderot.fr; lavelle@mnhn.fr
†Equivalent contribution.

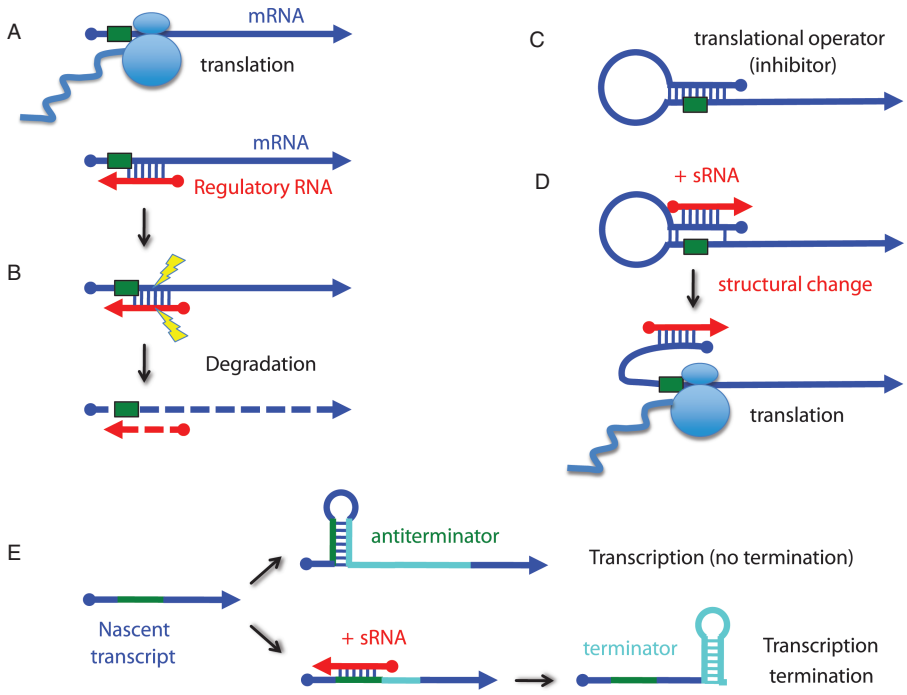

Figure 1. Heteromeric RNA–RNA assembly involving non-coding RNA. (A) Regulatory RNA:mRNA interaction affects translation by forming base-pairs with the Shine-Dalgarno ribosome-binding sequence (green box), thus occluding ribosome binding. (B) Base-pairing may also target both the mRNA and sRNA for degradation (stoichiometric turnover). (C) An mRNA that contains an intrinsic translational operator sequence in its 5′-untranslated region (5′-UTR) blocks the translation of mRNA. (D) In the presence of its cognate *trans*-antisense sRNA (+sRNA), the translational operator is paired to the sRNA, leading to a structural rearrangement of the 5′-UTR. The ribosome binding site becomes accessible to the ribosome and the mRNA is translated. (E) In the absence of a proterminator regulatory RNA, cotranscriptional folding blocks the formation of an intrinsic terminator and leads to transcription read-through (antitermination). Alternatively, interaction of a *trans*-proterminator regulatory RNA (below) with the nascent transcript leads to a structural rearrangement that favors the transcription terminator. Premature transcription termination results, and no protein is made. In these diagrams, RNA 5′-ends are denoted by a ball and 3′-ends are denoted by broad arrowheads. The mRNAs are in blue, except terminator sequences, which are in green and aqua, and the sRNAs are in red. Blue-shaded ovals symbolize translating ribosomes, with a 'tail' symbolizing protein synthesis.

Any RNA possessing stem–loop structures, for example, contains stretches of both a given sequence and its complement (see sequences P and P′ in Figure 2A). Even short stretches of natural RNAs are prone to contain stem–loops. If these sequences were to 'breathe,' melt, or misfold, and should the tertiary structure present the opportunity for access, intramolecular complementary base-pairs may then convert to intermolecular base-pairs with a complementary RNA sequence in another copy of the same RNA molecule (see sequences P and p′ as well as p and P′ in Figure 2B). Given the promiscuity of base-pairing in RNA (e.g. G·U, G·A, and other noncanonical base-pairings), the propensity to dimerize (Figure 2B) or multimerize (Figure 2C) may be innate to many RNAs, even if this capacity is not always functionally critical. Further, structures within individual RNAs may facilitate these pairing interactions, for example, loop–loop (Figure 2D) and loop–receptor (Figure 2E) interactions, wherein two single-stranded RNA sequences come together to form a helix structure. Intramolecular loop–receptor docking interactions (Figure 2F) could in principle facilitate the formation of intermolecular RNA–RNA interactions between RNAs either not fully folded or misfolded (Figure 2F–H), yielding RNA dimers or multimers. It is not presently known how commonly RNAs self-dimerize, or whether in most cases it is kinetically favorable. But in cases where multimerization contributes to function *in vivo*, there can be a solid basis for experimental study.

This review addresses the functional roles of specific dimer and multimer species in nucleic acid assemblies *in vivo*. It does not address the production of various engineered structures and devices that principally exist *in vitro*, materials (e.g. tectonics) or technical issues (e.g. RT-PCR), which are interesting but beyond the scope of this article (for recent reviews of nucleic acid objects *in vitro* see Bhatia et al. (2011) and Krishnan and Simmel (2011)). However, there is considerable and transformative applied work being done in the design of synthetic circuits, objects, and devices assembled from nucleic acids *in vivo*. Many designs are based on native nucleic acid molecules, on a *de novo* engineered basis, or on hybrids of the two. Accordingly, recent RNA and DNA nanotechnology systems that require or create RNA self-assembly as their basis of action *in vivo* are discussed in this review, including the potential merits and technical hurdles of building self-assembled objects *in vivo*.

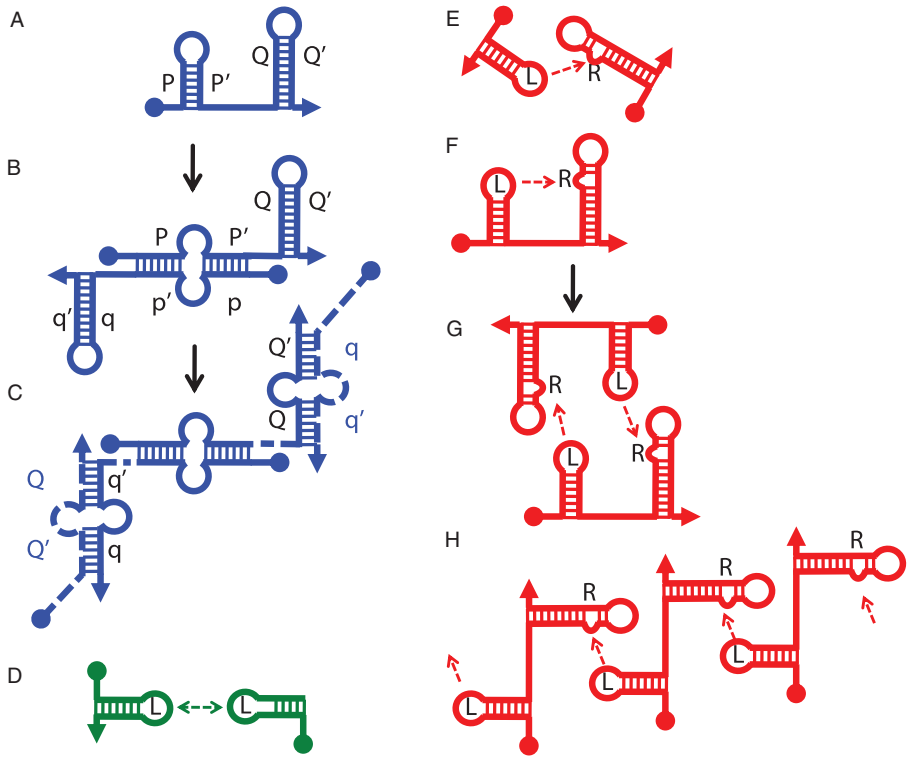

Figure 2. The basis of dimerization and multimerization by structured RNAs. The 5′-end of each RNA is labeled as a ball; the 3′-end is depicted as a broad arrowhead. (A–C) A stem–loop multimerization model is depicted in blue. (D) A loop–loop interaction involving Watson–Crick base-pairing between two complementary RNA loops is depicted in green. (E–H) A loop–receptor multimerization model, which usually involves non-Watson–Crick base-pairing, is depicted in red (Hansma et al. 2003). (A) Stem–loop structures are by definition largely self-complementary (here P–P′ and Q–Q′). (B) Dimerization occurs by replacement of intramolecular pairing (P–P′) with intermolecular base-pairing to an exact RNA copy (P–p′ and p–P′, where the second RNA complementary region is in lowercase). (C) Polymeric self-assembly of monomeric RNA is enabled by conversion of additional intramolecular pairing regions (Q–Q′) to intermolecular pairings (Q–q′ and q–Q′) with more copies of the same RNA (blue dashed lines). (D) For loop–loop interactions, the loop sequences must be complementary (see, e.g. Figure 3). (E–H) A scenario is depicted for loop–receptor interactions, wherein L–R indicates complementary loop–receptor interactions. In (E), the loop and receptor are on separate heterologous molecules. In (F), the RNA contains an internal loop–receptor interaction pair (red dashed arrow). In (G), a dimer forms by pairing (red dashed arrows) of loop and receptor between two copies of the same RNA. In (H), the RNA polymerizes via loop–receptor interactions.

Lessons and commonalities resulting from the study of naturally evolved systems of self-assembly that may apply to the design of synthetic systems are also considered.

Self-assembly of native RNAs

Versatile double helix interactions and dimerization of RNAs

Among RNA–RNA interactions, RNA dimerization plays an important role in diverse biological functions. RNA dimerization often involves the formation of a loop–loop or loop–receptor complex, which is then stabilized by the formation of an extended intermolecular duplex (Brunel et al. 2002). Different modes of accessory stabilization of RNA:RNA interactions are possible, for example, helix propagation, ribose zippers, formation of subsidiary canonical or noncanonical base-pairs, or tertiary interactions (Westhof et al. 2011).

There is a diversity of functional activity of RNA *in vivo*, and work in recent years has revealed an increasing number of RNA molecules that form dimers. These may also arise *in vitro* during transcription as well as from renaturation of RNA purified from sources *in vivo* or *in vitro*. One well-characterized example with impacts on human health is that certain tRNAs are able to self-assemble. A striking example involves human mitochondrial $tRNA^{Leu}$, where a mutation (A32-43G) induces the formation of a tRNA dimer due to the presence of a self-complementary dimerization motif in the D-stem of the tRNA mutant (Wittenhagen & Kelley 2002). One consequence of this self-assembly is a significantly reduced aminoacylation that may underlie human diseases (Finsterer 2007).

Retroviruses are another example of a human disease element that relies fundamentally on dimerization. Retroviral genomic RNAs dimerize *via* kissing loops, an interaction that governs essential steps in the viral life cycle (Paillart et al. 2004). The region responsible for the dimerization is

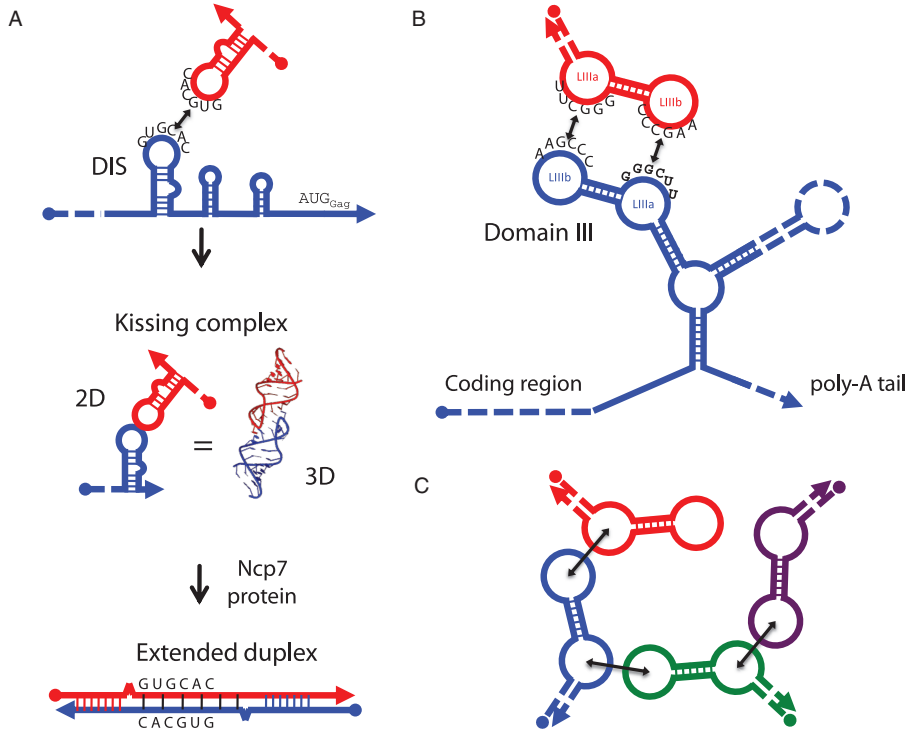

Figure 3. RNA dimerization. (A) HIV-1 RNA dimerization. This RNA dimerization process involves the dimerization initiation site (DIS) of two RNAs (Ennifar & Dumas 2006). The formation of a DIS loop–loop complex promotes dimerization, which is considered to be converted into a stable extended duplex in the presence of viral nucleocapsid protein Ncp7. The 3-D image was generated in Jmol software using PDB file 1XP7. (B) Self-assembly of *bcd* mRNA. The six-nucleotide complementary sequences in loops of domains III of *bcd* mRNA are shown. The RNA can form intermolecular loop–loop interactions leading to the formation of dimers or eventually (C) to multimers (Wagner et al. 2004). This process may be controlled by *bicoid* mRNA concentration and depends on the mRNA gradient in the embryo.

generally located in the 5′-untranslated region (5′-UTR) of the viral genomic RNA and is necessary for efficient replication and RNA packaging (Johnson & Telesnitsky 2010). One of the best-studied model systems for viral genomic RNA dimerization is the HIV dimerization initiation site (DIS) (Ennifar et al. 2001). The DIS adopts a structure with a loop consisting of nine nucleotides, of which six are self-complementary (Figure 3A). This allows the DIS to initiate dimerization by forming a loop–loop complex (*cf*. Figure 2D), which is then stabilized by the RNA chaperone activity of the nucleocapsid protein NCp7 to form a more compact and thermodynamically stable extended duplex (Figure 3A). Note that while the interaction between the loops of DIS is well-established (Figure 3A), formation of the extended duplex remains unclear and somewhat conjectural (Paillart, Marquet et al. 1996; Paillart, Skripkin et al. 1996; Ennifar et al. 2001). Mutations at the DIS site cause significant defects in replication and encapsidation, with diminished infectivity of virions. The necessity of dimerization to the viral replication cycle may be that genomic RNA has to circumvent translation in order to be partitioned correctly to the membrane of the cell for virion assembly. Moreover, dimerization enables these viruses to be genetically diploid. Because of the high error rate in HIV reverse transcription, along with viral recombination by template hopping, the progeny of a virus within a single cell generates viral genomic sequence diversity. Thus, dimerization doubles the ploidy of the viral genome and presents a genetic advantage as a hedge against the host immune system (Hill et al. 2012).

An additional example of an RNA that undergoes *in vivo* self-assembly is the *bicoid* (*bcd*) mRNA from *Drosophila*. In contrast to the 5′-UTR dimerization basis of HIV RNA, the ~900 nt structured regulatory element of *bcd* RNA is within the 3′-UTR and controls the production of a morphogen, the Bicoid protein. This mRNA is essential for early steps in organismal development: *bcd* mRNA localizes to the anterior pole of the *Drosophila* egg and its translation allows the formation of a morphogen gradient that governs appropriate head and thorax formation (Berleth et al. 1988). The *cis*-regulatory RNA element in the 3′UTR of the mRNA consists of four domains, among which the elements responsible for dimerization are localized in domain III (Figure 3B). This RNA dimerization domain contains two essential loops with self-complementary sequences of six nucleotides, which are phylogenetically conserved and can induce dimer assembly. Although *bcd* mRNA dimer formation proceeds via loop–loop interactions (Wagner et al. 2001), the initial, reversible complex is converted rapidly into a nearly irreversible one, and this conversion

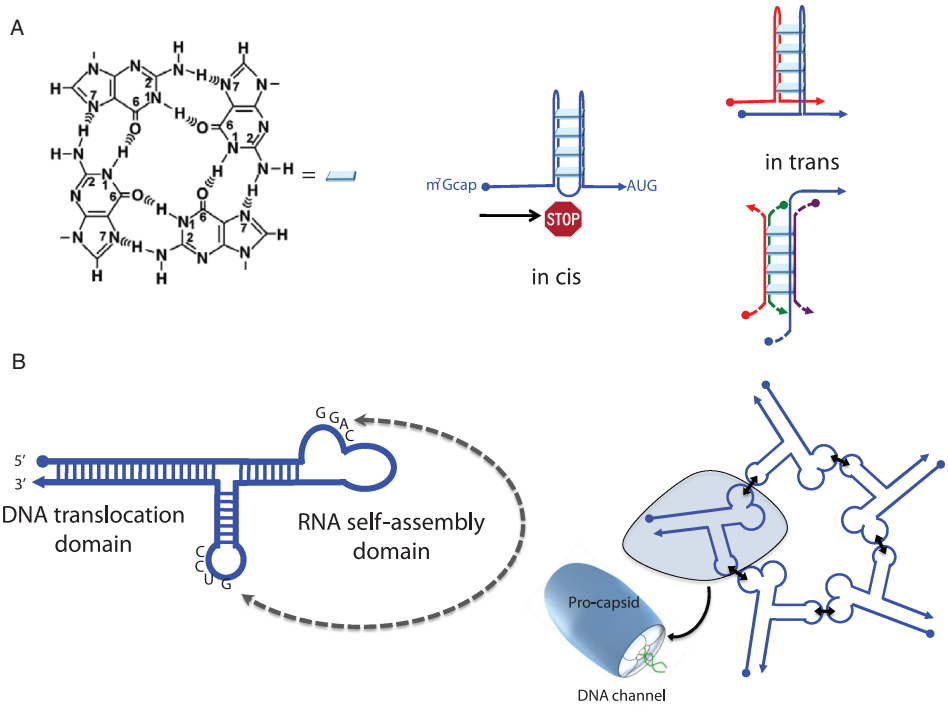

Figure 4. RNA multimerization. (A). Structure of G-quadruplexes and hypothetical functions for their roles in RNA UTRs. Presence of a stable RNA G-quadruplex may prevent translation by disrupting the scanning process toward the start codon or may cause RNA dimerization or tetramerization. Both parallel and antiparallel G-quadruplexes can form *in trans*. (B) Phage Φ29 pRNA self-assembly. pRNA is one component of the Φ29 DNA-packaging motor. Through the interaction between loops in adjacent RNAs, pRNA forms a multimeric ring-shaped structure. The pentameric or hexameric oligomerization state of pRNA in the prohead is still under debate. (G-quadruplex graphic image was contributed by R. Sinden.)

may involve sequences of RNA outside of domain III. Dimerization of *bcd* mRNA is important for its localization (MacDonald 1990), but the mechanism involved is still unclear. The possibility of assembly in higher-order oligomers exists (Figure 3C) and the concentration gradient of the *bcd* mRNA in the embryo may thus influence the order of self-assembly. RNA quaternary structure might additionally play a function in binding regulatory protein, such as Staufen (Ferrandon et al. 1997).

Beyond the basic double helix interactions: RNA polymers

In addition to the canonical (Watson–Crick) and noncanonical (e.g. Hoogsteen) pairing of two bases, the formation of planar four-membered rings of hydrogen-bonded bases also represents a self-assembly motif of RNAs (Halder & Hartig 2011; Millevoi et al. 2012). The G-quadruplex (also called a G-quartet) is a stable four-stranded secondary structure formed from tracts of three or more guanines separated by several bases, where rings of G-residues are held together by Hoogsteen-type hydrogen bond interactions (Figure 4A) (Simonsson 2001; Huppert & Balasubramanian 2005). A recent study demonstrated that DNA G-quadruplexes exist in mammalian cells *in vivo*, where the structures are a drug target (Rodriguez et al. 2012). While much is known about DNA G-quadruplexes, an interest in G-quadruplexes of RNA, particularly in the 5′-UTRs of mRNAs, has emerged recently (Huppert et al. 2008). Compared with DNA quadruplexes (e.g. in telomeres), the instances of native RNA quadruplexes appear to be relatively rare. Depending on the number and orientation of strands and the sequence content, G-quadruplexes can form intramolecular structures and can also represent a potential pattern of dimerization and tetramerization (Figure 4A, right) (Kim et al. 1991). Functions for RNA G-quadruplexes have been suggested in translational repression (Figure 4A) (Wieland & Hartig 2009; Bugaut & Balasubramanian 2012). Moreover, RNA quadruplexes may form during the dimerization of retroviral genomes (Yu et al. 2007) or respond to changes in temperature, thus operating as an RNA thermometer (Wieland & Hartig 2007). One specific aspect of RNA quadruplexes relative to DNA quadruplexes is that RNA can uniquely present additional U-tetrads at the ends of a G-quartet, forming an extended motif that greatly stabilizes the quartet structure (Xu et al. 2010).

One example of a naturally occurring oligomeric RNA that adopts a circular topology is a bacteriophage structural RNA called pRNA. During replication, the bacteriophage linear DNA genome is translocated into the procapsid, an energy-intensive process that utilizes ATP. In some phages, for example the *Bacillus subtilis* Φ29, an RNA molecule, together with an ATPase and a connector protein (Lee &

Guo 2006) form an essential RNA structural element of the DNA-packaging motor (Figure 4B). This bacteriophage prohead RNA (pRNA) is composed of two conserved secondary structural domains (Figure 4B), although only the first domain is essential for DNA packaging (~120 nt). While pRNA is able to form dimers and trimers *in vitro*, either five or six copies of pRNA assemble into a ring-shaped structure in the phage prohead (Figure 4B, right) (Xiao et al. 2008; Ding et al. 2011; Shu et al. 2011; Ye et al. 2012). Interestingly, in the case of both Φ29 and HIV, RNA self-assembly is required before packaging of genomes. This self-assembly step may incorporate a built-in metric of the cell's environment for viral reproduction, and thus a gauge of the external environment, and makes sense inasmuch as there may be an advantage to avoid packaging genomes if the threshold RNA concentration is not high enough.

Another apparent function of RNA self-assembly is to enact a quality control screen of RNA structure (Guantes et al. 2012). In recent work, it was demonstrated for the first time that bacterial small regulatory RNAs (sRNAs) may form polymers in the bacterial cell (Busi et al. 2009; Cayrol et al. 2009) (Figure 5A). Bacterial sRNAs are used by bacteria as environmental response elements and are commonly transcribed under cellular environmental stress (for a recent review, see Storz et al. (2011)). Many native prokaryotic sRNAs depend on the binding activity of the bacterial Sm-protein called Hfq, which has been called an RNA chaperone inasmuch as Hfq facilitates RNA pairing (reviewed in Brennan and Link (2007) and Vogel and Luisi (2011)).

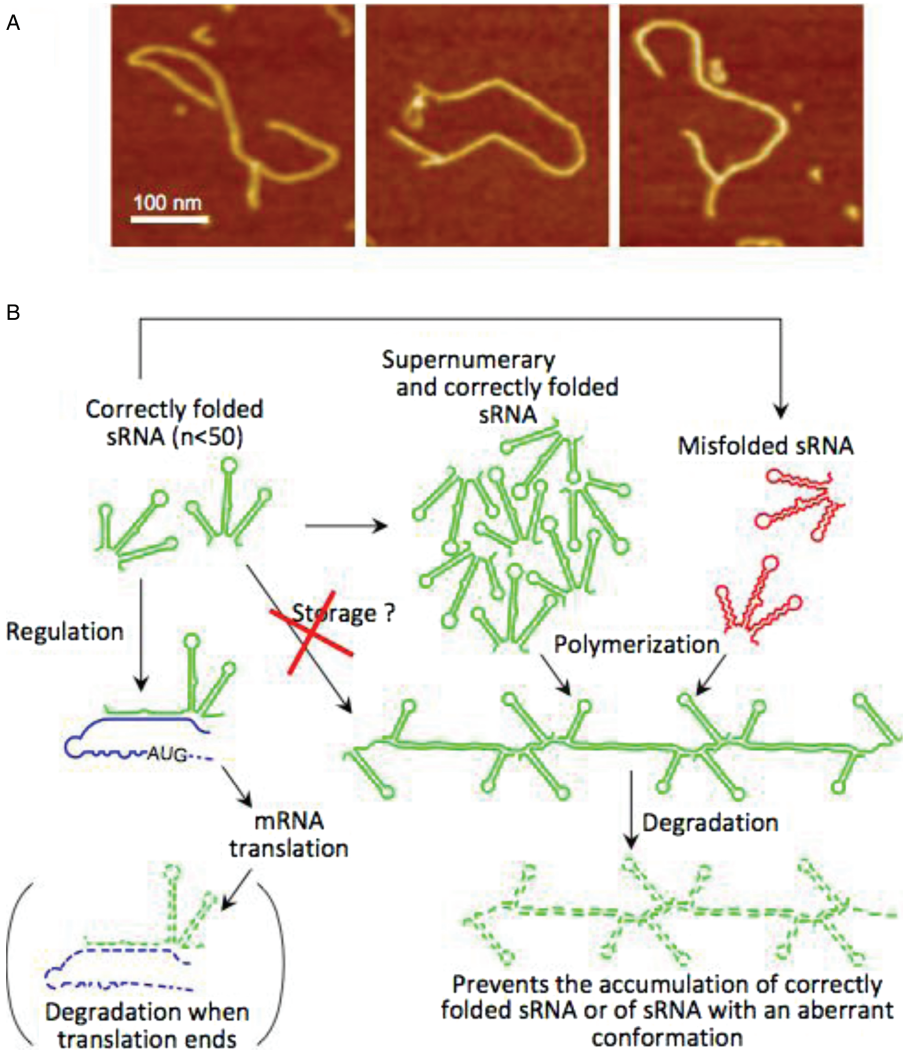

Figure 5. Polymerization of sRNA into long filaments. (A) Atomic force microscopy visualization of nanostructures formed by DsrA transcripts, an abundant 87nt long *Escherichia coli* ncRNA (O. Pietremont, IGR, Villejuif, personal communication). (B) Schematic drawing illustrating how RNA self-assembly could target supernumerary or misfolded small noncoding RNAs for degradation. As DsrA polymers are degraded much faster than monomers, the formation of DsrA polymers cannot be a storage form of the sRNA, but plausibly either regulates the sRNA concentration *in vivo* or helps to eliminate misfolded RNAs.

Hfq facilitates sRNA–mRNA heteromeric self-assembly, and although the mechanism is still incompletely understood, it melts secondary structures in bound RNAs and promotes strand displacement (Hwang et al. 2011). A complete discussion of Hfq involvement in sRNA function is beyond the scope of this review and is discussed elsewhere (Storz et al. 2011; Vogel & Luisi 2011). It is perhaps worth mentioning here that Hfq is not known to enhance sRNA self-dimerization.

Two different small regulatory RNAs (sRNAs) of *Escherichia coli*, the 87-nt DsrA and the 206-nt GcvB sRNA, each of which control the translation and the turnover of important bacterial mRNAs, have shown striking auto-assembly properties as assessed with various techniques (Busi et al. 2009; Cayrol et al. 2009) (Figure 5A). Because DsrA polymers are degraded much faster by RNase E than are monomers *in vitro* (Cayrol et al. 2009), the formation of DsrA polymers could plausibly regulate the sRNA concentration *in vivo*: supernumerary sRNAs could be deleterious to the cell because the polymers could bind and titrate cellular Hfq (Véronique Arluison and Christophe Lavelle, unpublished result), causing a disruption via the sRNA network (Hussein & Lim 2011). The DsrA cellular concentration is consistent with DsrA multimerization *in vivo*, given the intrinsic self-binding affinity (K_d) and the absolute quantification data (as RNA copies per cell (Guantes et al. 2012)). However, the low steady-state levels of DsrA found *in vivo* (<25/cell) suggest that long polymers should not be present *in vivo* and that only short oligomers may form. Taken together, it seems unlikely that self-assembly plays a major function for DsrA-based regulation *in vivo*. Rather, the likelihood is that misfolded sRNAs formed during transcription, instead of an excess of sRNA, are the origin of self-assembly. RNAs can adopt misfolded conformations (Woodson 2010) and misshapen nonfunctional RNA would be harmful for the cell. For example, sRNAs that cannot form correct ternary complexes with target mRNAs and Hfq could also titrate cellular Hfq and create a pleiotropic dominant network effect (Adamson & Lim 2011). RNA can thus self-assemble, as individual RNAs expose complementary sequences that would ordinarily be hidden in the correctly folded RNA and can be more rapidly degraded in the absence of any mRNA partner other than the sRNA itself (Figure 5B). Note that the sRNA polymer formation cannot be reversed by Hfq chaperone activity (Cayrol et al. 2009).

Applications of RNA self-assembly

RNA and synthetic biology

We will now discuss those RNAs that have been engineered in the laboratory and *in silico* for production and function *in vivo*. There has been an explosion of interest in the use of nucleic acids in synthetic biology. In one sense, synthetic biology has been with us since the dawn of recombinant DNA methods (Szybalski & Skalka 1978), but clearly there has been a sea change (Endy 2005). In the same way that molecular biology was invigorated by an influx of physicists after the discovery of the structure of DNA, there has been an increased involvement of engineers in designing synthetic biology applications. This is the result of several factors, including a combination of technological advances (low-cost oligonucleotide synthesis and PCR; low-cost and broadly applied genomic DNA sequencing) and the development of novel methods (e.g. the Gibson synthesis for the assembly of genomes (Gibson et al. 2009) and high-throughput RNAseq). This shift has come at a time of increased appreciation of the multitude of roles played by RNA in both prokaryotes and eukaryotes. The capacity to design and deliver RNA sequences *in vivo* presents an attractive series of opportunities for those interested in cellular and molecular biological engineering and is the subject of several reviews (Isaacs et al. 2006; Ellington 2007; Win & Smolke 2007; Liu & Arkin 2010).

Applications of RNA as dimers

A major goal of synthetic biology is to design or evolve robust genetic regulatory elements or circuits (reviewed in Sprinzak and Elowitz (2005), Dougherty and Arnold (2009), and Randall et al. (2011)). The pairing of small, ncRNA and mRNA is a heteromeric dimer interaction that is favored by mass action as the concentrations of either species increases. The small regulatory ncRNAs, such as prokaryotic sRNAs and eukaryotic miRNAs, present practical benefits as modular circuit elements owing to several factors (Lucks et al. 2008). Among the advantages of ncRNA are their capacity to target mRNAs by base-pairing with sufficiently high specificity that orthogonality of the interaction is likely. By orthogonality we mean that two similar but distinct regulatory ncRNAs may act on similar but distinct targets, yet avoid cross-activation. Further, as negative regulators, the *trans*-acting ncRNAs that act noncatalytically (i.e. stoichiometrically) (Masse et al. 2003) suppress mRNA transcript fluctuations occurring from 'noisy' transcriptional bursts and exhibit a threshold-linear dosage–response curve (Levine & Hwa 2008). This kind of response is advantageous from an engineering or rational design perspective because linear scaling of the mRNA levels and activity can be achieved with control of the small RNA concentration, above a threshold to be determined by transcript ratios, ncRNA–mRNA affinity, and the rate of ncRNA–mRNA degradation (reviewed in Levine and Hwa (2008); also see Mitarai et al. (2009) and Hussein and Lim (2012)). *Ab initio* simulations have predicted that the regulation of mRNA by sRNAs would present certain advantages and disadvantages relative to the use of protein-based transcription control (Shimoni et al. 2007; Mehta et al. 2008; Hussein & Lim 2012). Although sRNA

regulation of mRNAs can introduce significant intrinsic noise, particularly near a 1:1 sRNA-to-mRNA ratio, the sRNAs may offer advantages over transcription factors in state-switching, via their fast kinetics of onset and delay of deregulation (or fast return of deregulation; see Guantes et al. (2012) and Hussein and Lim (2012)). Indeed, experimental work with specific sRNAs verifies that there are parameters of sRNA and mRNA copy number and mutual turnover that can filter or mitigate the predicted sources of intrinsic noise in signal transduction (Mitarai et al. 2009). In principle, and to some extent in practice, the activity of the ncRNA against the translation or degradation of the mRNA can therefore be modulated.

Ab initio design specification of RNA–RNA interactions does present certain challenges, however. The prediction and design of RNA structure and function, like that of protein structure, is a major goal of computational biology (Beisel & Smolke 2009; Laing & Schlick 2011). *In silico* design of bacterial RNA-based regulation in *cis* has recently advanced considerably, for example, both the prediction of translation initiation rates and the design of mRNA 5′-UTR structures with a desired initiation rate have been facilitated by a thorough statistical mechanical consideration of the free energies of ribosomal RNA binding modes (Salis 2011). Further, by using a rational framework of biochemical rate constants and RNA folding models, systematic design of genetic regulation and expression via properly spaced ribozymes and aptamers has also been achieved in the 5′-UTRs of bacterial transcripts (Carothers et al. 2011). It is noteworthy that cotranscriptional, kinetic RNA folding models (Isambert 2009), rather than thermodynamic folding calculations, were required to obtain the predicted RNA device behaviors.

A computational approach to developing *trans*-acting RNAs that control transcription termination and antitermination has shown promising results using *in vitro* assays, but has not been implemented *in vivo* (Dawid et al. 2009). Efforts are underway to automate the design of *trans*-activating (positive) regulatory RNA networks (Rodrigo et al. 2012). Computational and mutagenic analysis of sRNA–mRNA interactions with the *E. coli* sRNA RhyB suggests that the strength of target repression correlates with the free energy of the sRNA:mRNA target duplex (Hao et al. 2011). However, not all ncRNA:mRNA interactions can be specified *ab initio*. The assumption that base-pairing is the principal or the only component of the free energy of complex formation may assume dsRNA helices or omit the contribution of diverse RNA structural elements. As an example, helical or loop stacking free energies (i.e. from two helices or loops stacking coaxially on each other or on a third strand) may play a role in the optimal targeting of an mRNA by a small RNA. Further, the differential free energies that are predicted are in many cases based on predicted structures, frequently in the absence of experimental secondary and tertiary structure data. Thus, prediction and design of customized ncRNAs solely via computational means remains somewhat incomplete, and yet seems tantalizingly within reach. A complementary approach that also utilizes native ncRNA structural elements as a basis and that combines rational modular design with combinatorial and/or randomized libraries together with a functional selection or screen has proven extremely fruitful (discussed below).

Several groups have focused on the bacterial sRNAs as modular, orthogonal circuit elements for mRNA translational/turnover control, transcriptional control, or both. The work of the Collins group includes engineering of the 5′-UTRs of mRNAs coupled with specific, synthetic *trans*-acting sRNAs that enhance translation (Isaacs et al. 2004; Callura et al. 2010). This design is based on the topology of the *E. coli* native *rpoS* (sigma-S) mRNA, wherein an ‘anti-antisense’ translational operator (Figure 1C and D) sequesters the ribosome binding site for translation and is activated by structural rearrangement driven by sRNA pairing (reviewed in Repoila et al. (2003)). A variety of translational operator mRNA variants were designed and functionally screened against multiple *trans*-acting engineered sRNAs. One potential shortcoming of this scheme is that the mRNA targets must also be altered in this engineered system, which may perturb their intrinsic stability in a cellular milieu. An advantage of the synthetic *trans*-antisense scheme is a decrease in ‘leaky’ protein expression due to the translational operator sequestering the ribosome binding site (Callura et al. 2010). This synthetic *trans*-antisense RNA system has been used to demonstrate metabolic engineering applications that alter metabolomic flux with orthogonal control (Callura et al. 2012), with the synthetic *trans*-sRNA and *cis*-mRNA under control of the same promoter. The strong dynamic range of this system also makes it very useful for perturbation studies of cells by controllably producing conditionally essential or toxic proteins.

A body of recent work from the Arkin group has described engineered circuits based on repurposed–natural sRNAs. In one system, synthetic sRNAs were developed from the pT181 plasmid regulatory antisense-sRNA of *Staphylococcus aureus* (Kumar & Novick 1985; Brantl & Wagner 2002), a riboregulator that acts by a transcription termination mechanism in *trans* (Figure 1E). The high specificity of the RNA–RNA loop–loop interactions permits orthogonality of control (Lucks et al. 2011), although it was seen that to attain sufficient specificity for orthogonality, extensive mutagenesis of the wild-type sequence ‘platform’ was required. The use of these circuit elements in tandem acts as an amplifier (of a low dynamic range for a single regulator) or as a basis of integration (to create logical gates). Similarly adapting a native sRNA mechanism, translational control by the IS10 transposon RNA-IN/RNA-OUT sRNAs was utilized in a large optimization study that combined rational design using thermodynamic calculations with a genetic screen in an iterative design framework (Mutalik et al. 2012). This work generated a large number of translational regulatory RNAs with orthogonal specificity.

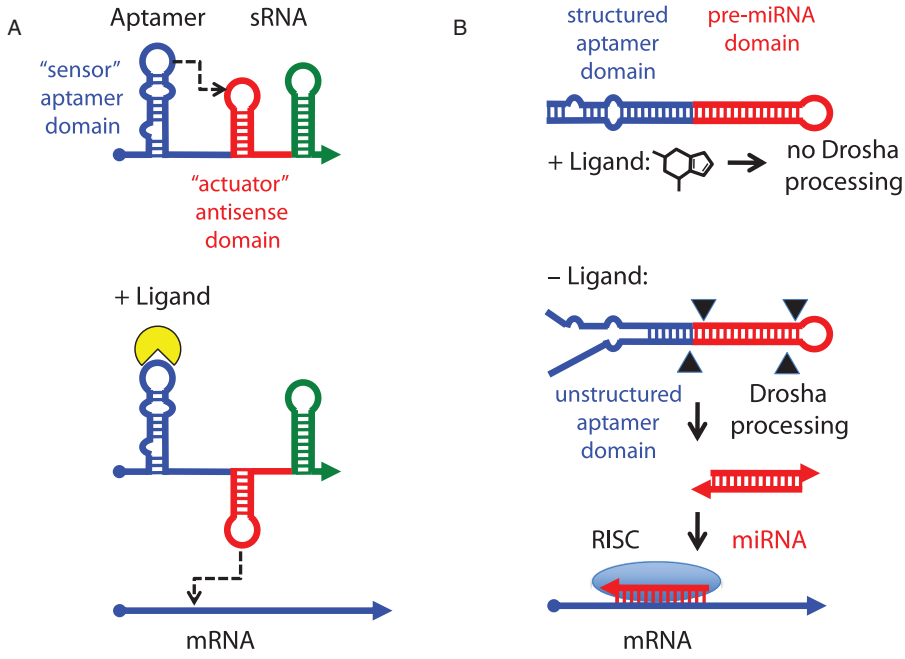

Figure 6. Hybrid sensor–actuator component design of RNA transduces RNA binding by ligands or proteins into regulatory outcomes. (A) A small RNA sensor–actuator binds either a small ligand or MS2 RNA-binding protein to activate regulation. (B) An engineered ligand-binding aptamer domain stabilizes pre-miRNA structures and blocks Drosha processing that produces the miRNA. Removal of the ligand restores the activity by creating an unstructured precursor recognized by Drosha that can be processed into a RISC complex for RNA interference. RNA 5′-ends are denoted by a ball and 3′-ends are denoted by broad arrowheads. The sensor components are in blue and actuators are in red.

Strikingly, the conserved native YUNR sequence motif, an element of the wild-type basis RNA–RNA interaction, was frequently lost during the selection/optimization of RNA–RNA interactions. A recent study from the Aiba group defined the antisense sequence structural constraints of the sRNA SgrS and redesigned mutant SgrS to bind and regulate several native transcripts as novel targets (Ishikawa et al. 2012). Studies suggest that regions of sRNAs may function as structural or sequence modules to target mRNAs (Lease & Belfort 2000; Papenfort et al. 2010).

Another modular RNA engineering approach utilizes an RNA aptamer or riboswitch as a sensor element, integrated together with an RNA genetic regulator into a chimeric riboswitch–regulator element (Figure 6A). The integration of sensor/aptamer and antisense output modules in engineered sRNAs was pioneered by the Smolke lab, working in eukaryotes (Bayer & Smolke 2005) with synthetic sensor regulators that integrated aptamers and antisense RNA regulatory sequence domains. These 'antiswitch' small RNAs block translation by binding near the 5′-cap regions of mRNA targets and can be turned either on or off by ligand binding, depending on the antiswitch configuration. Using the prokaryotic systems, the Arkin group similarly applied aptamers/riboswitches in tandem with either the transcriptional or translational sRNA control elements, mentioned above, to create a ligand-inducible or MS2-protein–inducible mRNA control element (Figure 6A) (Qi et al. 2012), again using a mix of rational design and screens of mutant library variants. In several ways, this is an extension of generalized sensor–actuator hybrid motifs designed as regulatory modulators of gene expression in eukaryotes, for example engineered introns, ribozymes, protein titration elements, or antisense RNA sequences (reviewed in Win et al. (2009) and Wittmann and Suess (2012)) but utilizes the specific prokaryotic biological function of these sRNA 'actuator' domains.

Along related lines, an understanding of the structural basis of miRNA precursor processing by Drosha protein was applied in engineering a riboswitch-responsive precursor miRNA (Beisel et al. 2010). Drosha protein RNA processing of a pre-miRNA was blocked by stabilizing an unstructured RNA loop outside of the miRNA–mRNA targeting antisense region via engineered aptamer ligand binding (Figure 6B). In the absence of a ligand, the miRNA was processed normally and created an miRNA–mRNA heterodimer via RISC (Figure 6B, bottom). In the presence of micromolar concentrations of a specific ligand, correct miRNA processing was reduced, miRNA production was decreased, and negative regulation of the mRNA was reversed. As a modular antisense regulator, the miRNA could be retargeted by altering the antisense sequences; further, exchanging the aptamer component with different ligand-binding aptamers met with qualified success depending on the aptamer structure. A related strategy was used to design a ligand-responsive platform technology that modulates the regulatory activity of RNAi. The latter study

included a fine-tuning design component guided by mathematical simulations as well as combinatorial mutation and assays (Beisel et al. 2008). Increasing the level of informational integration, a 'Boolean classifier' was built that integrates logical input from five exogenous RNAi signals (Rinaudo et al. 2007) or six endogenous miRNA signals (Xie et al. 2011) to precisely control the synthesis of a protein. In the latter case, this synthetic logic analyzer was used to analyze and 'classify' a cancer cell miRNA profile and then induce cancer cell apoptosis.

A completely different methodology is worth mentioning as it avoids structural design concerns by expressing sRNA sequences generated from a cloned, randomized library pool, with screens for growth phenotypes (Komasa et al. 2011). This approach to sRNA engineering is the exact antithesis of the *ab initio* rational design methodology and is akin to the selection of natural sequences over evolutionary time. Clearly, the constraints in this methodology shift almost entirely from structural concerns to the development of appropriate selections and screens for the desired phenotype. It will be interesting to see what structural motifs arise from further use of this method. A more conservative approach has been to use native sRNAs as a 'scaffold' with randomization in focused sRNA regions, together with selection of these partially randomized sRNA libraries to target specific mRNA leaders (Sharma et al. 2011). A strong caveat of this approach is that sRNAs can target other mRNAs either directly or indirectly, and the remaining conserved 'scaffold' may possess intrinsic antisense activity. Therefore, global validation of mRNA target orthogonality is crucial.

Engineered RNAs as multimers and polymers

Several laboratories have built novel systems of self-assembling nucleic acid multimers and polymers *in vivo* that are derivatives or mimics of natural systems. As an example, for many years the goal of DNA nanotechnology has advanced by building geometric objects *in vitro*, partially to take advantage of the specificity and predictability of DNA in creating useful or interesting shapes, but with a long-term goal of building devices. A variety of these systems and objects have been designed (for a review, see Seeman (2007)), including hybrid RNA/DNA assemblies (Ko et al. 2010). However, limitations of producing specified ssDNA within cells (Lin et al. 2008) has rather encouraged the use of transcribed RNA as a building material *in vivo*.

If a single transcript sequence is used as a 'tile' to self-assemble as a multimer (e.g. panels C and H in Figure 2), the object may scale in size with the amount of the 'tile' produced. An outstanding example of a human-designed object that takes advantage of RNA dimer and multimer self-assembly is an 'RNA scaffold' that directs the spatial organization of two tethered proteins (ferredoxin + hydrogenase) in a hydrogen-producing biochemical pathway *in vivo* (Delebecque et al. 2011). These two proteins were partitioned on the RNA–polymer scaffold to determined, periodic RNA structures at specific locations in the repeating scaffold subunit by fusion of each enzyme in the biochemical pathway with a specific RNA-binding protein domain (MS2 or PP7). The RNA elements were carefully designed according to formalized principles of helix nucleation and propagation (Yin et al. 2008) and utilized both dimerization and multimerization interfaces in order to favor the propagation of scaffold self-assembly, while avoiding collapsed-state folding traps (Thirumalai & Hyeon 2005; Woodson 2010). The resulting scaffolds can take the shape of sheets or nanotubes. Thus, an understanding of the potential RNA dimer- and multimerization outcomes, as well as the fundamentals of RNA folding, was used to guide the design of the subunits for self-assembly and subsequently tiled into superstructural RNA–protein objects for improved generation of hydrogen *in vivo*.

Going forward, it may be useful to consider the design of synthetic nucleic acid objects (that have been built *in vitro* as proofs-in-principle) for production *in vivo*. We should inquire, then, what advantage in cost, scale, function, or efficiency results from producing RNA or DNA nanotech objects *in vivo*? Does molecular crowding, or the capacity of cells to encode nucleases or ligases, either permit or facilitate assembly *in vivo*? Are single transcripts sufficient *in vivo* to produce spatially finite, structured and/or functional objects? In several well-known cases, structural folding of DNA objects *in vitro* needs to be directed or stabilized by additional smaller, sequence-specific oligonucleotides, for example, tiled objects and molecular origami shapes (Shih et al. 2004; Rothemund 2006). The assembly of 2-D and 3-D objects from volumetric pixels or 'voxels' of DNA (Ke et al. 2012; Wei et al. 2012) or the creation of 2-D and 3-D tiles of RNA built from multiple strands (Chworos et al. 2004; Afonin et al. 2010) should be considered as a basis for the construction *in vivo* of finite-lattice functional scaffolds. For both the 2-D and 3-D molecular tiles, the requisite *in vivo* production of many individual, unique small strands presents a technical challenge. It is encouraging that in the cases of self-assembling 2-D or 3-D molecular tiles, the stoichiometry of parts need not be precisely controlled so long as mass action favors self-assembly. To produce these objects *in vivo* from, for example, RNA would presumably require either coordinated production from multiple promoters and/or engineered nuclease-sensitive sites to separate from a longer polymer the 'voxel' or 'staple' strands that can then bind each other or a specified 'scaffold'. In this regard, perhaps future designers of complex self-assembled objects and devices *in vivo* will look to the ribosomal RNAs and tRNAs for inspiration, as these native assemblies are processed from longer precursors and ultimately bind proteins in a functional framework. Certain ribosomal and other RNA structural elements have already been appropriated for the design of objects *in vitro* (Chworos et al. 2004; Afonin et al. 2010).

Designing RNA self-assembly: practical issues and caveats

In creating RNA-based regulatory systems, it continues to be essential to consider potential roles of dimerization, for example in both the design and experimental analysis of synthetic riboregulators and their testable (output) function. Extensive self-similarity in stem–loops may give rise to dimers or multimers, as has been seen with native bacterial sRNAs (Busi et al. 2009; Cayrol et al. 2009), and the formation of such self-assemblies may be favored at higher RNA concentrations (Figure 5). Accordingly, the level of subunit RNA expression should be optimized, not necessarily maximized. False-negative results could occur from RNA overproduction that sequesters the RNA in an inactive state, that yields a lower-than-anticipated effective RNA concentration, or that creates Hfq network-titration effects (Adamson & Lim 2011). It is important to regard gel analyses with care, as a fraction of sRNA, for example, can multimerize and form large networks that may not enter the gel. This is especially problematic in native gels but also in denaturing or partially denaturing conditions, when the RNAs have been forcibly denatured and renatured at high concentration prior to fractionation or when there is free magnesium present (Uhlenbeck 1995; Lease & Woodson 2004; Cayrol et al. 2009).

When considering the use of a native sRNA as the basis of sequence modification for an engineering platform, it must be further kept in mind that sRNAs can target multiple mRNAs or even a single mRNA in multiple locations (Papenfort & Vogel 2009; Corcoran et al. 2012). Thus, here and in other instances, it is advisable both to consider well-characterized systems for further modification as well as to validate chosen systems and their variants for regulatory side-effects in the cell.

Other nucleic acid systems of self-assembly *in vivo*: RNA–DNA

The various RNA self-assembly mechanisms discussed in this focused review already provide evidence of important regulatory roles of this molecule and may provide productive avenues for engineering new RNA self-assembly technologies in the future. Also, due consideration of the versatile interactions of RNA with DNA, as for instance RNA/DNA hybrid associations and self-assembly by dimer and multimer formation, will open even broader perspectives on countless key roles in cell metabolism. Indeed, RNA/DNA assembly is thought to occur during various and ubiquitous events such as transcription elongation (Aguilera & Garcia-Muse 2012), class switch recombination (Mizuta et al. 2005), and the control of chromatin structure (Magistri et al. 2012). There is little doubt that further examples, perhaps fundamental ones, will be found as both pure and applied research continues in these important areas.

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