Methods for identifying and characterizing interactions involving RNA

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1. Introduction

1.1. Why target RNA?

Modern organic chemistry enables practitioners of the art to controllably synthesize an enormous array of structures with diverse molecular architectures, functional group representation, and physical properties. In addition to creating novel materials, sensor platforms and even new genomes, organic synthesis is most often performed to prepare naturally produced molecules (natural products), or molecules not found in nature, that have useful or potentially useful medicinal and therapeutic properties. Most notably over the course of the last two decades, labs with expertise in chemical synthesis have expanded their repertoire to include basic drug discovery efforts. Since the overwhelming majority of existing small molecule drugs target small molecule-binding pockets found in disease-related proteins, it is unsurprising that these drug discovery efforts have primarily focused on identifying small molecules (\(<1\, \text{kDa}\)) that bind disease-related proteins and modify their biochemical activity. Protein-targeted therapeutic discovery has generated a significant number of small molecule drugs and lead compounds.\(^2\)\(^,\)\(^3\) However, the increasing challenge of identifying new small molecules that regulate protein function requires an expansion of discovery efforts to other targets.\(^4\)\(^\text{–}^6\) The development of new therapeutic modalities and drug delivery platforms that expand the scope of therapies to currently ‘undruggable’ regions of the proteome is a growing area in modern chemical biology and biomedical engineering, and will almost certainly generate entirely new sectors within the global pharmaceutical industry. However, expanding the therapeutically accessible space of the proteome will likely require multiple fundamentally new technologies, which will undoubtedly take a significant amount of time to develop and integrate into the marketplace.

An alternative approach to therapeutic development, which does not necessarily require the development of fundamentally new technologies, is the application of small molecule therapeutics to disease-related nucleic acid targets. Relative to RNA, genomic DNA structure is generally uniform,\(^7\)\(^,\)\(^8\) limiting the specificity with which traditional small molecules bind DNA over a relatively short sequence. While the design of short-polymeric molecules that bind DNA in a sequence-selective manner represents a seminal achievement in molecular recognition and bioorganic chemistry,\(^9\) and these DNA-targeting short polymers may represent one potential next-generation therapy, the relatively homogeneous nature of DNA structure makes it very difficult to target selectively using traditional small molecules. In contrast, the diversity and complexity of RNA folding generate molecular structures with well-defined pockets that can bind a small molecule with high affinity and specificity.\(^7\) Numerous natural products bind RNA with good sequence-selectivity and affinity,\(^10\)\(^,\)\(^11\) and naturally occurring small molecule–RNA-binding interactions are commonly used in Nature to regulate the biochemistry of those RNAs.\(^12\)

Like many proteins, the biochemical function or expression level of RNAs has been directly linked to various diseases.\(^13\) While \(\sim 75\%\) of the proteome has been declared currently undruggable using small molecules,\(^14\)\(^\text{–}^18\) RNA is a largely untapped therapeutic target and RNA structure may be generally accessible to small molecule binding and small molecule-dependent regulation of function. Since mRNAs encode proteins, sequence-selective, small molecule-dependent regulation of mRNA translation is an obvious method to regulate the expression of a particular disease-related protein.

Largely due to the expanding and important role of RNA biochemistry in disease, the last decade has been witness to an explosion of research focused on the development of RNA-targeting therapeutics in both academic and industrial laboratories. Perhaps the greatest barrier to begin a drug discovery effort and identify a ‘hit’ is the lack of information and/or knowledge about how to begin screening compounds, and subsequently characterize identified interactions. The development and broad use of various spectroscopic methods during the 20th century drastically advanced the practice of organic synthesis. Similarly, various technologies have made it possible to examine properties of large numbers of compounds, identify a ‘hit’, and characterize the identified interaction. Taken together, this report is intended to serve as a general reference to equip chemists and biochemists with a ‘toolbox’ of methods to identify small molecule and biopolymer ligands for RNA, and regulators of RNA function. In addition, methods for characterizing an identified interaction are described. Obvious omissions from this report include the use of mass spectrometry and footprinting experiments to identify and characterize interactions involving RNA. Very good and thorough reviews on these methods have been published, and interested readers are advised to read those references.\(^17\)\(^,\)\(^18\) Throughout this report, the reader will be introduced to a number of RNAs with unique or disease-related biochemistry, as well as various classes of RNA-binding small molecules. Our discussion of RNA targets and RNA-binding molecules will not be exhaustive; however, extensive reviews on that topic are available.\(^19\)\(^\text{–}^21\) In addition, a recent and very good book on studying nucleic acid/drug interactions is available.\(^22\)

1.2. The ‘challenges’ associated with handling RNA

All biopolymers (DNA, RNA, peptides, and proteins) are susceptible to environmental and enzymatic degradation. Unlike most DNA and proteins, which are generally stable in near neutral pH buffered solutions, RNA can be unstable and generally cannot be kept for extended periods of time (\(\sim 1\) to \(3\) months) at \(>+4^\circ C\) or \(\lesssim -20^\circ C\). In addition, ribonuclease (RNAse) catalyzes the degradation of RNA. Unfortunately, RNAse contamination in laboratories can be common if sterile technique practices are not followed. Numerous simple protocols for sterile technique are available,\(^23\)\(^,\)\(^24\) and should be consulted before working with RNA. In addition, RNAse free water and various reagents to control RNAse contamination are available commercially. Following simple protocols when handling RNA should assure the integrity of the RNA for a period of time required to conduct a significant number of screening and characterization experiments.

2. Fluorescence-based methods

2.1. Fluorescence-based methods

Fluorescence-based methods are sensitive, quantitative, easily applied to high-throughput screening, and relatively inexpensive to perform. There have been various innovative applications of fluorescence-based approaches applied to the identification and characterization of small molecule–RNA-binding interactions.\(^25\)\(^,\)\(^26\) In addition, most of the fluorescence-based techniques reviewed in this report can be performed using a commercially available fluorescence spectrophotometer or fluorescence multi-well plate reader. Both of these instruments are popular items in central instrument facilities and independent research labs.

Unsurprisingly, the simplest fluorescence-based method to identify and/or characterize a small molecule–RNA-binding interaction involves using a molecule with inherent fluorescence. In such a case, the researcher monitors a concentration-dependent change in small molecule fluorescence as a result of increased levels of target RNA.\(^22\) This situation is ideal, as it minimizes the complexity of the analysis, and obviates the need for a fluorescent probe, which might alter or abrogate a binding interaction. If the small molecule ligand does not have appreciable fluorescence, a fluorescent tag must be added through chemical or enzymatic
synthesis.\textsuperscript{28,29} Appending a fluorescent tag to a large number of small molecules is both labor intensive and costly. Moreover, this requires that each molecule contains the same reactive group. Therefore, fluorescent tagging of small molecules is typically limited to focused libraries or a small number of ‘hits’ identified from a larger screen. Most commonly, a tag is selectively incorporated into the RNA target and small molecule-dependent changes in tagged RNA fluorescence is monitored. While numerous fluorescent modifications for RNA (and DNA) are available, and numerous labs have made contributions to this field,\textsuperscript{30–33} in the interest of space, the focus of the discussion will be limited to the use of 2-aminopurine as a sequence-selective fluorescent tag for RNA.

2.2. 2-Aminopurine tagging

Binding interactions between a molecule and RNA target can be measured by incorporating a fluorescent base proximal to the proposed binding site and measuring small molecule concentration-dependent changes in that fluorescence.\textsuperscript{34,35} Fluorescent tags can be sequence-specifically incorporated through chemical synthesis methods, or through the use of in vitro transcription using a labeled RNA primer.\textsuperscript{36,37} Modified nucleotides with inherent fluorescence, or a functional group amenable for orthogonal tagging, are commercially available. An obvious concern with tagging the target RNA is that a modification will alter or abrogate a binding interaction, which would result in a decrease in the number of ‘hits’ identified in a screen. Therefore, minimal changes to the target RNA are ideal. 2-Aminopurine (Fig. 1) is a commercially available\textsuperscript{38} 2’-deoxy nucleotide analog that has inherent fluorescence ($A_{\text{max}}$ 303 nm; $E_{\text{max}}$ 371 nm). This modified nucleotide has a relatively modest structural change from the canonical nucleotides and can substitute for adenine or guanine. 2-Aminopurine fluorescence is sensitive to the local environment, making it a useful probe for monitoring the structure and dynamics of nucleotide structure and binding interactions.\textsuperscript{39} Since significant changes in the fluorescence of 2-aminopurine result due to changes in the local environment (including small molecule binding), information on the general location of the binding site, or of the targeted site within the target RNA should be known. Once the targeted binding pocket is identified, it is relatively simple to synthesize (or order) a target RNA with a 2-aminopurine that is proximal to the targeted binding site. Unsurprisingly, a number of labs have used 2-aminopurine to identify and characterize binding interactions involving a RNA of interest.\textsuperscript{40–44}

![Fig. 1. 2-Aminopurine.](image)

A potential RNA target associated with HIV-1 replication is the RNA Rev responsive element (RRE), which is a part of the env gene contained within the HIV-1 RNA genome.\textsuperscript{45,46} Rev is a virally encoded sequence-specific RNA-binding protein responsible for transporting essential viral pre-mRNAs from the nucleus of the host cell to the cytoplasm for translation. Binding between Rev and RRE is required for HIV-1 to mature from a latent infection stage to active replication within the host cell. Small molecules capable of interrupting the interaction between Rev and RRE may have significant promise as HIV-1 therapeutics.\textsuperscript{47,48}

Marino and co-workers synthesized a truncated form of RRE RNA and incorporated 2-aminopurine into the purine rich stem-loop, which is the binding site of Rev.\textsuperscript{49} Incorporating 2-aminopurine within the purine rich stem-loop did not alter the binding interactions between Rev and RRE. Using this modified RNA, Rev binding was easily observed by monitoring small molecule-dependent perturbations in 2-aminopurine fluorescence. The screen was conducted using a fluorescence spectrophotometer to monitor changes in 2-aminopurine fluorescence emission at 371 nm upon addition of a molecule into tagged-RRE RNA. Changes in tagged RNA fluorescence corresponding to a single-site binding model was measured using Eq. 1, where $F_0$ and $F_1$ are the initial and final fluorescence intensities, respectively, $[\text{RRE}]_{\text{tot}}$ is the total RRE concentration,

\[
F = -\left( \frac{F_0 - F_1}{2[R\text{RE}]_{\text{tot}}} \right) \times \left[ b - \sqrt{b^2-4[L]_{\text{tot}}[R\text{RE}]_{\text{tot}}} \right] + F_0
\]

and $[L]_{\text{tot}}$ is the total ligand concentration. They selected one molecule from this screen, profavin, which bound to RRE with a dissociation constant ($K_d$) of 0.11 $\mu$M (Fig. 2). No change in 2-aminopurine fluorescence was observed when the modified RRE–profavin complex was treated with Rev, indicating that Rev was unable to bind RRE in the presence of profavin.

![Fig. 2. Sample polycationic aromatic heterocycles screened for potential RRE binding (A) acridine and (B) profavin.](image)

2.3. Ethidium bromide displacement

Sequence-specific tagging methods, such as 2-aminopurine tagging, typically require some knowledge about the structure of the targeted RNA-binding site. For large RNAs, the fluorescent probe(s) must be incorporated relatively close to the targeted binding site in order to observe binding. At the same time, the fluorescent probe must be placed in a position that does not abrogate RNA function and/or a binding interaction. In contrast, fluorescent probes that non-selectively bind multiple positions within the targeted RNA structure can be used to assess binding when you do not have information on the targeted binding site, but simply want to identify molecules that bind the RNA of interest.\textsuperscript{50,51} Once the targeted RNA is pre-complexed with a fluorescent molecule, libraries can be tested for an ability to bind the target RNA and either alter the fluorescent molecule–RNA interaction or displace the indicator entirely. Either event changes the fluorescent properties of the indicator, which suggests a binding interaction between the library member and RNA target. Ethidium bromide is an inexpensive and commonly used non-selective RNA and DNA intercalator (Fig. 3). Solvation by water quenches ethidium bromide fluorescence. Therefore, binding between ethidium bromide and the hydrophobic environment of a nucleic acid results in an increase in ethidium bromide fluorescence. Conversely, if a small molecule displaces ethidium bromide from RNA, a decrease in ethidium bromide fluorescence would be expected and this change in fluorescence can be measured to identify and characterize binding interactions between a small molecule and RNA (Fig. 4).
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Fig. 3. Ethidium bromide.

Fig. 4. Ethidium bromide displacement. Small molecule binding displaces the fluorescent intercalator ethidium bromide leading to solvation by water and concomitant decrease in ethidium bromide fluorescence.

Inspired by the molecular architecture present in naturally occurring compounds such as neocarzinostatin and synthetic analogs, which bind bulged RNA secondary structures, Hergenrother and Meyer synthesized and screened a targeted library of analogs, which bind bulged RNA secondary structures, Hergenrother and Meyer synthesized and screened a targeted library of ‘wedge-shaped’ small molecules.52 A series of sequence-defined RNA structures with sequence variation in the bulge were first pre-complexed with ethidium bromide then treated with small molecule library members. Binding was measured by a small molecule-dependent reduction in the fluorescence of ethidium bromide. Molecules identified in this screen were then evaluated for concentration-dependent activity and these data were fit to a single-site binding model to identify the dissociation constant of the interaction using Eq. 2, where y is the change in fluorescence relative to control, [L] is the aqueous ligand concentration and a is the asymptotic limit. If a change in fluorescence due to addition of a particular small molecule was sequence-selective, it could be inferred that the binding was sequence-selective. Like many fluorescence-based in vitro screens, this method is amenable to high- and moderate-throughput screening and can be performed on most commercially available fluorescence spectrophotometers and fluorescence plate readers. While this method does not provide any information on where binding occurs within a particular RNA sequence.

\[
y = \frac{a[L]/K_d + [L]}{a} \]

2.4. Fluorescence anisotropy

When a fluorophore is excited by a photon, an electron is promoted to an excited state. When this electron relaxes to its ground state through radiative decay, energy is emitted in the form of a photon and some residual heat. For most fluorophores, the timescale for excitation and emission is typically in the nanosecond range.53,54 Low molecular weight fluorophores typically undergo some significant rotation in solution within this excitation/emission timescale. Therefore, since the time it takes for a low molecular weight fluorophore to significantly rotate in solution is approximately the same as the timescale for excitation/emission, the emitted photon is not polarized. In contrast, if a small molecule fluorophore is bound to a macromolecule, such as RNA, the rotational timescale of the complex is increased.55,56 Thus, if the RNA-bound fluorophore is excited by polarized light, the rotational timescale is slower than the excitation/emission timescale and the emitted photon retains the polarity, or most of the polarity of the light source. A cartoon depicting the concept of fluorescence anisotropy is shown in Fig. 5. Since observing a change in anisotropy relies on a significant change in molecular weight, the fluorophore is typically conjugated to the small molecule and not the macromolecule (RNA target).

As stated previously, labeling a large molecule library with a fluorophore is laborious and costly. Thus displacement-based fluorescence anisotropy assays are commonly employed.57,58 In this approach a known ligand is tagged with a fluorophore and pre-incubated with the target RNA. Small molecules are then screened for their ability to displace the fluorescent ligand from the target RNA. Since displacement of the known ligand from the ligand–RNA complex results in a significant decrease in molecular weight (the weight of the RNA target), a large change in anisotropy results, which can be easily measured (summarized in Fig. 6).

Tor and co-workers used fluorescence anisotropy and ligand displacement to measure the affinity of guanidinoglycosides for RNA.59 A peptide consisting of the amino acids present in Rev34–50 was conjugated to fluorescein (Rev-Fl) and pre-incubated with a 67 nucleotide subunit of RRE RNA, thereby generating a Rev34–50–RRE RNA complex. The Kd of this known interaction can be measured by plotting the change in anisotropy of the known ligand versus concentration of the target RNA, to saturation, using Eq. 3, where A is the anisotropy of Rev-Fl, A0 is the

Fig. 5. Fluorescence anisotropy. The fluorophore tag on a small molecule will emit depolarized fluorescence signal due to the high rate of Brownian tumbling. Binding between the small molecule and an RNA target slows down this tumbling, leading to a polarized fluorescence emission signal.

Fig. 6. Application of fluorescence anisotropy to characterize RNA-binding small molecules. Displacement of the bulky fluorescent tagged ligand from the RNA increases the depolarization of fluorescence emission.

Tor and co-workers used fluorescence anisotropy and ligand displacement to measure the affinity of guanidinoglycosides for RNA.59 A peptide consisting of the amino acids present in Rev34–50 was conjugated to fluorescein (Rev-Fl) and pre-incubated with a 67 nucleotide subunit of RRE RNA, thereby generating a Rev34–50–RRE RNA complex. The Kd of this known interaction can be measured by plotting the change in anisotropy of the known ligand versus concentration of the target RNA, to saturation, using Eq. 3, where A is the anisotropy of Rev-Fl, A0 is the
anisotropy of Rev-Fl in the absence of RNA and \( \Delta A \) is the total change in anisotropy at saturation of Rev-Fl. The Rev-Fl–RNA complex was treated with a focused library of guanidoglycosides and affinity was indicated by displacement of fluorescein–Rev bound to RNA. Since the RRE-bound fluorescent Rev peptide has a slower Brownian tumbling motion relative to the free peptide, displacement of the fluorescent Rev peptide results in a decrease in anisotropy. When a change in anisotropy is measured to saturation over various small molecule concentrations, half maximal inhibitory concentration (IC\(_{50}\)) values can be measured using Eq. 4.

\[
A = A_0 + \Delta A \frac{([\text{RNA}]_{\text{total}} + [\text{Rev} - \text{Fl}]_{\text{total}} + K_d) - \sqrt{([\text{RNA}]_{\text{total}} + [\text{Rev} - \text{Fl}]_{\text{total}} + K_d)^2 - 4[\text{RNA}]_{\text{total}}[\text{Rev} - \text{Fl}]_{\text{total}}}}{2[\text{Rev} - \text{Fl}]_{\text{total}}}
\]  

(3)

2.5. Methods to measure changes in RNA structure and reactivity

There are numerous examples of RNA restructuring or RNA cleavage events that generate a change in RNA biochemistry. For example, riboswitches are mRNAs that change structure in a small molecule-dependent manner, resulting in a change in ribosomal translation of that mRNA. In addition, RNA cleavage by certain proteins is involved in newly identified RNA biochemistry, including RNA interference. Ribozymes are RNAs that catalyze the cleavage of another RNA or their own RNA sequence. These RNAs often have interesting biochemistry and therefore researchers are generally interested in identifying small molecule regulators of RNA structure and reactivity. The ability to measure changes in RNA structure or RNA cleavage is critical to understanding these processes, as well as identifying new molecules that control the activity of these biochemically important RNAs. Fluorescence-based techniques have been used to study RNA structural changes and cleavage. Most often, these approaches involve measuring the distance between two molecules present on the RNA. Changes in the spatial relationship between those two molecules can result in changes in a fluorescent signal, which is easily monitored using standard fluorescence spectrophotometers and multi-well plate readers. Below we describe two common techniques to study changes in RNA structure and reactivity, Förster Resonance Energy Transfer (FRET) and fluorescence quenching.

2.6. Förster Resonance Energy Transfer (FRET)

Förster Resonance Energy Transfer (FRET) refers to the phenomenon in which the emission wavelength of a donor fluorophore serves as the excitation wavelength of an acceptor fluorophore. Unsurprisingly, the effect \( E \) of the non-radiative dipole–dipole energy transfer between the donor and acceptor fluorophore is a function of the distance between the two. Typically high efficiency energy transfer is achieved when the donor and acceptor fluorophores are within 10–100 Å of each other. The distance between two fluorophores can be obtained by Eq. 5, where \( R_0 \) is the distance at which half the energy is transferred, referred to as the Förster radius, and \( r \) is the actual distance between the donor and acceptor. When calculating FRET, measurements are often taken at both the emission wavelength of the donor and of the acceptor. A ratio of these two values allows calculations to be made independent of the absolute concentration of both species.

\[
E = \frac{R_0^6}{R_0^6 + r^6}
\]

(5)

Tor and co-workers synthesized a fluorescent ribonucleoside derivative (5-aminoquinazoline-2,4(1H,3H)-dione, Fig. 7) that can be sequence-selectively incorporated into a RNA sequence, and acts as a FRET acceptor for tryptophan and amino acid with inherent fluorescence that is found in many RNA-binding peptides and proteins. Tor and co-workers purchased a truncated RRE RNA with the fluorescent ribonucleoside derivative site specifically incorporated. Concentration-dependent changes in Rev tryptophan fluorescence were measured while titrating fluorescently labeled RRE RNA. Fluorescence intensity corrections were determined using Eq. 6.

Intensity \( |V| = \lambda^2 \times \text{Intensity} [\lambda] \)

Using this method, these researchers were able to measure the quantum yield of the modified ribonucleoside, relative to a coumarin standard, using Eq. 7, where \( s \) represents the coumarin standard, \( x \) represents the nucleoside, \( A \) is the absorbance at excitation wavelength, \( F \) is the integral of the emission curve, \( n \) is the refractive index of the solvent, and \( \phi_S \) is the quantum yield. A large number of RNA-binding peptides and proteins contain a tryptophan within the RNA-binding site, which make this method well suited to study peptide or proteins RNA binders, since only the RNA must be tagged. As discussed below, however, FRET is commonly used to measure binding interactions involving molecules without a tryptophan.

\[
\phi_{F[S]} = \frac{(A_s/A_x)(F_S/F_X)(n_x/n_s)^2\phi_S}{F_S}
\]

(7)

The majority of modern antibiotic drugs target the bacterial ribosome in such a way that disrupts the translational machinery of the targeted organism. Numerous potent antibiotics cause a decrease in the fidelity of protein synthesis by binding to the decoding site (A-site) on the ribosome. The primary structure of the A-site on the 16S subunit of the prokaryotic ribosome is different from the A-site on the 18S subunit of the eukaryotic ribosome, but the secondary structure and aminoglycoside binding specificities are similar. Tor and co-workers sought to develop a fluorescence-based screen to examine binding selectivities for the A-site of the prokaryotic 16S A-site over the eukaryotic 18S A-site. They developed a three-component FRET system in which the 16S A-site from bacteria, the 18S A-site from humans, and a known A-site binder were each labeled with different fluorophores. As shown in Fig. 8A, the 16S bacterial A-site was labeled with a FRET...
2.7. Fluorescence quenching

A fluorescence quencher, or ‘dark fluorophore,’ is a molecule that absorbs the photon emitted from a fluorophore but does not emit energy in the form of light, thereby suppressing fluorescence emission. As the term suggests, collisional quenchers must be in close proximity to the donating fluorophore to act as a fluorescence quencher. Unlike FRET acceptors, collisional quenchers do not absorb at a specific wavelength and can therefore quench the fluorescence emission of fluorophores with diverse spectral properties. Given the broad range of fluorophores that can be used, this strategy is a common method for measuring changes in RNA structure.

In this experiment, the fluorophore and quencher are sequence-specifically incorporated into the target RNA at positions that are in close proximity when the RNA is folded. Under such conditions fluorescence is quenched. Changes in RNA folding, perhaps through the action of a small molecule ligand, separates the quencher from the fluorophore, leading to an increase in fluorescence. Like many in vitro fluorescence-based approaches, this method is amenable to high- and moderate-throughput screening and can be performed using commercially available instrumentation, such as fluorescence multi-well plate readers.

Davies and Arenz used a fluorescence quenching-based screening method to look for small molecules capable of disrupting a RNA–protein interaction involved in micro-RNA (miRNA) maturation (summarized in Fig. 9A). miRNAs are small non-coding RNAs that inhibit gene expression at the posttranscriptional level. Preliminary miRNAs (pre-miRNAs) are single-stranded RNAs of about 70–100 nucleotides in length, which form into hairpin structures. After being shuttled to the cytoplasm of the host cell, the protein Dicer binds the double-stranded portion of the hairpin and cleaves it on both sides, leaving about 21–25 basepairs of double-stranded mature miRNA. This mature miRNA is then used to sequence-selectively suppress the translation of complementary mRNA. In their assay, they chose to use the pre-miRNA let-7 from Drosophila melanogaster, an RNA known to be cleaved by Dicer. The experimental setup began by tagging the 5′-end of a pre-miRNA with fluorescein (Fig. 9A, FAM-EX-5′-linker) and the 3′-end with Dabcyl (Fig. 9B, Dabcyl linker). Given the hairpin structure of the RNA, the fluorophore and quencher are in close proximity, and fluorescence is quenched. Cleavage of this RNA hairpin by Dicer would effectively release the fluorophore–quencher pair from the RNA, allowing them to dissociate. As a result, a significant increase in fluorescence is observed.

In an effort to identify small molecule inhibitors of RNA cleavage by Dicer, Davies and Arenz pre-incubated a library of individual molecules each with the tagged RNA hairpin in a multi-well plate, then titrated in Dicer enzyme. The researchers measured small
molecule-dependent inhibition of fluorescence, which indicates inhibition of Dicer activity. As a positive control, they used a known RNA binder, kanamycin A, which suppressed Dicer activity by 73%. This proof-of-concept screen exemplifies a high-throughput application of a fluorescence quenching assay. The commercial availability of RNAs with 5' and 3' modifications, including fluorophores and quenchers, makes this assay well suited for broad use.

3. Microarrays

3.1. Microarray synthesis

A microarray is an ordered collection of molecules immobilized on a substrate. Typically, molecules are arrayed using commercial instruments that generate spots that are 50–200 µm in diameter. The most common substrate is glass; however, gold, nitrocellulose, polyacrylamide, and silicon have all been used. Once the molecules are immobilized on the substrate, the arrayed material is incubated with a solution containing fluorescently labeled target. Binding interactions involving a particular immobilized small molecule and the target are observed using a fluorescence slide reader and molecules with target affinity are identified by location on the microarray grid. One obvious requirement for microarray-based approaches is chemospecific immobilization of a small molecule library to the substrate. A functionalized glass microscope slide is the most common substrate, and microarraying is typically performed using homemade or commercially available microscope slides with a monolayer of a reactive group on the surface of the slide. Various examples of preparing functionalized glass microscope slides exist. Common commercially available slides contain N-hydroxysuccinimide activated esters and aldehydes. Slides containing surface epoxides are also commercially available, however, in our experience the conjugation efficiencies of these slides are far lower than N-hydroxysuccinimide or aldehyde functionalized slides.

Since very small amounts of material are used in a typical microarray experiment, this technology allows researchers to easily perform multiple experiments, or variants of experiments, without consuming large quantities of small molecule or RNA. For example, a ~10,000 member small molecule library can typically be arrayed multiple times on a single microscope slide, allowing multiple experiments to be performed on a single slide. In addition, since multiple slides are often arrayed at the same time, each slide can be incubated with a different concentration of target, allowing target concentration-dependent analysis resulting in the identification of highest-affinity ligands. While microarray-based experiments require access to a commercial microarrayer, these instruments are becoming common components of central instrument facilities.

3.2. Detecting small molecule–RNA interactions on microarrayed glass slides

Micro-RNAs (miRNAs) are short, naturally occurring RNAs that regulate translation of specific messenger RNAs (mRNAs). miRNA plays critical roles in various cellular processes, including development and response to stress. Since miRNAs control the cellular levels of particular proteins by suppressing translation of their encoding mRNAs, it is unsurprising that aberrant expression of miRNAs can lead to disease. miR-21 is an miRNA that targets the expression of tumor suppressor genes. Therefore, overexpression of miR-21 is associated with various cancers. Inhibiting miR-21 function has been shown to reestablish pro-apoptotic mechanisms. In an effort to identify a molecule that binds an miR-21 RNA stemloop (Fig. 10A) and potentially inhibit its expression of these molecules for the target RNA using fluorescence binding assays involving 2-aminopurine variants of the miR-21 RNA hairpin. Peptoids are peptidomimetic compounds with favorable properties such as cell permeability and resistance to proteolytic degradation. A library of 7680 thiol-terminated peptoid/peptide conjugates were prepared using standard solid phase synthesis and chemospecifically conjugated in an array on homemade maleimide-functionalized microscope slides. After blocking unreacted maleimide with a DMSO solution containing 1% (v/v) 2-mercaptoethanol, the immobilized peptoid/peptide conjugate library was incubated with only 80 µL of a 5 µM solution of 5'-fluorescently labeled target RNA. This demonstrates an important feature of microarray screening, in that very small quantities of material are used in each screen. Three compounds with the highest affinity for the target RNA were identified, simply by imaging the slide. Luebke and co-workers then measured the affinity for the target RNA is shown in Fig. 10B.

3.3. Detecting small molecule–RNA interactions on microarrayed agarose gels

Disney and co-workers have used small molecule microarrays on chemically functionalized agarose to study the
selectivity of interactions involving various aminoglycosides and RNA. For this application, agarose arrays are ideal because they provide a three-dimensional surface that allows high concentrations of ligand loading, and relatively large amounts of bound RNAs can be excised from the array and their sequence identified. In their approach, azide-functionalized agarose is coupled to an alkyne-functionalized molecule with known RNA affinity in an array format. This arrayed agarose slide is then incubated with a focused $^{32}$P radio-labeled RNA library, and spots with high levels of $^{32}$P signal are extracted, cloned into a plasmid, and sequenced using standard techniques (Fig. 11). Unlike SELEX (see Section 4.2), which is most commonly used to identify high-affinity RNAs from a large library of randomized nucleic acids, this approach focuses on relatively small RNA libraries generated from a single RNA secondary structure. Using this approach, Disney and co-workers have determined the sequence specificity between various functionalized aminoglycosides and RNA internal loops.

### 4. Selection-based methods

#### 4.1. Screens versus selections

In a screen, molecules are spatially segregated and individually assayed for a desired property, such as binding to a macromolecular target. As we have seen, various fluorescence- and microarray-based methods have been developed, which allow researchers to perform relatively high-throughput screens (typically $10^3$–$10^5$ molecules).

However, inherent to this approach is the discreet plating or arraying of compounds into multi-well plates or on a substrate. This requirement places a serious limitation on the number of compounds that can be reasonably screened. Screening very large libraries can often be very time consuming, laborious, and potentially costly.

An alternative to screening-based approaches is a selection. Unlike screens, selections amplify and enrich a small number of ‘hits’ from a single complex solution that often contains greater than a million different compounds (summarized in Fig. 12).

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**Fig. 11.** Small molecule microarrays on chemically functionalized agarose. Library members are easily immobilized using a Huisgen dipolar cycloaddition. Bound $^{32}$P-labeled RNAs are visualized by radiography and easily excised from the gel. RNAs are then identified by cloning and sequencing.
Since selections most often require enrichment of active compounds over the course of numerous rounds of selection, the active molecules must be amplifiable. Biological polymers (DNA, RNA, or proteins) are easily amplified using standard molecular biology techniques, so it is unsurprising that these molecules are most often used in selections. Below we will examine various selection-based approaches that are commonly used to identify RNA-binding molecules.

### 4.2. Systematic Evolution of Ligands by EXponential enrichment (SELEX)

Selections most often involve DNA, RNA, or their encoded protein sequences. One obvious selection-based method for identifying small molecule–RNA interactions is Systematic Evolution of Ligands by EXponential enrichment, or SELEX. As depicted in Fig. 13, SELEX involves incubating a large RNA library (typically >10^6 RNA molecules) with a small molecule and then selecting for high-affinity interactions through repeated rounds of incubation, amplification, and screening.
molecules) with an immobilized small molecule. After an incubation period, the immobilized small molecule is captured and unbound RNAs are washed away. Common methods of immobilization include biotinylation of the small molecule followed by complexation onto a streptavidin-coated magnetic bead and covalent conjugation to a substrate. The small molecule-bound RNAs are amplified using standard molecular biology techniques (reverse transcription (RNA → DNA), cloning this DNA into a plasmid, and in vitro transcription (DNA → RNA)). Enriched RNAs are incubated with the immobilized small molecule for another round of selection. Rounds of selection, enrichment, and amplification are performed until only the highest-affinity RNAs remain. The incubation time and concentration of RNA or small molecule can be changed in order to identify highest-affinity interactions. While SELEX is a relatively straightforward method for identifying selective small molecule–RNA interactions, it does require significant expertise in various molecular biology methods. In addition, perhaps most importantly, the random selection of small molecule–RNA interactions from a large RNA library makes it statistically improbable that an interaction involving a biologically relevant RNA will be identified.104 SELEX is, however, a good example of a selection-based approach to identify an interaction involving RNA. In addition, using SELEX on focused libraries, where only a few nucleotides are randomized, may be useful for identifying molecules that bind a particular RNA fold.

### 4.3. Phage display

The main limitation of SELEX is that the diversity lies on the RNA end, and therefore, it is improbable that a therapeutically relevant RNA will be enriched and amplified from a large completely randomized RNA library. An alternative approach is to select a ligand from a large library of amino acid-based biopolymers that target therapeutically relevant RNAs. Perhaps the most common method for identifying an amino acid-based biopolymer with affinity for a target is phage display. In phage display, a bacteriophage is genetically encoded to express a protein, antibody, antibody fragment, and circular or linear peptide on the terminus of a major or minor phage coat protein.105–108 When the DNA encoding the displayed biopolymer is randomized over some regions, a library is formed. A phage library is typically incubated with an immobilized target, and phage displaying a biopolymer with affinity for that target is enriched by affinity chromatography (summarized in Fig. 14). After each round of selection, enriched phage is amplified in *Escherichia coli* and isolated using standard methods. Targets are commonly immobilized to a substrate through the formation of a biotin–streptavidin complex. Biotinylated RNAs are available commercially, as are various streptavidin-coated beads and plates. Since phage that binds the substrate would be amplified in addition to phage that binds the target, a round of negative selection is commonly performed to remove phage with affinity to the substrate. In addition, additives such as non-target RNAs, such as tRNA, or proteins such as bovine serum albumin can be added to the solution to remove phage that binds non-selectively. Since these additional RNAs or protein additives are not biotinylated, phage that binds these added components are removed from the library during the affinity enrichment step.

A number of phage libraries displaying cyclic peptides (via a disulfide bond) and linear peptides are commercially available.109 Typically, commercial phage libraries display peptides that are 7–12 amino acids in length and commercial libraries are typically $10^7$–$10^9$ members in size. While performing a selection on a phage library does require basic molecular biology knowledge and equipment (the ability to culture *E. coli* being foremost among them), phage display represents a relatively 'simple' entry into molecular biology-based approaches to discover peptides or proteins that bind a target RNA. Detailed protocols for phage display selections are often provided with commercially available libraries. A number of papers describing the use of phage display are also available, as are numerous books.110–113

Dietrich, Pustowka, and co-workers performed a selection on three different commercial phage libraries to identify linear or cyclic peptides that bind ψ RNA, which is responsible for encapsidation of HIV-1 RNA genomes into virus particles during HIV-1 assembly.114 Immobilized full-length ψ RNA was prepared by in vitro transcription and immobilized on a streptavidin-coated plate via complexation to a 5′–biotinylated oligo that is complementary to the 3′ end of the full-length ψ RNA. During positive selections, phage libraries were incubated with the immobilized ψ RNA, unbound phage was removed through washing, and phage that bound the RNA target was eluted by treatment with a 0.2 M, pH=2.2 glycine solution. Negative selections were performed to remove RNA that bound non-ψ RNA. After four rounds of positive and negative selections, phage were amplified and titered, and the sequences of peptides displayed on the enriched phage were identified by DNA sequencing. A series of peptides with micromolar affinity for ψ RNA were identified, and peptide–RNA interactions were measured by Enzyme-Linked Immunosorbbent Assay (ELISA)115 and electrophoretic mobility shift assays (see Section 5.1).

More recently, Chow and co-workers utilized phage display to target methylated nucleotides on 16S ribosomal RNA, which are specific for bacterial ribosomes.116 They were able to identify two peptides with high nanomolar dissociation constants for the targeted RNA. Further characterization showed that the two peptides both bind the h31 hairpin of the 16S subunit ribosomal RNA, and inhibit in vitro protein synthesis. Therefore, these peptides have potential anti-bacterial activity.

### 4.4. Dynamic combinatorial chemistry

Dynamic combinatorial chemistry (DCC) shares features of high-throughput small molecule screens and biopolymer-based selections. Briefly, a dynamic combinatorial library (DCL) is formed from a library of small molecule or peptide building blocks that reversibly react with each other.117–120 Typical examples of reversible bond formation commonly used in DCC include disulfide, imine, Diels–Alder, and thioester chemistry. Since DCC relies on reversible bond formation to make DCLs, the distribution of library members in the absence of a target molecule is under thermodynamic control. If a target is added to this solution, that target can select the highest-affinity ligand from the DCL. This binding interaction effectively removes the target-bound molecule from the equilibrating solution. By the dictates of Le Chatelier's
principle, a shift in the equilibrium will occur that favors the preparation of the target-bound molecule(s). As a result, an increase in the mole fraction of high-affinity molecule(s) is observed after addition of the target (Fig. 15). In addition to its use in identifying novel protein and DNA ligands, catalysts, and materials, DCC has been used to identify novel RNA-binding compounds, perhaps most notably from Miller and co-workers.

In an early demonstration of DCC, Miller and Karan analyzed a library of salicylamide–copper complexes for affinity to a RNA stemloop 5′-UAGUCUUCGAGACUA-3′, which is derived from the GTP-binding P7 helix from the Pneumocystis carinii Group I intron. In their approach, salicylamide monomers were equilibrated with a Cu²⁺ solution and target RNA in a dialysis membrane. The RNA is sufficiently large that it cannot pass the dialysis membrane tubing. However, small molecules can pass in between the membrane. Therefore, if a particular salicylamide selectively forms a complex with copper and the target RNA, the concentration of that monomer will increase on the side of the membrane containing the RNA, compared to an experiment lacking target RNA (Fig. 16). After equilibration is reached, small molecule RNA ligands are removed from the dialysis tubing and their levels are measured using High-Performance Liquid Chromatography (HPLC). Miller and Karan identified a histidine-substituted salicylamide–copper complex that binds the target RNA with high affinity (152 nM) and greater than 300-fold selectivity over the homologous DNA sequence.

Like the above experiment, HPLC is commonly used to observe target-induced amplification of a species from a DCL. However, thorough analysis of the library by HPLC requires segregation of each compound on a chromatogram. This severely limits the theoretical size of a DCL. Unsurprisingly, most DCLs have been limited to <100 theoretical compounds.

More recently, Miller and co-workers have developed a method called Resin-Bound Dynamic Combinatorial Chemistry (RBDCC) to screen relatively large DCLs (>10,000 theoretical library members). The RBDCC method is summarized in Fig. 17. Beads bearing monomers are first incubated with a fluorescently labeled target, then washed and imaged by fluorescence microscopy. A concentration of target, and imaging exposure time, is chosen such that beads containing monomer building blocks do not appreciably bind the labeled target, as determined by bead fluorescence. Beads bearing monomer building blocks are then equilibrated with solution phase monomers and labeled target. Since the amount of material on the beads is much greater than the amount solution phase monomers, reactivity between solid and solution phase monomers is favored (disulfide exchange in Fig. 17), as opposed to a reaction between two solution phase monomers. Target-induced reactivity is therefore favored to occur between a monomer on the bead. Binding interactions can be observed by imaging the solid phase beads following incubation and washing steps. Bead fluorescence indicates that the monomer present on that bead is involved in target binding. Since the majority of the molecules on the bead are unreacted (remain monomers), cleaving the bead and identifying the composition of that monomer by mass spectrometry provide information on the makeup of a molecule that binds the target RNA, drastically simplifying the identifying of the highest-affinity ligand. Degeneracy among the fluorescent beads can be used to elucidate the formed ligand with target affinity.

Using disulfide exchange as a reversible reaction, Miller and co-workers screened a RBDCL with a theoretical size of 11,325 disulfides for affinity to HIV-1 frameshift-inducing stemloop, which controls the expression of proteins involved in HIV-1
replication, and (CUG)\textsubscript{n} RNA\textsuperscript{123} a causative agent of type 1 myotonic dystrophy (DM1). In their initial RBDCC screen, these researchers identified molecules that bind the HIV-1 frameshift-inducing stemloop with good affinity (4.1 \mu\text{M} by SPR, 350 nM by fluorescence titration), and selectivity over alternate RNA and DNA sequences was observed. In a second screen, that same team identified molecules that bind (CUG)\textsubscript{n} RNA with low micromolar affinity, and perhaps most importantly, inhibit binding between the protein MBNL-1 and (CUG)\textsubscript{n} RNA. Variants of the molecules identified in these initial screens with increased target affinity and/or drug-like properties have been reported\textsuperscript{124} and are currently being evaluated as potential HIV-1 and myotonic dystrophy therapies.\textsuperscript{125} While the initial RBDCC method relied on disulfide exchange chemistry, more recently, Miller and co-workers have reported the concept of ‘ternary RBDCC’ wherein monomers simultaneously undergo both disulfide and hydrazone exchange.\textsuperscript{120} This advance paves the way for the evaluation of much larger and structurally complex resin-bound dynamic combinatorial libraries.

5. Low-throughput methods for measuring ligand–RNA interactions

5.1. Electrophoretic mobility shift assay

Electrophoretic Mobility Shift Assay (EMSA) is a relatively simple way to measure binding between ligands and RNA.\textsuperscript{126–128} This approach requires simple and inexpensive instrumentation and is relatively simple to perform. RNA and ligand are mixed in a solution, then run on a polyacrylamide (commercially available) or agarose gel for a short period (0.5–2 h). If the ligand binds the RNA target, an increase in mass is observed as a band shift. RNA (or DNA) complexes are easily observed by staining the gel with a commercial nucleic acid stain such as ethidium bromide or SYBR green. Numerous commercial EMSA assays are available. While this method is useful for observing peptide or protein interactions with RNA, interactions involving small molecules may be difficult to observe. Alternatively, EMSA can be used to observe small molecule displacement of a known ligand from a target RNA.\textsuperscript{129,130} When a series of experiments are run using varied concentration of ligand, dissociation constants or inhibition constants can be determined (summarized in Fig. 18).

In a nice example, Tor and co-workers measured the ability of neo-acridine to inhibit the Rev–RRE complex, which, as we have already discussed, plays an important role in HIV-1 proliferation.\textsuperscript{131} 32P-labeled RRE RNA (25 nM) was incubated with 2 \mu\text{M} Rev protein. Serial dilutions of neo-acridine were added to aliquots of the Rev–RRE complex. Those solutions were incubated for a period of time and then loaded onto a 10% (m/v) polyacrylamide gel. The gel was run using standard commercially available electrophoresis equipment. These researchers were able to observe neo-acridine concentration-dependent displacement of Rev from RRE by measuring changes in the position of the RRE RNA band on the gel (similar to Fig. 18B).

Radiolabeling the target RNA with 32P is common for EMSA experiments, which requires special training, facilities, and disposal methods. Newer commercial imaging reagents have made this technique more accessible and practical. Meisel and co-workers\textsuperscript{132} employed a commercially available fluorescent Cyano dye (Cy5) tagged on the 5’ end of DNA to run EMSA, and showed comparable sensitivity to a 32P-labeled control. RNAs with various fluorophores, including Cy5, are commercially available through numerous vendors.

![Fig. 17. Resin-bound dynamic combinatorial chemistry. The high concentration monomer building block bonded to the resin reacts reversibly with the solution phase monomers, generating a resin-bound DCC. Deconvolution of the monomer involved in binding the fluorescently labeled target is simplified by identifying in building block bonded to fluorescent beads.](image)

![Fig. 18. Electrophoretic Mobility Shift Assay (EMSA). A. As the concentration of ligand is increased from lanes 1 to 7, a measure of the dissociation constant can be visualized by the equal ratio between the free RNA and RNA–ligand complex bands. B. A displacement assay allows the inhibitory constants of smaller ligands to be measured by observing the displacement of a larger ligand from the RNA.](image)
5.2. Nuclear magnetic resonance (NMR)

The use of NMR has gained significant momentum as a powerful tool in drug discovery efforts.\textsuperscript{133,134} Due to its inherent sensitivity, small molecules with weak dissociation constants can be identified and subsequently optimized to improve binding affinity. In contrast to fluorescence methods, which usually require chemical modification to attach a fluorophore, NMR gives highly quantitative ligand binding data using the native nucleic acid and unmodified small molecule. Moreover, by incorporating \textsuperscript{13}C\textsuperscript{15}N isopes during RNA biosynthesis, site-specific information about small molecule–RNA interaction surfaces can be determined with atomic resolution.

As an initial screening tool, 1D \textsuperscript{1}H NMR can be used to detect RNA–small molecule interactions. Typically, the imino chemical shifts of guanosine and uracil nucleobases are monitored using WATERGATE type pulse sequences.\textsuperscript{135} The imino chemical shifts provide the highest resolution (9.5–15 ppm) in a 1D \textsuperscript{1}H NMR spectrum; they are assigned with relative ease using conventional 2D NMR experiments, and they report directly on the RNA secondary structure. Changes to the RNA local environment induced by substrate binding often manifest as perturbations in the 1D imino chemical shifts. Thus, the imino protons are sensitive reporters of RNA–small molecule interactions. Fesik and co-workers used 1D imino chemical shift perturbation (CSP) to screen a library of ∼10,000 aminoglycoside mimetics against the E. coli 16S A-site RNA.\textsuperscript{136} By analyzing 1D imino spectra of 16S A-site targets combined with mixtures of 10 small molecules, several classes of lead binders were identified. Apparent dissociation constants (0.07–3 mM) were subsequently determined by monitoring well-resolved imino chemical shifts as a function of increasing small molecule amount. Following lead optimization, aminoglycoside mimetics with improved binding affinities (0.009–0.035 mM) for 16S A-site RNA were identified. The Fesik CSP method offers several advantages for identifying lead compounds that target RNA: (i) it is simple and fast, (ii) does not require isotope labeling, and (iii) is carried out using label-free RNA. In practice, the 1D imino CSP approach is only applicable to relatively small RNA molecules (<50 nts) that give rise to well-behaved NMR spectral properties. Larger RNAs display considerable spectral overlap that complicate analysis, and shorter T2 relaxation times that deteriorate resolution and sensitivity. These challenges can be mitigated via preparation of \textsuperscript{15}N(G/U)-labeled RNA constructs along with the application of transverse relaxation optimized\textsuperscript{137} (TROSY, where SY denotes spectroscopy) pulse sequences. Of course, the use of isotope-enriched rNTPs and the application of TROSY NMR experiments lead to an increase in cost per sample and a reduction in throughput.

The saturation transfer difference (STD) method,\textsuperscript{138} and its variant Water-LOGSY,\textsuperscript{135} is an alternative to 1D imino CSP that has gained popularity as a tool for screening RNA–small molecule interactions. In an STD experiment, the small molecules are present in large excess (typically 20-fold) over the RNA. This ensures that the NMR spectra are dominated by signals emanating from the small molecules to be screened. Further suppression of background RNA signals can be achieved by applying a spin lock pulse train. To achieve saturation transfer, on resonance pulses are applied to a chemical shift bandwidth where only RNA protons resonate. Magnetization is then transferred throughout the RNA as a result of spin diffusion and subsequently onto the bound small molecule via intermolecular NOEs. Subtraction of a reference 1D spectrum (off resonance pulse) from spectra acquired using the on resonance pulse readily yields small molecule NMR signals that originate from the saturation transfer. James and co-workers used STD to screen phenothiazines against several RNA drug targets, including the HIV-1 TAR stem loop.\textsuperscript{140} In their work, acetopromazine was shown to be a potent ligand for HIV-1 TAR and STD assisted epitope mapping clearly demonstrated that the aromatic ring system of acetopromazine forms the primary binding contacts. As a result of the high small molecule to RNA ratios used in STD, care must be taken to rule out non-specific binding events. Since most small molecule–RNA ligands target non-canonical surfaces, Williamson and co-workers proposed using an all A-form RNA duplex as a counterscreen for non-specific binders.\textsuperscript{141}

Potential small molecule binders identified from high-throughput 1D NMR screens need to be further verified by mapping their sites of interaction on the RNA surface. This is most easily accomplished via conventional 2D correlation NMR experiments. For smaller RNA constructs, 2D \textsuperscript{1}H–\textsuperscript{1}H total correlation spectroscopy (TOCSY) can be used. \textsuperscript{1}H–\textsuperscript{15}N TOSCY experiments correlate HS/ H6 chemical shifts and thus provide a map of all the pyrimidines present in the RNA. By comparing TOCSY spectra of the free RNA to the small molecule bound form, one can readily map the binding surface. Two-dimensional \textsuperscript{1}H–\textsuperscript{13}C or \textsuperscript{1}H–\textsuperscript{15}N heteronuclear single quantum coherence (HSQC) spectra can be recorded either at natural abundance (\textsuperscript{13}C) or using isotope-enriched rNTPs. The advantage of isotope enrichment is that it speeds up the total acquisition time, and it allows for site-specific detection of multiple probes with a high degree of accuracy. Given the increasing sizes of many functional RNAs that one hopes to make ‘drugable’, it is clear that multidimensional NMR methods along with advanced rNTP labeling patterns must be implemented into the NMR-based screening approaches.

5.3. Surface Plasmon Resonance

Surface Plasmon Resonance (SPR) is commonly used to measure binding interactions between solution phase and surface-bound molecules. SPR can be used to study small molecule–biopolymer and biopolymer–biopolymer interactions, including those involving RNA.\textsuperscript{142,143} The output of SPR binding assays are typically generated when the interaction is at equilibrium; however, since SPR data are obtained in real-time, binding kinetics can be measured as well. Immobilization of a nucleic acid onto the SPR chip surface can be achieved through a RNA–biotin streptavidin interaction, which is achieved by using a streptavidin-coated SPR chip. These chips are commercially available. SPR chips that contain a monolayer of carboxylic acids are also available, and RNAs functionalized with a terminal amine can be coupled to these surfaces using common amide bond coupling protocols. Since the size of the change in refraction, due to a binding interaction, scales relative to binding-dependent changes in the size of the surface conjugated molecule, SPR experiments involving a small molecule are typically performed by immobilizing that molecule to the SPR chip surface and using RNA as the solution phase component. If the ligand is a peptide, peptidomimetic, or larger molecule, measurements can be made with the RNA target conjugated to the surface. For the purposes of this discussion, we will assume that RNA is surface bound and the ligand is the solution phase component.

In a typical experiment, RNA conjugated to the SPR chip and solutions containing increased concentration of ligand are flowed over the SPR chip containing the immobilized molecule. As the ligand flows over the surface and binds the surface conjugated RNA, interaction-dependent changes in refraction angle are monitored until equilibrium is reached. After each solution containing ligand is incubated with the immobilized RNA, the bound complex is dissociated by introducing buffer that doesnt contain any ligand. Solutions containing higher concentrations of ligand are serially flowed over the surface conjugated RNA until binding saturation is observed. The change in refraction angle is reported as a change in resonance units (RU), which is plotted against time, thereby generating a sensogram. Fitting the binding data using a software program
is then required, and many examples of this data analysis are available.144–146 A 1:1 binding ratio can be fit to Eqs. 9 and 10, where the concentration of the RNA + L complex is equal to the signal in RU. The concentration of free ligand in the flow, [L], is equal to the constant C in Eq. 9, unbound RNA, [RNA] is equal to the maximum amount of immobilized RNA on the surface, which can be calculated as RU\textsubscript{max}, minus the amount of bound RNA (RU). The association and dissociation constants are defined as \(K_a\) and \(K_d\), respectively. The equilibrium association constant can be determined using Eq. 11, where \(n\) is the number of binding sites on the RNA.

\[
d\text{[RNA + L]} = K_a\text{[RNA][L]} - K_d\text{[RNA + L]} \quad (9)
\]

\[
d\text{RU} = K_aC(\text{RU}_{\text{max}} - \text{RU}) - K_d\text{RU} \quad (10)
\]

\[
\text{RU} = \frac{\text{RU}_{\text{max}} \cdot n \cdot K_a(L)}{1 + (K_a(L))} \quad (11)
\]

Wong and co-workers demonstrated the importance of dissociation rates of small molecule–RNA interactions by monitoring the binding kinetics of two aminoglycosides with similar binding constants using SPR.147 Unsurprisingly, a compound that demonstrated a greater than 100-fold higher anti-bacterial activity had a much lower dissociation constant. SPR is a very sensitive technique and requires relatively small amounts of ligand and target RNA. While SPR is typically low-throughput, high-throughput SPR instruments are available and their use is becoming more commonplace.

5.4. Isothermal Titration Calorimetry

Isothermal Titration Calorimetry (ITC) provides information on all thermodynamic properties of a binding interaction (enthalpic and entropic).148–150 In addition, unlike other techniques discussed in this report, which require conjugation of one of the interacting pairs to a surface, ITC is a solution phase technique that does not require conjugation of the ligand or substrate. Finally, ITC can be used to measure binding interactions in a wide range of conditions, including acidic, basic, high salt, and even some organic solvents. The basic instrumental setup, depicted in Fig. 19, includes a sample chamber and a reference chamber. The sample chamber contains the target and the reference chamber is filled with the same solution, minus the target. Ligand is injected into the sample chamber and the binding enthalpy (\(\Delta H\)) leads to a small temperature change within the sample chamber. For an exothermic reaction, the variable power to the sample chamber is decreased so that the temperature can equilibrate and reach that of the reference chamber. If the reaction is endothermic, variable power to the sample chamber increases. The energy applied to the reference chamber is recorded (reported as \(\mu\text{cal/s}\)) and plotted as a function of time (injection). Injection-dependent changes in heat, representing energy applied to the system after each ligand injection, are integrated (represented as \(q_i\)) and can then be related to binding stoichiometry (\(n\)), fractional saturation of the binding event (\(f_i\)), total concentration of the ligand [L], binding enthalpy (\(\Delta H\)), and the cell volume (\(V\)) by the following set of equations:

\[
q_i = nF[L]\Delta HV 
\] (12)

Solving Eq. 12 for \(F\) using a quadratic equation generates Eq. 13,

\[
F^2 - F \left(1 + \frac{[RNA]}{n[L]} + \frac{1}{nK_B[L]}\right) + \left(\frac{[RNA]}{n[L]}\right) = 0 
\] (13)

where \([RNA]\) is the initial RNA concentration, provides Eq. 14.

\[
q_i = n[L]\Delta H \left\{X - \left(\frac{X^2}{4} - \frac{4[RNA]}{n[L]}\right)^{1/2}\right\} 
\] (14)

This form of the equation can be fit using a nonlinear least-squares fit to give \(\Delta H\), \(n\), and the binding constant (\(K_B\)). The change in entropy (\(\Delta S\)) of the binding event can be found by first using \(K_B\) to solve for \(\Delta G\). Thus all the thermodynamic properties of ligand–target binding can be evaluated in a single experiment.

ITC measurements call for high concentrations of RNA and ligand. The appropriate amount of RNA required for an experiment can be estimated using Eq. 15, where \(c\)-values

\[
\text{Fig. 19. Isothermal Titration Calorimetry (ITC). A sample cell and reference cell are kept at the same temperature through communicating heaters. When a ligand is injected to the sample chamber, an exothermic or endothermic binding interaction can occur. The change in energy needed to maintain the same temperature in both chambers is plotted against time (injection number) until saturation is reached.} 
\]
higher concentration of ligand, research institutions. Other screening methods previously outlined in this report have been developed to resolve this. While ITC is not amendable for the RNA and small molecule can be cumbersome. Another glaring example. ITC provides a unique window into understanding the biological functions, among them is the purine riboswitch seen in this examples where changes in RNA folding directly affect some bi-

\[
\frac{n[RNA]}{K_D} = \frac{6.4}{c^2} + \frac{13}{c}
\]  

Using \( R_m \), the initial concentration of ligand can be calculated by Eq. 17.

\[
|X| = \frac{R_m[RNA]V_{cell}}{V_{injector}}
\]  

In the absence of good estimations of the dissociation constant, a general starting point would be 10 μM RNA and 10- to 30-fold higher concentration of ligand, ~100 μM.\(^{149}\) Solubility of the ligand may dictate the concentrations used in the experiment and indeed molecules with low solubility will preclude them from this application. Batey and co-workers used ITC to elucidate the functional groups that are essential for ligand recognition by a purine binding riboswitch.\(^{151}\) Using 2,6-diaminopurine (DAP, Fig. 20) as a starting ligand scaffold, they modified both the molecule and the RNA to individually disrupt hydrogen bond interactions thought to be involved in binding. The kinetic data they collected allowed them to fit a model for binding in which a ligand recognition event facilitates a structural change in the RNA to close up the binding pocket around the purine. They were able to determine the kinetics of the structural changes in the RNA as well as the binding kinetics of the DAP ligand. This exemplifies the utility of ITC in studying small molecule–RNA-binding events, which are commonly associated with folding changes in the RNA. As mentioned in Introduction, the growing knowledge base of RNA biochemistry has presented many examples where changes in RNA folding directly affect some biological functions, among them is the purine riboswitch seen in this example. ITC provides a unique window into understanding the biophysics of these events.

![Fig. 20. 2,6-Diaminopurine.](image)

The large amount of material required by this technique for both the RNA and small molecule can be cumbersome. Another glaring limitation is that binding constants of less than 10 nM are not readily measured with ITC, although some specialized techniques have been developed to resolve this. While ITC is not amendable for high-throughput screening, this technique nicely compliments other screening methods previously outlined in this report by providing full thermodynamic characterization of a binding event. The instrumentation required for this experiment is becoming increasingly available in central instrument facilities of many research institutions.

6. Conclusions

The vast desert of ‘undruggable’ space within the landscape of the proteome, and the increasing role of RNA in diverse biological processes, including those involved in disease, make RNA a particularly compelling target for current and future drug discovery efforts in academic and industrial laboratories. The development of diverse methods to identify interactions involving RNA has allowed researchers to find new small molecule and macromolecule RNA binders and regulators of RNA function. Various analytical methods have shown enormous utility, and make possible the careful characterization of small molecule and macromolecule interactions with various RNAs. Advances in existing technologies, such as high-throughput SPR instrumentation,\(^{152}\) will allow researchers to obtain rich datasets on a large number of interactions involving RNA. These large datasets may ultimately enable the formation of a set of ‘rules’ for the design of RNA sequence-selective ligands. Generating these large datasets on the selectivities and affinities for various molecules to structurally diverse RNAs will require the application of existing methods to RNA-focused drug discovery efforts, as well as the development and/or broader use of new technologies. For example, the development and broad use of new analytical methods with the potential to be very high throughput, inexpensive, and label free, such as nanopore-based analysis\(^{153}\) will likely allow researchers to identify RNA-binding molecules, and characterize those interactions, on a scale and at a cost that is not possible today. Applying powerful synthesis and screening methods such as diversity oriented synthesis\(^{154}\) and DNA-templated synthesis\(^{155}\) to RNA-focused drug discovery will undoubtedly result in the generation of new RNA-binding molecules, paving the way to new RNA-targeted therapies and basic research tools. Experimentally simple and reliable selection-based techniques particularly well suited for identifying RNA-binding molecules are needed as well.

Given the critical role RNA plays in various biological processes, and the limited scope of protein-targeted therapies, increased research focus and key advances in the next decade will likely result in the generation of RNA-targeted therapies for numerous human diseases. It is truly an exciting time to be involved in the identification and characterization of interactions involving RNA and RNA-targeted therapeutic discovery.

References and notes

Biographical sketch

Brett D. Blakeley received a BS in Chemistry from Central Connecticut State University in 2009. He concurrently did research in the group of Professor Challa Kumar at the University of Connecticut. He is currently a graduate student in the Department of Chemistry at Colorado State University, working under the direction of Professor Brian McNaughton. His research is focused on developing new methods for drug discovery.

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Ritwik Burai received a BS in Chemistry from the University of Calcutta in 2002. He moved to the Indian Institute of Technology, Bombay, India where he completed his MS degree in Chemistry in 2004 with Professor M. Ravikanth, and later completed his PhD at New Mexico State University with Prof. Jeffrey B. Arterburn. He then joined Professor Brian McNaughton’s lab at Colorado State University, working on the synthesis of heterofunctionalized polyvalent scaffolds. He is currently a postdoctoral fellow in Professor Hilal Lashuel’s lab at Ecole Polytechnique Fédérale de Lausanne (EPFL).
Blanton S. Tolbert received a BS in Chemistry from the University of South Carolina in 1999. He earned his PhD in Biophysics from the University of Rochester in the research groups of Professor Ravi Basavappa and Professor Doug H. Turner. He joined the group of Professor Michael F. Summers as a postdoctoral fellow where he developed NMR methods to study large RNA systems. In 2009, he moved to Miami University as an Assistant Professor of Chemistry and Biochemistry, and he recently relocated his laboratory to the Department of Chemistry at Case Western Reserve University. His research interests are in the area of structural biophysics of protein–RNA systems involved in viral gene expression.

Brian R. McNaughton received a BS in Chemistry from the Indiana University of Pennsylvania in 2001. In 2002 he began graduate studies at the University of Rochester and in 2007 completed his PhD in the group of Professor Benjamin Miller. Following postdoctoral studies at Harvard University in the group of Professor David Liu, he joined the faculty at Colorado State University in 2009 as an Assistant Professor in the Department of Chemistry and Department of Biochemistry & Molecular Biology, where he began a research program centered on the development and application of new methods for drug discovery and targeted drug delivery.