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RNA Structural Differentiation: Opportunities with Pattern Recognition

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Abstract

Our awareness and appreciation of the many regulatory roles of RNA have dramatically increased in the past decade. This understanding, in addition to the impact of RNA in many disease states, has renewed interest in developing selective RNA-targeted small molecule probes. However, the fundamental guiding principles in RNA molecular recognition that could accelerate these efforts remain elusive. While high-resolution structural characterization can provide invaluable insight, examples of well-characterized RNA structures, not to mention small molecule:RNA complexes, remain limited. This Perspective provides an overview of the current techniques used to understand RNA molecular recognition when high-resolution structural information is unavailable. We will place particular emphasis on a new method, pattern recognition of RNA with small molecules (PRRSM), that provides rapid insight into critical components of RNA recognition and differentiation by small molecules as well as into RNA structural features.

Graphical Abstract

The need to understand RNA molecular recognition has become increasingly pressing given the newly discovered roles for RNA in multiple disease states.^{1–7} These studies imply that selective RNA targeting could be a potential treatment method, yet nearly all medicinal, Food and Drug Administration (FDA)-approved compounds target proteins.^{8–11} Distinct from proteins, which are largely recognized through defined ligand binding pockets, identifying selective ligands for RNA is challenging due to the ability of RNA to sample

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multiple energetically stable conformations, the minimal chemical property differences among the four nucleobases, and the negatively charged backbone.^{11–18} In part because of these challenges, we are only beginning to discover the guiding principles behind the differential affinity and selectivity of small molecules for RNA motifs, including the topological and sequence dependence of small molecule:RNA interactions. It is clear that the use of several complementary methods will be needed to elucidate principles of RNA molecular recognition, which will in turn yield insight into the fundamental biology, disease relevance, and therapeutic targeting of RNA.

Numerous experimental approaches have been developed to examine the interactions of RNA with small molecules as well as biomacromolecules.^{13,19–22} Relevant methods can focus on RNA structure and dynamics as well as biophysical measurements of RNA interactions. High-resolution structural characterization, generally through nuclear magnetic resonance (NMR) spectroscopy or X-ray diffraction, can be highly informative but also difficult and occasionally impossible for some RNA molecules and complexes.^{23–27} Several additional techniques have been employed to probe RNA structure and recognition. These include, for example, two-dimensional (2D) and three-dimensional (3D) structure prediction that can be enhanced via chemical probing.^{28–33} Small-angle X-ray scattering (SAXS) and cryo-electron microscopy (cryo-EM) can experimentally characterize RNA structural ensembles.^{34–37} RNA dynamics have been investigated with techniques such as NMR spectroscopy and Förster resonance energy transfer (FRET).^{23,38–41} Methods for directly investigating RNA interactions are numerous and varied, ranging from large-scale screening methods to those that allow careful calculation of binding constants along with kinetic and thermodynamic parameters. A summary of select methods that have been used to investigate recognition and select references can be found in Table 1. While often highly successful in determining specific RNA binding partners, these techniques do not typically provide binding profiles for multiple RNA sequences and small molecules simultaneously, which would aid more rapid elucidation of general binding principles.

Our research in this area has included the implementation of a molecular-scale pattern recognition technique to reveal small molecule:RNA interaction principles, which we termed pattern recognition of RNA with small molecules (PRRSM).^{42,43} General pattern recognition illuminates complex binding properties through the use of differentially binding receptors that interact with a range of analytes of interest.^{44–47} Advantageously, selective receptor:analyte binding is not required, and commercially available or easily synthesized small molecule receptors can be utilized. In this context, our laboratory demonstrated that RNA binding small molecules acting as “receptors” could differentially sense RNA secondary structures as “analytes” (Figure 1). Additionally, PRRSM can provide insights into both small molecule properties privileged for RNA binding and the components of RNA topology that influence the molecular recognition of RNA. In addition to the focus on discerning the guiding principles of RNA recognition, PRRSM can provide RNA structural information. The work to date suggests a wide range of applications and areas of expansion for the PRRSM method moving forward.

In this Perspective, we discuss the current techniques used to gain insight into RNA structure and dynamics as well as small molecule:RNA interactions and how PRRSM can be used as a

complementary technique to investigate both RNA structure and molecular recognition. Given the difficulty of high-resolution 3D characterization for RNA and previous reviews on the subject,^{23,24,26,27,48–51} we will focus on alternative methods. Additionally, we will discuss future opportunities for understanding and harnessing small molecule:RNA interactions as well as the potential role of PRRSM in elucidating guiding principles of RNA recognition.

STRUCTURAL AND DYNAMICS CONSIDERATIONS FOR RNA RECOGNITION

RNA sequence, structure, and dynamics play important roles in RNA recognition. While high-throughput sequencing techniques have revolutionized the determination of RNA sequence, elucidation of RNA structure and dynamics can be more difficult. RNA folding is generally hierarchical, where the primary sequence forms secondary structure motifs based on Watson–Crick base pairs, which can form tertiary interactions in the presence of magnesium, leading to increasingly complex structures.^{52–55} RNA dynamic flexibility affects the structural complexity and increases the potential structural diversity for individual RNA sequences.⁵⁶ In addition, post-transcriptional modifications have recently been revealed to impact RNA structure, dynamics, and recognition.^{57–61}

For RNA secondary structure, advances in computational methods are constantly increasing the length of RNA sequence for which the structure can be accurately predicted.^{62–64} For example, 700 nucleotide RNA structures have been predicted with 73% accuracy.⁶⁵ Computational predictions are typically informed through structural constraints derived from chemical probing techniques, such as selective 2'-hydroxyl acylation analyzed by primer extension and mutational profiling (SHAPE-MaP).^{66–68} SHAPE reagents acylate more solvent-exposed 2'-OH groups at higher rates compared to less exposed 2'-OH groups, i.e., base-paired nucleotides (Figure 2).^{31,69,70} The modified RNA is then analyzed through error prone reverse transcription, where acylated nucleotides are more prone to mutations. These data are added to computational prediction programs to provide experimentally constrained structure predictions, thus enabling higher accuracy and elucidation of an impressive range of RNA structures.^{68,71–77} Several other chemical probing techniques also provide valuable insight, including dimethyl sulfate (DMS),^{78,79} terbium-induced cleavage,^{80,81} light-activated structural examination of RNA (LASER),⁸² and in-line probing techniques.^{83,84} Each technique mentioned utilizes different patterns of reactivity toward RNA structure, and the data can be combined to provide additional constraints for RNA computational predictions.⁶⁴ At the same time, *in vivo* RNA secondary structure determination techniques have been developed to understand important structural motifs in biological systems.^{31,68,85,86} Current research has been focused on merging 2D probing and tertiary predictions to contribute insight into RNA global structure and interactions.^{65,87}

The accuracy of computational predictions for three-dimensional RNA structure is currently limited due to a number of factors, including (1) the computational cost of probing the wide range of potential conformations and tertiary interactions within a given sequence, (2) the paucity of high-resolution structures from which to train the algorithms, and (3) the need for

improved force fields for RNA.^{50,88–90} Nonetheless, great progress has been made in this area. For example, 3D Motif Atlas can correctly predict the bacterial 16S rRNA subunit 3D structure and highlight the differences between the bacterial and eukaryotic 16S rRNA subunits.⁹¹ Future computational progress is expected to include analysis of conformationally flexible and electronically equivalent RNA structures. Experimentally, RNA interaction groups by mutational profiling (RING-MaP) can help identify potential tertiary contacts using data from DMS probing and single-molecule sequencing experiments.⁹² Specifically, if two or more sites have statistically similar mutation frequencies within an individual RNA molecule and are not predicted to form secondary structures, a through-space interaction is predicted and thus provides insights into RNA tertiary interactions and global folding of RNA structures. Mutate-and-map readout by next-generation sequencing (M2-Seq) is a complementary technique developed to analyze RNA structure in a one-pot experiment.⁹³ To achieve a low false positive rate for helix identification, a neural network-inspired algorithm termed M2-net is used to recognize helix-specific signatures. These current techniques are useful for *in vitro* RNA tertiary structure prediction, and future work will be focused on examining *in vivo* RNA tertiary structures with minimally biologically perturbing experiments.⁹⁴

RNA dynamics and conformational sampling are also critical to RNA molecular recognition.^{23,95–98} Techniques such as NMR spectroscopy, FRET, and molecular dynamics simulations have been invaluable in elucidating these processes.^{38–40,56,97,99–113} For example, residual dipolar coupling (RDC), heteronuclear single-quantum coherence (HSQC), and nuclear Overhauser effect (NOE) NMR investigations, combined with computational simulations, have revealed that despite the general flexibility of RNA, RNA structures sample only a specific subset of the potential conformations.^{104,114–117} Nucleotide identity, buffer composition, and magnesium concentration were each shown to alter this conformational sampling,^{97,118} and it has been proposed that both charge and topological constraints play important roles.^{109,119} These constrained dynamics can also impact binding interactions, including those with small molecules. Indeed, recent work has suggested that small molecules can bind to specific but minor RNA conformers, implying a conformational selection in small molecule:RNA interactions.^{97,120–122}

Recently, dynamics of more than 1000 RNA two-way junctions were interrogated by combining tectoRNA, a method for organizing RNA in 3D space using tetraloop-based tertiary contacts, and a high-throughput method termed quantitative analysis of RNA on a massively parallel array (RNA-MaP).^{123–125} In this application, an array of immobilized tectoRNA pieces were generated that included a comprehensive set of three-nucleotide two-way junctions (bulges and internal loops) along with others derived from X-ray diffraction structures. The binding equilibria between these sequences and another tectoRNA piece in solution were measured via FRET and were dependent upon the conformational dynamics of each junction. Through this method, “thermodynamic fingerprints” were generated that reveal patterns in two-way junction dynamics, including the general finding that the number and arrangement of unpaired residues primarily determine the conformations sampled while sequence identity plays a secondary role. These determinants also varied by secondary structure. Bulge conformations were found to be relatively independent of sequence identity, while mismatches (i.e., internal loops) were strongly dependent on nucleobase identity. At

the same time, unbiased clustering revealed that traditional secondary structure classes do not completely define the conformational landscape, with select junctions having conformational ensembles more similar to those of other classes. These data could further be used to predict tertiary folding energetics and, in the future, may reveal insights into the impact of small molecule binding and other factors on the conformational dynamics of large sets of RNA sequences.

Small Molecule:RNA Recognition.

In addition to a detailed understanding of RNA structure and dynamics, it is important to understand the specific interactions RNA utilizes in binding and selectivity. In general, small molecule:RNA recognition is achieved through a combination of noncovalent interactions such as electrostatics, π -stacking, and hydrogen bonding. Other biomacromolecules, including proteins, also utilize these noncovalent interactions to bind RNA, and multiple reviews that discuss these in greater detail are available.^{126–136}

One approach toward a better understanding of small molecule:RNA recognition is to analyze the properties of small molecules that have achieved selective recognition.^{11,16,137,138} To this end, we have examined the differences in the cheminformatic parameters of bioactive RNA-targeted small molecules, which we have consolidated into the RNA-targeted Bioactive ligand Database (R-BIND), as compared to FDA-approved small molecules, which are thought to largely target proteins.¹⁶ Although many “drug-like” properties were consistent across both libraries, bioactive RNA-targeted small molecules had statistically significant differences in structural and spatial properties. These included increased nitrogen counts compared to oxygen counts as well as an increase in the number of aromatic and heteroatom-containing rings. These and other differences underscore the importance of hydrogen bonding, stacking, and complementarity of both electrostatics and shape in small molecule:RNA recognition.

Computational and experimental techniques have been developed to analyze selective small molecule:RNA interactions. As computational docking studies have typically modeled a single biomacromolecule conformation with likely binding partners, the ability of RNA to adopt multiple conformations has hindered such investigations.^{106,139–146} To address this issue, the Al-Hashimi laboratory constructed an ensemble of RNA structures derived from NMR and molecular dynamics (Figure 3).^{98,101,122,147,148} Specifically, NMR studies are used to determine the structural constraints of RNA in solution, which are then utilized to select appropriate conformations found through molecular dynamics simulations of the RNA structure. Docking studies with this ensemble of conformations thus offer improved predictive power because small molecules are virtually screened against RNA structures that better resemble those present in solution. These studies have successfully identified small molecule leads for HIV-1 trans-activation response element (TAR) RNA and are being continually refined via comparison with experimental screens.^{122,148}

A range of experimental assays have also been used to elucidate small molecule:RNA interactions, including assays that compare multiple small molecule:RNA binding events as well as structural studies of complexes when possible. While certainly not exhaustive, a detailed list of select methods and references that have been used to investigate recognition

can be found in Table 1. Importantly, each of these methods has been employed to study multiple RNA constructs, and each can yield different biophysical insights. For example, ITC employs the heat of binding to calculate thermodynamic parameters for small molecule interactions, allowing for determination of dissociation constants. Thermal denaturation studies analyze the shift in RNA melting temperatures (T_m) in the absence and presence of a small molecule, which are presumed to reflect changes in the stability of the RNA structures. SPR experiments can be utilized to examine the kinetic parameters that influence small molecule:RNA interactions. Conformational changes are often investigated via FRET through the incorporation of paired fluorophores at RNA locations sensitive to such changes to reveal if the RNA structure is altered upon small molecule binding. FRET can also be employed within a two-body system where the FRET fluorophore pairs are covalently attached to the RNA and the small molecule to monitor ligand binding. Recently, conformational changes and their relationship to selectivity have also been elucidated via second harmonic generation (SHG). Additionally, chemical probing techniques such as SHAPE can be used to globally interrogate site-specific conformational changes in complex RNAs. NMR studies can determine the potential site specificity of small molecules for RNA secondary structures as well as small molecule-induced conformational changes. Recently, band-selective optimized flip-angle short transient heteronuclear multiple quantum coherence (SO-FAST-HMQC) has been used to rapidly identify small molecule-induced perturbations based on ^{13}C and ^1H peak shifts, suggesting potential binding sites of small molecules as well as the specificity of interactions. We note that high-resolution NMR and X-ray diffraction have also been used to reveal important interactions in small molecule:RNA complexes, including a recent example of time-resolved crystallographic measurements, though limited examples are available outside of native riboswitch ligands.

Numerous assays have been used to rapidly generate binding constants and investigate the associated selectivity. Traditional optical methods include (1) site-specific incorporation of a fluorophore into the RNA sequence, e.g., 2-aminopurine or other longer wavelength base mimics, which report on the base solvent exposure; (2) indicator displacement assays (IDAs), which utilize indicators that differentially fluoresce when bound to RNA, allowing for monitoring of small molecule binding without covalent labeling of either binding partner (Figure 4); (3) monitoring of intrinsic ligand fluorescence; and (4) FRET, mentioned above. Other valuable approaches include mass spectrometry and microscale thermophoresis.

Higher-throughput methods that investigate multiple small molecule:RNA interactions can yield valuable information regarding selectivity. In small molecule microarrays (SMMs), several thousand small molecules can be covalently attached to a glass microscope slide surface and then incubated with a fluorescently tagged RNA to rapidly assess binding interactions. Because the same small molecule set can be screened against multiple RNA targets, specificity can be readily determined and promiscuous small molecules identified. A related approach, two-dimensional combinatorial screening (2DCS), generally utilizes a smaller number of small molecules immobilized in agarose and incubation with RNA secondary structure libraries of randomized sequences. Bound sequences are isolated, cloned, and sequenced to reveal the most tightly bound RNA motifs for each small molecule. Several additional methods have been used for ligand discovery and recently reviewed elsewhere.^{8,11,15}

The invaluable techniques listed above are often combined to provide insight into small molecule:RNA binding modes, thermodynamic properties, and binding constants. With the exception of 2DCS, these techniques have examined individual RNAs and/or small molecules rather than elucidating general guiding principles of small molecule:RNA interactions. As discussed below, our laboratory has applied pattern recognition protocols to this question for the first time via PRRSM. This method can rapidly elucidate the ability of multiple small molecules to differentiate a range of RNA structures. We have also used PRRSM to evaluate the influence of environmental factors on this recognition and to determine RNA structural characteristics.

Pattern Recognition of RNA by Small Molecules.

Pattern-based sensing is a technique used for the determination of complex recognition properties in which a suite of receptors with reasonable affinity and selectivity can classify analytes of interest through differential interactions (Figure 5).^{44–47,201,202} Similar to the olfactory and gustatory senses that differentially sense an expansive number of stimuli,²⁰³ pattern-based sensing assays can discriminate among highly similar analytes, including nitrated explosives,²⁰⁴ ions in aqueous solutions,²⁰⁵ tannins in red wines,²⁰⁶ and normal, cancerous, or metastatic cells.^{207,208} While traditional detection methods utilize lock-and-key receptor design to achieve highly specific binding of a single analyte, receptors for pattern-based sensing need only show differential binding and thus can often be purchased commercially or designed with simple and rapid synthetic schemes. Our work has focused on extending pattern recognition research to classify RNA secondary structure and understand the fundamental properties that are important in small molecule:RNA recognition.

Initial experiments examined the ability of well-characterized RNA ligands, specifically aminoglycosides, to differentiate canonical RNA secondary structure motifs.⁴² The canonical secondary structure motifs of RNA have been shown computationally to have a wide range of conformations that are dependent on sequence and the number of nucleotides within the motif. We proposed that PRRSM could test whether the computationally derived topological distinctions were important for differential binding of small molecules. As a training set, we identified a set of 16 RNA sequences with a range of well-predicted secondary structure motifs and both variable sequence and nucleotide length (excluding the internal loops and stems, which maintain the same number of nucleotides within the secondary structures) (Figure 6A). To measure binding of this set, we developed a 384-well plate assay utilizing the solvatochromic chemosensor benzofuranyluridine (BFU).²⁰⁹ The BFU fluorophore mimics uridine in an RNA structure, allowing for replacement of a native uridine base with a minimal change in base pairing. Each RNA construct was thus chemically synthesized, and a BFU was inserted at a computationally predicted flexible site in the hopes of achieving maximal signal changes upon small molecule binding. The fluorescence was measured upon exposure to 11 aminoglycosides at various concentrations, and the data were used as input for principal component analysis (PCA), which revealed an unbiased clustering of each secondary structure class (Figure 6B). Additionally, the clusters were quantitatively analyzed by leave-one-out cross validation (LOOCV).²¹⁰ Specifically, an R-script was designed utilizing a naïve Bayes algorithm to remove randomly selected data

sets, and then the ability of the remaining experimental data to recapitulate the PRRSM clustering was examined iteratively. Through LOOCV, PRRSM could predict secondary structure motifs with 100% accuracy. In addition, some separation of individual sequences was observed (Figure 7). These results suggest that aminoglycosides, while often characterized as promiscuous RNA ligands, can sense topological differences among these RNA structures.

To understand why the RNA structures were clustered differently, we examined the small molecule and RNA structures. One of the most widely used small molecule structural comparison techniques is Tanimoto coefficient comparison, in which path-based fingerprints are compared between small molecules.²¹¹ On the basis of literature standards, structures with a Tanimoto coefficient of >0.85 were deemed to be significantly similar, any structure with values from 0.55 to 0.85 was not significantly similar, and any structure with a Tanimoto coefficient of <0.55 was seen to be structurally different (Figure 8). Because our receptors are within the same small molecule class, i.e., aminoglycosides, it is unsurprising that many of the receptors were structurally similar with major differences seen in a small subset of receptors (sisomicin and 2-deoxystreptamine). We then compared the Tanimoto coefficients to the loading factors of the principal components, which can be used to interpret the differential binding propensities of the receptors for the RNA motifs. In some cases, significantly similar structures revealed highly similar binding tendencies (kanamycin, neamine, and neomycin), but in other cases (guanidino-kanamycin and guanidino-paromomycin), similar structures displayed somewhat different binding tendencies. In addition, little correlation between loading factors and standard physicochemical properties (total charge, molecular weight, etc.) was identified. These results are in line with suggestions that aminoglycoside recognition depends on the three-dimensional properties of the ligand.

Insights into the RNA properties that allow for small molecule differentiation could also be gained through this initial PRRSM study. In general, differential RNA recognition is achieved by proteins through a combination of sequence- and topology-dependent binding, which allows for high selectivity and binding affinity.^{112,130,134,212} We used PRRSM to evaluate the importance of sequence and topology in small molecule differentiation of RNA, where far fewer interactions can be utilized to achieve affinity and specificity. By examining the PRRSM data for the 16 individual sequences, we identified rough correlations among the principal component axes for both sequence and motif size (Figure 7). The largest amount of variance within the data correlated with the motif size of the RNA secondary structures (PC 1, 81.22%), while sequence dependence was second in importance (PC 2, 12.09%). These data, along with motif-based clustering, support previous work showing that small molecule:RNA differentiation, at least among aminoglycosides, is primarily dependent on the topology of the RNA structure.¹⁰⁴

These initial findings inspired a range of other research directions, including how modulation of RNA topology impacts differentiation by small molecules. One way to modulate RNA topology is by changing buffer conditions.²¹³ For example, it was previously established that the stability of RNA secondary and tertiary structure can be altered through binding of monovalent and divalent cations, the presence of molecular crowders, and

variations in temperature.^{214,215} We thus evaluated the impact of environmental factors on RNA molecular recognition by employing the PRRSM assay under a range of different buffer conditions (Table 2).

Using predictive power as an indicator of RNA differentiation, we evaluated a set of common buffer conditions utilized in small molecule:RNA assays, biologically relevant conditions, and conditions expected to ablate aminoglycoside binding. Unsurprisingly, a high sodium concentration (140 mM Na) and a low pH (pH 5) were found to significantly decrease the level of differentiation as high salt screens electrostatic interactions and lower pH changes the protonation states of the aminoglycosides. Relative to the original conditions, removal of magnesium, a relatively neutral pH, and changing the buffer composition (phosphate vs Tris) had a minimal effect on differentiation. On the other hand, addition of polyethylene glycol (PEG) and increasing the temperature from 25 to 37 °C increased the level of differentiation. This result was surprising as PEG and an increased temperature are known to destabilize secondary structure motifs.²¹⁶ Conversely, a decreased level of differentiation was observed with an increased magnesium concentration, which would be expected to stabilize secondary structure, though an increased level of competition between the magnesium ions and the aminoglycosides for RNA binding cannot be ruled out.^{217,218} Taken together, this study suggested that conformationally dynamic RNA secondary structures are better differentiated by small molecules. In the context of RNA molecular recognition, these results imply that a conformational selection or induced fit mechanism is valuable for differential recognition of RNA structural motifs by small molecules.^{161,192,219} Furthermore, these findings are consistent with previous work suggesting that dynamic RNA structures sample a defined set of available conformations and that these defined sets can be unique for a given RNA structure, thus allowing differentiation.^{104,116,161,220}

In addition to providing a better understanding of RNA molecular recognition, we evaluated whether the classification power of PRRSM could generate insight into site-specific RNA structure. We analyzed biologically relevant RNA constructs of known structure with multiple secondary structure motifs and/or inducible conformational changes.²²¹ We fluorescently labeled each construct with BFU at nucleotide positions of interest. To begin, we examined a truncated version of the HIV-1 TAR RNA consisting of a three-nucleotide bulge and a six-nucleotide hairpin. Second, we assessed the prequeuosine-1 riboswitch (PreQ1) and fluoride riboswitch (FR), which undergo conformational changes in the presence of the respective analyte.^{102,107,108,113,222,223}

The TAR, PreQ1, and FR RNA constructs were synthesized with the BFU fluorophore at different, biologically important sites and were studied with the PRRSM assay (Figure 9). Within the RNA sequences, TAR was modified at a single position in two respective constructs, and both riboswitches were modified at three different positions within three respective RNA constructs. Of the eight RNA constructs analyzed, six of the constructs were classified as expected on the basis of the known ligand-bound structures. The two constructs that did not cluster as expected were the U6- and U11-modified fluoride riboswitches. In the folded state, the U6-modified fluoride riboswitch showed no folding via PRRSM, which was consistent with previous work showing that a U6–C6 mutation prevented proper folding and was confirmed via NMR.¹¹³ The U11–fluoride riboswitch construct did not cluster with any

known secondary structure classes, which could again indicate incomplete folding given the proximity of the modification to the binding site or perhaps a limitation of the training set used. The first possibility was supported by a PRRSM experiment using mixtures of RNA sequences closely representing the two states as a mimic of incomplete folding that demonstrated similar results, and this was also confirmed via NMR. On the basis of these results, we determined that PRRSM can classify a range of RNA structures, including folded and unfolded states, and provide insight into sites that are critical for these structural changes. Overall, PRRSM has provided a new understanding of RNA molecular recognition, evidence of differential binding of small molecules based on RNA topology, and an orthogonal technique for analyzing RNA secondary structure.

Future Work.

The insights gained via PRRSM to date and the robustness of the assay suggest a wide range of future applications for this technique. To date, we have utilized only aminoglycoside receptors, and although an important class of RNA binding small molecules, these receptors can be more difficult to tune for specificity and favorable biological activity relative to more traditional “drug-like” small molecules. Our next steps toward understanding general guiding principles for non-aminoglycoside small molecule recognition will utilize a new, chemically diverse set of receptors. For example, PRRSM has the potential to reveal the impact of the binding modes of aromatic, aliphatic, and/or charged ligands on RNA differentiation. Additionally, diverse receptors are likely to have greater differential binding, potentially creating more spatially separated clustering and allowing more complex structures to be analyzed. As well as aiding in understanding recognition differences, PRRSM can determine the environmental conditions that are important for modulation of RNA binding with more diverse chemical structures.

PRRSM is currently based on a fluorescent plate reader assay utilizing the covalently attached solvatochromic fluorophore BFU, which may impact tertiary interactions and must be incorporated through solid phase synthesis. For applications in which the label may interrupt native structure, one potential solution is an indicator displacement assay, allowing for classification of RNA secondary structure with unmodified RNA. For applications involving large RNAs that cannot be chemically synthesized, small molecule microarrays may be employed. These arrays can be used to visualize hundreds to thousands of small molecules binding to RNA structures, though with a single RNA at a time, via enzymatic modification of the 3'-end. For applications in which site-specific information about a large RNA is critical, ligation of a chemically synthesized fragment is under investigation.^{224–226}

PRRSM could also evolve to inform the recognition of RNA tertiary motifs. A wide range of tertiary structures is formed through secondary structure motif interactions, including pseudoknots, kissing loops, and G-quadruplexes. Similar to that of RNA secondary structure, utilization of a training set of previously characterized RNA tertiary structures with an array of differentially binding small molecule receptors would allow clustering based on distinctive interactions with these tertiary structures. After an initial RNA training set is designed and tested, RNAs with unknown tertiary structures can be analyzed. In addition to classification, recognition principles for RNA tertiary structures could be probed

and explored, allowing an improved understanding of important interactions that can be exploited for RNA-targeted small molecules.

We further envision the implementation of PRRSM to classify RNA function. An overwhelming number of RNA sequences thought to be important in regulating both normal and diseased states lack a known molecular function, in part due to the time-consuming nature of this process. Because PRRSM can classify RNA structure, and RNA structure is often related to RNA function, we propose that small molecules can be used to directly classify function. Such an assay will likely require a large set of diverse small molecule receptors such as those that can be displayed on a microarray, which will also allow for rapid analysis of binding. Again, on the basis of a known training set, this one step assay could be used to narrow the set of likely functions for a particular RNA of interest, significantly focusing more in-depth analysis of molecular function.

In conclusion, the field of RNA molecular recognition has multiple tools for examining single small molecule:RNA interactions. Our technique, PRRSM, can discretely assess a wide range of RNA secondary structures and small molecule receptors that together provide a better understanding of the guiding principles of these interactions. Our research, along with that of others, has shown that RNA topology as well as sequence is important for distinguishing small molecule binding, and modulating the RNA topology has a strong effect on the small molecule binding propensities. On the basis of initial data classifying biologically relevant RNA structure, PRRSM is a promising technique for examining tertiary motifs as well as for classifying the most likely function for new RNA structures. The future of RNA molecular recognition and RNA targeting is dependent on understanding small molecule binding in the context of RNA three-dimensional structure and dynamics, and PRRSM is uniquely positioned to contribute to this understanding.

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Figure 1.

Pattern recognition of RNA by small molecules (PRRSM). An array of small molecule receptors is titrated with RNA secondary structure analytes. Utilizing the small molecule differential binding and an unbiased statistical method allows for clustering based on the RNA structural motifs.

Figure 2. Overview of SHAPE reactivity. 2'-Hydroxyl acylation reactivity is dependent on the local environment, where constrained base pair nucleotides are unreactive compared to flexible nucleotides within secondary structure motifs. SHAPE reactivity based on nucleotide position provides additional constraints for structure prediction models. Adapted with permission from ref 70.

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Figure 4. Indicator displacement assay utilizing a peptide indicator with a covalent fluorophore, which, upon binding to RNA, can fluoresce. The displacement of the fluorophore peptide by λ N protein, inducing quenching of the peptide fluorophore. Adapted from ref 182.

Figure 5.

Example of a pattern recognition assay. (A) An array of receptors is tested for background interference. (B) An analyte (beer in this example) is examined with the receptor array, and the background signal is removed. (C) The resulting pattern is used to classify potential beer analytes. (D) Utilizing the same receptor array, additional analytes can be analyzed, such as whisky, for example. (E) After the assay is repeated and the background removed, a differential pattern will form for whisky as compared to beer. Reproduced from ref 202. Copyright 2010 The Royal Society of Chemistry.

Figure 6.

(A) Secondary structure of 16 RNA analytes determined computationally, with the BFU chemosensor shown in each structure (blue star). The remaining sequences outside the variable secondary structures were kept constant to allow consistent comparisons.

Abbreviations: Blg, bulge; AIL, asymmetrical internal loop; IL, internal loop; HP, hairpin.

(B) PCA plot of the RNA secondary structure motifs based on aminoglycoside differential binding. The predictive power of the assay was determined to be 100%.⁴²

Figure 7.

All 16 RNA training set sequences, stems, bulges (Blg), the internal loop (IL), the asymmetrical internal loop (AIL), and hairpins (HP). PC 1 correlated to the increasing motif size (from stem to AIL), while PC 2 correlated to the purine:pyrimidine ratio, which is dependent on the sequence of the RNA (HP to IL).⁴²

A.					B.			
> 0.85		< 0.55			Loading Factors			
Neom		2DOS	Siso			PC 1	PC 2	PC 3
0.931	Kana	0.257	0.215	Amik	2DOS	0.948	0.184	-0.177
0.889	Neam	0.321	0.490	Apra	Amik	0.902	-0.390	-0.108
		0.267	0.550	D-Strep	Apra	0.938	0.064	-0.230
Neam		0.300	0.454	G-Kana	D-Strep	0.823	0.539	0.024
0.926	Kana	0.284	0.469	G-Paro	G-Kana	0.876	-0.337	0.155
		0.403	0.463	Kana	G-Paro	0.871	-0.020	0.447
G-Paro		0.422	0.545	Neam	Kana	0.948	-0.299	-0.042
0.867	D-Strep	0.375	0.521	Neom	Neam	0.949	-0.289	-0.027
0.947	G-Kana	0.243	0.536	Strep	Neom	0.949	-0.291	-0.035
		0.215		Siso	Siso	0.869	0.433	-0.001
Strep					Strep	0.826	0.539	0.043
0.910	D-Strep							

Figure 8.

(A) Comparison of the Tanimoto coefficients of the aminoglycoside receptors. Significantly similar structures are colored dark green (>0.85), and structurally different structures light green (0–0.55). All other aminoglycosides were moderately similar based on the Tanimoto coefficients (0.55–0.85). (B) Loading factors of the 11 aminoglycoside receptors for the first three principal components. Abbreviations: 2DOS, 2-deoxystreptamine; Amik, amikacin; Apra, apramycin; D-Strep, dihydrostreptomycin; G-Kana, guanidino-kanamycin; G-Paro, guanidino-paromomycin; Kana, kanamycin; Neam, neamine; Neom, neomycin; Siso, sisomicin.⁴²

Figure 9. PCA plot of PreQ1 U9 and FR U9 BFU constructs with and without a ligand. Also, U25 and G33 TAR BFU constructs cluster for bulge and hairpin secondary structures, respectively.²²¹

Table 1.

Methods for Assessing Small Molecule:RNA Interactions

method	obtainable data	example refs
isothermal titration calorimetry (ITC)	binding constants, thermodynamic binding parameters, stoichiometry	149–152
thermal denaturing studies	small molecule stabilization of RNA structure	153, 154
surface plasmon resonance (SPR)	binding constants, kinetic parameters	155–159
Förster resonance energy transfer (FRET)	ligand-induced conformational change, binding constants	20, 38, 103
second harmonic generation (SHG)	ligand-induced conformational changes, binding constants	160, 161
selective 2'-hydroxyl acylation analyzed by primer extension (SHAPE)	site-specific ligand-induced conformational changes	162–165
nuclear magnetic resonance (NMR)	secondary and tertiary structures of RNA, insight into potential binding sites and conformational changes, binding constants	23, 40, 100, 101, 110, 111, 118, 147, 166–169
X-ray diffraction	secondary and tertiary RNA structures, small molecule binding sites, insight into targetable structures within the RNA	26, 27, 49, 170–174
fluorophore incorporation	site-specific binding interactions, binding constants	175–180
indicator displacement assay (IDA)	binding constants	181–185
intrinsic ligand fluorescence	binding constants	159, 186–188
mass spectroscopy	stoichiometry, binding constants	161, 189–191
microscale thermophoresis	binding constants	192
small molecule microarray (SMM)	specific small molecule ligands	164, 193, 194
two-dimensional combinatorial screening (2DCS)	specific small molecule RNA binders, insight into RNA-targeted small molecule chemical space	12, 138, 195–200

Table 2.

Predictive Power of the RNA Training Set under Varying Conditions

buffer	predictive power (%)
standard	59.5
pH 5 ^a	19.2
pH 6 ^a	50.8
pH 8 ^a	49.8
140 mM Na ^a	10.5
0 mM Mg ^a	47.9
10 mM Mg ^a	29.2
Tris ^b	57.1
37 °C ^a	39.6
PEG ^c	65
PEG/37 °C ^c	71
PEG no stem ^c	85
PEG/37 °C no stem ^c	92

^aIn 10 mM NaH₂PO₄, 25 mM NaCl, 4 mM MgCl₂, and 0.5 mM EDTA at pH 7.3 and 25 °C unless otherwise indicated.

^bIn 10 mM Tris, 25 mM NaCl, 4 mM MgCl₂, and 0.5 mM EDTA at pH 7.3.

^cIn 10 mM NaH₂PO₄, 25 mM NaCl, 4 mM MgCl₂, 0.5 mM EDTA, and 8 mM polyethylene glycol (PEG) 12000 at pH 7.3 and 25 or 37 °C.