Application Note
MAR 2024
AN-850-0142

Structural Characterization of RNA and Detection of RNA-ligand Binding Using Microfluidic Modulation Spectroscopy

- ○ Biosimilars
- ○ mAbs
- ○ ADCs
- ○ AAVs
- ✓ Ligand Binding
- ○ Protein/Peptide Analysis
- ○ VLPs
- ✓ Nucleic Acid
- ○ Fusion Proteins
- ○ Enzyme Analysis

- ⬡ Aggregation
- ⬡ Quantitiation
- ✓ Structure
- ⬡ Stability
- ✓ Similarity

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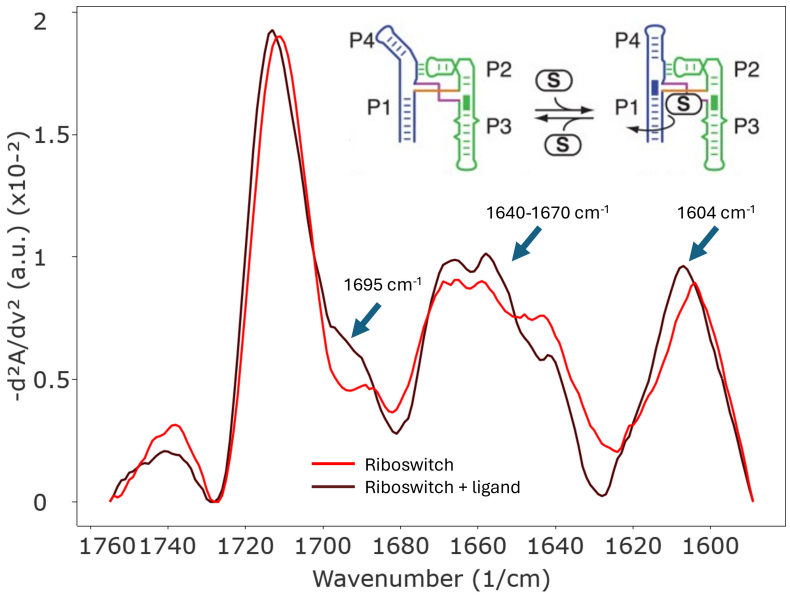
Abstract

Riboswitches are small, well folded mRNA sequences that control the translation of specific mRNA segments for regulation of protein production. Each riboswitch has a unique small molecule that induces structural changes upon binding and results in activation of the riboswitch. In this study, we used X-ray crystallography data from the literature and MMS to characterize the structural changes of the SAM-I riboswitch associated with ligand binding and determine the apparent dissociation constant, Kd. MMS provides a better overall understanding of not only binding affinity, but also structural change associated with binding. This technique can be useful in the development of novel small molecular regulators of RNA for therapeutic purposes.



Introduction

Within the complex network of molecular interactions occurring within cells, RNA molecules play versatile roles that extend beyond their conventional function as mediators in gene expression. Among these various functions, RNA riboswitches are known as critical regulatory components governing gene expression in reaction to small molecule ligands. The dynamic interplay between RNA and ligands lies at the heart of numerous biological processes, spanning from microbial pathogenesis to human disease pathways.[1] Understanding RNA-ligand binding through riboswitches may help elucidate the fundamental mechanisms of gene regulation and open avenues for targeted therapeutic interventions.

RNA molecules have transcended their traditional roles as mere messengers of genetic information, evolving into sophisticated regulators of gene expression through intricate structural motifs known as riboswitches. Among these, the S-adenosylmethionine (SAM)-I riboswitch regulates gene expression in response to fluctuations in intracellular SAM concentrations.[2] SAM, a key metabolite involved in numerous cellular processes, serves as both a cofactor and a regulatory molecule, exerting important effects on diverse biological pathways, such as transmethylation, transsulfuration, and polyamine synthesis.[3]

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Introduction, continued

The SAM-I riboswitch, with its unique specificity and affinity for SAM, serves as a molecular sentinel, modulating gene expression to maintain cellular homeostasis and adapt to environmental cues.

The SAM-I riboswitch comprises two distinct domains: an aptamer domain responsible for SAM recognition (Figure 1) and a downstream expression platform governing gene expression. Upon SAM binding, the aptamer domain undergoes conformational rearrangements, transmitting signals to the expression platform to modulate transcriptional or translational outputs (Figure 2). This allosteric regulation mechanism, inherent to riboswitches, provides cells with a rapid and precise means of sensing and responding to fluctuations in SAM levels, thereby fine-tuning metabolic pathways and ensuring cellular fitness.[4]

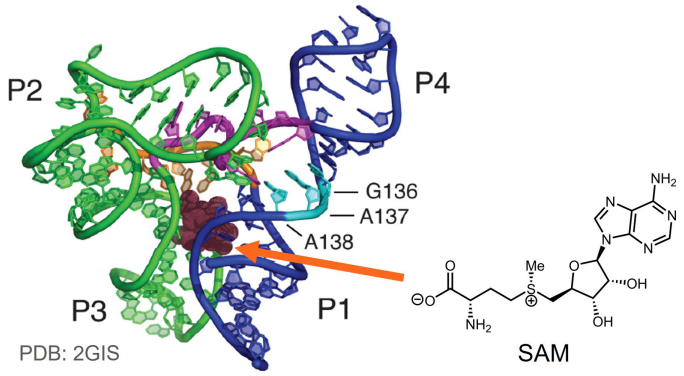

Figure 1. X-ray crystal structure of SAM-I riboswitch with a bound ligand (PDB: 2GIS) and the chemical structure of SAM. Adapted from ref. 4. Copyright 2017 by the RNA Society.

The structural and functional elucidation of the SAM-I riboswitch has offered unprecedented insights into the dynamic interplay between RNA and ligand molecules. Structural studies employing X-ray crystallography, nuclear magnetic resonance (NMR) spectroscopy, and cryo-electron microscopy (cryo-EM) have revealed the comprehensive picture of the SAM-binding pocket and the allosteric communication pathways within the riboswitch. Furthermore, biochemical assays, coupled with biophysical techniques such as fluorescence spectroscopy and single-molecule imaging, have unveiled the kinetic parameters governing SAM binding and riboswitch folding dynamics. However, all these techniques only provide either structural information or binding information on the RNA-ligand complex.

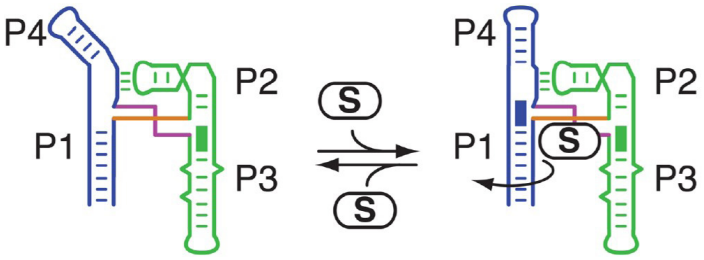

Figure 2. Binding induced conformational changes in SAM-I riboswitch. Upon binding of SAM at the four-way junction, the P1 domain rotates to bind the ligand, which in turn causes a large allosteric change on the P4 domain. Adapted from ref. 4. Copyright 2017 by the RNA Society.

In this study, Microfluidic Modulation Spectroscopy (MMS) is used to study the structural change of the SAM-I riboswitch upon binding. MMS probes the nucleic acid bases in the Amide-I IR-range to interrogate the base pairing/stacking upon RNA folding and unfolding. The Amide-I band has been conventionally used to study the secondary structures of proteins. Yet, this IR region also contains rich structural information on the nucleic acid bases which has been largely overlooked. For yielding accurate, real-time background correction, MMS continuously modulates against the reference buffer. The sensitivity provided by this technique makes it especially useful for quality control, and it also has compatibility with a broad variety of formulation buffers. the Apollo MMS system was used for this study. This instrument is equipped with a high-power Quantum Cascade Laser which, compared to traditional FTIR light sources, is substantially more intense. In combination with the modulating background subtraction, this makes MMS about 30 times more sensitive than FTIR and 5 times more sensitive than CD with respect to structural changes.[5]

Methods

SAM-I riboswitches (apo and ligand-bound) were obtained from Arrakis (Waltham, MA). To ensure buffer matching, the RNA samples were buffer-exchanged 3 times to their formulation buffer (20 mM HEPES pH 7.5, 100 mM KCl, 3 mM $MgCl_2$, 0.05% TWEEN-20, and 2% DMSO) and the final eluent was used as the reference buffer. The buffer-exchanged RNA samples were run in triplicate on the Apollo with a final concentration of 0.67 mg/mL (22 µM).

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Methods, continued

A backing pressure of 5 psi was used to move the samples into the flow cell where they were modulated at 1 Hz between sample and reference buffer (using the eluent at the end of the triple buffer-exchange cycle) for background subtraction. The differential absorbance was measured between 1580 -1765 cm^{-1}. Replicates were averaged and all samples were normalized for concentration and interpolated to get the absolute absorbance spectra.

Data processing followed the procedures used in our previous application note.[6] Briefly, the raw differential absorbance was converted to absolute absorbance which is normalized by concentration and optical pathlength. The second derivatives of the absolute absorbance spectra were taken to enhance spectral features. This plot was then inverted and baselined to result in the "similarity plot" where the area of overlap is calculated compared to a control to quantitate similarity between samples.[7]

Results and Discussion

MMS results indicate subtle but detectable structural changes as reflected by the spectral differences (Figure 3) due to ligand binding on the SAM-I riboswitch. The regions of change are peaks at 1690, 1640-1670, and 1604 cm^{-1}. These peaks are assigned mainly to guanine, uracil/cytosine, and adenine, respectively (Figure 4). Specifically, the increase in intensity at 1690 cm^{-1} indicates the guanine C=O stretch in a double-strand or base-paired state. The crystal structure of ligand-bound SAM-I riboswitch (PDB: 2GIS) shows that the binding involves the interactions of SAM to U57, A45, A46, G11, and G58 in the riboswitch. These base-ligand interactions likely contributed to the spectral change at the noted peaks in Figure 3 (with peak assignments in Figure 4).[8]

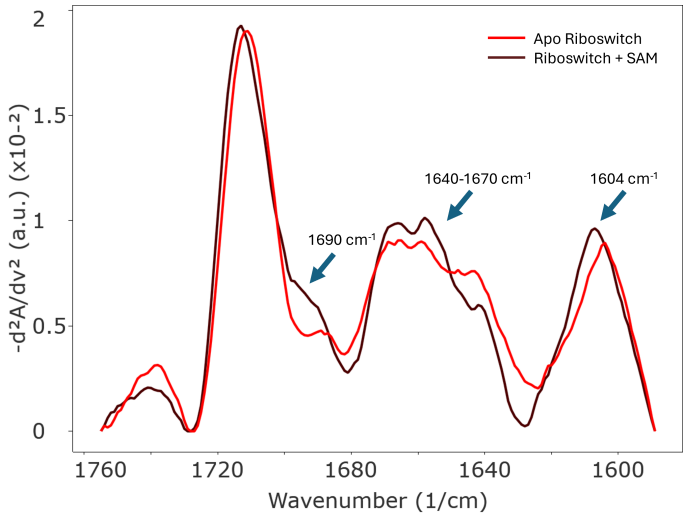

Figure 3. MMS spectra (similarity plot) of the apo riboswitch and ligand-bound riboswitch.

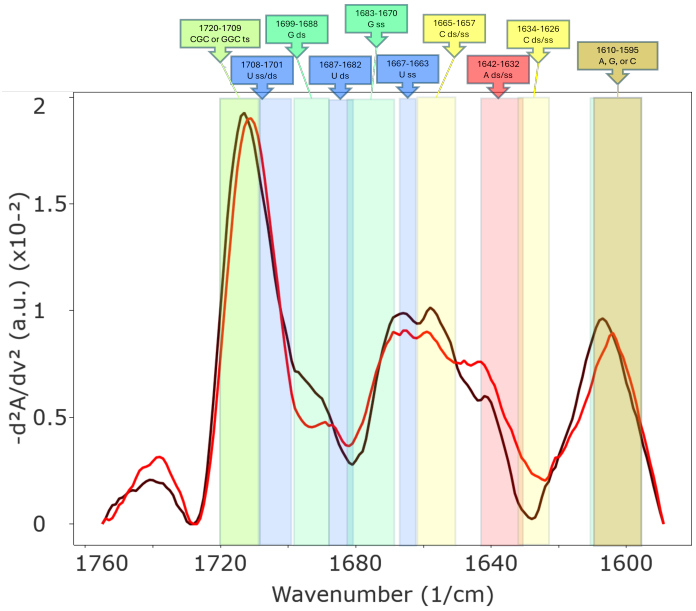

Figure 4. MMS spectra (similarity plot) of the apo riboswitch and ligand-bound riboswitch with peak assignments. (ss: single strand. ds: double strand. ts: triple strand.)

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Results and Discussion, continued

A dose-dependent titration between SAM and the SAM-I riboswitch was also conducted and the samples were structurally characterized using MMS. The MMS spectra in Figure 5 highlight two regions of gradual change upon SAM titration. The first one is the increase at 1695 cm^{-1}, suggesting guanine base-pairing according to Figure 4. In this case, the increasing concentrations of SAM resulted in more interactions between G11, G58 and SAM, and therefore gave rise to an increase in the 1695 cm^{-1} peak. The second region is the 3-wavenumber shift from 1604 to 1607 cm^{-1}. It is not well known in the literature, but in this case the shift is likely due to the interactions of the two adenines (A45, A46) with SAM. It is noteworthy that the spectral change in the 1640-1670 region did not exhibit a clear trend as the SAM concentration increased. The explanation for that may be of a complex nature and requires further investigation.

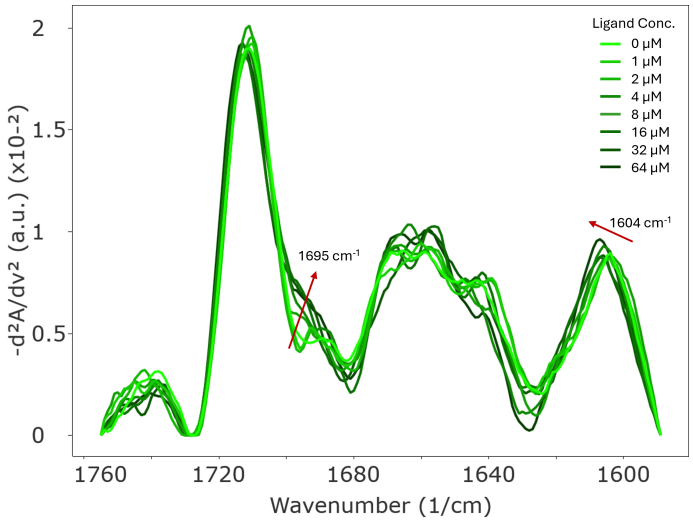

Figure 5. Dose-dependent titration of SAM into SAM-I riboswitch. Gradual change in spectra has been observed as the SAM concentration increases.

To illustrate that the spectral changes observed are significant rather than noises, we used the area of overlap (AO) method as established previously[5,7] to calculate the spectral differences in % similarity between samples. Table 1 shows the repeatability of measurements (in triplicate) versus the sample-to-sample similarity using the AO method. In general, repeatability indicates how well the spectra of the replicates overlay. Therefore, if the similarity between different samples drops below this value, there is significant spectral change. Starting at 4 µM SAM and onward, all the samples are significantly different from the reference (Apo riboswitch).

Table 1. Repeatability of measurement and sample-to-sample similarity (Apo riboswitch as reference: 100% similarity).

Ligand Conc. (µM)	% Repeatability	% Similarity
0	96.6	100
1	96.5	96.6
2	96.2	96.5
4	97.1	95.5
8	96.6	94.6
16	96.0	92.9
32	96.8	92.7
64	96.7	91.8

The question remains whether the changes in spectra were caused by conformational change in the RNA due to ligand binding or the IR signal change from the bound ligand. A plot of the spectral difference (1 – similarity) between the titrated samples and the apo riboswitch is shown in Figure 6. The spectral change (blue line) plateaued after ~20 µM of SAM concentration, which is around 1:1 ligand-to-riboswitch molar ratio, indicating that the spectral change observed in this study was indeed caused by ligand binding. Furthermore, the 1st derivative of the spectral difference (red line) in Figure 6 highlights the SAM concentration where the spectral change happened the fastest at 4 µM. Although this study used a concentration of riboswitch that is much larger than the Kd of the binding, it still demonstrates the utility of MMS as a quick and easy tool to detect both structural change and determine Kd (in the micromolar range) of RNA-ligand binding.

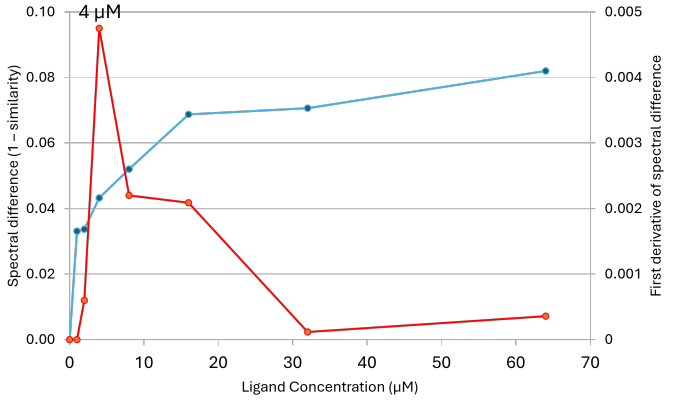

Figure 6. Spectral change in area of overlap (AO) in response to SAM titration. Apo riboswitch was used as the reference. Spectral change increases as the SAM concentration increases. Blue: spectral change. Red: 1st derivative of spectral change.

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Conclusions

This work highlighted the utility of MMS as a viable orthogonal assay for the detection of RNA structural change in the presence of small molecule ligands. It also demonstrated the potential to use MMS to determine apparent Kd of RNA-ligand binding in the micromolar range by sensitively distinguishing structural changes in the RNA riboswitch due to ligand binding.

Contributors

Richard Huang, Ph.D.
Valerie Collins, Ph.D.

RedShiftBio
80 Central Street,
Boxborough, MA 01719

Scott Gorman, Ph.D.

Arrakis Therapeutics
828 Winter Street
Waltham, MA 02451

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