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# Probing RNA Base Pairing and Ligand Interactions In Solution with Infrared-based Microfluidic Modulation Spectroscopy


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Richard Huang

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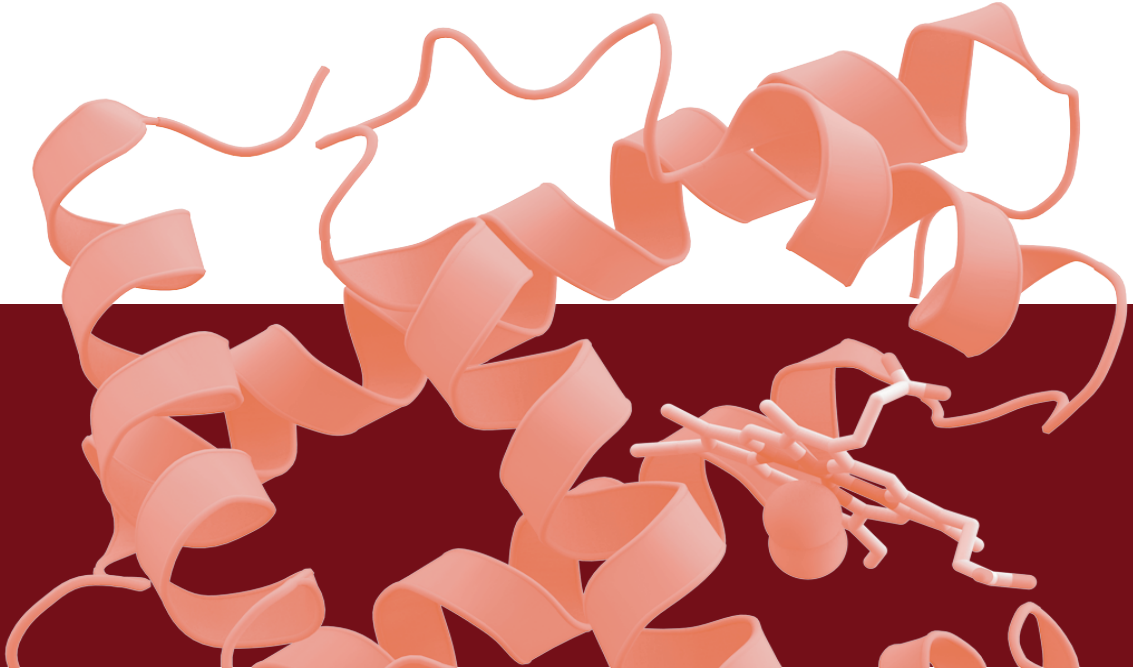
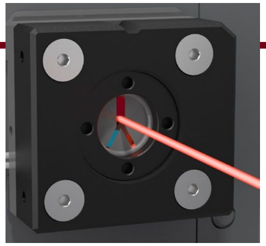
2025.03.11

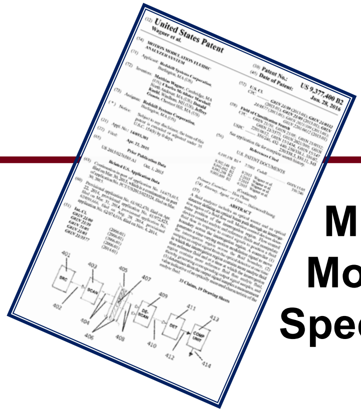


# About RedShift BioAnalytics, Inc.

- **RedShiftBio®:** Massachusetts-based biotech company backed by two of the largest life science instrumentation companies, one of which is Waters.
- **MMS:** Microfluidic Modulation Spectroscopy, a powerful new technology for characterizing biomolecules in fluids.
- **Aurora & Apollo:** Innovative instruments for enhanced characterization and monitoring of therapeutic proteins from development to manufacturing to product release.
- **delta:** Proprietary protein analytical software for both automated and streamlined data analysis.

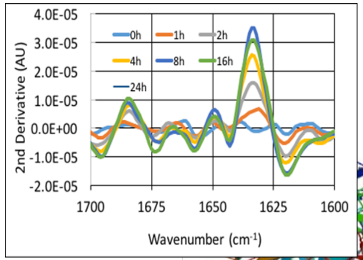


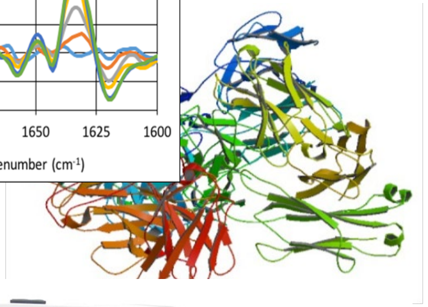
Infrared Optics



Microfluidic Modulation Spectroscopy

Protein Analytics Software



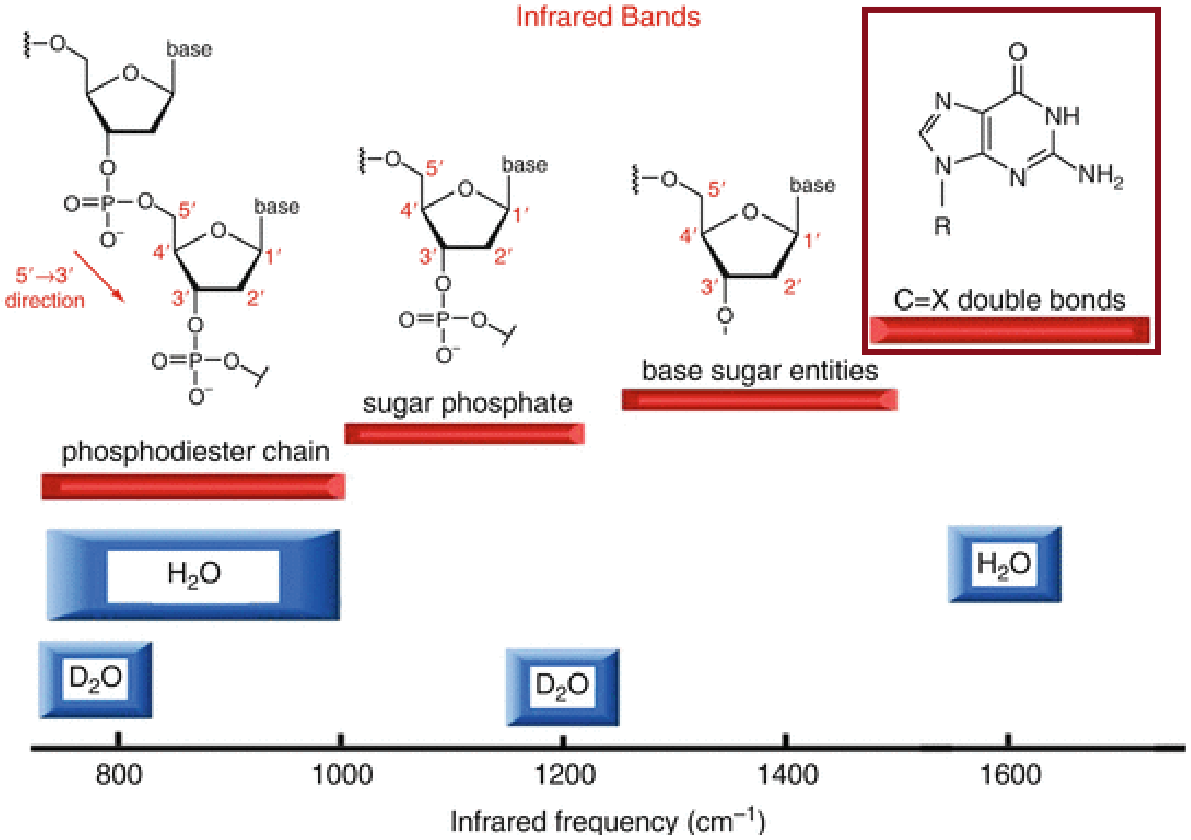



Protein Characterization Solutions


# Microfluidic Modulation Spectroscopy (MMS)

- Unique microfluidics alternates sample and buffer in flow cell
  - The absorbance of the sample and buffer are alternately measured across the Amide I band
  - Differential Absorbance (DiffAU) is recorded
  - Rapid sample-buffer referencing without cell movement provides >98% system repeatability.

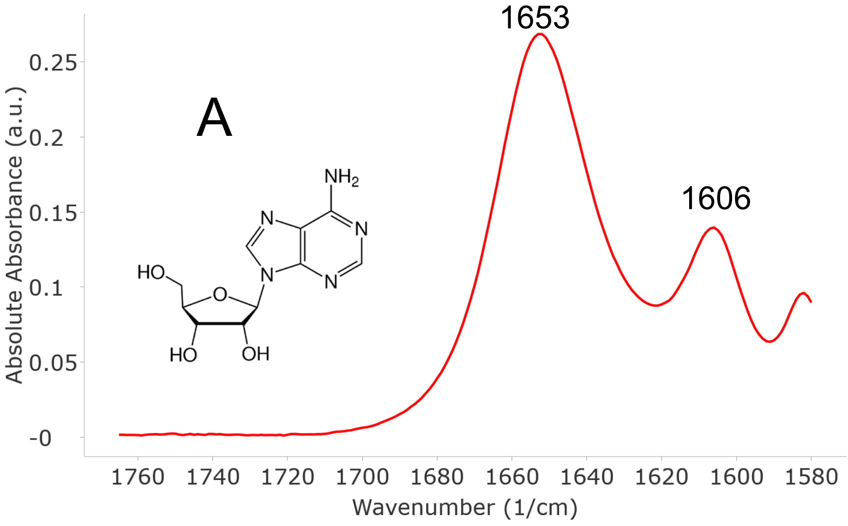
# Nucleic Acid Infrared Bands

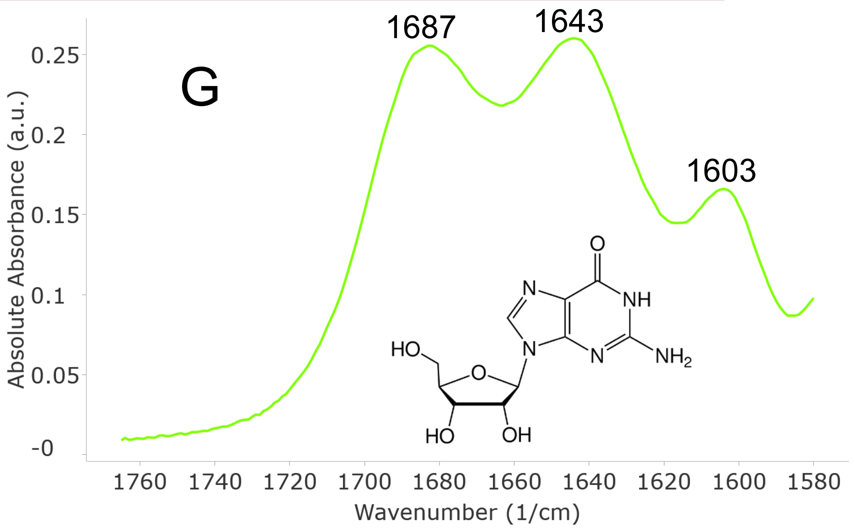


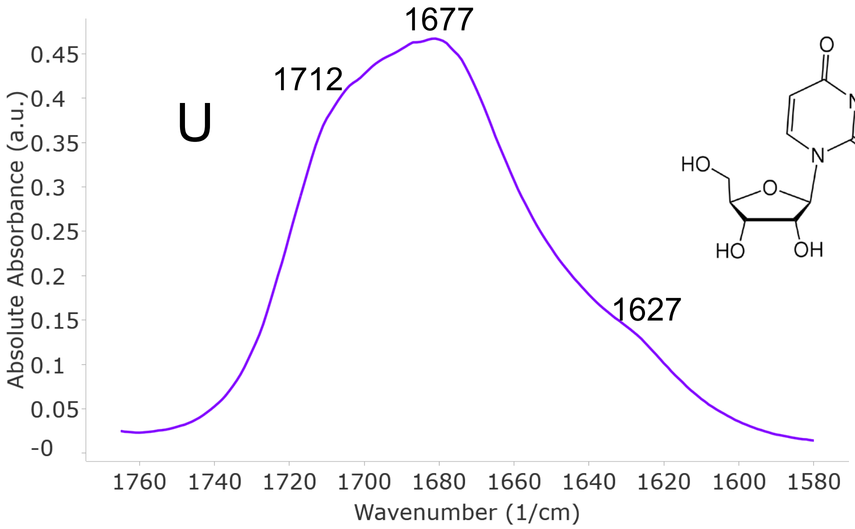

| Assignment | Wave number (cm-1) |
|---|---|
| **Base vibrations** | 1800-1500 |
| Double-helical structures | 1673-1660 to 1689-1678 |
| Thermal denaturation | 1696-1684, 1677-1653 |
| Triple-helical structures | 1800-1500 |
| **Base-sugar vibrations** | 1500-1250 |
| Interaction involving the N7 sites of purines | 1495-1476 |
| Anti/syn conformation | 1381-1369 |
| Sugar conformation | 1344-1328 |
| **Sugar-phosphate vibrations** | 1250-1000 |
| Backbone conformation, PO2- stretching band | B-form double helix ~1225 cm-1<br>A-form ~1240 cm-1<br>Z-form ~1215 cm-1 |
| **Sugar vibrations** | 1000-800 |
| Sensitivity to sugar conformation | N-type sugars, 882-877, 865-860<br>S-type sugars, 842-820 |
| Contribution from POP vibration | 840-800 |

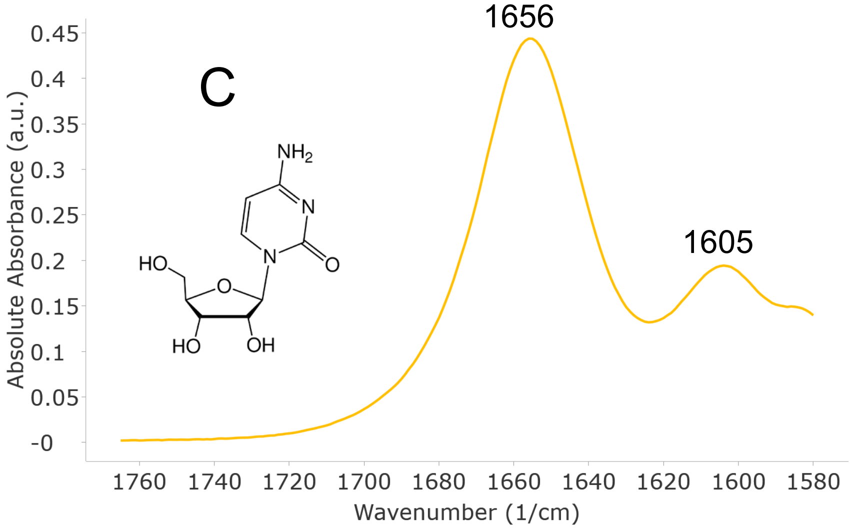
# RNA Building Blocks: Nucleosides MMS data

The nucleosides are the building blocks for RNA and DNA (we're showing A,U,C, and G, but we've also measured T!) and have signature peaks in the amide I band. Using these building blocks, we can predict what sequences will look like and compare to experimental data to observe base-pairing, Hoogsteen pairing, and other higher order structures like triple strands.




**Bonds responsible for these absorption bands:**
**C=O stretch**
**C=C and C=N ring vibrations**

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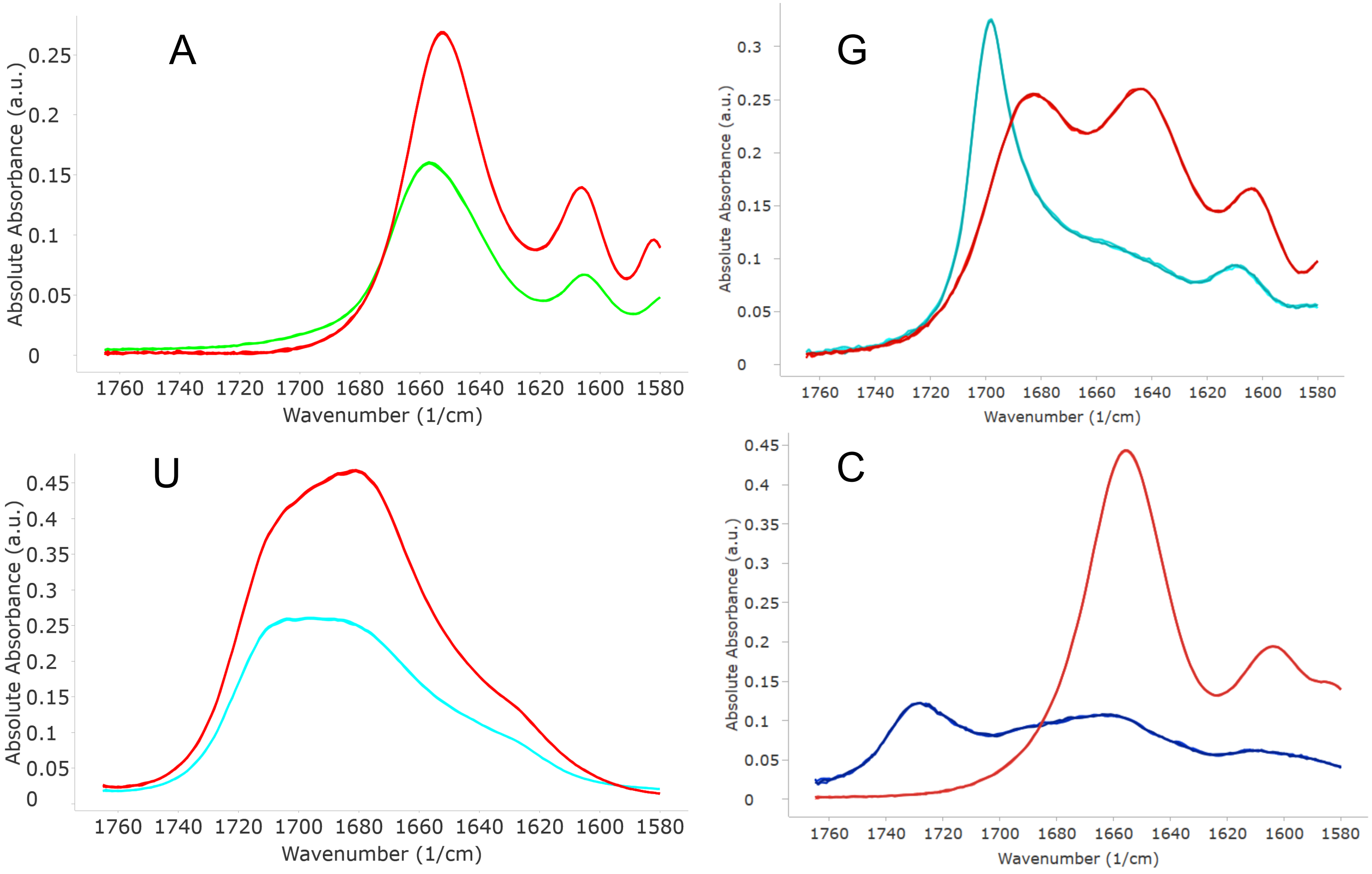
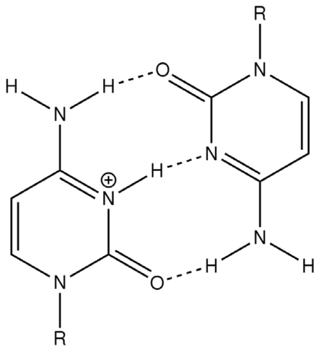
# RNA Polynucleotides gave us a hint that MMS can reveal base pairing patterns

- Red traces are the nucleosides
- Blues/Green colors are the polys
- A and U nucleosides vs polys are similar peaks just different intensities
- G and C have drastic shifts that may be due to more complex higher order structures
- G quadruplex and C i-motif

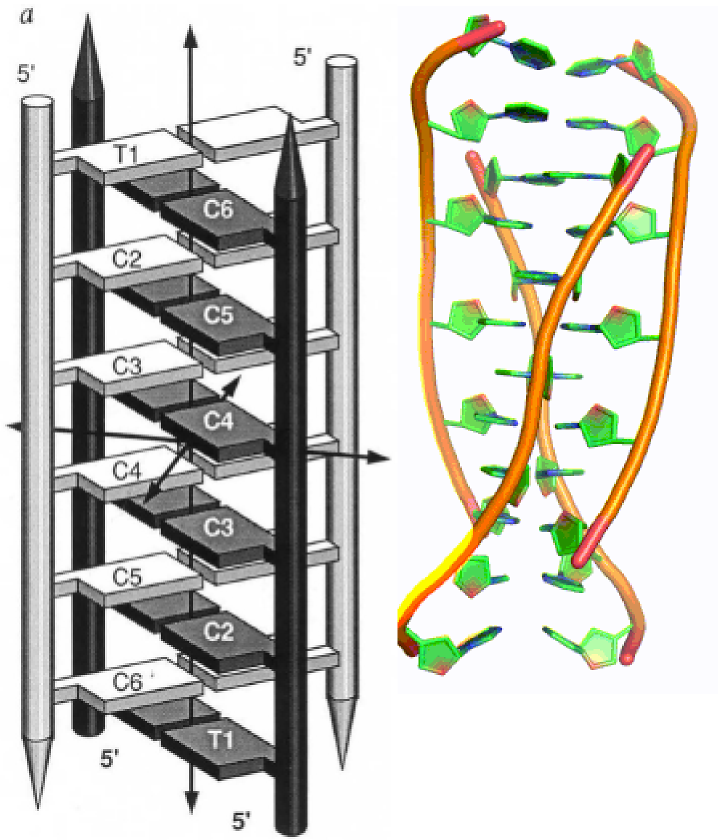


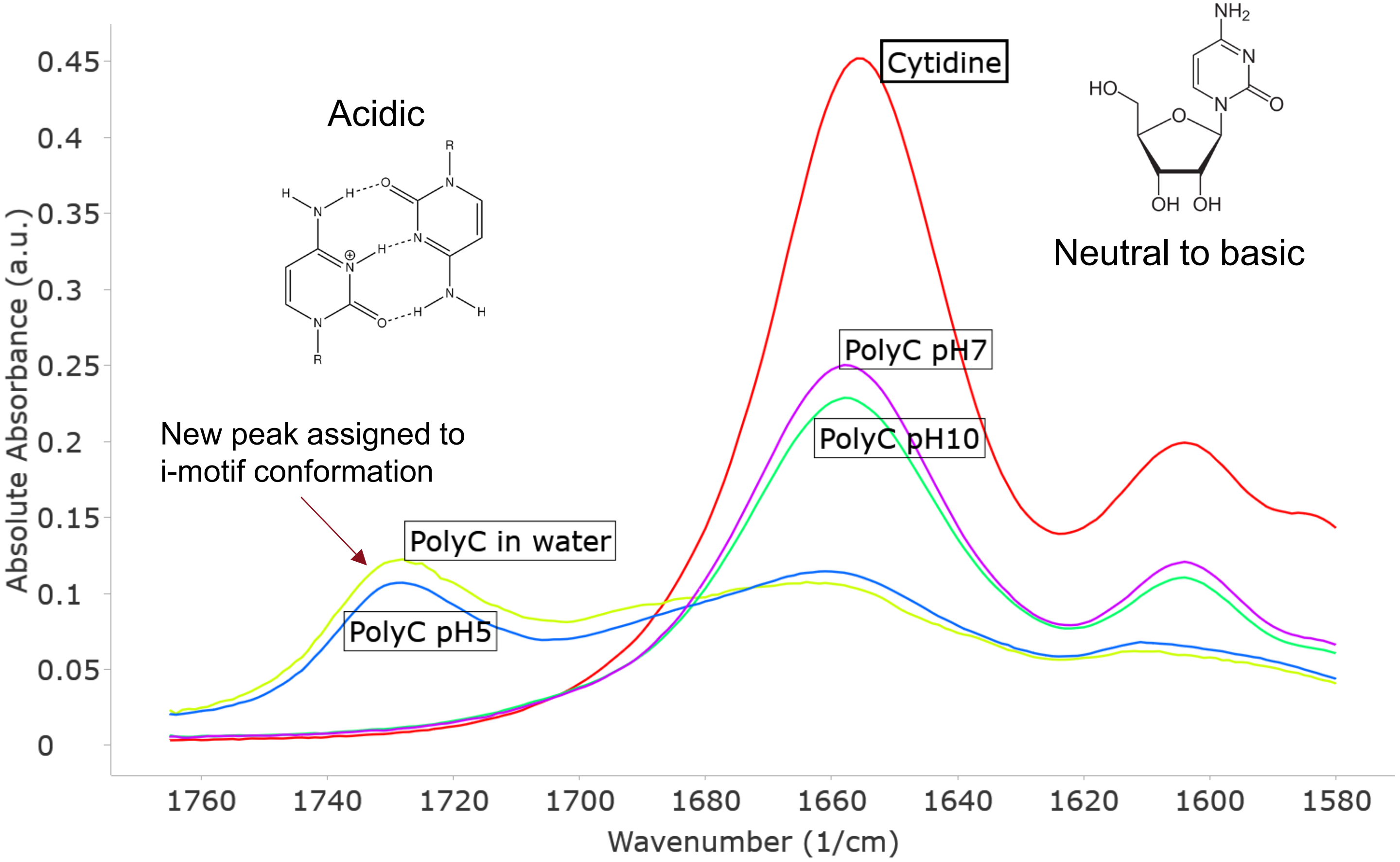
# i-motif and unstructured poly(C) is pH dependent

i-motif (C-quadruplex)



**pH-dependent**



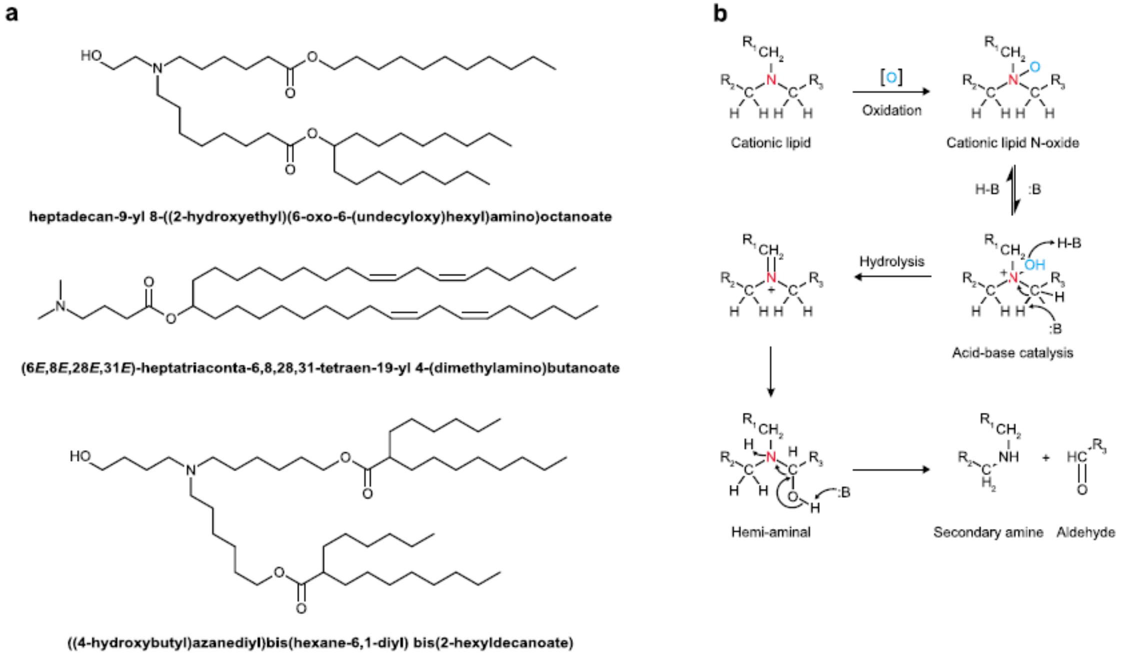

Gehring K, Leroy JL, Guéron M. A tetrameric DNA structure with protonated cytosine.cytosine base pairs. Nature. 1993 Jun 10;363(6429):561-5.

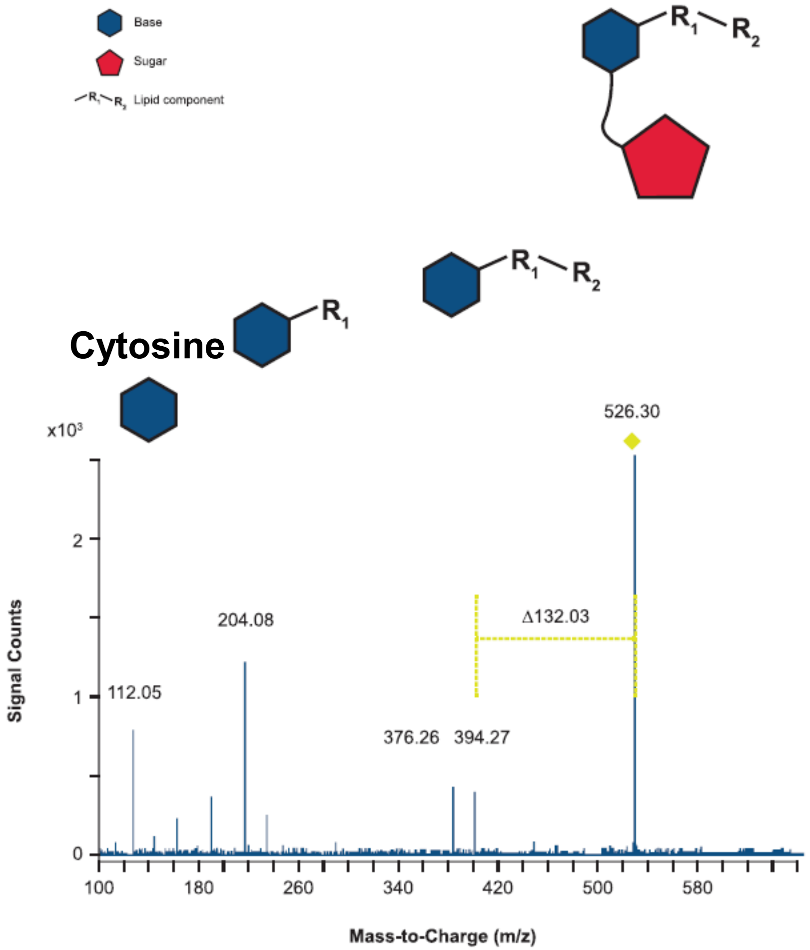
# Case Study: mRNA Impurities in Vaccine Development

Use of MMS to detect lipid adduct formation in formulated mRNA-LNP system

# Ionizable lipids that are necessary for RNA-LNP formation are almost always contaminated with RNA-lipid adduct-forming oxidation products.

- Packer *et al*. found that some mRNAs are modified in LNP formulation.
- Nucleobases are modified by covalently reacted to ionizable lipids.
- These lipid-mRNA adduct impurities reduce the activity of mRNA, causing significant loss of protein expression.
- Also affect the mRNA stability under refrigerated condition.




Packer, M., Gyawali, D., Yerabolu, R. *et al.* A novel mechanism for the loss of mRNA activity in lipid nanoparticle delivery systems. *Nat Commun* **12**, 6777 (2021).

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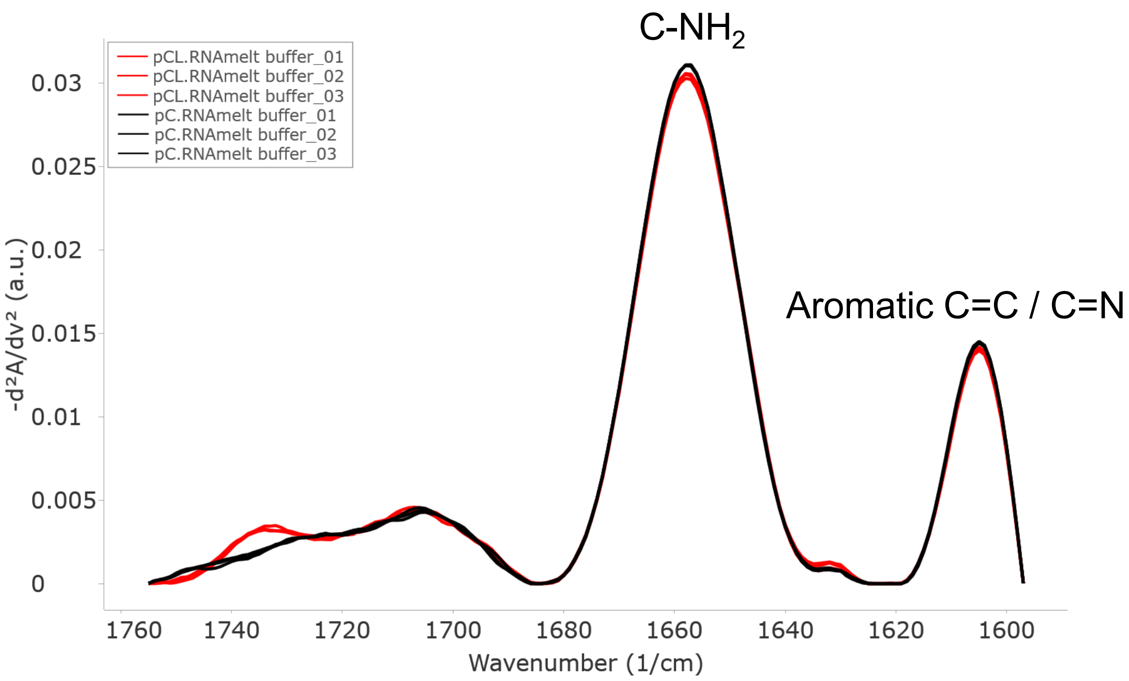
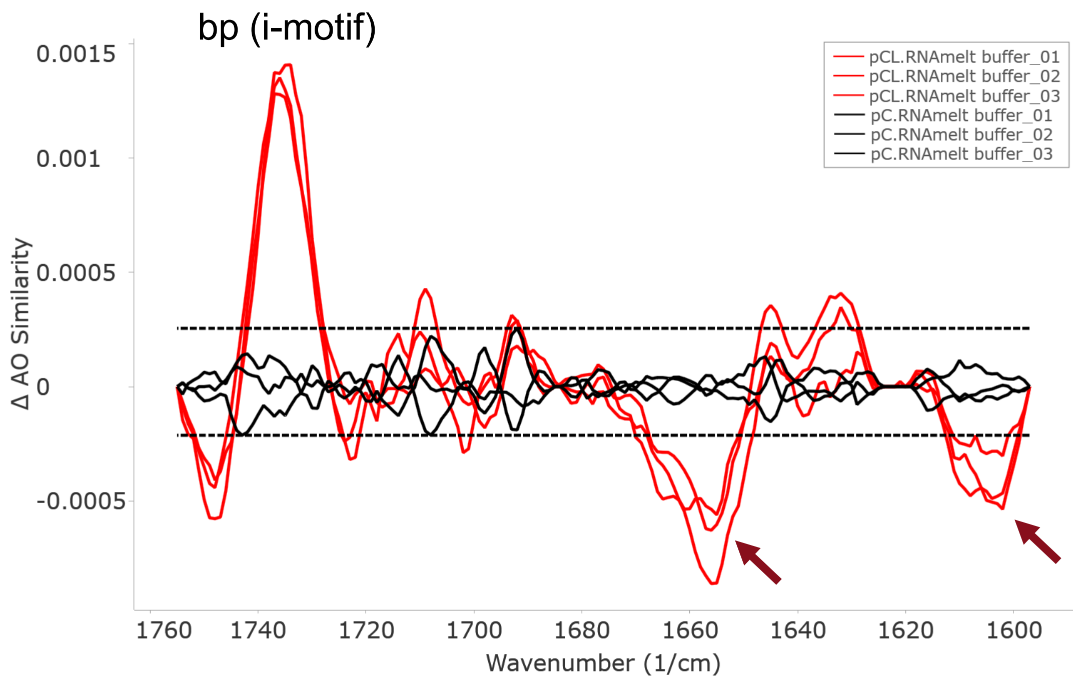
# After forming the adduct between the SM-102 lipid and polyC RNA, we observe minor spectral changes at neutral pH.

## Similarity Spectra



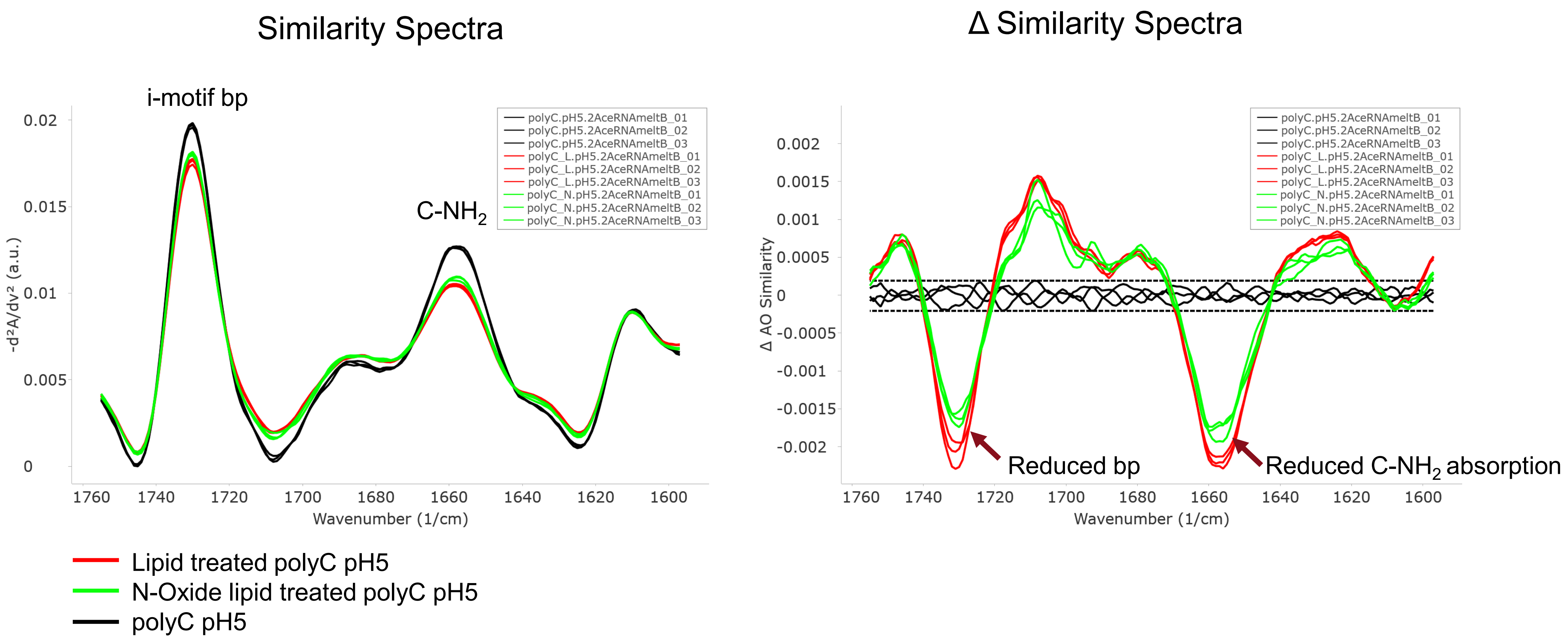

Lipid treated polyC pH7
polyC pH7

## Δ Similarity Spectra



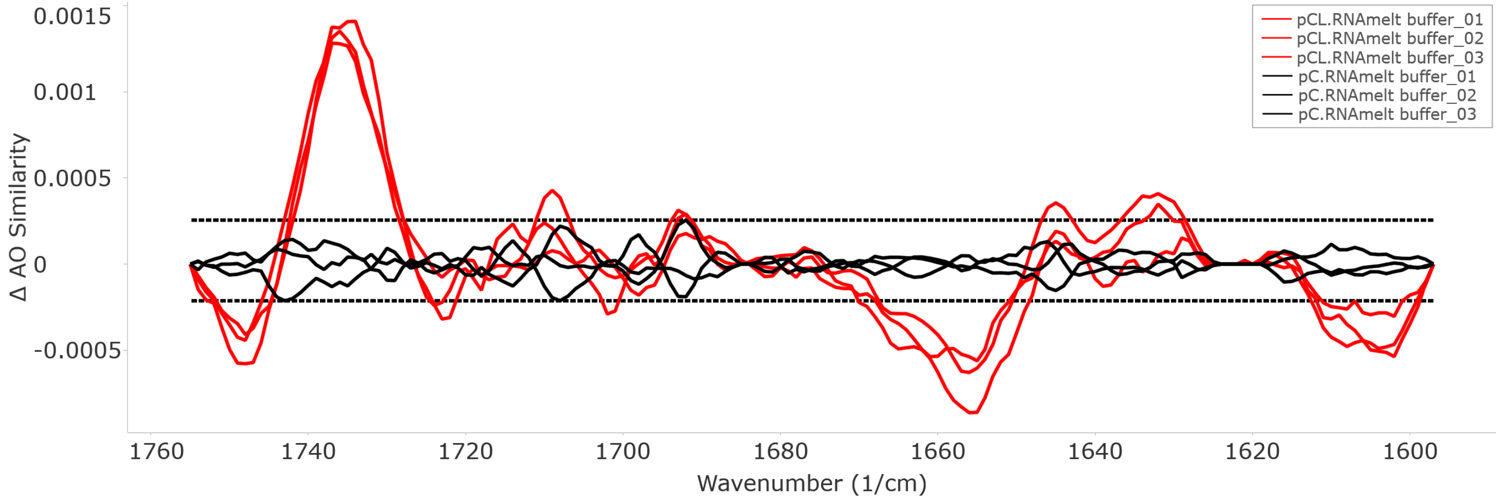

Weaker C-NH$_2$
and aromatic C=C / C=N

# Under acidic conditions that promote polyC i-motif formation (C-C base pairing), we observe an attenuation of C-C base pairing.

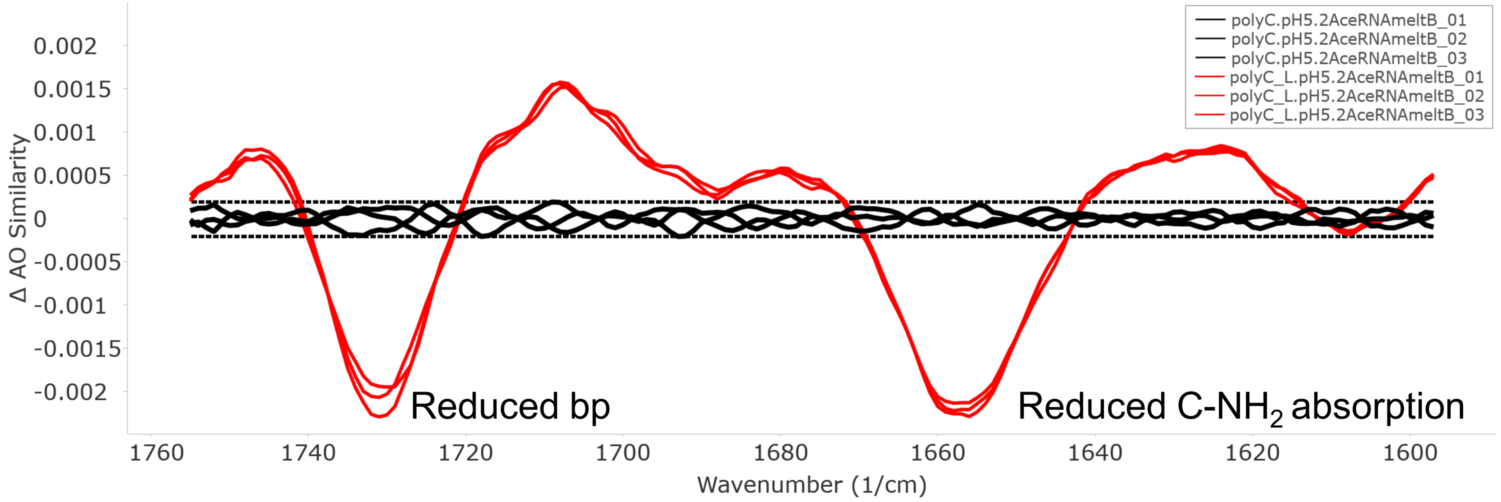


# polyC lipid adduct under neutral (very little or no C-C base pairing) and acidic (i-motif C-C base pairing) conditions:

pH 7




pH 5

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# Making sense of structured RNA

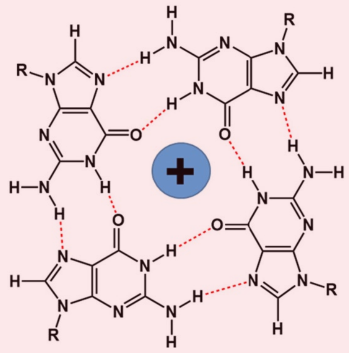
Building a library for spectral assignment

# What RNA structural elements do we need in our library?

- Anything with a unique hydrogen bond pattern to bases can provide a unique spectral signature.
- AU and GC Watson-Crick (WC) base pairing
- GU wobble base pairing
- G-quadruplexes (GG WC-Hoogsteen base pairing)
- Triple helices
- Loop and bulge residues- a mix of unpaired and noncanonically paired residues
- Mismatches- noncanonical pairing

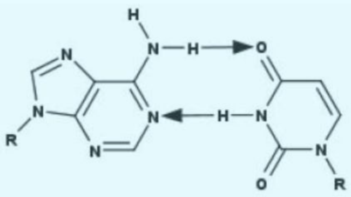
Currently works in progress

## G-quads (WC-Hoogsteen GG)

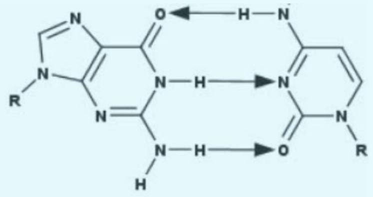


Frasson, I., Pirota, V., Richter, S. N., & Doria, F. (2022). *International Journal of Biological Macromolecules*, 204, 89-102.

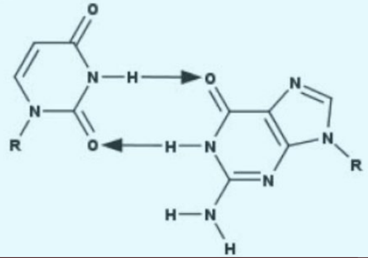
## AU base pairing (WC)



## GC base pairing (WC)

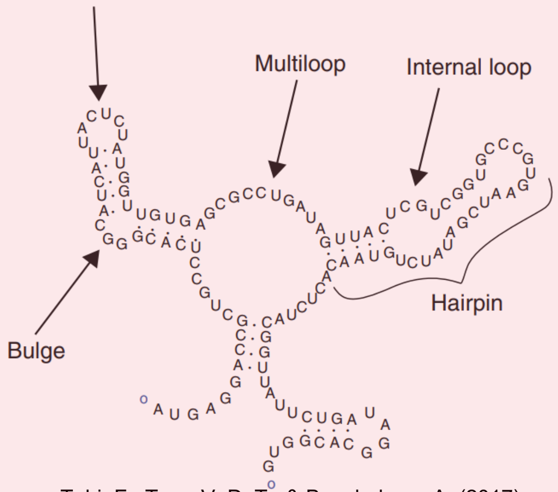


## GU base pairing (wobble)



Agris, P. F., Eruysal, E. R., Narendran, A., et al. (2017). *RNA Biology*, 14(4), 429-440.

## Loops and bulges, tetraloops
(Expect mostly unpaired, some noncanonical pairing)



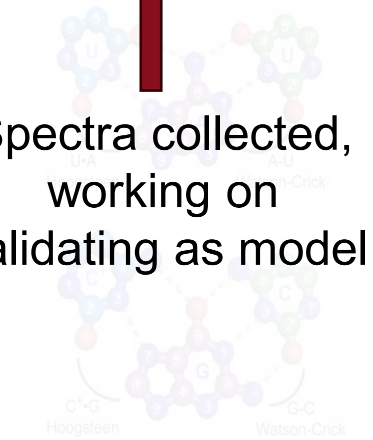

Tahi, F., Tran, V. D. T., & Boucheham, A. (2017). *Methods in Molecular Biology*, 1543, 145-167.

Common RNA mismatches
(Noncanonical pairing)



Davis, A. R., Kirkpatrick, C. C., & Znosko, B. M. (2011). *Nucleic Acids Research*, 39(3), 1081-1094.

RNA Triple Helices
(CG and UA Hoogsteen-WC)



Brown, J. A. (2020). *WIREs RNA*, 11(4), e1598.

Spectra collected, working on validating as model

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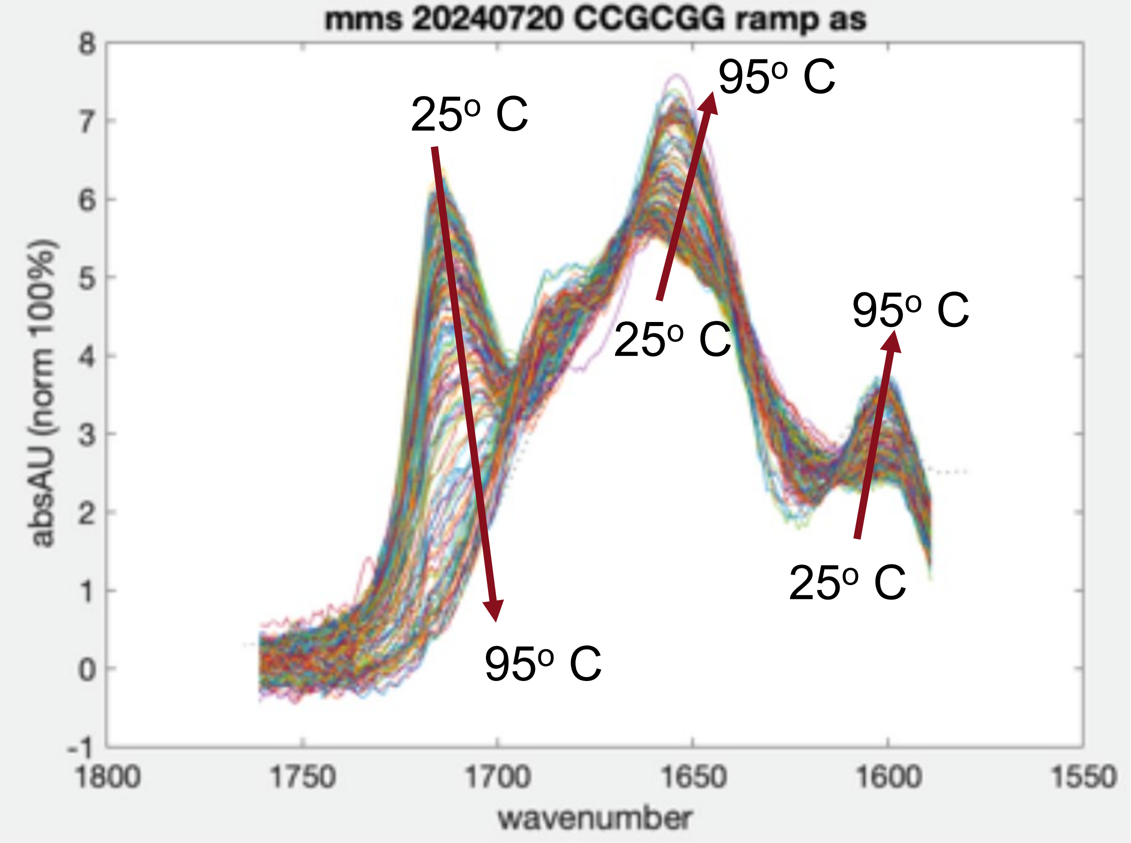
# GC base pairing model RNA construct: CCGCGG self-complementary duplex

100% Watson-Cric
predicted at 295 K:



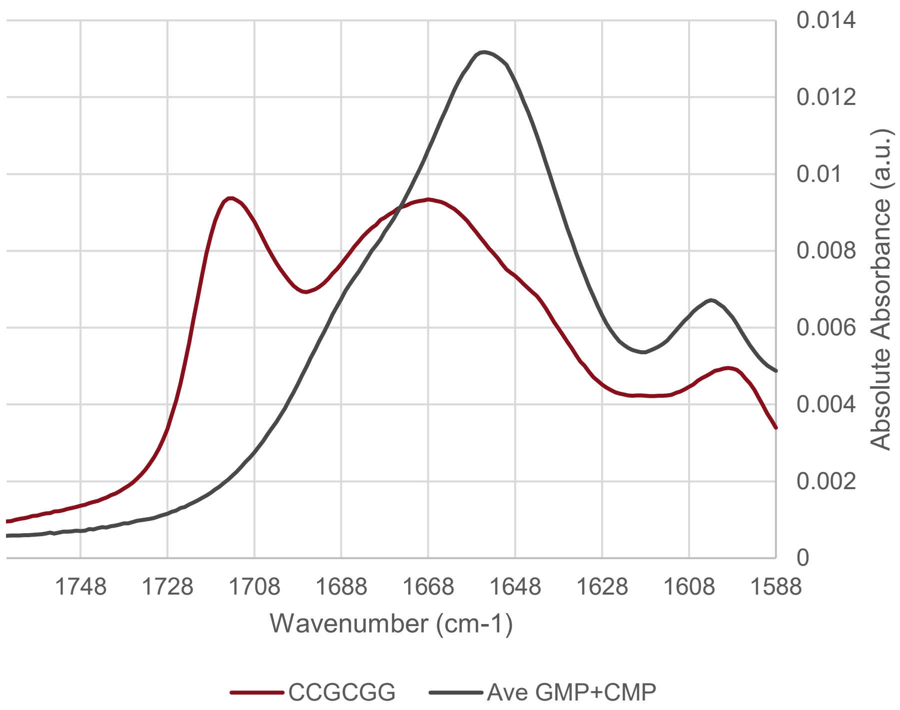

ENERGY = -13.1 CCGCGG_ CCGCGG

Duplexfold Web Serve
(https://rna.urmc.roche
u/RNAstructureWeb/Servers/
DuplexFold/Example.php)



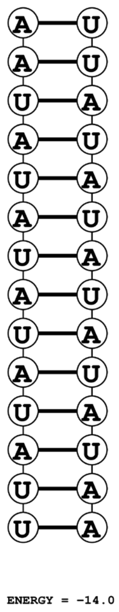

log concentration

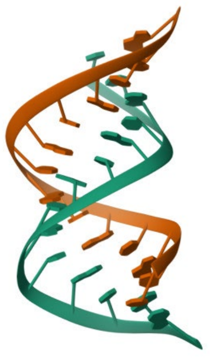
*All spectral integrals normalized to 1



# AU base pairing model RNA construct: 1RNA (UUAUAUAUAUAUAA) self-complementary duplex

100% Watson-Crick helix predicted at 295 K:

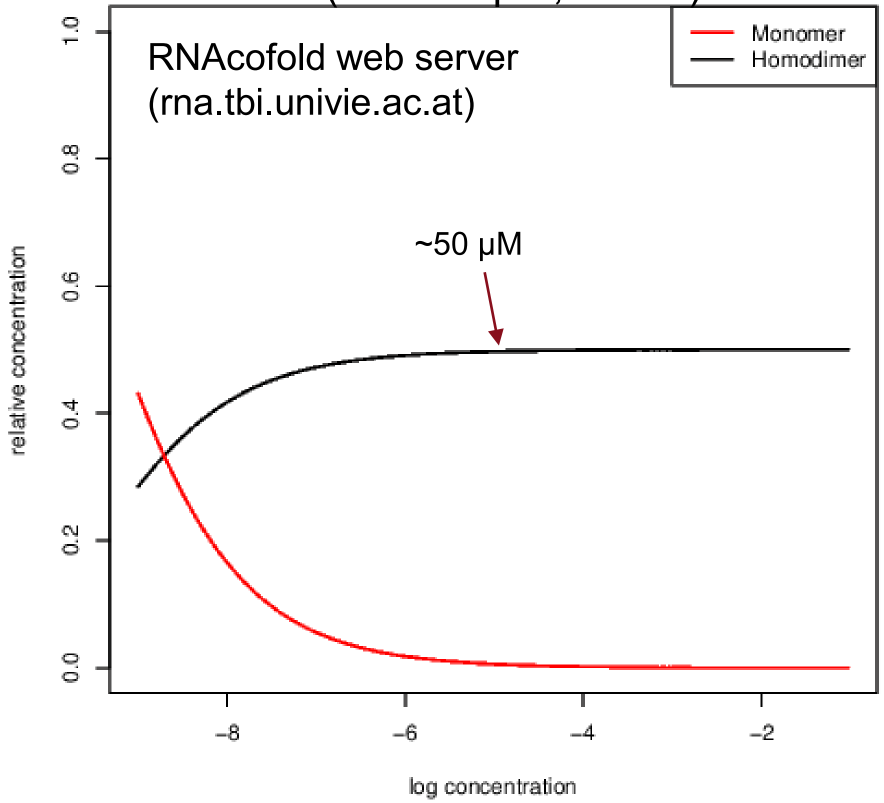


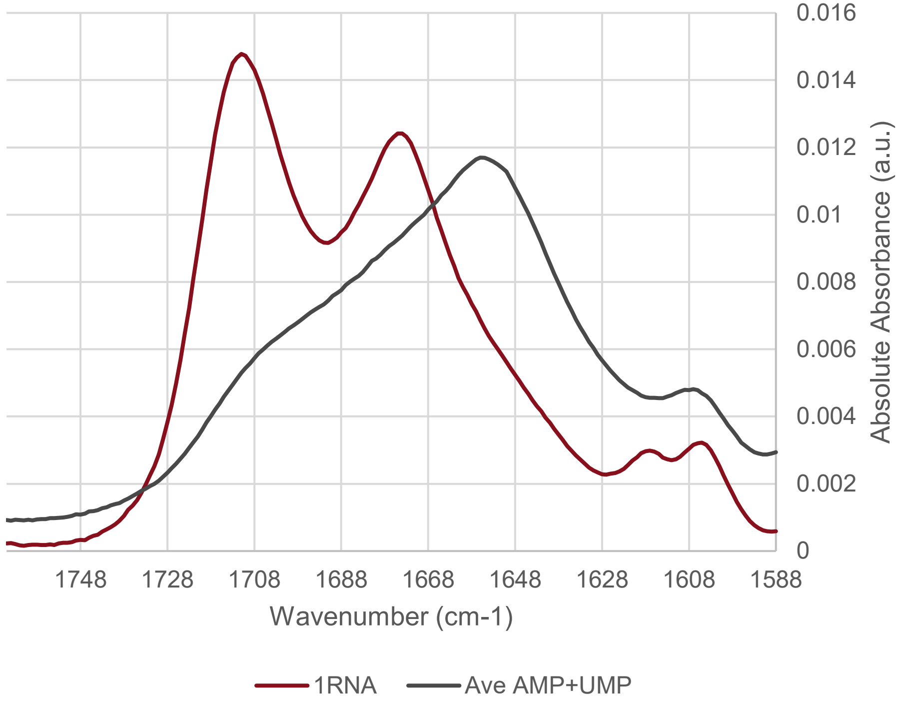
PDB: 1RNA

Duplexfold Web Server (https://rna.urmc.rochester.edu/RNAstructureWeb/Servers/DuplexFold/Example.php)

Very little monomer present under MMS conditions (i.e. >50 μM, 295 K)




*All spectral integrals normalized to 1

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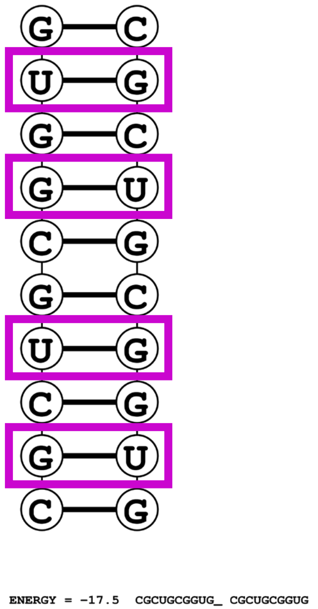
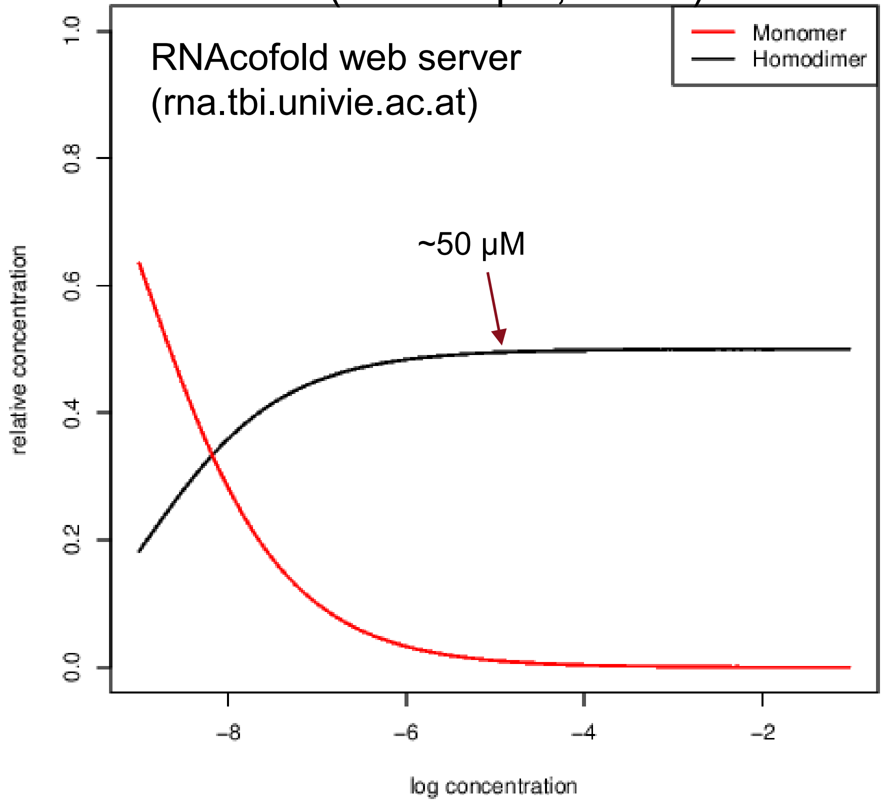
# GU base pairing model RNA construct: “GU Wobble” (CGCUGCGGUG) self-complementary duplex

100% base paired predicted at 295 K:

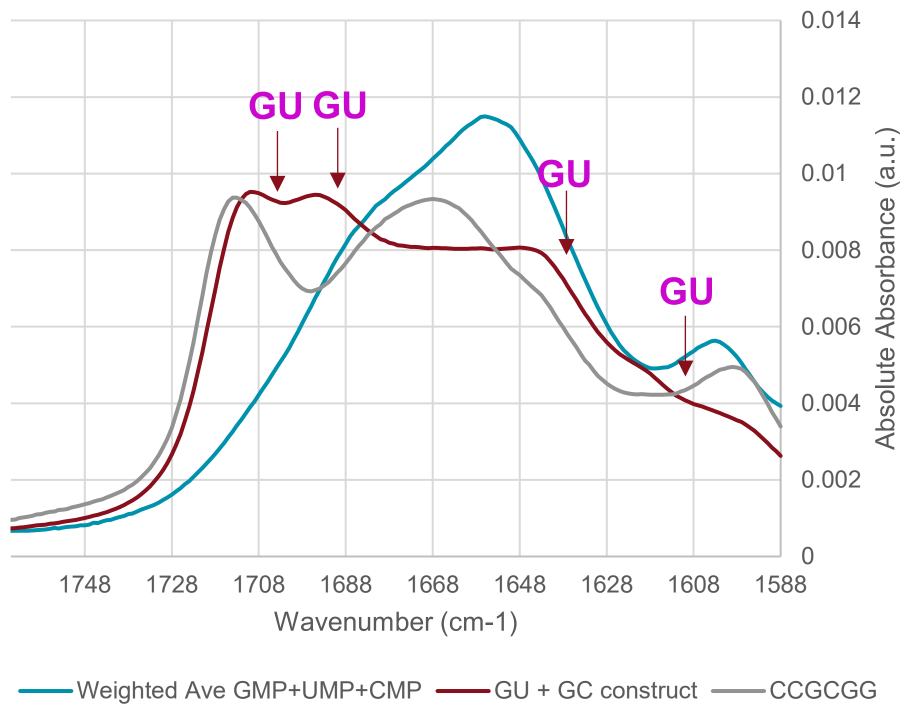



Duplexfold Web Server (https://rna.urmc.rochester.edu/RNAstructureWeb/Servers/DuplexFold/Example.php)

Very little monomer present under MMS conditions (i.e. >50 μM, 295 K)




*All spectral integrals normalized to 1

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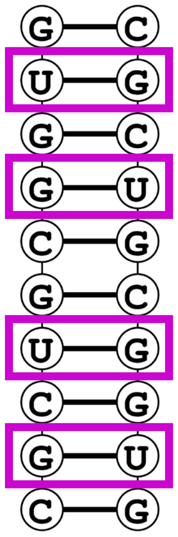
# GU base pairing signature after GC subtraction

100% base paired predicted at 295 K:

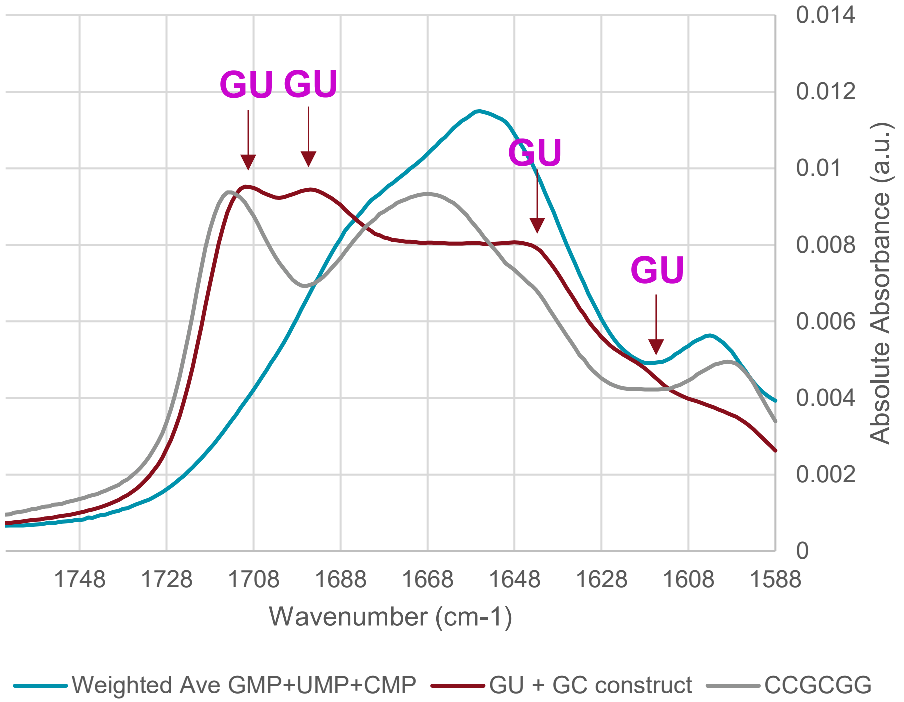



ENERGY = -17.5 CGCUGCGGUG_ CGCUGCGGUG

Duplexfold Web Server (https://rna.urmc.rochester.edu/RNAstructureWeb/Servers/DuplexFold/Example.php)

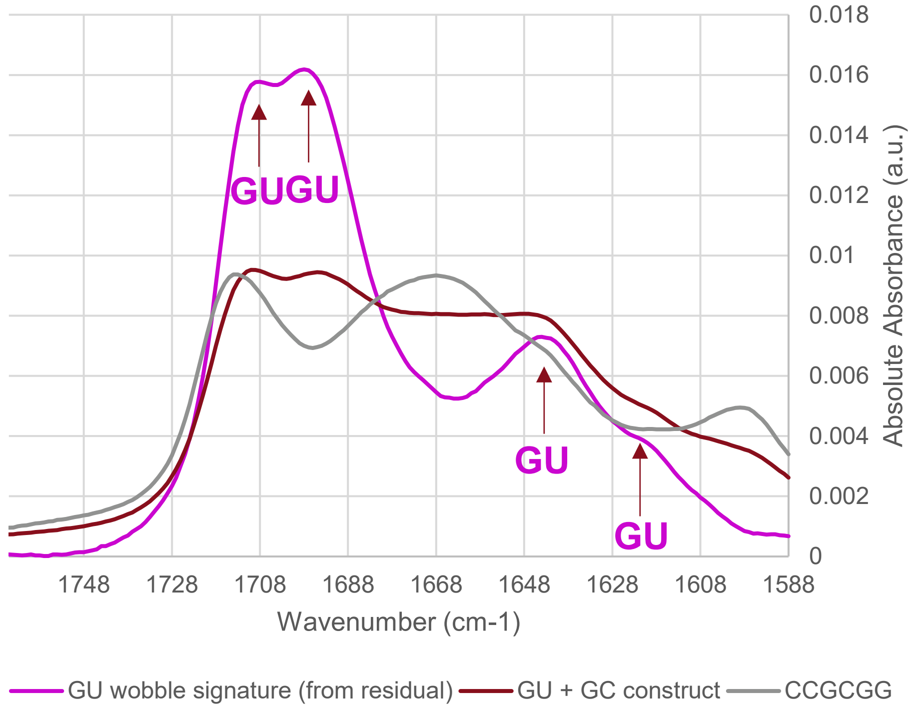
Comparison of CCGCGG, CGCUGCGGUG, and weighted GMP, UMP, and CMP MMS spectra

*All spectral integrals normalized to 1




Comparison of CCGCGG, CGCUGCGGUG, and residual GU wobble spectrum from subtraction

*All spectral integrals normalized to 1

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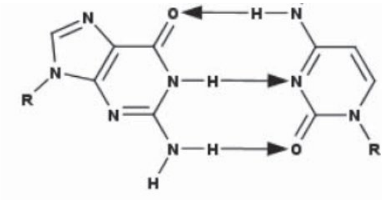
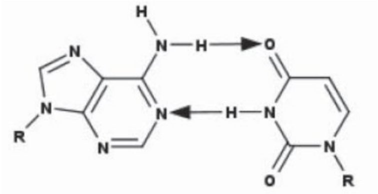
# Comparison of GC, AU, and GU base pairing signatures

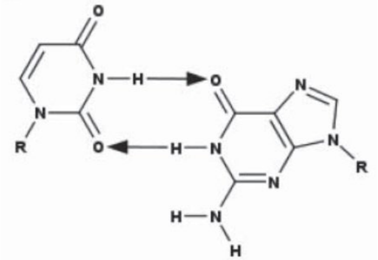
**GC base pairing (WC)**



**AU base pairing (WC)**

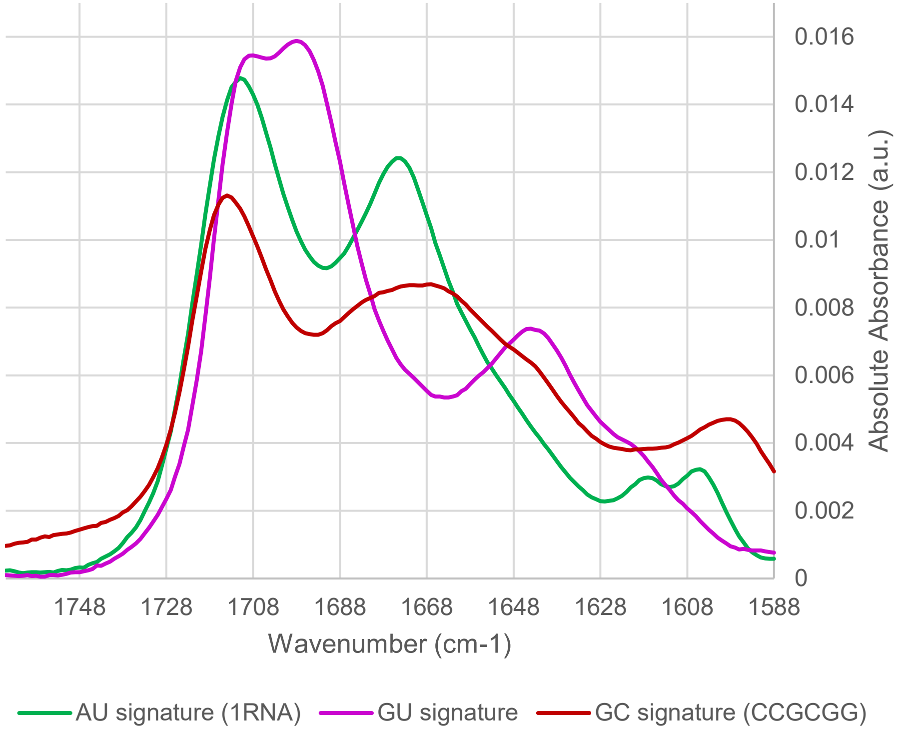


**GU base pairing (wobble)**

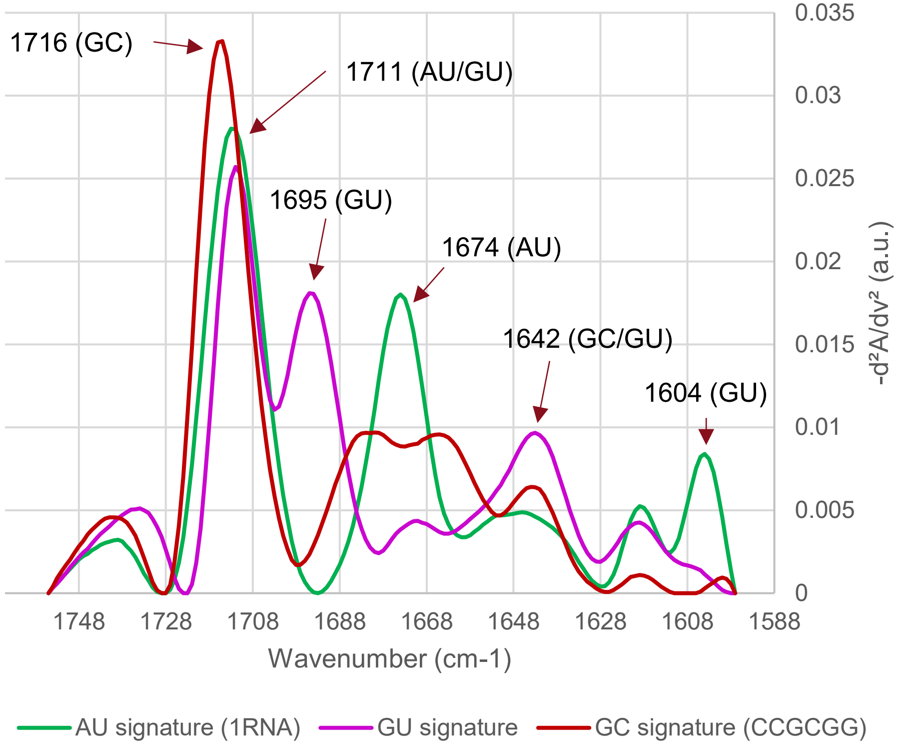


Absolute Absorbance Plots:

*All spectral integrals normalized to 1




Similarity Plots
(Sharpens peaks)
*All spectral integrals normalized to 1





Base pairing images:
Agris, P. F., Eruysal, E. R.,
Narendran, A., et al. (2017). *RNA Biology*, 14(4), 429-440.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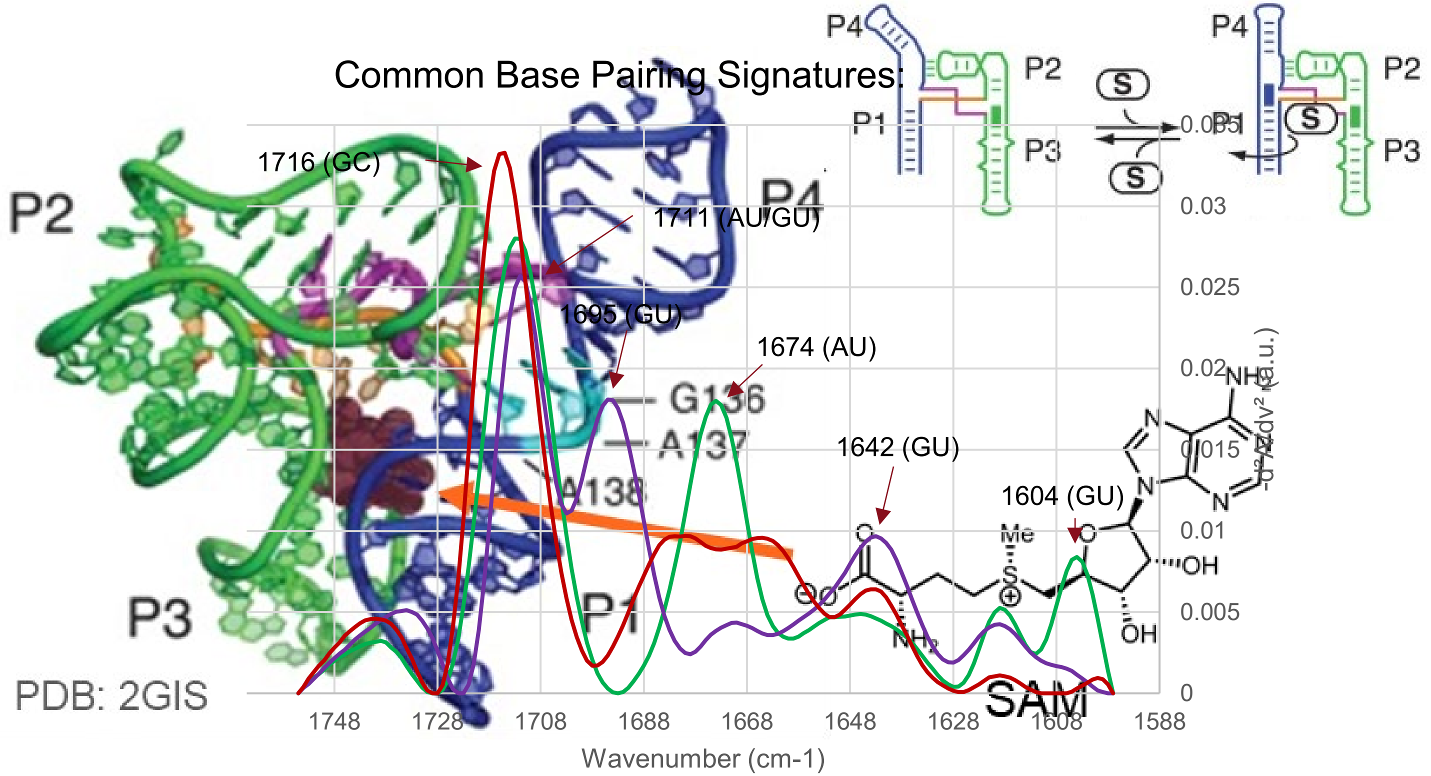
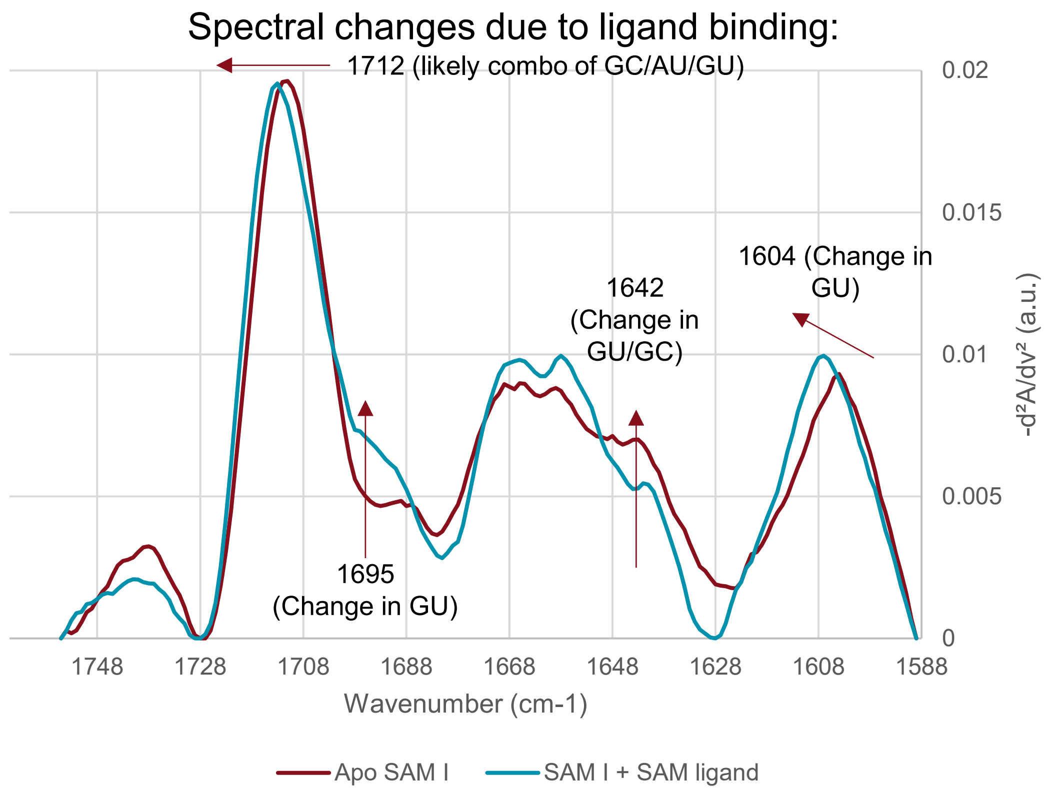
# Addition of SAM to the SAM-I riboswitch results in an MMS-observable ligand-induced conformation change.




Riboswitches are small, well-folded RNA that bind tightly to small molecular ligands. When bound, the RNA undergoes conformational change that controls whether the riboswitch is active or inactive.

# Summary

- MMS is able to distinguish minute changes in hydrogen bonding patterns to nucleobases:
  - Unpaired
  - C-C i-motif and G-G G-quad
  - A-U Watson-Crick, G-C Watson-Crick, and G-U wobble base pairing
- RNA-lipid adduct formation seems to attenuate polyC i-motif base pairing under acidic conditions.
- We can see RNA structural associated with ligand binding.
- Future focuses:
  - RNA therapeutic degradation
  - polyA tail quantification
  - Chemometrically fitting structured RNA spectra to measure base pairing populations
  - Further development of the use of MMS in the detection of RNA-lipid adduct formation