August 18, 2022

Dear Professor Carreira,

Here we wish to submit our manuscript “*The Metabolome Weakens RNA Thermodynamic Stability and Strengthens RNA Chemical Stability*” as an original research paper to the *Journal of the American Chemical Society*. RNA serves myriad key roles in biology and medicine, for example in mRNA vaccines, gene editing (CRISPR), molecular recognition (riboswitches), and catalysis (ribozymes). RNA is also an important target for small molecule and antisense oligonucleotide therapeutics. However, there is a chasm between the information provided by highly simplified conditions for RNA study *in vitro* and the true properties of RNA folding / function *in vivo.* As such, *in vivo* like systems that simulate multiple elements of the complex cellular environment have the potential to provide deep insight into the molecular properties of RNA. However, to date, *in vivo* like conditions to study RNA have focused on highly simplified conditions, such as non-biological crowders like polyethylene glycol (PEG) or just a few biologically relevant small molecules. A number of these foundational but limited studies have been published in JACS by our group and others.1–5

Our present manuscript describes a major step forward for studying the chemical properties of RNA with biological relevance. We describe the development of “Eco80”-- an artificial cytoplasm containing 80% of *E. coli* metabolites, which is a manageable, defined, and controllable surrogate for studying RNA *in vivo*. We demonstrate that Eco80 has a number of RNA-favoring molecular attributes: it buffers the free Mg2+ concentration, destabilizes potentially inhibitory RNA helices, protects RNA from chemical degradation, and supports RNA catalysis. Indeed, Eco80 is likely the most biologically relevant and chemically accessible artificial cytoplasm available, which should provide a new medium for the chemistry field for performing experiments on RNA. As such, we expect this work to have broad appeal to those studying the molecular properties of RNA, making it appropriate for *JACS*.

We recommend Professor Matthew Disney as an associate editor and Professors Gary Pielak, Danny Incarnato, Martin Gruebele, and Kristin Koutmou as reviewers.

This manuscript is not under consideration for publication and has not been published elsewhere.

Thank you very much for handling our manuscript.

Yours Sincerely,



Philip C. Bevilacqua

Distinguished Professor of Chemistry and of Biochemistry & Molecular Biology

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