**Reviewer: 1 Comments:**

Summary: In this brilliant work, Sieg and colleagues present Eco80, an artificial cytosol that mimics the cytosol of E. coli cells, by accounting for approx. 80% of the metabolome of E. coli. The systematic analysis of the different metabolites with Mg2+ cations is unprecedented. This work provides the first concrete step towards "filling the gap between in vitro and in vivo folding". I do not have any remarks on the technical side. The manuscript is extremely well written, and it can be, in my opinion, accepted and published as is. I have however a few small requests concerning points that, I believe, would be very important to mention in the discussion:

**Minor revisions:**

1.) As this cytosol only accounts for 80% of the metabolome, are there reasons to expect that the remainder of the metabolites (~20%) would have any opposite effect on the destabilization of RNA helices?

The reviewer points out that we only accounted for 80% of the metabolome and that the other 20% could have an effect, possibly opposite, of the metabolites in Eco80. We believe that the remaining 20% of metabolites will reinforce the effect of Eco80 rather than oppose it.

Insert an analysis of the remaining 20% of metabolites here.

1. Composition strong
2. Composition weak
3. Each individual component is less than X% of the metabolome
4. Spotty stabilizing effect of weak chelators in WMCM is already dominated by the destabilizing effects in Eco80.

While there is nothing apparently special about the remaining 20% of *E. coli* metabolites, and we predict that the remaining metabolites will behave much like Eco80, only an experiment can say for certain, which would be prohibitively expensive and difficult given the large number of remaining metabolites. We thus incorporated the following into the manuscript, providing a caveat that such an experiment is not going to be practical in the immediate future:

2.) How is this destabilization expected to impact the folding landscape of the RNA in vivo, in terms of structural heterogeneity and the ability to interconvert between alternative structural states? For instance, major structural rearrangements are normally unlikely to occur spontaneously, as they would involve disrupting a large number of base-pairs and reforming new ones, which, from an energetic perspective, is very "expensive". RNA chaperones are normally thought to facilitate these events, but, could the reduced stability of helices in vivo suggest that RNA structures might be more "dynamic" in the cell, independently of the action of proteins?

How the destabilization we observe is expected to impact the folding landscape of RNA *in vivo* is a topic of great interest to us. A number of *In vivo* chemical probing studies do indicate that RNA structures are more dynamic in the cell than *in vitro*. Helicases and RNA chaperones have been hypothesized to play a central role because disrupting a large number of base-pairs is so energetically “expensive” *in vitro*. The weak destabilization we observed in this work, about 0.6 kcal/mol, could have a large effect on RNA dynamics *in vivo*, when extrapolated to the entire transcriptome, outside of the impact of proteins. Importantly, formation of helices that are marginally stable *in vitro*, about 3-4 base-pairs, would become energetically unfavorable *in vivo*. Another consideration, is that helicases and chaperones do not work for “free”, often requiring the hydrolysis/expenditure of NTPs. A 0.6 kcal/mol decrease in the energy for every helix that a chaperone or helicase must unwind would provide major energetic savings for the cell.

3.) Can Eco80 be used to derive improved in vivo-like thermodynamics parameters (an alternative nearest neighbor set of parameters) to be incorporated in RNA structure prediction tools to yield more "realistic" structure models?

The reviewer hits on another topic that we are interested in. We believe that Eco80 can be used to derive improved *in vivo-*likethermodynamic parameters to be incorporated in RNA structure prediction tools and are actively working towards that goal. However, this discussion is beyond the scope of this paper.

**Reviewer 2 comments:**

This manuscript continues an important series of papers from Prof. Bevilacqua et al that has revealed how metabolites impact RNA structure and function in the cell (see refs 24-26). In earlier papers, the group looked at the impact of strong and weak magnesium-chelating metabolites individually, and now these authors have extended the work to a more native-like mixture of metabolites including strong and weak chelators, covering 80% of the E. coli metabolome by molarity.

The work and conclusions are presented clearly and the authors are commended for the transparency of their analysis. The work is suitable for Biochemistry and I recommend publication after the authors address the comments below.

**Major revisions:**

1.) The free magnesium is stated to be precisely 2 mM (e.g., Table 2), but it presumably has some error associated with it. Indeed, in Figure 1 the total magnesium concentrations align to regions where the free magnesium concentration changes more rapidly with the total concentration, and these concentrations appear to lie beyond the linear range of the HQS calibration curve. To properly interpret the results, it’s important to understand the error of the free magnesium concentration and the potential impact on RNA stability over that range.

Reviewer two points out that that it’s important to understand the error of the free Mg2+ and the impact of such errors on RNA stability. This was a glaring omission to the original manuscript and addressing free Mg2+ errors substantially improves the paper. We believe that there are four questions to address here:

(1) Do the total Mg2+ concentrations that provide 2 mM free Mg2+ align to regions where the free Mg2+ concentration changes more rapidly with total concentration Mg2+?

It may appear that the free Mg2+ concentration is changing rapidly with the total Mg2+ concentration near 2 mM free Mg2+ in Figure 1E because there is almost no change at lower total concentrations and the linear y-axis/log10 transformed x-axis. The actual slope for this region is not large, at 0.08 mM free Mg2+ for each 1 mM increase in total Mg2+. This buffering is evidence that the free Mg2+ is fairly constant.

(2) Do these concentrations lie beyond the linear range of the HQS calibration curve?

The biological free Mg2+ concentration range of 0.5 to 3.0 mM is within the linear range of the HQS calibration curve (R figure 4A). Propagation of the errors from the calibration curve fit used to determine the free Mg2+ concentration indicate that errors are minimized in the biological free Mg2+ concentration range, less than 5% of the calculated value (R Figure 2B). The HQS assay is less precise outside of this range of free Mg2+ concentrations, because large changes in Mg2+ concentrations only lead to small changes in HQS emission. For example, error bars, based on propagating uncertainty are smaller than the data points in the biological free Mg2+ concentration range but substantial above it.

Chart

Description automatically generated

R Figure 2 Analysis of free Mg2+ errors in Eco80.

(3) What is the uncertainty for the free Mg2+ concentration in our experiments?

The is true uncertainty for the Mg2+ concentration in our experiments is hard to calculate, but 10%, or twice the propagated uncertainty from the fit in the biological free Mg2+ range (R Figure 2B), is a conservative value. At 2 mM, this would be an uncertainty of 0.2 mM, which would require a total Mg2+ change of 2.5 mM to achieve given the buffering of 0.08 mM free Mg2+ for each 1 mM increase in total Mg2+ by Eco80 (R Table 1).

(4) Could this uncertainty impact our results?

A Mg2+ concentration error of 0.2 mM is unlikely to impact our results. For example, we

2.) The high level results of this manuscript seem to be at odds with those of ref 24 from the same group, although the details of the papers differ significantly. More discussion seems warranted to put these results in the context of this previous work.

3.) In the last sentence of the third paragraph of the results section, “identify” -> “identity”

**Minor revisions:**

1.) I think the use of the term “aufbau” to describe this approach is confusing given its previously established meaning in chemistry. (Also, I don’t know German, but it doesn’t seem to quite mean bottom-up.) I suggest swapping aufbau for “bottom-up” itself.

We agree with the reviewer and we removed the term “aufbau” from the manuscript.

2.) It’s surprising that chelated magnesium complexes decrease the stability of RNA structures given a constant concentration of free magnesium. The explanations given for how the complexes interact with RNA do not necessarily involve magnesium, so it seems that the chelating and RNA-destabilizing effects could be disentangled experimentally. For example, would a nucleoside or NMP mixture with 2 mM magnesium behave similarly to an NTP mixture with 25 mM magnesium? Such experiments are not required for publication but would be helpful to strengthen the mechanistic model.

**Reviewer 3 comments:**

The work presented by Sieg and colleagues tackles an important challenge in mechanistic biochemistry - how to ensure that in vitro insights recapitulate what is happening with biological molecules in cellular systems. This universal struggle for biochemists is particularly frustrating for those in the RNA field because 1) most experimental techniques for studying RNA structure/function cannot be applied in a cellular context, and 2) RNAs are highly dynamic molecules whose structure/function changes rapidly in response to subtle shifts in pH, ionic strength and divalent metal concentrations. To tackle this problem, the authors created a buffer that contains 15 most abundant metabolites, which comprise 80% of the E. coli metabolome (Eco80). I particularly appreciated their rigorous assessment of the binding constants for Mg(II) exhibited by each of the metabolites - this is the sort of careful system-wide thinking that is much needed, and the values they measure provide a valuable resource.

The authors comparison of RNA thermo- and chemical-stability in Eco80 sub-optimal buffers indicate that RNAs behave more like what has been reported in vivo in Eco80; their structures are less thermodynamically stable. Their findings generate a new tool/experimental condition for the field, and provide strong evidence for the importance of Mg(II) buffering in cellular systems on RNA structure and function. This work is incredibly thorough, rigorous, and extremely well written. I recommend it for publication, after the authors address a few very minor comments:

**Minor revisions:**

1) Can the authors discuss how (or if) they took into account other cellular components (e.g. RNAs and proteins) that also chelate Mg(II)? If they didn’t, it’s totally fine, but I’d put something in the discussion about why they made they choice. Similarly, did they consider adding other divalent ions to their buffer (e.g. Zn(II) or Ca(II)), which can also likely chelate the metabolites in cells? Free Zn(II) and Ca(II) concentrations are low (hundreds of micromolar) compared to that of Mg(II) in cells (and therefore likely don’t compete too much for these metabolites), but thinking about how might be relevant to cells in some situations, such as when Ca(II) is released during signaling.

2) The second period should be removed from this sentence:

“MeltR calculated folding energies using two Van’t Hoff methods: (1) directly fitting a Van’t Hoff plot as a function of temperature (Figure 3C) and (2) globally fitting raw fluorescence emission. .”

3) The second “2 mM Mg” term in following sentence should be “25 mM Mg” according to the values given in the associated SI Table 6.

“The predicted 19.7 +/- 0.2A radius of gyration. . . .”