Contents

[I. Reviewer: 1 Comments: 2](#_Toc114845270)

[A. Minor revisions: 2](#_Toc114845271)

[II. Reviewer 2 comments: 5](#_Toc114845272)

[A. Major revisions: 5](#_Toc114845273)

[B. Minor revisions: 8](#_Toc114845274)

[III. Reviewer 3 comments: 9](#_Toc114845275)

[A. Minor revisions: 9](#_Toc114845276)

[IV. List of revisions to the submitted manuscript 11](#_Toc114845277)

[IV. List of revisions to the submitted Supplemental Information 13](#_Toc114845278)

# I. 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:”

We are grateful for the exceptionally positive assessment of our work and helpful suggestions.

## A. 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.

We performed an analysis of the remaining 20% of the metabolome using estimated binding constants (R Figure 1).1

1. The remaining 20% of the metabolome has a similar, but slightly higher, composition of strong Mg2+ chelating metabolites (NTPCM) as Eco80, 19% and 14% respectively.
2. Only three of the 228 remaining metabolites are expected to carry a positive charge at cellular pH
3. Each individual component is less than 1.1% of the metabolome
4. Spotty stabilizing effect of weak chelators in WMCM is already dominated by the destabilizing effects in Eco80.

A picture containing diagram

Description automatically generated

**R Figure 1** Analysis of the other 228 metabolites that compose the 20% of the metabolome not included in Eco80. The NTPCM consists of strong Mg2+ chelators, meaning they have KDs for Mg2+ less than 2 mM, the approximate biological free Mg2+ concentration in *E. coli*. WMCM consists of weak or negligible Mg2+ chelators.

While there is apparently nothing 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 paragraph into discussion, along with a response to reviewer 3:

“Eco80 is a significant step towards reconstituting the cytoplasm *in vitro* but Eco80 is still a simplification. First, the cell contains 228 other metabolites, the 20% of the metabolome that was not included in Eco80. While it was not feasible to test in this study, we expect the remaining 20% of the metabolome to reinforce the effects of Eco80 because the remaining 20% of the metabolome has a similar, but slightly higher, composition of strong Mg2+ chelating metabolites to Eco80, 19% and 14% respectively, and because each individual component makes up less than 1.1% of the total metabolome.23 The remaining 20% of the metabolome also shares structural features with the metabolites in Eco80, with less than five of the remaining metabolites expected to carry a net positive charge at physiological pH. The second simplification is that Eco80 does not contain biological macromolecules such as RNA and proteins, or other biological divalent metal ions such as Zn2+ or Ca2+. While the effects of these cellular components were outside of the scope of our study, the effects could be interrogated using a similar theoretical and experimental treatment.”

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 0.69 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 2-4 base-pairs, would become energetically unfavorable *in vivo*. Another consideration, is that helicases and chaperones do not work for “free”, requiring the hydrolysis/expenditure of NTPs. A 0.69 kcal/mol decrease in the energy for every helix that a chaperone or helicase must unwind would provide major energetic savings for the cell. However, this is all speculative without experiments or modeling. To address the reviewers comments, we added the following paragraph to the discussion:

“Moreover, the destabilizing effect of Eco80 indicates that RNA structures are more dynamic in the cell than *in vitro*. Helicases and RNA chaperones have been proposed to play a central role in dynamics in cell because interconversion between RNA structures requires breaking base pairs, which is energetically “expensive”. The weak destabilization we observed in this work, about +0.69 kcal/mol, could have a large effect on RNA dynamics *in vivo*, when extrapolated to the entire transcriptome, outside of the impact of proteinous RNA chaperones. Another consideration is that helicases require the hydrolysis/expenditure of NTPs. A 0.69 kcal/mol decrease in the energy required for every helix that a helicase must unwind could 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.

# II. 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.”

We thank the reviewer for the careful and critical analysis of our work. They have identified oversights in the original manuscript, and we believe this improved our work.

## A. 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.”

The reviewer 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 an omission to the original manuscript and addressing free Mg2+ errors substantially improved 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. However, 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 constant near 2 mM in our artificial cytoplasm as errors om the determination of the total Mg2+ required to obtain 2 mM free Mg2+ will be buffered.

(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 2A). 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 free Mg2+ concentrations only lead to small changes in HQS emission. For example, error bars, based on propagating uncertainty from the calibration curve fit, are smaller than the data points in the biological free Mg2+ concentration range but substantial above it (R Figure 2C).

Chart

Description automatically generated

**R Figure 2** Analysis of free Mg2+ errors in Eco80. **(A)** Figure 1B modified with a x-axis to show that the biological free Mg2+ range is within the linear range of the HQS calibration curve. **(B)** Uncertainty in the free Mg2+ concentration calculated from HQS emission, estimated by propagating uncertainty in the calibration fit coefficients, as a function of the free Mg2+ concentration calculated from HQS emission. **(C)** Figure 1E modified with error bars to representing uncertainty in the free Mg2+ concentration calculated from HQS emission.

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

The true uncertainty for the Mg2+ concentration in our experiments is hard to calculate, but 10%, or twice the maximum 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+ error 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).

**R Table 1** Mg2+ concentrations used to obtain 2 mM free Mg2+ in artificial cytoplasm.

|  |  |  |  |
| --- | --- | --- | --- |
| Condition | [Total Mg2+] (mM) | [Chelated Mg2+] (mM) | [Free Mg2+] (mM) |
| Eco80 | 31.6 | 29.6 (±0.2)a | 2 (±0.2)a |
| NTPCM | 25 | 23 (±0.2)a | 2 (±0.2)a |
| WMCM | 6.4 | 4.4 (±0.2)a | 2 (±0.2)a |

aUncertainty is 10%, double the maximum %uncertainty for HQS determination of the free Mg2+ concentration in the biological free Mg2+ range, propagated from uncertainties in the fit coefficients.

(4) Could this uncertainty impact our results?

A free Mg2+ concentration error of 0.2 mM is unlikely to impact our results. For example, we applied a tightly bound ion (TBI) theoretical model for mixed Na+ and Mg2+ solutions to calculate the change in free energy (ΔΔG°37) from a 380 mM Na+ 2 mM free Mg2+ reference state as a function of the free Mg2+ concentration (R Figure 3).2 Accordingly, errors in the free Mg2+ concentration are unlikely to cause the free energy changes we observed in Figure 2, notebly the +0.69±0.12 kcal/mol we observed in Eco80. Indeed, a 1 mM free Mg2+ concentration error, an error of 50%, would be required to cause even a 0.25 kcal/mol fluctuation in the ΔG°37. A 1 mM free Mg2+ concentration error would require our estimate for the total Mg2+ required to provide 2 mM free Mg2+ in Eco80 to be off by 12.5 mM, given the free Mg2+ buffering capacity of Eco80. In summary, given the free Mg2+ buffering capacity of the artificial cytoplasm and precision of the HQS assay in the biological free Mg2+ range, the effects of artificial cytoplasm on RNA cannot be explained by errors in the free Mg2+ concentration alone.

Chart, line chart

Description automatically generated

**R Figure 2** TBI theoretical model prediction of the ΔΔG°37 as a function of the free Mg2+ concentration for a 8 nucleotide helix. The reference state is an approximation of the 2 mM free Mg2+ condition in this manuscript, 380 mM Na+ and 2 mM Mg2+.

In order to address the reviewer’s comment, we made the following changes to the manuscript:

* We summarized the above discussion a new supplemental file (Supplemental information 2).
* We added error bars to Figure 1E-G.
* We added a description of the error bars to the Figure 1 legend.

“Error bars represent the uncertainty in the free Mg2+ concentration from propagating errors in the HQS calibration curve fit.”

* We added uncertainty values to Table 2.
* We added the following footnote to Table 2:

“aUncertainty is 10%, double the maximum %uncertainty for HQS determination of the free Mg2+ concentration in the biological free Mg2+ range, propagated from uncertainties in the fit coefficients (See Supplemental file 2).”

* We added the following sentence to the methods.

“Uncertainty in the free Mg2+ concentration was calculated by propagating the uncertainties in the fit parameters, using the error propagation rules defined below in the description of the statistical model. A detailed error analysis is provided in Supplementary information 2.”

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.”

There is already considerable discussion of ref 24 in the discussion, but we interpret that the reviewer is referring to the fact that ref 24 found tertiary structure stabilization and we found helix destabilization. We agree that more discussion is required to put these results in the context but disagree that the high-level results of our manuscript are at odds with those of ref 24. We added the following paragraph to the discussion to address the reviewers concerns.

“The destabilizing effect of Eco80 on RNA helices, apparently contradicts a previous study that demonstrated that amino acid-chelated Mg2+ stabilized the tertiary fold and increased the folding cooperativity of RNA structures.24 However, this study only accounted for amino acid-chelated Mg2+, a weakly-chelated Mg2+ species, and used approximate binding constants. Moreover, our finding of less stable helices is more consistent with increased tertiary structure stabilization than it first appears, as secondary structure destabilization and tertiary structure stabilization is an important driving force of cooperative folding for biological RNA.12 Indeed, our SAXS analysis was consistent with increased tertiary compaction of the apo-guanine aptamer in Eco80 (SI table 6), even with the destabilization of RNA helices observed in Figure 2.”

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

We fixed the error.

## B. 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 word “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.”

We agree on two points: (1) that this experiment would be helpful to strengthen the mechanistic model and (2) that this experiment is not required for publication. We hope that a potential impact of this study will be a more systematic analysis of the mechanistic model we present, especially applied to nucleotides, but such an analysis is outside of the scope of this paper.

# III. 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:”

We thank the reviewer for the positive assessment of our work and helpful comments for improvements to the discussion.

## A. 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.”

We did not account for other cellular components (RNA and proteins) that also chelate Mg2+ or other divalent ions such as Zn2+ or Ca2+ because it was not practical at the time. This manuscript is the culmination of 5 years of work by JPS and not including other cellular components in our systems-wide analysis was a limitation that we had to accept. To address the reviewer’s comment, we added the following paragraph (which also includes a response to reviewer 1):

“Eco80 is a significant step towards reconstituting the cytoplasm *in vitro* but Eco80 is still a simplification. First, the cell contains 228 other metabolites, the 20% of the metabolome that was not included in Eco80. While it was not feasible to test in this study, we expect the remaining 20% of the metabolome to reinforce the effects of Eco80 because the remaining 20% of the metabolome has a similar, but slightly higher, composition of strong Mg2+ chelating metabolites to Eco80, 19% and 14% respectively, and because each individual component makes up less than 1.1% of the total metabolome.23 The remaining 20% of the metabolome also shares structural features with the metabolites in Eco80, with less than five of the remaining metabolites expected to carry a net positive charge at physiological pH. The second simplification is that Eco80 does not contain biological macromolecules such as RNA and proteins, or other biological divalent metal ions such as Zn2+ or Ca2+. While the effects of these cellular components were outside of the scope of our study, the effects could be interrogated using a similar theoretical and experimental treatment.”

If the reviewers and editor will accommodate a bit of speculation on the behalf of JPS, I believe the most important aspect of this manuscript was the theoretical and experimental treatment of Mg2+ speciation in presence or multiple cellular components. The obvious next step is adding more cellular components, and variables such as temperature and pH, to this theoretical framework. As such, I have provided a detailed methods section, all of the raw data, and all of the scripts without restrictions at https://github.com/JPSieg/JPSiegMetaboMetaloRNA, to help other researchers should they choose to join us on this path.

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. .””

We removed the aberrant period.

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. . . .”

We fixed the error.

# IV. List of revisions to the submitted manuscript

1.) Trivial adjustment to the placement of graphics/table were made and formats were fixed to accommodate the more substantial edits requested by the reviewers and editorial team.

2.) “Herein, we take a bottom-up, *aufbau*, approach” was changed to “Herein, we take a bottom-up approach” as per the request of reviewer 2. Page 2.

3.) “This *aufbau* approach allows us” was change to “This bottom-up approach allows us” as per the request of reviewer 2. Page 2.

4.) “environmental factors such as pH, ionic strength and identify, and temperature” was changed to ““environmental factors such as pH, ionic strength and identity, and temperature” as per the request of reviewer 2. Page 2.

5.) Add uncertainty to the free and chelated Mg2+ values in Table 2, in response to reviewer 2. Page 3.

6.) Add the footnote, “aUncertainty is 10%, double the maximum %uncertainty for HQS determination of the free Mg2+ concentration in the biological free Mg2+ range, propagated from uncertainties in the fit coefficients (See Supplemental information 2).” to Table 2 in response to reviewer 2. Page 3.

7.) Add error bars to Figure 1 E-G in response to reviewer 2. Page 3.

8.) Add the description “Error bars represent the uncertainty in the free Mg2+ concentration from propagating errors in the HQS calibration curve fit.” to the Figure 1 description in response to reviewer 2. Page 3.

9.) Change “globally fitting raw fluorescence emission. .” to “globally fitting raw fluorescence emission.” As per the request of reviewer 3. Page 5.

10.) Changed “27.1±0.2 Å in 2 mM free Mg2+ (SI Table 6).” to “27.1±0.2 Å in 25 mM free Mg2+ (SI Table 6).” as per the request of reviewer 3. Page 7.

11.) Changed “In this study, we used a bottom-up, *aufbau*, approach” to “In this study, we used a bottom-up approach” as per the request of reviewer 3. Page 8.

12.) Added the paragraph:

“Eco80 is a significant step towards reconstituting the cytoplasm *in vitro* but Eco80 is still a simplification. First, the cell contains 228 other metabolites, the 20% of the metabolome that was not included in Eco80. While it was not feasible to test in this study, we expect the remaining 20% of the metabolome to reinforce the effects of Eco80 because the remaining 20% of the metabolome has a similar, but slightly higher, composition of strong Mg2+ chelating metabolites to Eco80, 19% and 14% respectively, and because each individual component makes up less than 1.1% of the total metabolome.23 The remaining 20% of the metabolome also shares structural features with the metabolites in Eco80, with less than five of the remaining metabolites expected to carry a net positive charge at physiological pH. The second simplification is that Eco80 does not contain biological macromolecules such as RNA and proteins, or other biological divalent metal ions such as Zn2+ or Ca2+. While the effects of these cellular components were outside of the scope of our study, the effects could be interrogated using a similar theoretical and experimental treatment.”

In response to reviewer 1 and 3. Page 9.

13.) Added the paragraph:

“The destabilizing effect of Eco80 on RNA helices, apparently contradicts a previous study that demonstrated that amino acid-chelated Mg2+ stabilized the tertiary fold and increased the folding cooperativity of RNA structures.24 However, this study only accounted for amino acid-chelated Mg2+, a weakly-chelated Mg2+ species, and used approximate binding constants. Moreover, our finding of less stable helices is more consistent with increased tertiary structure stabilization than it first appears, as secondary structure destabilization and tertiary structure stabilization is an important driving force of cooperative folding for biological RNA.12 Indeed, our SAXS analysis was consistent with increased tertiary compaction of the apo-guanine aptamer in Eco80 (SI table 6), even with the destabilization of RNA helices observed in Figure 2.”

In response to reviewer 2. Page 9.

14.) Added the following paragraph:

“Moreover, the destabilizing effect of Eco80 indicates that RNA structures are more dynamic in the cell than *in vitro*. Helicases and RNA chaperones have been proposed to play a central role in dynamics in cell because interconversion between RNA structures requires breaking base pairs, which is energetically “expensive”. The weak destabilization we observed in this work, about +0.69 kcal/mol, could have a large effect on RNA dynamics *in vivo*, when extrapolated to the entire transcriptome, outside of the impact of proteinous RNA chaperones. Another consideration is that helicases require the hydrolysis/expenditure of NTPs. A 0.69 kcal/mol decrease in the energy required for every helix that a helicase must unwind could provide major energetic savings for the cell.”

In response to reviewer 1. Page 9.

15.) Labeled the abstract as requested by the editorial team. Page 1.

16.) Labeled an experimental details section that refers readers to the supplemental methods. Page 10.

17.) Updated the Associated Content section to include the second supplemental information file added in response to reviewer 2. Page 10.

18.) Labeled TOC “For Table of Contents use only”

19.) We identified and fixed the following errors in the original manuscript.

* Changed “‘NTPCM’ contains the four strong Mg2+ chelating NTPs, and ‘WMCM’ contains 11 other weak Mg2+-binding metabolites.” to “‘NTPCM’ contains the four strong Mg2+-chelating NTPs, and ‘WMCM’ contains 11 other weak Mg2+-chelating metabolites.” on page 3.
* Changed “Figure 1 E-G, blank lines” to “Figure 1 E-G, black lines” on page 4.
* Changed “We used a binding isotherm method, wherein decreasing concentrations” to “We used a binding isotherm method, wherein increasing concentrations” on page 5.
* Changed “The destabilizing effect of Eco80 appeared to be related to the AU content of the helix” to “The destabilizing effect of NTPCM appeared to be related to the AU content of the helix” on page 6.
* Changed “strong-Mg2+-chelating metabolites, and a mixture of stabilizing and destabilizing interactions for weak-Mg2+-chelating metabolites” to “strong Mg2+-chelating metabolites, and a mixture of stabilizing and destabilizing interactions for weak Mg2+-chelating metabolites” on page 6.
* Changed “Several studies indicated that weak and strong Mg2+ chelating metabolites” to “Several studies indicated that weak and strong Mg2+-chelating metabolites” on page 6.
* Changed “25 and 31.6 mM total Mg2+ concentration used” to “25 and 31.6 mM total Mg2+ concentrations used” on page 6.

# IV. List of revisions to the submitted Supplemental Information

1.) The sentence “Uncertainty in the free Mg2+ concentration was calculated by propagating the uncertainties in the fit parameters, using the error propagation rules defined below in the description of the statistical model. A detailed error analysis is provided in Supplementary information file 2.” was added to the supplemental information on page 4.

2.) We provided an analysis of the uncertainty in the free Mg2+ concentration in artificial cytoplasm as a separate document, “Supplemental Information 2”.

# V. References

(1) Yamagami, R.; Sieg, J. P.; Bevilacqua, P. C. Functional Roles of Chelated Magnesium Ions in RNA Folding and Function. *Biochemistry* **2021**, *60* (31), 2374–2386. https://doi.org/10.1021/acs.biochem.1c00012.

(2) Tan, Z.-J.; Chen, S.-J. RNA Helix Stability in Mixed Na+/Mg2+ Solution. *Biophys. J.* **2007**, *92* (10), 3615–3632. https://doi.org/10.1529/biophysj.106.100388.

(3) Yamagami, R.; Bingaman, J. L.; Frankel, E. A.; Bevilacqua, P. C. Cellular Conditions of Weakly Chelated Magnesium Ions Strongly Promote RNA Stability and Catalysis. *Nat. Commun.* **2018**, *9* (1), 2149. https://doi.org/10.1038/s41467-018-04415-1.

(4) Leamy, K. A.; Yennawar, N. H.; Bevilacqua, P. C. Cooperative RNA Folding under Cellular Conditions Arises From Both Tertiary Structure Stabilization and Secondary Structure Destabilization. *Biochemistry* **2017**, *56* (27), 3422–3433. https://doi.org/10.1021/acs.biochem.7b00325.