

April 12, 2018

Dear Dr Alisha DSouza,

Thank you for forwarding the reviewers' comments on our article titled "Analysing protein architectures and protein-ligand complexes by integrative structural mass spectrometry". We are delighted by the overall positive tone of the reviews. We would like to thank you and the two reviewers for their thoughtful reviews and comments. We have revised our manuscript in light of these comments and we think that the revisions have made the manuscript stronger. Please find attached our response to the referees together with the revised text.

Editorial comments:

We would like to thank the editor for their comments on our work and helpful suggestions. We agree with the important points raised, and hence we have addressed these points (1-18) in our revised manuscript.

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
2. Please use American English.
3. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes.
4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Sigma-Aldrich, Millipore, etc.
5. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.
6. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.
7. Please provide the volumes used throughout. We need specific values in order to film and publish. Generalized protocols are not compatible with a video article.
8. What containers are being used throughout? 1.5 mL microcentrifuge tube? 15 mL tube?
9. 1.7: How is the buffer exchange done?
10. Please specify all incubation times. We need specific values.
11. Please specify all experimental values and parameters used.

12. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step.

13. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

14. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

15. Please do not highlight calculations for the video: step 3.4-3.6, 4.3-4.7

16. Please include at least one paragraph of text to explain the Representative Results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. Data from both successful and sub-optimal experiments can be included.

17. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

- a) Critical steps within the protocol
- b) Any modifications and troubleshooting of the technique
- c) Any limitations of the technique
- d) The significance with respect to existing methods
- e) Any future applications of the technique

18. Please do not abbreviate journal titles.

Reviewers' comments:

Reviewer #1:

We would like to thank the first reviewer for their very positive comments on our work and helpful suggestions. We agree with important points raised by the first reviewer, and hence addressed these points in our revised manuscript.

Manuscript Summary:

The manuscript is intended to be a tutorial for the analysis of non-covalent protein complexes using native state nano-electrospray ionization coupled with ion mobility mass spectrometry. The interaction between the helicase-nuclease protein complex (HerA-NurA) with several ligands (DNA, ATP, ADP) studied in a previous publication [21] is used as a model compound for this tutorial. The

manuscript provides adequate details for sample preparation, mass spectrometer operation as well as data interpretation. I believe this manuscript would be of great utility to everyone interested in hands-on characterization of complex protein complexes using intact mass analysis, therefore I recommend the publication of this manuscript with several revisions.

Major Concerns:

We have now divided the previous 'part 3' into two parts (part 3 and 4) and therefore highlighted section 2-5 to be included in the video.

Part 1 of the Protocol section contains mostly general guidelines for optimizing the sample preparation before the analysis of the HerA-NurA protein complex interacting with several ligands. I don't think that Part 1 should be highlighted in yellow and included in the video material because is not as relevant as Parts 2-4.

Minor Concerns:

We have addressed all points (1-25) raised in the revised manuscript. We have revised our figures such that numbers used for subscript notation. Figures are now provided at a higher resolution.

Please find below a list of my suggested revisions and comments:

1. Page 3, Lines 127-128:

For HerA-NurA studies 200 nM ammonium acetate pH 7 was used.

2. Page 4, Line 143:

Please indicate the upper m/z value of the quadrupole installed in the Synapt G2-Si instrument used for these experiments (e.g. 4,8 or 32 k).

3. Page 4, Lines 147-149:

For IM separations, Nitrogen (60 mL/min) and Argon (8.4mL/min for the Trap region) were used. However, Helium can be used for IM separations as well.

4. Page 4, Lines 152-153:

... and insert it into a capillary holder.

5. Page 4, Line 156:

Please indicate the pressure units for the nano-flow pressure gauge.

6. Page 4, Line 159:

Apply a capillary voltage in the range of 0.9-1.6 kV.

7. Page 4, Lines 161-162:

... adjusting the gas flow in the Trap (2-8 mL/min), He Cell (180 L/min) and the IMS cell (90mL/min)

...

8. Page 4, Line 168:

The Synapt G2-Si instrument does not have an extraction cone. Please provide instead the

optimized values of other nano-ESI source parameters like source offset, source temperature and cone gas flow.

9. Page 5, Lines 180-182:

To quantify the relative abundance of species use the corresponding ion intensities observed in the raw ESI-MS spectra (for example ligand bound, different oligomers, etc). Alternatively, quantification can be performed using specialized software like UniDec and Massign34 (Figure 1 and 3).

10. Page 5, Line 209:

... use the SUMMIT software...

11. Page 6, Line 230:

... by subjecting it to CIU.

12. Page 6, Line 231:

Specialized software packages such as PULSAR32,

13. Page 6, Line 245:

... transitions and stabilizations of protein ions.

14. Page 7, Line 306, Figure 1 caption:

... composition and topology of the HerA-NurA non-covalent complex.

15. Page 8, Line 313, Figure 2 caption:

Native ESI-MS reveals the mechanism ...

16. Page 8, Lines 334-335:

... (after considering the typical uncertainty in the resolution of travelling wave ion mobility mass spectrometry of $\pm 5-8\%$).

17. Page 8, Lines 338-339, Figure 5 caption:

Colored circles indicate conditions where each sub-complex was observed: native (blue, prior to disruption), ...

18. Page 8, Line 348, Figure 7 caption:

There is a discrepancy between the Figure 7 caption and the actual Figure shown on Page 19.

The figure caption needs to be corrected (Figure 7A is mentioned 3 times in the caption).

19. Page 9, Lines 376-377:

... careful sample preparations steps are essential, ...

20. Page 9, Line 382:

... electrospray mass spectrometers.

21. Page 10-12, References section:

Please reformat references no 7, 16, 18, 28, 38, 40 and 44 to match the format used for the the authors's names in all other references.

22. Page 11, Line 461:

Reference 27 has several typos.

23. Page 16, Figure 4C:

Please provide the Figure 4C with higher resolution, the labeling of the oligomeric species is not clear at all! The numbers used for subscript notation are barely distinguishable.

24. Page 17, Figure 5:

Please provide the Figure 5 with better resolution, the numbers used for subscript notation are barely distinguishable.

25. Page 20, Table 1:

Please avoid the decimals for the theoretical and experimental CCS values of the gas phase complexes and sub-complexes. Use instead the integer numbers because the IMS method is not that precise for this large molecular weight complexes.

Reviewer #2:

We would like to thank the second reviewer for their comments on our work and supportive suggestions. We agree with important points raised in the minor and major concerns sections and have addressed these points in our revised discussion. Moreover, all parts now have a paragraph in the beginning that provides a brief explanation of the section. .

Manuscript Summary:

The manuscript addresses an important aspect of analyzing biomolecular structure using mass spectrometry, and should be published.

Major Concerns:

The manuscript suffers from a lack of clear structure and organization, which makes it difficult to follow - particularly the breakdown into sections and the logic flow of the experiment with options such as ligand binding studies clearly described. For example the main exp part starts with preparation of ATP in a very specific way, without explaining the goal and limitations of the intended experiment first.

Generally many points might well apply to the particular complex but other statements of apparent universal applicability are mixed in with this, which makes the instructions very confusing.

Statements such as "To obtain sufficient DNA binding, screen different DNA concentrations." are not of general validity - is this complex particularly difficult to form?

He gas can indeed be used for ion mobility, but not easily on the instrument described here or by the non-expert user, so it would be best if the authors describe a "standard" workflow, and clearly highlight which points are specific to the system studied here and why.

The instrument tuning strategy makes no sense to me, it should start with the inlet (Cone) and only increase coll. energies if "front end" voltage offsets are insufficient. What does the used need to look out for when setting these parameters? How do we know if conditions are still "native"?

Also section titles such as "Part 4: Investigating protein-ligand interactions using collision induced unfolding (CIU)" are not very clear, why would CIU only inform on ligand interactions rather than e.g. protein stability towards unfolding? Please re-organise the sections to create a logic flow.

I would strongly discourage reporting of ccs values as "average of all 358 charge states." - please discuss and justify which charge states should be used.

Minor Concerns:

We have addressed these points in our revised manuscript. With regards to proteins used for calibration, we have rephrased the sentence to be clearer "Select four calibrants, two with a mass above and two with a mass below that of the protein under investigation". To make Figure 4 clearer, we have split the content into two Figures (Fig.4. and Fig.5).

Why are ammonium acetate buffers non-physiological? Why not use MgATP? Part II 20 - I would not recommend making any calibration with just two reference points?

Figure 2: please check if the labels got mixed up

What are the curves representing in Fig 3B? Symbols in Fig4 are too small and blurred.

Yours sincerely,

A handwritten signature in blue ink, appearing to read "Argyris Politis".

Argyris Politis