**Response to editor and reviewers' comments:**

**Editorial comments:**

*1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.*

Manuscript has been proofread.

*2. Please shorten the title if possible. For instance, “― a method for” may be removed from the title.*

Title was shorted to: "Ion Exchange chromatography (IEX) coupled to Multi Angle Light Scattering (MALS) for protein separation and characterization."

*3. 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. You may use the generic term followed by “(see table of materials)” to draw the readers’ attention to specific commercial names. Examples of commercial sounding language in your manuscript are: AKTA, GE Healthcare, Wyatt Technology, ASTRA, Optilab, Millipore, Stericup, etc.*

Manuscript has been rephrased and commercial language was deleted.

*4. Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm).*

Speed units were converted from RPM to g.

*5. Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.*

Manuscript was revised to the imperative tense. "Notes" were added where it was necessary.

*6. 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. Use sub-steps as necessary. Please move the discussion about the protocol to the Discussion.*

Paragraphs were shorted to simplify the protocol.

Discussion parts were removed to the discussion section.

*7. 2.2, 2.3, 3.1, 3.2, etc.: Please provide specific values to be used here. We cannot film a generalized protocol; we need specific settings of a specific experiment. For instance, please specify the protein sample used, the pH and ionic strength that are adjusted to, and quantity of protein to inject.*

We specified the protocol and gave specific values along all the protocol.

*8. 3.3.1: Please specify the sample used and sample volume injected. What is used to wash?*

Sample and volume and buffers were specified.

*9. 3.4.1-3.4.10: Please write the text in the imperative tense that describes how to optimize different parameters.*

Manuscript was revised to the imperative tense.

*10. 4.2.1: Please specify the flow rate.*

Flow rate was specified.

*11. 4.2.2: Please specify the concentration and sample volume.*

concentration and volume were specified.

*12. 4.3.2: Please specify the buffers used. How many washes are needed?*

Buffers and washes were specified.

*13. After you have made all the recommended changes to your protocol (listed above), 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.*

Pages to film were highlighted.

*14. 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. Notes cannot usually be filmed and should be excluded from the highlighting.*

Complete parts to film were highlighted (Notes were excluded).

*15. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.*

Complete parts to film were highlighted

*16. Discussion: Please discuss critical steps within the protocol.*

The discussion referring several steps in the protocol. For example: "The relatively large amount of protein used in IEX-MALS (detailed in section 2.3) compared to SEC-MALS is important….." ; "This requires additional blank run for each IEX-MALS experiment and analysis with baseline subtraction (as described in section 5.2), unless concentration analysis is limited to UV detection" ; "optimization by varying different parameters such as gradient slope (see section 3.4) can be performed…".

*17. Please note that the illustration figure of IEX-MALS is not referenced in the manuscript. If it is not intended to be used in the manuscript, please remove it from the submission files.*

Illustration figure was referred in the introduction. For filming we suggest to show this illustration as animation (slide after slide).

*18. Table of Materials: Please use the micro symbol µ instead of u and include a space between all numerical values and their corresponding units (10 mm, 0.1 µm, etc.). Please sort the items in alphabetical order according to the name of material/equipment.*  
Table of materials was updated as required.

**Reviewers' comments:**

**Reviewer #1:**

*Line 83: "degraded" analysis - I suggest the word "flawed".*

*The legends in Fig1 are a bit fuzzy, so a higher resolution file would be useful.*

We thank the reviewer for these comments. We rephrased the line 83 as was suggested and improved the resolution of figure1.

**Reviewer #2:**

*In par 2.3 the authors mention that for a measurement 0.3-0.5 mg of sample is needed of a 60 kDa protein. Here they should also mention the size of the column used for the experiment. Furthermore, it would be useful if the authors could give an indication of the amount of smaller and larger protein needed instead of only mentioning higher or lower (par. 2.4). So that the reader can make an educated guess how much of his target protein he/she would need.*

We thank the reviewer for the comment. We added in the manuscript section 2.3 the size of the column as was suggested, and also added quantity of injection for smaller and larger proteins (in the discussion).

*In par. 3.3.2 the authors mention the option to split the measurement into two gradients. This should be explained better.*

We rephrased this sentence and elaborate on this split gradient.

*In general, it would be useful if the authors could give more information about IEX columns that could be used for this method. A table of commercially available columns would be useful. Did the authors look into the use of weak versus strong IEX resins (e.g. DEAE vs Q)?*

We used several IEX columns, AIEX such as monoQ, miniQ, sepharose Q (from GE), and CIEX such as monoS (GE) and SCX-NP5 (sepax). We did not look into a comparison between strong and weak IEX column, comparing same sample. However, this protocol describes the use of a specific column (mono Q HR GL). Separation and analysis of other proteins with other columns for IEX-MALS experiments can be seen in our previous paper, at Amartely et al, Scientific Reports 2018. We added to the discussion that preparative IEX columns can also be used with MALS – *"Non-analytical IEX column are also stable, with low degree of particle releasing, therefore can be used with MALS. "*

*In par 4.2.2 the authors mention the standard dn/dc for proteins (0.185 ml/g). It would be very useful if the standard dn/dc values for non-protein compounds could be added, such as DNA, RNA, glycosylation residues, etc. These values are needed to be able to accurately calculate the mass of e.g. a protein-DNA complex.*

dn/dc values of sugars, DNA and lipids were added to the manuscript, to section 5.1.3.

*In par. 4.3 the authors do not mention how they prepare their columns before the measurements or regenerate it in between measurements. Do they clean the column or just wash with binding buffer?*

Step 4.3.2 describes the procedure required for washing the column for each IEX-MALS experiment: *"Wash column and valves with the relevant buffers: 20 mM Tris-HCl pH 8 with 50 mM NaCl for washing buffer (A valve) and same buffer with 500 mM NaCl for elution buffer (B Valve). Make sure that the final column wash uses the binding buffer, containing low salt concentration, to enable binding of the protein to the column matrix."* So column is washed with high salt buffer first (elution buffer) and then by the washing buffer between measurements. We also added additional optional wash step in this section: "For massive wash of strongly bound impurities, use 0.5 M NaOH before washing with the relevant buffers, followed by a buffer neutralization wash".

*In par. 5.2 the authors describe data analysis including baseline subtraction. This is done by performing a separate baseline experiment. Unfortunately, not all available LS equipments are able to perform this type of baseline subtraction. Therefore, would it be possible to correct the baseline within the experiment as is done for SEC-MALS?*

Experiments that are performed with a linear gradient and enough protein quantity can be analyzed without buffer subtraction, just by adjusting the baseline of the RI (can be tricky). For high quality analysis and for this protocol (that use also low quantity of protein) RI baseline subtraction is very recommended (for analysis that utilize RI as concentration source). Therefore, the procedure describes the baseline subtraction as well. However, we did emphasize that subtraction is required only if RI signal is used as a concentration source. If UV signal is used for measuring the concentration, this step is not required (see section 5.2 and discussion).

*Furthermore, the authors mention that is may be necessary to adjust the dn/dc values when the NaCl concentration well exceed 200 mM. In IEC chromatography this is not unusual. Even in the BSA experiment the authors use a gradient from 75 to 350 mM NaCl. Therefore, the authors should explain how the adjustment of the dn/dc values is done.*

Dn/dc of each peak should be adjusted according to the buffer conductivity at the eluted peak. If protein eluted at [NaCl] < 200 mM (like in the BSA example) this adjustment is not very necessary. However, description of how the adjustment is done can be found in our previous paper (Amartely et al Scientific Reports 2018) as we cited in the manuscript. We also added this point in the discussion.

*In the BSA experiment the authors should mention which buffer and which quality BSA were used*.

Manuscript has been updated and we added the buffers that were used for the run. Quality of commercial BSA is mentioned in table of material.