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Live Webinar Q&A Sheet:

Towards Well-Characterized Proteins and Protein Conjugates Using SEC- MALS
Dr. Michelle Chen

The recorded webinar may be viewed from the Biotherapeutics webinars page. These questions were submitted by live viewers. Additional information on SEC-MALS, DLS, FFF, CG-MALS and RT-MALS may be found on the Wyatt web Library under Webinars, Application Notes, Featured Publications and Bibliography, as well as on the corresponding Product page and Solutions page of our web site.

Please contact info@wyatt.com with any additional questions.

Questions & Answers

GENERAL MALS QUESTIONS

Q: *How accurate are the extinction coefficients from SEC-MALS-UV-RI method?*

A: The accuracy of the extinction coefficients from SEC-MALS-UV-RI method is high, approximately 5% or better in the solvent used as the mobile phase. Protein molecular weight measured by the SEC-MALS-UV method can be used to further confirm the accuracy of the extinction coefficients. For more information, refer to the following two publications:

- Wen, J., et al. *Techniques in protein chemistry*, vol. 8, pp. 113-119. Academic Press, 1997.
- Miranda-Hernández, M., et al. *Analytical and bioanalytical chemistry* 408, no. 5 (2016): 1523-1530.

Q: *You mentioned the accuracy in MW of SEC-MALS is usually 1-5%. How precise is SEC-MALS in measuring the diameter of the particles?*

A: For a particular SEC-MALS system, its accuracy for MW is 5% or better. The error in MW is often systematic and the precision of MW measurement is 1% or better under optimal conditions. The MALS detector can also measure the size (in terms of RMS radius) of a particle with radius greater than 10 nm. The precision of RMS radius can be as good as 0.2 nm. For proteins, their radii are typically too small to be measured by MALS. The online DLS detector, however, is used to measure the hydrodynamic radius of proteins with 0.1 nm resolution under optimal conditions. Here is a good NIH reference on MALS precision and accuracy:

- Soman, Gopalan, et al. "Analytical characterization of ch14. 18: a mouse-human chimeric disialoganglioside-specific therapeutic antibody." MAbs. Vol. 4. No. 1. Taylor & Francis, 2012.

Q: *Can SEC-MALS be validated to use as a test method for quality release of a protein or conjugate?*

A: Yes, SEC-MALS has been validated as a release assay for complex protein samples for which a simple concentration detector alone is insufficient to reveal quality variations. We provide GMP compliant software and services to facilitate our customers using SEC-MALS for release assay or extended characterization as part of release panel.

Q: *How can I confirm my SEC method?*

A: By adding a MALS detector to an existing SEC method, absolute molecular weight (MW) can be directly measured without the dependence on retention, column calibration, and making assumptions on protein shape. In fact, the combination of absolute MW and retention time can help us judge the quality of the SEC method. If you determine that the SEC method is not optimal, you can reach out to Waters colleagues for assistance in optimizing your SEC method.

Q: How do you run an experiment with two UV traces and load these signals into the Wyatt software?

A: If you use an LC that is digitally integrated by Wyatt ASTRA through HPLC CONNECT, multiple UV signals could be collected. For other LCs, not supported by HPLC Connect, two UV traces could be collected through analog outputs of the UV detector. You can register on the Support Center to download the Technical Note on this topic or start your reading here: https://www.wyatt.com/products/software/hplc-connect.html

Q: *Some of the slides have two lines of MWs over the peaks, how can there be two different MWs at the same time slice?*

A: The two lines of molecular weights across the antibody-oligonucleotide conjugate (AOC) peaks are the molecular weights of the respective protein and RNA from ASTRA's Protein Conjugate Analysis module.

SEC & COLUMNS

Q: *Do I need a high-resolution column to analyze MW for each part of a complex by MALS?*

A: The SEC-MALS method measures weight-averaged molecular weight (MW) of the molecules eluted at each data slice. If the molecule is a binary conjugate, the method can measure not only the total MW but also the MW of each moiety. Further, MALS helps us determine whether the peak contains homogeneous molecules/conjugates or contain different species from the MW perspective. When the peak is heterogeneous in MW, which means the peak contains multiple molecules or conjugates, a high-resolution column helps with more detailed and accurate analysis and quantification of these different species.

Q: *Will hydrophobic interactions play a role with column and membrane protein interaction? Is there a specific column for membrane proteins?*

A: When an analyte interacts with the column, whether by hydrophobic or electrostatic interaction, the analysis resolution of different species will be reduced. However, the addition of MALS can still help reveal the presence of non-ideal SEC effects and more insights of the sample. Waters XBridge Premier columns can work well with both soluble proteins and membrane proteins due to their inert surfaces.

Q: *Which column pore size and matrix could be better for SEC-MALS on Virus like particles (empty virions)?*

A: To select the right columns matching with your VLP's size, please visit either the Waters or Wyatt Technology website. https://store.wyatt.com/sec-columns/

Q: Does mobile phase gradient alter the initial baseline noise in the MALS detector? Do you need additional hardware to carry out gradient chromatography-MALS analysis apart from UV, dRI and MALS detector?

A: Mobile phase gradient does not alter MALS baseline noise much but does alter MALS baseline reading. For a linear gradient, MALS baseline drifts slightly but linearly. Therefore, by setting a linear MALS baseline, we can simply remove the gradient effect on the MALS detector without requiring any additional hardware.

Q: *Will Waters be updating Empower to be able to run SEC MALS?*

A: Yes, we appreciate that many of our customers have requested Empower running SEC-MALS to simplify workflow and facilitate method transfer to QA/QC. We are working hard to deliver the solution in the near future.

AEX/RPC-MALS

Q: *I haven't seen too many papers on anion exchange chromatography (AEX)-MALS, which was used in the antibody oligo-conjugate experiment. What is the hardware configuration that is needed and some "watch-outs" if I want to run this type of assay?*

A: Using AEX or other adsorption-based chromatography modes for proteins or protein conjugates, dRI detector is not suitable to provide concentration data, but we can use a UV detector for concentrations, single wavelength UV for proteins and TUV or PDA for protein conjugates. Please contact support@wyatt.com or your Wyatt regional manager for more details.

Q. *Previously I heard MALS could not be used with reverse phase gradient methods - how straightforward is the workflow for getting MW of protein in a reverse phase method? Can MALS detect MW differences of glycosylated and non-glycosylated fractions of a protein?*

A: RPC-MALS has a precision of roughly 1-2%. If the change in molecular weight due to glycosylation is less than 1%, then MALS cannot differentiate between the glycosylated and non-glycosylated protein.

Q: *Are there any limitations to column selection for SEC-MALS hyphenation? Especially for flow rate?*

A: We have two sets of SEC-MALS detectors, one optimized for the HPLC platform and the other for the UHPLC platform. Depending on your needs, one platform may be more appropriate than the other. For example, if you are limited in sample amount or would like to speed up your analysis, then the UHPLC system may be the best choice for you. It also allows you to use a low flow rate. Please contact support@wyatt.com to discuss more details.

Q: How can I analyze the MW of each part of a complex in the same peak in SEC-MALS? I have MW data only for the complex.

A: We use the signals from MALS and two concentration detectors to calculate MW of each component in a binary complex.

Q: *Could you elaborate how you measured both siRNA and antibody molecular weight simultaneously?*

A: The measurement is based on the signals from MALS and UV at 280 and 260 nm. Please contact us directly to go over more details.

Protein Conjugates & Complexes

Q: *Can we use SEC-MALS to determine AlexaFluor/Antibody ratio in dye labeled antibodies? (label/antibody ratio)*

A: This may not be so straightforward when the modifier MW is small (less than 1%) compared to MW of antibody. In addition, the fluorescence from the dye affects the light scattering signals and needs to be removed first.

Q: *When working with the antibody-siRNA conjugates, did you find that the dn/dc value for antibody was appropriate? Did addition of the siRNA have a large effect?*

A: We only need to know the dn/dc values of antibody and siRNA, ***a priori***.

Q: *If we have a conjugated protein but we don't have any information on the conjugate molecule - how can we address this (practically) in SEC-MALS?*

A: I'd suggest to analyze it as an unconjugated protein first. Based on the apparent MW and extinction coefficient, we get more clues on conjugated molecule.

Q: *For a protein-polysaccharide conjugate, would we have to separately measure dn/dc? For an RI analysis? Is dn/dc 0.185 for membrane proteins too?*

A: For protein conjugates, we only need to enter the dn/dc values for protein and modifier, respectively. In the case of protein-polysaccharide conjugate, we enter the dn/dc values for the respective protein and glycans; for membrane protein-surfactant complex, dn/dc values for the respective protein and surfactant.

Q: *When running gradient methods such as reverse phase, does sample dn/dc change as mobile phase aRI changes? Can you please comment on how to deal with this when analyzing data in ASTRA?*

A: The dn/dc value does vary with mobile phase composition, but only slightly. Depending on your needs for accuracy and precision, you may or may not need to refine your dn/dc values. Please feel free to reach out if you would like to discuss more.

Q: *Can your method apply to hydrophobic interaction chromatography (HIC) for antibody drug conjugates ADCs? How does it compare to mass spectrometry (MS)?*

A: Yes, MALS can also be applied to HIC for ADCs. It is not as accurate as MS, however, it can be used for routine analysis and identify the aggregates. Please check back in April 2024 when we have more data to share.

Q: *Ribonucleoproteins (RNPs) are not covalently conjugated, can SEC-MALS still be able to analyze them?*

A: SEC-MALS analyzes both covalently bond and non-covalently bond complexes in the same way.