Live Webinar Q&A Sheet:

RT-MALS: Downstream PAT for Biologics, Vaccines and Gene Vectors

The recorded webinar may be viewed from the RT-MALS webinars page. These questions were submitted by live viewers. Additional information on RT-MALS may be found on the corresponding Product page and Solutions page of our web site.

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

Viruses and nanoparticle applications

Q: *What are the limits of detection for viruses and lipid nanoparticles?*

A: The limits depend on size and refractive index of the particle. For 100 nm diameter viruses the lower and upper limits of detection are approximately $1x10^6$ and $4x10^{12}$ particles / mL, respectively. For 200 nm lipid nanoparticles, I would estimate the limits of detection as $1x10^5$ and $1x10^{11}$ particles / mL.

Q: *Have you seen applications in which RT-MALS for process monitoring was used to generate real-time estimates for virus size and concentration?*

A: Yes, and in fact such a real-time MALS process monitoring test was recently reported by scientists at Janssen, in collaboration with Wyatt, in the 2021 ISPPP conference. The results were remarkably close to those obtained by offline methods performed on fractions.

Q: *Will turbidity in the solution affect the analysis?*

A: Unlike dynamic light scattering, where turbidity leads to incorrect size measurements as a result of multiple scattering, MALS is surprisingly robust to turbidity. As long as the solution is not opaque and light actually penetrates the sample in the flow cell, size measurements will be quite accurate. Concentration measurements, which depend on the ratio of scattered to incident light, must be corrected to account for light lost to turbidity, and we do that using the ultraDAWN's Forward Monitor which measures transmission through the cell.

Q: *Can ultraDAWN analyze viruses or lipid nanoparticles (LNP) to determine if they do or do not contain nucleic acid?*

A: This capability is implemented in our analytical MALS-based systems for AAV and LNP, with ASTRA® software. So far we have not addressed quantifying the nucleic acid content of viruses much larger than AAV, especially enveloped viruses. Determination of AAV nucleic acid content using a DAWN® MALS instrument requires either two UV wavelengths or one UV channel plus

refractive index (RI) detection using an Optilab® differential refractometer, while the payload analysis of LNP requires absorption data from one UV wavelength plus RI.

An ultraDAWN can perform the same analyses as DAWN in 'post-processing' mode, i.e. analyzing the data using ASTRA after the run has been completed, much like you would do in an analytical system incorporating a DAWN. The benefit of the ultraDAWN is its ability to connect to high flow rate systems; while UV detectors for such systems are available, so far high-quality RI detectors are not, so under such conditions we can perform AAV content analysis but not LNP payload analysis. With the availability of high-flow-rate/high sensitivity RI detectors, we would be able to analyze LNP payload as well.

So far, this nucleic-acid-content analysis has not been incorporated into the OBSERVER real-time software, so only post-processing mode is available for this application. But we do expect to add it to OBSERVER in the future, so stay tuned!

Q: *Intermolecular interaction affects light scattering at high concentration - any example how high the concentration of virus particle is?*

A: Intermolecular interactions primarily affect light scattering from concentrated macromolecular solutions, e.g. several g/L of protein or polysaccharide. Most viral particles do not reach those concentrations. For example, for adenovirus, the upper MALS detection limit of 6×10^{12} viral particles/mL, where the viral molar mass is 160 MDa, has a mass concentration of 1 g/L. With AAV it is possible to reach high enough concentrations – 10^{15} particles/mL is equivalent to ~ 7 g/L – but this AAV titer is fairly uncommon. To date we have not incorporated a light scattering correction to size or concentration for interparticle interactions, but it is certainly doable.

Q: *What are the typical error ranges for estimates of virus concentration using MALS? Does that error range depend on size of the particle, and in what way?*

A: Other than small viruses like AAV, where concentration detectors are used, we need accurate values of size and refractive index to calculate virus concentration. Viral size measurement by MALS is accurate to roughly 2-3% for spherical viruses, and refractive index to roughly 2% as well. The typical error range for concentration corresponding to these uncertainties is about 20-30%. In percentage terms these errors are independent of particle size within the specified measurement range of the ultraDAWN, i.e. radius from 10 to 250 nm.

This is the situation for relatively homogeneous solutions, e.g. adenovirus spans a quite narrow size range. However, if the solution is heterogeneous, which is the case for lentivirus, accuracy declines since in the absence of separation, MALS is biased to the larger sizes and tends to underestimate the total number of particles. The degree of error depends on the size distribution. For example, measurement of equal populations of 100 and 120 nm particles will underestimate the total population by 15%; if the size are 100 and 200 nm, the measurement will

basically not see the smaller particles at all, but if the 100 nm particles are 10x more abundant than the larger particles, the final tally is low by about 35%.

Q: *On slide 33 showing separation of virus from protein and DNA, which virus was it and which chromatography process was used?*

A: This was ion-exchange chromatography. Unfortunately I cannot go into the details.

AAV applications

Q: *How is MALS used to get estimates for viral genome titer in AAVs and small VLPs?*

A: MALS data are combined with UV and/or RI to determine viral genome titer. The combination of UV + RI or two UV wavelengths provides the total concentrations of protein and nucleic acid, and these may be integrated over the peak to calculate the total protein mass and nucleic acid mass. When MALS data are added, the molar masses of the protein capsid and the encapsidated DNA or RNA may be calculated. Finally, dividing the total protein mass by the capsid molar mass gives the capsid titer, and dividing the total nucleic acid mass by the encapsidated molar mass gives the viral genome titer.

Q: *For AAV measurement, does your result agree with the traditional way of qPCR/ELISA combination?*

A: Yes, though with much better precision than those methods which require reagents and extensive sample prep. Please request the AAV Solutions Guide for details.

Q: *In the case of AAV process monitoring, if the nucleic acid co-elutes with the empty capsids, can the MALS measurement tell if the nucleic acids are encapsulated within the capsid, or if it is outside the capsid?*

A: If the nucleic acid is not encapsidated and co-elutes with the empty capsids, the MALS signal will be relatively low compared to the concentration signals, resulting in an apparent molar mass that is below that of the empty capsid. If you had previously tested empty capsids or otherwise knew their molar mass, you could conclude that either the capsids are ill-formed and below their expected molar mass, or the nucleic acid is not encapsidated, or perhaps both are true.

Protein applications

Q: *For proteins, how large of an effect does the gradient baseline change have on assessing aggregates?*

A: This depends on the analysis mode, whether post-processing or real-time, as well as the specific gradient. In post-processing you can set the baseline to account for the gradient. In our current real-time software this is not possible, but we do plan on adding some fairly significant capabilities to overcome this issue. Currently I would estimate that a strong gradient can impact

the apparent molar mass by 4-6%, so if you assume that the aggregates are all dimers, the uncertainty in estimating their abundance would be 2-3%.

Polymer applications

Q: *Can RT-MALS be used to monitor the conjugation reaction of polysaccharides with proteins? Can the polysaccharide:protein ratio be calculated?*

A: RT-MALS most certainly can monitor polysaccharide-protein conjugation, since the solution weight-average molar mass increases as the reaction proceeds. The molar mass values may not be very accurate because the mixture of materials with different dn/dc constants and the formation of unknown stoichiometries results in underspecified math, but the signal will be robust and can be calibrated against offline analytical measurements using SEC-MALS or FFF-MALS.

Calculating the polysaccharide:protein ratio in the conjugates cannot be done without separation.

Q: *Can we use RT-MALS in offline mode along with OBSERVER software to monitor the reaction using an existing MALS systems where we have multiple sample preparation steps (due to use of very high concentration of inorganic acids in the process)?*

A: You could perform, and possibly even automate, a series of injections of aliquots into your MALS detector, without separation and using a sequence in ASTRA, which would result in a real-time series consisting of molar mass points every few minutes.

OBSERVER software does not work with MALS instruments other than ultraDAWN.

Q: *Are there any reported applications where RT-MALS has been used in monitoring polymerization reaction progress?*

A: Yes, in fact the raw light scattering signal has long been used to monitor polymerization reactions. Converting the MALS data to an apparent molar mass is straightforward.

Q: *Regarding the protein aggregation with respect to concentration 2 g/L , is this concentration the effective concentration in the flow cell or is it the test concentration? How can we make a decision to include the impact of A2 coefficient effect on Rg/MW if the concentration is >2g/L?*

A: The apparent onset of interaction effects at 1-2 g/L refers to the concentration in the flow cell, and if the concentration reaches that level then it is certainly worth at least measuring A_2 under your buffer conditions to see how it might affect the M_w result. Please note that 'interactions' does not necessarily mean aggregation. In fact, the typical onset around 1-2 g/L pertains to excluded-volume interactions, which are repulsive and do not lead to aggregation. If the molecule does have a strong tendency to aggregate, then it is entirely possible that A_2 effects become significant at lower concentrations.

General technical questions

Q: How might an ultraDAWN integrate with a third-party chromatography system?

A: In terms of flow path, we generally adapt the tubing diameter to that of the chromatography system, up to an internal diameter of 1.0 mm (0.040”). In terms of synchronization and control, we set up an exchange of digital pulses and analog signals, via analog and digital input/output connections. For example, in AKTA systems we use the IE9 I/O box, and in BioRad systems, the SIM module. Then we program the chromatography method to issue digital pulses at the appropriate times, and to monitor the analog signals sent by the ultraDAWN.

Q: For in-line measurement, do you provide aseptic connectors/tubings, etc.?

A: We use standard lab-scale chromatography tubing and fittings such as 10-32 cone fittings and 1.6 mm PEEK tubing. We do provide a few of each, but they are easy to purchase via your labware distributor.

Q: How can the ultraDAWN be sterilized? Does it have a single-use option?

A: No, the ultraDAWN does not have a single-use option as it is primarily directed at the process development lab (even though it can also be used online in full GMP manufacturing scale). The instrument may be sterilized by flowing 1 M NaOH, 10% nitric acid or similar sterilizing solvents. We are, however, looking into a single-use version that would be suitable for scaled-up, full GMP bioprocessing. Please contact us if you have near-term needs for such an instrument.

Q: How do the error ranges for concentration and size estimates change when you move from preparative to analytical methods for unit ops, like chromatography or AF4?

A: In a preparative operation, samples are not separated and the error mentioned above due to heterogeneous populations comes into effect. Separating by size and measuring each size fraction independently is highly beneficial for obtaining accurate size distributions and concentrations, since detection of the smaller particles is not overwhelmed by scattering from the larger particles. The error related to heterogeneity is no longer in effect and you return to an overall accuracy of 20-30%, given a reasonably accurate virus particle refractive index.

Q: What are the challenges in qualifying and validating RT-MALS PAT measurements for GMP runs?

A: That depends, of course, on the particular product and process. Essentially you would want to collect aliquots from the process and perform offline analytics such as SEC-MALS, DLS, NTA or other techniques to validate the RT-MALS results, and vary the process conditions so as to introduce whatever variability you expect to see during production. The biggest challenge is offline analytics on many, many aliquots if you have long runs or many possible variations.

Q: *What is the typical acceptable range for the measurement analysis of a peptide of 7-8K Daltons?*

A: From about 50 µg/mL and up for analytical measurements, and for real-time I would go with at least 500 µg/mL.

Q: *How can we integrate RT-MALS signals in chromatographic system software (e.g. UNICORN by GE/Cytiva) for evaluation without OBSERVER control software?*

A: OBSERVER provides an analog output signal in real time that can be read into preparative chromatographic software like UNICORN, for example via the AKTA IE9 I/O Box. The signal is proportional to one of the attributes calculated by OBSERVER: molar mass, size or particle concentration. The user simply designates the output channel and the constant of proportionality in OBSERVER, then instructs UNICORN using e.g. a 'watch' instruction to monitor that signal for a specific value, much as would be done for a UV signal.

Q: *Do you use different wavelengths of light? When detected: do you use a CCD detector for increased speed?*

A: Only one wavelength is used for light scattering, usually 660 nm.