Live Webinar Q&A Sheet:

Expanding the characterization toolkit with FFF-MALS: Developments, techniques and applications

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

Request information about FFF-MALS instrumentation

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FFF-MALS application

Q: *Can you share the advantages and disadvantages of FFF-MALS compared to DLS and SEC-MALS for AAV analysis?*

A: Utilizing all of these orthogonal techniques is desirable for AAV analysis. Because AAVs are relatively small, they are actually quite suitable for SEC-MALS and DLS. And with SEC-MALS, we've developed a robust method for determining critical quality attributes like identity, particle concentration, capsid content, and purity for many serotypes. To learn more about the SEC-MALS and DLS AAV Method Implementation & Training, visit us online. The advantage of FFF-MALS for AAVs is confirming the purity. If you have AAV aggregates, FFF-MALS is particularly advantageous for characterizing those, as there is no stationary phase that can degrade or filter off larger aggregates as you might see with SEC.

Q: *Can FFF-MALS be used to measure empty/full AAVs and what is the accuracy and resolution compared to SEC-MALS?*

A: Both FFF-MALS and SEC-MALS can be used as biophysical characterization techniques for AAVs, with FFF typically capable of higher selectivity but lower resolution than SEC. It's worth noting that both SEC and FFF separate by size, so would not be able to resolve empty from full AAVs; however, as a biophysical characterization technique they can both quantify capsid content, identity, and particle concentration. SEC-MALS is a simpler, more robust technique that benefits from the relatively smaller size of AAVs, and offers detailed characterization, which you can learn more about online. However, FFF-MALS can be used to investigate the presence of aggregates, which SEC columns may filter off.

Q: *How do you compare FFF versus SEC for AAVs analysis ex. aggregation?*

A: AAVs are relatively small compared to other viruses, and so are quite compatible with SEC. However, AAV aggregates could potentially be filtered off by the SEC column, and because they would not elute, you would not be able to quantify or analyze them with SEC-MALS. The best way to confirm is to run the sample on both FFF-MALS and SEC-MALS—if the FFF-MALS data reveals AAV aggregates than you know the SEC column likely filtered them off. So, the techniques benefit each other.

Q: *What types of absorbances can you achieve with DCM? Particularly for the lentivirus and exosomes?*

A: The concentration signal you achieve will depend on several factors like loading, peak broadening, etc. but the DCM can give you a relative enhancement to that signal. For example, a split ratio of 1:1 generates a 2x enhancement in concentration compared to no DCM. A split ratio of 3:1 generates a 4x enhancement in concentration. With some loss to resolution, you can achieve 10x concentration enhancement or more. It is worth noting, however, that UV absorbance with lentivirus and exosomes is vulnerable to UV scattering due to the large size of these species, which can overdetermine the concentration. This is where MALS is particularly advantageous for particle concentration, as we analyze the scattering dependence on angle in combination with intensity to determine particle concentration.

Q: *What is the lowest concentration of sample that needs to be used to effectively separate using EF4 to separate based on size and charge?*

A: I encourage you to reach out to us so that we can discuss your specific sample of interest. The lowest concentration in EAF4- or FFF-MALS depends more on the sample properties and the detection methods than the technique itself. EAF4 can separate from the nanogram scale to the multiple milligram scale depending on the channel size. The lower concentration limit depends on the detection methods—the DAWN® MALS detector for example can detect a few hundred nanograms of a small protein standard, and likely you need less for larger samples that scatter more light or that have a higher dn/dc.

Q: *What particle concentrations of exosomes can be achieved with MALS?*

A: As MALS provides both the radius and the number density (with inputs for the refractive index of the sample and solvent), it becomes possible to determine the number density as a function of radius as we demonstrated in the webinar. In this example, the particle concentration for the entire peak was around 10^9 particles/mL, though depending on light scattering data quality, suitable data may be acquired for peaks down to 10^7particles/mL or maybe 10^6 particles/mL.

Q: *What was the difference between the traces comparing free RNA and LNP-RNA blue and red curves?*

A: In this example, the red trace was the LNP-RNA complex of interest and the blue trace was the free RNA control. By identifying where the free RNA would elute relative to the LNP-RNA (using the same method), we can more confidently identify the presence or lack of free RNA in the sample.

Q: *Can FFF be used to purify large quantities of AAV for manufacturing?*

A: We offer a few different channel sizes, the largest being the semi-preparative channel that can be loaded with multiple milligrams of sample. Unfortunately, as a preparative technique in manufacturing, this amount of material, which would have to be purified in "batches" instead of a continuous process, may not be suitable for large quantities (15+ mg). However, for monitoring your manufacturing processes, you can still use MALS for real-time monitoring of AAV production. You can learn more online about our RT-MALS benefits.

Q: *Any case of combining FFF with zeta potential for antibody like molecules?*

A: We do have case studies exploring AF4 and EAF4 with antibodies—please reach out to us to discuss further. We have measured mAbs, including Human IgG, with EAF4, where mobility can potentially be used to improve the monomer-dimer separation with a positive polarity applied as an orthogonal technique to SEC-MALS.

Q: *In the separation of free RNA and LNP, the light scattering signal of free RNA was too low to be observed when in the LNP sample. How do you verify the free RNA, by collecting fractions?*

A: Collecting fractions, especially with DCM is a strategy. In this case, the UV peak (if the RI or LS peaks are too weak) can be integrated to determine free RNA—provided it is large enough to be retained by the membrane. With the new Eclipse, improvements in signal and system pressure stability provide better baselines and more robust quantitation, and the addition of DCM enables weaker signals like free RNA to be enhanced beyond what was shown in this webinar.

FFF practical

Q: *You mentioned there are new fixed height channels. What are the advantages and disadvantages of these channels?*

A: Most commonly, an interchangeable spacer made out of a polymeric material is used to define the height of the interior channel. This variable-height channel design is the most common design, and offers excellent flexibility for method development. You can easily switch between different heights using spacers of different thicknesses as part of method development. However, if you have a method already or routinely use just one channel height, the fixed-height channels

become much more convenient. In these new channels, the spacer thickness is integrated into the top block. Because the top and bottom blocks are in direct contact, you can assemble them finger-tight with a screwdriver instead of using a torque wrench. The effective height of the channel is also closer to the specified height because there is no spacer that gets compressed. And if you do need multiple heights, you can buy multiple top blocks.

Q: *Are there limitations for what analyte or what range can be used with the dilution control module?*

A: The DCM functionality is not dependent on analyte properties or solvent properties. It draws off solvent from the top of the channel, where we would expect any amount of cross flow will drive most of the sample to the bottom 10 - 50 microns of the channel. It can work with a broad range of different samples. For the range, the DCM reduces dilution by siphoning off liquid, so removes some fraction of the channel flow. This results in lower detector flow as you go to higher split ratios, which has some additional benefits like improving DLS data. However, we typically do not recommend going below detector flow rates of 0.1 – 0.15 mL/min, which may lead to suboptimal mixing in the flow cell or excessive band broadening. We've explored 10:1 split ratio and higher with fraction collectors with some loss of resolution; or 5:1 split ratio with no effect on resolution.

Q: *Which carrier solvents are compatible with AF4? Can you run AF4 using DMSO?*

A: The Eclipse FFF system is compatible with both aqueous and organic solvents like THF, toluene, hexane, alcohols and others. Strongly polar organic solvents like DMSO, DMAC, and such are problematic because the traditional membranes used in FFF like regenerated cellulose can degrade in these solvents. You could try cutting alternative membranes, such as PTFE but in our experience, DMSO is one of the more challenging solvents to use successfully.

Q: *Can you run an on-line viscometer with the new Eclipse?*

A: This is something we are continuing to look into and develop, as with the DCM control over flow rate and pressures, we would expect viscometry data to be of much higher quality. At this point, we have not done validation experiments.

Q: *What is the tolerance of FFF for in process samples, which may have cellular debris that would otherwise foul up a SEC column?*

A: Because there is no stationary phase and the only consumable is the membrane, FFF-MALS is quite suitable for process samples which may have cellular debris. In this presentation, we provided an example with lentivirus (LVs), which was purified separately to remove the very largest cell debris—those debris that are in the multiple micron range; however, there was a significant amount of cellular debris in the sub-micron range, as well as smaller serum albumin contaminants, that FFF-MALS was able to separate from LVs.

Q: *Does the mobile phase need to be the same for the entire separation when coupled to MALS? Will a change in the mobile phase result in an incorrect molar mass?*

A: In FFF-MALS, separation is achieved by exploiting size differences. Because there is no stationary phase, solvent gradients are not typically used in FFF and usually the same mobile phase is used for the entire separation. Instead, separation is governed by controlling the amount of cross flow during elution.

Q: *How many stationary phases can be used in the FFF-MALS system?*

A: FFF is a one-phase separation technique that resolves species by controlling two flow streams—the channel flow parallel to the membrane and the cross flow perpendicular to the membrane. Although the membranes have a surface chemistry, these interactions are ideally minimized in FFF-MALS as we do not want membrane interactions to influence the separation mechanism. As a result, there are no stationary phases like required for SEC, RPC, or IEX but rather separation is achieved by exploiting the diffusion differences between samples, therefore the hydrodynamic size.

Q: *How many running pumps do I need to enable FFF? How many solvent channels?*

A: With the Eclipse FFF separation system, you need only a single pump. The advantage of the Eclipse compared to other manufacturers is that a single pump flow is diverted in multiple ways inside the Eclipse to achieve channel flow, injection flow, cross flow, DCM flow, and can even switch flows between a channel and a column. If you have multiple solvent channels, you can use different mobile phases for experiments or utilize them for automating the flushing or cleaning of the system, but ultimately only a single pump and a single solvent reservoir are needed for FFF-MALS experiments.

Q: *Is there any path for increasing FFF peak efficiency?*

A: Compared to other techniques, FFF-MALS has much higher peak selectivity—by tuning the cross flow, you can achieve baseline resolution between peaks that would never be possible with SEC or other column-based techniques, especially for samples larger than 30 – 50 nm. However, one of the key limitations is the lower resolution compared to techniques with higher theoretical plates like SEC, which results in broader peaks. With MALS, you can absolutely characterize these peaks regardless of peak width. To improve peak efficiency, you can steeply drop the cross flow to promote elution once the sample is separated. The more time sample spends in the system, the broader the peaks become, but the advantage of FFF is how tunable the system is.

Q: *Does the DCM changes the detector flow?*

A: Yes—fundamentally, the DCM operates by siphoning off pure solvent from the channel flow but the detector flow would be constant throughout the run, so you'll still achieve very stable signals.

The higher split ratios that you configure, the more flow that is removed and the lower the detector flow. Lower detector flows benefit DLS data, and we've seen no loss in resolution for 5:1 split ratio but if you are willing to sacrifice some resolution, you can achieve higher concentrations for fraction collection, up to 10:1 or even 20:1 split ratio.

Q: What are typical sample recoveries with DCM and with focusing?

A: With the newest variable-height and fixed-height channels, the channels seal much more reliably than ever before thanks to polycarbonate spacers with a PTFE coating in the variable-height channels and the new design in the fixed-height channels, which consistently improve sample recovery. With our control tests, we've seen 90 – 100% mass recovery of protein standards with the short and long channels. Both the DCM and the focusing step do not typically cause significant loss of sample. Most often, sample is lost by sticking to the membrane, through conditioning the membrane with a standard material can improve subsequent sample recoveries.

Q: Can the software control and incorporate data from FLD modules?

A: Currently VISION, the software for controlling the Agilent 1260 Infinity II modules and Eclipse instruments, is capable of controlling FLD module. You can configure the FLD method, the gain, the excitation and emission wavelengths, and more in VISION RUN. Additionally, you can view 3D spectral data of FLD spectra in VISION DESIGN. Unfortunately, ASTRA does not natively feature a dedicated FLD configuration for use as a concentration detector; however, you are able to collect analog FLD signals in ASTRA masked as a generic signal that can be used for data analysis, peak area analysis, and for record-keeping. We are continuously looking for ways to improve FLD support and welcome your feedback.

Q: How is lab solution software compatibility from Shimadzu?

A: The combination of Agilent module control and Eclipse instrument support in VISION provides the most seamless experience when running FFF experiments as you only need to create one sequence to perform you experiments and generate both Eclipse and ASTRA data files. Unfortunately, with the release of the new Eclipse and VISION 3, we no longer support Shimadzu HPLCs for FFF-MALS. We also no longer support the Lab Solutions Plugin (or other Plugins for Chromeleon, etc.) for all third-party chromatography software with FFF-MALS.

Q: Will increasing the flow rate in the beginning dilute the later eluting species significantly?

A: Dilution is a challenge in FFF-MALS experiments; however, there are a number of strategies that were discussed, such as DCM and focus flow steps, that can significantly reduce the effects of dilution. As for the effect of high flow rates, this depends on several factors. After focusing, when the sample is most concentrated (often focusing concentrates the sample into a few microliters volume), the subsequent elution step will witness a gradual broadening of the sample volume as it diffuses and travels through the system. Stronger cross flow, which in turn causes the sample to

be retained in the channel longer, can gradually broaden the peaks the longer it is applied and the longer the sample is in the channel. Part of the "art" of FFF-MALS experiments is tuning the method to maximize the separation in the shortest amount of time. This can be achieved by reducing the cross flow after some time to promote elution of the separated species.

Q: *What strategies would you recommend for samples with unknown composition (ie size, mass fraction not known for the sample)?*

A: The more you know about your sample going into FFF-MALS method development, the faster the method development process can be, but the advantage of FFF is its flexibility even if you don't know much about your samples. Although DLS measurements, like those performed by the NanoStar or Plate Reader, can quickly give you a hydrodynamic radius that can be used for computer-aided method development, you can still get a rough idea about size by running a screening method, or a method that applies a broad range of cross flows. Based on the elution time, you can then optimize the method by focusing on that relative cross flow rate. With a few iterations, you can generate a suitable method for your unknown sample. One of the advantages of the suite of characterization tools that comes with FFF-MALS like the Optilab RI detector is the ability to determine sample parameters like dn/dc, extinction coefficient, etc. that can be used for analysis.

Q: *On your slide #33, why the small first eluting peak without DCM (blue trace) disappears in the green trace with DCM?*

A: Great observation! —that first small peak is actually a system peak—the detectors can sometimes get an artifact signal from the pressure change between focus mode and elution mode, and that often generates a system peak. With DCM, the detector flow is reduced and these pressure changes may stay the same or become more/less pronounced as the system pressures change with the flow conditions. The sample peaks, including the albumin impurity, cell debris, and lentivirus all increase with DCM.

Q: *Can high chaotropic concentrations like urea or guanidine be used in FFF as a mobile phase for denaturing conditions?*

A: These are additives we have seen used with FFF-MALS in the past. From a chemical compatibility viewpoint, these additives should be okay as long as the pH of the mobile phase is safe for the regenerated cellulose membrane and flow cell, usually in the pH 2 – 10 range. From a practical standpoint, higher concentrations of additives can lead to more instability in the detector signals, especially the RI signal. Other things to keep in mind are the changes to normalization and *dn/dc* with additives, viscosity (if using DLS), etc.

Q: *What are the minimum and maximum sample volumes that can be applied to a given membrane?*

A: Sample volume is quite flexible. During focusing, the sample volume is concentrated and the sample is essentially dialyzed into the system solvent. We have some customers injecting large volumes either with a multi-draw kit or multiple injections on the channel, with a long focus step that concentrates the sample. On the other hand, we can inject just a few µL of sample (i.e., at 0.25 – 2 mg/mL) for a total loading in the µg scale and get sufficient signal.

Q: *How long would it take to separate viral vector from media of a large scale 200L batch? Can you run multiple channels on one system to decrease separation time?*

A: At this time, our FFF-MALS systems are primarily designed for analytical or semi-preparative scale separation and analysis, so may not be suitable for large scale batch purification. However, for your processes, tangential flow filtration (TFF) may be more suitable and we offer real-time MALS solutions that enable you to monitor the process.

Light scattering practical

Q: *What is the difference between measuring size by standard DLS and online DLS?*

A: Standard DLS, also referred to as Batch DLS, typically covers a larger size range and does not dilute the sample compared to online DLS; however, batch DLS offers lower size resolution. Online DLS takes advantage of upstream separation, enabling higher resolution size distributions but the measurable size range is lower and the sample is inherently diluted. There are strategies to improve the upper size limit for online DLS, such as utilizing the wide-bore flow cell, as utilizing online DLS in conjunction with MALS can infer particle shape or structure from the R_g/R_h ratio where R_g is determined by MALS and R_h by DLS.

Q: *How is the measurement of hydrodynamic radius R_h affected by the flow of the molecule due to elution flow rate?*

A: At higher flow rates, there are non-ideal transit and flow shear affects in the flow cell that reduce the upper limit of hydrodynamic radius determination. We can improve this range by lowering the flow rate or widening the flow cell bore (thus reducing mean velocity in the cell). With the standard bore flow cell, you can reliably achieve an upper hydrodynamic radius of 50 nm. With the wide-bore flow cell, you can determine hydrodynamic radius up to 300 nm.

Q: *For a rigid polymer whose chain does not fold in solution as flexible polymers, its hydrodynamic radius can be very different from flexible polymer at the same molecular weight. Can MALS still accurately measure its absolute molecular weight?*

A: Absolutely—MALS can accurately determine molar mass from first principles independent of shape or hydrodynamic volume. There are shape-independent formalisms such as Debye, Berry,

and Zimm that determine the rms radius and molar mass. You can do more advanced analyses by comparing the hydrodynamic radius (from DLS or viscometry) to the rms radius from MALS to understand more about how extended or compact the polymer chains are.

Q: *Which was the injection loading?*

A: The lower limit we usually target for particle concentration is 10^7 particles per milliliter for MALS analysis, below which you may get noisier MALS peaks due to particle fluctuations. For the lentivirus particle concentration example, we injected about 50 µL of sample and determined a particle concentration in the $10^8 – 10^{10}$ particles/mL range.

Q: *How well does MALS and other light scattering methods work for nanoparticles/species that absorb/emit the light used for scattering? Are there ways to address this?*

A: To learn a little more about our strategies for overcoming fluorescence, please take a look at our white paper, WP9002, that discusses characterization of fluorescent macromolecules and nanoparticles. If the sample just absorbs the laser wavelength, the data can be corrected for the forward monitor in ASTRA quite readily. If the sample absorbs but also emits, there are two strategies—if the emission wavelengths are different enough from the laser wavelength, you can install interference filters on the photodiodes that only allow laser wavelength photos through, while filtering off the fluorescent light. Otherwise, you can consider a near-IR 785 nm laser where absorbance/emission spectra may not have an effect.

Q: *What flow rate limitations exist for in-line DLS analysis?*

A: In general, the higher the flow rate, the lower the upper limit for hydrodynamic radius determination via DLS. Depending on the sample being measured, there is some flexibility. With a standard bore flow cell, the upper limit is 50 nm radius at a flow rate of 0.3 mL/min. With a wide bore flow cell, the upper limit is 300 nm radius at a flow rate of 0.5 mL/min. As you increase the flow rate, the upper limit decreases.

Q: *If I already have a DAWN with 18 angles that is not the Eclipse instrument, can I still enable FFF by acquiring the separation bed?*

A: The combination of the Eclipse and separation channel enable FFF experiments, and can readily be added to your existing DAWN. If you are already performing SEC-MALS experiments with the DAWN, you can also consider the Eclipse with SEC Switching option, which enables you to change between FFF and SEC mode without having to change the fluidics.