



## Live Webinar Q&A Sheet:

### **Field-Flow Fractionation 2.0: Obtaining high resolution distributions of nanoparticle size and zeta potential, with less time, less effort and more confidence**

The recorded webinar may be viewed from the [FFF-MALS](#) webinars page. These questions were submitted by live viewers. Additional information on MALS, DLS, and FFF 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 [Theory](#) page of our web site.

Please contact [info@wyatt.com](mailto:info@wyatt.com) with any additional questions.

### **SCOUT Method Development and Size Determination**

*Q: Does **SCOUT<sup>®</sup> DPS** need any information about the particles besides size?*

A: No. According to FFF theory, retention only depends on the diffusion coefficient, which is equivalent to hydrodynamic radius. Note that retention does depend on shape but, as in dynamic light scattering, size is always expressed in the radius of the equivalent sphere: the sphere which has the same diffusion coefficient as the particle you measure.

*Q: Do metallic nanoparticles fractionate on AF4 the same as protein aggregates of the same size, and if not – does SCOUT DPS know how to differentiate between their behaviors?*

A: In the absence of interactions and electrostatic effects, a protein aggregate and a metallic nanoparticle of the same size should elute at the same retention time. In most practical applications, there will be differences. This is why we use light scattering to measure the size in an absolute way as fractions elute from the FFF channel. But with respect to



planning an experiment, it is ok to neglect these effects. There is a second important value in using SCOUT DPS: we can interpret the deviations between the theoretical prediction and the experimental outcome as the influence of non-ideal interactions. If the sample elutes earlier, we have repulsive interactions with the accumulation wall, if they come later, the opposite effect is probable. This allows us to make intelligent decisions how to modify the carrier solution, e.g. increase ionic strength or change the pH.

*Q: Does the shape of the particle affect the size measurement?*

A: Shape affects the diffusion coefficient and therefore also affects the retention time. We measure always the diffusion coefficient which is transformed to the hydrodynamic radius if the equivalent sphere.

*Q: You have only shown linear decay in the cross-flow. Is it also possible to have an exponentially decaying cross-flow?*

A: Yes, this is possible. In the method editor you can choose for each step of the elution between constant, linear decay and exponential decay and select the exponent. In this way you can have any combination of these in a given method.

## **Particle analysis**

*Q: How accurate is the number density calculation, and how does it correspond to particle counting techniques such as NTA or TEM?*

A. The number density calculation is accurate, this has been shown in a number of publications with comparison to TEM and AFM. The analysis will generally be more accurate than NTA because in NTA the system may be blind to small particles when large particles are present, something that does not happen when the particles are first fractionated by size.



It does require good quality light scattering data and spherical particles. The analysis relies on Mie scattering equations and will not be accurate for non-spherical particles.

Q: *What is the lower detection limit for the number of particles that can be counted?*

A: This depends on a number of factors including particle size and refractive index. Roughly, for particles such as viruses in aqueous buffer with a rms radius ( $R_g$ ) of 45 nm, the limit is about  $10^5$ /mL and the limit varies inversely with the 6<sup>th</sup> power of the radius. This actually translates to one particle in the laser beam for particles above 55 nm in  $R_g$  or roughly 70 nm in physical radius.

Q: *In the course of the webinar you mentioned a lower size limit for particle counts of 30nm. Is this lower limit based on latex beads? Can you "see" and separate sub-10nm particles like metallic particles, which will scatter more light?*

A: The 30 nm lower limit was only for determining the number density, i.e. the number of particles per mL. In terms of size determination, we can go down to 10 nm with MALS and 0.5 nm with DLS. So yes, gold and silver particles can be separated and measured with DLS down to 0.5 nm in hydrodynamic radius.

The molar masses of macromolecules such as polymers and peptides can be measured down to 200 Da, even if the physical dimensions cannot be determined.

Q: *With mixtures containing inorganic particles, will settle ability at various regions affect measurements?*

A: This should not be the case in the sub-micron size range, unless we have particles of very high density. If they settle, it means that they do not diffuse any more by Brownian motion. In that case the FFF equation will not apply. Heavier particles will elute later, because they stay closer to the



accumulation wall as expected by their size. With a MALS detector, we are able to measure the size of eluting fractions and we are able to detect such a behavior.

#### **EAFF4**

*Q: What types of samples have been characterized with EAFF4?*

A: We have published data on polystyrene latex, BSA and antibodies. We have run many other types of samples, like liposomes and their complexes with RNA, virus particles, pectins, high molar mass polyacrylamides, soil particles. As this is a new method, the number of sample types we have explored is limited but expanding rapidly.

*Q: Can you elaborate on how you calculated the net charge of latex and proteins?*

A: The primary parameter we measure is the electrophoretic mobility: the relationship between particle drift velocity and electric field strength, which is also the case for PALS, our [Mobius®](#) instrument. We use the identical equations to calculate net charge from mobility such as the Debye-Hückel equation. Ionic strength and size need to be entered in this equation.

*Q: Are you aware of applications of FFF with electrophoretic mobility to the separation of empty/full viral particles--not containing/containing DNA?*

A: It depends on the sample - this will work if the charge difference is big enough. So far we have not seen a good example, but we have not had much experience with this application – feel free to contact us to try it out with your samples!

*Q: Can you comment on the upper size limit for charge particles?*

A: This depends on the upper size limit of normal FFF retention, which is around 500 nm in radius.



Q: *How different do particles with the same MW need to be in terms of charge?*

A: This depends on the size. If we are up to 100 nm (which covers most macromolecules), the mobility values need to differ by about 0.3  $\mu\text{m}/\text{Vs}$ .

Q: *Can Mobility<sup>®</sup> be added on to an older Eclipse<sup>®</sup> system?*

A: It can be added to any Eclipse from 3+ or newer.

## **FFF**

Q: *What is the largest size disparity that can be managed by the technique?*

A: The range of separation is from a few nanometers to a few micrometers in one injection

Q: *What is the highest resolution that can be achieved if particles differ in size by ~10%, in terms of separation?*

A: We cannot separate two populations which differ by 10% down to the baseline, but we can certainly distinguish peaks to 10 – 15%. For example, the VLP example shown clearly resolved peaks that differed in radius by 15%.

Q: *What is the range of shear rates in FFF?*

A: The shear forces are roughly equivalent to what we have in the arteries of the blood circulation. They are very low compared to shear in the inter-column packing of a SEC column.

Q: *How do you ensure that the unwanted VLP fragments shown (2.5 MDa) are not pressed through the membrane on the FFF channel?*

A: This fragment is much too large to permeate through the pores of the 10 kDa-cutoff membrane. It is important to select a proper cut-off in view of sample components which need to be analyzed.



- Q: *Can you do an analysis of a mixture of organic and inorganic species?*
- A: Yes. FFF separation does not depend on chemical composition, so in principle we do not care if the sample is organic or inorganic, provided we can prevent interactions between sample components and/or the membrane
- Q: *Can FFF be added to an existing HPLC system or does one need to buy a completely new system?*
- A: We can integrate an Eclipse with existing HPLC systems from Agilent, Thermo and Shimadzu. We are working on integrating Waters as well—stay tuned.
- Q: *How will the samples need to be processed to inject into the ribbon?*
- A: FFF does not require extensive sample prep. We need to assure that our solution or suspension is stable and we need to exclude oversized particles, which would block the tubing, e.g. with a mild centrifugation.
- Q: *What is the typical vol % or mass % concentration of the prepared sample?*
- A: Volume is very flexible, ranging from 1  $\mu\text{L}$  to hundreds of  $\mu\text{L}$ . The sample amount is more limited to avoid overloading: typical is a few  $\mu\text{g}$ , but we can use a semi-prep channel and then increase the load by a factor of 100 or more.
- Q: *Is there a physical separation from the sample? Can you collect fractions?*
- A: Yes, FFF results in a physical separation and you can collect fractions. The Eclipse system works with standard HPLC systems and therefore the corresponding fraction collector can be used.



Q: *What is the best way to concentrate the samples after preparative fractionation?*

A: Currently, post-fractionation centrifugation. We are working on a more sophisticated way for sample concentration, directly in the semi-preparative channels.

Q: *Can we use organic solvent, such as chloroform, tetrahydrofuran?*

A: Yes, we have a version of the Eclipse for organic solvents such as THF and chloroform. DMSO, DMF and DMAC are a problem because of lack of membrane stability.

### **Downstream detection**

Q: *Why do we have such low RI signal with our latex samples from Invitrogen?*

A: This is usually because you are loading a small amount of sample. Latex particles scatter intensely, so you get a nice MALS signal, but RI will be extremely low. If you are only measuring particle size the RI signal is not used.

Q: *How do you ensure reproducibility even with additional backpressure due to the RI connected behind the Viscometer?*

A: It is fairly uncommon to connect a differential viscometer such as the [ViscoStar®](#) to FFF along with MALS and RI detectors. However, the additional backpressure is low and should not affect reproducibility of FFF separations. The enhanced immunity to pump pulses of the ViscoStar III makes it a good candidate for working with an FFF-MALS system.

Q: *How high can we increase the molecular weight of polymeric nanostructures with AF4-MALS method relative to SEC-MALS?*

A: SEC-MALS is limited by the SEC column pore size, which usually only go up to a few millions in molecular weight. With FFF you can separate



particles up to about 500 nm in radius which roughly corresponds to 1 billion ( $10^9$ ) in molecular weight.

*Q: Will the MALS library and software only be available with the Eclipse or is it able to be applied to other devices?*

A: The **ASTRA**<sup>®</sup> software that is provided, free of charge, with each Wyatt MALS instrument is appropriate for use with SEC, FFF and unfractionated (“batch”) light scattering measurements.

*Q: If you run solvent mixtures, how can you use light scattering? The  $dn/dc$  changes with composition and hence the  $dn/dc$  in light scattering would also change?*

A: Mixed solvents are certainly a complication; if they differ much in refractive index, it will be difficult to calculate absolute molar mass and radius without ascertaining the  $dn/dc$  value at each elution volume. However, it is possible to estimate the  $dn/dc$  theoretically if you know the  $dn/dc$  in pure solvent, the specific volume of the analyte and the refractive indices of the pure and mixed solvents.