Live Webinar Q&A Sheet:
Probing Submicron Protein Aggregation Using Asymmetrical Flow Field-Flow Fractionation (AF4) and Light Scattering

The recorded webinar may be viewed from the FFF webinars page. These questions were submitted by live viewers. Additional information on MALS, DLS, CG-MALS, and ELS 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 with any additional questions.

Q: *Can you compare and contrast AF4-MALS and SEC-MALS for protein aggregation studies?*

A: SEC and AF4 are usually considered as complementary techniques with SEC providing better resolution in the lower molar mass (MM) region and AF4 able to handle the higher MM region of macromolecules. Protein aggregates can be quite polydisperse and the wide size range that spans nanometers to one micrometer and beyond are more suited for the open channel used in AF4. Furthermore, SEC has limited size range and mobile phase composition compared to AF4.

Q: *Are there benefits to using a multi-detector approach?*

A: Multiple detectors are essential when analyzing heterogeneous samples. We need to not only separate but to use orthogonal methods to measure size and to confirm chemical identity. This talk showed mainly AF4-MALS results. Unfortunately, I did not have the time to show our AF4-DLS results. I would love to be able to couple AF4 with a composition sensitive detector.

Q: *A recent review alluded to AF4 having difficulties at higher protein concentrations (>50 mg/mL), can you comment on this?*

A: First of all, I think the units are ug/mL. This question may be in reference to sample overloading, a possibility in separations techniques, that can subsequently lead to decreased separation resolution. Analytical scale separations usually involves injected protein amounts of ~20 ug. In 2017, we published a work with semi-preparative AF4 showing that up to 10 mg of silica can be injected without overloading the channel. In our latest paper on lipoproteins in human plasma, semipreparative AF4 produced > 650 ug of total proteins in a single separation.

Q: *Do the proteins interact with the AF4 membrane and how do you address this, if it's an issue?*

A: This is an important question, particularly, if one is using AF4 theory to calculate hydrodynamic sizes from measured retention times. If there are non-ideal interactions, MALS and DLS can compensate as these detectors measure the MM and size of eluting components.

Interaction of proteins with membranes is depending on charge and the solvation state reflected in the second virial coefficient. The membranes used in Flow-FFF are negatively charged at physiological pH, so proteins with a pI around 7 or higher will have a higher potential of absorption on the membrane, either via electrostatic attraction to negative sites on the membrane or through hydrophobic interaction if they have no net charge near the pI. If possible, one adjusts the pH to be above the pI. In terms of solvation state, for many proteins solubility is decreased above a critical ionic strength. Buffers commonly used for SEC applications with 150 mM of higher salts added, may reduce solubility (shifting the virial coefficient to more negative values). It is recommended to use lower salt conditions, it could increase recovery.

In the drastic situation of complete sample loss, i.e., nothing elutes, the first thing is to decrease the field strength or cross flow rate. With new samples, I usually recommend doing an initial run with no crossflow to make sure that a very large peak elutes just to make sure that enough sample has been injected. And then incrementally increase Vc and determine sample recovery (peak area with Vc versus peak area with no Vc). Other ways to decrease interaction include 'preconditioning' the membrane by injecting a protein solution at the beginning of each day.

Q: *When would you use AF4-MALS instead of an imaging technique such as microflow imaging?*

A: Microflow imaging is great because one can see the particle and obtain visual verification of size, shape, transparency, and particle count. However, this technique is usually used for large aggregates in the 1 to 100 um size range.

Q: *Analytical ultracentrifugation is very popular as an orthogonal method to SEC for protein aggregate characterization, how does FFF compare to AUC?*

A: SEC is the most commonly used method for soluble aggregates. However, the heterogeneity of the protein aggregates has resulted in regulatory agencies recommending the use of orthogonal

methods to SEC. The two orthogonal methods that have received the most attention are AUC and AF4.

AUC and AF4 similarities include:

1) Samples usually do not need to be filtered
2) Both have a dependence on shape due to frictional drag.
3) Both have a larger size range than SEC.
4) Both have more flexibility than SEC with respect to the fluid used to do the separation. In other words, formulation buffer can be used.

AUC and AF4 main differences:

1) AF4 is an elution technique (sedimentation velocity SV-AUC is not). AF4 can be coupled with other detectors such as MALS and stoichiometric information can be obtained from MM of aggregates/MM of monomer as shown in this talk.
2) SV-AUC - Difficult to reliably assign stoichiometry to protein aggregates (especially for minor components present at the level of a few percent) because sedimentation coefficients depend on aggregates conformation and mass.
3) SV-UC can be significantly affected by excipients present in the sample. Sugars and polyol at more than a few wt% can establish a density and viscosity gradient across the AUC cell that then affects the sed coef of the aggregates. This could lead to false peaks in size distribution (when using the c(s) method for data analysis).
4) SV-AUC data analysis is more complicated.

Q. *The introduction showed the software supported method development. How useful is that for a real-life application, like a protein aggregate sample?*

A. The method development tool has three benefits. It helps to plan for the first initial experiment, which channel geometry and flow conditions to use. This is especially useful to decide on the spacer height. The second benefit is to compare the theoretical prediction to the experimental outcome. If it close, we know that we have ideal FFF conditions and no interactions with the membrane. If there are differences, we can learn from them and understand what is going on (repulsion or attraction to the membrane). This does help to make decisions on improving the carrier solution composition (see answer above on protein interaction with the membrane). Then the third benefit is to use the method development to improve separation. You can ask the software what happens if you change flow conditions or spacer height.

To summarize, you should not run an experiment, which theory tells you will not have useful results (e.g not resolving the peaks you want to separate).

Q: *During the FFF suppress stage, do you have a concern of particles self-interaction?*

A: Our results show that the anti-streptavidin IgG is not affected by the sample focusing stage used in AF4. A key point here is to not use unnecessarily high focusing times. Our study shown on Slide 22 involves reinjecting a 1.25 mL protein aggregates fraction into the AF4 channel. Using a sample injection flow rate of 0.2 mL/min translates to a focusing time of ~6.5 minutes for all the sample to be loaded onto the channel and focused. Since the objective was to examine the effect of focusing time, we doubled the focusing time. In a regular analysis, we would increase the focusing time by only ~20% over what is needed/calculated.

Q: *When using FFF for measuring broad Mw polymers in THF, how is the mass recovery and is the dn/dc value universe?*

A: The mass recovery depends on the experimental conditions. For a broad Mw polymer, it is not uncommon to use a programmed cross flow rate like that shown in our protein aggregates work to improve mass recovery. For proteins > 100 kDa, calculated dn/dc values are shown to be narrowly distributed around 0.190 +/-0.003 mL/g (see Zhao et al., "On the distribution of Protein Refractive Index Increments" Biophys J. 2011, 100, 2309-2317). Our protein MWs were >100,000 and we assumed a constant dn/dc value. Having said this, the best approach is to measure the analyte's dn/dc in the separation solution whenever possible.

Q: *It looks like very large aggregates (>20 um) eluted before, and after, the monomer. Why is this? This from analysis of heat stressed and (un)centrifuged sample analyses.*

A: There are two FFF separation modes – normal mode (typically < ~1 um with small size eluting first) and steric mode (typically > ~ 1 um with large size eluting first). We chose heat stress conditions to produce mainly < 1 um aggregates that allowed us to examine submicron aggregates. A small amount of >20 um was also formed and these eluted in the steric mode

(before the monomer). For more details, please refer to our paper (Bria et al., J. Pharm. Sci. 2016, 105, 31–39).

Q: *Is it correct to assume that the larger dilution of AF4 at the elution step results in higher limits of detection than SEC?*

A: Under the AF4 conditions employed, the AF4 sample plug experiences only ~6% of the channel volume and undergoes only 2-4x dilution as it is transported through the AF4 channel. In SEC, the sample plug distributes across the entire cross section of the column and experiences higher dilution as it moves through the column. Most of the dilution in AF4 occurs at the channel outlet as the ~6% volume with sample elutes with the remaining 94% volume of fluid. These are two very different dilution processes and timeframes. The bottom line: If the aggregate has a slow dissociation rate when diluted, it may still be an aggregate when it elutes from the AF4 channel into the MALS/DLS detectors.

Q: *I would like to separate Amyloid beta 1-42 peptide aggregation, is it available to apply this AF4 system? What is the proper channel dimension for this small peptide aggregation behavior monitoring?*

A: What is the MW range of the Amyloid beta 1-42 aggregates and what is the question you are trying to answer? High order/MW aggregates can be separated by AF4, although it is likely there will be one continuous broad peak (rather than individual peaks signaling the addition of one peptide). The MALS detector confirms that a separation has indeed happened. If you are also interested in the monomer (MW of 4514 g/mol), you will need to use a lower MW cutoff membrane than the 30 kDa that is usually provided with the channel. You can use the standard AF4 channel provided by the instrument manufacturers. Here is a publication which describes the separation of Amyloid beta 1-42 from its aggregates and works out the aggregation kinetics: DOI: [10.1021/bi3016444]

Q: *Why did the void volume peak contain large 20um particles? How did know the void volume was large particles?*

A: 1) We suspected that large particles may have been produced in the heat stress step and thus we did AF4 experiments with polystyrene latex standards with sizes from 20 nm to 20 um. We then selected conditions where < 1 um particles eluted in the normal mode (small sizes elute first) and > 1 um particles eluted in the steric mode (large particles elute first; elution order is reversed). We correlated retention times with the particle size, so we knew where 20 um particles would elute (i.e., with the void peak).

2) We knew the void peak was large particles because this peak disappeared when the sample was centrifuged. Furthermore, the light scattering intensity of the void peak was a lot larger than the corresponding dRI signal intensity which suggested the presence of a small amount of large particles.