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Live Webinar Q&A Sheet:

Mass, Size, Composition: Field Flow Fractionation of Biomolecules and Nanoparticles plus Characterization by Light Scattering and ICP-MS

These questions were submitted by live viewer. Additional information on FFF-MALS and Wyatt instrumentation may be found on the Wyatt Library under Webinars, Application Notes, Featured Publications and Bibliography, as well as on the corresponding Product and Theory pages of our website.

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

Q: Can FFF separate a double stranded molecule into a single stranded molecule that can be then characterized; i.e. DNA?

A: The FFF system likely wouldn't cause double stranded DNA to break into single stranded DNA unless this would happen naturally by the dilution of the double stranded DNA during elution. If there was a shift of some double stranded DNA to single stranded DNA, the smaller single stranded versions would emerge earlier than the double stranded version since it would have a higher diffusion rate.

Q: Can particles with shape other than spherical be analyzed?

A: Yes, asymmetrical particles can also be fractionated by FFF. The mode of fractionation is diffusion based. Carbon nanotubes have been fractioned by FFF, for example, as have linear polymers.

Q: What is the lowest MWCO membrane that you have been able to use practically? You mentioned 5 kDa, which would allow your smallest (1 nm) size to pass through.

A: My experience with 1 kDa MWCO membranes was not particularly good. We have seen better results using 3 kDa MWCO membranes over 1 kDa. The challenge here is that the reason for wanting to use low MWCO membranes is to retain small molecules. Small molecules will also require very high crossflow rates to be fractionated. The tiny pores of these low MWCO membranes put up a lot of resistance to the high cross flow rates needed for small molecules. I have had some success by lowering the overall total flows so that I can still maintain high crossflow to channel flow ratios without having to also have high crossflow rates through the membrane.

Q: What is the detection method?

A: The fractionation does not depend on the downstream detector. Some users have only a UV or RI detector downstream, and calibrate the system by using size standards. This relative method depends on only running samples of the same conformation and same ratio of mass to R_h in order to get close to the size. We recommend a Multi-angle Light Scattering detector, which determines M_w and R_g by absolute vs. relative means, as an ideal detector to pair with FFF. Any slide in my presentation showing M_w or R_g or R_h results included the use of a MALS detector.

Q: Can you analyze smaller molecules, such as those in the 1-5 kDa region?

A: As mentioned above, this is a challenging application because it requires the use of a membrane with small pores to retain the sample, which naturally makes it highly resistant to high flux rates needed to retain small molecules in the channel. Some success in this area has been made by maintaining high crossflow to channel flow ratios, for example, a 5:1 ratio of channel flow to crossflow. By dropping the combined flow down to lower values, such as 0.2 mL/min channel flow and 1 mL/min crossflow, you can maintain the correct ratio and actually get the desired level of liquid flow through the membrane to make things work.

Q: Is the Hollow Fiber Cartridge reusable?

A: Yes, the hollow fibers are re-usable. Typically one can expect to achieve 50 – 100 injections or more per channel before separation performance declines to the point where replacement is warranted. They are easy to replace and condition and are ready for use quite quickly. They are much less expensive than an SEC column by comparison.

Q: I was wondering, from a practical point of view, how often the semi-permeable membrane has to be replaced and how easy that process is.

A: A flat channel membrane is also good for estimated 50 – 100 injections or a few weeks exposure to liquid, before replacement is warranted. Replacement is not complicated. Tools are provided to remove the upper block of a flat channel from the lower block, giving the user access to the membrane. The old membrane is removed and discarded and a new membrane installed, and the channel reassembled according to provided instructions. Conditioning new membranes with some molecule to block non-specific binding sites is recommended. It takes only about 10 minutes to change the membrane, and a little more time to flush out any air bubbles from the system and run a sample like BSA to condition the membrane.

Q: In terms of the effort involved in method development, how does FFF compare to SEC?

A: It might seem like there are a lot of variables to consider when it comes to optimizing FFF methods, but they are manageable. Users of the Wyatt Eclipse FFF system receive software that can simulate the fractionation of species based on input variables such as the estimated size and flow and geometry conditions. This helps speed up the process greatly and results can be near optimal after just a few scouting runs.

Q: Once method is setup, how scaleable is method to the larger membrane cartridges?

A: One of the main factors in the separation are the ratio of the channel flow through the membrane to the degree of crossflow, which is the force field. Crossflow can be kept constant throughout the elution period, or it can be relaxed, either in a linear, step, or exponential gradient fashion. Once these types of conditions are sorted out using a small capacity device such as the small flat channel or hollow fibers, these same conditions are great starting points when moving up to larger capacity units like the long channel or semi-preparative channel.

Q: Please describe what is forming the force field.

A: The force field used during the elution step inside the channel is formed by permitting some amount of the total fluid entering the channel or fiber to permeate through the semi-permeable membrane inside the system (which is in the form of a tube in HF5). For example, to get BSA to fractionate into monomer and dimer species (and higher order

aggregates if present) using a flat channel, I would typically need a ratio of detector flow, which stays above the 10 kDa MWCO membrane, of 1 mL/min, and then use 3 mL/min of force flow (crossflow). In the Wyatt Eclipse, what happens is that when you set up these conditions in the software, our one-pump approach knows that the pump should output 4 mL/min at the start of the elution step (1 mL/min for channel flow and 3 mL/min for crossflow). All 4 mL/min come in the inlet end of the channel. The fact that all liquid for the channel flow and crossflow come in asymmetrically at one end of the separation device is why this technique is often referred to as AF4, for asymmetric flow FFF. The 3 mL/min of liquid which forms the force field carries the BSA to the surface of the 10 kDa MWCO membrane lining one wall of the channel (the bottom in most arrangements). The BSA will try to diffuse away from the accumulation wall, and back into the detector flow. The monomer is smaller and can diffuse on average further from the membrane surface than the bigger dimer and higher species. The further away from the accumulation wall it can diffuse, the higher velocity flow from one end to the other will be experienced. So monomers emerge before bigger ones.

Q: What form factor has been used to evaluate the radius of particles which are not spherical (e.g. Adenovirus)? The radius from SLS is usually calculated from Guinier analysis and it's called gyration radius. How geometric radius relates to gyration radius?

A: Using multi-angle light scattering to determine size, the Wyatt ASTRA software allows you to select a math formalism that can either be conformation independent, such as the Zimm, Debye, or Berry formalisms for determining the RMS Radius from angular dependence of scattered light. That is also called R_g. You have the option of using a form factor to determine geometric radius by selecting conformation dependent formalisms like the sphere or coated sphere models. The geometric radius will be comparable to R_h in that case. In the case of the Adenovirus example slide I showed. This math did impose a spherical shape factor onto the derivation of the radius. In this case, the geometric radius calculated using a spherical shape factor couldn't have been too far off because the MALS derived geometric radius was pretty close to the radius determined from electron microscopy.

Q: The majority of these flow fff techniques seem to be for aqueous samples. do you know of flow fff examples involving organic moble phases (hexanes, toluene, etc)?

A: The technology also works for organic polymers in organic solvents. THF is the most commonly used solvent for this application with the regenerated cellulose membranes. Dr. Stepan Podzimek, who was the author of one of the books I showed in my penultimate

slide, is an expert in organic phase FFF with THF. His work on branched polystyrene fractionation showed that heavily branched PS tends to get physically entangled in SEC resin as shown by a strange asymmetric curve when plotting the log of the RMS radius against the log of M_w. When the same branched polystyrenes are fractionated on FFF, the trend between M_w and RMS radius is nice and linear, suggesting FFF-MALS is a superior fractionation technology to SEC-MALS for these types of polymers.

Q: Can a FFF unit be installed on a regular GPC line with RI and UV detectors to analyse nano and sub micron pigment particle distribution?

A: There are major differences in the approach to solvent delivery used with main commercial flow FFF systems. At Wyatt Technology, our Eclipse product line uses a high quality HPLC on the front end to provide all flow. We support Dionex/Thermo HPLCs, Agilent and Shimadzu HPLCs for providing all flow. These are the same HPLCs being used for traditional chromatography. Other commercial FFF units are not compatible with regular GPC systems and you would have to use the generic syringe pump provided with the FFF unit. I do not see any issue with using flow FFF to look at pigment particle distribution using a Wyatt Eclipse; nano to sunmicron level size particles are the ideal size range for this technology.

Q: Is the population at ~55 min in the 800/1600 µg BSA slide real?

A: There is some material at 55 minutes that scatters light in the prep BSA separation slide #35). This bump either appeared when the crossflow drops to zero, permitting any very large material trapped in the channel to emerge, or when the injection pathway is briefly opened again for cleaning. We recommend ending Eclipse methods in a state that prepares the channel for a followup injection. It might be that the LS producing sample at 55 minutes is some large material that did not get eluted in the first purge and now became washed out.

Q: Can you please remind me of the advantage of hollow fiber FFF vs conventional rectangular channel FFF.

A: Hollow fiber cartridges require very little sample while still producing sufficient signal for detection. So it is good for quantity limited samples to start developing optimal separation methods. Conditions that work well on hollow fibers can generally be scaled up and used with flat channels for examination of larger amounts of material.

Q: What type of membranes due you use when you're working with non-polar solvent systems?

A: The most frequently used non-polar organic solvents are toluene and THF. The regenerated cellulose membranes work well with these two solvents. The challenge with other organic solvents is to find a suitable membrane for the channel.

Q: If a protein can change conformations from an alpha helix to a beta sheet, how is this transformation characterized by FFF?

A: The main separation force is primarily a disffusion-based process. If this change in conformation also results in a change in D_t, then the spiecies with the smaller R_h value will elute prior to the species with the larger R_h. If the change in conformation does not change the structure or D_t value substantionall, these two forms may co-elute together.

Q: I am looking for a reference for FFF LIBS (for elemental analysis) of colloidal particles in water, specifically radiocolloids that transport nuclear reactor waste (e.g. plutonium) from repositories. Can you send a recent literature reference or two?

A: Please feel free to visit our website and check the searchable Bibliography for any citations that might be relevant to your interests. This database is open to anyone and does not require any passwords or equipment ownership.

Q: Can you tell a little bit more about the interface of FFF to ICP-MS? What is the dead volume?

A: There will be some type of electronic trigger to start the whole process of running the HPLC front end with Eclipse, and also starting data acquisition from the ICP-MS. Normally a small internal diameter PEEK line would be used from the last detector used with FFF-MALS to the ICP-MS. If the two instruments are not separated by a large distance, the dead volume between the last detector and the introduction into the ICP-MS will be quite low.

Q: Are there any official methods under development using FFF?

A: Sorry, I don't know whether this is the case or not.

Q: Polyethersulfone doesn't work for most solvents. Are there any other membrane materials available with your system?

A: If you are referring to organic solvents, the preferred membrane for use with solvents such as toluene and THF would be made of regenerated cellulose.

Q: Application in organic solutions?

A: Please see above. We have produced good results on branched polystyrene molecules which can become entangled in SEC packing material; these molecules behave much better in terms of eluting in the proper order by FFF than SEC.

Q: Can you talk briefly about the pros/cons of different membrane materials (PES/Cellulose)?

A: Regenerated cellulose is the most commonly used membrane for many protein and polymer applications. It is a hydrophilic membrane with low protein binding properties. The PES membranes have a more hydrophobic nature and can be a good alternative to regenerated cellulose when samples adsorb to this membrane. Both regenerated cellulose and Poly Ether Sulfone membranes come pre-cut and are available in molar mass cutoff values between 3 kDa and 30 kDa, with 10 kDa being most popular. The molar mass cutoff was determined with polymers so one should keep that in mind for membrane selection.

Q: What is the composition of the membrane? Do you know of any possible interaction of the membrane with hydrophobic proteins?

A: The goal is to avoid absorption of the sample to the membrane. If a hydrophobic protein

did happen to absorb to the regenerated cellulose membrane, then it might be repealed by the more hydrophobic PES membrane. One of these formulations should work.

Q: Have you tried separating nucleic acids such as RNA by FFF?

A: Not personally. I recommend checking the searchable FFF Bibliography on the Wyatt website. If someone has published the use of FFF with nucleic acids, I think you would have an excellent chance of finding that information here.

Q: Can the membrane tolerate organic solvent at high temeprature?

A: Dr. Stepan Podzimek prefers to use THF at 50°C. I don't know if this is what you think of as high temperature. I do not know of anyone using this with TCB at 135°C for example.

Q: Is it possible to run FFF in high throughput?

A: It is possible to use an autosampler to running many samples in an unattended fashion. A membrane generally lasts for over a hundred injections before needing replacement.

Q: How do you accurately determine the refractive index of your material?

A: I am assuming you mean the actual refractive index of a pure sample for the purpose of measuring number densities. There are refractometers available to measure the refractive index of solids and semi-solids that could be used for determining the refractive index that our ASTRA software needs as part of the number density analysis. If the pure sample forms a solution rather than a solid, the Wyatt Optilab T-rEX Refractometer can measure the absolute RI of a solution in addition to performing differential RI measurements of samples in solution as the emerge from fractionation from FFF or SEC.

Q: How shall we eliminate effect of particle/molecule interaction on the result?

A: We often run a series of sample amounts to see if there is evidence of self-association at

some concentration. If yes, it might be possible to find some additive that will help mitigate this challenge. That isn't always possible however and sometimes you will see co-eluting species buy FFF.

Q: Is FFF used for Quantum dots separation? Can it be used with strong acids and bases? Can it be used for prep at kg scale?

A: I don't see why this technology wouldn't work for quantum dots. It would be worth looking in our searchable FFF database to see if anyone has published anything about these molecules. As far as strong acids and bases, that depends on your definition of strong. We use 10% nitric acid for cleaning our FFF system in situ; we can also use 0.1 M NaOH for cleaning. If a Wyatt MALS detector is online with the FFF unit, minimizing exposure to alkaline conditions is a good idea because its flow cell is glass that can be etched by long term exposure to alkaline solutions. Short exposure for cleaning is permitted.

Q: How could gold particles be delivered to cancer cells?

A: In my research for this presentation I read that cancer cells are leakier than normal cells, and someone suggested that they may preferentially accumulate gold nanoparticles.

Q: In your examples there were never any adsorption phenomena to be seen, which occur excessively in the analysis of liposomes. Can you recommend any membranes or have other experiences which could help reducing the adsorption effects of liposomes?

A: I would recommend searching our FFF database for citations on liposomes that will have the specifics of their FFF separation conditions, including membrane type and any excipients that might have been added to reduce or prevent liposome interaction on the membrane.

Q: Have you tried or know of anyone who has tried gradient elution (similar to HPLC) FFF? Any advantages?

A: FFF will use a gradient of flow rate of the cross flow, which is quite common for particle applications. The channel flow that stays above the membrane and passes through any downstream detectors is typically kept constant during the fractionation. I am not aware of anyone using a composition gradient during elution steps. There is no desired adsorbtion step during FFF that we would use a composition gradient during fractionation.

Q: What about focusing and dilution effect with hollow fibers?

A: Samples still undergo focusing with the hollow fiber cartridge. The Wyatt DualTec is optimized for these devices and splits and measures the flow going into both ends of the fiber. The focused sample forms a ring around the hollow fiber inner wall before elution starts. The dilution factor during elution is much lower with hollow fibers than with flat channels. That is why the signal intensity from 1 μg of a homogeneous protein in a hollow fiber is equivalent to approximately 10x that much sample after dilution in a flat channel like the Wyatt small channel.