

## Editor's Page


**Robert**  
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**FFF Crosses the Chasm: Report on FFF-MALS Focus Meeting**

For the last three years, **Wyatt Technology Corp.** (Santa Barbara, CA) has appended a one-day symposium on field flow fractionation (FFF) to the annual International Light Scattering Colloquium. This is an opportunistic response to the unique complementary nature of FFF with light scattering detection. In 2009, about 35 scientists met on October 21st at the historic Biltmore Hotel in Santa Barbara, CA to discuss recent advances in instrumentation and applications. This meeting is significant, since the majority of the papers came from scientists who are representative of the early majority, not the technophiles who were characteristic of the early adopters.<sup>1</sup> Thus, after 40 years, FFF seems to have crossed the chasm.

Dr. Christoph Johann of **Wyatt Technology Europe** (Dernbach, Germany) opened the meeting by introducing the Eclipse 3+, which upgrades the Eclipse 3 and earlier models of asymmetric flow FFF (AF4). The most important upgrade is the addition of the CORI-FLOW<sup>®</sup> digital mass flowmeter (**Bronkhorst High-Tech B.V.**, AK Ruurlo, The Netherlands), which uses the Coriolis effect to instantaneously measure and control the cross-flow in AF4. The CORI-FLOW sensor consists of two small parallel tubes with the liquid flowing through them. The tubes are twisted slightly, which produces a small phase shift between the two tubes. The magnitude of shift is linear with mass flow rate. This novel flow sensing element has no pressure drop and very small band broadening or dead volume. The inlet is located below the cross-flow membrane, so it provides a direct, continuous measurement of the transmembrane flow. Dr. Johann reports that this is superior to prior flow monitoring devices, especially since the flow rate is not a function of the fluid properties. While improved convenience is the major benefit, the CORI-FLOW also decreases the apparent peak width. The improvement is small for Eclipse 3 instruments, but very significant for Eclipse 2 and F models. **Wyatt** is planning an upgrade program for some older models.

**AF4-MALS-ICP-MS is a powerful and useful tool since particles and molecules can be separated on the basis of size and chemical composition, including isotopic tracking.**

Preventing growth of organisms in the mobile phase (MP) reservoir is a significant problem in AF4, since the organisms are trapped by the cross-flow membrane. Conventionally, this has been controlled by adding a few micrograms of sodium azide to the MP reservoir. However, sodium azide is toxic and mutagenic. In Japan, it is not allowed to be used as a preservative in reagents. As an alternative to azide, the Eclipse 3+ uses a small (4-W) UV lamp in the MP reservoir to eliminate microbe growth. If the reservoir is large (over 2 L), **Wyatt** recommends using a magnetic stirrer to circulate the MP around the lamp.

Improved compatibility between **Wyatt** ASTRA software and the **Agilent** ChemStation (Wilmington, DE) is the third major upgrade. Now, the samples are entered into the setup page of the ChemStation. As the run proceeds, ASTRA collects and processes the data. The goal is to use the best points of the two software programs to provide seamless control and reports, which seems to be successful.

**Wyatt's** flow channels have also been expanded by adding new spacers of 600 and 800  $\mu\text{m}$  thickness. Now channel thickness can be selected over the range of 250–800  $\mu\text{m}$ . Lengths are 115, 152, and 240 mm. Dr. Johann reports that the short channel has become very popular recently since the resolution is the same as the longer units, but run time is cut in half. He points out that in AF4, resolution does not improve with length, in contrast to chromatography. Increasing the temperature from 20  $^{\circ}\text{C}$  to 80  $^{\circ}\text{C}$  provides an additional 50% reduction in run time for the samples that can withstand high temperature.

Most of the lectures during the day were on FFF of biopolymers, especially proteins. Prof. Jason Unrine (University of Kentucky, Lexington) was the conspicuous exception for his report on AF4-MALS (multiangle light scattering) and ICP-MS detection for the assay of inorganic materials including nanoparticles. In one study, the problem was to elucidate the mechanism of uranium contamination of groundwater. ICP-MS showed that uranium was associated with the UV-absorbing organic material in the water, rather than the inorganics in clays. The next example probed the toxicity of nanogold particles. These are taken up by cells, where they lead to changes in cell structure, resulting in death. They showed that the 55-nm nanoparticles are toxic. They found no evidence that larger aggregates were involved. In summary, Prof. Unrine has shown that AF4-MALS-ICP-MS is a powerful and useful tool since particles and molecules can be separated on the basis of size and chemical composition, including isotopic tracking.

Dr. D. Richard White (**Procter & Gamble [P&G]**, Mason, OH) described the use of AF4 for characterizing starch. The two main components of starch are amylose (AMY) and amylopectin (AMP). The size and amounts of these vary with the origin and preparation of the starch. Both are large molecules, with AMY usually ranging from  $10^5$  to  $10^6$  Da and AMP from  $10^8$  to  $10^9$  Da. AMY is usually linear, while AMP is typically highly branched off a long, common backbone. These are too large for reliable steric exclusion chromatography (SEC). Since starch appears in about 90% of **P&G** products, the company is very interested in controlling

starch composition. However, information from vendors can be unreliable, if provided at all.

Yet, **P&G** must supply a consistent product. Its processes are controlled based on assaying starch for the mass ratio of AMY:AMP, the distributions of molar mass, weight average molecular weights ( $M_w$ ) of both AMP and AMY, and the  $z$ -average mean square radius of AMP. He uses an Eclipse 3 AF4 instrument with a frit inlet and outlet for the characterization of starch. The frits aid in controlling the separation by adding a flow of MP to the top of the cell without diluting the sample. Typical fractograms have two large humps—the first for AMY and the second for AMP. Run times are about 20 min. Channel effluent is monitored with a DAWN EOS 18-angle MALS detector coupled in series with an Optilab® rEX dRI (both from **Wyatt**). The signals from the MALS and RI provide measurement of AMY:AMP ratio,  $M_w$ ,  $R_z$  (nm), and  $M_w/M_n$ . Plots of  $R_z$  versus  $M_w$  usually have a slope in the range of 0.39–0.41, which indicates that the AMP is highly branched. All of this agrees with legacy data, but with AF4, the results are more precise.

There are still a few problems: AMY has a long tail that can interfere with the AMP peak. The signal from AMY can be weak, which precludes estimation of the  $R_z$ . Also, the virial coefficient ( $A_2$ ) for AMP may be non-zero, which could lead to error in the estimation of  $M_w$ , especially if it is negative, which favors association.

### **AF4 separations in the steric mode**

AF4 has two distinct modes of separation separated by a cross-over or transition region. Analytes lower than about 500 nm in size are separated by differences in diffusion from the bottom plate. Recall the flow profile in an AF4 cell, with very low flow at the bottom and a parabolic flow profile extending down the flow cell. The smaller molecules diffuse more rapidly away from the bottom membrane and are swept into the main stream flowing down the cell. This gives an elution order of small to large. In contrast, the particles with a size of a few microns and larger are never really in the slow-moving MP due to their size. They just roll down the cell; the largest are in the fastest stream and emerge first. Thus the order is large to small. There is an ambiguous range, typically about 1  $\mu\text{m}$ , where FFF transitions between these two modes. Fortunately, a few experiments can readily resolve the ambiguity.

Ms. Julia Ferullo of **Wyatt** described AF4 in the steric mode using a 250- $\mu\text{m}$  spacer. Analytes were polystyrene particles in the range of 250–400 nm. She was able to promote the steric mode by decreasing the spacer thickness and channel flow rate while increasing the cross-flow rate. The fractograms generally looked as expected, except there was significant scatter or noise in the smaller peaks. The scatter decreased rapidly as the number of particles increased. The scatter was attributed to rapid fluctuations in the number of particles in the measurement cell. The fluctuations are statistical at low concentrations where one is counting particles, but at higher numbers the percentage variation is smaller due to the higher population. This has led to an update in the signal processing algorithm for ASTRA to include a modified Lorenz-Mie treatment that improves determination of particle size in the single-digit micrometer size range.

I had expected the focus of this program to be on aggregates of biopolymers, especially protein aggregates. This is an older, but still developing, topic. Since immunogenicity is more empirical than predictive, one needs to experimentally gather data from all corners of the room (experimental space). Dr. Shawn Cao (**Amgen**, Thousand Oaks, CA) discussed using AF4 for measuring the quality attributes of protein drugs, with a particular focus on visible and subvisible contaminants. Visible contaminants are simply not defensible. Any defense to “You want to inject me with that?! It doesn’t look right” starts off with a weak case, including a legitimate, presumptive fear of the unknown.

AF4 is not yet in the regulated QC environment, but the FDA has purchased and is evaluating instruments in its laboratories. Thus we can expect a rapidly increasing sophistication related to the power and limitations of AF4 technology, according to Dr. Cao. At first, AF4 was seen as an orthogonal method to SEC and analytical ultracentrifugation (AUC). However, with experience, AF4 has become the preferred analytical technology since it is faster and easier. The major difficulty with AF4 is that the sample concentration is high, typically mg/mL, though SEC is usually limited to much more dilute samples, which may not be representative of formulations and processing solutions. With these considerations in mind, both are thought of as useful orthogonal methods. AUC is a third orthogonal method, but is quickly falling from favor primarily due to low productivity.

At **Amgen**, FFF is used in the early stages of drug discovery and early stages of development, especially to characterize the product. Data have been submitted to the FDA as part of investigational new drug (IND) packages. However, AF4 has not been used in product release, but probably will be in the future. **Amgen** also uses AF4 over the size range of 0.001–1.0  $\mu\text{m}$ . Detection varies depending on size. Analytes larger than about 0.1  $\mu\text{m}$  are characterized by counting technology, while those that are smaller use conventional optical detectors, including UV and RI.

Dr. Cao presented several case studies comparing AF4 with SEC. One involved a monomer and small oligomer. In this case, SEC and AF4 both provided mutually consistent results. However, AF4 was superior in studying self-association of the protein, due to better tolerance of high concentrations. Another case involved characterization of a self-associating antibody. SEC could resolve the monomer, dimer, and trimer. AF4 provided discrete peaks for the tetramer and pentamer, plus a bleeding tail that contained 26% of the injected mass that was not resolved into discrete bands. Next, Dr. Cao showed use of AF4 for an in-process sample of an antibody. The problem was to measure the monomer:dimer ratio. This was done neatly with only an 8-min run. In another case, the problem was to detect submicron particles in a formulation. This failed due to inability to detect the particles since the signal was below the detection limit required for GMP samples.

A similar report was presented by Dr. Jan-Martin Hamelink of **GlaxoSmithKline** (Montreal, Canada), who compared SEC and AF4 for the characterization of protein antigens in vaccine development. The antigens are recombinant proteins and polysaccha-

**The combination of AF4 with light scattering detection is a very powerful tool.**

rides that have very low solubility in aqueous buffers. The data from AF4 were accepted as support for lot release. In a related study, it was shown that the formulation was stable for up to three freeze-thaw cycles. Another study dealt with high-molecular-weight polysaccharide particles. UV detection was a problem due to light scattering adding to the absorbance at 280 nm ( $A_{280}$ ). Since light scattering decreases strongly with increasing wavelength, the measured absorbance can be corrected to  $A_{280}$  (Eq. [1]) by subtracting twice the absorbance at 333 nm:

$$A_{280 \text{ corrected}} = A_{280 \text{ measured}} - 2A_{333} \quad (1)$$

Dr. Hamelink reports that the correction needs to be considered for UV absorbance detection of molecules larger than 100 kDa.

Still another study showed that AF4 was the method of choice for fractionation of vesicle particles that contained proteins, since it did not degrade the particles by shearing. There was no interaction with the matrix, and it operated over the required size range. The hollow sphere vesicles eluted in a window from about 10 to 15 min. Light scattering data showed that these vesicles had a shell thickness of 10 nm, which is similar to a monolayer of the lipopolysaccharide monomer.

Andrej Citowicz of **Bayer Corp.** (Richmond, CA) used AF4 for the characterization of virus-like particles (VLPs) assembled from proteins. VLPs are designed to encapsulate and deliver DNA as a vector in gene therapy. Recombinant VP1 proteins (~40 kDa) were expressed and grown in insect cells. Under the right conditions,

one can assemble pentamers of the VP1 protein into a VLP that contains a DNA strand inside. The protein part has a theoretical molar mass of 14,300 kDa. With the DNA inside, the mass is about 17,000 kDa, and is about 20 nm in diameter.

### Summary and credits

This report shows that FFF and AF4, in particular, are clearly delivering unique and useful information across the biopharm market segment. The combination of AF4 with light scattering detection is a very powerful tool. For access to a complete indexed listing of papers on FFF technology and applications, please visit [www.wyatt.com/literature/FFFbibliography.cfm](http://www.wyatt.com/literature/FFFbibliography.cfm). This complete bibliography was started by Dr. Mark Schure of **Dow Chemical Co.** (formerly Rohm & Haas) (Spring House, PA).

**Wyatt** deserves special thanks for organizing and sponsoring this series of users' meetings. The next meeting will be held October 20, 2010, in the same location. Please monitor the URL, [www.wyatt.com](http://www.wyatt.com).

### Reference

1. Moore, G.A. *Crossing the Chasm: Marketing and Selling High-Tech Products to Mainstream Customers*; HarperCollins: New York, NY, 1991; revised 1999.

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