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Editor's Page

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Update on Field Flow Fractionation

After 40 Years, Still the Best-Kept Secret in Analytical Chemistry

In middle age, field flow fractionation (FFF) is having an identity crisis, although no one seems to notice. Yet, the adventurous early adopters seem to have very positive experiences within a few weeks or months from a cold start. With today's instruments, the FFF is just not that difficult. And the need is now. Just look at biotechnology and nanotechnology. These must be the top two interest areas in chemistry. There is a crying need for new analytics.

The 4th Annual Eclipse FFF-MALS Focus Meeting was held October 20, the day after the 21st Annual International Light Scattering Colloquium, at The Four Seasons Resort, The Biltmore, in Santa Barbara, CA. Both are open user meetings organized by **Wyatt Technology Corp.** (Santa Barbara, CA). The meeting gave 19 scientists an opportunity to spend an extra day in Santa Barbara to talk shop. In contrast to prior meetings, the unseasonably cold, wet weather made it much easier to attend the lectures, which were uniformly excellent.

Although FFF encompasses several modes, **Wyatt** focuses on its Eclipse™ Asymmetric Flow Field Flow Fractionation (A4F) augmented by a stable of detectors. For example, MALS (multiangle light scattering) detection is ideally suited to provide independent and very accurate measurement of molar masses of the samples measured. The Optilab® T-rEX™ refractive index (RI) detector from **Wyatt** has exceptionally low noise and a dynamic range about 50 times greater than models from other vendors. For a more complete description of the T-rEX, please see "Highlights from the 22nd International Ion Chromatography Symposium (IICS 2010)," at www.americanlaboratory.com/webexclusive/IICS2010.

Chromatography benefits from several competing simulation programs designed to assist the chromatographer with developing and optimizing robust methods quickly. Dr. Dierk Roessner of **Wyatt Technology Europe GmbH** (Dernbach, Germany) described *Isis* simulation and optimization software. *Isis*, named for the Egyptian goddess of simplicity, is intended to facilitate optimization of the control settings and channel construction for A4F. For example, increasing the cross-flow will generally increase retention time of the analytes. Retention can also be improved by increasing the channel thickness. The software provides some error trapping, especially when a setpoint would probably be out of range. The printout from the Eclipse using ChemStation (**Agilent**, Santa Clara, CA) provides a summary of the plates, resolution, and selectivity for each separation. This is useful in method control and diagnostics.

A4F of polymers

Branching of polymers affects polymer performance, but it is often difficult to measure. A lecture and poster by Dr. Stepan Podzimek of **SYNPO** (Pardubice, Czech Republic) compared the results obtained from steric exclusion chromatography (SEC) and A4F both with MALS and RI detection for polymers. When the results from SEC-MALS were plotted using the conventional conformation plots of root mean square (RMS) radius vs molar mass over the range of 10^4–10^9 Da, the results showed a fishhook shape. The A4F plots were nearly linear over the same range. Dr. Podzimek attributed the abnor-

mal behavior of SEC to the ability of the branches of the molecules to penetrate and perhaps even mechanically anchor into the pores of the SEC stationary phase. As a result, some of the large branched polymers elute more slowly than normal, leading to underestimation of the molecular weight. With A4F, there is no stationary phase, the abnormal elution is eliminated, and the conformation plots are devoid of artifacts. Hence, A4F is useful for measuring conformation and branching.

Dr. Podzimek's lecture compared A4F with SEC in more detail. The sample in A4F is concentrated during the focusing step, in contrast to dilution only in SEC. The elution order is inverted between the two: With SEC, the largest molecules emerge first, followed by the smaller, in order. For A4F, the elution order depends on the size of the analyte. Over the range of 100 kDa to about 500 nm, which is called the diffusion region, the elution order is small to large. However, at about 1–20 µm, the particles are so large that they extend into the fast current, even when touching the sidewall. This is called the steric region, with an elution order of large particles followed by smaller. The transition or ambiguous zone is for analytes with a particle size near a micrometer or so. One can adjust the channel thickness to change the boundary between the normal diffusion and steric regions or modes. This is one of the attractive features of *Isis* software.

Several authors, including Dr. Podzimek, found that SEC of molecules larger than about a million daltons is unreliable. In addition to the mechanical delay mentioned above, they also reported that some large molecules may not even elute. Worse, many suspect that the mechanical interaction with the stationary phase is strong enough to break the polymer. This has been confirmed by collection and reinjection.

Large proteins

In addition to the problems with SEC above, Dr. Janice Davis of **Althea Technologies** (Camarillo, CA) expanded the list of assay problems for large analytes to include dynamic light scattering and analytical ultracentrifugation (AUC). The latter is too slow, and the former needs fractionation prior to the measurement since it responds preferentially to the largest molecules in the sample cell. Because **Althea** is a contract laboratory, Dr. Davis sees a variety of problems and samples. Some are relatively simple, if one has the right instruments. A case in point: A fractogram with MALS detection of a large multiunit protein showed peaks that corresponded to the expected mass, but some of the peaks presented nonflat responses with the MALS detector, indicating that the peaks were not monodisperse.

Another more extensive study involved a formulation study of an Fc fusion protein. Fusion protein is a protein made by connecting the Fc region of an antibody with another protein segment. The Fc region may reduce adverse host response, and the next region may contain a therapeutic agent. These are often called chimeric proteins since they are designed to combine the best of two species. Large aggregates were of particular concern since they were probably too large for SEC. The first problem encountered with FFF was a sample that overloaded the channel, even with only 100 µg. In an effort to improve detection, the cross-flow was increased from 1 to 3 mL/min. This improved detectability, as expected. Next, aged samples were examined. These showed large peaks eluting after the active pharmaceutical ingredient (API) peak. The magnitude of these peaks was used as a measure of instability to rank-order the formulations, with tall peaks being the worst. Several formulations were much better than the others and passed on to the next stage. Dr. Davis concluded, "Although it remains challenging to use quantitatively, A4F can add value in real-world studies of proteins."

Protein carbohydrate conjugates

Mr. Cliff Entrican of **Pfizer** (Andover, MA) described a program to characterize two different

protein–carbohydrate conjugates. Prior work with SEC–MALS led to a need to develop a robust analytical technique capable of providing the entire mass distribution without exclusion or loss of sample. Mr. Entrican's team looked at A4F and quickly adopted it as the analytical technique of choice for **Pfizer's** protein–polysaccharide conjugates.

He presented one study comparing two samples. A4F with MALS, RI, and UV quickly showed that one sample had a mass of about one megadalton, and the other was 10 times larger. Further, using UV to provide the protein content and RI for the total mass, it was clear that the samples were heterogeneous in composition over their size distribution range. Circular dichroism (CD) spectra showed that conjugation of the polysaccharide forces the protein to make significant changes in secondary and tertiary structure. Secondary protein structure refers to major elements such as sheets and helixes; tertiary structure refers to the overall three-dimensional structure of the protein.

Mr. Entrican went on to study the conjugates under denaturing conditions. This effort was confounded by the difficulty of running A4F with concentrated denaturants. However, it was concluded that A4F provides information that historically required several different characterization methods. It is now used consistently at **Pfizer** to characterize complex conjugates.

A4F-ICP-MS

Nanoparticles (NPs) are appearing more frequently in many commercial products. Silver, titania, and zinc oxide NPs are common. The toxicity profiles of the NPs for humans and the environment are not available, if they exist at all. Dr. H. Hagendorfer of the Swiss Federal Laboratories for Materials Testing and Research (Duebendorf, Switzerland) reported on the construction and validation of A4F with ICP-MS for studying metal-containing nanoparticles. The system included an Eclipse A4F coupled to an Element2 ICPMS (**Thermo Finnigan**, Waltham, MA). The system was calibrated with NIST gold particle standards (10 nm [NIST 8011], 30 nm [NIST 8012], and 60 nm [NIST 8013]). The curve was linear. To validate the new method, results from the A4F assay were compared to image analysis results from transmission electron microscopy (TEM) with $n = 160$ particles. The correlation looks good to the eye, but particle counting may have offered a bit better resolution at very high mass. The new method was used to analyze silver particles in a consumer spray product. Silver is probably added as a bactericide. The results show that the silver is polydisperse with a diameter range of 6–30 nm. However, the size also increases during spraying by agglomeration. Compared to TEM, the A4F method is faster and much less expensive.

Credits

To me, the yearly update on A4F is a case of mixed emotions. A few are using it with success as reported above. I asked why there were so few papers and posters, and the response was that several potential speakers had to cancel due to intellectual property considerations. This should not detract from the main message for the day, i.e., A4F works and is probably the most viable alternative to AUC and is certainly superior to SEC above a million daltons.

The **Wyatt** team, and especially Dr. Michelle Chen and Dr. Jeff Ahlgren, deserve special recognition for organizing and chairing a stimulating program. The administrative skills of Ms. Lindsey McGowan ensured an almost seamless presentation. It really helped that the creature comforts were also exceptional. I'm looking forward to the next meeting, which is expected for next October. Please visit www.wyatt.com for further information.

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