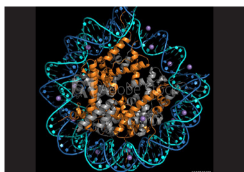
Advancing HPLC/ UHPLC Analysis with Multi-Angle Light Scattering Technology

JUNE 2019

Next-Gen Multi-Angle Light Scattering
Interview with Daniel Some



Applications of Analytical Light Scattering in a Biophysics Core Facility
Ewa Folta-Stogniew

UHP-SEC-MALS: Absolute Characterization of Polymers with Light Scattering and UHP-SEC
Daniel Some

Sponsored by

This custom ebook is sponsored by Wyatt Technology and presented in partnership with *LCGC*.

SPONSORED CONTENT

Next-Gen Multi-Angle Light Scattering

New HPLC/UHPLC product line offers more robust measurements and increased uptime.

Interview with Daniel Some

Images under license from stock.adobe.com

In March 2019, Wyatt Technology Corporation launched its next generation of online multi-angle light scattering (MALS), refractive index, and differential viscometry detectors for high performance liquid chromatography (HPLC) and ultrahigh-pressure liquid chromatography (UHPLC) systems. *LCGC* recently asked Daniel Some, PhD, Principal Scientist at Wyatt Technology, about the advancements made in Wyatt's product line for absolute macromolecular characterization.

LCGC: Can you explain what is size-exclusion chromatography (SEC)-MALS and why it is of interest to protein and polymer scientists?

Some: SEC-MALS couples online multi-angle light scattering detection and other online detectors (such as refractive index and differential viscometry) to size-exclusion chromatography. With this technique, the only purpose of the SEC column is to separate the different molecules from each other. The actual characterization of the molecules takes place solely within the detectors, which allows absolute characterization to be performed. This method does not depend on the retention time within the column, the conformation of the molecule, or a molecule's interactions with the column. Thus, in SEC-MALS we do not encounter the errors of typical analytical SEC where reference molecules are run even though they might (and often do) behave differently on the column than your molecules.

This technique allows us to analyze monodisperse molecules, such as proteins, or polydisperse macromolecules, such as heterogeneous polymers, to determine their molecular weight, size, conformation, and branching ratio. The oligomeric state of proteins in native solution can be determined, resulting in a much better understanding of the essential bio-

physical properties of the macromolecules than can be obtained from analytical SEC.

LCGC: What would you say is new and improved in Wyatt's DAWN, Optilab, and ViscoStar products launched in March 2019?

Some: In March, we launched a re-envisioned product line of the DAWN®, Optilab®, and ViscoStar® online detectors for SEC with multi-angle light scattering. While these detectors offer the same industry-leading sensitivity, range of measurements, and other features that our customers are used to for maximum characterization of their macromolecules, the new products have a sleek modern look and feel. For example, there is a large capacitive touchscreen that allows users to interact more intuitively with the instrument and access the information that they need from the front panel.

The instruments also have improvements in serviceability and maintainability, achieved by making them more modular. In fact, individual modules can be swapped out on-site. In addition, CheckPlus™ software performs a full diagnosis and sends those diagnostics to an engineer at Wyatt for a more in-depth look. Depending on what the engineer decides, a technician can come on-site and swap out the modules with very little downtime.

LCGC: What are some of the newest innovations in the DAWN line, which has been Wyatt's flagship product for 37 years?

Some: In previous generations, we worked on improving the technical specifications, getting higher sensitivity, expanding the range of measurements, and adding user interface improvements. Key in the new generation of DAWN detectors is the built-in intelligence that assists users in knowing when their SEC-MALS system is ready for optimal measurements, when the noise level is low enough, and when the system is fully equilibrated. In addition, swappable flow cells allow for a new flow cell to be swapped in without the need for laser alignment. Opto-mechanics are more robust, and modifications to the optical

> "Across all these products—DAWN, Optilab, and ViscoStar—the key added value is enhanced productivity arising from the new Smart Services™ Platform. The platform includes the System Ready Monitor and System Health Indicators, ensuring users do not waste runs due to sub-optimal chromatography conditions."

design further reduce stray light. Dedicated slots for the WyattQELS™ dynamic light scattering module have been added so that, rather than sacrificing one of the MALS angles as with the previous models, WyattQELS gets its own slot, and the software automatically identifies into which angle the user has placed the WyattQELS optical fiber.

LCGC: What do you see as the main value to customers in the updated product line?

Some: Across all these products—DAWN, Optilab, and ViscoStar—the key added value is enhanced productivity arising from the new Smart Services Platform. The platform includes the System Ready Monitor and System Health Indicators, ensuring users do not waste runs due to sub-optimal chromatography conditions. The platform's self-diagnostics and CheckPlus instrument log application permit remote evaluation by our service team, and full on-site repair service.

LCGC: What can Wyatt offer to those who use UHPLC?

Some: microDAWN is the online multi-angle light scattering instrument for use with UHPLC. microOptilab is the refractive index detector for UHPLC, and microViscoStar is the differential viscometer for UHPLC. Users can get the complete range of characterization of molecular weight, size, and conformation, with all the benefits of UHPLC, which means faster runs, lower sample consumption, lower mobile phase consumption, and enhanced productivity.

LCGC: Where can readers go to learn more about SEC-MALS technology and applications?

Some: The best place to start is our website, which is www.wyatt.com, and there we have information about the theory of SEC-MALS, light scattering, and other technologies. Folks can learn about the various solutions that the instruments offer, the applications they provide, the different types of analytes that can be analyzed, the industries served, and the products' features and benefits. There is also an extensive library of webinars that can be viewed to learn more.

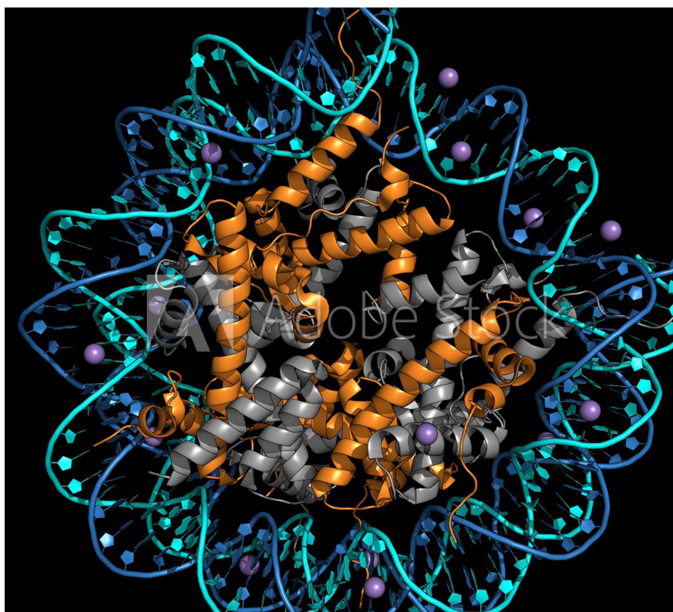
Daniel Some, PhD
Principal Scientist
Wyatt Technology

SPONSORED CONTENT

It's the DAWN of a new day.

Learn more at wyatt.com/NextGen

WYATT
TECHNOLOGY



SPONSORED CONTENT

Applications of Analytical Light Scattering in a Biophysics Core Facility

SEC-MALS is a powerful technique for probing the composition, stoichiometry, kinetics, and concentration dependent behavior of proteins and protein complexes.

Ewa Folta-Stogniew

Overview

Although mass spectrometry is a popular technique, its ability to determine molecular mass is limited by often-aggressive ionization conditions and it is generally not applicable to protein aggregates and complexes. Size exclusion chromatography with multi-angle light scattering (SEC-MALS) using a Wyatt DAWN® MALS detector, in contrast, can analyze protein complexes under native aqueous conditions. It is a powerful technique that can probe more than simply molecular weight (MW). When combined with ultraviolet absorption (UV) and Wyatt's Optilab® refractive index (RI) detectors, it can also characterize the composition, kinetics, morphology, and concentration-dependent behaviors of proteins, protein complexes, and mutation variants. A series of case studies will demonstrate the various strengths of this technology.

Protein Nucleic Acid Complexes

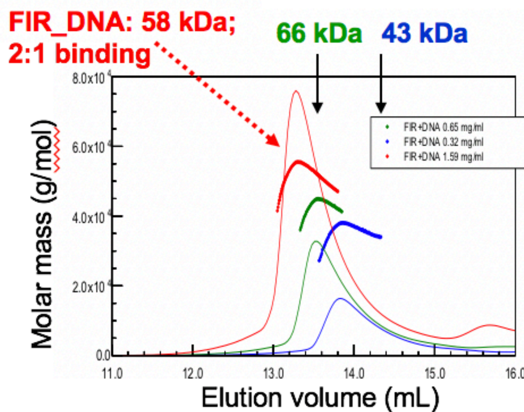
FIR protein is a repressor involved in regulating the transcription of c-myc oncogene, which is often over-expressed in cancerous cells. X-ray crystallography showed the protein as a dimer, but it was unknown if the protein exists in this state when in solution. When analyzed by SEC-MALS, it was surprising to find that even under fairly concentrated conditions, the protein exists primarily in the monomeric form in solution.

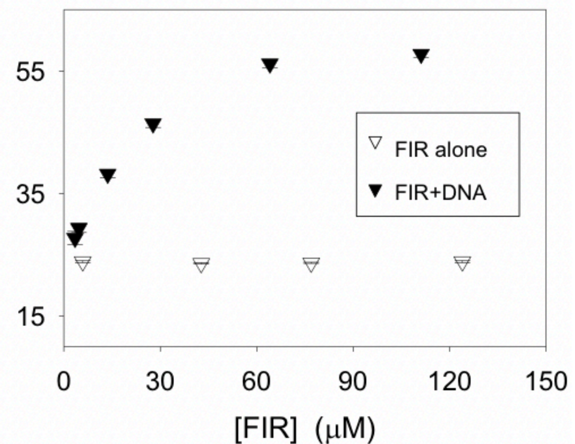
The FIR protein is known to have an interaction with the 8-kDa DNA fragment known as FUSE, which is a P1 promoter. With increasing concentration, the prevalence of protein complexed with the DNA and it becomes the predominant form. It was clear based on light scattering that the complex contained two protein molecules. However, the precision of the determination was not high enough to tell whether there were one or two copies of the much smaller DNA segment (**Figure**

Figure 1: Dimerization of FIR depends on DNA binding event.

ssDNA fragment upstream of the P1 promoter, known as FUSE: 8 kDa

FIR+DNA complex; task: *determine stoichiometry of the FIR+DNA complex in solution*





FIR-DNA complexes	MW (kDa)
FIR+DNA (2:1) complex	54.7
FIR+DNA (2:2) complex	62.8
Observed MW	57.7

Concentration dependent measurements reveal that, in solution, FIR dimerization is driven by DNA binding

Crichlow, G. V., Zhou, H., Hsiao, H-h., Frederick, K. B.,, Debrosse, M., Yuande Yang, Y., Folta-Stogniew, E. J., Chung, H-J., Chengpeng Fan, C., De La Cruz, E., Levens, D., Lolis, E., and Braddock, D. (2008) "Dimerization of FIR upon FUSE binding suggests a mechanism of c-myc inhibition", *EMBO J* 27: 277-289

1). The measured molecular weight falls between 2:1 FIR-to-DNA ratio and a 2:2 ratio.

Fortunately, having multiple in-line detection modes provides further information about the complex stoichiometry. The ratio of responses between the UV absorption and RI signals is telling. FIR has a weaker UV response when compared to DNA, and the UV/RI ratio is just 0.14. The DNA strand, in contrast, has a strong UV/RI ratio of 2.34. A 2:1 complex would be expected to have a UV/RI of 0.47 while a complex of 2:2 (or 1:1) would be expected to have a ratio of 0.72. At all but the lowest concentration, both the observed molecular weight and the UV/RI ratio were consistent with a 2:1 complex of FIR to DNA. However, at the lowest concentration (4 μM), both the observed molecular weight and the UV/RI ratio matched a 1:1 complex, indicating that the mechanism for complex formation is most likely that the DNA first interacts with a single molecule which then induces dimerization of the proteins. Per

the SEC-MALS-UV-RI results, it appears that FIR's propensity to dimerize when in crystal form is only seen in solution in the presence of the DNA strand.

Analysis of Membrane Proteins

SEC-MALS analysis is also well suited for the analysis of membrane proteins, which are generally not directly water-soluble. They must be purified in the presence of detergents, and unknown amounts of detergent molecules are bound to the protein in a complex. The analysis by standard analytical SEC can be challenging due to anomalous retention behavior, as they will elute according to the hydrodynamic size of the entire complex, not just the protein.

For modified proteins, such as glycoproteins, PEGylated proteins, or detergent-associated membrane proteins, a three-detector approach is often called for. Some constants are needed such as the UV extinction coefficient and the RI increments (*dn/dc*) of both the protein and the modifier. With those available, the software can solve a system of three equations to determine the molecular weight of the entire complex, the polypeptide portion, and the modifier. The system takes advantage of the fact that the UV signal is entirely determined by the concentration of the polypeptide, while the refractive index and light scattering are dependent on both the protein and the surfactant concentrations to different degrees. However, zero UV absorption by the modifier is not a requirement for the calculation.

As a case study, porin LamB and hemolysin alpha-HL were analyzed. LamB has a mass of 47 kDa and appears as a trimer in crystallographic studies. The alpha-HL is somewhat smaller at 33 kDa, but appears as a heptamer by crystallography. Both proteins were purified in the presence of dodecyl maltoside. With the three-detector system, the average molecular weight of the eluents can be plotted with elution volume on top of the UV chromatographic plot. When this was applied to the LamB sample, a fairly uniform mass of 141 kDa was seen across the width of the peak, indicating that the complex is a trimer in solution, in line with the crystallographic data. When the same analysis was performed on alpha-HL, the molecular weight was determined to be 225 kDa, which is close to the expected value of 231 kDa for a heptamer.

"SEC-MALS analysis is also well suited for the analysis of membrane proteins, which are generally not directly water-soluble."

Unexpectedly, when the two plots are overlaid (**Figure 2**), the smaller LamB trimer elutes earlier than the alpha-HL heptamer. Using the three-detector method, it was found that the LamB complex contained considerably more detergent, amounting to 1 gram per gram of protein, and resulting in a total complex weight of 285 kDa. The alpha-HL complex, by comparison, only associated with 0.26 grams of detergent per gram of protein,

bringing its complex to only 271 kDa. These findings are much more in line with the elution order seen of the two complexes, and a good demonstration of why SEC-MALS-UV-RI triple-detection system is often necessary to properly analyze protein-detergent complexes.

Analysis of Protein Complex Morphology

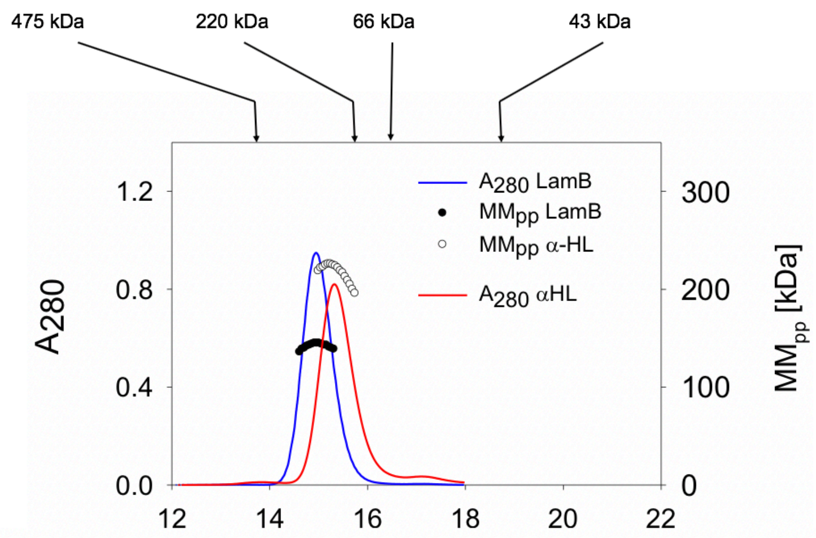
The combination of static and dynamic light scattering can also reveal important information about the morphology of protein assemblies. PopZ is a bacterial scaffolding protein involved in cell division. It was desirable to understand the oligomeric state of the wild type of the protein in comparison to two mutant strains. The protein has a monomeric mass of 21 kDa, however it is often found to be in oligomeric form. Under

"The combination of static and dynamic light scattering can also reveal important information about the morphology of protein assemblies."

Figure 2: Oligomeric state of detergent-solubilized proteins.

Proteins: porin **LamB** 47 kDa **trimer = 141±3 kDa** (141 kDa)

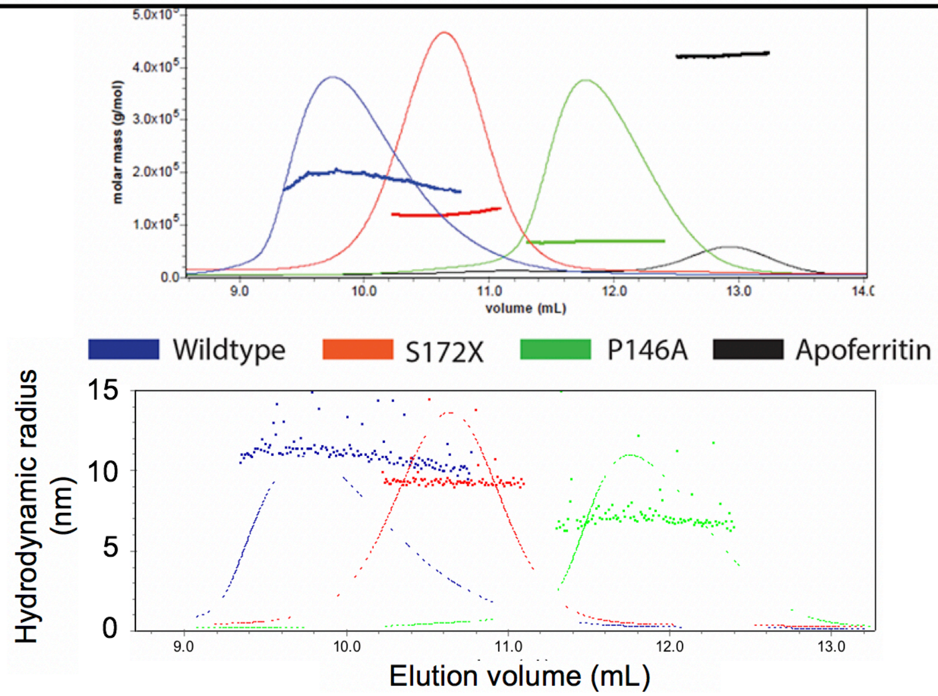
hemolysin **α-HL** 33 kDa **heptamer = 225±13 kDa** (231 kDa)



Yernool D, Boudker O., Folta-Stogniew E., and Gouaux E. (2003) Trimeric subunit stoichiometry of the glutamate transporters from Bacillus caldotenax and Bacillus stearothermophilus. *Biochemistry* 42: 12981-12988

Figure 3: Oligomerization of the PopZ polar scaffold protein.

	monomer	measured	oligomer	R_h
WT	21.3 kDa	202 kDa		11.5 nm
S172X (SN)	20.7 kDa	125 kDa	**hexamer**	9.1 nm
P146A (PA)	21.3 kDa	71 kDa	**trimer**	6.8 nm
Apo	20.0 kDa	475 kDa	24-mer	6.9 nm



Bowman, G. R., Perez, A. M., Ptacin, J. L., Ighodaro, E., Folta-Stogniew, E., Comolli, L. R., and Shapiro, L. (2013) Oligomerization and higher-order assembly contribute to sub-cellular localization of a bacterial scaffold. *Mol Microbiol*

gel electrophoresis, the wild type has an apparent migration consistent with a 600 kDa MW. One of the mutants (SN), is a C-terminal deletion, and appears to assemble much like the wild type. A second mutant (PA), with a mid-chain substitution, migrates with an apparent molecular weight of around 240 kDa.

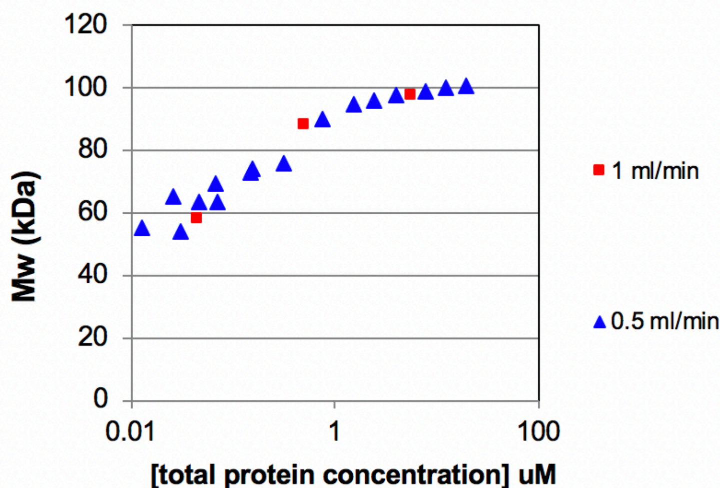
In the SEC separation, all three proteins elute surprisingly early, well before apoferritin, a 475 kDa protein. In spite of the early elution, three-detector analysis revealed that the protein molar masses were relatively small, ranging from 60 to 200 kDa, and corresponding to trimers for the PA mutant, hexamers for the SN mutant, and hexamers or larger complexes for the wild type, all considerably smaller than either gel electrophoresis or SEC alone indicated.

SPONSORED CONTENT

Figure 4: Determination of dimerization constant from SEC-MALS measurements: Mw vs. concentration.

Nucleobindin 1 (NUCB1) is a widely expressed multidomain calcium-binding protein whose precise physiological and biochemical functions are not well understood;

soluble form of NUCB1 (*sNUCB1*): Monomer 51 kDa



0.001 mg/ml 1 µg/ml

1 mg/ml

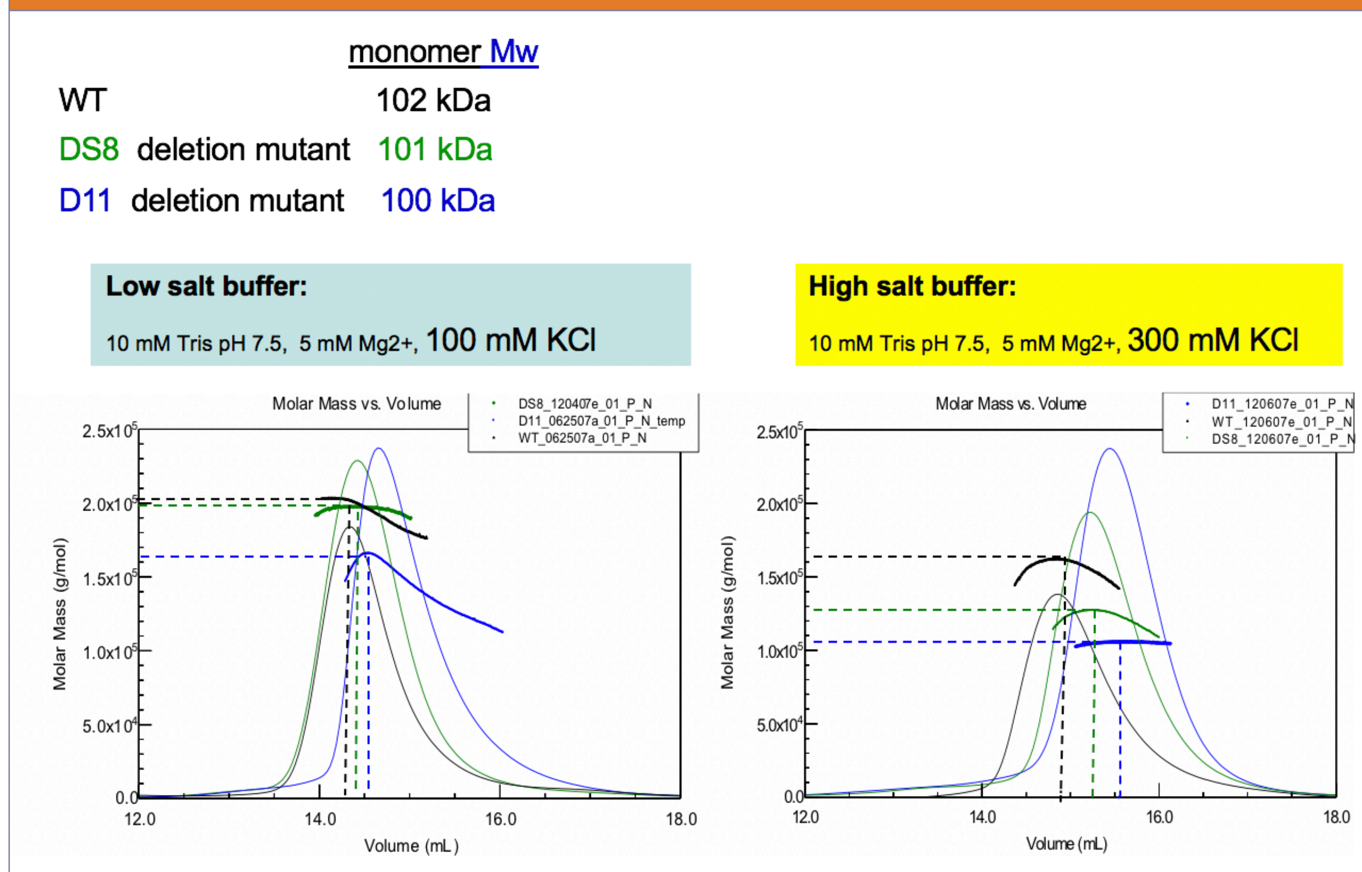
Kapoor N, Gupta R., Menon S. T., Folta-Stogniew E., Raleigh D. P., and Sakmar T. P. (2010) Nucleobindin 1 is a calcium-regulated guanine nucleotide dissociation inhibitor of G{alpha}i1. *J.Biol.Chem.* 285; 31647-31660

Shape of the complex may play a role in the abnormal elution. On-line dynamic light scattering is used to determine the hydrodynamic radii of the protein complexes, which ranged from 6.8 nm for the PA mutant to 11.5 nm for the wild type (**Figure 3**). The measured mass for the PA mutant corresponded with a trimer at 71 kDa, but the measured value of the hydrodynamic radius would be expected for a protein of 475 kDa. This indicates that the protein structures are highly asymmetric and non-globular. Interestingly, the elution time, but not the calculated mass, of the PA mutant changes with concentration, suggesting there might be concentration-dependent changes in the shape of the complexes. Analysis using circular dichroism spectroscopy indicated that the proteins tend to exist in a linear, unfolded state when in oligomeric form, consistent with a high-aspect-ratio complex.

Determination of Dimerization Constants

As a final case study, SEC-MALS was used to determine the dimerization constant of the protein Nucleobindin 1 (NUCB1). The first clue that NUCB1 has a concentration-

Figure 5: Determination of dimerization constant from SEC-MALS measurements: SecA Mw concentration dependence.



dependent dimerizing behavior can be seen by making sequential injections of varying sizes. At low concentrations, the eluted peak's MW corresponds to that of the monomer (51 kDa), but as the concentration is increased, the MW approaches that of a dimer. It appears that this dimerization is an equilibrium process with rapid kinetics. The MW depends only upon the concentration in the eluted peak (as determined by UV absorption) and not in the initial injection, and is independent of flow rate (**Figure 4**). With a plot of concentration versus average MW, it was possible to perform curve fitting to determine the dimerization constant.

The technique was also applied to study the effect of N-terminal deletion in SecA protein on the dimerization constant. Two mutants were tested, corresponding to 8- and 11- amino acid deletions. Literature values for the dimerization constants are inconsistent, and appear to be dependent on ionic strength, so an analysis was performed at low and high salt concentrations. It was confirmed that high ionic strength destabilizes the dimer η with the effect being more pronounced for the shorter mutants (**Figure 5**). The D11 mutant was almost completely destabilized

SPONSORED CONTENT

at the higher salt concentration.

Conclusion

A SEC-MALS system incorporating a DAWN multi-angle light scattering detector and Optilab differential refractive index detector can analyze MW over a range of concentrations of four orders of magnitude. Measurements are fast and accurate, making it a critical tool for the characterization of often-difficult samples such as non-globular macromolecules and protein complexes with anomalous behavior under SEC or gel electrophoresis. The availability of the MALS-UV-RI multi-detection system means that information-rich data can be obtained, going well beyond just molecular weight. Protein complex stoichiometry, shape information, and dimerization constants can all be determined using only 50 µg or less of protein to perform the analysis.

Ewa Folta-Stogniew, *PhD, MSc, is the director of the Keck Biophysical Resource at Yale University.*

UHP-SEC-MALS: Absolute Characterization of Polymers with Light Scattering and UHP-SEC

Daniel Some

Ultrahigh-pressure size-exclusion chromatography (UHP-SEC) offers multiple benefits for synthetic polymer characterization. However, despite the many advantages of UHP-SEC, its rapid, low-volume separation is more sensitive to column calibration errors and drift than traditional high performance (HP)-SEC. Additionally, only a small selection of column chemistries are available. It is therefore essential to combine UHP-SEC with online, low-volume multi-angle light scattering instrumentation (UHP-SEC-MALS) to overcome these challenges.

Ultrahigh-pressure size-exclusion chromatography (UHP-SEC) is growing in popularity as the technique of choice for analysis of synthetic polymers. With greatly reduced run times and consumption of both sample and solvent, the time- and cost-saving benefits are compelling. Factoring in better resolution in separation and the consequential improvement in quantification of monomers and oligomers, as well as the flexibility to revert to standard gel permeation chromatography (GPC) as necessary, the case for upgrading to a highly productive UHP-SEC system is clear.

With all the advantages of UHP-SEC, several drawbacks need to be addressed. The first is the implicit assumption of a column calibration curve: that different elution times necessarily imply different molar masses, with decreasing molar mass corresponding to increasing elution time. By this logic, a narrow standard would elute in a very narrow peak—narrower than the inherent broadening caused by the finite column volume. In reality, even a narrow standard elutes with a width that—theoretically, by column calibration—corresponds to at least a two- to fourfold span in molar mass.

The second drawback is surface chemistry. When optimizing a separation method, it is important to find conditions where both the reference markers and sample to be tested do not interact non-ideally (for example, via hydrophobic or charge interactions) with the column packing. This

is because column calibration depends on the assumption that the reference molecules and sample undergo only ideal (steric) interaction with the column, and any additional "stickiness" will render erroneous results. Compared with high performance liquid chromatography (HPLC)-SEC columns, there are relatively few surface chemistries available to UHP-SEC for method optimization, hence a higher probability that the method will not be fully optimized.

The third is column creep and drift in chromatography conditions. With such a fast separation, even a minor difference in elution time because of column ageing, sample loading, or changes to the pump operation conditions will lead to a relatively large error in estimated molecular weight.

"Online multi-angle light scattering (MALS) measures polymer molar mass at each elution volume, absolutely, without reference to retention time."

Light Scattering

Online multi-angle light scattering (MALS) (1,2) measures polymer molar mass at each elution volume, absolutely, without reference to retention time. It does so by a first-principles physical relationship between the scattered intensity (I_{sc}), molar mass (M), concentration (c), and scattering angle θ:

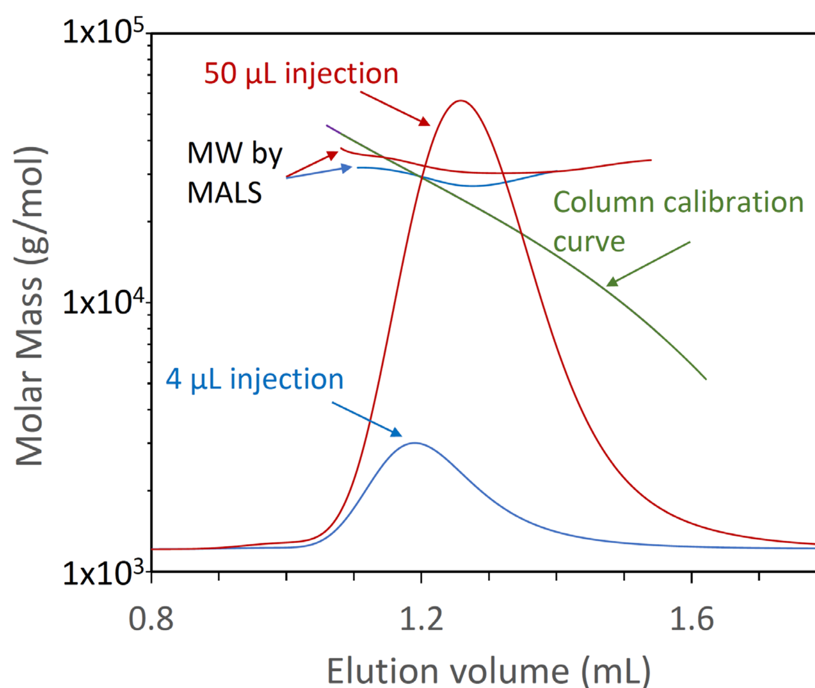
$$I_{sc} \propto M \cdot c \cdot \left(\frac{dn}{dc}\right)^2 P(\theta) \qquad [1]$$

where *dn/dc* is usually known or readily measurable for a polymer in any given solvent, $P(\theta)$ is an angular dependence that is dependent on the molecule's rms radius R_g, and the constant of proportionality may be calculated from the measurement system properties such as laser wavelength. Hence measurement of I_{sc} and c directly yields the values of M and R_g, contiguously, as the solution passes through the MALS detector's flow cell. The determination of molecular weight is independent of conformation or shape as well as elution properties. For these reasons, SEC-MALS has long been the de facto standard for rigorous analysis of proteins and polymers in solution separated by standard HPLC-SEC.

With the addition of an embedded dynamic light scattering (DLS) module, SEC-MALS adds a second, independent determination of molecular size (hydrodynamic radius, R_h). The relationship between molar mass and molecular size may be analyzed to determine polymer conformation such as random coil, branched, or elongated.

MALS and refractive index (RI) detectors for UHP-SEC have recently become available (3), conferring the benefits of MALS and embedded DLS on the newer separation technology. UHP-SEC-MALS maintains the central benefits of UHP-SEC while

Figure 1: Elution of two injections of 30 kDA polystyrene (4 and 50 µL) by UHP-SEC overlaid with molar masses derived from light scattering. In contrast to the broad and disparate molar mass ranges implied by column calibration, MALS proves that both contain narrow distributions around 30 kDa.



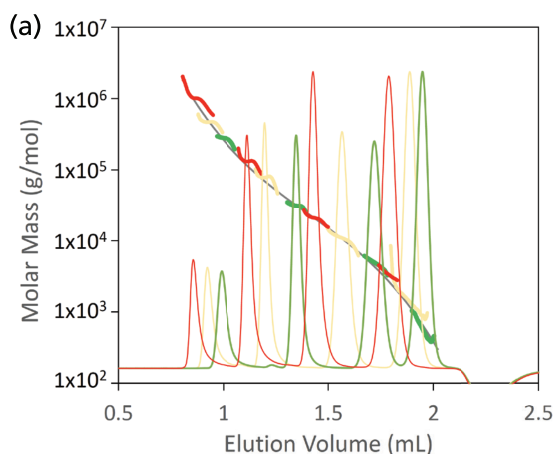
preserving chromatographic resolution and providing absolute molecular weight and size of the eluting species.

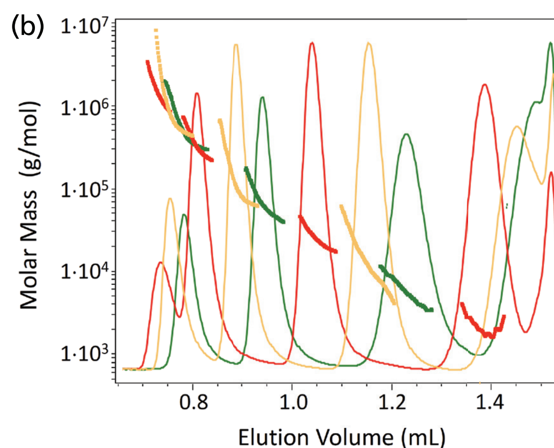
The importance of UHP-SEC-MALS is exemplified in **Figure 1,** where the chromatograms of a 30 kDa polystyrene standard with two injection volumes are presented. A naïve interpretation of the chromatograms based on elution time would suggest (incorrectly) that the 50-µL injection contains primarily smaller species than those in the 4-µL injection. It would also indicate (incorrectly) a broad molar mass range covering 10–40 kDa or 20–40 kDa in the respective peaks; yet MALS analysis shows that (correctly) both injections consisted of narrow distributions around 30 kDa.

Knowns and Unknowns

It is always helpful and even essential to validate an unfamiliar technique against commonly accepted standards. For UHP-SEC, such standards are linear polystyrenes, and SEC-MALS analyses of a series of polystyrene standards are quite informative. As shown in **Figure 2(a),** the molar masses derived by light scattering align perfectly with the column calibration curve. However, the

Figure 2: (a) Overlay chromatogram of a series of linear polystyrene standards. MALS analysis indicates high correspondence of calculated molecular weights with the calibration curve and in addition provides the true homogeneity within each peak. (b) Overlay chromatogram of a series of PMMA samples. MALS analysis shows the deviations from column calibration which result from branched conformations as well as internal heterogeneity for each peak.heterogeneity for each peak.





flat MALS results across most of the peaks provide absolute evidence that these polystyrene standards are quite homogeneous, something that could not be proven with column calibration alone.

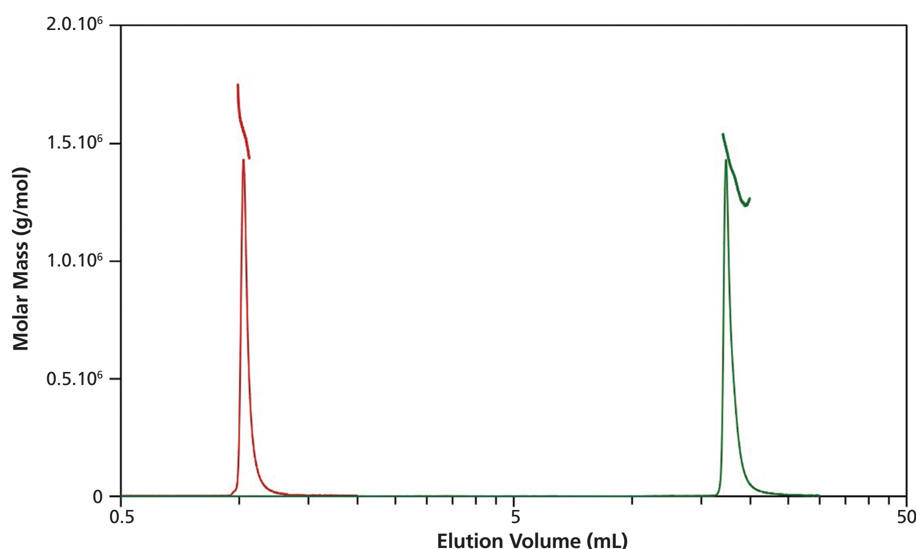
PMMA, on the other hand, elutes quite differently from linear polystyrene because of branching. As shown in **Figure 2(b),** the molar masses determined by MALS for these nominally "unknown" samples do not align nicely with each other as for the polystyrene standards, and each peak is quite polydisperse. Nevertheless, MALS provides the full picture in terms of correct, accurate molar masses and distributions across the entire range from hundreds to millions of g/mol.

"Not only is it essential to validate a new technique against standards, it is also important to compare it against the old technique."

SEC-MALS and UHP-SEC-MALS

Not only is it essential to validate a new technique against standards, it is also important to compare it against the old technique. For decades, SEC-MALS has been the gold standard for evaluating polymer molar mass and size. While current UHP-SEC-MALS instrumentation does

Figure 3: SEC-MALS (green, late eluting) and UHP-SEC-MALS (red, early eluting) chromatograms of a 1.5M MW polymer, both at 0.5 mL/min. MALS analyses of weight-average molar mass and z-average rms radii indicate no degradation on the UHP-SEC columns compared to standard GPC columns.



not cover the entire size range of standard SEC-MALS (MW: 200–10^9 Da, rms radius: 10–500 nm), it does cover the entire range of UHP-SEC columns (MW: 200–10^6 Da for linear polymers and higher for branched polymers, rms radius: 10–50 nm). Cross-validation has consistently shown excellent agreement between SEC-MALS and UHP-SEC-MALS.

The comparison is particularly illuminating in light of the concerns that have been raised about the potential for shearing polymers under high pressure on a tightly packed UHP-SEC column. **Figure 3** presents the analysis of a 1.5 MDa polystyrene sample, characterized by SEC-MALS (green, late-eluting chromatogram) and UHP-SEC-MALS (red, early eluting chromatogram), both at flow rates of 0.5 mL/min. In both analyses the weight-average molar masses came out to precisely $(1.56 \pm .01) \times 10^6$ g/mol, and the *z*-average rms radii were nearly identical at 63.0 and 61.5 nm, respectively. We could therefore conclude that no shearing occurs, at least under these conditions. Potential for shear at higher flow rates remains to be tested.

Nonlinear Conformation—Not An Obstacle

Polymers may take on a variety of conformations, including random coil, branched,

Figure 4: (a) The elution behaviour of stiff, rod-like epoxy (EP) versus linear polystyrene (PS) or slightly branched PMMA by UHP-SEC-MALS. At each elution volume, the molar masses of PS and PMMA are quite similar while that of EP is significantly lower, a consequence of its stiff, rod-like conformation. (b) UHP-SEC-MALS analysis of two epoxy resins samples. While one sample exhibits quite distinct low-molar-mass peaks, the molar masses overlay perfectly along the chromatogram, indicating that the two samples possess the same conformation.

(a)
PS
PMMA
PMMA
Molar Mass (g/mol)
1.10^6
1.10^5
1.10^4
10^3
100
1
1.5
2
2.5
3
3.5
Elution Volume (mL)

(b)
Molar Mass (g/mol)
1.10^5
1.10^4
1.10^3
100
10
1.25
2.25
2.75
3.25
Elution Volume (mL)

Table 1: Molar mass moments and polydispersity values along with experimental uncertainties (precision) for two epoxy resins, each with two replicates. The agreement between subsequent runs is quite good.

	Mn (kDa)	Mw (kDa)	PD (Mw/Mn)
EP1(001)	1.55 ±0.02	3.26 ±0.01	2.10 ±0.03
EP1(002)	1.55 ±0.02	3.25 ±0.01	2.10 ±0.03
EP2(001)	3.58 ±0.07	7.90 ±0.02	2.21 ±0.04
EP2(002)	3.47 ±0.06	7.96 ±0.02	2.30 ±0.04

and rod-like. Each conformation presents a different relationship between molar mass and hydrodynamic volume, and therefore a different relationship between molar mass and elution volume. Unlike PMMA or polystyrene, some epoxy resins are stiff and elongated, similar to rigid rods. Their elongated shape produces a comparatively large hydrodynamic volume and they therefore elute much earlier for a given molar mass than either of these macromolecules.

Figure 4(a) presents three UHP-SEC chromatograms corresponding to these three polymer types along with the molar masses determined by MALS. While this particular PMMA sample is not heavily branched and therefore its molar mass–elution volume relationship diverges only slightly from that of polystyrene, the epoxy resin (EP) is clearly distinguished from the other two. At each elution volume, EP exhibits a molar mass that is about 40–50% lower than the corresponding polystyrene species. The discrepancy in elution behaviour is a direct consequence of the differing conformations. While standard analytical SEC would not recognize the discrepancy, it is highlighted and revealed immediately by MALS and the true molar mass values are determined.

Figure 4(b) presents the chromatograms and overlaid molar mass values determined for two EP samples. The excellent matchup of molar masses along the chromatogram indicate that these resins possess the same conformation, even though they span different ranges and exhibit quite different distribution shapes. The moments and polydispersity values presented in **Table 1** indicate very good repeatability for each sample.

One of the most challenging tasks for polymer characterization is the analysis of copolymers. Since no well-characterized, narrow reference standards exist for these quite heterogeneous complexes, analysis by SEC with column calibration is impossible.

Triple-detection combining MALS, ultraviolet (UV), and differential refractive index (dRI) is the most common method for analyzing copolymers (4). The combination of two distinct concentration signals is sufficient to determine the copolymer ratio and weight-average *dn/dc* value, which are then plugged into the light scattering equation (Equation 1) to determine the molar mass of each component in the complex as well as the overall molar mass. All

the key components of a triple-detection system for copolymer analysis are readily available and integrate well in a UHP-SEC format. Hence the standard techniques developed for SEC-MALS are readily extendible to UHP-SEC-MALS.

Experimental

The data presented here were acquired using an Acquity UPLC system with APC 125 and 450 Å SEC columns (Waters Corp.), a μDAWN MALS detector (Wyatt Technology Corp.), and an Optilab UT-rEX refractive index detector for UHPLC (Wyatt Technology), and were analyzed with the ASTRA software (Wyatt Technology). Typical conditions included 4–20 μL injections and 0.5–1.0 mL mL/min flow rate. Additional tests have shown that the elution volume of any given species may vary with flow rate, but molar mass and size as determined by light scattering do not, over a range of flow rates from 0.05 μL/min to 2 mL/min.

Conclusions

UHP-SEC provides many benefits for the characterization of synthetic polymers and other macromolecules ranging in size from hundreds to millions of g/mol. However, these can only be fully realized with the addition of an online light scattering detector for absolute determination of molar mass and size to overcome the inherent limitations of SEC, which may be exacerbated in the context of UHPLC. In addition, UHP-SEC-MALS characterizes complex molecules, such as copolymers, branched, or elongated polymers, that do not possess available reference markers. Light scattering is therefore an essential tool for analytical, process development, and quality control laboratories implementing UHP-SEC.

References

1. P.J. Wyatt, *Analytica Chimica Acta* **272**, 1–40 (1993).
2. For brief overview of light scattering as applied to macromolecules and size-exclusion chromatography, see http://www.wyatt.com/Theory
3. V.H. Hsieh and P.J. Wyatt, *Scientific Reports* **7**, 10030 (2017).
4. S.M. Rowland and A.M. Striegel, *Analytical Chemistry* **84**(11), 4812–4820 (2012).

This is an edited and updated version of an article that was originally published in LCGC The Column, 14 (16), 24–28 (2017).

Daniel Some, PhD is Principal Scientist at Wyatt Technology Corporation, where he has spent the last 13 years in various roles including R&D, software development, product management, and marketing. Some was previously involved in the development of light-scattering-based patterned wafer inspection tools for the semiconductor industry.

E-mail: dsome@wyatt.com

Website: www.wyatt.com

“Don’t you know of any other separation techniques?”

The unique combination of FFF-MALS-DLS

Moses made separating the Red Sea look easy enough, but if you’re trying to separate your macromolecules or nanoparticles with conventional chromatography—*and it isn’t working*, maybe it’s time to look at field-flow fractionation (FFF)—an alternative separation technology that has no stationary phase. We’ll show you how FFF combined with multi-angle and dynamic light scattering (MALS-DLS) can determine absolute molecular weights and particle sizes with no column calibration or reference standards.

Learn more at www.wyatt.com/separation

WYATT
TECHNOLOGY