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International Light Scattering Colloquium 2008: Molecules Happen

Light scattering has a long history, dating back to Lord Rayleigh (1842–1919) (Nobel Laureate, Physics, 1904), who studied the elastic scattering of light by particles smaller than the wavelength of incident light. **Wyatt Technology Corp.** (Santa Barbara, CA) was founded over 26 years ago to design instruments for light scattering. The company is proud of its history of developing instruments that it refers to as "an evolution of intelligent design!" Indeed, the American Physical Society awarded its 2009 Prize for Industrial Application of Physics to Dr. Philip J. Wyatt, Founder and CEO of **Wyatt**, with the citation:

> For pioneering developments in the physics of the inverse scattering problem: new applications of laser light scattering and the successful sustained commercialization of new related analytical methods and instrumentation.

Few would argue with this characterization of the company's continually evolving instrumentation. Today, **Wyatt** instruments are used in the laboratories of at least five Nobel Laureates and a dozen additional members of the National Academy of Sciences. For the last 19 years, **Wyatt Technology Corp.** has sponsored the International Light Scattering Colloquium, which is focused on the technology and applications of light scattering. This year, about 75 scientists congregated at the 19th Annual International Light Scattering Colloquium, October 20–21, at the exclusive Bacara Resort in Santa Barbara, CA, to talk shop. And, there was a lot to talk about.

The basics

Classical light scattering involves measuring the intensity of light scattered as a function of the angle, hence the name, multiangle light scattering, or MALS. For macromolecules, and especially proteins and related biologicals, the MALS measurements produce the molecular mass, root mean square (RMS) radius (aka "radius of gyration"), and second viral coefficient. The latter is a measure of the molecule–solvent interaction and its consequences. A negative number indicates propensity of the suspended molecules to aggregate. Dynamic light scattering is a different measurement, recording the time-dependent light fluctuations resulting from particles undergoing Brownian motion within the solution. The output provides the diffusion coefficient of the molecules/particles, which is related to an associated hydrodynamic radius.

Some applications

Dr. John Philo, cofounder of **Alliance Protein Laboratories** (Thousand Oaks, CA), described several case studies he had worked on over the last 10 years. Although he fogged the details to honor confidentiality agreements, his lecture pinpointed many of the problems caused by aggregates and uncharacterized particles in biotherapeutics. First he discussed aggregates of the product, but later pointed out that particulate contamination can be even more worrisome. It seems that nearly all formulations have a background of particles that are present and uncharacterized. Most are benign, but some could be nucleation sites, or even catalytic. And since they are uncharacterized and uncontrolled, who knows what evil lurks in that vial? While the regulators and regulated seem to recognize the importance of measuring and understanding biotech product aggregates, they seem much less concerned with particulate contamination. Fortunately, suitable instrumentation exists for detecting these particles. In addition to its MALS systems, whose unique sensitivity permits the detection of even a single particle, **Wyatt's** dynamic light scattering instruments, such as the DynaPro™ Titan and DynaPro Plate Reader, quickly show the presence of particle populations, even in fresh formulations. Some of the larger ones may be removed by short spins on a centrifuge.

Dr. Philo explained that product aggregates are often degradation products that decrease potency and shelf-life while increasing the potential for increased immunogenicity. The latter is a particular problem since it can be individual specific. Often aggregates are visible, such as the "snow" that appears in some formulations after a few months. While annoyingly visible, this flocculent precipitate is usually much less than 0.1% of the active material dose. Even if you cannot see an aggregate, it can be there. MALS is certainly the most sensitive means for detection, but dynamic light scattering (DLS) is very good and often easier to perform. However, if the concentration of degradation products is high, it may be hard to disentangle the contributions from the various fractions, which can be confusing at best. However, if you couple DLS to size exclusion chromatography (SEC), you can get around this problem.

Several lectures reported on the use of light scattering in basic research.

He explained that aggregation mechanisms are often very complex, involving nucleation-controlled mechanisms with long induction phases. During the induction phase, the concentration of degradation products slowly increases in quantity and size, and also may involve making intermediates. Eventually, the induction phase runs its course, leading to the formation of visible precipitates (aka snow, floaters, or white amorphous material [WAM]).

Next, Dr. Philo described results obtained by his consulting laboratory over the years using SEC with multiple detectors in series. The most popular combination is MALS plus UV and RI. This enables measurement of the mass and concentration of the protein and mass of the glycoconjugate. SEC provides the fractionation. In one example, he found that the proteins in the aggregate peak were much less glycosylated than the main product peak.

A flat mass line from the MALS detector is usually taken as proof of peak purity. Occasionally one sees a wiggle at the leading or trailing edge, particularly if the concentration is high. Rerunning the experiment at a lower concentration provides a flat line. Thus, the wiggle is the signature of reversible and rapid association and dissociation. Here is Dr. Philo's take-home message in three parts: 1) No one analytical method covers the entire range of aggregate types and sizes, 2) DLS is useful in detecting trace amounts of aggregates and contaminating particles, and 3) our ability to detect and characterize aggregates exceeds our knowledge of how these affect product safety and efficacy.

Dr. Mark Witmer of **Bristol-Myers Squibb** (**BMS**, Princeton, NJ) provided highlights of his 10+ years of experience with DLS and SEC-MALS. He is currently in the Gene Expression and Protein Biochemistry Group at **BMS**, which is early in drug discovery. DLS plays a central role in the laboratory, since changes in molecular size are useful metrics of a reaction. DLS provides the hydrodynamic radius, provided the viscosity is known via the Stokes–Einstein equation. In one case study, he used DLS to characterize vesicles produced by extrusion of phospholipids through a polycarbonate membrane. The size distribution of vesicles needed to be determined since the activity of the enzyme (cytosolic phospholipase A2) seemed to be higher with larger vesicles. The relationship was confirmed: 25-nm-diameter vesicles showed low activity and also rapidly fused in the presence of 10 mM Ca^{++}. In contrast, the 400-nm vesicles maintained activity longer and were more active.

Light scattering is beautifully noninvasive.

A second example focused on characterizing a protein (BACE-1) produced using *E. coli*, Chinese hamster ovary (CHO) cells, and S2 insect cells. The product from *E. coli* was the smallest since it was not glycosylated. CHO cells produced a glycoproduct that was larger and had a broader molecular width distribution, due to the range of glycol forms. SEC-MALS and SEC-DLS were compared. The results were within about 10% of each other. For the nonglycosylated proteins, the mass derived from the DLS radius measurement was very close to the actual mass of 50.03 kDa. The mass of the glycoforms was not independently verifiable. However, DLS was able to show that the polydispersity was less than 10%. The study was extended to measure the binding of several peptides to BACE-1. They were found to be noncompetitive, indicating different binding sites, but other tests suggested that they were useful therapeutically.

Perhaps Dr. Witmer's most disturbing report was that peptide solutions are frequently not what they seem. One can prepare a solution that does not demonstrate visible signs of aggregation, yet DLS will show that this is not the case. He recommends using isothermal calorimetry, analytical ultracentrifugation, circular dichroism, and surface plasmon resonance (SPR) in combination, especially if peculiar behavior is observed. For example, SPR binding plots have a normal signature pattern. If this is not observed, then look at the analyte solution for aggregation. His point, which was well taken, is that the more independent characterization methods one has, the more confidence one has in the results.

A further example reported how DLS was instrumental in explaining peculiar results obtained from the testing of 17 drug leads that were classed as high binders to human serum albumin (HSA) from equilibrium dialysis assays. When submitted for SPR assays, the result gave nontraditional binding results, including stoichiometries. Assay of the drug stock solutions with DLS and long wavelength scattering showed that the drugs were aggregates with RH >100 nm, yet with no visible signs of precipitation, etc. The large size in solution prevented passage through the dialysis membrane, which had been mistakenly attributed to high binding with HSA. Dr. Witmer concludes that DLS is a central technology in his laboratory for the evaluation of large and small biomolecules.

Basic research: Protein folding for proteostasis

Several lectures reported on the use of light scattering in basic research. Prof. Jeffery W. Kelly of Scripps Research Institute (San Diego, CA) opened with a lecture entitled, "Maintaining Protein Homeostasis or Proteostasis for Healthy Aging." Protein folding is a key event in proteomics since the protein takes a snakelike form and arranges into a specific shape that is biologically active. Although folding can occur in vitro, it is much too slow and inefficient to support function. Specifically, the rate of formation of disulfide bonds that pin together many proteins is much too slow. He feels that protein folding is an underappreciated step in the central dogma of molecular biology. Over evolutionary time, life has developed a complex system for maintaining proteostasis. Proteins are synthesized and folded into active enzymes. Mistakes are detected and destroyed, as is the protein after it is no longer needed. Various signaling pathways have evolved. Small mistakes in signaling arising from genetics, age, or stress can perturb the signals, leading to misfolding, which leads to proteotoxicity, itself manifest as one of the degenerative diseases such as Alzheimer's and Parkinson's. Prof. Kelly sees the opportunity to restore proteostasis by focusing on the signaling molecules that control the concentration, conformation, quaternary structure, etc. Light scattering is used as the primary readout since aggregation of natural proteins is believed to be the mode of action. The number of nucleation sites can be very small (single-digit nanogram range) but, once formed, the protein mass is impervious to proteolytic degradation and clearance. It will continue to grow as long as it has the nutrients.

Although the role of folding is certainly appreciated by many researchers, the problem is that there are not many analytical techniques that can monitor folding without tagging, which can affect the very process one wishes to monitor. Light scattering is beautifully noninvasive.

Engineering a blood substitute

Prof. Andre F. Palmer of Ohio State University (Columbus) described the key role that light scattering plays in his work in developing a hemoglobin-based oxygen transporter for use in transfusion medicine. The problem with hemoglobin (Hb) is that it is able to pass around the epithelial cells lining the vascular surface to the next layer of cells that are involved in maintaining blood pressure with the NO pathway. Hb reacts with NO, which causes constriction of blood vessels, leading to high blood pressure. Prof. Palmer's approach is to increase the size of Hb to keep it confined by the epithelial cells. Encapsulating within liposomes is one promising approach. However, liposomes are fragile and often have a short half-life in the body. He proposed strengthening the liposome with an actin network. This was characterized with a combination of asymmetric flow field flow fractionation with MALS detection.

Virus-like particles

A poster from Jason Payne and Jeff Allen of **Dow Chemical Co.** (San Diego, CA) reported using SEC plus MALS to characterize the stability of virus-like particles in three candidate buffers. The particles are used in Pfenex Expression Technology™ (**Dow**). The coat protein is derived from Cowpea Chlorotic Mottle Virus, which has 180 subunits. The particle is about 25 nm in diameter, which is similar to the wild type. Pfenex is designed as a carrier for peptide-based vaccines and immune stimulation. The storage buffers provide very good stability with two of the three buffers for 30 days or more at 4 °C.

Credits

In his opening remarks, Prof. Kelly thanked **Wyatt Technology Corp.** for creating the remarkable instruments that give such fine and consistent results so important for the country's ongoing research efforts. Clearly the firm is doing well. It has catalogued over 4100 articles citing the use of its instruments. More than 900 of these were added in the past 12 months alone. Just before the meeting, I read a report that the company was chosen as one of the top four places to work. This shows up in the **Wyatt** team. They are sharp, proud, and friendly. All deserve credit for organizing a stimulating meeting on what could have been a rather esoteric subject. However, each speaker avoided this trap by relating the work to the real world. Congratulations to all.

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