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Highlights from the 21st Annual International Light Scattering Colloquium

The new world of very large molecules requires advanced analytics, particularly for structures with a molar mass larger than one million daltons extending up to particles with diameters of 1000 nm. Many interesting biologicals and nanotech objects fall into this range. At the 21st Annual International Light Scattering Colloquium, held in Santa Barbara, CA, October 18–19, 2010, the current state-of-the-art was illustrated in the opening lecture by Dr. David Narum of the NIAID Laboratory of Malaria Immunology and Vaccinology (Rockville, MD). His laboratory is using a range of techniques, including size exclusion chromatography-multiangle light scattering-quasielastic light scattering (SEC-MALS-QELS), atomic force microscopy (AFM), analytical ultracentrifugation (AUC), and gel electrophoresis to develop vaccine leads for malaria.

Dr. Narum noted that, after centuries of neglect, fighting malaria is finally drawing attention from governments and large funders such as The Gates Foundation. Globally, malarial infections kill over a million people per year. The infection cycle consists of three distinct stages, each offering a possible vaccine target. Since each stage has been studied, some basic information is available to guide rational vaccine design and development. Examples included two large rod-like malarial proteins that were targeted based on their ligand receptor interactions. None of the candidate vaccines was particularly effective. Efficacy improved significantly when candidates affecting two stages were combined. This was attributed, at least partially, to differences in the infection stage of the patient cohort. A vaccine against the pre-erythrocyte stage is not effective with subjects who are already infected. For late-stage patients, the vaccine target is to block retransmission back to the mosquito to prevent further spread.

The development of chemistry is a mixture of fads and particularly opportunism. New technologies make it easier to explore areas that were previously difficult. Progress is especially intense when two or more new technologies share a common focus. This is happening today with the confluence of nanotechnology, biotechnology, and light scattering, as described in various lectures and posters at the meeting.

Drug conjugates

Several lectures focused on developing drug conjugates, many of which are generally larger than one million daltons. Light scattering is the technology of choice following the preparation. Researchers in the laboratory of Prof. Roger Tsien* of the University of California (San Diego) used light scattering to follow the development of smart chemistry to extend the use of Doxil® (**Ortho Biotech**, Raritan, NJ) as a chemotherapeutic. Doxil is a pegylated liposome containing doxorubicin as the active pharmaceutical ingredient (API) distributed by **Johnson & Johnson** (New Brunswick, NJ). First, adding sugars to the surface improved solubility.

While pegylation improves the duration of doxorubicin in the body, it does little to enhance specificity. To improve the specificity, the surface of the liposome was modified. Polycations on a particle's surface can trigger endocytosis. Also, metastatic tumors often have high levels of proteases, since these are necessary for the cells to leave the primary site and enter the distal tissue. With these facts in mind, the researchers constructed a hairpin of a block polymer consisting of a polyanion region bonded to a polypeptide linker segment linked to a polycation segment and finally a tether group for coupling to Doxil. The idea is when the modified Doxil is near a tumor site, the high protease activity associated with the metastatic process would cleave the hairpin at the polypeptide linker. This would free the polyanion and expose the tethered polycation. Free polycations would trigger endocytosis, taking the doxorubicin into the cell, where it interrupts DNA duplication and ultimately cell death. This chemistry appears to reduce solid tumors in mice, but not consistently.

Dr. Richard Vandlen of **Genentech, Inc.** (South San Francisco, CA) also sought to improve specificity, but with antibody drug conjugates (ADCs). Antibodies provide exquisite targeting ability, which takes care of tissue or organ targeting, and the drugs can do their job at a potentially much lower dose. This is important since many drugs have very narrow therapeutic windows. But with antibodies, there are many ways to slice and dice them, often producing unexpected results. Throughout the process, one needs good analytics to follow the synthesis and subsequent metabolism. Since antibodies are relatively large, dynamic light scattering is frequently used to determine their average size since, for most SEC separations, they may remain unseparated within the exclusion volume of a size exclusion chromatogram. One case study showed delivery of a cytotoxic ADC to a tumor cell; another delivered siRNA to silence expression of a particular gene.

The new world of very large molecules requires advanced analytics.

Transmembrane cell signaling often involves the close association of two or more signal transmitters on the exterior of the cell that assemble transmembrane proteins in close proximity. The transmembrane proteins then attract and arrange the receiver proteins in the cell's interior. The key is that only when the right proteins on the exterior are in close proximity is the signal transmitted to the interior of the cell. Dr. Vandlen pointed out that antibodies have divalent hypervariable regions closely joined at the hinge. Could one make a conjugate drug that has two different signaling proteins at the

*Prof. Tsien was awarded the 2008 Nobel Prize in Chemistry for his contributions to the development of green fluorescent protein (GFP).

end of the Fab region of the antibody and thus deliver an engineered message (turn the process on or off) to cells? This was confirmed by sequentially joining two different Fab arms with a bis-malimido crosslinker near the distal end. One simple study involved a 4 × 4 matrix of bis-Fab fragments usually associated with inhibition. Surprisingly, it was found that a few of the constructs were agonists, rather than antagonists. What is going on? Dr. Vandlen did a bit of hand-waving to explain the phenomenon. The required contortions seem to be difficult; however, the data are irrefutable.

Mr. Cliff Entrican of **Pfizer** (Andover, MA) investigated the influence of conjugation with polyethyleneglycol (PEG) on the pharmacokinetics of the associated protein conjugate. PEG is available in a variety of forms and sizes. In addition, the structure can be varied from linear to branched. The branched PEGs are also available as isomers. Using SEC-MALS, combinations of a drug with various PEGs were evaluated. This led to the determination that a tri-PEG construct was superior to all other options. SEC-MALS showed that the branched structures were significantly more compact than the conjugates with linear PEGs. Pegylation of any sort did not affect the pharmacokinetic profile, but proximity of the drug conjugate and PEG was quite important for in vivo use.

Protein folding

Prof. Christopher M. Johnson of the Center for Protein Engineering & Laboratory of Molecular Biology (Cambridge, U.K.) described fluctuation correlation spectroscopy (FCS) with a particular emphasis on protein folding. FCS probes the conformation and movement of large molecules. It is particularly useful in measuring the interaction of large molecules, such as proteins, with DNA. Experimentally, a small chip processes the signal to provide the cross-correlation of a noisy signal with itself, which improves the signal-to-noise ratio. Typical determinations of the method include the diffusion time constant and the number of molecules within the viewing volume. Because of diffusion, signals fluctuate on a millisecond time scale. Faster fluctuations arise from intrachain movement. Quenching of fluorescence resonance energy transfer (FRET) and other techniques can also provide measures of relative movement, even with single, large molecules. In contrast, QELS requires higher concentration, which is often precluded by induced self-association effects. Nevertheless, in favorable cases, there is a strong correlation between the hydrodynamic radius (R_h) determined by both FCS and QELS.

Prof. Johnson described the use of FCS to measure protein folding in solution. Typically, native proteins occupy only about 30% of the volume of an unfolded protein in denaturing conditions (8 M GdmCl [the hydrochloride salt of guanidine]). Since FCS can measure R_h with an uncertainty of 0.05 nm or less, it is practical to follow the dynamics of folding through three stages: intrachain diffusion (faster than 1 µsec, ultrafast folding ($\tau = 10^{-6}$ to 10^{-4} sec), and molecular diffusion (τ slower than 10^{-4} sec). His model suggests that protein folding is driven by burial of hydrophobic patches within the interior of the protein. Hydrogen bonding along the peptide backbone is probably also important, but less so than hydrophobic burial. Prof. Johnson went on to discuss the formation of p53 tetramers and the determination of the structure of a small protein called SepF. Because this protein did not crystallize, no crystal structure was available. The shape factor from SEC-MALS was 1.1, which is very high. The value for a sphere is 0.77. The large value was attributed to an open ring structure, which was subsequently confirmed with electron microscopy.

Protein folding, aggregation, and particulates were mentioned by other speakers as well. Dr. Linda Narhi of **Amgen** (Thousand Oaks, CA) discussed the use of light scattering during development of protein therapeutics. In the initial discovery stages, assays are used for screening a large number of compounds for biological properties. Generally, at the beginning, expediency is favored: Usually qualitative or simple rank ordering is sufficient. But once the decision to proceed with development is made, the game changes to include quantitative assays, except for formulation studies, which initially still resemble discovery assays.

Aggregation (both homo- and hetero-) is a real issue. Sample stress, including heat, cold, freeze-thaw, pH change, and subvisible particles, are all potential factors. In one study, candidate protein drugs were subjected to a transitory pH cycle. Some showed irreversible changes and were dropped from the candidate list. Viscosity is another important consideration. Proteins and excipients with a high viscosity generally do not process well. A similar report by Dr. Tia Estev of **Biogen IDEC** (San Diego, CA) discussed the use of differential scanning calorimetry, dynamic light scattering, and other techniques to elucidate the role of conformation and colloidal stability on aggregation rates.

Dr. Narhi also pointed out the need for improved analytical technology, especially for particles/molecules in the range of 10 nm to 10 µm. She mentioned using asymmetric flow field flow fractionation (A4F)-MALS-UV for characterizing an engineered antibody. The main product peak showed a slightly sloping MALS plot across the UV trace of the product peak, but this was followed by a large "lumpogram" of very heterogeneous materials that were attributed to aggregation phenomena. Remember that the elution order in FFF is small to large, which is just the opposite of SEC.* She reports that the main problem with FFF is detection sensitivity.

As in the past, the quality of the technical presentations was better than excellent.

Heparin: Three years later and still a problem

Do you recall the heparin problem from early 2008? Heparin was introduced as an

*The elution order for FFF depends on the separation mechanism. In the lower size range, generally less than 500 nm in diameter, the particles are separated as a result of diffusion effects, with the smaller diffusing further from the accumulation wall than the larger particles. However, depending on the established flow conditions, an inversion condition may develop, wherein the separation mechanism changes to the steric mode. In the steric mode, the particles are confined closely to the accumulation wall with the larger protruding further into the channel flow stream. The larger particles are thus swept ahead of the smaller that remain closer to the accumulation wall. This changes the elution order with the larger particles leading the smaller. The exact flow condition for such reversal may also be achieved by changing the temperature and thickness of the separation channel.

anticoagulant in 1935. Despite the huge advances in analytics during the intervening 75 years, little was done to bring the assay methods up to date, even when the source was switched from bovine to porcine in the 1990s as a result of concerns about bovine spongiform encephalopathy (BSE). China grows about two-thirds of the pigs in the world. One pig intestine produces one dose of heparin. When the swine flu or blue ear epidemic hit, there were contracts that needed to be filled, but pig supply was insufficient. Adulterants were substituted, but these were toxic to a few humans. Efficacy and safety were compromised, and the FDA became involved.

Dr. Ed Moore of **Baxter** (Deerfield, IL) discussed the new analytics developed for heparin over the last two years. These may be included in a new USP monograph due in 2011 or, at the latest, 2012. Since larger heparin is more active, he advocates that the characterizations include SEC-MALS. This gives the molecular weight distribution. SEC alone is simpler experimentally and less expensive, but less reliable. The question for the USP is: Is better analytics worth the money?

Refractive index detector

For the past 40 years, advances in detection technology applied to refractive index (RI) detectors have been evolutionary at best. Legacy beam deflection detectors used a split photodiode to measure small changes in beam position caused by changes in RI in the sample cell. The T-rEX™ from **Wyatt** (Santa Barbara, CA) uses a 512-element photodiode array and inboard computer for an 80-msec response time. The 512-element array means that the light beam is focused somewhere on the array. There is no need to hunt for and adjust the beam position manually. The optics also breaks the light beam into 10 parallel beams. These are averaged on the array, further reducing the noise. The result is an improvement in range and signal to noise ($\pm 7.5 \times 10^{-10}$ RIU) (noise measured by ASTM-E1303-95 [2000] with a 4-sec time constant). The light source is a high-powered heterojunction light-emitting diode (LED). The service life of LEDs is legendary, but should the need occur, replacement requires only a few minutes. One attractive option is to connect the detector to **Wyatt's** Orbit™ to recycle the mobile phase at the end of the sample set. The instrument will recycle mobile phase until the next set of samples are ready. Since the flow system has stabilized, the samples can be run immediately. A side benefit is the reduction of wasted mobile phase. The T-rEX is a new benchmark in RI detection.

Update on mobility instrument

In an example of improved analytics for the 1 nm to 10 µm particles, **Wyatt** introduced the Möbiuζ™ mobility instrument, which uses a 50-mW solid-state laser to illuminate a small (5, 45, or 170 µL) flow cell. To measure electrophoretic mobility, a low-voltage square wave is applied across the flow cell. The electrophoretic mobility is recorded as a positive or negative Doppler shift of the detector. With appropriate math, the zeta potential and molecular charge are calculated. In contrast to more conventional techniques for measuring electrophoretic mobility, the analyte is not subjected to high voltage, which can denature proteins. The instrument is easily used to measure mobility as a function of pH. In this manner, the isoelectric point may be determined at the pH where mobility is zero. With the optional **Wyatt** QELS, the Möbiuζ can report also the hydrodynamic radius by using the backscattered light to measure the translational diffusion coefficient.

Credits

The 21st International Light Scattering Colloquium attracted about 90 scientists to The Four Seasons Resort, The Biltmore in Santa Barbara, CA, for an intense, two-day update on advances in analytics for the nanoworld, with a particular focus on light scattering technology. As in the past, the quality of the technical presentations was better than excellent. The premium location is a proper setting for truly a high-quality agenda. **Wyatt** deserves special credit for setting the delightful tone of the meeting. Ms. Lindsey McGowan certainly deserves recognition for organizing and then managing the logistical portion. This allowed the delegates ample opportunity for conversations/discussions with each other, as in the narrow meaning of "colloquium." Please monitor the **Wyatt** Website http://wyatt.com/ for news on next year's meeting.

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