

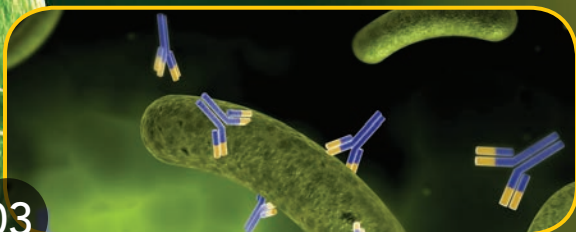


ADVANCING BIOPHARMA ANALYSIS WITH LIGHT-SCATTERING DETECTION

JUNE 2015

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03



BACKGROUND

The Use of Light-Scattering Detection with SEC and HPLC for Protein and Antibody Studies, Part I: Background, Theory, and Potential Uses

Simion Kreimer, Anurag S. Rathore, and Ira S. Krull

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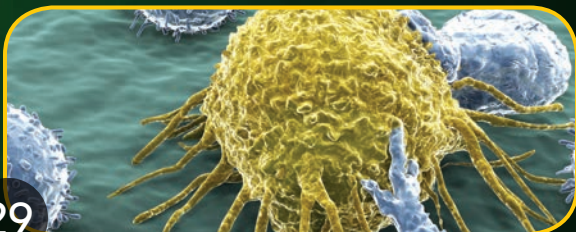


EXAMPLES

The Use of Light-Scattering Detection with SEC and HPLC for Protein and Antibody Studies, Part II: Examples and Comparison to Mass Spectrometry

Simion Kreimer, John Champagne, Anurag S. Rathore, and Ira S. Krull

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TOOLKIT

Characterizing Antibody-Drug Conjugates for Biotherapeutics with the Light-Scattering Toolkit

Chris Broomell

The Use of Light-Scattering Detection with SEC and HPLC for Protein and Antibody Studies,

PART I: BACKGROUND, THEORY, AND POTENTIAL USES

S. Kreimer, Anurag S. Rathore, and Ira S. Krull

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SEC-MALS of proteins



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The technique of SEC-MALS



The use of light-scattering detection for the characterization of proteins and antibodies is increasing. Examples of applications include determining absolute molecular weight, studying PEGylated proteins, characterizing protein–drug and antibody–drug conjugates, and studying protein aggregation. This two-part article explains how light scattering works and assesses its capabilities for biotechnology applications.

Light-scattering (LS) detection, particularly multiple-angle light scattering (MALS) is an important tool for the characterization of proteins and antibodies, and in a number of applications, its performance exceeds that of mass spectrometry (MS) detection. Here in part I of this two-part article, we outline the development of light scattering in biotechnology, summarize its current uses and advantages, and explain the theory of how light scattering works. In part II, we provide a few detailed examples of its use and discuss how its performance compares to MS and other methods.

History of Light Scattering

In 1974, Ouano and Kaye ushered in a new era for macromolecular characterization with the first use of on-line light-scattering detection (1,2).

Despite the intrinsic appeal of this concept, however, many improvements in both the chromatography and detection were required for further implementation with relative ease. Important developments included the detection and quantitation of branching, precise measurement of the polydispersity of so-called “standards,” conformation plots, precise extraction of M-H-S coefficients by combining light-scattering and viscosity measurements with reversed-phase chromatography to detect and quantify multimeric formation, field-flow fractionation (FFF) for an alternative separation combined with light scattering, temperature rising elution fractionation (TREF) measurements, capillary electrophoresis (CE) combined with light scattering, and the quantitation of aggregation phenomena and microgel formation.

Light Scattering in the Study of Biopolymers

In the mid-1980s there were few, if any, applications of chromatography combined with any form of light-scattering detection for the study of biopharmaceuticals described in the literature (3–6). This was true even for conventionally derived proteins from

natural sources (that is, not recombinant proteins). Most detectors used in tandem with size-exclusion chromatography (SEC) or high performance liquid chromatography (HPLC) for biopolymers were ultraviolet (UV), differential refractive index (DRI), photodiode array (PDA), ultraviolet–visible (UV–vis), or fluorescent. Back then, MS was not yet conveniently interfaced with any form of SEC or HPLC. There was no general usage of electrospray ionization (ESI) or matrix-assisted laser desorption–ionization–time of flight (MALDI-TOF) MS developed or applied for biopolymers. Most vendors of light-scattering instrumentation had not yet developed instrumentation or applications aimed at the nascent, but rapidly growing biopharmaceutical industry. This situation would rapidly change.

Our own light-scattering work (Krull and colleagues) in those days was supported and encouraged by a firm known as Laboratory Data Control/Milton Roy (LDC/MR), in Florida, which no longer exists. This company was involved in both HPLC and light-scattering instrumentation, but it manufactured only a low-angle laser light scattering (LALLS) instrument and an off-line, differential refractometer for measuring the differential or incremental refractive index. Both instruments used a 633-nm laser. Of course, LALLS had been used for synthetic organic polymer characterizations but there were very few, if any, applications in the literature for using LALLS or other light

scattering, with biopolymers separated using HPLC or SEC (3–16). In our initial discussions with the management of LDC/MR, we proposed to study such new and novel applications, and to bring LALLS/DRI into a more common usage for biopharmaceutical proteins, antibodies, and related analytes. Herb Kenny and colleagues were influential in bringing about this collaboration, which lasted several years. Eventually LDC/MR was purchased by Thermo Electron Corporation, and the light-scattering product line was subsequently abandoned. Other vendors picked up the slack of course, and today there are at least two major companies offering such instrumentation with numerous applications for biopharmaceuticals. There are some other, smaller firms as well, but these do not offer the same extent of instrumentation diversity, in-house applications literature, or technical expertise.

In the late 1980s and early 1990s, Krull and others used SEC or HPLC with isocratic elution conditions interfaced on-line and in real-time with LALLS, using off-line DRI for dn/dc measurements of the protein analytes. Later, we moved to using either isocratic or gradient elution in ion-exchange chromatography (IEC), hydrophobic interaction chromatography (HIC), or reversed-phase chromatography (3–16). We also began using on-line, 633-nm DRI with UV detection (concentration detector) to obtain at least three chromatograms (light scattering, UV, and DRI). Such

approaches then provided direct light-scattering, dn/dc , and c (concentration) measurements on every injection of protein products and mixtures. With this approach, we could derive very accurate and precise measurements of molecular weights (weight-average, z -average, and n -average) but we could not yet calculate the radius of gyration (R_g) or the hydrodynamic radius (R_h). We could also obtain the second virial coefficient term (A_2) in the Zimm equation, suggesting the best solvents to use for nonaggregation or disaggregation prevention and overall stability or compatibility of the proteins with the HPLC solvents for their elution and separations. Eventually, we were able to use viscometry instrumentation, in an on-line format with the light-scattering, UV, and DRI detectors, and then derive R_h in addition to the measurements previously described. These were studies in the late 1980s and early 1990s, using instruments that are by and large no longer commercially available. However, the techniques were soon readily adopted and adapted by various biotechnology firms for their own recombinant protein or antibody products, perhaps using other commercial instrumentation and methods, but especially SEC–MALS (17).

Current Uses of Light Scattering in the Biotechnology Industry

The use of SEC–MALS and similar techniques is still growing within the biotechnology industry. The most common uses are outlined below.

Determining Absolute Molecular Weight

In general, today most applications of SEC–LS involve the determination of absolute molecular weights, as described above, plus either R_h or R_g . By absolute, we mean without reference to standards whose molecular weights are often known and used to calibrate the instrumentation. Nor does absolute refer to any technique that makes use of calibration curves such as SEC or intrinsic viscosity based on so-called “universal calibration.”

That is, light scattering can be used to determine absolute molecular weights ± 2 –3% (standard deviation) or better for individual proteins after HPLC or SEC separations, and for covalent (oligomers) or noncovalent aggregates of such proteins with one another or with their antibodies. As long as the conditions being used in HPLC or SEC do not change the nature of the protein samples through the chromatographic process, then light-scattering analysis will provide a true picture of what was in the injected sample in terms of monomer, dimer, or higher-order aggregates of the parent protein or antibody.

Of the four absolute methods for measuring molecular weights, light scattering is applicable to the greatest range of samples. The other three methods capable of absolute molecular weight determination are sedimentation equilibrium, vapor osmometry, and MS (although the latter method is not as useful if the “solution” properties

of the molecules, such as noncovalent aggregates, are being measured). For light scattering, the range of accessible molecular weights is from several hundred to tens of millions of daltons (grams per mole). Vapor osmometry measurements span a much smaller range, up to only a few hundred thousand daltons under the most favorable of conditions. The lower limit, however, can be an order of magnitude smaller. Sedimentation equilibrium, also called *analytical ultracentrifugation*, often requires several days of measurement and has a range of applicability considerably smaller than light scattering. MS has an upper limit between 500,000 and 1,000,000 Da, although today, it can be considerably higher, in the tens of millions, with special instrumentation.

Light-scattering techniques also have another interesting advantage when combined with fractionation techniques such as SEC: They can be used to determine the molecular weight distribution and various other molecular properties, as we shall see in the following sections and in part II.

Studying PEGylated Proteins

PEGylation (adding a molecule of polyethylene glycol [PEG]) is a widely used technique for modifying protein drugs to make them more water soluble and provide different pharmacokinetic properties. HPLC–LS or SEC–LS can be used to determine the nature of PEGylated proteins, or of proteins that

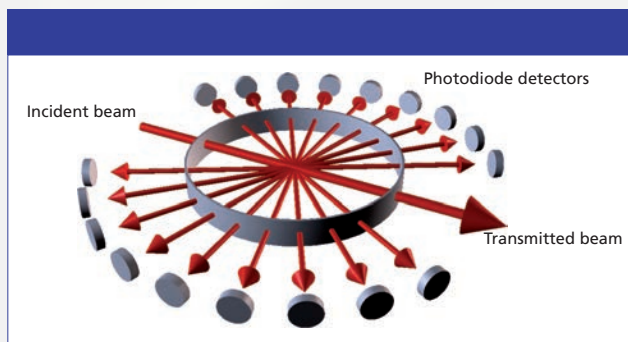


Figure 1: An array of collimated detector elements used to detect scattered light at discrete scattering angles.

have been PEGylated and perhaps then modified with another, synthetic organic polymeric reagent (17,18). It is now possible to determine the molecular weight ratio of protein to PEG in a PEGylated protein. This is also true for virtually any polymer used to modify a protein by covalent or even noncovalent steps. Electron-transfer dissociation (ETD) in MS has also proven useful for identifying the specific site and nature of PEGylation in certain peptides (19). However, this technique does not work when trying to determine the molar ratio of protein to PEG for a mixture of PEGylated proteins.

Characterizing Drug Conjugates

Another very important and growing area of biotechnology characterization relates to antibody–drug and protein–drug conjugates, which are synthetically derived combinations of an antibody or protein and a small-molecule drug against a specific disease related to that protein target. Using LS-based techniques, it is possible to measure, with a high

degree of accuracy, how much drug (or PEGylation) has been incorporated, on the average of all such species present. It cannot, however, indicate the specific points of such attachments. On the other hand, if these derivatives can be adequately resolved before MS detection, MS can indicate exactly where a drug or PEG molecule is located on a (specific) protein sequence; however, MS cannot accurately measure how much of the drug has been incorporated into all the protein molecules unless each such species can be resolved one from the next. This is yet another area where the capabilities of light scattering are superior to those of MS.

Studying Aggregation

Perhaps the most useful information for the biotech industry is that which indicates the level of aggregation present in any protein or antibody sample, as well as the specific nature of each aggregate (dimer, trimer, and so on), and the R_h or R_g of each aggregate. Another piece of information of value will be a demonstration that the aggregates being measured by SEC with LS detection were indeed present in the original sample, and that their amounts (relative percent peak areas) have not changed through the chromatographic and detection steps.

Light scattering by itself does not change the nature of aggregation in any given sample, but that may not always be true for SEC and HPLC or other modes of separation, or when using MS detection

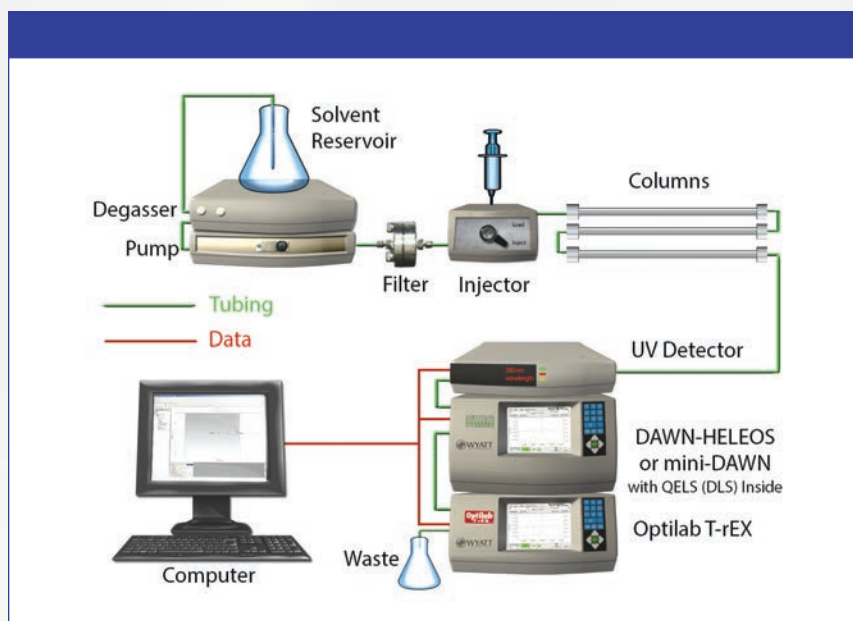


Figure 2: A typical SEC arrangement combining UV, MALS, and RI detection in series.

method. In essence, that makes all data and conclusions questionable at best and, in reality, worthless.

Even gel permeation chromatography (GPC) and SEC are known to change sample composition, especially in the case of noncovalent aggregation. (In the case of covalent aggregates, other than for disulfide rearrangements, there are usually no changes

methods. At times, SEC and MS have both been shown to change the nature of aggregates detected after initial injection into a separation-MS scheme (20–22). Furthermore, the level of aggregates could be made to increase or decrease depending on the conditions used in both the separation (SEC) and MS steps.

This, of course, is not a desirable occurrence. If an analytical method changes the original sample components, then it becomes useless in determining what was originally in the sample before analysis. Analysts must demonstrate using accepted methods that their analytical methods have not in any way altered the original samples through the analytical scheme. If this cannot be done, then one should assume the worst: that there are indefinable alterations of the sample components during that analytical

in the sample composition by HPLC or ultrahigh-pressure liquid chromatography [UHPLC] methods.) In the case of a complex mixture of synthetic, organic, or natural polymers, changes in elution times because of the particular GPC or SEC conditions being used may suggest differing molecular weights as a function of concentrations injected, which is artifactual. This concentration dependence will mainly occur when using a reference calibration method but should not occur when using an absolute technique such as light scattering. Of course, if some of the injected biopolymer mixture remains on the SEC column, then light scattering will provide the true molecular weight for what is being eluted but not necessarily what was in the sample injected. Knowing when this is happening is, of course, extremely

important in characterizing the original sample.

In part II of this article, we provide further discussion of some of the possible pitfalls in attempting to characterize noncovalent protein aggregates using today's MS methods. Such approaches can be fraught with danger, unless the analyst has very clearly and definitively demonstrated that his or her methods do not and will not alter the sample components or their relative amounts.

Today, most applications of light scattering in the biopharmaceutical industry are done using MALS–RI–UV combinations, or some other type of instrumentation arrangement (such as RALS–LALS).

Light-Scattering Theory

Before we enter into further detail (in part II) about the application of light-scattering methods to the analysis of biopharmaceuticals, let us review how light scattering works.

Classical light-scattering measurements, in the absence of initial separations (such as SEC), are performed by first preparing a series of samples at different, decreasing concentrations in a suitable solvent. The solvent chosen must be fully compatible with the analytes and should not in any way change the nature of the original sample. Each sample is then illuminated sequentially by a collimated light source, such as a monochromatic laser, and the scattered light from each

sample is measured as a function of scattering angle (1,2,23–29). This is shown schematically in Figure 1, where an array of collimated detector elements detect scattered light at discrete scattering angles (30).

Historically, such measurements were first made using a single detector that rotated about the sample cell, stopping at each angular location, where measurements were made. Using an array of detectors speeds up the measurement process significantly. The relation between the measured intensities and the weight-average molecular weight (M_w) is given to the second order in the sample concentration (c), by the equation developed by Zimm:

$$R(\theta) = K^* M_w c P(\theta) [1 - 2A_2 M_w P(\theta) c + \dots] \quad [1]$$

Zimm showed that for such small concentrations, equation 1 may be written in its reciprocal form as

$$K^* c / R(\theta) \approx 1 / M_w P(\theta) + 2A_2 c + \dots \quad [2]$$

In equations 1 and 2,

$$K^* = 4\pi^2 (dn/dc)^2 n_0^2 / (N_A \lambda_0^4) \quad [3]$$

where c is the concentration of the solute molecules (g/mL), n_0 is the refractive index of the solvent, and dn/dc is the refractive index increment of the solution (that is, the solution refractive index changes an amount dn for a solute concentration change of dc). Furthermore,

$$R(\theta) = f_{\text{geom}} [I(\theta) - I_s(\theta)] / I_0 \quad [4]$$

where θ is the scattering angle, $I(\theta)$ is the intensity scattered by the solution into the collimated detector solid angle about θ , $I_s(\theta)$ is the intensity scattered by the solvent into the collimated detector solid angle about θ , and I_0 the light intensity incident on the sample. f_{geom} is a geometrical factor depending on the structure of the scattering cell, the refractive indices of the solvent and the cell, as well as the field of view of the corresponding detector and its acceptance angle for scattered light. N_A is Avogadro's number, λ_0 is the incident wavelength in vacuum, M_w is the weight-average molecular weight, and A_2 is the second virial coefficient (a measure of the solvent-solute interaction). The form factor, $P(\theta)$, may always be written as an infinite series in $\sin^2\theta/2$:

$$P(\theta) = 1 - \alpha_1 \sin^2(\theta/2) + \alpha_2 \sin^4(\theta/2) - \dots \quad [5]$$

where α_1 and α_2 are constants and

$$\alpha_1 = (4\pi n_0 / \lambda_0)^2 \langle r_g^2 \rangle / 3 \text{ (always!)} \quad [6]$$

and

$$\langle r_g^2 \rangle = \sum_i r_i^2 m_i / \sum_i m_i = 1/M \int r^2 dm \quad [7]$$

the integration is taken over all mass elements, dm , of the molecule. The distance of mass element dm from the molecule's center of gravity is r .

Measurements of the scattered light intensity from different sample concentrations form the basis of Zimm's method to extract the molecular parameters M_w , $\langle r_g^2 \rangle$, and A_2 . Further details may be found in the original Zimm papers (26,27,33). Note from equation 2 above, in the limit, as c and the scattering angle, θ , go to zero, $K^*c/R(0^\circ) = 1/M_w$. Although the molecular weight derived in this manner is the M_w of the sample, the derived mean square radius $\langle r_g^2 \rangle$ is, in general, some type of average that is only easily defined for the case of random coil molecules in a theta solvent ($A_2 = 0$). In that case, it may be shown quite easily that the measured mean square radius is a z-average value.

Combining Light Scattering with Chromatography for Full Characterization

Although light-scattering measurements performed on unfractionated samples will yield average values for both mass and size, following Zimm's method (discussed briefly in the previous section), the true characterization of any nonmonodisperse sample requires that the differential and cumulative distributions be measured. To achieve this, the sample must first be fractionated, for instance by SEC or GPC. Figure 2 shows a typical SEC arrangement combined with a light-scattering detector.

The light-scattering detector is usually placed between the concentration detector (a DRI or UV detector) and the SEC columns (31). Samples separated

by the columns are generally diluted between 10 and 100 times from the injected concentration (which, in itself, can often lead to varying degrees of disaggregation, if aggregates were first present in the injected sample), so the second virial coefficient term in equation 2 may generally be neglected. Therefore, for GPC or SEC, equations 1 and 2 both reduce to the form

$$R(\theta) = K^* M_w c P(\theta) \quad [8]$$

It is important to note that the baseline adjusted light-scattering signal (excess Rayleigh ratio) is proportional to the product of the molecular weight times c . Because c is proportional to the number density multiplied by the molecular weight, the excess Rayleigh ratio is proportional to the number of molecules per unit volume multiplied by the square of the molecular weight. Thus, the excess Rayleigh ratio, which is measured at each eluted fraction (slice), may be extrapolated to zero scattering angle to determine the molecular weight for that fraction from the intercept with the ordinate axis.

Because the fractionation process itself is assumed to yield monodisperse fractions at each collection, the weight-, number-, and z-average molecular weights should be the same at each such slice. The concentration (UV or RI) detector measures a concentration at each slice; therefore, one can calculate the differential and cumulative-weight

fraction molecular weight for each sample injected. The mean square radius may be calculated at each slice from the slope of the excess Rayleigh ratio as a function of $\sin^2(\theta/2)$ extrapolated to zero scattering angle, and thus the distributions of the mean square radius may also be calculated. Note that the mean square radius may be calculated without any knowledge of the sample concentration, provided that the result of equation 8 holds.

From a measurement of the root mean square radius and the corresponding mass at each slice, the conformation of the molecules making up the distribution present in the sample ensemble may be determined, as well. There are two requirements for this to be possible: First, the molecules must be large enough to permit a meaningful measurement of the mean square radius and, second, the distribution present must be polydisperse and span a reasonable range of molecular weights. Finally, it should be noted that for certain types of copolymers, the mean square radius cannot be calculated immediately from the variation of the excess Rayleigh ratio with $\sin^2(\theta/2)$.

Conclusion

Here in part I of this two-part article, we have discussed the history of light-scattering detection in tandem with chromatography for biopolymer separations and characterizations, and briefly discussed current applications. We have also provided a summary of

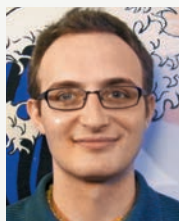
the theory of light scattering and how it is combined with chromatography. In part II, we will provide a more in-depth discussion of several applications of light scattering in biotechnology: measuring protein stability, measuring aggregates in formulation studies, and characterizing low-molecular-weight heparin. We also compare the performance of light-scattering methods with MS detection and other methods.

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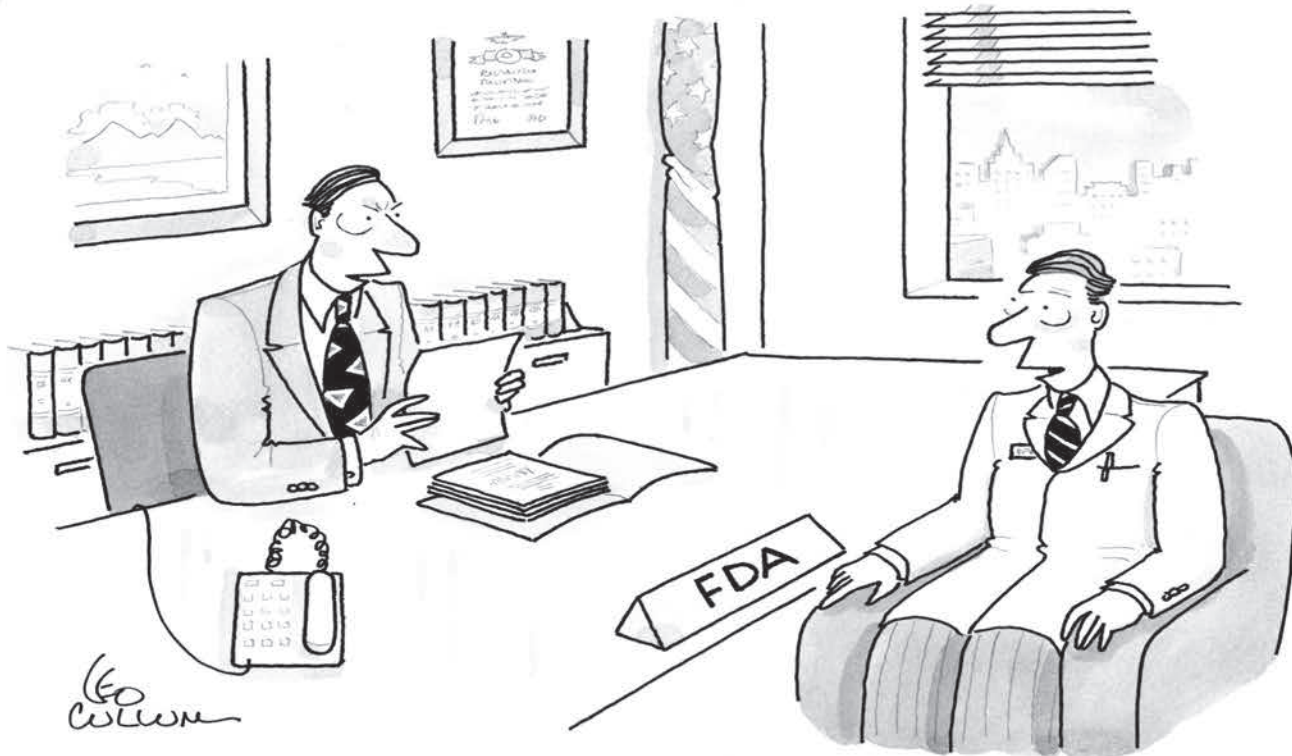
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"So you're sure this protein has a molecular weight of 150,000?"
"According to our assumptions, that's correct."

□ □ □

That's the problem with relying on column calibration to characterize macromolecules. You don't really know if you're right—you can only make assumptions. Which is why every major pharmaceutical and biotechnology company is switching from *relative* methods to Wyatt Technology's *absolute* measurements. Our DAWN® multi-angle light scattering (MALS) instruments allow you to determine absolute molecular weights and sizes *without* relying on so-called standards and tedious column calibration. Wyatt's instruments measure *all* of the quantities required for determining absolute molar masses *directly*. So visit **www.wyatt.com** and request our free 32-page *Ultimate Guide to Light Scattering*. You'll learn how to end your dependence on reference standards, which will make you and your products a whole lot more convincing.



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The Use of Light-Scattering Detection with SEC and HPLC for Protein and Antibody Studies,

PART II: EXAMPLES AND COMPARISON TO MASS SPECTROMETRY

Simion Kreimer, John Champagne, Anurag S. Rathore, and Ira S. Krull

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Analysis of conjugated proteins



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Light scattering tools for proteins



The use of light-scattering detection for the characterization of proteins and antibodies is increasing. Examples of applications include determining absolute molecular weight, studying PEGylated proteins, characterizing protein–drug and antibody–drug conjugates, and studying protein aggregation. This two-part article explains how light scattering works and assesses its capabilities for biotechnology applications.

Light scattering detection, particularly multiple-angle light scattering (MALS), can be very useful for the characterization of proteins and antibodies. In a number of applications, its performance exceeds that of mass spectrometry (MS) detection. In part I of this two-part article (1), we outlined the development of light-scattering detection in biotechnology, summarized its current uses and advantages, and explained the theory of how it works. Here in part II, we provide some detailed examples of its use and discuss how its performance compares to MS and other methods. As we

will see in the examples, light-scattering analysis particularly excels at studying the bulk properties of proteins and antibodies in highly complex solutions.

Where Mass Spectrometry Falls Short, Light Scattering Steps In

When addressing the characterization of biopharmaceuticals or other complex biological samples, it is impossible to ignore analytical methods involving the combination of liquid chromatography and mass spectrometry (LC–MS) (2). These techniques range from high performance liquid chromatography (HPLC)–MS, where a relatively simple mixture is separated into several components and characterized by the intact masses and relative abundances (or even absolute concentrations, with the use of reference standards), to multidimensional LC techniques paired with high-resolution MS systems, which separate complex biological mixtures into thousands of peaks and perform in-depth analysis of individual species with multiple rounds of fragmentation. The information that is gained by in-depth LC–MS–MS analysis includes identification of post-translational modifications (PTMs), oligosaccharide structure, and sequencing of peptides or even proteins with masses as large as 30 kDa.

In essence, LC–MS techniques are strongest for determining specific details about individual species. This ability is important for characterizing a substance with a limited number of variants, such as

a small protein drug product. However, LC–MS falls short in quantitating the bulk properties of extremely complex substances, including PEGylated protein drug products (where PEG is polyethylene glycol), biologically derived substances such as low-molecular-weight heparin (LMWH), and inherently complex pharmaceutical substances such as copaxone. Light-scattering techniques pick up some slack in these areas.

Two additional challenges that cannot currently be addressed by LC–MS techniques are the determination of the tertiary or quaternary structure of a protein, and the study of protein–protein interactions (including aggregation). This information is irreversibly lost when proteins are ionized and vaporized by electrospray ionization or matrix-assisted laser desorption ionization (MALDI) for MS detection. Light-scattering techniques have the advantage of analyzing the protein in solution where the native conformations and protein–protein interactions are intact. In particular, size-exclusion chromatography (SEC)–MALS is especially valuable for very complex mixtures of proteins, antibodies, and aggregates (noncovalent or covalent), as well as highly derivatized proteins or antibodies as drug conjugates or PEGylated species.

Some Practical Biotechnology Applications of Light-Scattering Techniques

Below, we present several specific

applications where light-scattering techniques adequately solve biotechnology challenges that are not currently fathomable by any LC–MS technique. All three involve studying highly complex solutions. Because many current protein-based pharmaceutical products are relatively large and complex, the development of therapeutic formulations at the high concentrations required can lead to a variety of complex protein stability issues (3). Many of these issues involve the interaction of protein therapeutics in vitro, leading to various degrees of mass-action associated and non-mass-action associated aggregation states.

Formulation Studies:

Ensuring Protein Stability

A significant biotechnology application of light scattering technology is in

formulation studies, investigating the stability of proteins in the solution used to deliver them to the patient. This can be accomplished by monitoring the protein's hydrodynamic radius (R_h), which will decrease or increase if the protein's native conformation collapses or expands because of disruption of stabilizing tertiary interactions. Figure 1 presents a study conducted using a Wyatt DynaPro plate reader, which simultaneously monitored an antibody formulation at a range of temperatures and various concentrations on a 384-well plate matrix (4). The R_h of the antibody is indicated as a function of temperature and concentration. The hydrodynamic radius exhibits a sigmoid relationship as a function of temperature for all antibody concentrations at pH 9.5. The change in midpoint temperature and

radius as a function of concentration may indicate nonspecific attraction or oligomerization. Similar studies can be conducted with a range of pH levels and excipient concentrations to establish the most thermally stable formulation for any protein or antibody biopharmaceutical.

Peptide mapping by reversed-phase ultrahigh-pressure liquid chromatography–mass spectrometry (UHPLC–

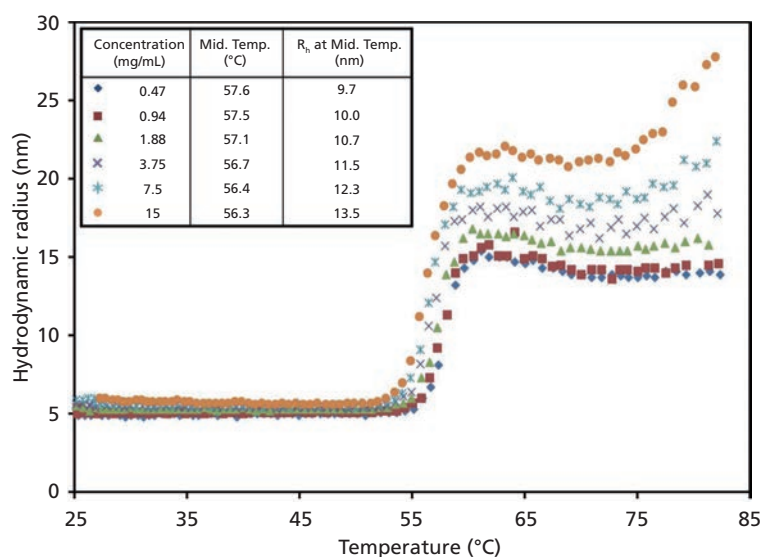


Figure 1: The hydrodynamic radius (R_h) of an antibody as a function of temperature and concentration.

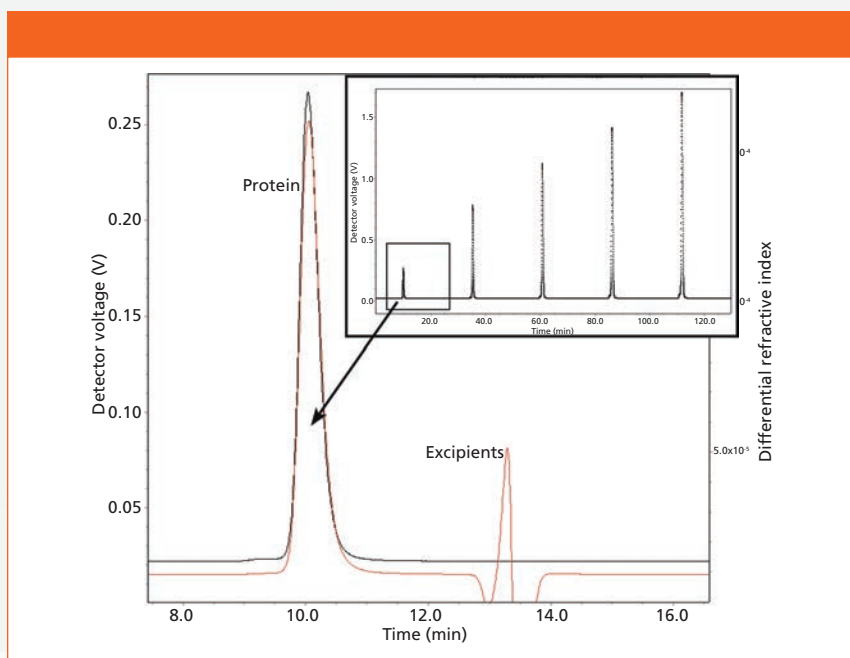


Figure 2: Light-scattering and refractive index measurement of serial injections.

MS) is an alternative approach to investigating protein stability. This technique specifically detects chemical degradations that involve a change in charge or mass, such as deamidation or oxidation of certain residues. However, this technique does not detect conformational changes that involve the physical rearrangement of the protein's three-dimensional structure. Therefore, using LC-MS alone, it is possible to miss protein population shifts from functional to nonfunctional states or noncovalent aggregation of denatured proteins. Conversely, a residue modification in the protein's active site may affect activity without resulting in a major change in conformation; in this case LC-MS may identify this substitution, whereas light-scattering techniques will not.

Formulation Studies: Minimizing Aggregation

Another key aspect of biopharmaceutical formulation is the prevention and detection of aggregation. Aggregation occurs if protein-protein interaction is thermodynamically favorable; this interaction varies with protein concentration and buffer pH, salinity, temperature, and overall composition. One measure of bulk protein-protein

interaction in solution is the second virial coefficient (A_2). The ability to rapidly and accurately measure this parameter aids in establishing the optimal buffer composition for drug products to mitigate aggregation. The automated SEC-MALS-refractive index (RI) method presented in Figures 2 and 3 is one such method of measuring A_2 . Light-scattering and refractive index measurements are obtained for each peak in a series of dilutions of one stock protein sample, and from this information the A_2 coefficient is calculated for each peak (5). In Figure 2, the chromatogram in the upper right part of the figure shows the light-scattering signal of the 20–200 μ L antibody solution injection series. The expanded boxed region shows the light-scattering signal in black and the differential RI signal in red, demonstrating complete dialysis of

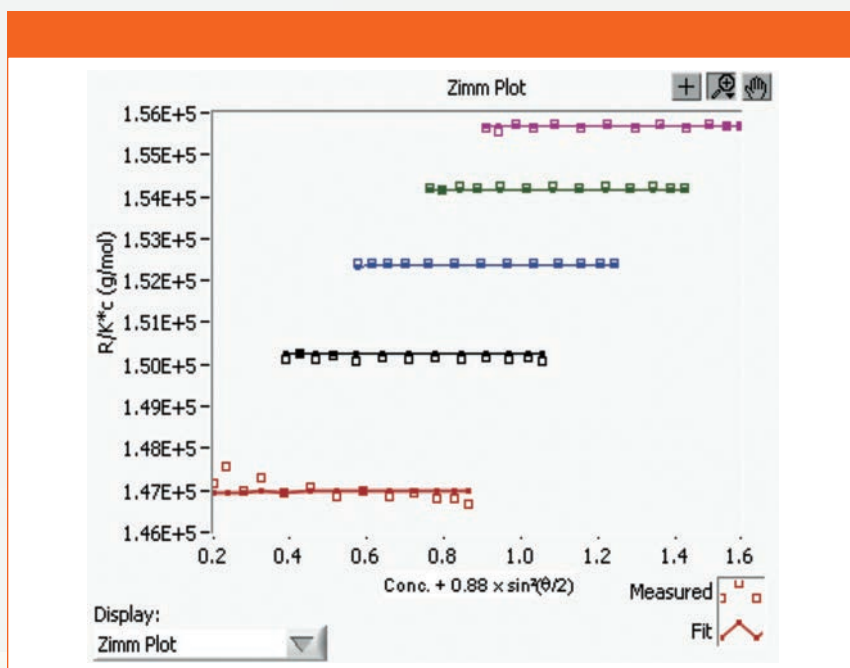


Figure 3: Zimm plot of an antibody in phosphate-buffered saline.

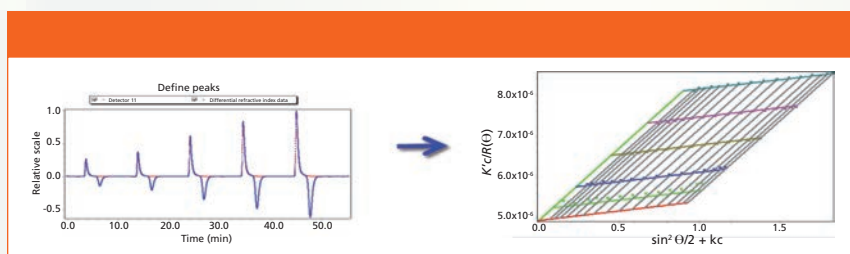


Figure 4: On-line A_2 analysis using a buffer-exchange column, with UV and DRI.

injected antibody with the mobile phase on the SEC column. Only the protein peaks are used in the on-line A_2 analysis. Figure 3 shows a Zimm plot from the light scattering and concentration data of the protein peaks from Figure 2. Each color represents a different peak. From this Zimm plot, it can be determined that the antibody in phosphate-buffered saline (PBS) has an A_2 of $-5.46 \times 10^{-5} \text{ mol} \cdot \text{mL/g}^2$.

Figure 4 shows another type of Zimm plot, perhaps the more customary format. Figure 4 illustrates an on-line A_2

experiment to determine the molar mass, root mean square radius, and second virial coefficient of a sample (6). To do so, it is necessary to measure the light-scattering signal as a function of angle and concentration. The angular and concentration-dependent light-scattering data are then fit to the basic light-scattering equation using a Zimm plot (Figure 4, right). It is important to note that the global Zimm fit takes the data as a whole and extrapolation to zero angle or concentration is unnecessary. Together, these measurements yield insight into a number of properties of the protein and represent one of the

most comprehensive determinations of the aggregation state.

Using these methods, different buffer compositions can be sequentially analyzed to find the buffer that produces the optimal (small, positive) A_2 coefficient. Another application for this method is in molecular biology, for finding the optimal conditions for protein crystallization that have a low, negative A_2 coefficient.

Figure 5 illustrates an SEC chromatogram in which the molar masses (y-axis) are plotted as the

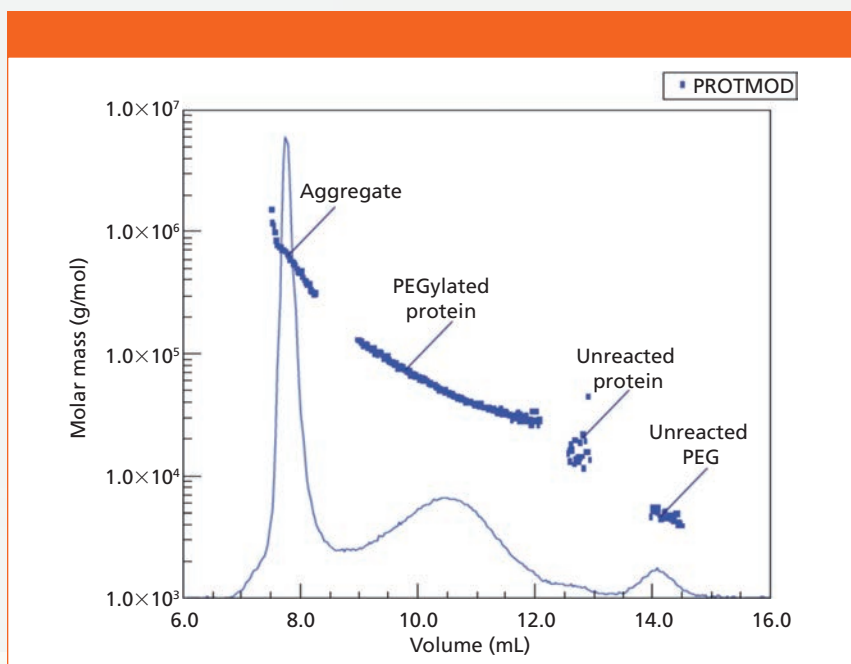


Figure 5: Plot of mass vs. volume from an SEC–MALS analysis of a protein–PEG complex.

MALS detector 90° light-scattering signal versus the elution volume (x-axis), indicating the presence of a large range of molar masses (7). The peaks in this chromatogram are likely a result of different degrees of protein modifications, as well as aggregate formation, residual unreacted protein, and unreacted PEG, as indicated.

Characterization of Low-Molecular-Weight Heparin by SEC–MALS

LMWH is manufactured by depolymerizing animal heparin, a heterogeneous substance, resulting in a mixture of linear polysaccharides of varying lengths. Heparin is made up of repeating disaccharide subunits that are sulfonated to various degrees. It is not a trivial challenge to characterize any individual species in the enormous

multitude of structures that exist in this mixture. Sophisticated separation techniques would have to be used to isolate a single structure, and then multiple rounds of MS–MS may be necessary to unambiguously identify the linkages and levels of sulfonation of each saccharide of that single variant (although locations and anomericity of these modifications may be impossible to identify without nuclear magnetic

resonance [NMR] spectroscopy). Biologically derived substances like LMWH are best characterized using bulk properties, and these features should be routinely examined between manufactured batches to ensure consistency. One of the most basic bulk features of a heterogeneous substance is the distribution of molar masses. Figure 6 shows how SEC–MALS was used to examine the molar mass distributions of three LMWH products generated by different depolymerization reactions. Three commercial LMWH products were examined by Scientific Protein Laboratories using a Waters 510 pump and 401 refractometer, a Shodex Ohpak SB-803HQ SEC analytical column (300 mm × 8.0 mm) with an SB-G guard column, and a Wyatt miniDawn triple-angle light-scattering instrument. The

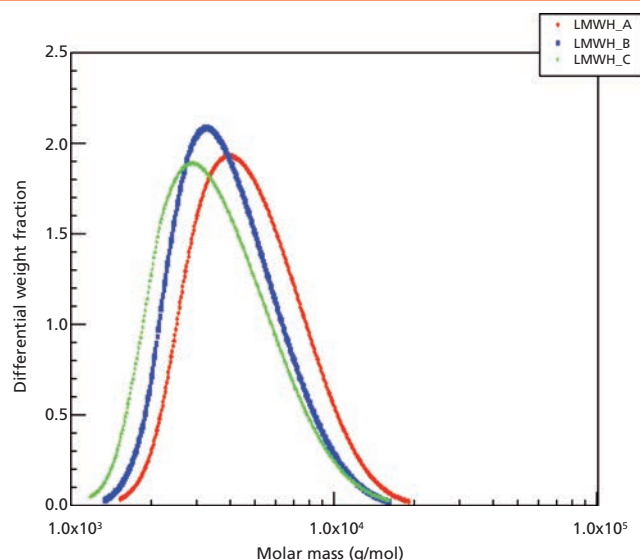


Figure 6: Molar mass distributions of three low-molecular-weight heparin products.

experiment was carried out in 0.1 M ammonium acetate buffer and dn/dc was measured off-line in a Wyatt Optilab DSP instrument operating at the same wavelength as the triple-angle instrument. The three LMWH products are distinguishable by their molar mass distributions (8).

Molar mass distributions alone, however, are insufficient to characterize LMWH. Potency should be tested in vitro to confirm efficacy, and other key features that affect the activity of this substance, such as the concentration of the biologically active, unique pentasaccharide sequence that is known to interact with antithrombin, should be carefully monitored (9). Along with other analytical techniques, SEC-MALS is a key tool in characterizing LMWH and is used routinely in the quality control of these products.

Light Scattering Complements MS and Other Techniques

It is important to restate that the strength of light-scattering techniques is the ability to measure bulk properties of mixtures, such as the average molecular weight, the level of protein-protein interactions (A_2), and hydrodynamic radius (R_h) and gyration radius or radius of gyration (R_g).

When dealing with high-complexity mixtures like LMWH, these bulk properties provide the most useful information about the substance, rather than in-depth characterization of a few species.

On the other hand, light-scattering techniques do not provide great detail. For example, R_h is not as useful as complete protein structure by NMR, MS, or X-ray crystallography, and quantitation of PEGylation is not complete characterization, if the exact sites of PEGylation are not determined by LC-MS analysis. In summary, light-scattering techniques are essential for filling in the analytical gaps left behind by LC-MS techniques, and vice-versa.

Ensuring an Analytical Method Does Not Change the Nature of the Samples

When studying proteins in solution, it is also important that the analytical

methods do not adversely affect the thermodynamics of the protein therapeutic so as to change the solution behavior during or after analysis. Because of its high degree of resolving power and relatively high-throughput nature, SEC has long been the industry standard for analyzing proteins in solution. This technique employs hydrostatic pressure along with a mechanical sieve to separate molecules based on their hydrodynamic characteristics. Although SEC has many advantages, there is concern over its analytical accuracy because proteins can interact with the stationary sieving matrix. It has been observed that the prolonged interaction between the proteins and the SEC matrix may lead to adsorption of the protein onto the column and possibly contribute to the artificial dissociation of preformed, weakly associated aggregates. In addition, the extent to which higher-order aggregates can permeate the pores of the stationary phase can also lead to an under-representation of these species. SEC also functions to dilute the injected sample, to varying degrees, which can also lead to disaggregation effects. Such defects in the SEC method itself are unwanted, because they change or alter the nature of the injected sample and distort the detector measurements, be these UV, refractive index, or light scattering.

It is therefore critical to use a first-principle technique to characterize and quantitate the protein samples eluted from the chromatography column, as

well as techniques that do not involve mechanical sieving for preliminary separation. In other words, one should use direct MALS with no initial SEC or other separation step (as discussed further in the next section). MALS is one of the few first-principle techniques that can be used to determine the molecular weight and structure of proteins in solution, and it is applicable over the broadest range of any analytical method (10).

However, direct MALS or other direct light scattering methods, without SEC, are unable to determine how many dimers, trimers, or higher-order aggregates are present in the protein or antibody sample. Nor can they derive information about the size or shape (R_h or R_g) or A_2 term of individual species in an unresolved mixture of such species.

Also, MALS and other light-scattering techniques produce a weight-averaged molecular weight and the corresponding z-average, square radii $\langle r_g^2 \rangle_z$ (11,12), another reason why they also should be used after a separation method (such as SEC).

Using Light Scattering Without Chromatography in Free Solution

As mentioned above, to adequately characterize whether a method is changing the nature of the samples during characterization, it is essential to get a preliminary baseline of the free-solution nature of the protein complex (that is, without any initial separation

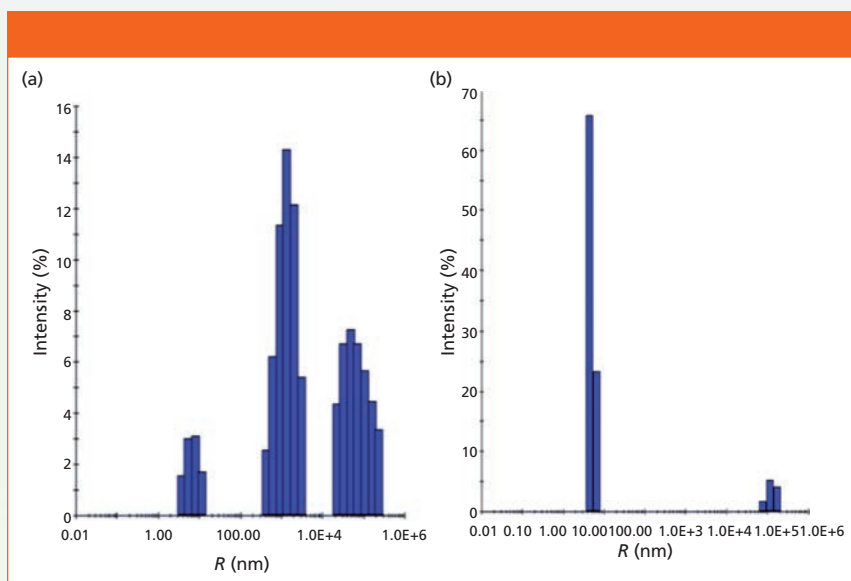


Figure 7: DLS analysis of pre- and post-method non-mass-action aggregation.

step). For this measurement, another Rayleigh light scattering technique can be employed in a “batch” mode without using any separation mechanism. If the light-scattering intensity is measured over time, the resulting intensity fluctuations can be used to directly determine the translational diffusion coefficient (D_T) for a protein, or protein complex in solution. Referred to as *dynamic light scattering* (DLS), this technique can be used to then convert the measured D_T to a hydrodynamic radius (R_h) through the Stokes-Einstein equation. Although a more complete review of this technique is available through many sources, it is sufficient to say that this technique usually does not have the resolving power of SEC–MALS (2,12,13). Although a 3–5 fold difference in D_T is required for resolution of species in solution when determining the mass-action aggregation of proteins, it is an invaluable tool when

assessing the pre- and post-method, non-mass-action aggregation state. In Figure 7, the association state of an IgG1 antibody was measured using DLS at the time of preparation (pre-method) and after SEC analysis (post-method) (14). The DLS analysis clearly shows the degree of non-mass-action aggregation before analysis and the extent to which the SEC technique can artificially

alter this aggregation state. The width of the histogram peaks represent the degree of polydispersity for each. Again, it must be understood that chromatographic conditions, even in SEC or gel permeation chromatography (GPC), can alter the nature of the original species injected onto the column, often in unknown ways. In this particular instance, the number of aggregates have decreased during the very method of analysis. Methods of analysis that change the original nature of a sample are meaningless to determine what was originally present in any such sample.

Limitations to SEC–MALS

Naturally, there are limitations to any analytical technique, and it is important to point these out when considering SEC–MALS characterization. Based on the theory of Rayleigh scattering, static light-scattering measurements can resolve

the size of a protein complex (r_g^2)^z down to about 1/20th of the incident wavelength (a radius of ~10 nm at 660 nm) and up to 1000 nm. Above 1000 nm, Rayleigh scattering does not hold, as the refractive index of the particles and that of the solvent produces a distortion of the electric field of the incident light that is solved only through the use of the Mie equation (2,12). As for molecular weight, for a given concentration c , the scattered light at zero angle is proportional to c multiplied by molecular weight. Thus, for protein samples with relatively low molecular weights (~1000 D_a), relatively high concentrations are required to achieve a suitable signal-to-noise ratio. Alternatively, high-molecular-weight proteins and non-mass-action aggregates require very low concentrations to yield a suitable signal-to-noise ratio. This phenomenon raises the obvious issue of obtaining an adequate parallel mass concentration for each eluted fraction. For this reason, it is advisable to employ a differential refractometer detector as a universal concentration detector, along with a UV detector for its increased sensitivity, and selectivity, for low-protein-concentration species. These parallel concentration detectors can also provide valuable insight about the mass recovery of the injected protein samples onto the column and assist in the selection and conditioning of the stationary column matrix to minimize adsorption. A low mass recovery is not an acceptable set of SEC or GPC conditions for that particular

analyte, and it should be rectified to ensure observing all the various species possible from a biotechnology product, adducts, aggregates, and derivatives. Provided that these conditions are achieved, SEC-MALS represents one of the most accurate and powerful tools available for characterizing the aggregation state of protein therapeutics in solution.

When to Use Mass Spectrometry and When to Use Light Scattering

In the preceding sections, we have described possible uses of SEC-MALS in the biotechnology industry, and where it can fill in some gaps left by SEC-MS methods. As already mentioned, MS is better for certain types of analysis and MALS is better for others. They are complementary techniques and should be used for those analyses for which they perform best or better than the other. Let us review some specific applications of each approach, especially MS, to highlight when to use each method (1).

Three Examples Where MS Works Best

Figure 8 first illustrates two SEC-UV-electrospray ionization (ESI)-MS chromatograms for a reduced and alkylated antibody, showing the heavy chain (HC), HC-HC dimer, and free light chain (LC) (15,16). The top chromatogram is the UV trace, and the bottom is the MS trace and spectra. Because the HC is a much higher molecular weight species (approximately 50 kDa), it shows

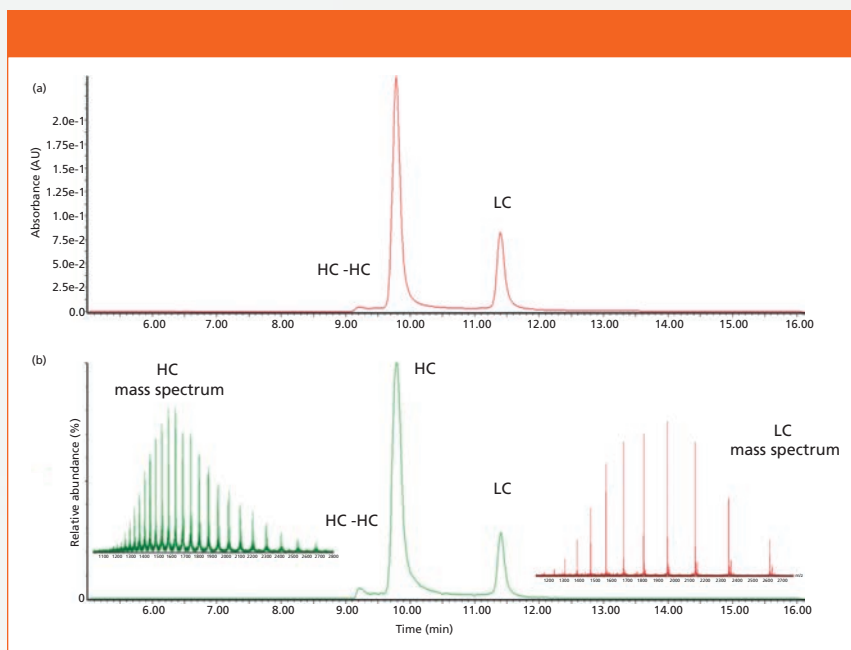


Figure 8: SEC-UV-MS analysis of a reduced and alkylated monoclonal antibody, showing both UV and MS (TIC) chromatograms and mass spectra for both heavy chains (HC) and light chains (LC).

a much more complex MS spectrum via ESI, with more multiply charged ions. Each of these individual peaks could be characterized by its molecular ion as well as by various fragmentation techniques involving high-resolution MS (such as Fourier transform MS), using collision-induced dissociation (CID) or electron-transfer dissociation (ETD) to sequence the amino acids and attachments (such as glycans), in each species. Because there are only three distinct species present and fully baseline resolved from one another, each of these can be readily, easily, and fully characterized. There is no need to use SEC-MALS on such species, even if the HC has several glycoforms present, as expected. Glycoprofiling can be performed easily by baseline-resolving the intact HC variants using

other HPLC-MS conditions or by releasing all glycans and characterizing these, quantitatively and qualitatively, by conventional glycoprofiling using 2AB-tagging with hydrophilic interaction chromatography (HILIC), reversed-phase LC, capillary electrophoresis, and fluorescence detection (17,19). Problems with isobaric species in such studies do not generally arise, depending on the specific nature of the glycovariants. Between the

high resolution obtained using UHPLC and the lack of isobaric variants in high-resolution MS, each such species should be identifiable. MALS will not provide any better characterizations for such species.

Figure 9 illustrates a slightly different scenario, somewhat artificially created, again for LC- and HC-derived species but now wherein the HCs are partially (not fully) alkylated on their residual thiol groups (15–17). This partial alkylation leads to a much larger number of HC species, in addition to glycovariants. Again, UHPLC conditions, whether with reversed-phase LC or ion-exchange chromatography (IEC), together with high-resolution MS, should be able to fully characterize each of these variants. It is, once again, not clear that MALS can play a significant role in adding to the

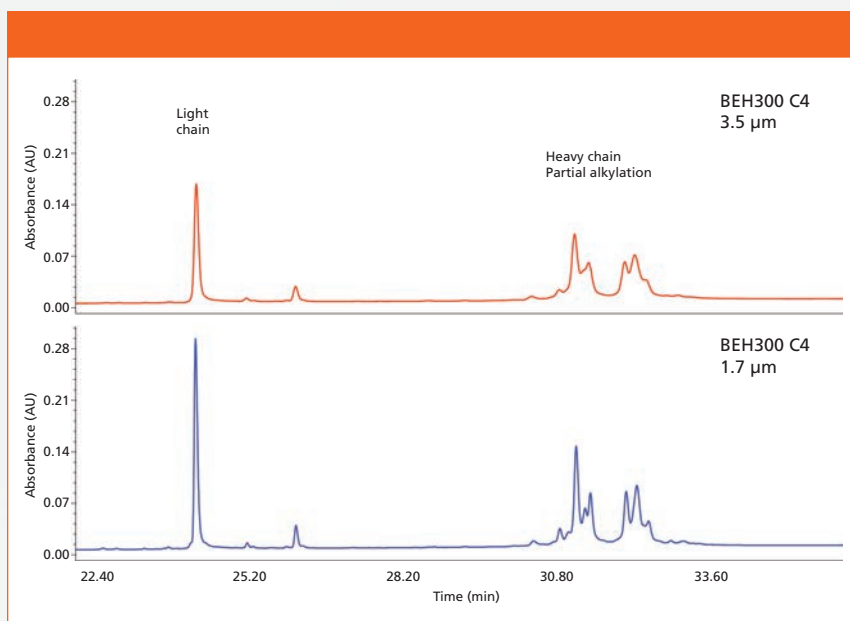


Figure 9: UHPLC separation of IgG heavy and light chains. Sample: Reduced and partially alkylated monoclonal antibody (IgG).

characterization already possible using combined UHPLC–ESI–MS methods.

Figure 10 illustrates typical UHPLC–ESI–MS chromatograms for reduced monoclonal antibody (MAb) samples, comparing an innovator MAb to a generic or “biosimilar” product (19–20). The data were processed using Waters BiopharmaLynx software. In Figure 10a, we see that the light chain of the innovator and biosimilar MAb are identical. In Figure 10b, the heavy chain of the biosimilar candidate MAb shows a consistent mass shift of ~32 Da for all protein glycoforms compared to the reference sample (the innovator product). Glycosylation pattern differences were also detected. The molecular weight of each resolved species present can be accurately derived and sequenced, showing even very small differences between the HC species.

Such results are clearly the forte of MS and not MALS, which is why it is important to recognize what each technique offers and is routinely capable of providing for a given analyte.

Where MALS Excels: Complex Mixtures

On the other hand, as we have described in the preceding sections of this column and in part I, MALS provides informational content for mixtures of protein- and antibody-derived species that are more complex than those described above, such as higher order aggregate species. (MS has problems, to unknown or undefined degrees, in determining any protein aggregate state, and surely not how much of each species is present.) MALS also provides R_h or R_g , unique numbers that indicate size, shape, and conformation for these species, and this is not possible by MS or other techniques now available. And, as the protein or antibody undergoes changes upon prolonged standing under stability testing conditions, under forced oxidation testing conditions, or on complexing with its antigen, MALS provides specific numbers that indicate changes in conformation and perhaps also changes in its biological activity. MS is not yet able to compete in such arenas. Such numbers

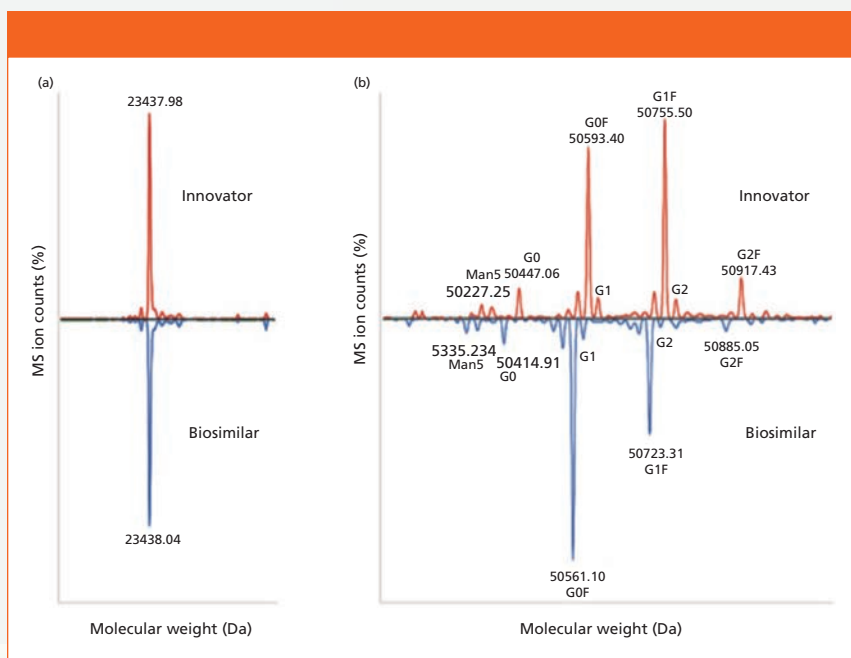


Figure 10: Typical UHPLC-MS chromatograms for reduced monoclonal antibody variants, comparing an innovator drug to a generic (biosimilar).

become unique characteristics for identifying active versus inactive proteins or antibodies, and together with intact molecular weights obtained by MALS or MS, can be used to suggest retention or loss of bioactivity. Such information about size and shape is crucial information in the biopharmaceutical industry, not easily available by other, existing analytical techniques (such as surface plasmon resonance, enzyme-linked immunosorbent assay, or affinity capillary electrophoresis).

Summary

In summary, for easily resolved individual species, as shown in Figures 8–10, UHPLC-SEC-ESI-MS is perhaps the ideal approach for characterizing what species are really present. On the other hand, for

more complex mixtures of proteins (synthetic or native), protein-antibody conjugates or aggregates (covalent or noncovalent), PEGylated proteins, antibody-drug conjugates, and related species, UHPLC-SEC-MALS appears to be the more advantageous analytical method.

Acknowledgments

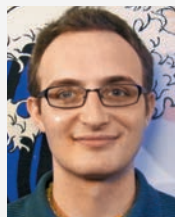
This two-part article has been a group effort, involving several unsung contributors, as well

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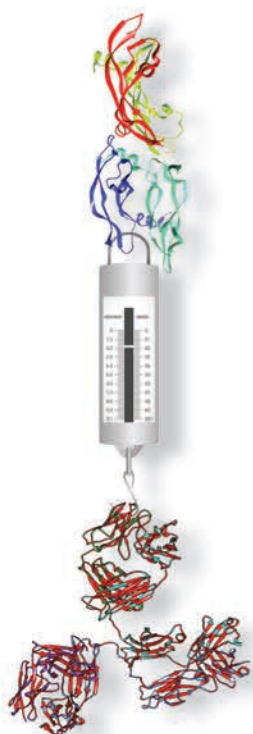
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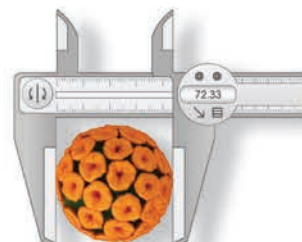
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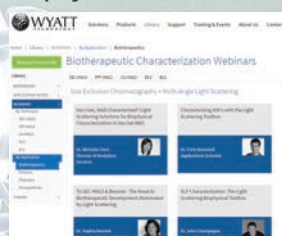
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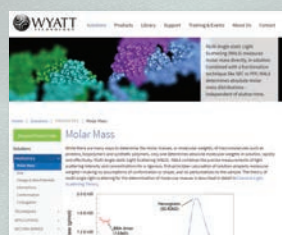


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Absolute molecular weight determination



This article is an overview of the different types of characterization of antibody-drug conjugates (ADC) that can be performed with a light scattering toolkit to support the development of biotherapeutics.

Interest in the use of antibody-drug conjugates (ADCs) as targeted therapeutic agents for the treatment of cancer and other diseases has increased significantly. Treatments can be administered with greater accuracy and sensitivity by targeting disease cells, reducing the dose value and therefore the damage caused to healthy tissues.

However, ADCs often exhibit increased aggregation propensity because of non-specific interactions arising from attached drug-linker moieties. To ensure the efficacy and safety of these conjugates, the drug-antibody ratio (DAR) must be determined and optimized. The DAR directly affects both the potency and toxicity, as well as having a considerable influence on other properties such as stability and aggregation. Furthermore — as is the case for unmodified biotherapeutics — the development of a viable ADC product requires careful determination

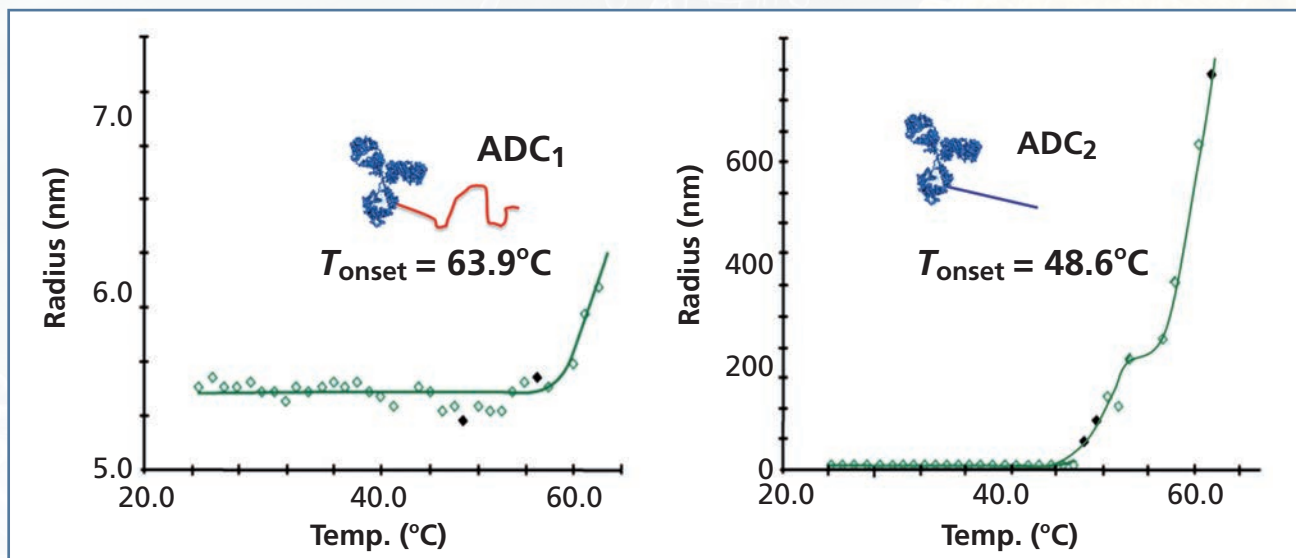


Figure 1: Aggregation pathways of two antibody-drug conjugates ADCs studied by dynamic light scattering (DLS) to demonstrate linker-induced instability.

of formulation conditions that maintain conformational and colloidal stability. This can be an extensive process because it requires empirical screening of a range of buffer and excipient combinations, often without a prior knowledge of degradation pathways and the compounds that might best prevent them. Therefore there is a growing need for more efficient techniques to accelerate the screening process and ensure that only the most promising compounds are advanced into development stages.

There are a number of techniques that are already widely accepted for characterization of ADCs and other biotherapeutics. DAR is often assessed by mass spectrometry (MS), but characterization relies on uniform ionization efficiencies and recovery of all sample compounds, which is often not the case with heterogeneously modified

ADCs. Samples containing particles across a wide size distribution can also pose a problem when performing MS because it is difficult to differentiate between species of significantly differing sizes. UV spectroscopy is an alternative technique that can determine DAR by exploiting the unique absorption properties of the antibody and modifier. By assessing absorption ratios at wavelengths specific to each component, DAR can be calculated from molar extinction coefficients. However, this method does require that the antibody and drug/linker exhibit distinct behavior in the UV spectrum. For example, the modifier needs to absorb at a wavelength where the antibody does not. The results can also be confounded because of Rayleigh scattering of aggregate in the sample, which appears as an increase in absorption — note that this is more

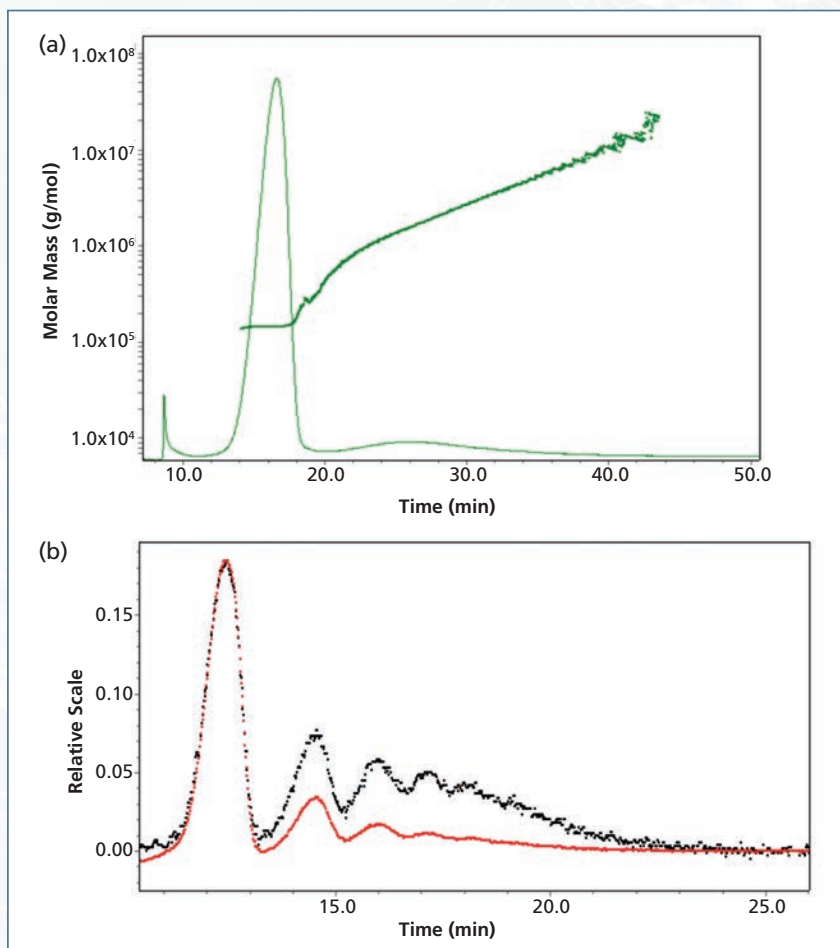


Figure 2: AFFF elution profiles for an antibody solution. Fractionation conditions can be optimized to detect and quantify (a) aggregate or (b) low-order oligomeric species without the need for multiple columns.

pronounced at shorter wavelengths.

Light scattering detection is an alternative method that can characterize aggregates and the propensity for aggregation, as well as perform advanced characterization such as determining DAR. Dynamic light scattering (DLS) can assess aggregation and unfolding, and multi-angle light scattering (MALS) can give an absolute value of M_w entirely in solution based on fundamental relationships between molar mass and the intensity of light scattered

by a molecule. Coupling MALS to a fractionation regime such as size-exclusion chromatography (SEC) or asymmetric field-flow fractionation (AFFF) allows mass determination without the need for conventional calibration as well as characterization of oligomers and aggregates in complex samples.

Formulation Screening by High-Throughput Dynamic Light Scattering

Short and long-term stability must be assessed under different chemical and thermal conditions to ensure integrity of the formulation is maintained. Myriad excipients should be screened to identify conditions that support

stability, including surfactants, sugars, salts, antioxidants, and amino acids. High-throughput DLS can be a powerful tool for characterizing the presence and degree of aggregation, thermal and conformational stability, and colloidal stability. DLS directly determines the diffusion coefficient (D_t) by measuring the rate of intensity fluctuations of light scattered by the sample, which can then be used to calculate the hydrodynamic radius (R_h) using the Stokes-Einstein equation:

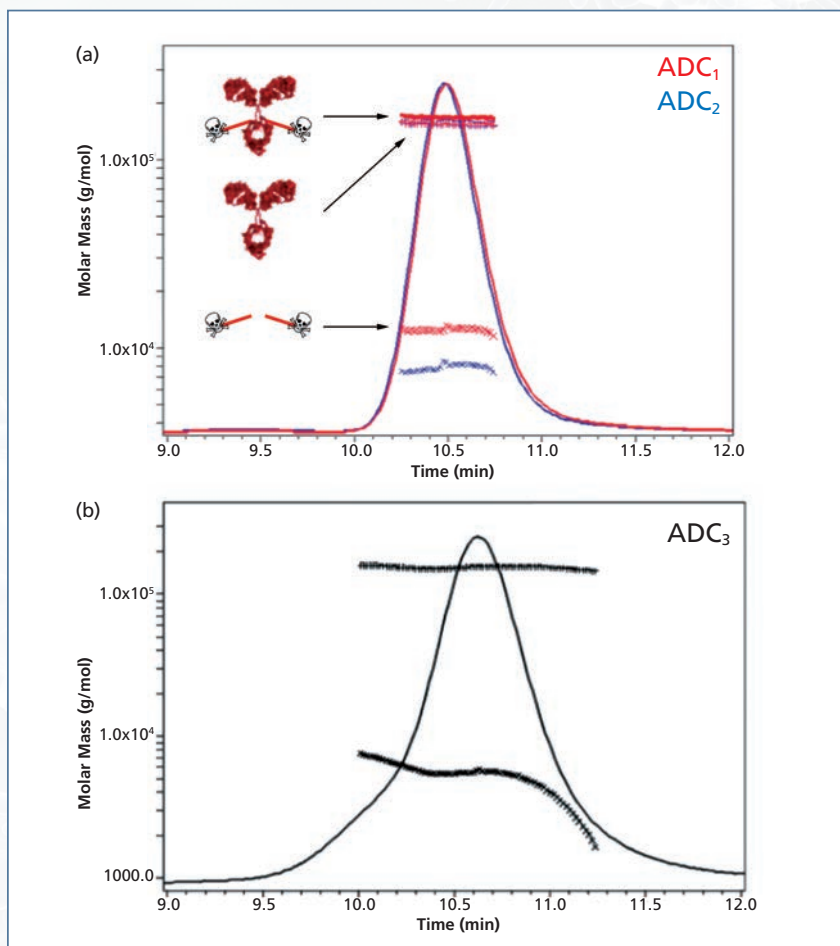


Figure 3: Determination of the mAb, drug-linker, and total molar masses of three distinct ADC constructs using SEC-MALS.

$$R_h = \frac{kT}{6\pi\eta D_t} \quad [1]$$

A more detailed analysis of the fluctuation signals gives a complete size distribution without sample fractionation. Although a low resolution technique, DLS is useful for biotherapeutics characterization because it is very sensitive to small quantities of large aggregates and there is sufficient information in DLS to assess both

degree of aggregation and propensity for aggregation via temperature and concentration dependent studies.

Linker-Induced Stability Studied by DLS

In addition to determining general formulation stability of an antibody platform, the impact of chemical modification on overall ADC stability must be assessed. DLS can be used to gauge shifts in sample behavior caused by the addition of pendant groups to the molecular surface. Figure 1 shows the unfolding and aggregation of two different ADCs when exposed to high temperature conditions as indicated by DLS. Both ADCs consist of the same

IgG platform and small-molecule drug (at similar DARs); the only difference is in the composition of the cross-linking agent itself. In this case, ADC₂ exhibits decreased unfolding and aggregation temperatures relative to ADC₁, suggesting it to be a less desirable modificant. Potential dissociation of reversible aggregates with dilution, sometimes seen in SEC, is not an issue when performing DLS, and the low sample volume required for screening saves on preparation time and resources.

Table 1: Calculation of the molar mass of three distinct ADC formulations.

	M_w (kDa)			
	ADC	IgG	Drug	DAR
ADC₁	167.8 (±1.2%)	155.2 (±1.8%)	12.6	10.1
ADC₂	163.7 (±1.2%)	155.6 (±1.2%)	8.1	6.5
ADC₃	159.5 (±8.0%)	155.2 (±8.0%)	4.3*	~1 – 7

Size Distribution by FFF–MALS

MALS, which allows direct determination of molar mass, can be particularly useful when coupled with SEC or AFFF where samples can be fractionated for identification and quantitation of resident oligomeric species. The relationship between the intensity of scattered light, molar mass, and weight concentration is well-established and can be represented by the following basic relationship:

$$I_{\text{scattered}} \propto M_w \cdot c \quad [2]$$

Scattered intensity and concentration are simultaneously determined on-line with a MALS detector and UV or refractive index detector. The calculated molecular weight is independent of the macromolecule's shape. For molecules with a radius exceeding 10 nm, the angular dependence of the scattering intensity can be used to calculate the root mean square (RMS) radius of the molecule. Most ADCs will be below this

threshold, and so on-line DLS must be used for accurate sizing.

Fractionation of antibodies and ADCs by SEC is ubiquitous, and coupling SEC to MALS has proven a great boon to biophysical characterization. There are, however, some circumstances where alternative fractionation techniques are required. For example, if quantitation of aggregates typically filtered out by SEC columns is required or when modification results in significant hydrophobic/non-ideal interaction between the ADC and SEC column packing. In these cases, ADCs can be resolved and characterized by field-flow fractionation (FFF). This technique separates particles based on their diffusion in a variable laminar flow and in the absence of a stationary phase.

The benefit of FFF is twofold because surface interactions are eliminated and a wide range of oligomers and particles can be resolved without the need for multiple columns and extended separation times. Figure 2 shows fractograms of a

monoclonal IgG formulation separated on a single FFF channel by two distinct methods, one optimized for aggregate detection and the other for speciation of low-order oligomers.

One of the most promising applications of light scattering to ADC characterization is the calculation of DAR. It can be performed by combining MALS with simultaneous UV absorption and differential refractometry. Similar to DAR determination by differential UV absorbance, this method exploits the distinct UV and dRI behavior exhibited by the antibody and modifier. As long as UV extinction coefficients and refractive index increments are known for the antibody and modifier (both of which are determined empirically), the molar masses and stoichiometric ratios of each group are established via a simple calculation. Figure 3 shows examples of three ADC constructs, based on the same IgG platform and modified with the different drug-linker compounds; each sample presents a distinct DAR as evaluated. Notably, ADC₁ and ADC₂ comprise homogeneous DAR across the eluted peak, as evidenced by the horizontal M_w profile (Figure 3[a] and Table I). In contrast, ADC₃ bears a distribution of modifier additions, from ~7 to ~1 (Figure 3[b] and Table I); such information would not be attainable via bulk assessment by MS or differential UV spectroscopy.

Conclusion

The light scattering toolkit provides

multiple, complementary methods for effective characterization of ADCs and determination of their chemical, thermal, and colloidal stability. The high-throughput capability of DLS is essential for accurately assessing the stability and aggregation of ADCs to evaluate product potential in linker technologies with the greatest efficiency.

The application of MALS to various fractionation regimes allows speciation of samples into subcomponent populations along with molecular weight characterization and quantitation. Most notably, FFF–MALS offers broad-resolution characterization capability for the accurate determination of very small quantities of aggregates as well as a more comprehensive understanding of the oligomeric subcomponents on a single platform.

MALS can also be used alongside UV spectroscopy and refractive index determination to quantify the drug-to-antibody ratio, an important parameter that has considerable influence on the efficacy, safety, and toxicity of ADC therapeutics.

As no single technique can independently quantify all of the necessary factors in determining the suitability of a therapeutic agent, light scattering techniques provide a comprehensive characterization platform for the screening of ADCs.

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