WHITE PAPER

WP1615: SEC-MALS for absolute biophysical characterization

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Summary

Accurate characterization of biomacromolecules is essential for successful programs of research and development in the life sciences and biopharmaceuticals. The basic biophysical properties of these molecules include molecular weight, size, conformation, degree of conjugation, aggregation and complex-forming interactions.

Size-exclusion chromatography is commonly used to separate and analyze proteins and other biomacromolecules. However, in order to reliably determine their basic biophysical properties in solution, an absolute, independent means of characterization must be added downstream of the separation step.

Multi-angle light scattering and dynamic light scattering instruments, combined with UV and RI detectors, fulfill that need, making them essential in every lab that produces, uses or characterizes proteins, peptides, nucleic acids, polysaccharides or bionanoparticles constructed of these building blocks. This article explores the technology, capabilities and applications of light scattering paired with size-exclusion chromatography for biophysical characterization.

Introduction

The need for biophysical characterization

Reliable analysis of the molecular weight (MW) of proteins in solution is essential for biomolecular research[1–4]. MW analysis informs the scientist if the correct protein has been obtained and if it is suitable for use in further experimentation[5,6]. As described on the web sites of protein networks P4EU[7] and ARBRE-Mobieu[8], protein quality control must characterize not only the purity of the final product, but also its oligomeric state, homogeneity, identity, conformation, structure, post-translation modifications and other properties.

Biophysical properties determined by light scattering

A solution-based measurement of MW identifies the form of the protein that is present in an aqueous environment. While for many proteins the goal is to produce monomers, for others a specific native oligomer is key to biological activity[9–12]. Incorrect oligomeric form or the presence of non-native aggregates will adversely impact structural determination by crystallography, NMR or small-angle x-ray scattering (SAXS); they may also create artifacts or inaccuracies in functional assays that quantify binding and interactions, e.g. isothermal titration calorimetry or surface plasmon resonance[2,13].

For biotherapeutics such as monoclonal antibodies (mAbs), solution-based MW analysis serves a similar purpose of quality control and product characterization. Excessive aggregates and fragments are indicative of an unstable product that is not suitable for human use. Regulatory agencies require careful characterization, not only

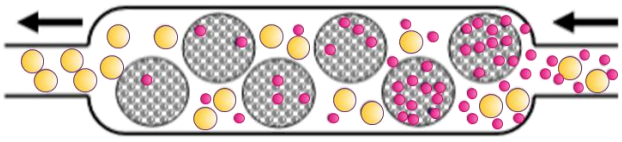
of the therapeutic molecule but also potential degradants that may be present in the final product[14–17].

Some of the most widespread methods for analyzing protein MW are SDS-PAGE, capillary electrophoresis (CE), native PAGE, mass spectrometry (MS), size-exclusion chromatography (SEC) and analytical ultracentrifugation (AUC). Of these, SDS-PAGE, CE and MS are not performed in the native state and typically lead to dissociation of oligomers, complexes and aggregates. Often they are unable to correctly analyze glycoproteins and other modified forms.

Although native PAGE does, theoretically, retain the native state, it is difficult to optimize for many proteins, and results are not very reliable. AUC, whether by sedimentation velocity or sedimentation equilibrium, is quantitative and can determine MW from first principles, but it is quite cumbersome; AUC involves much manual labor and requires significant expertise in data interpretation, long experiment time and a very expensive instrument.

Analytical SEC: promising, with caveats

SEC is a quantitative and relatively robust, simple and fast method for separating macromolecules[18–20]. However, separation of different species by SEC does not depend directly on MW; it depends on size and diffusion properties[21].



Size-exclusion chromatography separates molecules by hydrodynamic size

In analytical SEC a calibration curve, such as that in Figure 1, is constructed using a series of reference molecules, relating the MW of the molecule to its elution volume. For proteins, the reference molecules are well-behaved, globular proteins that do not interact with the column via charge or hydrophobic surface residues.

Notably, the analysis of MW in SEC relies on two key assumptions regarding the proteins to be characterized:

1. They share with the reference standards the same conformation and specific volume (in other words, the same relationship between diffusion properties and MW);
2. Like the reference standards, they do not interact with the column except by steric properties—they do not stick to the column packing via electrostatic or hydrophobic interactions.

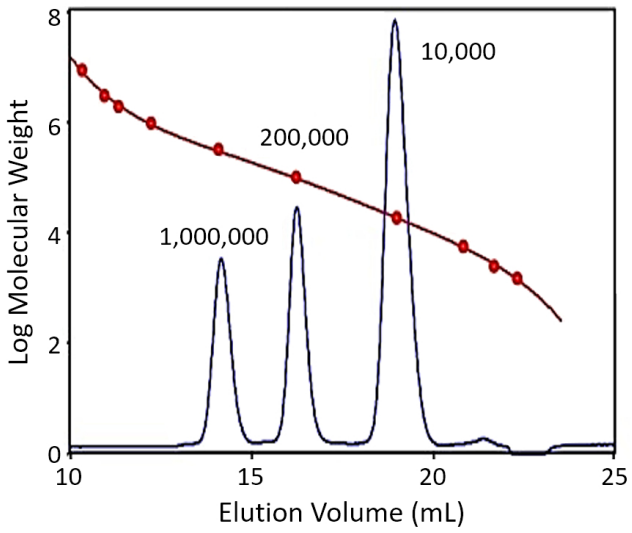


Figure 1. SEC calibration curves use reference standards to relate molecular weight to elution volume, assuming globular conformation and ideal steric interactions with the SEC column.

When these assumptions are not fulfilled, the calibration curve is invalid and its use will lead to erroneous MW values. Many classes of protein, including the ADH tetramer and kinase fragment examples in Figure 2, do not meet the assumptions:

- Intrinsically disordered proteins have comparatively large hydrodynamic radii due to their extensive unstructured regions[22,23];
- Non-spherical or linear oligomeric assemblies[10] are, by definition, non-globular;
- Heavily glycosylated proteins are also larger than pure proteins with the same overall MW[19], since glycans are generally linear rather than compactly folded;
- Detergent-solubilized membrane proteins elute from SEC according to the total size of the polypeptide–

detergent or -lipid complex rather than the oligomeric state and molar mass of the protein alone[24,25];

- Proteins with charged or hydrophobic surface residues may interact with the stationary phase and elute non-ideally depending on column chemistry, pH and salt conditions [26,27].

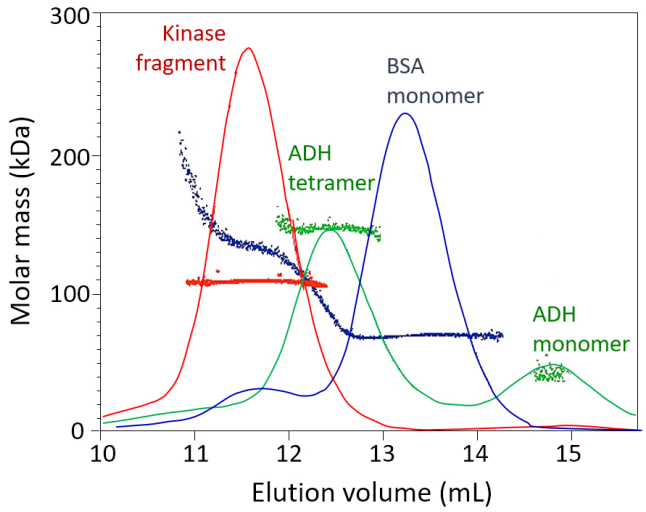


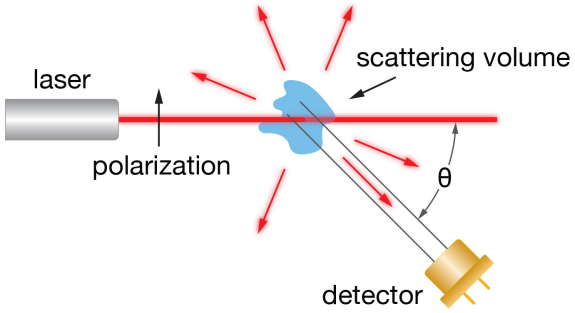
Figure 2. Elution volumes of various proteins and molar mass determined by MALS. ADH tetramer elutes later than BSA dimer even though it has larger molar mass, while a lower molar mass kinase fragment elutes at the same volume as BSA dimer. See AN1607.

The solution: light scattering

SEC becomes much more versatile and reliable for MW determination when combined with multi-angle light scattering (MALS), UV_{280} and differential refractive index (dRI) detectors[3,4,11,28–31]. The UV detector measures protein concentration via absorbance at a wavelength of 280 nm. The dRI detector determines concentration based on the change in solution refractive index due to the presence of the analyte. The MALS detector measures the proportion of light scattered by an analyte into multiple angles relative to the incident laser beam. Collectively known as SEC-MALS, this configuration determines MW independently of elution time since MW can be calculated directly from first principles using Equation 1,

$$M = \frac{R(0)}{Kc\left(\frac{dn}{dc}\right)^2} \qquad (1)$$

where M is the molecular weight of the analyte, $R(0)$ the reduced Rayleigh ratio (i.e., the amount of light scattered by the analyte relative to the laser intensity) determined by the MALS detector and extrapolated to angle zero, c the weight concentration determined by the UV or dRI detector, dn/dc the refractive index increment of the analyte (essentially the difference between the refractive index of the analyte and the buffer), and K a system constant[28].



Multi-angle light scattering measures light scattered by the analyte into several angles relative to the laser beam.

In SEC-MALS, the SEC column is used solely to separate the various species in solution so that they enter the MALS and concentration detector cells individually. The actual retention time has no significance for the analysis except as far as how well the proteins are resolved. Since the instruments are calibrated independently of the column and do not rely on reference standards, SEC-MALS is considered an 'absolute' method.

MALS can also determine the size (physical dimension) of macromolecules and nanoparticles with diameter larger than about 25 nm by analyzing the angular variation of the scattered intensity[28]. For smaller species such as monomeric proteins and oligomers, a dynamic light scattering (DLS) module may be added to the MALS instrument in order to measure radii from 0.5 nm and up[32].

While either UV or dRI concentration analysis may provide the value of c in Eq. 1, use of dRI is preferred for two reasons: 1) dRI is a universal concentration detector, suitable for analyzing molecules such as sugars or polysaccharides that do not contain a UV chromophore; and 2) the concentration response dn/dc of almost all pure proteins in aqueous buffer is the same to within one or two percent (0.185 mL/g)[33], so there is no need to guess or calculate from sequence the UV extinction coefficient.

Instrumentation

SEC

SEC-MALS detectors generally work with any good-quality size exclusion chromatograph including HPLC, UHPLC or FPLC systems. In most cases the detectors may be simply added downstream of the LC's UV detector with appropriate interfacing to the UV analog output signal and an auto-inject contact closure switch. Wyatt detectors are most commonly used with HPLC or UHPLC systems from Agilent, Waters, Thermo, and Shimadzu, and with FPLC systems from GE and Bio-Rad.

The DAWN 18-angle MALS detector provides the highest sensitivity and widest measurement range for HPLC-, FPLC and FFF-MALS.

MALS detectors

Wyatt Technology's DAWN® is the premier MALS detector for HPLC and FPLC, offering the highest sensitivity, widest measurement range and most options:

- Range of molar mass: 200 Da -1 GDa (SEC typically works for proteins up to a few million Daltons, but the DAWN may be used with other separation techniques such as FFF to address the upper range)
- Range of rms radius R_g: 10 – 500 nm using the angular dependence of scattering
- Sensitivity rating: 200 ng injected mass of monomeric BSA in PBS on a standard 7.8 mm x 300 mm SEC column
- Number of detection angles: 18, which determine the size range covered and add built-in redundancy to overcome the most common source of noise in SEC-MALS, particulates shed by the column
- Temperature control options: ambient,-20 °C to +150 °C and room temperature to +210 °C

Wyatt's miniDAWN® is a basic MALS detector that offers a slightly lower measurement range and fewer options than the DAWN, but is still appropriate for most SEC work:

- Range of molar mass: 200 Da - 10 MDa
- Range of rms radii R_g: 10 - 50 nm
- Sensitivity rating: 500 ng of BSA monomer in PBS, injected on a standard SEC column.
- Number of detection angles: 3

The miniDAWN 3-angle MALS detector covers the entire size range of standard HPLC- or FPLC-SEC.

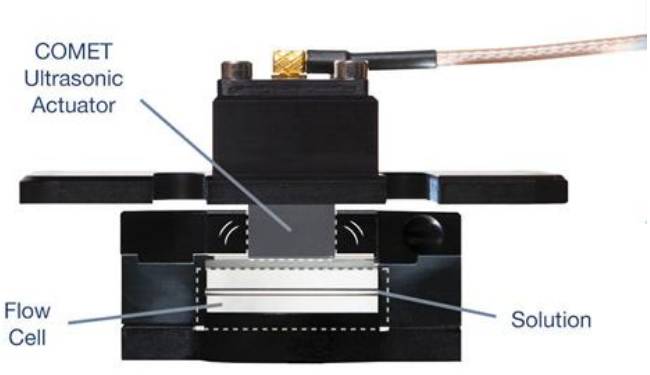
The only MALS detector designed specifically for UHPLC's low-volume peaks is Wyatt's microDAWN®. It is similar to the miniDAWN in terms of number of angles and the ranges of molar mass and size, with a sensitivity rating of 70 ng of BSA monomer when injected on a 4.6 mm x 150 mm UHP-SEC column with sub-2 µm beads.

The microDAWN 3-angle MALS detector works with UHP-SEC.

Additional MALS features

A unique feature common to all three MALS instrument is the Forward Monitor (FM) detector which measures light transmitted through the cell. While the FM has several uses, one of the most important is for analysis of molecules that absorb at the instruments' laser wavelength of 660 nm, e.g. heme-containing proteins. The FM detects and compensates for this absorption phenomenon in order to report the correct MW, which otherwise would be incorrect.

The DAWN, miniDAWN and microDAWN all include a built-in ultrasonic flow cell cleaner to minimize manual cell cleaning, and are modular for rapid field service. Indicators on the front panel let the user know when the SEC-MALS system is equilibrated, clean and ready to make high-quality measurements.



The COMET module applies ultrasonic agitation to dislodge particles from the glass, reducing optical noise.

The DAWN may also be fitted with fluorescence-blocking filters in case of fluorescently-tagged molecules or other analytes that fluoresce under 660 nm excitation, in order to provide accurate molecular weights.

DLS detectors

In order to minimize flow paths and dispersion, Wyatt's online DLS detection options utilize the MALS flow cell and laser beam. DLS detection may be configured in two ways:

1. A WyattQELS™ embedded DLS module, connected via optical fiber to the flow cell, resides inside the MALS detector; or
2. An external, stand-alone DLS detector is reconfigured to connect via optical fiber to the MALS flow cell. Both the DynaPro® NanoStar® cuvette-based DLS detector and the Mobius® flow-through DLS/PALS detector offer this interoperability.

dRI detectors

The preferred dRI detector for use with SEC-MALS is Wyatt's Optilab®. Benefits of the Optilab:

- Sensitivity rating: $7.5{\cdot}10^{-10}$ RIU, equivalent to the best HPLC dRI detectors on the market
- Wavelength-matched to the DAWN and miniDAWN for maximum accuracy in molar mass determination;
- Read digitally by Wyatt's chromatography software in order to take full advantage of its sensitivity
- Range: $\pm 4.7{\cdot}10^{-3}$ RIU, 10-20x more than standard HPLC dRI detectors, with no need to switch gain settings or loss of sensitivity
- Hardware timebase synchronization with Wyatt MALS detectors to eliminate drift between the signals
- Never needs recalibration

A high-concentration version of the Optilab is available for specialized measurements such as semi-preparative MALS and coupling of MALS to ion-exchange chromatography[34,35]. It is similar to the Optilab save for a higher range: $-2.6{\cdot}10^{-3}$ to $+3.4{\cdot}10^{-2}$ RIU, and slightly lower sensitivity, $1.5{\cdot}10^{-9}$ RIU.

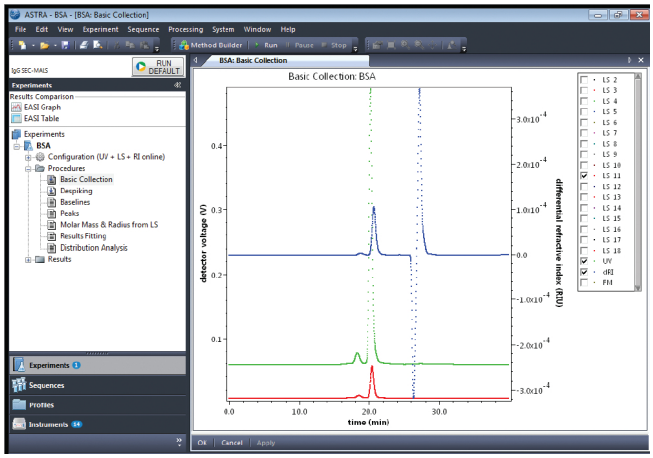
For UHPLC SEC-MALS, Wyatt offers the microOptilab® which is similar to the Optilab save for reduced volume and slightly lower sensitivity, $1.5 \cdot 10^{-9}$ RIU. The microOptilab couples with the microDAWN in UHPLC SEC-MALS.

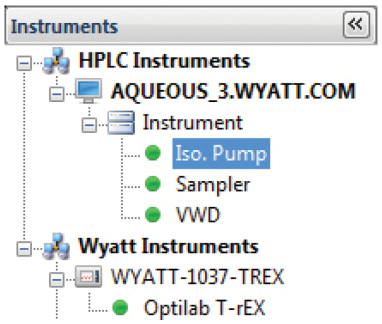
In lieu of an Optilab or microOptilab, standard (U)HPLC dRI detectors may be used. However, they require use of analog output signals which may reduce effective sensitivity. They will usually use a broadband light source or a narrow-band source at a wavelength different from the MALS detector and are not timebase-synched in hardware, reducing MW accuracy.

Software

ASTRA® software for SEC-MALS is required for use with Wyatt's MALS, DLS and dRI detectors. It offers robust data acquisition, straightforward data processing and a comprehensive set of analyses for biophysical characterization including molar mass, size, distributions and averages, percent aggregate, percent recovery, conjugate analysis, conformational analysis and determination of extinction coefficient. Key results for multiple samples may be consolidated into one table (EASI Table) and the graphical data such as chromatograms, absolute MW or size versus elution volume, and distributions consolidated into one graph for side-by-side comparison (EASI Graph).

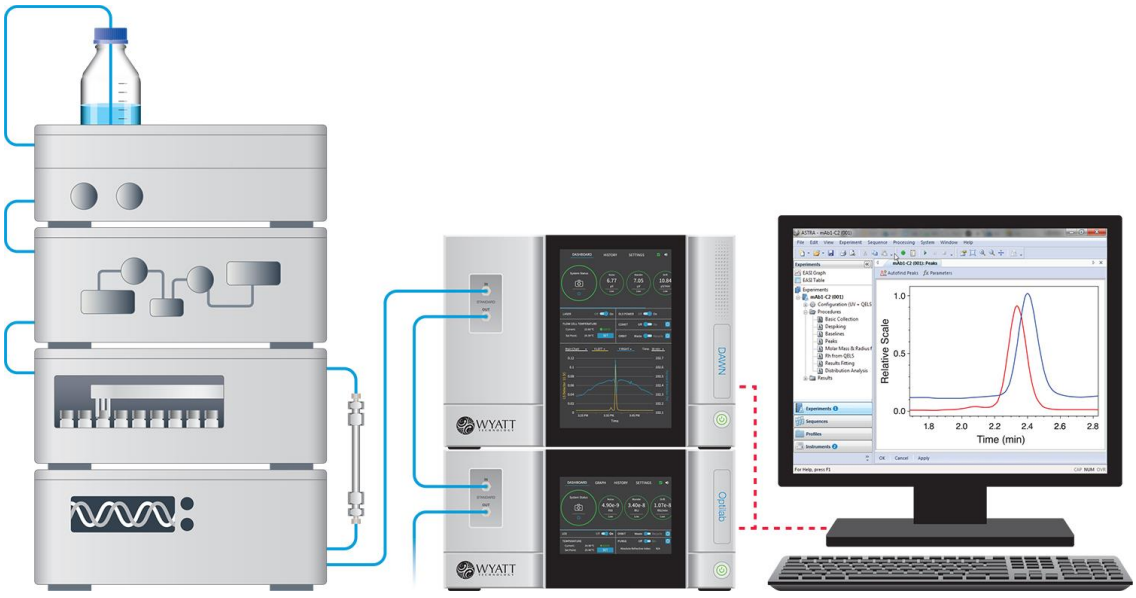
ASTRA may be set up to control select HPLC modules such as pumps, UV detectors and autosamplers or may be used side-by-side with native HPLC software.





Control of industry-leading HPLC modules is integrated into ASTRA alongside control of Wyatt instruments.

Reports are customizable, allowing for as much or as little information as desired. For GMP use, 21CFR(11) data integrity and administrator hierarchy support is available.



A complete SEC-MALS experimental setup for protein analysis includes a standard HPLC or FPLC system with UV detector, an appropriate column, a DAWN or miniDAWN MALS instrument and an Optilab dRI instrument. The microDAWN and microOptilab are used with UHPLC-based SEC.

Applications of SEC-MALS

Monomers, oligomers, aggregates and impurities

The use of SEC-MALS in protein research is quite extensive. By far the most common applications are establishing whether a purified protein is monomeric or oligomeric and the degree of oligomerization, and assessing aggregates[3,10,11,17,30,36–38].

Quality control

A protein purification run often does not completely eliminate all undesirable forms or impurities. As shown in Figure 3, SEC-MALS readily identifies and quantifies the purity and homogeneity of the protein. Uniform molar mass, calculated independently at each elution slice, is found across the monomer peak and the well-resolved soluble oligomers. Where the species are not fully resolved by SEC, the molar masses determined by MALS decrease with increasing elution volume.

Of particular note is the shoulder on the trailing edge of the monomer peak. Such shoulders can arise from a few causes:

- Tailing resulting from protein sticking to the column;
- Dynamic dissociation of complexes as the concentration decreases;
- Low-molecular-weight species.

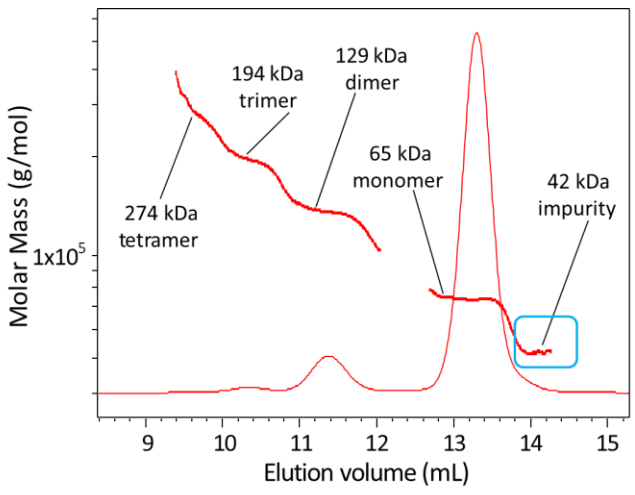


Figure 3. BSA monomer, soluble aggregates and a low-molecular-weight shoulder identified as a fragment using FPLC and a GE Increase SEC column. Molar masses determined by MALS overlaid with UV chromatogram.

While simple SEC-UV cannot determine to which of these the shoulder corresponds, MALS-dRI immediately provides the answer – here a 42 kDa fragment. Though the protein is unknown *a priori* and hence the UV extinction coefficient is unknown, dRI can always be used to analyze unknown proteins.

Monoclonal antibody aggregates

The aggregates produced upon stress or aging of therapeutic IgG must be thoroughly characterized for regulatory filings and biosimilarity assessments. This need is met by separating on UHP-SEC and analyzing online by MALS. In particular, a microDAWN-microOptilab setup paired with 30-cm BEH UHPLC column provides excellent characterization capabilities, as shown in Figure 4, where the very low dispersion of the instruments preserves peaks that are very close in molecular weight.

The instruments' sensitivity permits robust characterization even when the height of each aggregate peak is less than 1% of the main monomer peak. The molar masses of the distinct peaks correspond to those of a complete dimer as well as dimers missing one heavy chain (or two light chains), one heavy + one light chain, and two heavy chains.

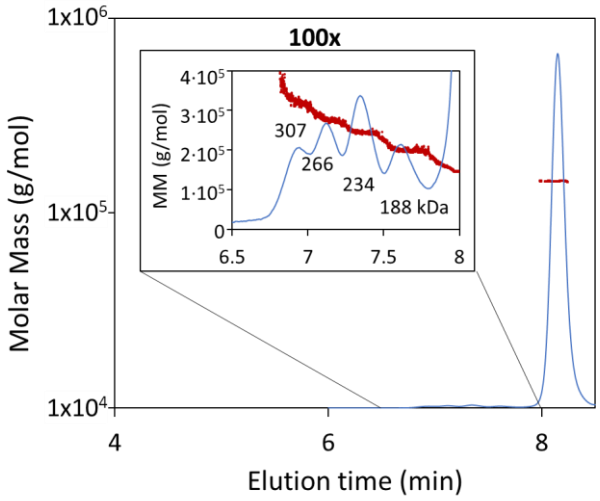


Figure 4. High-resolution UHP-SEC separation of aggregates of a stressed IgG, molar masses determined by MALS (red) overlaid with dRI chromatogram. The various peaks correspond to full dimer (307 kDa) and combinations of dimer with missing light and heavy chains.

Monoclonal antibody fragments

Stressed IgG may degrade into fragments as well as aggregate. UHP-SEC using 30-cm BEH columns provides excellent resolution of monoclonal antibodies, aggregates and fragments, demonstrated in Figure 5. Here peaks eluting later than the IgG monomer at 8 minutes are suspected to be fragments based on their molar masses, which correspond to dual heavy chain, single heavy chain or dual light chain, and single light chain. The analysis utilizes dRI measurements for concentration since the species are not known *a priori*.

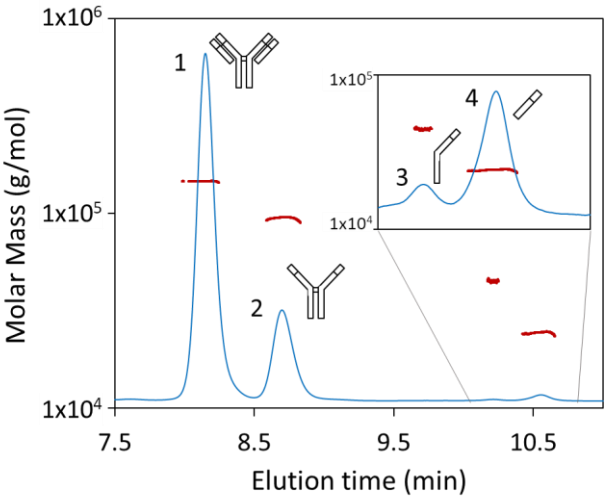


Figure 5. Fragments produced upon stressing a monoclonal antibody are well-separated by UHP-SEC and a 30-cm BEH column with 1.8 µm beads, molar masses determined by MALS (red) overlaid with dRI chromatogram. The inset shows the late-eluting portion magnified 50x. Molar masses determined by MALS and dRI correspond to the expected degradation products.

Peak	Mw [kDa]	Extinction Coefficient [mL/(mg·cm)]
1	145	1.53
2	95	1.54
3	45	1.53
4	24	1.46

Table 1. UV280 extinction coefficients determined from SEC-UV-dRI analysis of the monomer and purported fragment peaks. The nearly identical values confirm that these are, in fact, fragments of the monomer.

Confirmation of this assignment is provided by analyzing the UV extinction coefficient. The analysis consists of comparing the areas of the peaks in UV and dRI. Table 1 lists the calculated extinction coefficients, showing that the late-eluting peaks have the same extinction coefficient as the monomer and therefore are, in fact, fragments.

Insulin oligomerization under different buffer conditions

Figure 6 shows the results of analyzing insulin in two buffers, one of which (Sample 1, red) maintains mostly monomers while the other (Sample 2, blue) promotes self-association[39]. MALS clearly identifies the uniform molar mass across the main peak of Sample 1, including the trailing edge which in this case is simply tailing. Conversely, for Sample 2, the primary peak—including its trailing edge—is hexameric, which would not be deduced from the UV trace alone.

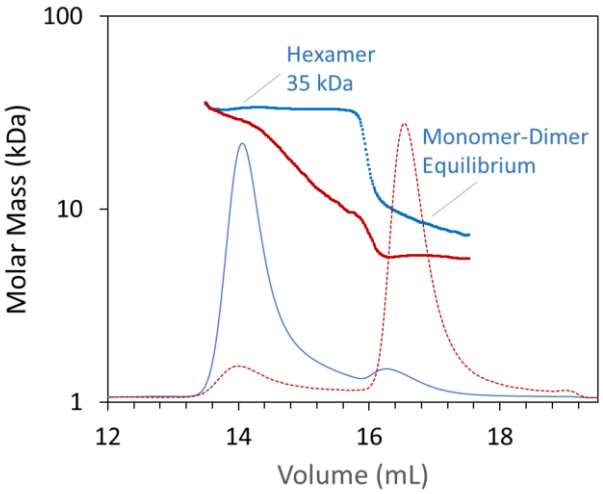


Figure 6. UV chromatograms and molar masses from MALS of insulin under two different buffer conditions. Sample 1 (red dashed line) is primarily monomeric with small aggregates that reach hexamer. Sample 2 (blue solid line) is primarily hexameric in form, with a small amount of protein in monomer-dimer equilibrium. See AN1605.

The secondary peak of Sample 2 is shown by MALS to transition from dimer to monomer. While a single experiment cannot determine if this shift is a result of poorly-resolved, irreversible dimers or dynamic equilibrium, a further experiment presented in the application note injected different concentrations of Sample 2 and showed

unequivocally that the equilibrium shifts with concentration, a hallmark of self-association in dynamic equilibrium.

Large aggregates and different conformation

In contrast to the low-molar-mass protein of Figure 6 that do not aggregate beyond hexamer, Figure 7 presents the SEC-MALS analysis of two high-molar-mass proteins that aggregate extensively. Both apoferritin and IgM exhibit well-resolved monomer, dimer and trimer peaks with uniform molar masses across each as determined by MALS, with unresolved aggregate tails extending into the tens of millions of Dalton.

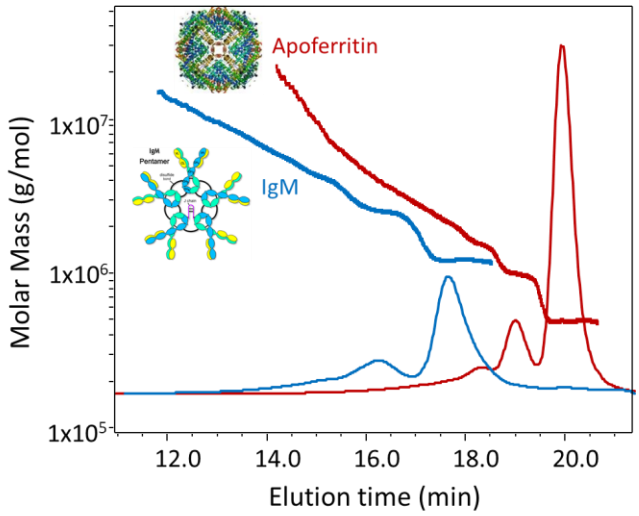


Figure 7. SEC-MALS analyses of two proteins with very different conformations that exhibit extensive aggregation, well beyond dimer and trimer, into the tens of millions of Da. The dimer of apoferritin elutes at a very different volume than the monomer of IgM even though they have approximately the same molecular weight, due to different conformations.

Notably, the apoferritin dimer has about the same molar mass as IgM but elutes at a very different time. This is a consequence of their very different conformations – apoferritin is globular while IgM is extended and partially glycosylated. Despite the different elution behavior, MALS has no problem ascertaining the correct MW values.

Aggregation due to labeling

Labeling a protein can often affect its behavior in solution and on SEC. As described in detail in AN1606: Protein Aggregate Assessment of Ligand Binding Assay (LBA) Reagents Using SEC-MALS, ELISA-based ligand-binding assays used to measure levels of biologic drugs or anti-drug antibodies depend on reliable reagents. The reagents are antibodies labelled with biotin and digoxigenin. SEC may be used for LBA reagent quality control, but SEC-MALS is required for reliable interpretation of the purity and aggregate forms present. Figure 8 shows the difference in retention time induced by the label (despite maintaining an identical and fully homogeneous molar mass) as well as different aggregation levels and forms present, relative to the unlabeled antibody.

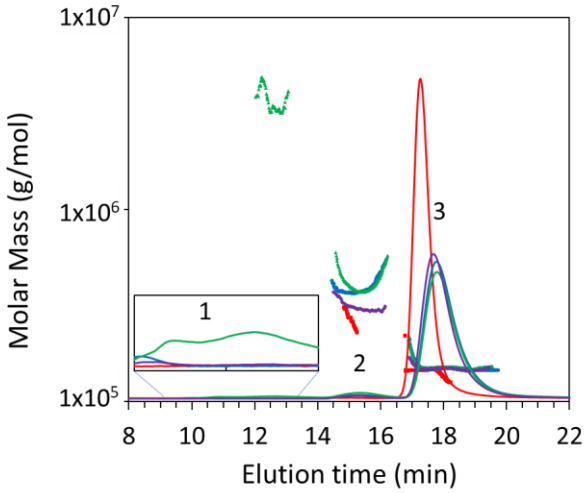


Figure 8. SEC-MALS results for a monoclonal antibody drug, unconjugated (red), and three different lots conjugated to digoxigenin for use in ELISA-based ligand-binding assays (blue, purple, green). LS chromatograms overlaid with MALS data (symbols). The conjugate increases retention time of the monomeric species and increases aggregate levels, affecting the efficacy of the assay. See AN1606.

Protein complexes

SEC-MALS is used productively in structural biology and structural virology to investigate the formation and absolute stoichiometry of biomolecular complexes[9–12,23,25,40–44]. A key benefit is the ability to determine the molecular weight of all types of complexes, whether non-globular or inherently disordered, even if the components are not entirely proteinaceous; the formation (or lack thereof) and absolute stoichiometry (as opposed to stoichiometric ratio) of heterocomplexes including protein-protein, protein-nucleic acid and complexes[23,41,42,44–47] and determining the monomer-dimer equilibrium dissociation constant.[41,43,48]

Oligomerization of wild type and mutants

The native oligomeric state of many proteins is dimeric, trimeric, tetrameric or hexameric. Mutations are often used to probe the specific domain responsible for oligomerization, exemplified in AN1610: Stoichiometry of Intrinsically-Disordered Protein Complexes. As shown in Figure 9, different mutations can modify the native oligomer from tetramer to dimer and even monomer. However, the tetramer is not extremely stable under these conditions and the SEC-MALS-derived MW exhibits dissociation at decreased concentrations, on the leading and trailing edges of the peak.

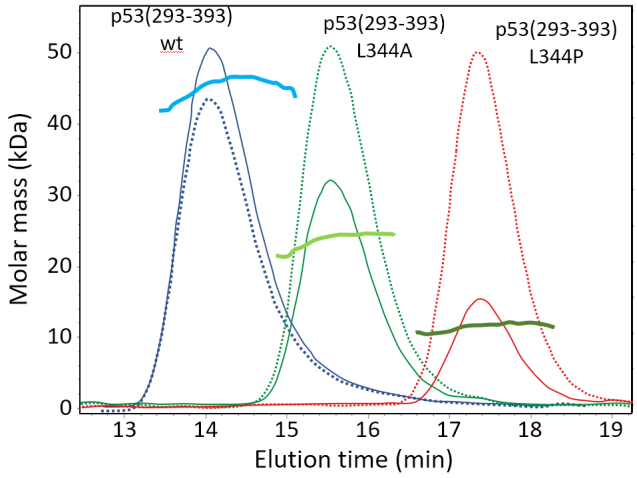


Figure 9. Wild-type p53 DNA-binding protein forms tetramers in solution while the L344A and L344P mutations only form dimers and monomers, respectively. Solid chromatograms are light scattering intensity while dashed chromatograms are refractive index signals. Symbols indicate molar mass from MALS. The pronounced concentration dependence of the w.t. molar mass indicates dynamic equilibrium, presumably between dimers and tetramers. See AN1610.

Protein-protein complexes

While traditional titration assays can only determine the molar ratio of proteins in a heterocomplex, the additional information provided by SEC-MALS enables the confirmation of absolute stoichiometry, i.e. the number of copies of each type of protein in the complex. This is accomplished by incubating different ratios of the two proteins and measuring the resulting molar masses by SEC-MALS. AN1610: Stoichiometry of Intrinsically-Disordered Protein Complexes further describes a series of experiments designed to study the complexes formed by p53 wild type and mutants, with S100B, a native dimer.

Figure 10 presents the SEC-MALS results for the L344P mutant. At excess L344P, substantial amounts of dimeric S100B and monomeric L344P are found, along with small amounts of complex. As the relative amount of S100B increases, more and more complexes form, though in all cases only one species is identified: one dimer of S100B bound to a single monomer of p53 mutant. The results of the complete set of experiments are summarized in Table 2.

For both mutants the complex consists of a S100B dimer and a p53 monomer, even though the L344A mutant dimerizes in the absence of S100B. Apparently the affinity of L344A for a S100B dimer is much greater than for another L344A mutant protein.

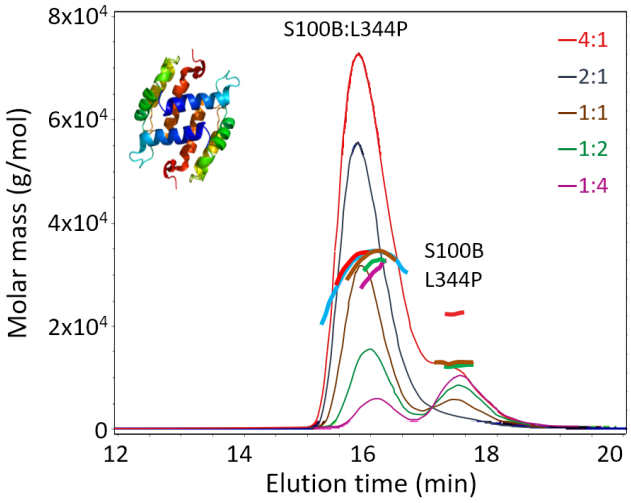


Figure 10. Formation of S110B:L344P complexes upon incubation of various stoichiometric ratios of S100B and L344P. LS chromatograms (solid lines) overlaid with MW determined by MALS (symbols). See AN1610.

Though wild type p53 binds to S100B in the same stoichiometric *ratio* as the mutants, the complex that forms is much different: four S100B dimers bind to a tetramer of p53, the functional oligomer. The overall affinity of S100B for p53 is not very high: relatively weak dynamic equilibrium is indicated by the decrease of molar mass away from the apex of each peak.

Native state:	S100B dimer	w.t. tetramer	L344A dimer	L344P monomer
Stoichiometry	Complex forms with S100B?			
1:1		-	-	-
2:1		-	√	√
2:2		-	-	-
4:1		-	-	-
8:4		√	-	-

Table 2. Absolute stoichiometry of complexes that form between S100B and p53, wild type and mutants. For both mutants the complex consists of a S100B dimer and a p53 monomer, even though the L344A mutant dimerizes in the absence of S100B.

Protein-nucleic acid complexes

ASTRA offers a powerful method for analyzing binary complexes, *Protein Conjugate Analysis*, described in more detail below. This method is applicable when the two components differ sufficiently in either UV extinction coefficient, differential refractive increment, or both. While the analysis is not suitable for most protein-protein complexes, it often is for protein-nucleic acid complexes because of strong absorption at 280 nm by nucleic acids relative to proteins.

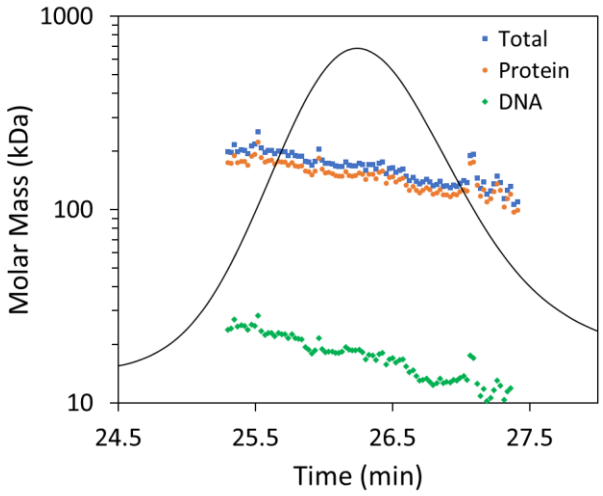


Figure 11. Analysis of prototype foamy virus intasome bound to U5 DNA using ASTRA's *Protein Conjugate Analysis* method. UV chromatogram (solid line) overlaid with molar mass values of protein, DNA and total, at each elution slice (symbols). See WP3001.

The analysis of a complex between the prototype foamy virus integrase (PFV IN) protein and a DNA segment, U5, is described in WP3001: SEC-MALS and CG-MALS characterize protein-DNA interactions. PFV IN is a native ~170 kDa tetramer. U5 consists of 19 base pairs, equivalent to 11 kDa. Figure 11 presents the results of the analysis, indicating that the intasome tetramer binds two strands of U5 to form a ~ 200 kDa complex, though some dissociation is present and the smaller PFV IN complex binds just one U5 strand. Similar analyses may be performed for small viruses[49], or for larger viruses using FFF separation.

Transient complexes

As an aliquot of solution containing protein complexes in dynamic equilibrium passes through a size-exclusion column, the complexes are diluted and possibly sheared, resulting in partial dissociation. On the one hand, this phenomenon complicates analysis of the complex itself, but on the other hand is beneficial in probing the presence of dynamic equilibrium and binding affinity. In some instances is may be utilized to estimate the monomer-dimer equilibrium dissociation constant[41,43,48] as shown in Figure 12 and demonstrated for a domain antibody in AN1608: Transient Protein Self-Association Determined by SEC-MALS. For more robust characterization of self-associating and hetero-associating proteins, to determine K_d and absolute stoichiometry, MALS is used with composition gradients, CG-MALS[50].

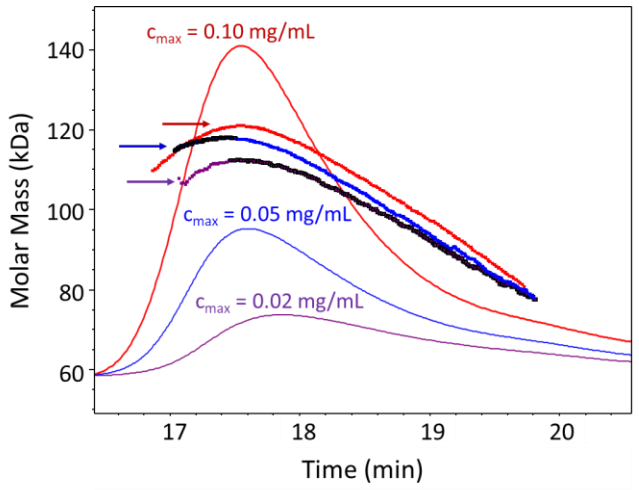


Figure 12. Analysis of a transiently-associating dimer with injection of three protein quantities. Each quantity results in a different concentration profile across the peak. The molar masses determined by SEC-MALS reach a maximum in the vicinity of the apex of the peak and decreases on either side, indicating dissociation. See AN1608

Conjugated proteins

Proteins are often conjugated to other materials, whether naturally (as in glycoproteins) or synthetically (as in PEGylated proteins or antibody-drug conjugates, ADCs). Conjugation typically causes great deviation from the MW/R_h ratio of unmodified globular proteins, imparting large uncertainties to methods such as analytical SEC, SDS- or native PAGE. Conversely, the added moiety could interact with the SEC column and change the elution properties for other reasons.

Standard two-detector SEC-MALS cannot usually provide the most accurate characterization of such conjugates because the concentration response of the specific detector (UV or RI) is different for each component. In this case, a three-detector technique, combining MALS, UV *and* RI is applied[4,30]. The results provided upon analysis in ASTRA are not just the molecular weight of the entire complex, but the masses of the protein and modifier individually as well. The analysis also provides the protein fraction and the overall weight-average specific refractive index increment *dn/dc*. This analysis can be applied to establishing the degree of post-translational modification and polydispersity of glycoproteins, lipoproteins and similar conjugates[4,30,46,51–53]. The ability to analyze detergent-solubilized membrane proteins that cannot be characterized by traditional means is especially prized, and detailed protocols for this have been published[30,54–58].

Post-translational modifications in different cell lines

Choice of cell line for protein expression is crucial for glycoproteins, since the degree of glycosylation will vary with cell type. Figure 13 illustrates the differences and similarities of a glycoprotein expressed in two different cell lines, one insect and the other mammalian. ASTRA's *Protein Conjugate Analysis* method indicates that the protein components of both samples are, as expected, identical in molar mass and uniformity across the chromatographic peaks (solid lines). On the other hand, the degree of glycosylation varies about 50% between the cell lines, with mammalian cells producing higher degrees of glycosylation. In addition to the total amount of glycans, the heterogeneity is also determined through the glycan mass at each elution volume.

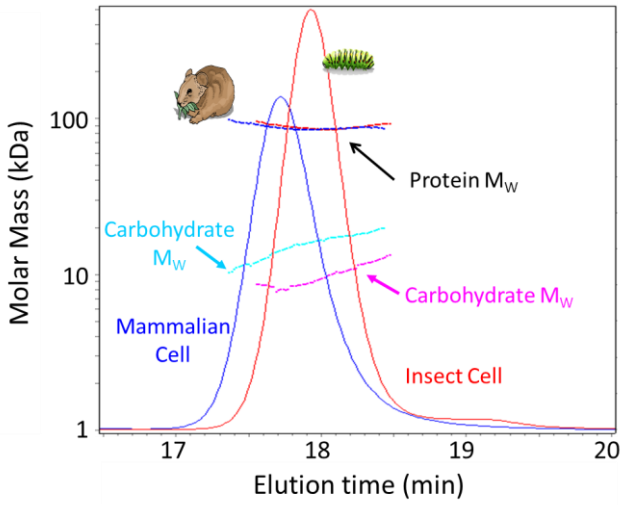


Figure 13. Conjugation analysis by SEC-MALS of a glycoprotein expressed in two different host cells, insect and mammalian. UV chromatograms (solid lines) overlaid with molar mass values (symbols).

Membrane proteins

Detergent-solubilized membrane proteins are partially enveloped by amphiphilic molecules that enlarge their hydrodynamic volume greatly relative to the molar mass of the pure protein. Therefore it is impossible to rely on column calibration with globular proteins, or native PAGE, to determine the molar mass and quaternary state of the protein. These complexes must be analyzed by means of SEC-MALS-UV-RI, which calculates not only the protein mass but also that of the detergent or other modifier. An example is provided in Figure 14, which tests the most appropriate detergent for retaining the native/functional oligomeric state of the protein.

In the analysis, described in more detail in the Application Note, LDAO is found to lead to monomeric CorA protein. However, the functional configuration is a pentamer, which was maintained with DDM (though some dissociation is observed). Hence DDM is a suitable detergent for solubilizing functional CorA. AN additional example is provided in AN1602: Lipid-Membrane Protein Complexes.

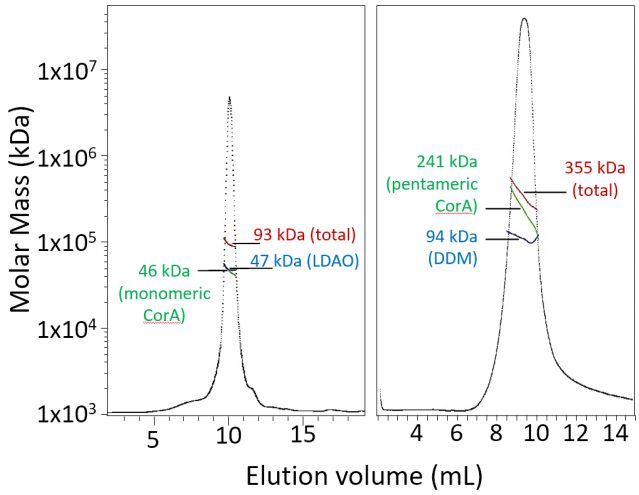


Figure 14. UV Chromatograms of CorA membrane protein solubilized in LDAO (left) and DDM (right) overlaid with molar mass values of the protein, detergent and total determined by ASTRA's protein conjugate analysis. Despite the sharp elution profile in LDAO, only DDM maintains the functional, pentameric form. See Application Note.

PEGylated proteins

PEGylation is used to enhance PK/PD properties of therapeutic proteins and peptides, increasing the half-life in the blood stream. SEC-MALS-UV-dRI analysis is uniquely suited to provide quantitative analysis for process development and quality control of PEGylated drug product, since it indicates the nature of the molecule in each elution volume (protein, PEG or PEGylated protein), the degree of PEGylation, and the monomeric or aggregation state.

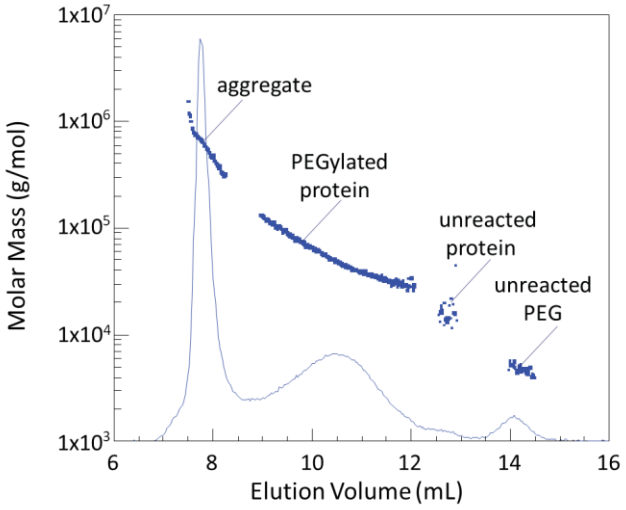


Figure 15. SEC-MALS analysis of a PEGylation process, showing the LS chromatogram (solid line) overlaid with molar mass determined at each elution volume by MALS-UV-dRI analysis (symbols). See AN1612.

The results of such an analysis in the course of process development is presented in Figure 15, with further details provided in AN1612: Protein PEGylation Processes Characterized by SEC-MALS. Similar analyses may be performed for protein-polysaccharide complexes.[45,47]

ADC drug-antibody ratio

Modifiers that make up as little 5% of the total mass in a conjugated protein may be quantified by SEC-MALS-UV-dRI. Application note AN1609: ADC drug-antibody ratio by SEC-MALS describes the results of analyzing two antibody-drug conjugates (ADC) samples based on the same mAb and drug-linker system but different conjugation processes. As seen in Figure 16, reproduced from that note, the molar masses calculated for the mAb are identical to well within experimental precision. The drug-antibody ratio (DAR), calculated from the known linker-drug mass of 1260 g/mol, is 12.6 for ADC1 and 8.1 for ADC2. Separate experiments, not shown, determined the modifier's UV extinction coefficient and dn/dc value for use in the conjugate analysis algorithm.

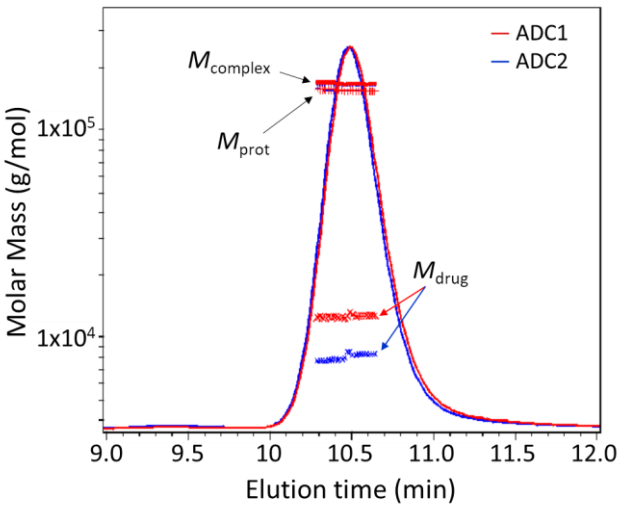


Figure 16. Conjugate analysis of two antibody-drug conjugates, with UV chromatograms (solid lines) overlaid with protein, drug and total molar masses (symbols). See AN1609.

Protein conformation

Information provided by SEC-MALS-DLS is invaluable in evaluating overall protein conformation in solution, even if circular dichroism does not indicate changes.[59,60]

Conformational stabilization by ligand binding

It is not unusual for protein-protein complexes to elute earlier than the constituent proteins due to the increased size of the complex. Later elution is not very common, but it does occur and may result either from non-ideal interaction with the column matrix, or from a reduction in overall hydrodynamic size when the ligand stabilizes a partially-disordered protein. The latter behavior is exhibited by the interleuken-4 trap : interleukin 4 (IL4) complex, depicted in Figure 17. The cause of later elution—stabilization of the partially-disordered trap by the much-smaller IL4—may be deduced from the simultaneously-acquired DLS data which show a smaller hydrodynamic radius for the complex than for the trap.

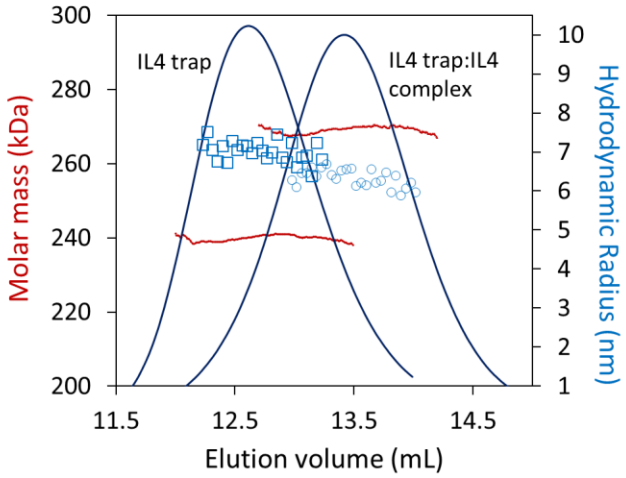


Figure 17. Conformational change in interleuken-4 trap due to binding of interleuken 4. The complex elutes later despite its higher molar mass. This counterintuitive behavior is explained by the decrease in hydrodynamic radius, measured by online DLS, rather than column interactions.

Evaluating chromatographic conditions

Of the three mAb peaks shown in Figure 18, acquired as UV chromatograms on UHP-SEC, only Peak 1 appears in the elution volume corresponding to its expected molecular weight with a nicely symmetric shape. Peak 2 is delayed and stretched as a result of hydrophobic adhesion to the SEC column packing, while Peak 3 is symmetric but elutes late due to electrostatic repulsion from the column material.

Despite the non-ideal behaviors of Peaks 2 and 3, SEC-MALS correctly identifies their molar masses. SEC-MALS often accompanies method development for optimization of the SEC column and buffer, guaranteeing that the eluting peaks continue to represent intact, unaggregated and pure protein (or other macromolecule, as the case may be).

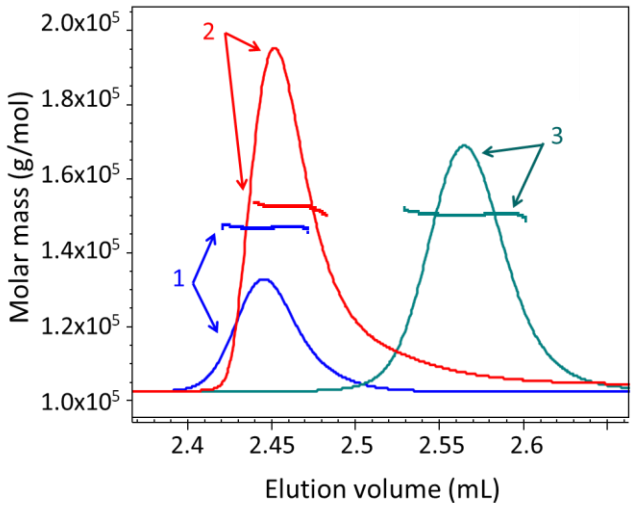


Figure 18. UV UHP-SEC chromatograms (solid lines) of three monoclonal antibodies overlaid with molar mass values determined by MALS (symbols). Despite their different elution behavior, all three have molar masses that are close in value.

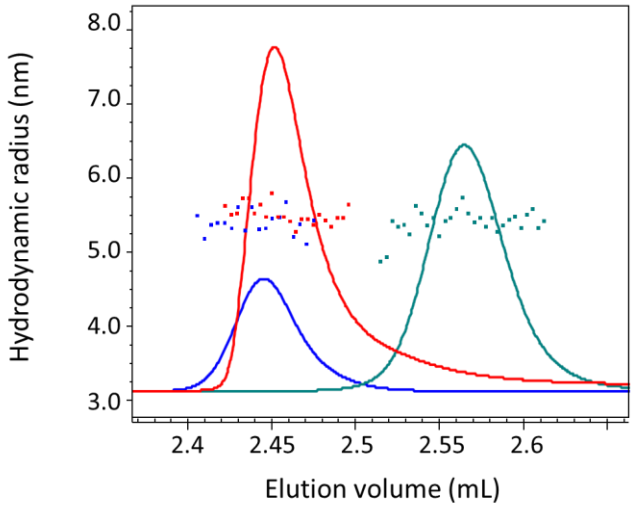


Figure 19. Same as Figure 18, with hydrodynamic radius (symbols) instead of molar mass. All three mAbs have same R_h, indicating that their different elution volumes do not derive from different conformations.

Additional information about the molecular properties and the possible cause of non-ideal elution is provided by adding online dynamic light scattering, e.g. with a WyattQELS embedded DLS module. As seen in Figure 19,

the hydrodynamic radii of all three mAbs is the same, confirming that the different elution volumes are not related to differences in conformation, but to protein-column interactions.

Additional biomolecules

Beyond proteins, SEC-MALS is invaluable for characterization of peptides[61,62], broadly heterogeneous natural polymers such as heparins[63] and chitosans.[64,65]

Small peptides

Multi-angle light scattering covers a very broad range of molar mass, from hundreds of Daltons to hundreds of millions. While most often used to characterize proteins and polymers above 10 kDa, smaller molecules are readily measured as well (as long as they can be properly separated on the column). AN1613: Peptide Characterization by SEC-MALS presents two examples of therapeutic peptides, Bradykinin (a 1060 Da peptide according to sequence) and Leucine-Enkephelin (556 Da according to sequence).

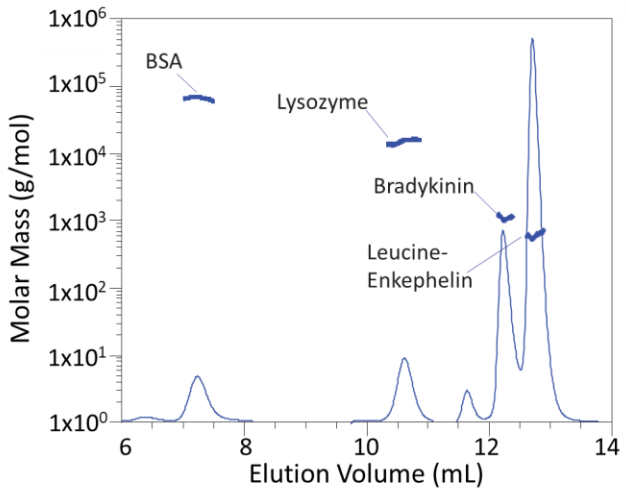


Figure 20. SEC-MALS analysis of a mixture of two standard proteins and two therapeutic peptides, Bradykinin and Leucine-Enkephelin. UV chromatogram (solid line) overlaid with molar mass values (symbols). See AN1613.

The chromatograms and molar masses are seen in Figure 20. The measured values differed by just a few percent from the sequence weights, possibly a consequence of uptake of counterions from the solution. The results were repeatable to within just 2-3%. Since small peptides do not usually have the same universal dn/dc values as proteins, their refractive increments were measured using an Optilab (they could also have been calculated from the sequence, just like the UV extinction coefficient).

Mono- and disaccharides, low- and high-molecular weight polysaccharides

Polysaccharides are, by nature, quite heterogeneous and span a broad range in molar mass. Analysis of three injected masses of maltodextrin demonstrate just a portion of the DAWN's measurement range as well as its exquisite sensitivity: even the monomer mass can be quantified with a moderate injected mass of 200 µg (because of its low mass, it scatters very little light relative to its concentration).

The molar masses of all three sample loadings overlay quite closely. This is a sign of the ideality of the chromatography, the absence of intermolecular interactions and excellent repeatability of the detectors. The observed log linearity, together with the slope of the line, are indicative of uniform, random coil conformation with no branching. With an appropriate series of columns or separation by AF4, the instruments can cover a range into the hundreds of millions of g/mol.

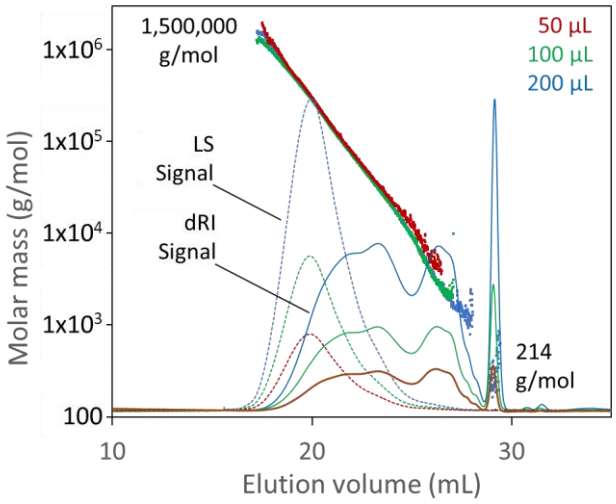


Figure 21. Maltodextrin solution, 1 mg/mL, injected at three volumes to assess sensitivity. Light scattering plots are dashed, refractive index plots are solid. Dots indicate molar masses. Molar mass values of the monomer peak were only obtained for the largest injection, 200 µg.

Supporting QC of multivalent polysaccharide vaccines

Multivalent polysaccharide vaccines contain many immunogenic components, each of which must be characterized separately. While SEC-MALS is not suitable for quality control of these multi-component mixtures, the final quality control technique must be traceable to reliable analytical methods such as SEC-MALS. In AN1306: Polyvalent pneumococcal polysaccharide vaccine by SEC-MALS, the use of SEC-MALS to characterize individual serotypes used in Merck's PNEUMOVAX 23 product is described. The analysis quantifies the reduction of polymer weight-average molar mass from 270 kDa to 110 kDa upon ultrasonication and their results correlated with rate nephelometry, an empirical method appropriate for quality control purposes.

Summary

Multi-angle and dynamic light scattering, combined with size-exclusion chromatography, are essential biophysical characterization technologies applicable across a wide range of analytes. SEC-MALS instrumentation informs research and development, both fundamental and applied, from quality control to understanding interactions.

The examples of biomolecular characterization mentioned in this document are just a few of thousands of published instances. An extensive bibliography may be found in the literature[66] and online at http://www.wyatt.com/bibliography, while application notes are available on the Wyatt web site at www.wyatt.com/AppNotes.

Acknowledgements

We greatly appreciate sharing of data and examples of using SEC-MALS to overcome characterization challenges in the application notes contributed by our customers, including (but not limited to) Tesal Desai, John Hennessey Jr., Chris Broomell, David Veesler, Manish Burman, Oliver Schoen, Kushol Gupta, Jan van Dieck, Justin Low, Mehraban Khosraviani, Sylvia (Kyung-Joo) Lee, Jihong Yang, Per-Olof Wahlund, Joey Pollastrini and Shawn Cao.

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