Video Article

Characterization of Proteins by Size-Exclusion Chromatography Coupled to Multi-Angle Light Scattering (SEC-MALS)

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URL: https://www.jove.com/video/59615
DOI: doi:10.3791/59615

Keywords: Biochemistry, Issue 148, size-exclusion chromatography, multi-angle light scattering (MALS), protein characterization, molecular weight, bovine serum albumin, oligomers, aggregates, protein-protein complexes, quality control

Date Published: 6/20/2019

Citation: Some, D., Amartely, H., Tsadok, A., Lebendiker, M. Characterization of Proteins by Size-Exclusion Chromatography Coupled to Multi-Angle Light Scattering (SEC-MALS). *J. Vis. Exp.* (148), e59615, doi:10.3791/59615 (2019).

Abstract

Analytical size-exclusion chromatography (SEC), commonly used for the determination of the molecular weight of proteins and protein-protein complexes in solution, is a relative technique that relies on the elution volume of the analyte to estimate molecular weight. When the protein is not globular or undergoes non-ideal column interactions, the calibration curve based on protein standards is invalid, and the molecular weight determined from elution volume is incorrect. Multi-angle light scattering (MALS) is an absolute technique that determines the molecular weight of an analyte in solution from basic physical equations. The combination of SEC for separation with MALS for analysis constitutes a versatile, reliable means for characterizing solutions of one or more protein species including monomers, native oligomers or aggregates, and heterocomplexes. Since the measurement is performed at each elution volume, SEC-MALS can determine if an eluting peak is homogeneous or heterogeneous and distinguish between a fixed molecular weight distribution versus dynamic equilibrium. Analysis of modified proteins such as glycoproteins or lipoproteins, or conjugates such as detergent-solubilized membrane proteins, is also possible. Hence, SEC-MALS is a critical tool for the protein chemist who must confirm the biophysical properties and solution behavior of molecules produced for biological or biotechnological research. This protocol for SEC-MALS analyzes the molecular weight and size of pure protein monomers and aggregates. The data acquired serve as a foundation for further SEC-MALS analyses including those of complexes, glycoproteins and surfactant-bound membrane proteins.

Video Link

The video component of this article can be found at https://www.jove.com/video/59615/

Introduction

Reliable analysis of the molecular weight (MW) of proteins in solution is essential for biomolecular research[1,2,3,4]. MW analysis informs the scientist if the correct protein has been produced and if it is suitable for use in further experimentation[5,6]. As described on the web sites of protein research 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.

MW measurement in non-denaturing solution identifies the form of the protein that is present in an aqueous environment, whether monomeric or oligomeric. While for many proteins the goal is to produce the monomeric form, for others a specific native oligomer is key to biological activity[9,10,11,12]. Other oligomers and non-native aggregates are undesirable and will lead to flaws in structural determination by crystallography, nuclear magnetic resonance (NMR) or small-angle X-ray scattering, as well as artifacts or inaccuracies in functional assays to quantify binding by isothermal titration calorimetry or surface plasmon resonance[2,13].

In the case of 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[14,15,16,17].

Some of the most widespread methods for analyzing protein MW are sodium dodecyl sulfate polyacrylamide gel electrophoresis (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 and aggregates, hence are not suitable for determining the native oligomer or quantifying aggregates. Although native PAGE does, theoretically, retain the native state, in our experience 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, requiring much manual labor and significant expertise in data interpretation, long experiment time and a very expensive instrument.

Analytical SEC is a quantitative and relatively robust, simple method that separates macromolecules during flow through a packed column. The principles and applications of SEC are well presented in several reviews[18,19,20] and in the handbook "Size Exclusion Chromatography: Principles and Methods"[21]. The differences in retention are due to different amounts of time spent diffusing into and out of the pores in the stationary phase before eluting from the end of the column. The differences arise (nominally) from the relative sizes and diffusion coefficients of the molecules[22]. A calibration curve is constructed using a series of reference molecules, relating the MW of the molecule to elution volume. For proteins, the reference molecules are generally well-behaved, globular proteins that do not interact with the column via charge or hydrophobic surface residues. Elution volume is measured with an ultraviolet (UV) absorbance detector. If the UV extinction coefficient is known-often calculated from the sequence-the protein peak total mass may also be quantitated.

Notably, the analysis of MW by 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) and 2) like the reference standards, they do not interact with the column except by steric properties-they do not adhere to the column packing by charge or hydrophobic interactions. Deviations from these assumptions invalidate the calibration curve and lead to erroneous MW determinations. This is the case for intrinsically disordered proteins that have large Stokes radii due to their extensive unstructured regions[23,24] or non-spherical/linear oligomeric assemblies[10]. Glycosylated proteins will generally have a larger Stokes radius than the non-glycosylated form, even when the added carbohydrate mass is taken into account[19]. Detergent-solubilized membrane proteins elute differently than calibration proteins because their elution from SEC depends on the total size of the polypeptide-detergent-lipids complex rather than the oligomeric state and molar mass of the protein[25,26]. Column chemistry, pH and salt conditions all affect elution volumes of proteins with charged or hydrophobic surface residues[27,28].

SEC becomes much more versatile and reliable for MW determination when combined with multi-angle light scattering (MALS) and differential refractive index (dRI) detectors[3,4,11,29,30,31,32]. A dRI detector determines concentration based on the change in solution refractive index due to the presence of the analyte. A 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 instrumentation determines MW independently of elution time since MW can be calculated directly from first principles using Equation 1,

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

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 an optical constant that depends on the system properties such as wavelength and solvent refractive index[29].

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 it resolves the protein species. The instruments are calibrated independently of the column and do not rely on reference standards. Hence, SEC-MALS is considered an 'absolute' method for MW determination from basic physical equations. If the sample is heterogeneous and not completely separated by the column, then the value provided at each elution volume will be a weight average of the molecules in each elution volume that flows through the flow cell per time slice, approximately 75 µL.

By analysis of the angular variation of scattering intensity, MALS can also determine the size (root-mean-square radius, R_g) of macromolecules and nanoparticles with geometric radius larger than about 12.5 nm[29]. 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 hydrodynamic radii from 0.5 nm and up[33].

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[34]; 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)[35], so there is no need to know the UV extinction coefficient.

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[3,10,11,17,31,36,37,38]. The ability to do so for detergent-solubilized membrane proteins that cannot be characterized by traditional means is especially prized, and detailed protocols for this have been published[31,39,40,41,42,43]. Other common applications include establishing the degree of post-translational modification and polydispersity of glycoprotein, lipoproteins and similar conjugates[4,31,44,45,46,47]; the formation (or lack thereof) and absolute stoichiometry (as opposed to stoichiometric ratio) of heterocomplexes including protein-protein, protein-nucleic acid and protein-polysaccharide complexes[24,46,48,49,50,51,52]; determining the monomer-dimer equilibrium dissociation constant[49,53,54]; and evaluating protein conformation[55,56]. Beyond proteins, SEC-MALS is invaluable for characterization of peptides[57,58], broadly heterogeneous natural polymers such as heparins[59] and chitosans[60,61], small viruses[62] and most types of synthetic or processed polymers[63,64,65,66]. An extensive bibliography may be found in the literature[67] and online (at http://www.wyatt.com/bibliography).

Here, we present a standard protocol for running and analyzing a SEC-MALS experiment. Bovine serum albumin (BSA) is presented as an example for separation and characterization of protein monomers and oligomers. The BSA protocol determines certain system constants that serve as a foundation for further SEC-MALS analyses including those of complexes, glycoproteins and surfactant-bound membrane proteins.

We note that SEC-MALS may be performed using standard high-performance liquid chromatography (HPLC) or fast protein liquid chromatography (FPLC) equipment from many vendors. This protocol describes the use of an FPLC system commonly found in labs that produce proteins for research and development (see **Table of Materials**). Prior to running the protocol, the FPLC system, MALS and dRI

detectors should have been installed, along with their respective software packages for control, data acquisition and analysis per manufacturers' instructions and any requisite calibration constants or other settings entered into the software. An inline filter should be placed between the pump and injector with a hydrophilic, 0.1 µm pore membrane installed.

Protocol

1. Preparation of the system

1. Connect the MALS and dRI detectors downstream of the FPLC's UV detector. Bypass the pH and conductivity detectors since they will add significant interdetector volume between the UV and MALS detectors. Use capillary tubing of 0.25 mm i.d. from the column to and between the detectors, and 0.75 mm i.d. capillary tubing on the output of the detectors to waste or fraction collector.
2. Ensure that the necessary signal connections between the FPLC and detectors have been established, including analog output from the UV detector to the MALS analog input, and digital output from the FPLC to the MALS Autoinject, via the FPLC's I/O Box.
3. Install a suitable analytical SEC column covering a fractionation range of at least 20 kDa to 500 kDa. Check the product info to determine if the column is suitable for the range of MW, pH and other properties of the sample and mobile phase.

2. Preparation of buffer, f lushing the system overnight and checking cleanliness

1. Using HPLC-grade reagents, prepare 1 L of phosphate-buffered saline with 50 - 100 mM NaCl. Filter the buffer to 0.1 µm using a bottle-top polyether sulfone filter or similar. Filter the first 50-100 mL of buffer to a waste bottle and discard, in order to eliminate particulates from the dry filters, and then filter the remainder to a clean, sterile bottle that has been washed thoroughly with filtered, de-ionized water and capped to prevent dust from entering.
   NOTE: Other mobile phase solvents such as a Tris buffer may be used if additional proteins that are preferentially dissolved in those solvents are to be analyzed.
2. Flush overnight at a flow rate of 0.5 mL/min, or as otherwise recommended by the column manufacturer, to equilibrate the column in the buffer and remove particulates. Use the FPLC's **Continuous flow** mode and ensure that the flow does not stop until all SEC-MALS runs are complete.
   1. Place the dRI flow cell in **Purge** mode during the overnight flush. Turn the purge off before beginning sample runs.
   2. When beginning the flush, gradually ramp the flow rate to prevent "column shedding" effect (or release of particles) caused by a sudden change of pressure in the column.
   3. If the system is known to be quite stable and particle-free, and in equilibrium with the desired mobile phase, replace the overnight flush with a shorter, 2-3 h flush.
3. Check system cleanliness by lightly tapping the tubing downstream of the column to release accumulated particles and observing the signal in the 90° detector on the front-panel display of the MALS instrument. Verify that the peak-to-peak noise is no more than 50 -100 µV.
4. Perform a 'blank' injection to verify that the injector is clean of particles. A 'blank' is simply the running buffer, prepared in a fresh, sterile vial.
   1. If the particle peak is no more than 1 mL in volume and no more than 5 mV above baseline, then the system is ready for samples. Otherwise, perform additional blank injections until clean, or perform maintenance to clean the injector.

3. Preparing and loading the sample

1. Prepare at least 200 µL of BSA at 1-2 mg/mL in the SEC buffer.
   NOTE: In order to prevent precipitation, BSA should never be dissolved in pure water.
2. Filter the protein to 0.02 µm using a syringe-tip filter.
3. Discard the first few drops of filtrate in order to eliminate particles from the dry filters.
4. Alternatively, centrifuge the sample at 10,000 x *g* for 15 min to enable precipitation of non-soluble aggregates and other large particles.
5. Inject 100 µL of the BSA solution into the loop.
   NOTE: This is the recommended amount of material, and more or less may be injected according to circumstances of the sample such as stability or availability. The quantity of protein required per injection varies inversely with molecular weight - twice as much protein mass is needed if the molecular weight is 33 kDa, or half that of BSA.

4. Preparation of the MALS software

1. Open **New | Experiment** from **Method** in the MALS software menu and select the **Online** method from the **Light Scattering** system methods folder. If a DLS detector is present, select the **Online** method from the **Light Scattering | With QELS** folder.
2. In the **Configuration** section, set parameters of the sample and mobile phase.
   1. In the **Generic Pump** view, set the flow rate to that used in the FPLC.
   2. In the **Generic Pump** view, **Solvent** branch, **Name** field, select **PBS**.
   3. In the **Injector** view, **Sample** branch, enter the **Name** as **BSA**, and set *dn/dc* = 0.185 (the standard value for unmodified proteins), A2 = 0, and UV extinction coefficient = 0.667 mL/(mg-cm).
      NOTE: For other proteins, the UV_{280} nm extinction coefficient may be found in the literature or calculated from its sequence using various public-domain software tools.
3. In the **Procedures** section, **Basic Collection** view, select the checkbox **Trigger on Autoinject** and set the duration of the run to 70 min so that data are collected for the entire elution until the total permeation volume of the SEC column is reached.

NOTE: The necessary amount of time may vary with column and flow rate - 35 min of collection are required for a standard 7.8 mm x 300 mm HPLC-SEC column at 0.5 mL/min.

4. Start the experiment in the MALS software by clicking on the **Run** button. It will start reading the data after receiving the pulse signal from the FPLC instrument via the MALS detector.
5. Zero the dRI signal by clicking the **Autozero** button on the instrument's front panel.

5. Preparation of the FPLC software

1. Insert the name of protein and the run in the FPLC software, in **Manual | Execute manual instructions | Set mark**.
2. Switch the injection valve from **Manual load** to **Inject** under **Flow path | Injection valve**.
3. Include a pulse signal by inserting a 0.5 s pulse under **I/O box | Pulse digital out**. This will trigger data collection in the MALS software.

6. Inject the sample into the loop. Click Execute in the FPLC software to start the experiment run.

7.Analysis of SEC-MALS BSA data

1. Perform analysis, step by step, under the **Procedures** section in MALS software.
    1. Verify that peaks appear, at approximately the same elution volume in UV, MALS and RI, by checking the **Basic Collection** view.
    2. In the **Baseline** view, define baseline for all signals (all LS detectors, UV and dRI). The baselines should be defined to indicate the level of pure solvent, preferable stretching from one side of the sample peaks to the other.
    3. In the **Peaks** view, define the peaks to be analyzed by clicking and dragging the mouse. Select the central 50% of each peak. First select the monomer peak ('Peak 1') and then the dimer peak ('Peak 2'). Verify correct values of *dn/dc* = 0.185 and UV 280 nm extinction coefficient = 0.667 for BSA under each peak.

2. Perform peak alignment, band-broadening correction and normalization procedures.
NOTE: Normally the SEC-MALS method is periodically calibrated for peak alignment, band broadening and normalization of the angular detectors to the 90° detector using a monodisperse protein with radius of gyration R_g < 10 nm such as BSA monomer. In this example, BSA serves both as the calibration molecule and is itself the subject of MW analysis.
    1. In the **Procedures | Alignment** view, select the central region of the peaks by clicking and dragging the mouse, click **Align Signals** and then **OK**.
    2. In the **Procedures | Band Broadening** view, choose the central 50% of the monomer peak. Make sure the RI detector is specified as the **Reference Instrument**, then click **Perform Fit** and **Apply** to match the UV and LS signals to the RI signal.
        1. Zoom in to the peaks to verify that they overlap very closely within the central 50-70%, then click **OK**.
        2. If the overlap is not perfect, (it may be necessary to) perform the fit and "Apply" one or two more times until the overlap is excellent.
    3. In the **Procedures | Normalization** view, select Peak 1, enter 3.0 nm as the R_g value, click **Normalize** then **OK**.
    4. In the **Procedures | Molar Mass and Rg from MALS** view, review the data to determine which, if any, detection angles should be deselected from the analysis due to excessive noise. Typically, these will be the lower angles into which dust particles scatter a relatively high intensity. Select individual slices within the peaks from the graph on the right and view the angular dependence of the inverse reduced Rayleigh ratio in the graph on the left. If the lowest (and sometimes the highest) angles consistently deviate greatly from the fit, then deselect them from the list at the bottom of the view.

3. View the graph of the results in the **EASI Graph** view. Select **Molar Mass** from the **Display** drop-down at the top of the window. Use Ctrl + click and drag to zoom in on the peak region.
4. View the final tabulated weight-average molar mass results for the monomer and dimer peaks in the Results | Report (summary) view under **Peak Results | Molar mass moments (g/mol) | Mw**. Purity is reported under **Peak Results | Mass fraction(%)**.
NOTE: Other molar mass moments are also shown; these are usually relevant to heterogeneous polymers, but not to proteins with discrete sizes. Many other results produced by the software may be included in the report such as percent mass recovery (the fraction of protein that eluted via the concentration detector relative to the amount injected), peak statistics, polydispersity, R_g and R_h moments, etc. When provided, the measures of uncertainty reflect the precision of the value cited based on the noise within the measurement series and should not be considered to represent the accuracy. The true accuracy of the reported values depends on various factors such as the accuracy of the provided *dn/dc* and extinction coefficient values, instrument calibration, etc.
5. From the **File** menu, select **Save as Method** and save the analyzed BSA data as a standard method for future measurements of all types of proteins. The normalization and band-broadening parameters determined for BSA will be carried over in the analysis.

Representative Results

Figure 1a,b show that three oligomeric forms of BSA: monomer, dimer and trimer, were well-separated on the 200 Å pore column with baseline resolution of monomer and dimer, while **Figure 2a** shows that separation on a 75 Å pore column did not achieve good monomer-dimer resolution. The latter example was included to illustrate a "poor" result; these differences in separation for the two columns may, in fact, be expected according to the manufacturer's stated separation ranges. The trimer is not fully separated from the dimer and higher oligomers are not well separated from the trimer and each other. **Figure 2b** is an example of noisy light scattering signal with particles present throughout the chromatogram, which precludes accurate MW determination.

Henceforth we focus on **Figure 1b**. The monomer, which eluted at 13.8 mL, exhibits a weight-average molar mass M_w of 64.1 ± 0.4 kDa determined by MALS and hydrodynamic radius R_h of 3.54 ± 0.01 nm. These results are in agreement with the sequence mass and known hydrodynamic radius of BSA, 66.4 kDa and 3.5 nm respectively[68], to within the usual accuracy of SEC-MALS, 5%[3,69]. The dimer, which eluted at 12 mL, exhibited a M_w value of 127 ± 1 kDa determined by MALS-as expected, twice that of the monomer to within experimental precision- and R_h of 5.68 ± 0.06 nm. The trimer peak was also observed at 11 mL with M_w of 194 ± 9 kDa determined by MALS, three times that of the monomer to within experimental precision, as expected. R_h of the trimer could not be determined due to low intensity of the DLS signal.

The molar mass points calculated across the monomer peak are uniform to within 2-5%, indicating homogeneity. It is not unusual to find a trailing shoulder with molar mass in the range of 38-50 kDa, corresponding to BSA fragments[70]. The molar mass points across the dimeric and trimeric peaks are not uniform, indicative of heterogeneity. The dimer peak is somewhat heterogeneous due to traces of trimer that bleed into the dimer peak, and the trimer peak is heterogeneous due to co-elution of poorly-resolved higher oligomers.

The signal-to-noise level of the monomer peak is quite acceptable in all three signals, over 100:1, as is the dimer peak with signal-to-noise of 40:1. Chromatogram regions beyond the protein peaks are flat, with the exceptions of a peak due to particulates near the total exclusion (void) volume in the LS trace and a (positive) salt peak and a (negative) dissolved air peak in the dRI trace, near the total permeation volume. These are pointed out in **Figure 1a**.

Level of purity can also be calculated in a SEC-MALS experiment: mass fraction of the monomeric peak in the report represents the percent of purity of the monomeric form. For BSA monomer, the calculated purity is 88%.

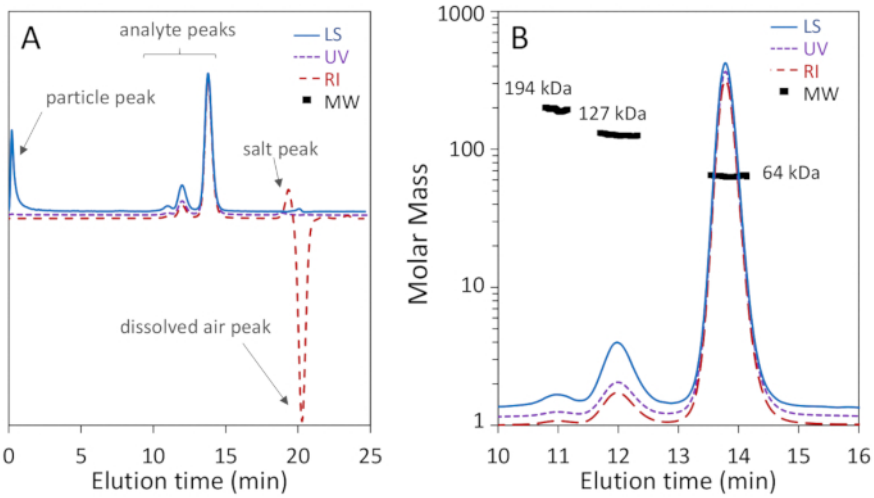

Figure 1: SEC-MALS analysis of bovine serum albumin (BSA) using a 200 Å pore size-exclusion column. Chromatogram traces are normalized to the monomer peak and offset for clarity. (**A**) Common artifacts that may be ignored are pointed out, including a particle peak near the beginning of the light scattering signal as well as salt and dissolved air peaks near the total permeation volume in the refractive index signal. (**B**) The chromatogram exhibits excellent monomer-dimer-trimer separation and the light scattering signal exhibits high signal-to-noise. The monomer and dimer MW values exhibit high homogeneity. Please click here to view a larger version of this figure.

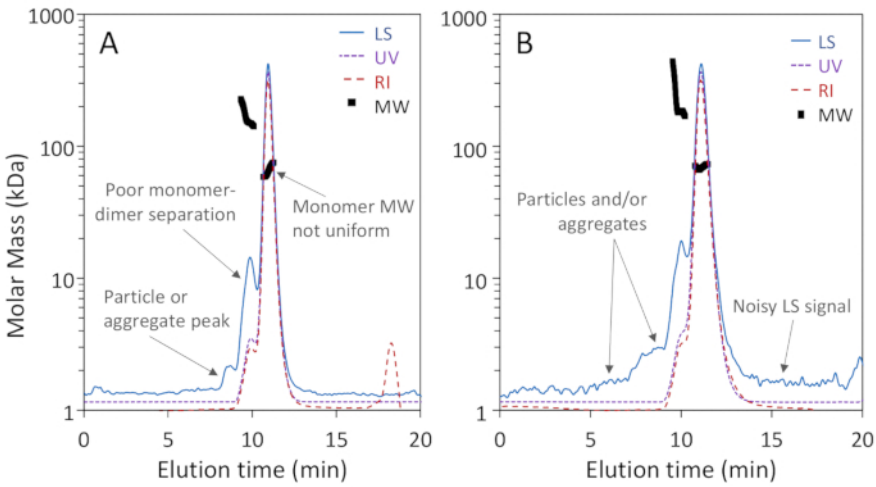

Figure 2: Examples of low-quality SEC-MALS analyses. Chromatogram traces are normalized to the monomer peak and offset for clarity. (**A)** Inadequate separation on a 75 Å pore size exclusion column; a particle peak between 8 - 9 min is not well separated from the proteins. (**B)** Inadequate signal-to-noise ratio and extensive particles adjacent to the proteins are apparent in the light scattering (LS) signal. Please click here to view a larger version of this figure.

Discussion

The SEC-MALS experiment has provided good separation of monomer, dimer and trimer, and quantitative results for the molar masses and hydrodynamic sizes of each peak. This in turn clearly identifies and characterizes each species present, as well as quantifying purity. Usually the results obtained are accurate to within 5%, and precise and repeatable to within 1-2%[3,69]. This level of precision and repeatability makes it possible to confidently distinguish between species that may be close in MW, as long as they are separated by SEC (may partially overlap within the same peak). The benefits for protein quality control and fundamental biophysical characterization are apparent.

Verification of the absence of particulates is quite important for sensitive, repeatable SEC-MALS measurements. Particle peaks generally appear as large MALS signals unaccompanied by comparable UV or RI signals. The mobile phase and sample should be prepared carefully to eliminate such particles. Use of HPLC-grade reagents or better, filtration of mobile phase and dilution buffer to 0.1 - 0.2 µm (pre-washing the filter to eliminate particles that are always present on dry membranes), maintenance of extra-clean mobile phase bottles and other glassware for SEC-MALS and extended column equilibration under flow (to remove particulates and aggregates that may have accumulated when flow was stopped or a column not in use) are all recommended. The sample should be filtered to the smallest pore size that does not remove the material of interest, usually no larger than 0.1 µm and, if possible, 0.02 µm. If the filter quickly clogs, the sample may be centrifuged, filtered in stages of descending pore size, and/or re-purified. When systems are consistently maintained with high-quality, fresh and filtered mobile phase, column shocks are prevented, samples are clean and do not adhere to the column, the measurements will not exhibit the aforementioned particulate noise and will provide high-quality data.

MALS is agnostic to typical SEC buffers for proteins; many other buffer systems besides PBS can be used to optimize separation and stability, including a variety of excipients[71]. MALS is also agnostic to the specific column, which should be selected for optimal separation and recovery. The primary concerns for excipients regarding analysis are significant changes in refractive index, leading to modification of the specific refractive index increment *dn/dc*, and excipients that absorb 280 nm when UV analysis is needed. For example, arginine is a common aggregation-reducing excipient that can dramatically affect the *dn/dc* of a typical protein, even bringing it into the negative regime (a protein with negative *dn/dc* can still be analyzed by SEC-MALS if *dn/dc* is determined empirically, but if *dn/dc* = 0 the intensity of light scattered by the protein will be null and MW analysis will be impossible). The topic of *dn/dc* values for proteins is discussed extensively by Zhao et al.[35] where it is shown that for standard aqueous buffers, the vast majority of unmodified proteins fall within 2-3% of the standard value (0.185 or 0.186 mL/g at = 660 nm), though proteins below ~ 10 kDa are more variable, and there are a few species that may go as high as 0.21 mL/g.

The MW profiles across the BSA monomer and dimer peaks in the data presented were both quite homogeneous to within 2% or less, indicating monodisperse species. Non-uniform MW values across a peak may arise from heterogeneity or improper analysis. In particular, a BSA MW profile that is concave ('smile') or convex ('grimace') could result from not correctly applying the band-broadening correction. For other proteins, a convex profile could also arise from dynamic equilibrium between monomers and oligomers, where the ratio of monomer to oligomer-and hence the apparent MW value-depends on peak concentration (BSA does not exhibit this behavior and is often used as a control sample to verify correct band broadening parameters). A MW profile that varies from the leading to the trailing edge and does not change with sample concentration is typical of a distribution of molecular weight species that are partially resolved by SEC. Dynamic equilibrium is readily distinguished from the other sources of apparently inhomogeneous distributions by injecting different total quantities of protein-the distribution will vary with dynamic equilibrium but not with a fixed distribution or incorrect band-broadening correction.

Given the constraints described above, SEC-MALS is not suitable for various tasks. It is not suitable for analysis of crude samples; the samples should be well-purified by standard affinity and polishing methods. It does not have sufficient accuracy or resolving power to identify mutants and variants of a protein or mAb with same or very close mass, and cannot be used with analytes that do not elute from or separate on a SEC column, though recently it has been shown that ion-exchange or reverse-phase chromatography can be combined with MALS to separate and characterize species that are not resolved by SEC[72,73,74]. Where protein quantities are severely constrained, SEC-MALS may not be feasible

since it typically requires 10 - 200 μg^3 and even more may be required by an FPLC system with tubing inner diameter greater than 0.25 mm; however, smaller quantities may be analyzed by UHPLC-SEC-MALS. Highly unstable proteins that aggregate upon introduction to the mobile phase are not suitable for SEC-MALS analysis, though buffer optimization using off-line dynamic light scattering may overcome this problem[75].

Despite the additional effort that SEC-MALS entails, it is invaluable for protein research and is used extensively by the academic and biopharmaceutical communities. In addition to characterization of monomers, oligomers and aggregates as described in the protocol above, SEC-MALS can characterize modified proteins such as glycoproteins (determining the MW of the protein and glycan components individually), surfactant- or lipid-solubilized membrane proteins (determining the MW of the protein and solubilizer components individually), protein assemblies such as virus-like particles, protein-protein and protein-nucleic acid complexes, polysaccharides, protein-polysaccharide conjugates, peptides and many other biomacromolecules.

Disclosures

DS is an employee of Wyatt Technology Corporation, whose MALS products are utilized in this protocol. AT is an employee of Danyel Biotech, a distributor of Wyatt MALS and AKTA FPLC instruments.

Acknowledgments

We thank Dr. Tsafi Danieli (Wolfson Centre for Applied Structural Biology, Hebrew University) for advice and collaborations. We also thank Daniel Biotech Ltd. (Rehovot, Israel) for the assistance and establishment of the analytical FPLC-MALS system utilized in this study.

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