Characterization of proteins by size-exclusion chromatography coupled to multi-angle light scattering (SEC-MALS)
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Corresponding Author:	Daniel Some, Ph.D. Wyatt Technology Corp Santa Barbara, CA UNITED STATES
Corresponding Author's Institution:	Wyatt Technology Corp
Corresponding Author E-Mail:	dsome@wyatt.com
Order of Authors:	Daniel Some, Ph.D.
	Hadar Amartely
	Mario Lebendiker
	Ayala Tsadok
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1   **TITLE:**
2   Characterization of Proteins by Size-Exclusion Chromatography Coupled to Multi-Angle Light
3   Scattering (SEC-MALS)
4
5   **AUTHORS AND AFFILIATIONS:**
6   Daniel Some[1], Hadar Amartely[2], Ayala Tsadok[3] and Mario Lebendiker[2].
7   [1]Wyatt Technology Corporation, Santa Barbara, CA, USA
8   [2]Wolfson Centre for Applied Structural Biology, The Alexander Silberman Institute of Life Science,
9   The Hebrew University of Jerusalem, Jerusalem, Israel.
10 [3]Danyel Biotech Ltd., Rehovot, Israel
11
12 Corresponding Author:
13 Daniel Some
14 dsome@wyatt.com
15
16 Co-author e-mail addresses:
17 Hadar Amartely (hadar.amartely@mail.huji.ac.il)
18 Ayala Tsadok (ayala@danyel.co.il)
19 Mario Lebendiker (mario.l@mail.huji.ac.il)
20
21 **KEYWORDS:**
22 size-exclusion chromatography, multi-angle light scattering (MALS), protein characterization,
23 molecular weight, bovine serum albumin, oligomers, aggregates, protein-protein complexes,
24 quality control
25
26 **SUMMARY:**
27 This protocol describes the combination of size exclusion chromatography with multi-angle light
28 scattering (SEC-MALS) for absolute characterization of proteins and complexes in solution. SEC-
29 MALS determines the molecular weight and size of pure proteins, native oligomers,
30 heterocomplexes and modified proteins such as glycoproteins.
31
32 **ABSTRACT:**
33 Analytical size-exclusion chromatography (SEC), commonly used for the determination of the
34 molecular weight of proteins and protein-protein complexes in solution, is a relative technique
35 that relies on the elution volume of the analyte to estimate molecular weight. When the protein
36 is not globular or undergoes non-ideal column interactions, the calibration curve based on
37 protein standards is invalid, and the molecular weight determined from elution volume is
38 incorrect. Multi-angle light scattering (MALS) is an absolute technique that determines the
39 molecular weight of an analyte in solution from basic physical equations. The combination of SEC
40 for separation with MALS for analysis constitutes a versatile, reliable means for characterizing
41 solutions of one or more protein species including monomers, native oligomers or aggregates,
42 and heterocomplexes. Since the measurement is performed at each elution volume, SEC-MALS
43 can determine if an eluting peak is homogeneous or heterogeneous and distinguish between a
44 fixed molecular weight distribution versus dynamic equilibrium. Analysis of modified proteins

45 such as glycoproteins or lipoproteins, or conjugates such as detergent-solubilized membrane
46 proteins, is also possible. Hence, SEC-MALS is a critical tool for the protein chemist who must
47 confirm the biophysical properties and solution behavior of molecules produced for biological or
48 biotechnological research. This protocol for SEC-MALS analyzes the molecular weight and size of
49 pure protein monomers and aggregates. The data acquired serve as a foundation for further SEC-
50 MALS analyses including those of complexes, glycoproteins and surfactant-bound membrane
51 proteins.
52
53 **INTRODUCTION:**
54 Reliable analysis of the molecular weight (MW) of proteins in solution is essential for
55 biomolecular research[1–4]. MW analysis informs the scientist if the correct protein has been
56 produced and if it is suitable for use in further experimentation[5,6]. As described on the web sites
57 of protein research networks P4EU[7] and ARBRE-Mobieu[8], protein quality control must
58 characterize not only the purity of the final product, but also its oligomeric state, homogeneity,
59 identity, conformation, structure, post-translation modifications and other properties.
60
61 MW measurement in non-denaturing solution identifies the form of the protein that is present
62 in an aqueous environment, whether monomeric or oligomeric. While for many proteins the goal
63 is to produce the monomeric form, for others a specific native oligomer is key to biological
64 activity[9–12]. Other oligomers and non-native aggregates are undesirable and will lead to flaws in
65 structural determination by crystallography, nuclear magnetic resonance (NMR) or small-angle
66 X-ray scattering, as well as artifacts or inaccuracies in functional assays to quantify binding by
67 isothermal titration calorimetry or surface plasmon resonance[2,13].
68
69 In the case of biotherapeutics such as monoclonal antibodies (mAbs), solution-based MW
70 analysis serves a similar purpose of quality control and product characterization. Excessive
71 aggregates and fragments are indicative of an unstable product that is not suitable for human
72 use. Regulatory agencies require careful characterization, not only of the therapeutic molecule
73 but also potential degradants that may be present in the final product[14–17].
74
75 Some of the most widespread methods for analyzing protein MW are sodium dodecyl sulfate
76 polyacrylamide gel electrophoresis (SDS-PAGE), capillary electrophoresis (CE), native PAGE, mass
77 spectrometry (MS), size-exclusion chromatography (SEC) and analytical ultracentrifugation
78 (AUC). Of these, SDS-PAGE, CE and MS are not performed in the native state and typically lead to
79 dissociation of oligomers and aggregates, hence are not suitable for determining the native
80 oligomer or quantifying aggregates. Although native PAGE does, theoretically, retain the native
81 state, in our experience it is difficult to optimize for many proteins, and results are not very
82 reliable. AUC, whether by sedimentation velocity or sedimentation equilibrium, is quantitative
83 and can determine MW from first principles, but it is quite cumbersome, requiring much manual
84 labor and significant expertise in data interpretation, long experiment time and a very expensive
85 instrument.
86
87 Analytical SEC is a quantitative and relatively robust, simple method that separates
88 macromolecules during flow through a packed column. The principles and applications of SEC are

89   well presented in several reviews[18–20] and in the handbook "Size Exclusion Chromatography:
90   Principles and Methods"[21]. The differences in retention are due to different amounts of time
91   spent diffusing into and out of the pores in the stationary phase before eluting from the end of
92   the column. The differences arise (nominally) from the relative sizes and diffusion coefficients of
93   the molecules[22]. A calibration curve is constructed using a series of reference molecules, relating
94   the MW of the molecule to elution volume. For proteins, the reference molecules are generally
95   well-behaved, globular proteins that do not interact with the column via charge or hydrophobic
96   surface residues. Elution volume is measured with an ultraviolet (UV) absorbance detector. If the
97   UV extinction coefficient is known—often calculated from the sequence—the protein peak total
98   mass may also be quantitated.
99
100 Notably, the analysis of MW by SEC relies on two key assumptions regarding the proteins to be
101 characterized: 1) they share with the reference standards the same conformation and specific
102 volume (in other words, the same relationship between diffusion properties and MW) and 2) like
103 the reference standards, they do not interact with the column except by steric properties—they
104 do not adhere to the column packing by charge or hydrophobic interactions. Deviations from
105 these assumptions invalidate the calibration curve and lead to erroneous MW determinations.
106 This is the case for intrinsically disordered proteins that have large Stokes radii due to their
107 extensive unstructured regions[23,24] or non-spherical/linear oligomeric assemblies[10]. Glycosylated
108 proteins will generally have a larger Stokes radius than the non-glycosylated form, even when
109 the added carbohydrate mass is taken into account[19]. Detergent-solubilized membrane proteins
110 elute differently than calibration proteins because their elution from SEC depends on the total
111 size of the polypeptide–detergent–lipids complex rather than the oligomeric state and molar
112 mass of the protein[25,26]. Column chemistry, pH and salt conditions all affect elution volumes of
113 proteins with charged or hydrophobic surface residues[27,28].
114
115 SEC becomes much more versatile and reliable for MW determination when combined with
116 multi-angle light scattering (MALS) and differential refractive index (dRI) detectors[3,4,11,29–32]. A dRI
117 detector determines concentration based on the change in solution refractive index due to the
118 presence of the analyte. A MALS detector measures the proportion of light scattered by an
119 analyte into multiple angles relative to the incident laser beam. Collectively known as SEC-MALS,
120 this instrumentation determines MW independently of elution time since MW can be calculated
121 directly from first principles using Equation 1,
122

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

124
125 where M is the molecular weight of the analyte, $R(0)$ the reduced Rayleigh ratio (i.e., the amount
126 of light scattered by the analyte relative to the laser intensity) determined by the MALS detector
127 and extrapolated to angle zero, c the weight concentration determined by the UV or dRI detector,
128 dn/dc the refractive index increment of the analyte (essentially the difference between the
129 refractive index of the analyte and the buffer), and K an optical constant that depends on the
130 system properties such as wavelength and solvent refractive index[29].

131

132 In SEC-MALS, the SEC column is used solely to separate the various species in solution so that
133 they enter the MALS and concentration detector cells individually. The actual retention time has
134 no significance for the analysis except as far as how well it resolves the protein species. The
135 instruments are calibrated independently of the column and do not rely on reference standards.
136 Hence, SEC-MALS is considered an 'absolute' method for MW determination from basic physical
137 equations. If the sample is heterogeneous and not completely separated by the column, then the
138 value provided at each elution volume will be a weight average of the molecules in each elution
139 volume that flows through the flow cell per time slice, approximately 75 μL.

140

141 By analysis of the angular variation of scattering intensity, MALS can also determine the size
142 (root-mean-square radius, R_g) of macromolecules and nanoparticles with geometric radius larger
143 than about 12.5 nm[29]. For smaller species such as monomeric proteins and oligomers, a dynamic
144 light scattering (DLS) module may be added to the MALS instrument in order to measure
145 hydrodynamic radii from 0.5 nm and up[33].

146

147 While either UV or dRI concentration analysis may provide the value of c in Eq. 1, use of dRI is
148 preferred for two reasons: 1) dRI is a universal concentration detector, suitable for analyzing
149 molecules such as sugars or polysaccharides that do not contain a UV chromophore[34]; and 2) the
150 concentration response dn/dc of almost all pure proteins in aqueous buffer is the same to within
151 one or two percent (0.185 mL/g)[35], so there is no need to know the UV extinction coefficient.

152

153 The use of SEC-MALS in protein research is quite extensive. By far the most common applications
154 are establishing whether a purified protein is monomeric or oligomeric and the degree of
155 oligomerization, and assessing aggregates[3,10,11,17,31,36–38]. The ability to do so for detergent-
156 solubilized membrane proteins that cannot be characterized by traditional means is especially
157 prized, and detailed protocols for this have been published[31,39–43]. Other common applications
158 include establishing the degree of post-translational modification and polydispersity of
159 glycoprotein, lipoproteins and similar conjugates[4,31,44–47]; the formation (or lack thereof) and
160 absolute stoichiometry (as opposed to stoichiometric ratio) of heterocomplexes including
161 protein-protein, protein-nucleic acid and protein-polysaccharide complexes[24,46,48–52];
162 determining the monomer-dimer equilibrium dissociation constant[49,53,54]; and evaluating protein
163 conformation[55,56]. Beyond proteins, SEC-MALS is invaluable for characterization of peptides[57,58],
164 broadly heterogeneous natural polymers such as heparins[59] and chitosans[60, 61], small viruses[62]
165 and most types of synthetic or processed polymers[63–66]. An extensive bibliography may be found
166 in the literature[67] and online (at http://www.wyatt.com/bibliography).

167

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

173

174 We note that SEC-MALS may be performed using standard high-performance liquid

175 chromatography (HPLC) or fast protein liquid chromatography (FPLC) equipment from many
176 vendors. This protocol describes the use of an FPLC system commonly found in labs that produce
177 proteins for research and development (see **Table of Materials**). Prior to running the protocol,
178 the FPLC system, MALS and dRI detectors should have been installed, along with their respective
179 software packages for control, data acquisition and analysis per manufacturers' instructions and
180 any requisite calibration constants or other settings entered into the software. An inline filter
181 should be placed between the pump and injector with a hydrophilic, 0.1 μm pore membrane
182 installed.

183
184 **PROTOCOL:**

185
186 **1.   Preparation of the system**

187
188 1.1   Connect the MALS and dRI detectors downstream of the FPLC's UV detector. Bypass the
189 pH and conductivity detectors since they will add significant interdetector volume between the
190 UV and MALS detectors. Use capillary tubing of 0.25 mm i.d. from the column to and between
191 the detectors, and 0.75 mm i.d. capillary tubing on the output of the detectors to waste or
192 fraction collector.

193
194 1.2   Ensure that the necessary signal connections between the FPLC and detectors have been
195 established, including analog output from the UV detector to the MALS analog input, and digital
196 output from the FPLC to the MALS Autoinject, via the FPLC's I/O Box.

197
198 1.3   Install a suitable analytical SEC column covering a fractionation range of at least 20 kDa
199 to 500 kDa. Check the product info to determine if the column is suitable for the range of MW,
200 pH and other properties of the sample and mobile phase.

201
202 **2.   Preparation of buffer, flushing the system overnight and checking cleanliness**

203
204 2.1   Using HPLC-grade reagents, prepare 1 L of phosphate-buffered saline with 50 – 100 mM
205 NaCl. Filter the buffer to 0.1 μm using a bottle-top polyether sulfone filter or similar. Filter the
206 first 50-100 mL of buffer to a waste bottle and discard, in order to eliminate particulates from
207 the dry filters, and then filter the remainder to a clean, sterile bottle that has been washed
208 thoroughly with filtered, de-ionized water and capped to prevent dust from entering.

209
210 NOTE: Other mobile phase solvents such as a Tris buffer may be used if additional proteins that
211 are preferentially dissolved in those solvents are to be analyzed.

212
213 2.2   Flush overnight at a flow rate of 0.5 mL/min, or as otherwise recommended by the column
214 manufacturer, to equilibrate the column in the buffer and remove particulates. Use the FPLC's
215 **Continuous flow** mode and ensure that the flow does not stop until all SEC-MALS runs are
216 complete.

217
218 2.2.1   Place the dRI flow cell in **Purge** mode during the overnight flush. Turn the purge off before

219   beginning sample runs.
220
221   2.2.2   When beginning the flush, gradually ramp the flow rate to prevent "column shedding"
222   effect (or release of particles) caused by a sudden change of pressure in the column.
223
224   2.2.3   If the system is known to be quite stable and particle-free, and in equilibrium with the
225   desired mobile phase, replace the overnight flush with a shorter, 2-3 h flush.
226
227   2.3   Check system cleanliness by lightly tapping the tubing downstream of the column to
228   release accumulated particles and observing the signal in the 90° detector on the front-panel
229   display of the MALS instrument. Verify that the peak-to-peak noise is no more than 50 -100 µV.
230
231   2.4   Perform a 'blank' injection to verify that the injector is clean of particles. A 'blank' is simply
232   the running buffer, prepared in a fresh, sterile vial.
233
234   2.4.1   If the particle peak is no more than 1 mL in volume and no more than 5 mV above baseline,
235   then the system is ready for samples. Otherwise, perform additional blank injections until clean,
236   or perform maintenance to clean the injector.
237
238   3.   **Preparing and loading the sample**
239
240   3.1   Prepare at least 200 µL of BSA at 1-2 mg/mL in the SEC buffer.
241
242   NOTE: In order to prevent precipitation, BSA should never be dissolved in pure water.
243
244   3.2   Filter the protein to 0.02 µm using a syringe-tip filter.
245
246   3.3   Discard the first few drops of filtrate in order to eliminate particles from the dry filters.
247
248   3.4   Alternatively, centrifuge the sample at 10,000 x g for 15 min to enable precipitation of
249   non-soluble aggregates and other large particles.
250
251   3.5   Inject 100 µL of the BSA solution into the loop.
252
253   NOTE: This is the recommended amount of material, and more or less may be injected according
254   to circumstances of the sample such as stability or availability. The quantity of protein required
255   per injection varies inversely with molecular weight – twice as much protein mass is needed if
256   the molecular weight is 33 kDa, or half that of BSA.
257
258   4.   **Preparation of the MALS software**
259
260   4.1   Open **New | Experiment** from **Method** in the MALS software menu and select the **Online**
261   method from the **Light Scattering** system methods folder. If a DLS detector is present, select the
262   **Online** method from the **Light Scattering | With QELS** folder.

263

264 4.2   In the **Configuration** section, set parameters of the sample and mobile phase.

265

266 4.2.1   In the **Generic Pump** view, set the flow rate to that used in the FPLC.

267

268 4.2.2   In the **Generic Pump** view, **Solvent** branch, **Name** field, select **PBS**.

269

270 4.2.3   In the **Injector** view, **Sample** branch, enter the **Name** as **BSA**, and set *dn/dc* = 0.185 (the
271 standard value for unmodified proteins), A2 = 0, and UV extinction coefficient = 0.667 mL/(mg-
272 cm).

273

274 NOTE: For other proteins, the UV 280 nm extinction coefficient may be found in the literature or
275 calculated from its sequence using various public-domain software tools.

276

277 4.3   In the **Procedures** section, **Basic Collection** view, select the checkbox **Trigger on**
278 **Autoinject** and set the duration of the run to 70 min so that data are collected for the entire
279 elution until the total permeation volume of the SEC column is reached.

280

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

283

284 4.4   Start the experiment in the MALS software by clicking on the **Run** button. It will start
285 reading the data after receiving the pulse signal from the FPLC instrument via the MALS detector.

286

287 4.5   Zero the dRI signal by clicking the **Autozero** button on the instrument's front panel.

288

289 **5.   Preparation of the FPLC software**

290

291 5.1   Insert the name of protein and the run in the FPLC software, in **Manual | Execute manual**
292 **instructions | Set mark.**

293

294 5.2   Switch the injection valve from **Manual load** to **Inject** under **Flow path | Injection valve**.

295

296 5.3   Include a pulse signal by inserting a 0.5 s pulse under **I/O box | Pulse digital out**. This will
297 trigger data collection in the MALS software.

298

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

301

302 **7.   Analysis of SEC-MALS BSA data**

303

304 7.1   Perform analysis, step by step, under the **Procedures** section in MALS software.

305

306 7.1.1   Verify that peaks appear, at approximately the same elution volume in UV, MALS and RI,

307　by checking the **Basic Collection** view.

308

309　7.1.2　In the **Baseline** view, define baseline for all signals (all LS detectors, UV and dRI). The
310　baselines should be defined to indicate the level of pure solvent, preferable stretching from one
311　side of the sample peaks to the other.

312

313　7.1.3　In the **Peaks** view, define the peaks to be analyzed by clicking and dragging the mouse.
314　Select the central 50% of each peak. First select the monomer peak ('Peak 1') and then the dimer
315　peak ('Peak 2'). Verify correct values of *dn/dc* = 0.185 and UV 280 nm extinction coefficient =
316　0.667 for BSA under each peak.

317

318　7.2　Perform peak alignment, band-broadening correction and normalization procedures.

319

320　NOTE: Normally the SEC-MALS method is periodically calibrated for peak alignment, band
321　broadening and normalization of the angular detectors to the 90° detector using a monodisperse
322　protein with radius of gyration R_g < 10 nm such as BSA monomer. In this example, BSA serves
323　both as the calibration molecule and is itself the subject of MW analysis.

324

325　7.2.1　In the **Procedures | Alignment** view, select the central region of the peaks by clicking and
326　dragging the mouse, click **Align Signals** and then **OK**.

327

328　7.2.2　In the **Procedures | Band Broadening** view, choose the central 50% of the monomer peak.
329　Make sure the RI detector is specified as the **Reference Instrument**, then click **Perform Fit** and
330　**Apply** to match the UV and LS signals to the RI signal.

331

332　7.2.2.1 Zoom in to the peaks to verify that they overlap very closely within the central 50-70%,
333　then click **OK**.

334

335　7.2.2.2 If the overlap is not perfect, (it may be necessary to) perform the fit and "Apply" one or
336　two more times until the overlap is excellent.

337

338　7.2.3　In the **Procedures | Normalization** view, select Peak 1, enter 3.0 nm as the R_g value, click
339　**Normalize** then **OK**.

340

341　7.2.4　In the **Procedures | Molar Mass and Rg from MALS** view, review the data to determine
342　which, if any, detection angles should be deselected from the analysis due to excessive noise.
343　Typically, these will be the lower angles into which dust particles scatter a relatively high
344　intensity. Select individual slices within the peaks from the graph on the right and view the
345　angular dependence of the inverse reduced Rayleigh ratio in the graph on the left. If the lowest
346　(and sometimes the highest) angles consistently deviate greatly from the fit, then deselect them
347　from the list at the bottom of the view.

348

349　7.3　View the graph of the results in the **EASI Graph** view. Select **Molar Mass** from the **Display**
350　drop-down at the top of the window. Use Ctrl + click and drag to zoom in on the peak region.

352　<mark>7.4　　View the final tabulated weight-average molar mass results for the monomer and dimer</mark>
353　<mark>peaks in the **Results | Report (summary)** view under **Peak Results | Molar mass moments**</mark>
354　<mark>**(g/mol) | Mw**. Purity is reported under **Peak Results | Mass fraction(%)**.</mark>

355

356　NOTE: Other molar mass moments are also shown; these are usually relevant to heterogeneous
357　polymers, but not to proteins with discrete sizes. Many other results produced by the software
358　may be included in the report such as percent mass recovery (the fraction of protein that eluted
359　via the concentration detector relative to the amount injected), peak statistics, polydispersity, R_g
360　and R_h moments, etc. When provided, the measures of uncertainty reflect the precision of the
361　value cited based on the noise within the measurement series and should not be considered to
362　represent the accuracy. The true accuracy of the reported values depends on various factors such
363　as the accuracy of the provided *dn/dc* and extinction coefficient values, instrument calibration,
364　etc.

365

366　<mark>7.5　　From the **File** menu, select **Save as Method** and save the analyzed BSA data as a standard</mark>
367　<mark>method for future measurements of all types of proteins. The normalization and band-</mark>
368　<mark>broadening parameters determined for BSA will be carried over in the analysis.</mark>

369

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

380

381　Henceforth we focus on **Figure 1b**. The monomer, which eluted at 13.8 mL, exhibits a weight-
382　average molar mass M_w of 64.1 ± 0.4 kDa determined by MALS and hydrodynamic radius R_h of
383　3.54 ± 0.01 nm. These results are in agreement with the sequence mass and known
384　hydrodynamic radius of BSA, 66.4 kDa and 3.5 nm respectively[68], to within the usual accuracy of
385　SEC-MALS, ± 5%[3, 69]. The dimer, which eluted at 12 mL, exhibited a M_w value of 127 ± 1 kDa
386　determined by MALS—as expected, twice that of the monomer to within experimental
387　precision—and R_h of 5.68 ± 0.06 nm. The trimer peak was also observed at 11 mL with M_w of 194
388　± 9 kDa determined by MALS, three times that of the monomer to within experimental precision,
389　as expected. R_h of the trimer could not be determined due to low intensity of the DLS signal.

390

391　The molar mass points calculated across the monomer peak are uniform to within 2-5%,
392　indicating homogeneity. It is not unusual to find a trailing shoulder with molar mass in the range
393　of 38-50 kDa, corresponding to BSA fragments[70]. The molar mass points across the dimeric and
394　trimeric peaks are not uniform, indicative of heterogeneity. The dimer peak is somewhat

395 heterogeneous due to traces of trimer that bleed into the dimer peak, and the trimer peak is
396 heterogeneous due to co-elution of poorly-resolved higher oligomers.

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

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

408 **FIGURE AND TABLE LEGENDS:**
409 **Figure 1: SEC-MALS analysis of bovine serum albumin (BSA) using a 200 Å pore size-exclusion**
410 **column.** Chromatogram traces are normalized to the monomer peak and offset for clarity. (**A**)
411 Common artifacts that may be ignored are pointed out, including a particle peak near the
412 beginning of the light scattering signal as well as salt and dissolved air peaks near the total
413 permeation volume in the refractive index signal. (**B**) The chromatogram exhibits excellent
414 monomer-dimer-trimer separation and the light scattering signal exhibits high signal-to-noise.
415 The monomer and dimer MW values exhibit high homogeneity.

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

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

433 Verification of the absence of particulates is quite important for sensitive, repeatable SEC-MALS
434 measurements. Particle peaks generally appear as large MALS signals unaccompanied by
435 comparable UV or RI signals. The mobile phase and sample should be prepared carefully to
436 eliminate such particles. Use of HPLC-grade reagents or better, filtration of mobile phase and
437 dilution buffer to 0.1 – 0.2 μm (pre-washing the filter to eliminate particles that are always
438 present on dry membranes), maintenance of extra-clean mobile phase bottles and other

439 glassware for SEC-MALS and extended column equilibration under flow (to remove particulates
440 and aggregates that may have accumulated when flow was stopped or a column not in use) are
441 all recommended. The sample should be filtered to the smallest pore size that does not remove
442 the material of interest, usually no larger than 0.1 μm and, if possible, 0.02 μm. If the filter quickly
443 clogs, the sample may be centrifuged, filtered in stages of descending pore size, and/or re-
444 purified. When systems are consistently maintained with high-quality, fresh and filtered mobile
445 phase, column shocks are prevented, samples are clean and do not adhere to the column, the
446 measurements will not exhibit the aforementioned particulate noise and will provide high-quality
447 data.

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

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

479 Given the constraints described above, SEC-MALS is not suitable for various tasks. It is not
480 suitable for analysis of crude samples; the samples should be well-purified by standard affinity
481 and polishing methods. It does not have sufficient accuracy or resolving power to identify
482 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[72–74]. Where protein quantities are severely constrained, SEC-MALS may not be feasible since it typically requires $10 - 200 \, \mu g^3$ 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.

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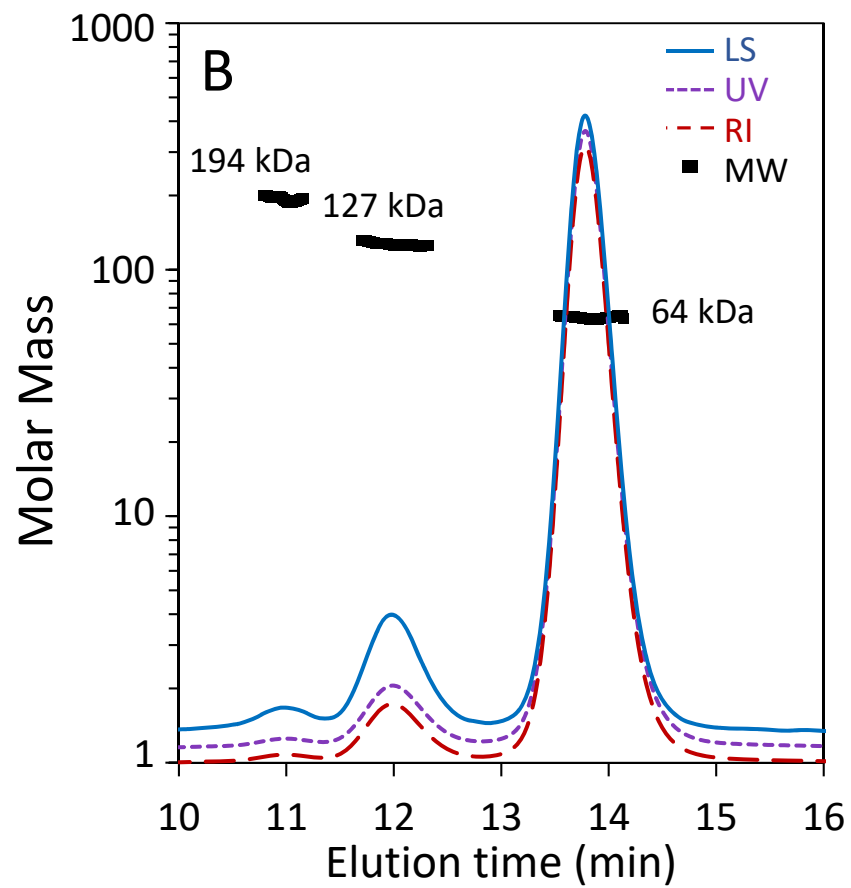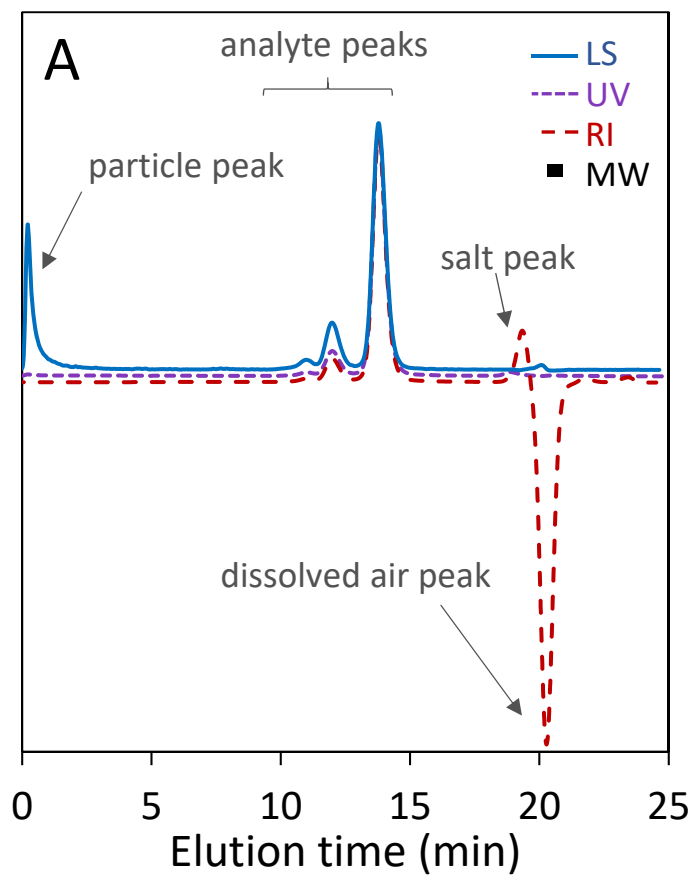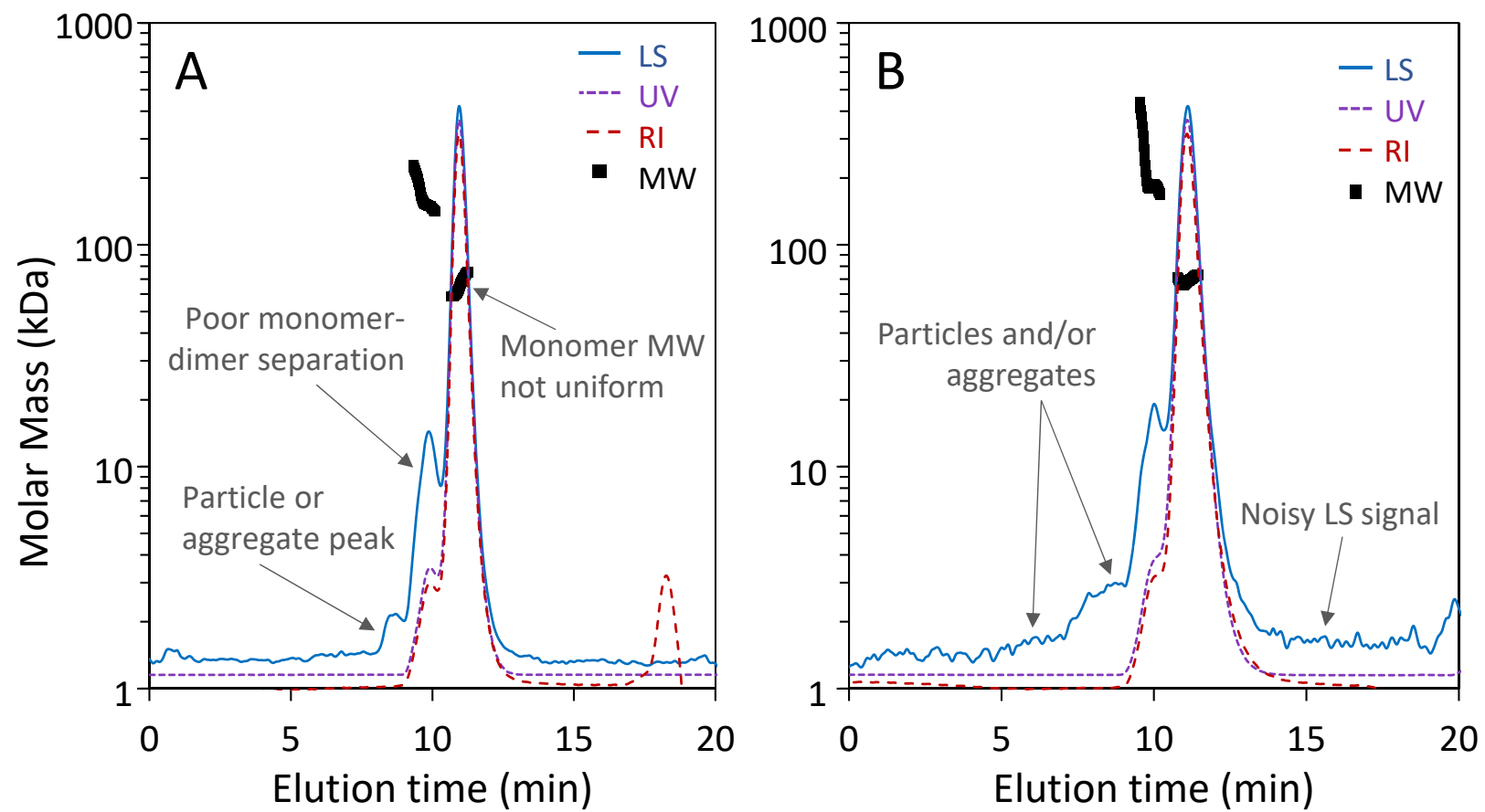
Table of Materials

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
AKTA pure	GE Healthcare	29-0182-26	fast protein liquid chromatograph (FPLC)
AKTA UNICORN	GE Healthcare		FPLC control software
ASTRA	Wyatt Technology		MALS data acquisition and analysis software
Bovine serum albumine (purity >97%)	Sigma	A1900	analyte
DAWN or miniDAWN	Wyatt Technology	WH2 or WTREOS	multi-angle light scattering (MALS) detector
Increase 200 10/300	GE Healthcare		size exclusion column, 200 Å pores, 10 mm i.d., 3
Optilab	Wyatt Technology	WTREX	differential refractive index (RI) detector
Sodium chloride NaCl	Sigma	71382	HPLC grade NaCl
Stericup bottle top filter polyether sulfone 0.1 μm 1000 mL	Millipore	SCVPU11RE	mobile phase filter
Whatman Anotop 10 syringe filter 0.02 μm	GE Healthcare	6809-1002	sample filter
Whatman Anotop 10 syringe-tip filter, 0.1 μm pore, 10 mm diameter	GE Healthcare	6809-1012	sample filter
Whatman Anotop 25 syringe filter 0.1 μm	GE Healthcare	6809-2012	mobile phase filter
WyattQELS	Wyatt Technology	WIQ	dynamic light scattering detector

00 mm length

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1 Alewife Center #200
Cambridge, MA 02140
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CORRESPONDING AUTHOR

Name: Daniel Some

Department:

Institution: Wyatt Technology Corp.

Title: Principal Scientist

Signature: *[signature]*   Date: Dec. 26, 2018

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<u>Response to editor and reviewers' comments:</u>

We thank the reviewers and editors for their helpful comments which have served to improve the manuscript. Detailed responses are provided below.

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

    Manuscript has been proofread.

2. Please upload each Figure individually to your Editorial Manager account as a .png or a .tiff file. Please combine all panels of one figure into a single image file.

    These will are provided in the current resubmission.

Reviewer #3's comments:

1. MW, Mw and Mw are used interchangeably - are these different or referring to the same unit?

    As noted in line 56 of the manuscript, 'MW' is an abbreviation of the term 'molecular weight'. As noted in line 384, M_w is the symbol for weight-average molar mass. 'Mw' is used only in line 356 to indicate a specific selection in the software, because this is the way it appears there. MW and M_w are not interchangeable since one is a molecular property and one is a moment of a distribution which has units of g/mol or Daltons.

2. Parts of the submitted manuscript are highlighted in yellow - is there a reason for the highlight?

    Per editorial instructions, the highlighted sections will be used in the video script. They will not be highlighted in the published article.

3. Line 141 - "approximately 75 uL." Is this constant for a particular instrument? Eg. Treos vs Heleos?

    In general, the protocol pertains to the instruments noted in the Table of Materials addendum that accompanies the manuscript. Specifically to this question, it would be the same for both DAWN and miniDAWN across multiple models (e.g., EOS, Tri-Star, HELEOS, HELEOS II, TREOS, TREOS II and next-generation models). The editorial guidelines specifically rule out "commercial language" in the text, including use of any brand names, so this cannot be clarified in the text.

4. Lines 143-147 - In the beginning of the paragraph, it is mentioned MALS can determine diameter of 25 nm, but towards the end of the paragraph, it can measure hydrodynamic radii of 0.5 nm. It is better to keep them either both diameter or both radii, for effective comparison.

MALS text has been revised to 'geometric radius larger than about 12.5 nm'. Please note that the geometric radius, rather than rms radius, is used here to provide some intuition about the limitations of the system with the understanding that many, if not most, protein chemists may not be familiar with R_g.

5. Line 183 - More common to filter SEC-MALS buffers with a 0.1 uM filter as inline 0.1 uM filter can lead to over-pressuring the FPLC system.

We recommend both pre-filtering the buffer (line 207) *and* inline-filtering (line 183), both of which are essential. While pre-filtering removes potentially large loads of particulates which might in fact overload the FPLC system if only inline filtering is used, inline filtering is still necessary to remove the smaller number of particles generated by the pump or shed from the degasser and other tubing.

6. Line 192-193 - Vague. Specify which detectors. 0.25 mm tubing between UV-LS, and LS-RI detector, and 0.75 mm tubing from output of RI detector to waste. It is useful to mention what FPLC system and what LS-RI detectors were used in the analysis in the body of the text. "In this study, we use a HELEOS etc."

The editor's guidelines specifically rule out "commercial language" in the text, including use of any brand names. All terms in the manuscript must be generic with the specific products listed in the Table of Materials addendum. The MALS and FPLC instruments appear in the Table of Materials.

7. Line 202 - It is useful to mention which SEC column was used in the analysis in the body of the text. "In this study, we use a GE Superdex S200 10/300 increase column." etc

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8. Line 241 - Suggestion to dialyze the proteins overnight in desired SEC-MALS buffer prior to running on SEC-MALS to have best signal-noise.

We agree that overnight dialysis is a good practice, but did not include it here for two reasons. The recommended BSA is lyophilized and contains just 2% salts, so the extra effort is not justified. For other proteins, there is a concern that prolonged exposure to the mobile phase may lead to precipitation or other changes in state which might be prevented if exposure to the mobile phase is shorter.

9. Line 387 and 429 - Reference for 5%

References added.