Determination of Molecular Weight and Dispersity of Antibody Samples Using Bio-Rad's NGC Chromatography System and Wyatt's MALS and RI Detectors

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Abstract

Size exclusion chromatography (SEC) is a common laboratory technique employed to estimate the molecular weight of biomolecules. To achieve an empirical determination of a protein's molecular weight, samples must be subsequently validated using an orthogonal method. Here, SEC in combination with multiangle light scattering and refractive index (MALS/RI) inline biophysical analytics were used to determine the molecular weights of multiple antibody samples. The data herein validate the use of SEC-MALS/RI to determine not only the molecular weight but also the dispersion state of a sample. These data were collected using the Bio-Rad NGC Chromatography System in tandem with Wyatt TREOS and T-rEX instrumentation. Peak analysis was carried out utilizing Bio-Rad ChromLab Software and Wyatt ASTRA Software. The ease of system integration between the NGC System and Wyatt hardware provided rapid, accurate, and reliable mass determination of proteins separated by SEC.

Introduction

SEC is a common laboratory technique that utilizes a porous resin to separate molecules based on their size through a nonadsorbing mechanism. The resin retains smaller molecules longer due to their ability to enter its pores, increasing the overall distance these molecules travel through the column. Larger molecules, unable to enter pores, have a shorter flow path relative to smaller molecules and therefore elute earlier. Researchers take advantage of this technique to estimate the molecular weight of biomolecules based on retention time or volume with respect to the molecular weights of known standards.

SEC is routinely used as a final polishing step for laboratory-scale chromatography to ensure the target molecule is of the expected molecular weight and buffer exchanged for downstream applications. Although this practice is commonly implemented, a limitation of SEC is the inability to determine small changes in the dispersity of a sample. Monodisperse protein samples post-SEC are ideal for downstream experiments because they are a pure and homogenous biomolecule solution. It is a common assumption that peaks on an SEC column are a single molecular species; however, if the biomolecule is polydisperse, the downstream finding may be invalid due to this incorrect assumption.

Underappreciation of small molecular weight changes could result in incorrect analysis of the data and thus incorrect conclusions being drawn and reported. Furthermore, as research moves toward interrogating complex biomolecules, such as cell and gene therapy products, researchers are in need of an inline microfluidics method that can empirically determine sample integrity.

One common way of investigating the weight, size, and conformation state of a biomolecule is MALS/RI. MALS is a technique for measuring the light scattered at specified angles when a macromolecule, such as a protein, is examined using collimated light. There are multiple types of MALS detectors with a variety of detection angles, with the simplest detecting three angles and the most advanced detecting 18 angles. In this example, we used the Wyatt TREOS MALS detector, which detects refracted light from three different angles. In order to use the data collected from MALS to calculate molecular weight, the RI must also be obtained. RI is the ratio of the speed of light in a vacuum to the speed of light in a medium, which changes depending on the medium or solution composition. A reference cell with buffer composition identical to that of the target sample is used to determine the refracted changes induced by only the sample. The information gained

from both MALS and RI allows unambiguous determination of sample size and dispersion.

Herein we provide an example of how monoclonal antibody (mAb) samples can be analyzed to ensure purity and monodispersion. Through coupling the Bio-Rad NGC Chromatography System in tandem with Wyatt TREOS and T-rEX instrumentation, we have provided a solution for sample interrogation on a preparative chromatography system. This technology offers researchers insight into purification consistency when experimental conditions vary.

Materials and Methods

Hardware

Data were collected using an NGC Quest 10 Chromatography System (Bio-Rad Laboratories, Inc.) equipped with a buffer blending valve, two buffer inlet valves, a sample pump, two column switching valves, a BioFrac Fraction Collector (Bio-Rad), and a signal import module (SIM). The SIM allows communication, data export, and data import to third-party devices. In this instance, TREOS and T-rEX instruments (Wyatt Technology Corporation) were used in conjunction with the NGC Chromatography System to collect MALS and RI data. These Wyatt devices were able to import the A_{280} signal from the NGC System via the SIM to allow analysis in ASTRA Software. For more information on integrating Wyatt instrumentation with the NGC System, reference Bio-Rad bulletin 7208.

Sample Preparation

Antibody samples (Bio-Rad) were purified to greater than 95% purity prior to being analyzed by SEC-MALS/RI. Antibodies were provided in 1x PBS at concentrations between 1 and 6 mg/ml (Table 1). A BSA control sample with 2 mg/ml concentration and greater than 99% purity was used for system calibration.

Prior to analysis, samples were clarified by high-speed centrifugation. Samples with concentrations greater than 2 mg/ml were diluted 1:1 in running buffer.

Table 1. Molecular weight and concentration of antibody samples.

Sample	Isotype	Monomer MW, kD (by sequence)	Concentration, mg/ml
1	IgG1	144	5.5
2	IgG1	146	2.4
3	IgA1	148	1.4
4	IgM	170	1.0

NGC Chromatography Method

For MALS/RI analysis, a Bio-Rad ENrich SEC 650 Column (10 x 300 mm, 23.56 ml, 600 psi maximum pressure limit, catalog #7801650) was used for sample separation. The running buffer used in these experiments was 140 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4. The column was manually equilibrated prior to starting the method.

The method used was the same for each sample with a constant flow rate of 1.0 ml/min and contained three steps: equilibration, sample application, and elution. Since the column was manually equilibrated prior to the start of the series of runs, each subsequent equilibration step was truncated to 0.3 column volumes (CV). The sample application step was completed using a 100 µl static loop, with the full 100 µl of sample injected to the ENrich SEC 650 Column. At the start of sample application, a digital signal was sent via the NGC SIM to Wyatt ASTRA Software to begin data collection. The final step of the method was elution, which continued with running buffer for 0.85 CV. The total run time for each method was 27 minutes.

Data Analysis — Size Determination by SEC

In order to determine size by SEC, the Bio-Rad Gel Filtration Standard (#1511901) was run on the ENrich SEC 650 Column to generate a standard curve. The Bio-Rad Gel Filtration Standard contains four proteins and one small molecule of known molecular weights. Using the Bio-Rad ChromLab Software Size Exclusion Calibration analysis tool, a standard curve was generated using the known sizes and experimentally determined retention volumes of the four proteins in the standard. The small molecule was not used in the generation of the standard curve because its molecular weight is outside the resolution range of the ENrich 650 Column. The generated standard curve was applied in the analysis of each sample within ChromLab Software to determine the calculated molecular weight of the unknown sample based on its retention volume on the column.

ASTRA Software Method

For data acquisition and analysis within ASTRA Software, a new experiment or method was performed for each sample. This experiment was based on the parameters obtained from the method saved from the BSA standard run (data not shown). Within ASTRA Software, the generic UV instrument was modified to match the NGC Chromatography System, including the cell length and UV response. Within the procedures, the trigger on Auto-Inject was selected so that data collection would begin at sample injection and the duration was set for 20 minutes, the length of the elution step. The experiment was then started in ASTRA Software prior to the method being started in ChromLab Software.

Data Analysis — Size Determination by ASTRA Software

Analysis of the collected MALS, RI, and A_{280} data was carried out within ASTRA Software. The baselines for the MALS and RI was manually adjusted when needed. Peaks were then selected and molar mass as well as radius were calculated based on the acquired data. The resulting analysis was exported to Microsoft Excel Software for graphing purposes.

Results

To check the proper functionality of the system, a BSA control sample was run. This sample was used to align and normalize the MALS, RI, and A_{280} signal. BSA is commonly used as a standard in the field, but any monodispersed protein or small macromolecule could have been used. BSA eluted with one major monodispersed peak corresponding to the calculated size of 68 kD (data not shown).

In order to use retention volume as a way of estimating molecular mass, a gel filtration standard was run on the ENrich SEC 650 Column. Using the size exclusion calibration curve feature in ChromLab Software, a standard curve was generated using the retention volumes and known molecular weights of the four proteins in the gel filtration standard ($y = -0.223x + 0.785$, $R^2 = 0.999$; Figure 1).

In the subsequent experiments, a series of antibody samples were characterized. The first sample to be tested was a human IgG1. The results obtained here show a single peak elution at 12.52 ml (Figure 2A). By applying the SEC calibration curve generated from the gel filtration standard (Figure 1B), the approximated size based on retention volume corresponds to 162 kD (Table 2). Using the MALS/RI data generated concurrently, the molar mass was calculated to be 160 kD, agreeing with the SEC data. Moreover, the Mw/Mn was shown to be 1.000, demonstrating a monodispersed peak (Figure 2B and Table 2).

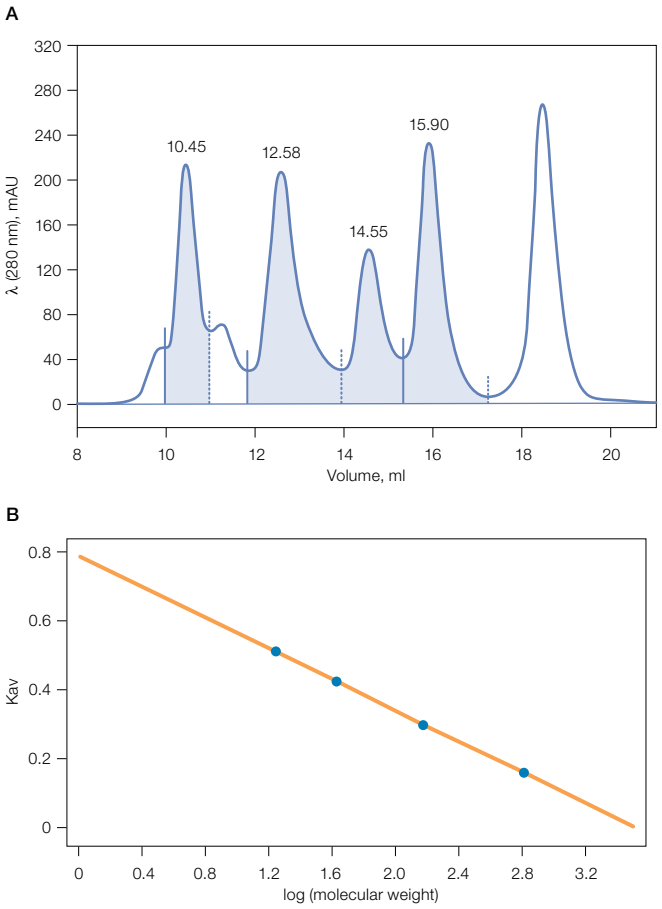

Fig. 1. SEC standard curve generation. A, chromatogram of SEC standard run showing distinct peaks at 10.45, 12.58, 14.55, and 15.90 ml corresponding to molecular weights of 670, 158, 44, and 17 kD, respectively. **B**, standard curve generated by analyzing the peak elution profile and matching to known molecular weights in the standards vial (as provided in the instructions). Kav, average particle coefficient equal to (Ve – Vo)/(Vc – Vo), where Ve is the elution volume of the sample, Vc is the packed bed column volume, and Vo is the void volume.

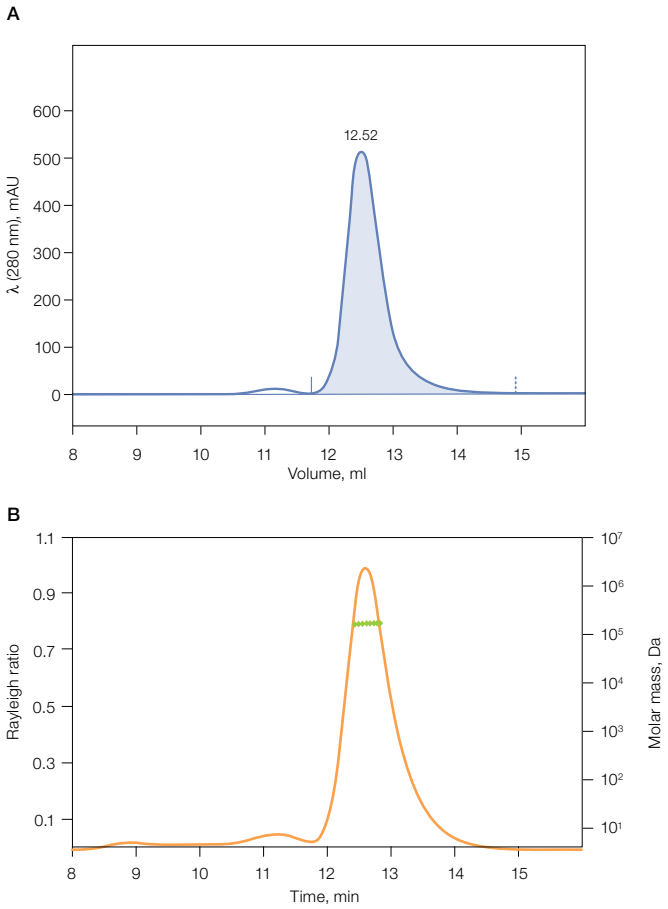

Fig. 2. Control IgG antibody appears as a monodispersed monomer by SEC-MALS/RI. A, chromatogram of IgG running on an ENrich SEC 650 Column, eluting with a single major peak at 12.52 ml. **B**, MALS data showing the Rayleigh ratio (—) and molar mass (◆).

Table 2. Summary of SEC and MALS data for the IgG sample in Figure 2.

	Size, kD	R^2	Mw/Mn
SEC	162	0.9996	N/A
MALS	160	N/A	1.000

The next sample was also a human IgG1. However, while similar in size to the previous sample, the elution profile had a broader peak width, suggesting either variation in molecular weight throughout the peak or interaction of the antibody with the SEC resin. The increase in peak width was easily seen when overlaying sample 1 and sample 2 within the same chromatogram (Figure 3A). Interestingly, the retention volume of this sample was 12.74 ml, resulting in a calculated molecular weight of 141 kD, which was smaller than anticipated but not unexpected due to the shift in the peak-height maxima (from 12.52 ml for first IgG1) (Figure 3B). The MALS analysis was encouraging because not only did the calculated molar mass appear to be invariant, but also the Mw/Mn was 1.000, again indicating a monodispersed sample throughout the peak (Figure 3C and Table 3).

Table 3. Summary of SEC and MALS data for the IgG sample in Figure 3.

	Size, kD	R^2	Mw/Mn
SEC	141	0.9996	N/A
MALS	153	N/A	1.000

The third sample assayed was human IgA. The chromatogram produced by SEC includes the elution of two minor peaks followed by a major peak, as seen in the A_{280} chromatogram (Figure 4A). All three peaks were analyzed for their molecular weights as well as the dispersion within the peaks. By SEC analysis of the retention volumes, the molecular weights of the peaks were calculated to be 1,035, 610, and 270 kD (Table 4). Using these data alone, one could conclude that the major peak at 11.74 ml consists of an IgA dimer. The less abundant, larger molecular weight peaks at 9.73 and 10.53 ml could be additional aggregates thereof. When analyzing the data generated by MALS/RI, the Rayleigh ratio also generated three peaks (Figure 4B). There was molecular weight variance in the two largest peaks, indicating polydispersity; however the major peak at 11.74 ml has a single molecular weight. Interestingly, by MALS the peaks have molecular weights of 784, 399, and 170 kD in order of elution. These molecular weight data deviate from those acquired by SEC. In this instance, mass determination by SEC alone leads to underestimation of the molecular weight of the sample resulting in an inaccurate stoichometry for IgA. However, the MALS/RI data agree with the calculated theoretical molecular weights of a multimer, dimer, and monomer (Table 4). These data and conclusions would not have been possible without the additional value provided by the MALS/RI.

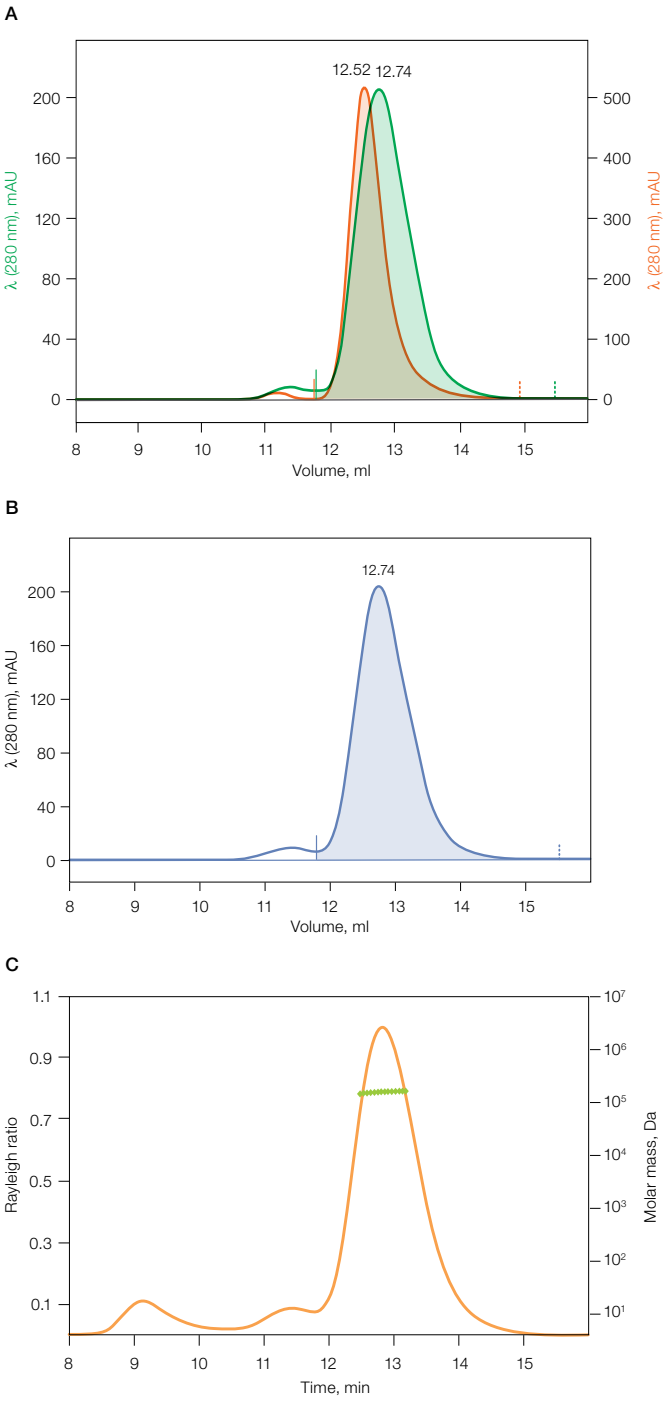

Fig. 3. A single species of IgG exists in an elongated elution, as confirmed by MALS. A, overlay of the sample from Figure 2 (—) and the current sample (—). **B**, chromatogram of IgG running on an ENrich 650 SEC Column, eluting with a single major peak at 12.74 ml. **C**, MALS data showing the Rayleigh ratio (—) and molar mass (◆).

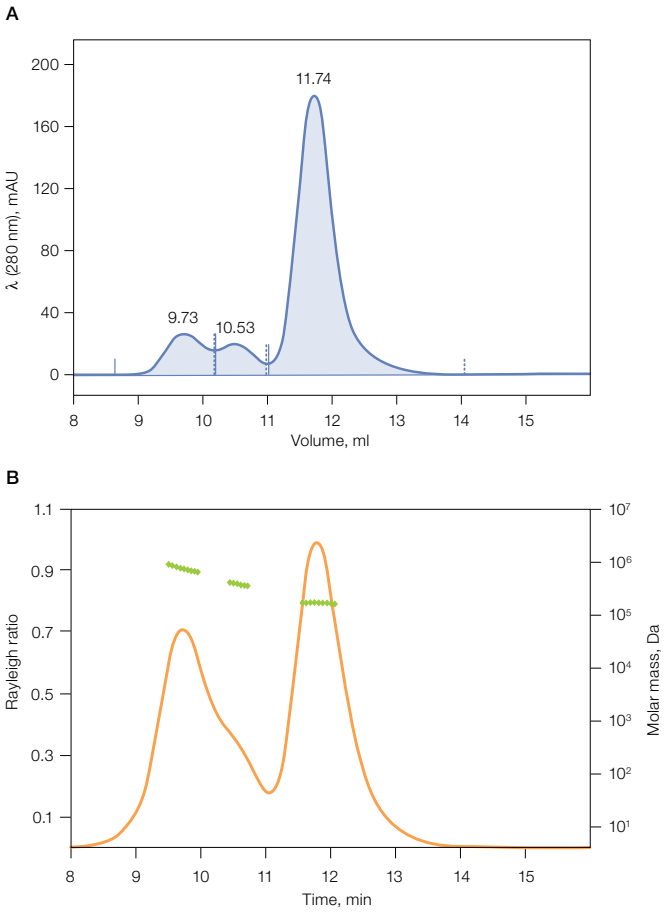

Fig. 4. MALS facilitates the detection of multiple polydisperse IgA species. **A**, chromatogram of IgA running on an ENrich SEC 650 Column eluting with peaks at 9.73, 10.53, and 11.74 ml. **B**, MALS data showing the Rayleigh ratio (—) and molar mass (◆).

Table 4. Summary of SEC and MALS data for the IgA sample in Figure 4.

Peak Elution, ml		Size, kD	R^2	Mw/Mn
9.73	SEC	1,035	0.9996	N/A
	MALS	780	N/A	1.011
10.53	SEC	610	0.9996	N/A
	MALS	400	N/A	1.003
11.74	SEC	270	0.9996	N/A
	MALS	170	N/A	1.000

The final data obtained were for an IgM sample. Due to the molecular weight of an IgM, a single peak eluted in the void volume. This is not unexpected as the Bio-Rad ENrich 650 Column resolves samples from 5 to 650 kD. The theoretical molecular weight based on the sequence of IgM is approximately 970–1,020 kD (pentamer vs. hexamer). SEC analysis suggested that the molecular weight is 1,252 kD; however, because this was outside the resolution range of the column, these data are not reliable (Figure 5A). Analyzing the sample by MALS/RI, the contents of the peak were found to be homogeneous and to have a molecular weight of approximately 1,360 kD, a reasonable size for an IgM that has been glycosylated (Figure 5B and Table 5). By using SEC analysis alone, it would not have been possible to determine whether a peak in the void volume was of uniform molecular weight. However, by investigating this sample using MALS/RI, the sample was determined to have a single molecular weight, even when occurring outside the recommended resolution range of the column.

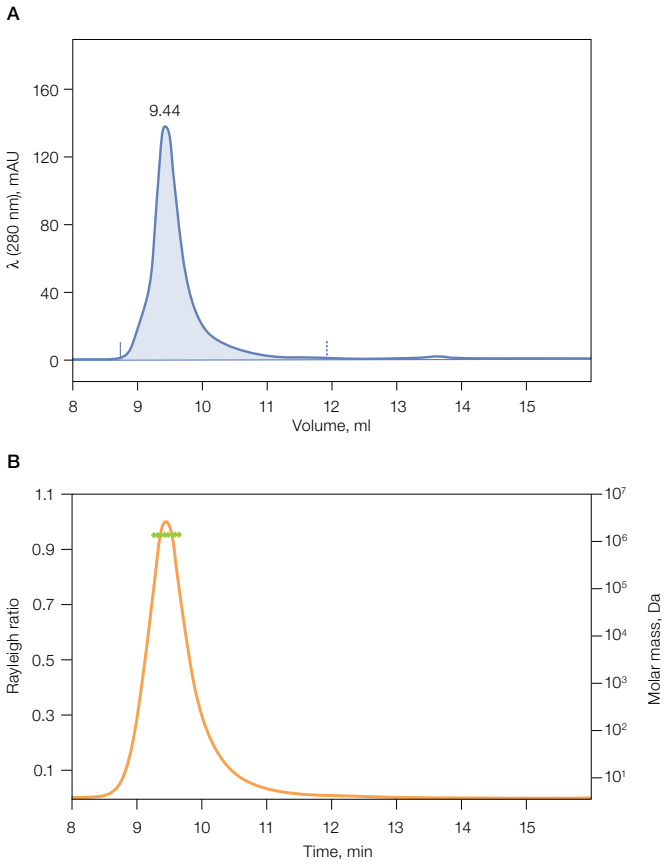

Fig. 5. Confirmation of the monodispersed IgM by MALS. A, chromatogram of IgM running on an ENrich SEC 650 Column eluting in the void volume at 9.44 ml. **B**, MALS data showing the Rayleigh ratio (—) and molar mass (◆).

Table 5. Summary of SEC and MALS data for the IgM sample in Figure 5.

	Size, kD	R^2	Mw/Mn
SEC	1,250	0.9996	N/A
MALS	1,360	N/A	1.000

Conclusion

SEC-MALS/RI is a useful technique utilized to determine a biomolecule's molecular weight and positively identify whether a sample is mono- or polydispersed. While MALS/RI instruments are commonly used in ultra high performance liquid chromatography (UHPLC) applications, herein we show how the medium-pressure Bio-Rad NGC Chromatography System and an SEC column can be used to generate reliable mass determination data in conjunction with the Wyatt MALS/RI detectors. The data demonstrate not only the functionality of this system but also how an SEC-MALS/RI application allows recognition of monodispersion of a large product, even when elution occurs outside the resolution range of an SEC column.

As research applications move toward large, complex biomolecules, such as cell and gene therapy products, coupling microfluidics with a reliable inline technique to quantify molecular size is going to be essential in judging the quality and integrity of biomolecule purification.

Visit **bio-rad.com/NGC-SEC-MALS** for more information.

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