A Comprehensive Workflow to Optimize and Execute Protein Aggregate Studies

Combining Size Exclusion Chromatography with Method Development and Light Scattering

Application Note

Biotherapeutics and Biosimilars

Authors

Andy Coffey and Matthew Rain

Agilent Technologies, Inc

Abstract

This Application Note illustrates a comprehensive aggregate analysis workflow to:

- Optimize mobile phase conditions for high-performance size exclusion chromatography (SEC) of monoclonal antibodies
- Characterize aggregation profiles that include monomers, dimers, and higher-order aggregates

We used Agilent Buffer Advisor software to automate complex SEC optimization experiments that use the full capabilities of the bio-inert quaternary pump of the Agilent 1260 Infinity II bio-inert LC system to mix a wide range of buffer compositions, automatically, in real time during a series of fast LC runs. The Agilent 1260 Infinity Bio-MDS multidetector suite provided dynamic light scattering detection capability to reveal higher-order protein aggregates, determine absolute molecular weights, and augment quantitative measurements made by a UV detection system.

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Introduction

Some monoclonal antibodies (mAbs) and proteins are prone to aggregating spontaneously in solution[1,2]. For many biopharmaceutical applications, the extent of such aggregation must be characterized and quantified precisely under a variety of conditions. Size exclusion chromatography (SEC) is a powerful technique to characterize and quantify protein aggregation, but accurate measurements require excellent chromatography under conditions that accommodate natural protein conformation. To improve chromatographic peak shape for a particular protein, and thus improve resolution, it is often necessary to evaluate a variety of different mobile phase conditions.

The utility of optimizing buffer conditions is sometimes overlooked with SEC techniques. Historically, buffer conditions were optimized to overcome undesirable nonspecific interactions with stationary phase materials, but optimizing for column deficiencies can introduce a risk of disrupting the very aggregation that the technique seeks to measure. However, the inert surface coating of Agilent AdvanceBio SEC columns helps to reduce secondary interactions across a wide range of buffer conditions, and provides greater flexibility to optimize buffer chemistry for protein conformation and chromatographic resolution. The sheer complexity of parameter-scouting experiments has been another impediment to routinely optimizing SEC buffer conditions for aggregate analysis. Optimization experiments required the design of complex tables of mobile phase possibilities, and the tedious manual creation of many different solutions to evaluate the matrix of salts, buffers, and pH variations experimentally. However, modern tools have greatly streamlined workflows for optimizing SEC conditions, characterizing and quantifying aggregates, and deploying optimized techniques in daily use.

This Application Note demonstrates the utility of a full workflow solution for aggregation studies to:

- Automatically mix a specified list of LC buffers from four simple stock solutions and to adjust pH and buffer concentrations in real time using Agilent Buffer Advisor software and a high-resolution bio-inert quaternary LC pump
- Measure higher-order protein aggregates with dynamic light-scattering detection, complementing UV detection to extend high analytical sensitivity across a broad mass range
- Characterize absolute molecular weight and hydrodynamic radius of aggregated and monomeric proteins by light-scattering detection

For the present analyses, we used an Agilent AdvanceBio SEC 15 cm column to provide fast separations for rapid screening. It is important to note that AdvanceBio SEC columns come in longer (and shorter) variations, so the methods can easily be adapted to the 30 cm format, or even multiplexed columns, where additional resolution may be required. Monoclonal antibody samples in this study include commercially available rituximab and a commercially available rituximab biosimilar.

Materials and Methods

Reagents, samples, and materials

Monobasic and dibasic sodium hydrogen phosphate and sodium chloride were purchased from VWR. All the chemicals and solvents used were ≥ 99.7 % pure. >18 MΩ Water was used from a Milli-Q A10 water purification system (Millipore, USA). Solutions were prepared fresh daily and filtered through 0.22 µm membrane filter prior to use.

Instrument

Agilent 1260 Infinity II Bio-inert LC System for aggregate analysis, comprising:

- Agilent 1260 Infinity II Bio-inert Quaternary Pump (G5654A)
- Agilent 1260 Infinity II Bio-inert Multisampler with sample cooler and multi-wash (G5668A)
- Agilent 1260 Infinity II Multicolumn Thermostat with bio-inert heat exchangers (G7116A)
- Agilent 1260 Infinity II Diode Array Detector WR with bio-inert standard flow cell (G7115A)
- Agilent 1260 Infinity Bio-SEC Multi Detector System (G7805AA)
- Agilent AdvanceBio SEC 300Å, 7.8 × 150 mm, 2.7 µm column (p/n PL1180-3301) or
- Agilent AdvanceBio SEC, 2.7 µm 300Å 7.8 × 300 mm column (p/n PL1180-5301)

Instrument conditions

Parameter	Value
Mobile phase	See Table 1
Flow rate	0.8 mL/min
Temperature	25 °C
Injection	1–25 µL (dependent on sample concentration)
Detection	220 nm, 280 nm, LS 90°, and DLS
Samples	Rituximab innovator, rituximab biosimilar, and BSA

Results and Discussion

To identify optimal mobile-phase compositions for each analyte mixture, we evaluated three different mobile phases across four different pH levels, representing a matrix of 12 experimental conditions. Buffer compositions were:

- 150 mM Sodium phosphate
- 10 mM Sodium phosphate + 140 mM NaCl (emulating PBS at different pH values)
- 100 mM Sodium phosphate + 150 mM NaCl

Each mobile phase was tested at pH 6.2, 6.6, 7.0, and 7.4.

To execute each experiment, we specified buffer composition and pH for each treatment in Agilent Buffer Advisor. The software automatically calculated the appropriate mixtures of stock solutions A–D to achieve the desired mobile phase specifications in real time during the LC parameter-scouting run. Table 1 summarizes the 12 experimental conditions.

These conditions were used to analyze a commercial sample of rituximab, a sample of rituximab biosimilar, and a commercial BSA protein standard mixture (10 mg/mL solution for instrument calibration purposes).

By using an Agilent AdvanceBio SEC 300Å 150 × 7.8 mm column, we were able to perform screening experiments in less than 10 minutes per sample.

Table 1. Experimental conditions and corresponding mobile phase compositions.

	User-specified parameters				Software-calculated parameters			
Experiment	pH	Buffer (mM)	NaCl (mM)	Total conc. (mM)	% A	% B	% C	% D
1	6.2	150	0	150	25.0	0.0	57.0	18.0
2	6.6	150	0	150	25.0	0.0	42.3	32.7
3	7.0	150	0	150	25.0	0.0	26.3	48.7
4	7.4	150	0	150	25.0	0.0	13.8	61.2
5	7.4	10	140	150	67.0	28.0	0.9	4.1
6	7.0	10	140	150	67.0	28.0	1.8	3.2
7	6.6	10	140	150	67.0	28.0	2.9	2.1
8	6.2	10	140	150	67.0	28.0	3.9	1.1
9	6.2	100	150	250	20.0	30.0	36.3	13.7
10	6.6	100	150	250	20.0	30.0	26.1	23.9
11	7.0	100	150	250	20.0	30.0	15.6	34.4
12	7.4	100	150	250	20.0	30.0	7.8	42.2

A = Water
B = 500 mM NaCl
C = 200 mM NaH_2PO_4
D = 200 mM Na_2HPO_4

An initial review of the chromatographic data revealed that the mobile phase conditions impacted the peak shapes of the rituximab innovator and rituximab biosimilar during the experiment, as shown in Figure 1 and Figure 2. The two versions of the molecule appear similar in profile, and demonstrate the same unusual behavior with the mobile phase composition of 10 mM sodium phosphate with 140 mM NaCl. This mobile phase composition produced a noticeable increase in peak tailing with a reduction in peak height. This type of behavior may also be seen with other proteins, illustrating the need to carefully evaluate the effect of mobile phase composition for both method development and method robustness.

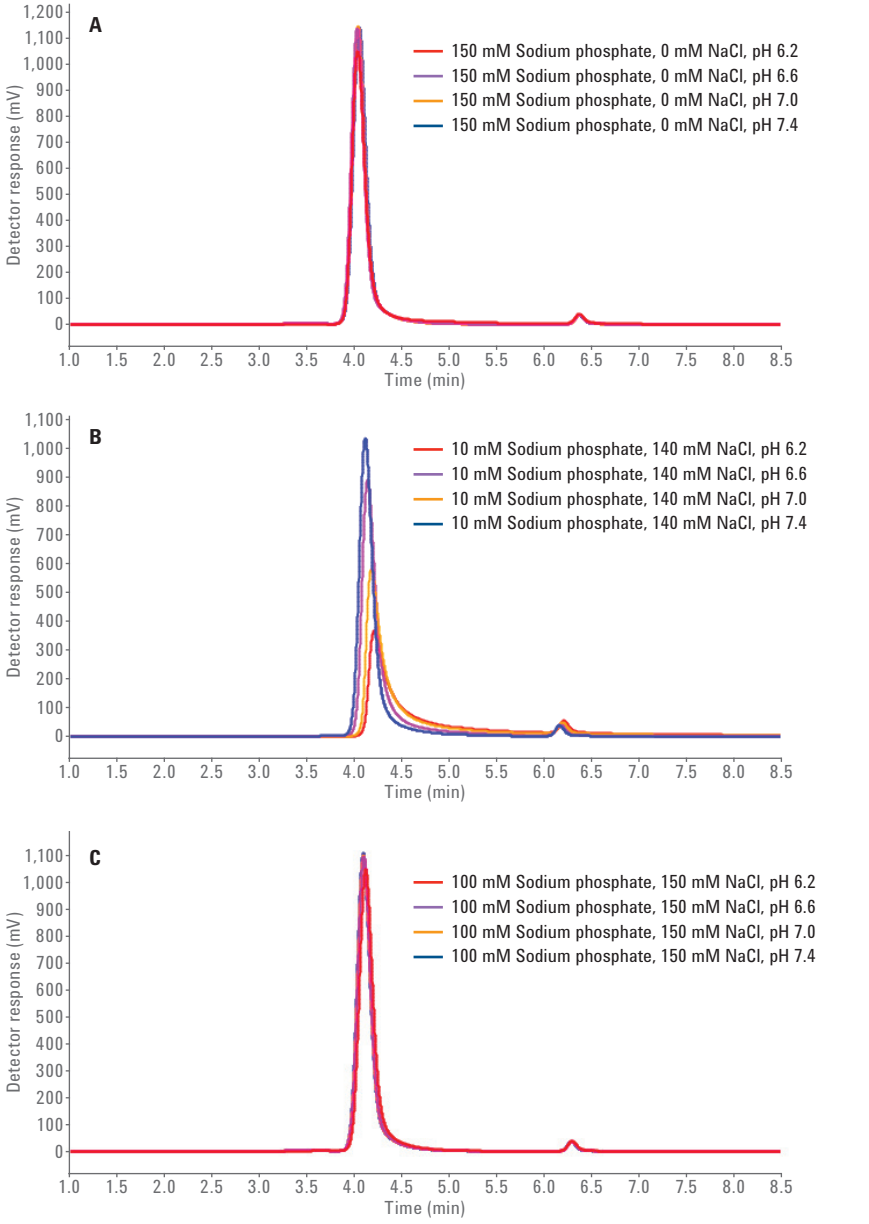

Figure 1. Rituximab innovator (1 µL injection) run consecutively at 12 different mobile phase conditions (Experiments 1–12: see Table 1).

For quantification of aggregate content, it is necessary to use the UV detector. Integration of monomer peak and aggregate peak (where detected) enables the peak area aggregate percentage to be determined. Results are reported in Figure 3A for rituximab innovator and Figure 3B for rituximab biosimilar. The most consistent results were obtained at pH 7.0 using 150 mM sodium phosphate or 100 mM sodium phosphate with 150 mM NaCl (Experiments 3 and 11).

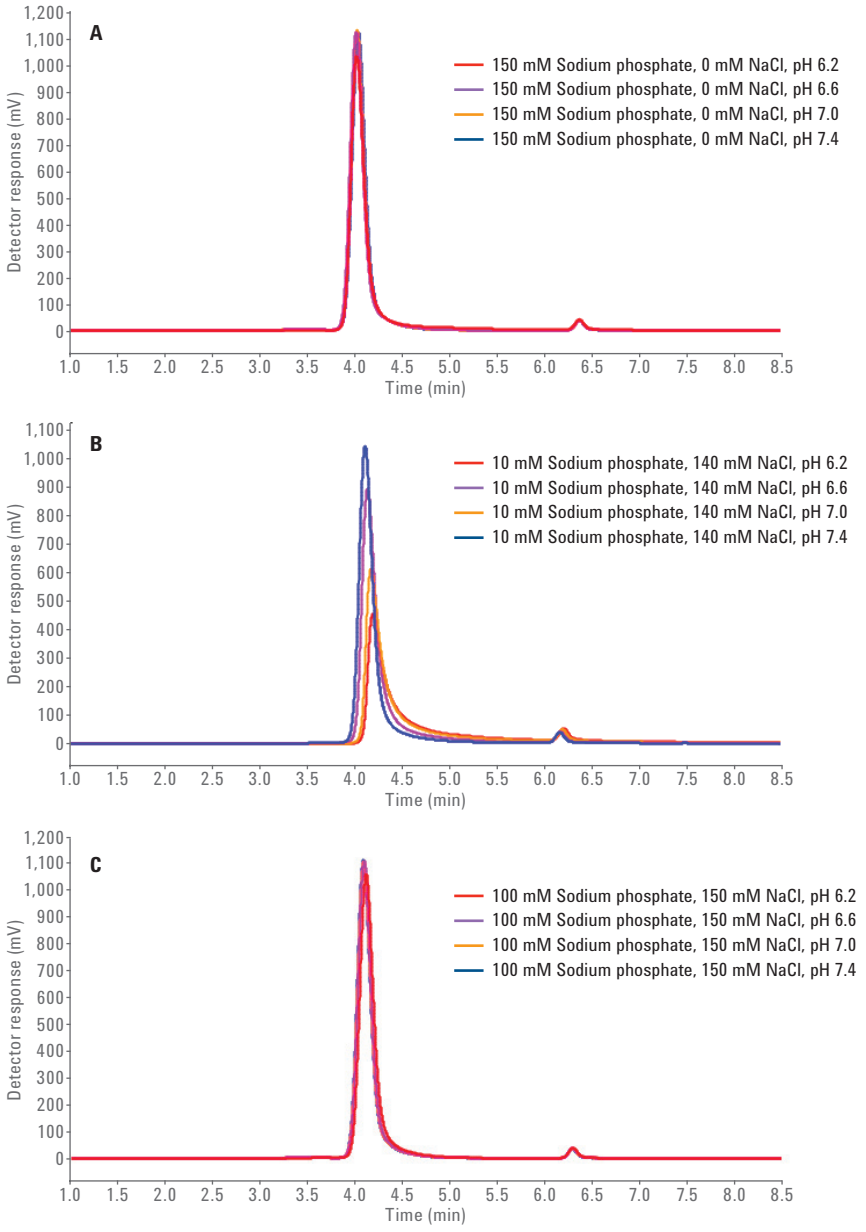

Figure 2. Rituximab biosimilar (1 µL injection) run consecutively at 12 different mobile phase conditions (Experiments 1–12: see Table 1).

Figure 4, showing chromatograms obtained at pH 7.0 in the three different buffer compositions, clearly illustrates that aggregates (highlighted with an arrow) were not detected when 10 mM sodium phosphate with 140 mM NaCl was used as mobile phase for the analysis of this molecule.

Adding a light scattering detector to the aggregate analysis workflow is optional, but it reveals more useful information about protein aggregates. Following a simple instrument calibration with a single injection of a well-defined molecule, in this case BSA, the inter-detector delay and instrument constants can be determined rapidly. Agilent Bio-SEC software can be used to report molecular weight information for individual peaks from any other chromatogram obtained under the same column and flow rate conditions.

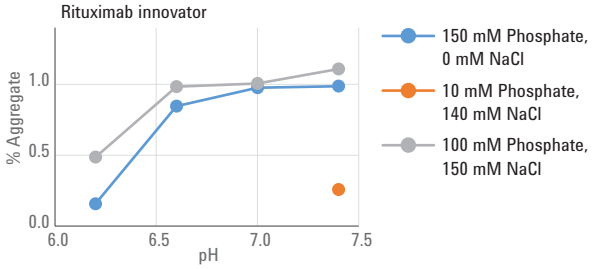

Figure 3A. Peak area percentage of aggregate content of rituximab innovator (Experiments 1–12: see Table 1). Points are shown for experiments where aggregates were detected.

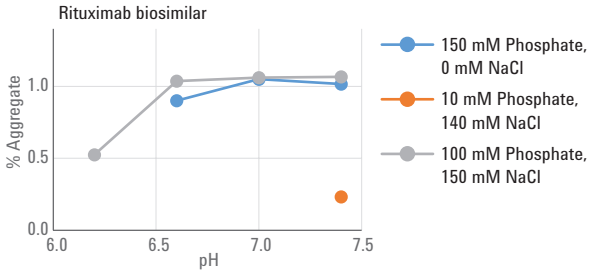

Figure 3B. Peak area percentage of aggregate content of rituximab biosimilar (Experiments 1–12: see Table 1). Points are shown for experiments where aggregates were detected.

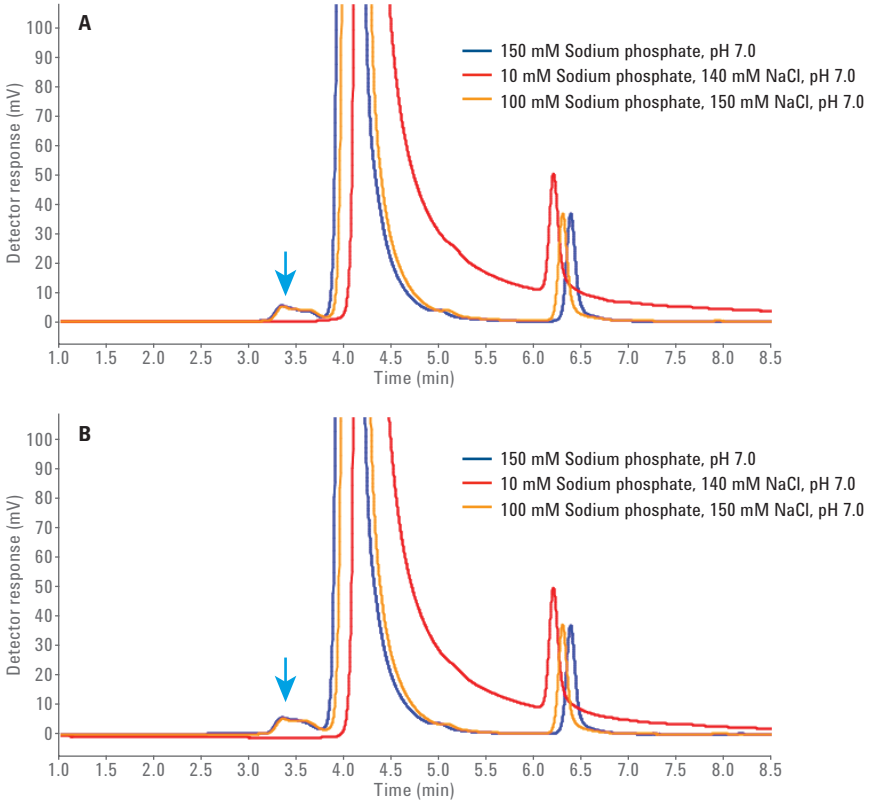

Figure 4. Baseline zoom of UV 220 nm signals of rituximab innovator (A) and rituximab biosimilar (B) run with different buffer salt concentrations at the optimized pH 7.0 (Experiments 3, 6, and 11 in Table 1).

Figure 5 shows the light scattering analysis results of the rituximab innovator versus rituximab biosimilar run under the high salt conditions of Experiment 11. Focusing analysis on the monomer peak only, the light scattering analysis reported molecular weights close to the accurate mass values seen in a previous Application Note[1]. The biosimilar molecule is expected to have a slightly higher mass due to the presence of C-terminal lysine variants not evident in the originator molecule.

The formation of larger aggregates and subvisible particles is a particular concern in biopharmaceutical applications. The analytical sensitivity of light-scattering detection toward highly aggregated samples complements the concentration data obtained from UV detection.

Despite the very similar results for monomers and dimers analyzed by UV detection, the light scattering detector proved more responsive to higher order aggregates, and revealed some differences in the more extensive aggregation of the rituximab innovator and biosimilar under certain mobile phase conditions as shown in Figure 6.

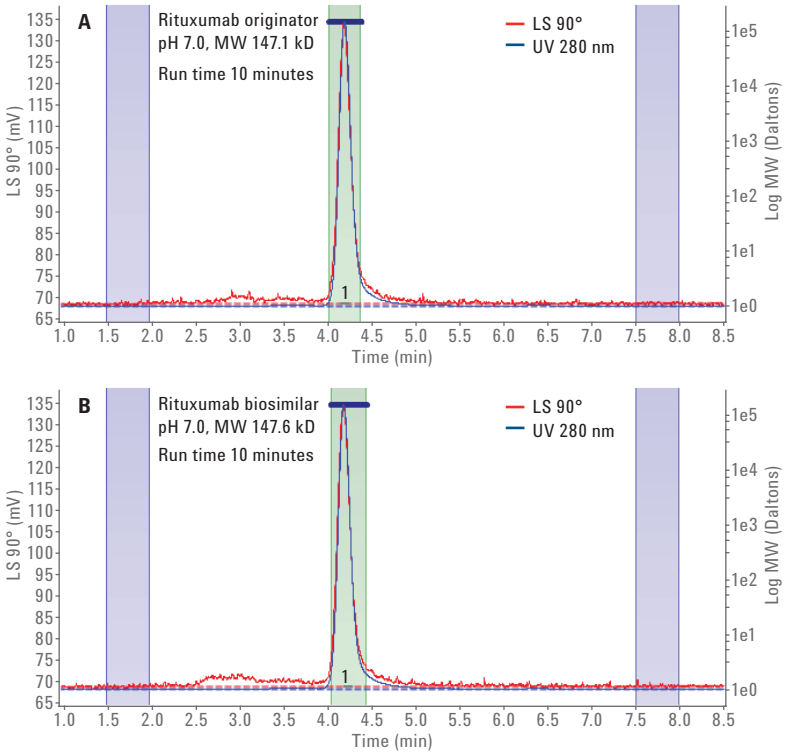

Figure 5. Comparison of LS analysis rituximab innovator (A) and rituximab biosimilar (B) run at 100 mM sodium phosphate with 150 mM NaCl, pH 7.0 (Experiment 11 in Table 1).

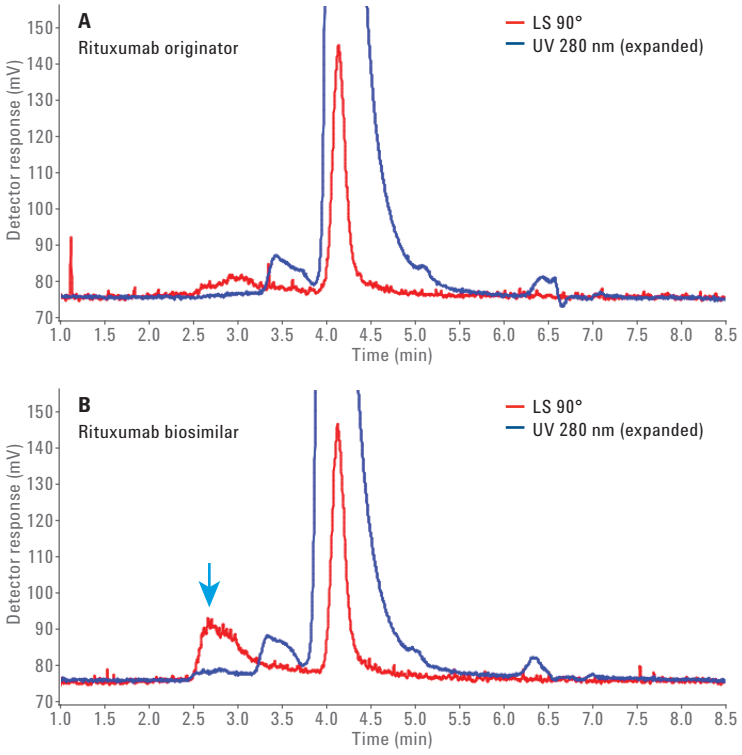

Figure 6. Comparison of LS analysis rituximab innovator (A) and rituximab biosimilar (B) run at 150 mM sodium phosphate, pH 7.0 (Experiment 3 in Table 1).

The addition of DLS capability further enhances the level of information that can be gained, providing hydrodynamic radius measurements to be made (Figure 7).

Conclusion

The Agilent 1260 Infinity II Bio-inert LC System containing a fully bio-inert flow path, with Agilent Buffer Advisor software, provides a simple way of performing method optimization for size exclusion chromatography for protein aggregate quantitation. Faster separations are possible using a shorter Agilent AdvanceBio SEC 300Å 15 cm column, which greatly increases throughput and reduces the time required for screening a wide range of analysis conditions. To gain more resolution and higher accuracy, use a longer 30 cm column. The AdvanceBio SEC column shows additional benefits such as low nonspecific binding. The Bio-MDS Multidetector Suite with Bio-SEC software can be used to reveal low levels of high molecular weight aggregates that are difficult to detect by any other means. Ultimately, the Bio-MDS can be used to determine protein molecular weight, or determine hydrodynamic radius information in conjunction with DLS detection. This suite of technologies comprises a comprehensive workflow solution to optimize SEC conditions rapidly, quantify aggregates accurately across their entire molecular weight range, and characterize the aggregation dynamics of monoclonal antibodies in relevant buffer conditions.

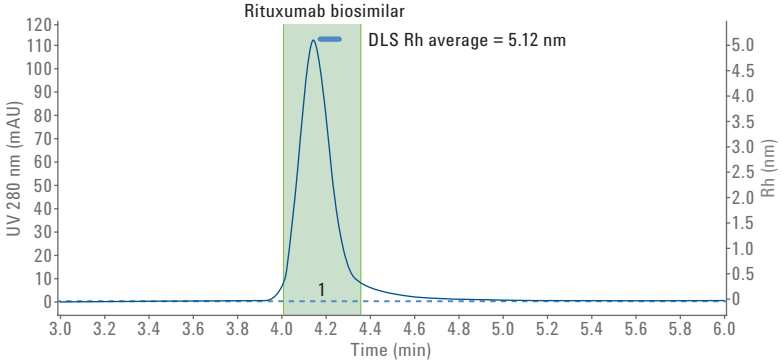

Figure 7. DLS analysis of rituximab biosimilar showing hydrodynamic radius results from LS detector.

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