Detailed Aggregation Characterization of Monoclonal Antibodies Using the Agilent 1260 Infinity Multi-Detector Bio-SEC Solution with Advanced Light Scattering Detection

Application Note

Biotherapeutics & Biosimilars

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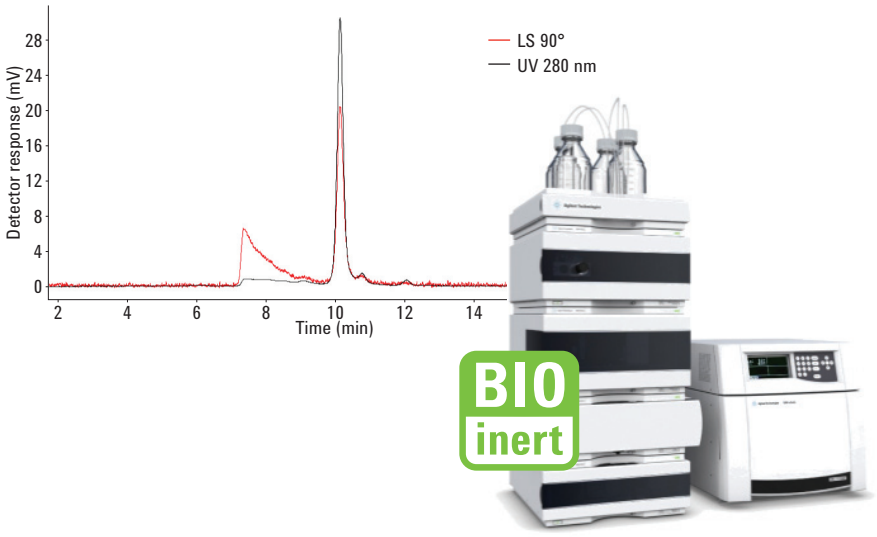
Abstract

This Application Note shows detailed size exclusion analysis of monoclonal antibodies and their aggregates using the Agilent 1260 Infinity Multi-Detector Bio-SEC Solution. Clear advantages for molecular weight determination and sensitivity for aggregates are observed with the 90° light scattering detector compared to UV detection. In combination with sensitive and linear DAD detection (R^2 = 0.9999), detailed aggregation analysis of monoclonal antibodies becomes possible.



Verified for Agilent
1260 Infinity II LC
Bio-inert System

Agilent Technologies

Introduction

The efficacy of protein-based therapeutic agents such as monoclonal antibodies (mAbs) is highly dependent on the correct primary, secondary, ternary, and quaternary structure. This includes correct amino acid composition, post translational modifications, conformation, and aggregation status. During production, storage, and transportation, the proteins can undergo aggregation processes. The presence of any type of aggregated therapeutic proteins is undesirable because they can lead to activity loss, decreased solubility, and enhanced immunogenicity. During development and production of those pharmaceutical biomolecules, it is essential to monitor the product stability. Even low aggregate levels of pharmaceutically used proteins might lead to negative physiological effects. Therefore, convenient, reliable, and especially sensitive analytical tools are required to detect and quantify trace protein aggregates.

Size exclusion chromatography (SEC) has been widely used as the standard method to characterize protein aggregates present in immunoglobulins. In traditional SEC analysis, the molecular weight (MW) is determined using a concentration-measuring detector such as an ultraviolet (UV) or refractive index (RI) detector relative to a column calibration. However, the analytical accuracy is limited due to interactions of the analytes with the SEC column[1]. For proteins sticking to the column, retention times can be delayed and inaccurate MWs calculated.

Further detection methods, such as light scattering detection, can be used to gain additional information about molecular weight and molecular size. The MW is determined using static light scattering (LS), where the scattered light intensity is measured. In addition, dynamic light scattering (DLS) enables the molecular size to be determined by measuring the hydrodynamic radius (R_H). DLS detects the fluctuations of the scattered light intensity due to the Brownian motion of the molecules in solution[2].

The Agilent 1260 Infinity Bio-inert Multi-Detector Suite with static and dynamic LS detection, enables the measurement of both MW and R_H. In combination with the Agilent 1260 Infinity Bio-inert Quaternary LC with the Agilent 1260 Infinity Bio-inert Diode Array Detector (DAD), a complete metal-free flow path is ensured.

In this Application Note, the characterization of a mAB is illustrated with respect to MW, R_H, and aggregation analysis. The advantage of using LS detection is demonstrated for the analysis of mAb aggregates when compared with UV detection. Reliable determination of MW is achieved independent of the amount of sample injected. When combined with high sensitivity DAD detection, detailed aggregation analysis of mAbs becomes possible.

Experimental

The Agilent 1260 Infinity Multi-Detector Bio-SEC Solution consists of the following modules:

- Agilent 1260 Infinity Bio-inert Quaternary Pump (G5611A)
- Agilent 1260 Infinity High Performance Bio-inert Autosampler (G5667A)
- Agilent 1290 Infinity Thermostat (G1330B) for sample cooling
- Agilent 1290 Infinity Thermostatted Column Compartment (G1316C) with bio-inert solvent heat exchanger
- Agilent 1260 Infinity DAD VL (G1315D) with a 10-mm bio-inert standard flow cell
- Agilent 1260 Infinity Bio-inert Multi-Detector Suite (G7805A) featuring dual-angle static and DLS detection (G7809A)

Column

Agilent Bio SEC-3, 300Å,
7.8 × 300 mm, 3 µm (p/n 5190-2511)

Inline filter in front of the LS detector (p/n G7808-64001) with 0.2-µm filter membranes (Supor-200 13 mm p/n 60298, Pall Life Sciences, Port Washington, NY, USA).

Software

Agilent Bio-SEC Software Version A.01.01 Build 4.30989

Solvents and Samples

All solvents used were LC grade. Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22-µm membrane point-of-use cartridge (Millipak). The phosphate buffered saline (PBS) tablets and the sheep IgG were purchased from Sigma-Aldrich, St.Louis, MO, USA. Gel filtration standard was purchased from Bio-Rad Laboratories, Inc., Hercules, CA, USA.

The monoclonal antibodies used were:

- RAT Anti-DYKDDDDK Tag Antibody
- Anti-c-Myc Monoclonal Antibody (Clone 9E10, mouse IgG1)

The prepared PBS buffer was triple filtered using a 0.2-µm membrane filter. In addition, the samples were filtered using an Agilent Captiva premium syringe filter with a regenerated 4-mm cellulose membrane, 0.2 µm pore size (p/n 5190-5106).

Even small pressure changes can cause disturbances in the column, which may result in increased baseline noise on the LS detector signal. To improve the pump/column noise and therefore to optimize signal-to-noise (S/N) ratios, it is important to consider following pump settings:

1. Bypass the multi-channel gradient valve (MCGV) in the quaternary pump. The quaternary pump has to be converted into an isocratic pump by directly connecting the solvent inlet tubing from the degasser or solvent bottle to the active inlet valve of the pump (use PEEK adapter 1/4-28 to 10-32 (p/n 0100-1847).

2. Edit the advanced parameters of the pump as seen in Figure 1, with special emphasis on the compressibility settings (46×10^{-6}/bar for water-based solvents). A low maximum flow gradient (even down to 0.2 mL/min²) is recommended for sensitive columns) as it prevents the column from damage due to strong pump strokes.

Results and Discussion

Traditional SEC analysis uses a column calibration of standard proteins of different sizes with a concentration detector (UV or RI). To illustrate the advantages of LS detection versus conventional SEC analysis, both methods were compared. Figure 2 shows the UV chromatogram of the gel filtration standard from Biorad. The masses from 670,000 Da (thyroglobulin) down to 1,350 Da (vitamin B12) were used to create a column calibration at 280 nm.

The hydrodynamic radii in nm (right axis) were measured for the three largest proteins (thyroglobulin plus aggregates, IgG plus aggregates, and ovalbumin).

Table 1. Chromatographic conditions.

Chromatographic conditions	
Mobile phase	PBS, pH 7.4
Flow rate	0.75 mL/min
Run time	25 minutes
Injection volume	Application dependent from 5 to 100 µL
Thermostat autosampler	5 °C
Temperature TCC	30 °C
DAD	280 nm/4 nm Ref.: OFF
Peak width	> 0.05 minutes (1.0 second response time) (5 Hz)
LS detector	30 °C, 5 Hz
DLS operational parameters	
Correlator run time	5 seconds
Correlator function clip time	10 µs
R^2	0.80
Eluent viscosity	0.0079 p (viscosity of water at 30 °C)
Eluent refractive index	1.333 (refractive index of water)

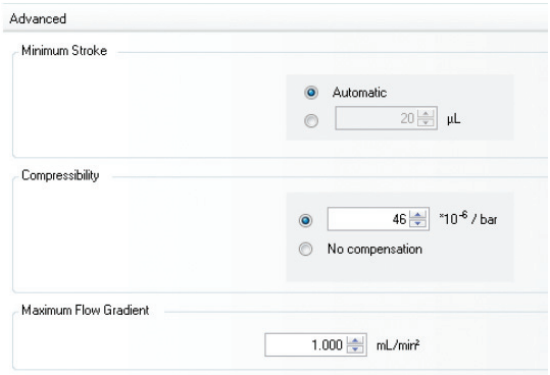


Figure 1. Advanced pump parameter.

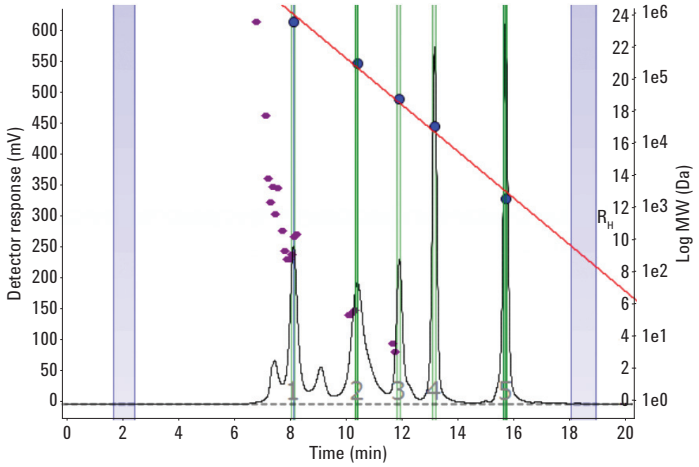


Figure 2. Column calibration for traditional SEC analysis at 280 nm using the Biorad gel filtration standard, containing different proteins from thyroglobulin (670,000 Da), IgG (158.000 Da), ovalbumin (44,000 Da), and myoglobin (17,000 Da), down to vitamin B12 with 1,350 Da.

Two different mABs were analyzed using the generated column calibration, and static LS detection at 90°. Figure 3 shows the analysis of the first mAB, anti-DYKDDDDK (Figure 3A = DAD signal at 280 nm together with the column calibration, B = LS signal at 90°). The resulting MW was approximately 153,000 Da after both methods of analysis. For this antibody, the results were absolutely comparable.

The situation was completely different for the second antibody (anti-c-Myc). Figure 4 shows the MW analysis of the anti-c-Myc antibody using (A) traditional SEC analysis with UV detection and column calibration, and (B) LS analysis at 90°.

Three peaks are clearly visible in both chromatograms. Using traditional SEC analysis, the first peak has a MW of 145,000 Da, the second 1,000 Da, and the third peak 100 Da. LS analysis at 90° (Figure 4B) reveals a MW of approximately 150,000 for all three visible peaks. Presumably, all three peaks represent the same antibody, but portions of it elute at different retention times due to the unspecific reaction of the antibody with the column. This result was confirmed after DLS analysis of the third peak with an average R_H of 5.23 nm (5.29 nm literature value[3]). According to the column calibration, this molecular size should elute at approximately 10 minutes (where the first peak of the anti-c-Myc antibody eluted). The anti-c-Myc antibody did not elute properly because it may have adhered to the column. Other possible explanations for this special elution behavior could be conformational changes due to denaturation or different folding processes (for example, nonglobular).

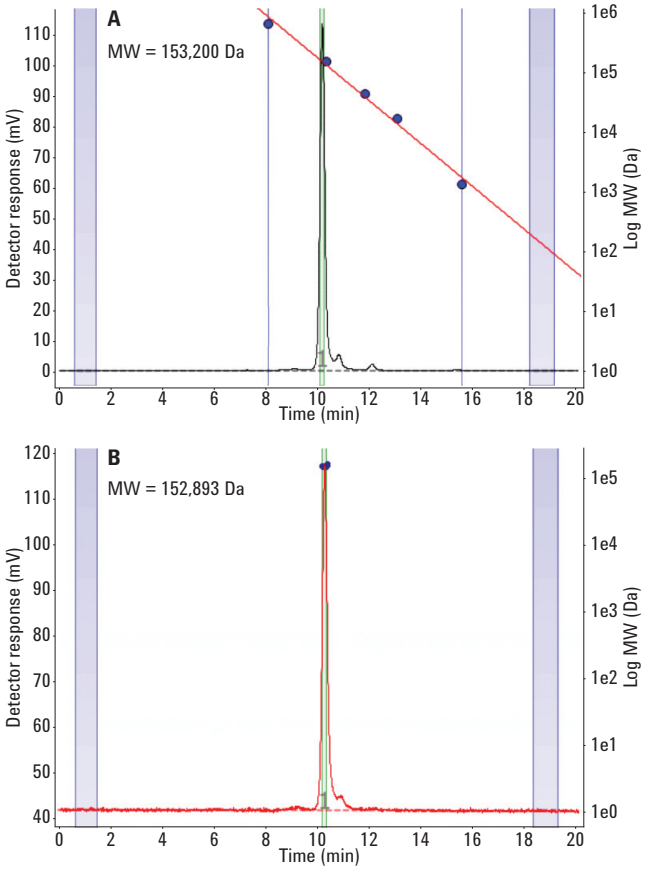


Figure 3. Comparison of traditional SEC analysis with UV detection and column calibration (A) and LS analysis at 90° (B) for anti-DYKDDDDK. Both MW results lie in the same range of approximately 153,000 Da.

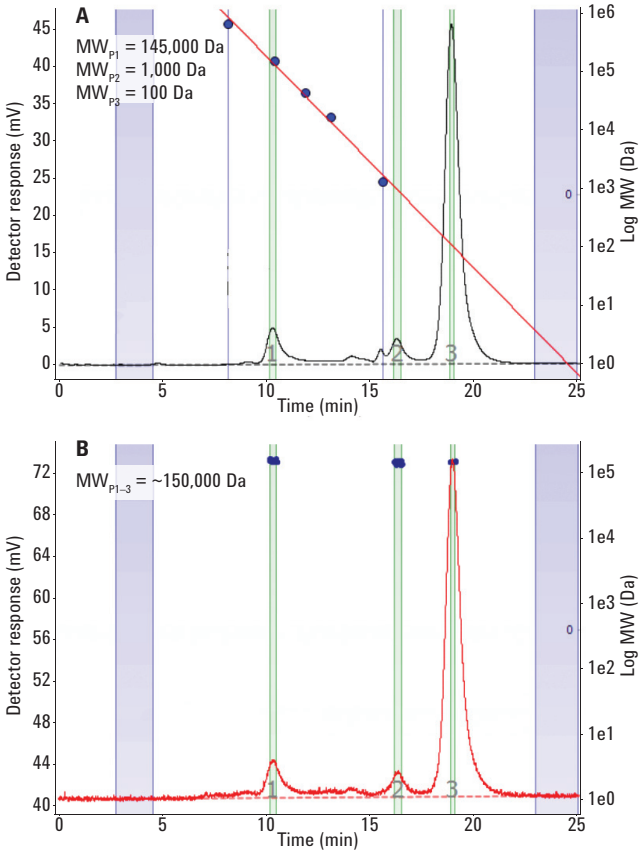


Figure 4. Comparison of traditional SEC analysis with column calibration (A) and LS analysis at 90° (B) for anti-C-Myc. The results for the second and third peak are completely different.

Due to the direct measurement principle of the LS detector, determination of the MW is truly independent of the amount of sample injected (Figures 5 and 6).

Table 2 shows the detailed MW determinations for all injected sample amounts. From 1 µg up to 120 µg, the MW values are between 145,000 and 155,000 Da (deviation of 3.33 %). The resulting MWs differed to a higher extent, with a deviation of ~17 % only for the two smallest injection amounts. The S/N of the smallest amount is 3 for the 90° signal, which also represents the standard detection limit, similar to the example observed with UV signals. Therefore, exact MW determination is difficult for these low concentrations. The limit of exact MW determination should be in the range of S/N = 10 to enable correct MW determination.

Table 2. MW determination for different injected amounts of monoclonal antibody.

µL injected	Amount on column (µg)	MW (Da)	R_H (nm)
0.1	0.2	127,584	
0.2	0.4	124,326	
0.5	1	146,225	
1	2	146,620	
2	4	148,750	
3	6	150,282	
4	8	151,515	
5	10	152,893	
6	12	152,659	
7	14	152,318	
8	16	152,419	
9	18	152,900	
10	20	150,327	
20	40	153,795	4.59
30	60	154,719	5.44
40	80	154,961	4.75
50	100	154,988	5.22
60	120	155,727	4.95

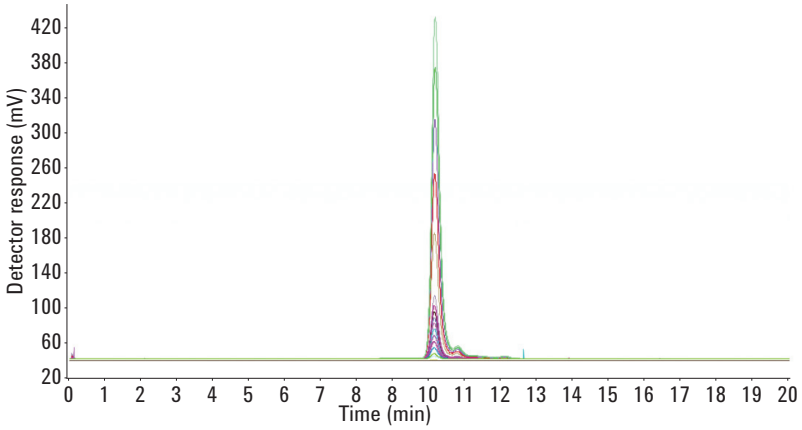


Figure 5. Overlay of 90° signals of different injected mAb amounts (anti-DYKDDDDK) from 0.2 up to 120 µg on column.

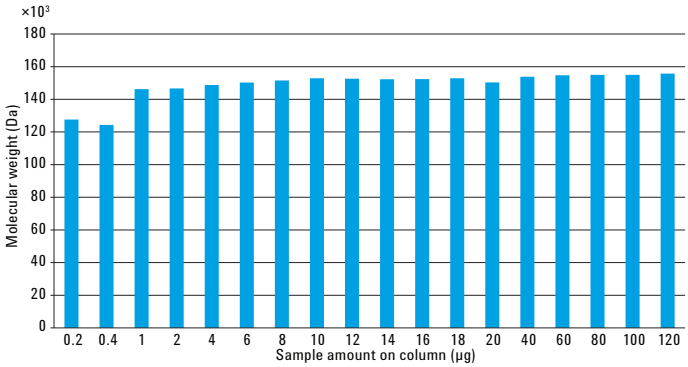


Figure 6. Determination of MW in Da for different sample amounts (anti-DYKDDDDK) on column.

For an amount of 100 µg mAb on column, the S/N value was 1,669 with a noise of 0.2 µV, and a signal of 334 (Figure 7).

To demonstrate the high sensitivity for antibody aggregates, a small amount of aggregates were generated for the anti-DYKDDDDK antibody. The antibody was heated to 60 °C for 1 hour before injection into the HPLC system. Figure 8 shows the analysis of the heated anti-DYKDDDDK antibody. A significantly higher sensitivity for aggregates was observed for the 90° LS signal.

DAD linearity for immunoglobulins was shown with a 1:2 dilution series of a sheep IgG from 10 mg/mL down to 78 µg/mL. At this range, an excellent linearity was achieved, with a coefficient of determination $R^2 = 0.9999$ (Figure 9).

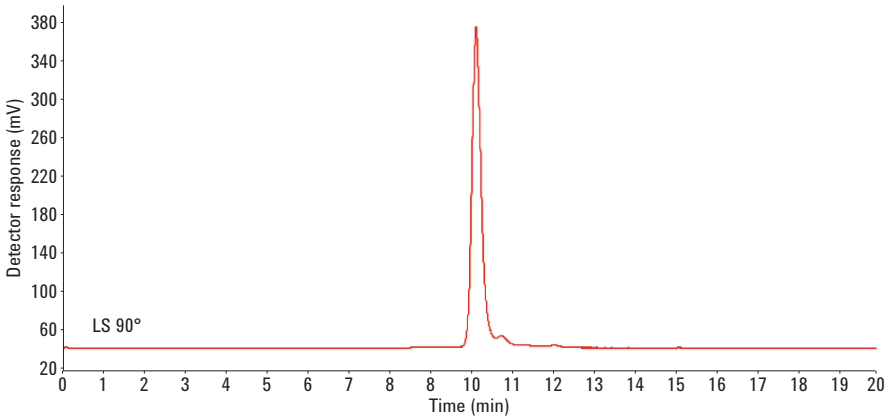


Figure 7. 100 µg mAb on column – S/N of 1,669 with a noise of 0.2 µV and a signal of 334 for the 90° LS signal.

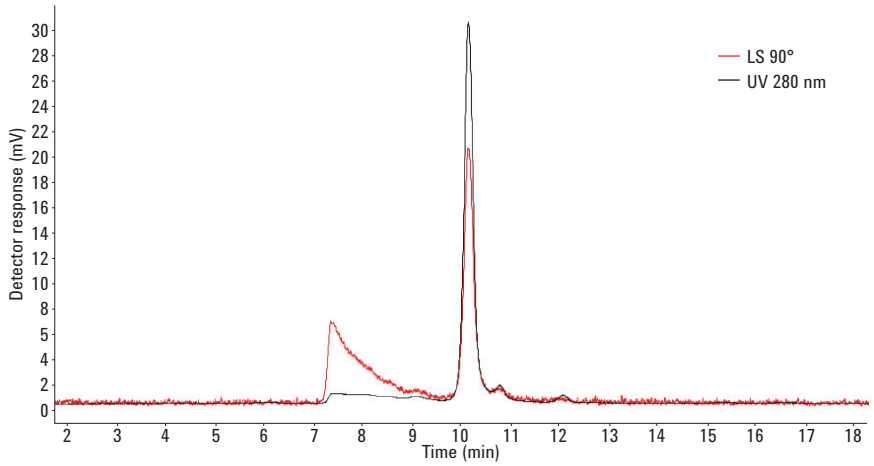


Figure 8. Analysis of anti-DYKDDDDK aggregates with DAD at 280 nm (black) and LS analysis at 90° (red). The sensitivity for aggregates is significantly higher for the LS detection at 90°.

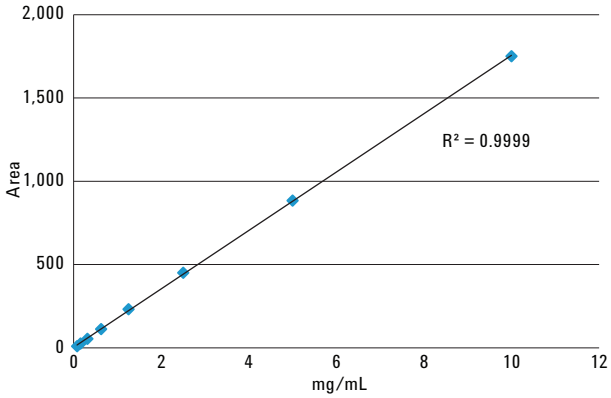


Figure 9. DAD linearity for a sheep IgG from 10 mg/mL to 78 µg/mL. R^2 was 0.9999, implying excellent linearity.

Conclusion

This Application Note characterizes two monoclonal antibodies and their aggregates in detail, using the Agilent 1260 Infinity Bio-SEC Solution. This solution comprises the Agilent 1260 Infinity Bio-inert Quaternary LC with the Agilent 1260 Infinity Bio-inert Diode Array Detector (DAD) in combination with the Agilent 1260 Infinity Bio-inert Multi-Detector Suite with dual-angle static and DLS detection.

The comparison of traditional SEC analysis (column calibration) and the direct determination of MW using LS detection at 90° revealed distinct advantages for using LS analysis. For samples showing unspecific interactions with the SEC column, the MW determination can be adulterated using traditional SEC analysis. Using an LS detector with direct measurement principle prevents inaccurate results caused by unspecific reactions between the sample and the column.

The determination of MW is completely independent of the sample amount injected. From 1 µg to 120 µg, the deviation of the MW from 150,000 Da was only 3.33 %. The S/N value was found to be 1,669, with a noise of 0.2 µV and a signal of 334 for an amount of 100 µg mAb on column.

The LS detector provided significantly higher sensitivity for mAb aggregates at 90° compared to UV detection. In combination with sensitive and linear DAD detection (R^2 = 0.9999), detailed aggregation analysis of mAbs was possible.

References

1. Gabrielson, J.P., *et al.* Quantitation of Aggregate Levels in a Recombinant Humanized Monoclonal Antibody Formulation by Size-Exclusion Chromatography, Asymmetrical Flow Field Flow Fractionation, and Sedimentation Velocity. *Journal of Pharmaceutical Sciences*, **2007**, 96 (2), 268-279.

2. Nobbmann, U., *et al*. Dynamic light scattering as a relative tool for assessing the molecular integrity and stability of monoclonal antibodies. *Biotechnology and Genetic Engineering Reviews*, **2007**, 24:1, 117-28.

3. Armstrong, J.K., *et al.* The Hydrodynamic Radii of Macromolecules and Their Effect on Red Blood Cell Aggregation. *Biophysical Journal*, **2004**, 87, 4259-4270.

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