Determination of Protein Molecular Weight and Size Using the Agilent 1260 Infinity Multi-Detector Bio-SEC Solution with Advanced Light Scattering Detection

Application Note

Biotherapeutics & Biosimilars

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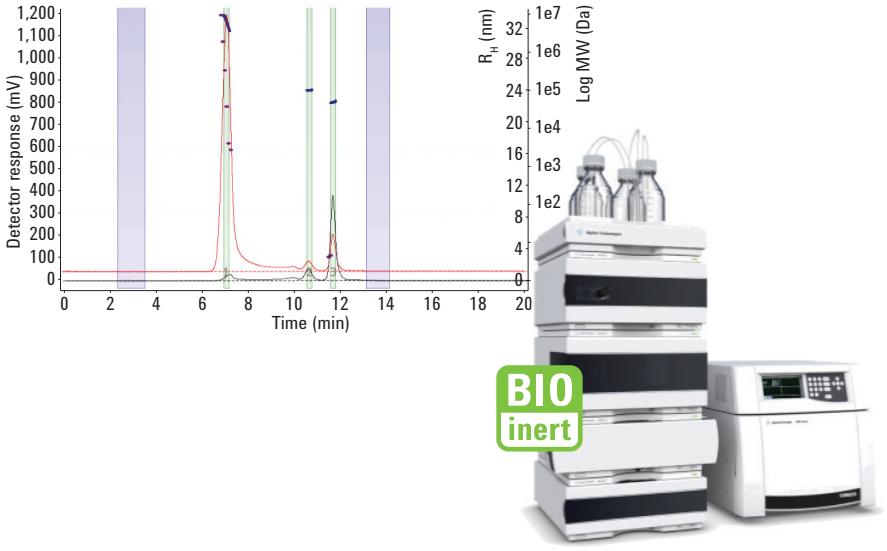
Abstract

This Application Note shows the determination of protein molecular weight and size, together with linear quantification using the Agilent 1260 Infinity Multi-Detector Bio-SEC Solution. The molecular weights and sizes of proteins, ranging from cytochrome C (11 kDa) to thyroglobulin (670 kDa) were correctly determined, independent of sample loading. Using the dual angle light scattering detector enables high sensitivity aggregation determination when compared to UV detection.



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Introduction

In protein chromatography, size exclusion chromatography (SEC) is often used to determine the molecular weight (MW) of proteins. To explain sample differences in more detail, the molecular size can also be determined. The molecular size can be depicted by the radius of gyration (R_g) or the hydrodynamic radius (R_H). The R_g is a mathematically defined dimension and it describes the overall spread of the molecule, calculated as the root mean square distance from each atom of the protein and its center of gravity. The R_H is also called Stokes radius and describes the phenomenological molecule properties as the radius of a hypothetical hard sphere that diffuses in the same manner as the solute to be analyzed. The practical aspect of the R_H makes it the more useful measurement, especially for biomolecules.

Traditional SEC uses a column calibration in combination with a concentration detector such as a refractive index (RI) or ultra-violet (UV) detector. The limitation of this method is revealed when samples with conformational changes (denaturation or post-translational modifications leading to different protein folding) are analyzed. Also, nonspecific interaction of the samples with the column (sticky samples) may result in inaccurate results for MW or size.

Static light scattering (LS) detection enables the direct determination of MW independent of column interactions or sample loading. In addition, sensitivity to detect protein aggregates is enhanced with LS detection at 90° compared to UV detection at 280 nm. Dynamic light scattering (DLS) measures R_H directly and accurately without the need for a column calibration. The Agilent 1260 Infinity Multi-Detector Bio-SEC Solution combines static and dynamic light scattering detection, and UV detection with a completely metal-free flow-path.

This Application Note presents the determination of molecular weights and molecular sizes (R_H) independent of sample loading with LS at 90° for protein samples ranging from 11 to 670 kDa. Linear and sensitive quantification was enabled by using a Diode Array Detector (DAD) within the setup of the 1260 Infinity Multi-Detector Bio-SEC Solution.

Experimental

The Agilent 1260 Infinity Multi-Detector Bio-SEC Solution comprises 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 Column Compartment (G1316C) with bio-inert solvent heat exchanger
- Agilent 1260 Infinity DAD VL (G1315D) with bio-inert standard flow cell, 10 mm
- Agilent 1260 Infinity Bio-inert Multi-Detector Suite (G7805A) featuring dual-angle and DLS detection (G7809A)

Column

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

Inline filter between 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 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 standard proteins were purchased from Sigma-Aldrich, St.Louis, MO, USA. Gel filtration standard was purchased from Bio-Rad Laboratories, Inc., Hercules, CA, USA.

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

The dilution series of the protein samples were carried out using the Agilent 7696A Sample Prep WorkBench.

Table 1. Chromatographic conditions

Chromatographic conditions	
Mobile phase	PBS, pH 7.4
Flow rate	0.75 mL/min
Run time	20 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)

Results and Discussion

The MW and size (measured as R_H) were determined for five different proteins ranging from cytochrome c, with a MW of ~11 kDa, to thyroglobulin with ~670 kDa. Figure 1 shows the analysis of ovalbumin with LS detection at 90°. Peak 3 represents the monomeric peak of ovalbumin with a MW of 47,928 Da after 90° static LS analysis (red signal trace). Peak 1 (multi-aggregate with an average MW of 5,744,499 Da) and peak 2 (Dimer, 97,531 Da) are aggregates of the ovalbumin monomer. The black signal trace is the UV signal from the DAD at 280 nm. The comparison of the 90° static LS signal and the UV signal at 280 nm showed a significantly higher sensitivity for the aggregates with the 90° LS signal compared to UV. The purple spots on peaks 1 and 3 represent the R_H values with 23 nm for peak 1, and 3 nm for the monomeric ovalbumin (peak 3).

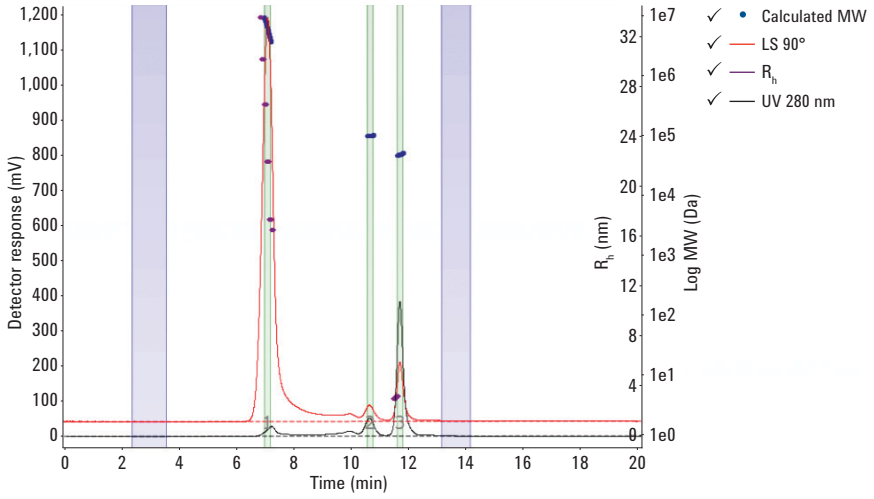

Figure 1. MW and size determination of ovalbumin. The monomer (peak 3) reveals a MW of 47,928 (blue spots) and an R_H (purple spots) of 3 nm. The 90° LS signal is displayed in red.

The MW of the monomeric ovalbumin peak was determined with different injection amounts ranging from 2 µg up to 960 µg protein on column. With an injection amount of 2 µg on column, the signal-to-noise (S/N) ratio was 7.5. Table 2 and Figure 2 show the determination of the MW from 2 to 960 µg total sample amount on column. The MW ranged from 46,079 to 47,975 Da, resulting in an average MW of 47,198 Da with an RSD of 2 %.

Table 3 gives an overview of the MW determination together with the related relative standard deviations (RSDs) for five different proteins from cytochrome c up to thyroglobulin. Different amounts of each protein were injected, and the MW determined. The RSDs were calculated over several injections resulting from MW determinations with different sample loadings, see Table 3 (injections). Depending on the sensitivity of the 90° static LS detector, the injection range was adjusted for every protein. The sensitivity of the LS detector increased with the size of the protein. Except for thyroglobulin, all MW determinations matched the literature values. For thyroglobulin, the deviation (702 kDa instead of 670 kDa) was higher due to insufficient separation of the thyroglobulin monomer and its aggregates (Figure 3). The better the resolution within a chromatogram, the more exact was the MW determination, as seen for ovalbumin (Figure 1).

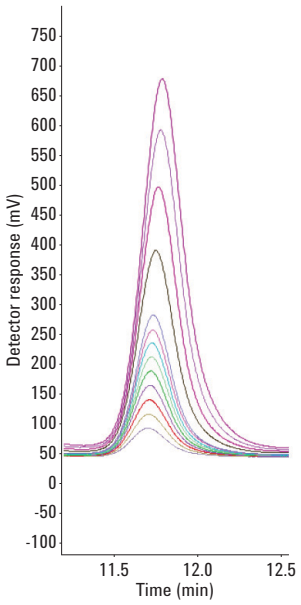

Figure 2. Overlay of 17 injections of sample amounts injected over a range of 2 to 960 µg ovalbumin on column.

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

µL injected	Amount on column (µg)	MW (Da)	R_H (nm)
0.25	2	46,079	
0.5	4	45,162	
1	8	44,763	
0.5	12	47,399	
1	24	46,795	
2	48	47,603	
4	96	47,385	3.13
5	120	47,420	2.76
6	144	47,651	2.77
7	168	47,928	2.81
8	192	47,716	2.71
9	216	47,811	2.8
10	240	47,975	2.98
15	360	47,588	2.96
20	480	47,881	2.97
25	720	47,665	3.03
30	960	47,540	2.99

Table 3. Determination of MW of five different proteins ranging from 11 kDa to 670 kDa compared to literature values.

	MW literature (Uniprot) in Da	MW experimental at 90° in Da	RSD in %	Determination range (µg on column)
Cytochrome c	11,749	11,674	2.5	180–750
Lysozyme	16,239	16,272	0.7	15–600
Ovalbumin	44,000	47,198	2	2–960
IgG	150,000	151,944	1.9	1–120
Thyroglobulin	670,000	702,652	2.9	3–76

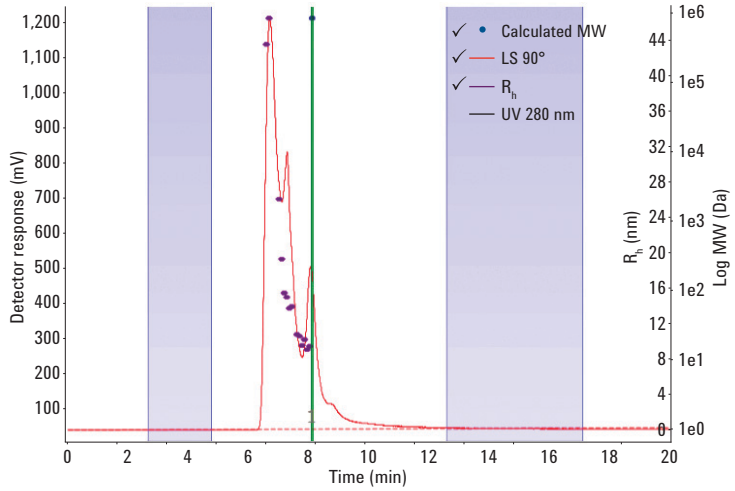

Figure 3. Analysis of thyroglobulin with 90° static LS (red signal trace) and DLS (purple dots). Due to high amounts of aggregates leading to bad resolution within the sample standard, exact determination of MW was difficult for the monomeric peak of thyroglobulin (peak 1).

Table 4 gives an overview of the determinations of molecular size as R_H together with the related RSDs for the five standard proteins. Different amounts of each protein were injected and R_H determined. The RSDs were calculated over several injections resulting from R_H determinations with different injected quantities (Table 4) (injections). Depending on the sensitivity of the DLS detector, the injection range was adjusted for every protein. The sensitivity of the LS detector increased with the size of the protein. All R_H determinations showed close agreement to the published literature values. Due to the limited sensitivity for smaller proteins, the RSD values decreased as the molecule size increased from cytochrome c to thyroglobulin.

In addition to the evaluation of LS detection for protein analysis, linearity of the DAD was also evaluated for ovalbumin and thyroglobulin. Both proteins were diluted in a 1:2 dilution series from 10 mg/mL to 39 µg/mL. For this range, excellent linearity was achieved with a coefficient of determination (R^2) = 0.9994 for ovalbumin and R^2 = 0.9999 for thyroglobulin (Figure 4).

Table 4. Determination of molecular size measured as hydrodynamic radius of five different proteins ranging from 11 kDa to 670 kDa compared to literature values.

	R_H literature in nm	R_H experimental with DLS in nm	RSD in %	Determination range (µg on column)
Cytochrome c	1.7	2.2	13	180–750
Lysozyme	1.9	2	8	270–600
Ovalbumin	2.8	2.9	5	96–960
IgG	5.1	5	7	40–120
Thyroglobulin	8.6	8.4	6	25–76

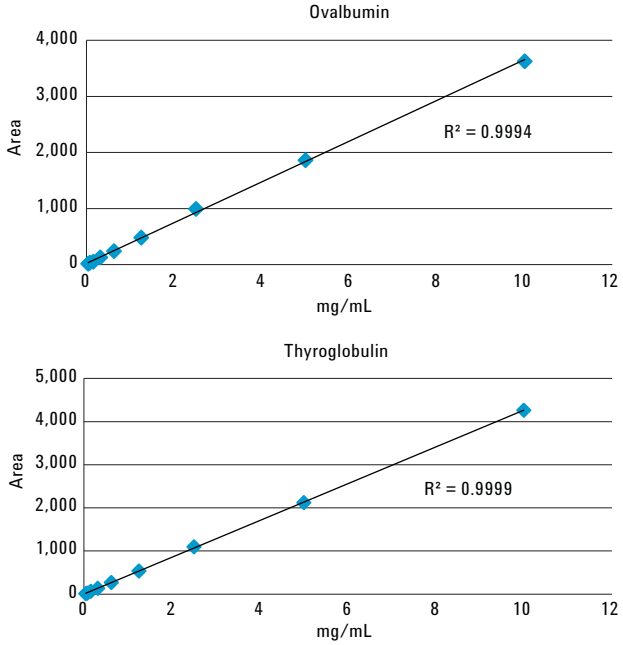

Figure 4. DAD linearity, exemplarily demonstrated for ovalbumin and thyroglobulin.

Conclusions

This Application Notes presents the Agilent 1260 Infinity Multi-Detector Bio-SEC Solution for the determination of protein molecular weight and size using size exclusion chromatography. It employs the Agilent 1260 Infinity Bio-inert Multi-Detector Suite featuring dual-angle and DLS detection together with the Agilent 1260 Infinity Bio-inert Quaternary LC System.

Five different standard proteins were analyzed for their MW and size (R_H) ranging from cytochrome c, with a MW of ~11 kDa, up to thyroglobulin with ~670 kDa. MW was determined using the static LS detector at 90°, whereas the hydrodynamic radius was determined using DLS detection. The MW and size of the standard proteins were determined with different sample loadings. The results showed good agreement to the values found in literature, provided that sufficient resolution of the measured peak was observed. The sensitivity of both static and DLS detection increased with the MW and size of the proteins. The comparison of the 90° static LS signal and the UV signal at 280 nm showed a significantly higher sensitivity for aggregates with the 90° LS signal compared to UV. In addition, highly linear and sensitive DAD detection enabled detailed protein aggregation analysis.

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