Precise Determination of Protein Molecular Weight using the Agilent 1260 Infinity Multi-Detector GPC/SEC System

Application Note

Proteomics & Protein Sciences

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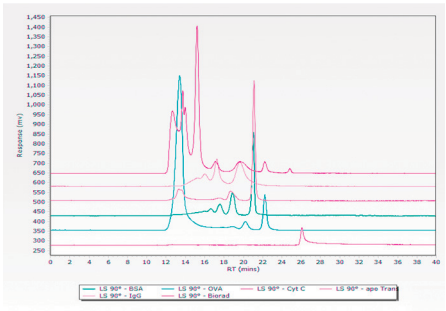
Abstract

This Application Note shows the expansion of the Agilent 1260 Infinity Bio-inert Quaternary LC System with the Agilent 1260 Infinity Multi-Detector GPC/SEC System for the determination of molecular weight, size, and relative concentration of protein samples after size exclusion chromatography. Accurate identification and quantification of proteins ranging from 11 kDa to 670 kDa was obtained using a tetra detection setup with concentration detectors (refractive index and UV), dual angle light scattering, and viscometer.



Verified for Agilent
1260 Infinity
GPC/SEC System

Agilent Technologies

Introduction

Size exclusion chromatography (SEC) (also known as gel permeation chromatography (GPC)) in biomolecule analysis comprises separation on the basis of molecular size. Molecules of different hydrodynamic volumes are distinguished on the basis of diffusion in the fine pores of the stationary phase. Larger sizes are excluded from most of the pores and, therefore, elute from the column first. In contrast, the smaller molecules diffuse into more of the pores, which results in longer retention times the smaller the molecules are, see Figure 1. In traditional SEC analysis, the molecular weight (MW) is determined using a detector measuring concentration such as an ultraviolet (UV) or refractive index (RI) detector relative to a column calibration. This detection method is limited as the molecular weight is only comparative with respect to the standards employed.

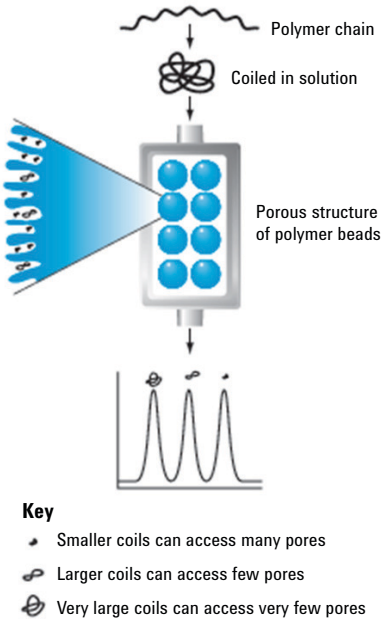


Figure 1. SEC principle.

Light scattering analysis for direct MW determination can be performed on a dual angle light scattering detector. The employment of a viscometer in the setup enables the user to determine intrinsic viscosity, and thereby molecular density, and ultimately the hydrodynamic radius (Rh).

The Agilent 1260 Infinity Multi-Detector GPC/SEC System contains an Agilent 1260 Infinity MDS RID, 1260 Infinity MDS Viscometer and an Agilent 1260 Infinity MDS Dual Angle Light Scattering Detector enabling the combination of concentration analysis and absolute determination of molecular weight along with size and intrinsic viscosity. Adding it in-line to the Agilent 1260 Infinity diode array detector (DAD) enables a tetra detection setup, allowing very sensitive protein quantification. Providing absolute determination of molecular weight, time-intensive calibration becomes obsolete. One single standard with a narrow peak is sufficient for system calibration, including accounting for inter-detector delay effects.

This Application Note presents the determination of molecular weights, molecular size (Rh, from Viscometer), and relative concentrations (if oligomers are present, using UV and/or RI) of different proteins ranging from 11 kDa (cytochrome C) to 670 kDa (thyroglobin) using the 1260 Infinity Multi-Detector GPC/SEC System in combination with the 1260 Infinity Bio-inert Quaternary LC.

Experimental

Instrumentation

The Agilent 1260 Infinity Bio-inert Quaternary LC system consisted 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 bio-inert standard flow cell, 10 mm
- Agilent 1260 Infinity Multi-Detector Suite (G7800A) featuring:
  - Agilent 1260 Infinity MDS RID (G7801A)
  - Agilent 1260 Infinity MDS Viscometer (G7802A)
  - Agilent 1260 Infinity MDS Light Scattering Detector (G7803A)

Columns

(2x) Agilent Bio SEC-3, 300Å, 7.8 × 300 mm, 3 µm (p/n 5190-2511)

Inline Filter between columns and detector (p/n 5067-4638) with 0.2-µm filter frits (p/n 5067-1562)

Software

Agilent GPC/SEC Software Version 1.2 (G7850AA) with multi-detector upgrade (G7852AA) and instrument drivers (G7854AA)

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 standard proteins and phosphate buffered saline (PBS) tablets were purchased from Sigma-Aldrich, St.Louis, 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 Syringe Filter, regenerated cellulose membrane, 4-mm diameter, 0.2-µm pore size (p/n 5190-5106).

Table 1. Chromatographic conditions.

Chromatographic conditions	
Mobile phase	PBS, pH 7.4
Flow rate	0.8 mL/min
Run time	20 (one column) or 40 minutes (two columns)
Injection volume	10 µL
Temperature TCC	35 °C
Thermostat autosampler	4 °C
DAD	280 nm/ 4 nm Ref.: OFF
Peak width	> 0.05 minutes (1.0-second response time) (5 Hz)
RID	35 °C, 1 Hz
Viscometer	35 °C, 1 Hz
Light scattering	35° C, 1 Hz

Results and Discussion

Figures 2 and 3 show the separation of bovine serum albumin on one Agilent Bio SEC-3, 300Å, 7.8 × 300 mm, 3 µm column. Figure 2 shows all recorded signals, with excellent signal-to-noise (S/N), of the 1260 Infinity Multi-Detector GPC/SEC System, the RI (zoom of RI signal, see Figure 3), both VS (Inlet and Differential Pressure) and both LS signals (15° and 90°) together with the UV signal from the DAD. The concentration of the complete sample solution was 20 mg/mL. To calibrate the system, only the monomeric peak of BSA was used, see Figure 2 or Figure 3 - its concentration was determined over the peak area percentage, resulting in 13.714 mg/mL.

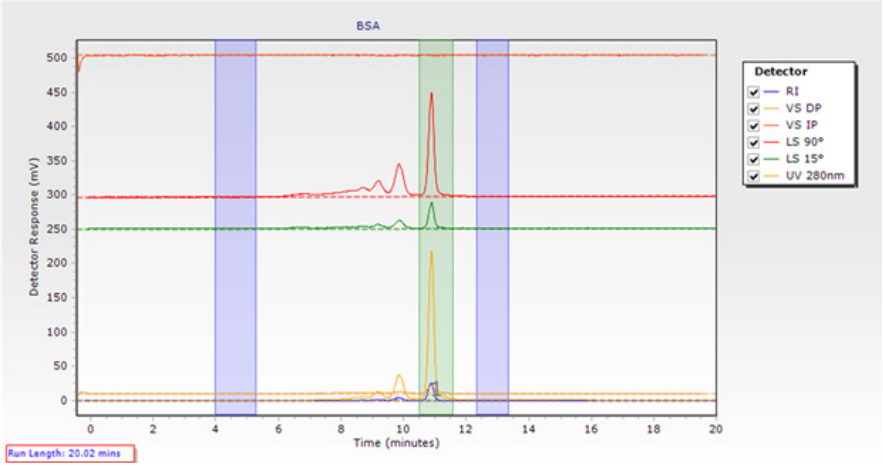


Figure 2. All signals of the separation of BSA monomer and polymers. The monomeric peak was used for system calibration of the multi-detector system.

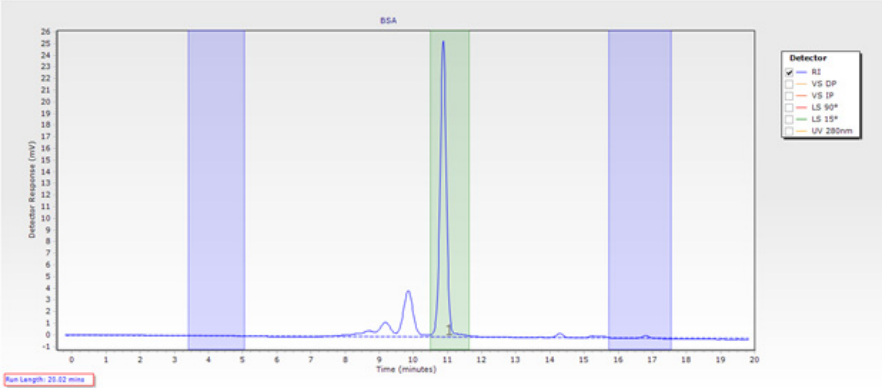


Figure 3. RI signal of the separation of BSA monomer and polymers.

The system calibration, besides the inter-detector delay (IDD) adjustment, includes the calibration of the concentration detectors (RI and UV), the light scattering detector, and the viscometer, see Figure 4. To calibrate the single detectors, it is important to enter the concentration, the refractive index increment (dn/dc), the UV extinction coefficient (ε) for the RI and UV detector, the molar mass for the light scattering detector, and the intrinsic viscosity (IV) for the viscometer. All these parameters can be entered at point of data collection and will be stored with the sample data.

After the system is calibrated, all signals can be used for the triple distribution calculation, where the software calculates the molecular weight, size, and relative concentration of the samples. The molecular weight was determined using the 90° LS detector, the relative concentration was determined using 280 nm UV (DAD) and RI detection, and the hydrodynamic radius (Rh) was determined using VS detection (triple distribution: LS, VS, and concentration detector). The 90° angle light scattering signal is sufficient for standard protein analysis due to the fact that, below one million Da, proteins are isotropic scatterers negating the requirement of multiple angles. However, adding a second angle (15°) for the detection of high MW protein aggregates improves sensitivity. In addition, the combination of both angles enables very exact MW determination for high MW protein aggregates (above one million Da).

To obtain correct results, it is essential to get best possible resolution of the single peaks in the chromatograms. Therefore, two columns were used for the determination of the molecular weight, relative concentration and size. Figure 5 shows the molecular weight distribution of the BSA monomer, dimer, trimer, and so forth, using the 90° angle signal of the LS detector.

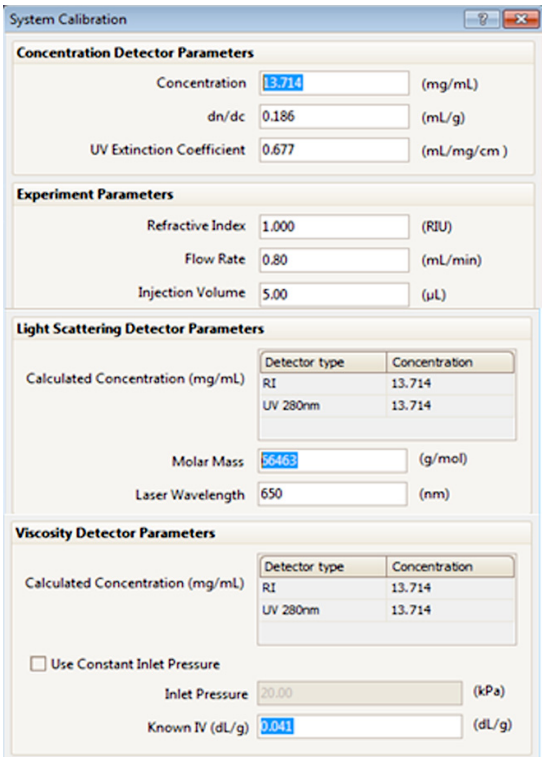


Figure 4. System calibration for the concentration detectors (UV and RI), the light scattering detectors, and the viscometer.

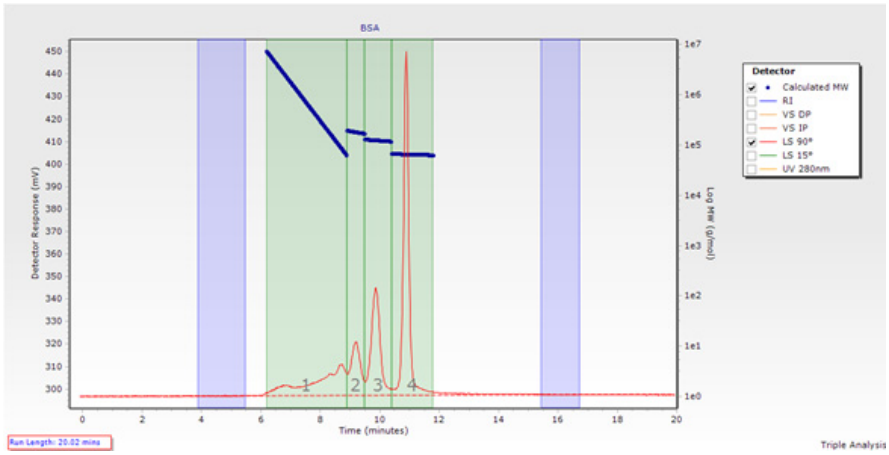


Figure 5. Molecular weight distribution of BSA monomer, dimer, trimer, and so forth, calculated using the 90° angle light scattering signal.

Six proteins, ranging from 11 kDa to 670 kDa, were analyzed for their molecular weight (MW) and size. The 90° LS signal revealed excellent sensitivity regarding S/N for all proteins, even for small proteins such as cytochrome C with a MW of 11 kDa, see Figure 6. The values were compared to those found in literature, and Table 2 displays the results for MW and their hydrodynamic radius Rh.

The comparison between the experimental data and the data from literature revealed an excellent correlation, see Figure 7. Depending on the methodology used for the determination of the Rh values from literature, the Rh values deviate, more or less, from the experimental data, resulting in slightly worse correlation compared to the molecular weight.

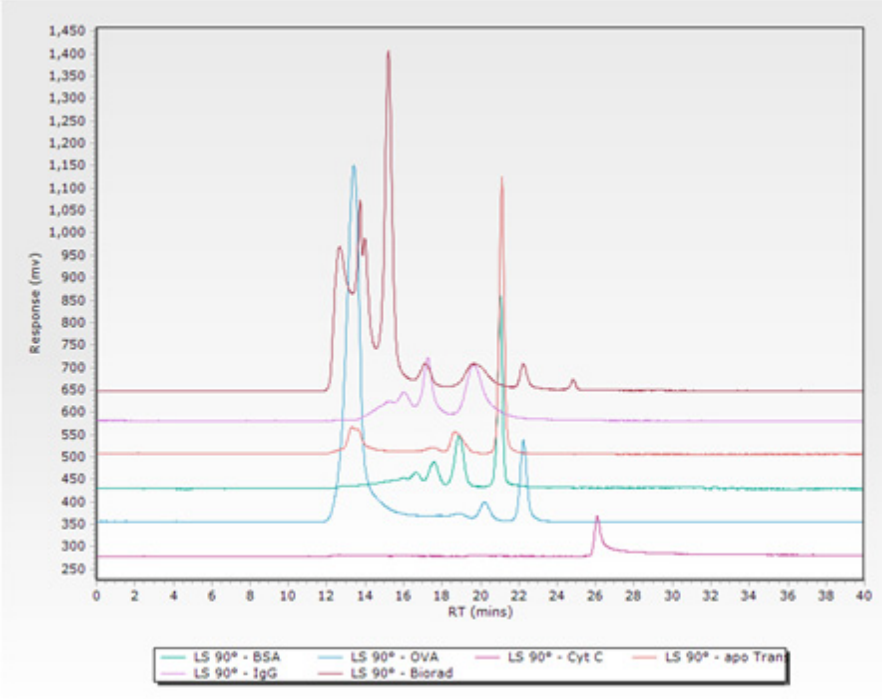


Figure 6. Overlay of the 90° light scattering signals of all six analyzed proteins.

Table 2. Molecular weight and size, experimental results from triple calculation in addition to those from literature.

	MW_{lit}	MW_{exp}	Rh_{lit}	Rh_{exp}
Cytochrome C	11,749	11,200	1.66	1.48
Ovalbumin	42,881	42,000	2.70	3.15
BSA	66,463	66,000	3.48	3.59
apoTransferrin	77,753	75,200	4.02	3.88
IgG	150,000	151,600	5.29	5.4
Thyroglobulin	670,000	672,400	8.58	8.34

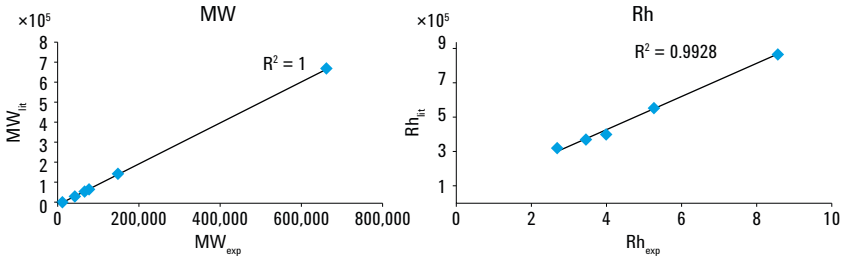


Figure 7. Comparison between experimental molecular weight (MW, from 90° light scattering) and hydrodynamic radius (Rh, from viscometer), and literature values.

Determining the molecular weight and size becomes especially important concerning biomolecule characterization, especially in the field of monoclonal antibodies (mAbs) used as biopharmaceuticals. mAb aggregates have a grave impact on the safety and efficacy of the drug. Therefore, it is of critical interest to quantify and identify the aggregates. Using light scattering, an identification of the protein aggregates can be achieved regarding molecular weight, for example for thyroglobulin (gel filtration standard from BIORAD), see Figure 8.

Table 3 shows the molecular weight of the monomer (Peak 4) and aggregates of thyroglobulin calculated using light scattering. The molecular weight of Peak 3 equals roughly the dimer of thyroglobulin, whereas Peak 2 equals roughly the tetramer of thyroglobulin with a monomeric mass of approximately 670 kDa. The molecular mass of Peak 1 is tenfold higher than the molecular mass of the tetramer, representing a high-mass aggregate of thyroglobulin. The peak area %, calculated using the UV signal at 280 nm from the DAD, represents the relative concentration of the monomers and the aggregates. From 20 mg/mL total concentration, the relative concentration results in 15.35 mg/mL for the monomeric peak, and 4.65 mg/mL for the aggregates.

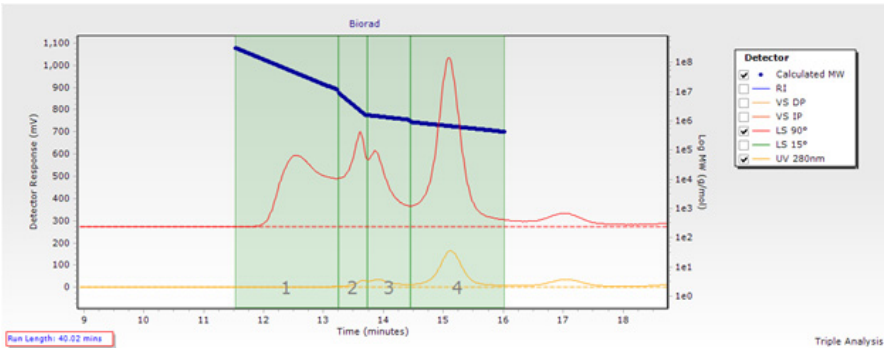


Figure 8. Separation of thyroglobulin monomer and its aggregates, 90° LS (red) and UV 280 nm (yellow).

Table 3. Molecular weight and relative concentration of thyroglobulin monomer (Peak 4) and its aggregates.

Thyroglobulin (Biorad)					
	Mwlit	Peak 1	Peak 2	Peak 3	Peak 4
MW *LS*	670,000 Da	26,999,000 Da	2,766,000 Da	1,342,000 Da	672,000 Da
Peak area % *UV 280 nm*		1.15 %	7.00 %	15.03 %	76.46 %

Conclusion

This Application Note shows the employment of the Agilent 1260 Infinity Multi-Detector GPC/SEC System together with the Agilent 1260 Infinity Bio-inert Quaternary LC System for the determination of protein molecular weight and size after size exclusion chromatography. Using this tetra detection setup, containing RI, VS, LS, and UV detection, it was possible to accurately identify and quantify proteins from 11 kDa, representing small proteins, up to big proteins such as thyroglobulin with a molecular mass of 670 kDa.

After the system calibration, all signals were used for triple distribution calculation using LS, VS, and concentration detectors to calculate molecular weight, size, and relative concentration of the samples. The comparison of the molecular weight determination with literature values revealed an excellent correlation with a coefficient of determination of $R^2 = 1$. In addition, good correlation, 0.99, could be achieved for the determination of the hydrodynamic radius Rh, representing molecular size. The relative concentration of thyroglobulin aggregates could be identified and relatively quantified.

The combination of the 1260 Infinity Multi-Detector GPC/SEC System together with the 1260 Infinity Bio-inert Quaternary LC System is ideal for the determination of MW, size and relative concentration of protein samples across a wide molecular weight range.

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