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APPLICATION NOTE

CHARACTERIZATION OF RECOMBINANT PROTEIN BIOTHERAPEUTICS BY UHPLC-SEC-MALS

INTRODUCTION

Biotherapeutics are generally larger molecules such as peptides, proteins and monoclonal antibodies with monomer molecular weight ranging from 3,000-150,000 Da. The drug must remain free from impurities such as fragment, dimer and other higher order aggregates as they may cause severe immunogenic response. This becomes even more important if the biotherapeutic protein is thermally susceptible. Historically, size exclusion is the preferred mode of chromatography used for separation and characterization of such applications. Here we report the online detection of absolute molecular weight of two recombinant protein samples using a UHPLC size exclusion chromatography (SEC) column directly connected to the LenS3™ Multi-Angle Light Scattering (MALS) detector.

MATERIALS AND METHODS

Samples: BSA (Calibration standard)
Sample 1: Re. Protein (~90 kDa) at 1.72 mg/mL
Sample 2: Re. Protein (~90 kDa) at 3.64 mg/mL

The samples were stored at -20 °C and thawed to 8 °C just before analysis. The concentration was adjusted by diluting the sample in mobile phase pre-chilled at 8 °C.

CHROMATOGRAPHIC CONDITIONS

Instrument: ThermoFisher Ultimate® 3000 UHPLC
Column: TSKgel UP-SW2000 (P/N 0023514), 2 µm, 4.6 mm ID × 30 cm L
Mobile phase: BupH modified Dulbecco's phosphate buffer prepared in light scattering grade water and filtered through a 0.1 µm PES membrane
Flow rate: 0.20 mL/min
Detection: UV @ 280 nm and LenS3 MALS detector (positioned in series: → UV → MALS)
Temperature: 25 °C
Injection vol.: Sample 1: 25 µL; ; Sample 2: 10 µL

RESULTS AND DISCUSSION

The multi-detector setup was calibrated using a freshly prepared bovine serum albumin (BSA) solution. Figure 1 shows the overlay trace of UV and MALS detectors. The one-step calibration procedure in the SECview™ software adjusted the dead volume between the detectors and corrected for the band-broadening effect caused by the in-series detector configuration while determining the detectors' calibration constants and offsets.

Figures 2 and 3 illustrate the UV detector overlays for two consecutive injections of Sample 1 and Sample 2 respectively. Zoomed-in figures (in set) show the excellent separation resolution between the monomer and the aggregates obtained from TSKgel UP-SW2000 column.

CALIBRATION OF THE DETECTORS USING BSA PROTEIN STANDARD

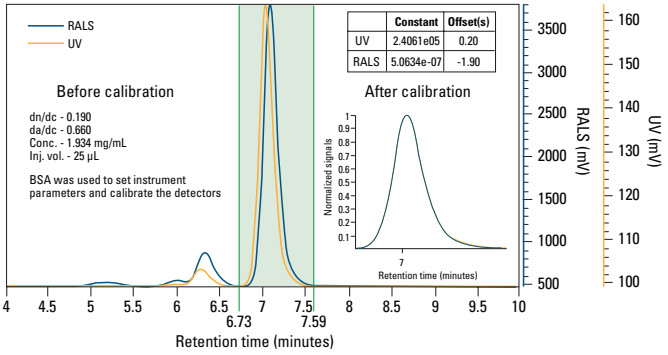

Figure 1

UV DETECTOR OVERLAY FOR SAMPLE 1 - TWO CONSECUTIVE INJECTIONS

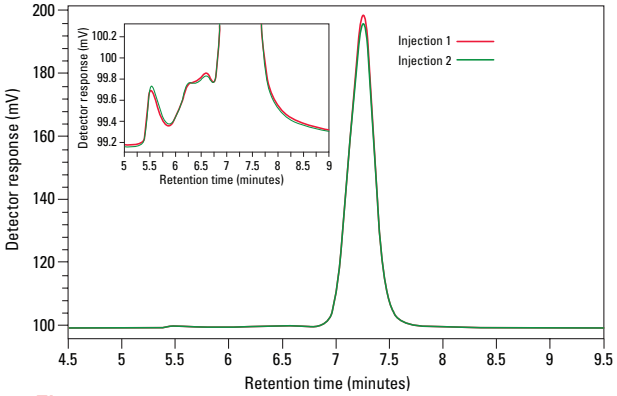

Figure 2

UV DETECTOR OVERLAY FOR SAMPLE 2 - TWO CONSECUTIVE INJECTIONS

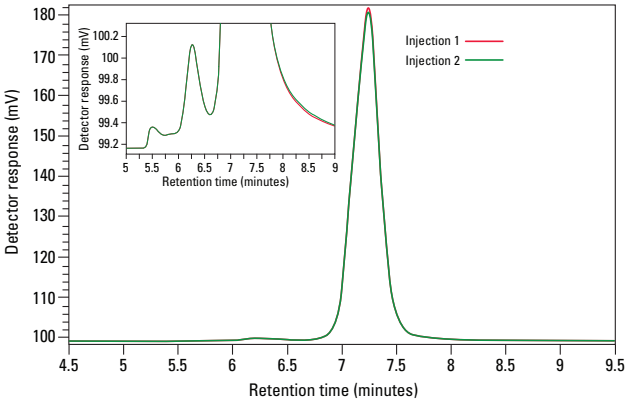

Figure 3

Figures 4 and 5 demonstrate the molecular weight (green) profiles for Sample 1 and 2 respectively. The concentrations of the aggregate contents differ in the two samples. Looking closer at the UV trace, it appears that the monomer peaks in both samples illustrate a slight shoulder on the higher molecular weight region, suggesting a bimodal shape. Further analysis using the molecular weight trace by the MALS detector reveals two separate populations of molecular weights. Figures 6 and 7 zoom in on the monomer peaks and demonstrate the two molecular weight plateaus, 1a and 1b, in both samples. Considering the sensitive nature of these recombinant proteins to the ambient conditions, the shoulder peak (1b) suggests the beginning of temperature-induced modifications, which varies in extent in both samples.

MOLECULAR WEIGHT PROFILE FOR SAMPLE 1

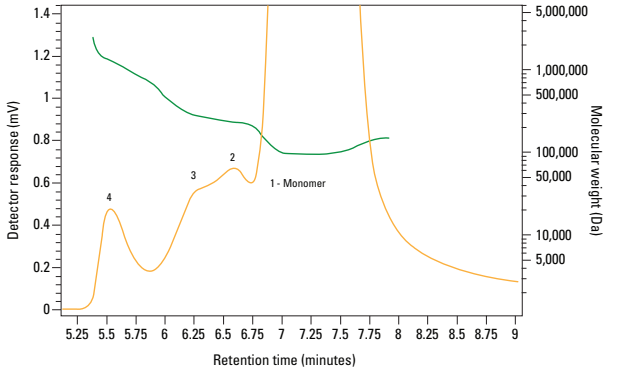

Figure 4

MOLECULAR WEIGHT PROFILE FOR SAMPLE 2

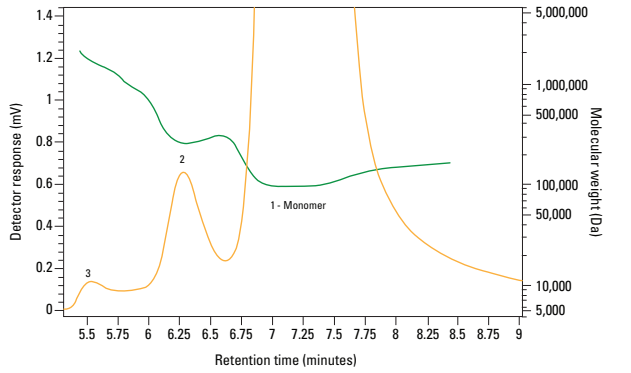

Figure 5

MOLECULAR WEIGHT PROFILE OF THE MONOMER PEAK FOR SAMPLE 1

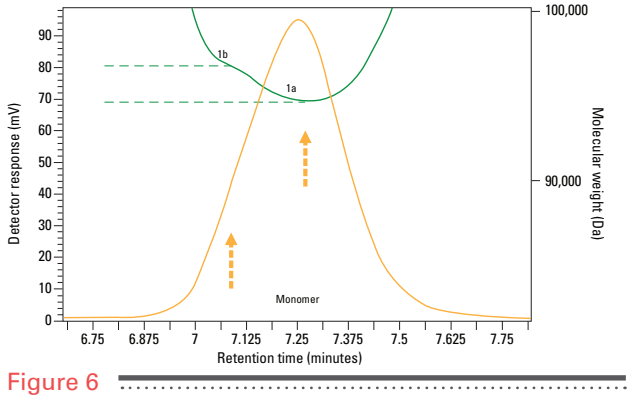

Figure 6

Tables 1 and 2 list the results including molecular weight and percent content, for the identified aggregate peaks.

MOLECULAR WEIGHT PROFILE OF THE MONOMER PEAK FOR SAMPLE 2

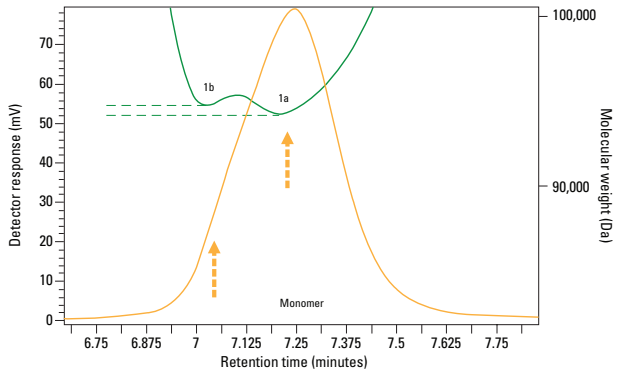

Figure 7

Peak	Retention time (min)	Peak MW Da	Area % UV
1a	7.220	93,564	98.06
1b	-	95,811	
2	6.593	229,024	0.69
3	6.355	276,217	0.73
4	5.533	1,285,996	0.51

Table 1

Peak	Retention time (min)	Peak MW Da	Area % UV
1a	7.242	93,106	98.73
1b	-	93,602	
2	6.282	251,078	1.04
3	5.540	1,631,227	0.23

Table 2

CONCLUSIONS

This study shows that the molecular weight species including monomer and multiple aggregate levels present in the recombinant protein therapeutics could be determined and quantified using a SEC-MALS configuration. TSKgel UP-SW2000 demonstrates excellent separation of the higher order aggregates from the monomer in both Sample 1 and Sample 2, as well as the slightly higher molecular weight temperature-induced variants that almost co-elute with the monomer. The MALS detection using LenS3 accompanied by the SECview software produces reproducible, accurate results in terms of MW determination for all peaks, in addition to area calculations, even at extremely low concentrations/presence of the aggregates.