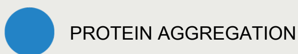
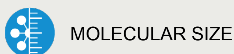
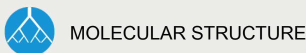
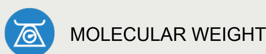
Characterization of IgG monomers & their aggregates

A comparison between column calibration & multi-detection SEC









Introduction

In biopharmaceutical formulations one of the key factors that manufacturers need to understand is the propensity of their samples to aggregate. Aggregates in biologic drugs are undesirable for two reasons. They reduce the amount of active ingredient in the sample, thereby reducing efficacy, and they can stimulate immunogenic responses in the body. This in turn can lead to more rapid clearance of the drug and again reduce efficacy, or in some cases can lead to severe immune responses. Full characterization of protein drug samples is therefore necessary both for those developing formulations and those characterizing final products. It is therefore extremely important to have a technique which can both measure the amount of different protein aggregate components and identify and characterize each of them. In this way a sample can be fully characterized and understood.

Traditionally, the determination of such information has employed size exclusion chromatography (SEC). Using SEC, sample components can be identified by their molecular weights in a process that compares the retention volume of analyte molecules through a size exclusion column against that of a series of standards with known molecular weights. In addition, the relative amounts of each component can be simply extracted from the respective peak areas providing that a concentration detector is used to record the elution and the relevant extinction coefficients are known.

Although a well-established technique and an industry standard for the characterization of proteins, the use of column calibration SEC for the determination of molecular weight is not without issues. Principally amongst these are the inaccuracies introduced into the analyses through shape-dependent elution. In this application note we characterize two antibody samples using column calibration and multi-detection SEC. Here we show the outcomes of

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both analyses and demonstrate how multi-detection SEC - a technique that combines the resolving power of chromatography with the revealing power of light scattering detectors and a viscometer. In a multi-detection SEC system, the light scattering detectors measure absolute molecular weight (as opposed to a column calibration system that can only provide a r*elative* measurement) and the differential viscometer provides information about the molecular structure of the protein being analyzed. This wider range of accurate data provides a more complete characterization of the protein mixtures under study is obtained.

Materials and methods

A protein molecular weight marker kit (Sigma Aldrich) comprising six globular proteins and an exclusion marker was prepared in phosphate buffered saline (PBS, pH 7.4) at final concentrations of approximately 1-3 mg/mL. In addition, two polyclonal immunoglobulin G samples (IgG, Sigma Aldrich) from different sources were also prepared in PBS at final concentrations of 2-3 mg/mL. All samples were filtered (cellulose acetate membrane, 0.2 µm) and three replicate injections of 100 µL were applied to the size exclusion column set (2x Viscotek P3000, Malvern, UK) using the zero-waste mode for the most volume-economic sample delivery. All measurements were undertaken on an OMNISEC system at a flow rate of 1 mL/min using phosphate buffered saline as the mobile phase. The sample tray, column and detector compartments were maintained at 25°C throughout data acquisition.

Results and discussion

IgG chromatograms: ultraviolet absorbance

The elution profiles of two IgG samples were monitored using an ultraviolet (UV) absorbance detector (Figure 1). An overlay of these chromatograms provides a qualitative comparison between samples and allows the minor differences in their relative composition to be discerned.

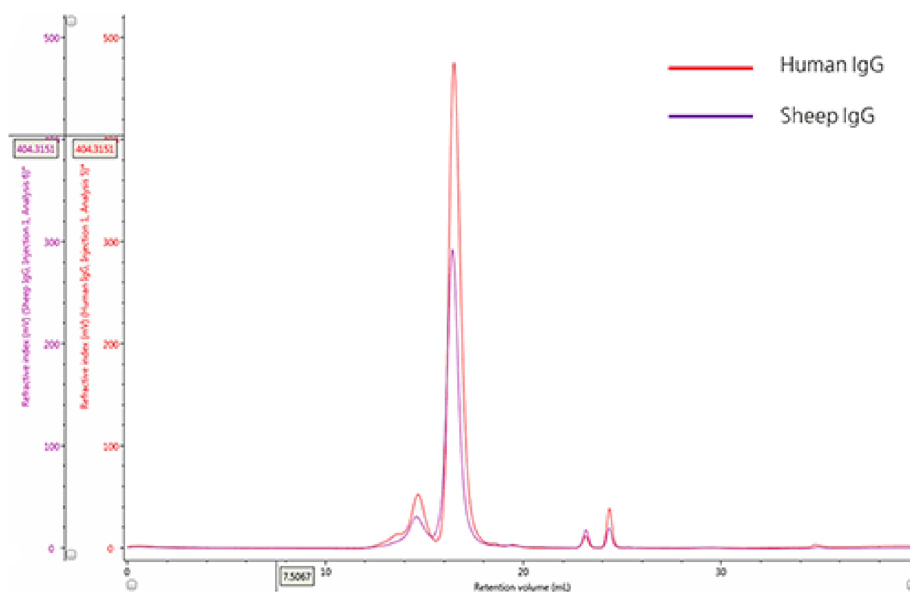

Figure 1: Overlay of representative UV chromatograms of human IgG (red) and sheep IgG (purple)

The chromatograms of both IgG samples show a major peak with a retention volume (V_R) at approximately 16.0 mL and also reveal the presence of multiple species at lower retention volumes. From size exclusion theory, we conclude that the peaks upstream from the main peak correspond to species that have a progressively larger hydrodynamic size and are most likely aggregates of the IgG monomer. Conversely, the minor peaks downstream of the main peak, with V_R ~ 24mL, correspond with molecules with small hydrodynamic radii and are most likely salt molecules.

Characterization of IgG samples by SEC column calibration

The logarithm of the molecular weight (Log Mw) of each protein standard was plotted as a function of the ratio of the protein retention volume to the column void volume (V_o). A linear fit of the data provided the calibration parameters necessary to estimate the molecular weight values for peaks in the chromatograms of both IgG samples (Figure 2). A summary of these estimated Mw values and other quantitative attributes identified by column calibration are provided in Table 1.

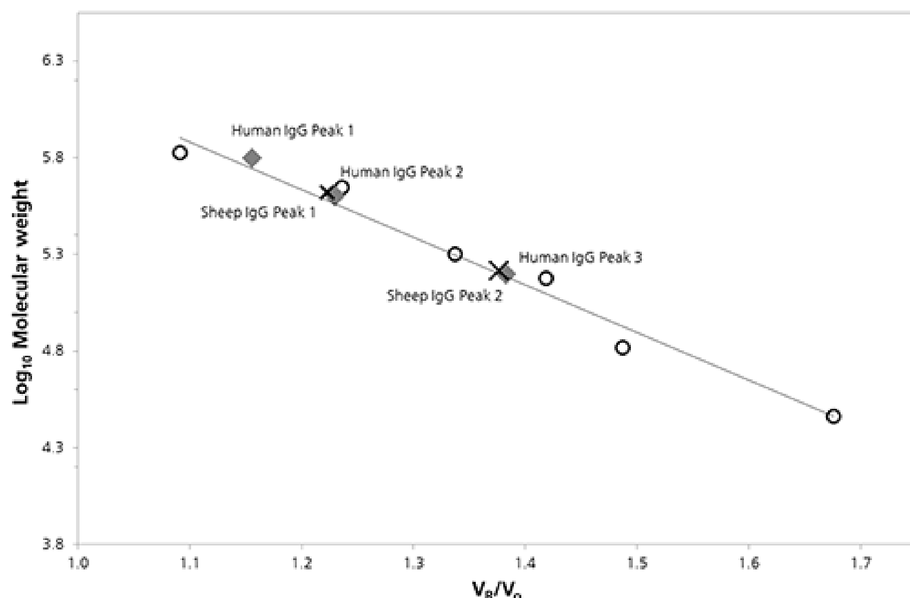

Figure 2: SEC Column calibration curve with sheep (cross) and human (diamond) IgG data superimposed

Table 1: Summary of results from SEC column calibration

	Human IgG				Sheep IgG		
	Peak 1	Peak 2	Peak 3	Peak 4	Peak 1	Peak 2	Peak 3
V_R (mL)	> 13.00	13.80	14.69	16.51	>13.80	14.60	16.44
Mw (kDa)	-	627.2	398.9	158.1	-	417.6	163.8
Identity	higher order aggregate	tetramer	n/d	Monomer	higher order aggregate	n/d	monomer

Antibody monomers have a nominal molecular weight in the region of 150 kDa. From column calibration data we observe that the estimated Mw determined for Peak 3 (human) and Peak 2 (sheep) most likely corresponds to the monomer species; although both values are slightly greater than the nominal molecular weight at 158.1 and 163.8 kDa, respectively. The peaks at lower retention volumes, 14.69 mL (Peak 2 human) and 14.60 mL (Peak 1 sheep), are not in good agreement with the dimer or trimer masses and thus cannot be definitively identified. Peak 1 (human) has an estimated molecular weight that may correspond with the tetramer mass (632.4 kDa).

Characterization of IgG samples by chromatography coupled multi-detection

The same IgG samples were analyzed using multi-detector SEC. The resulting quantitative properties of human and sheep IgG samples are summarized in Table 2.

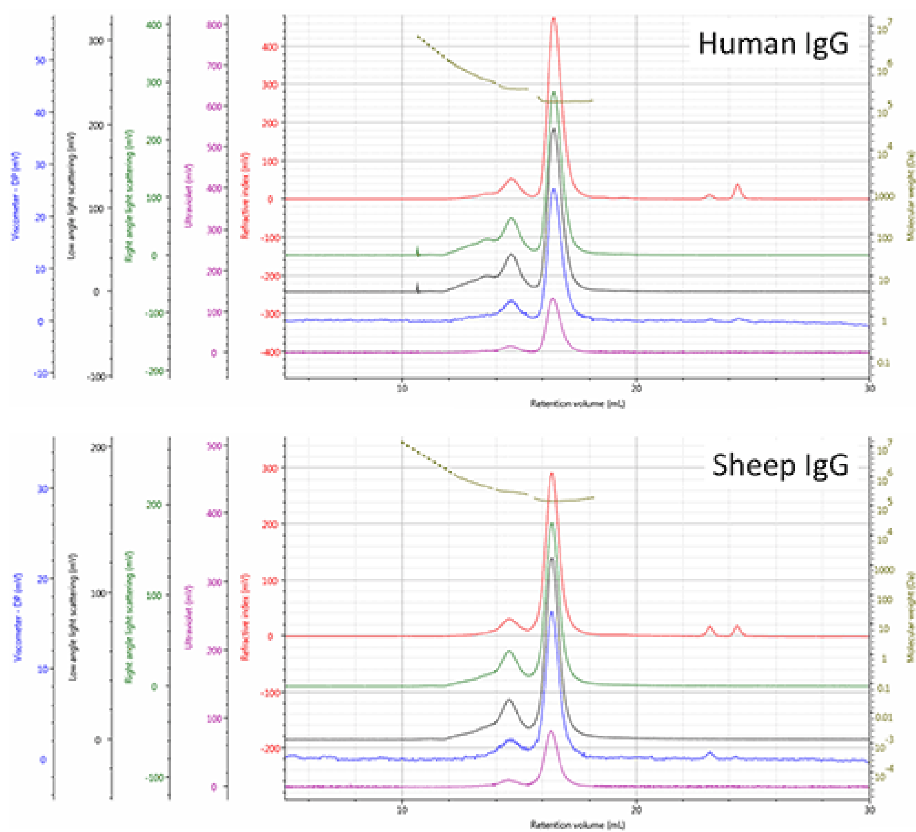

Figure 3: Multi-detector SEC chromatograms of human and sheep IgG samples - refractive index (red), right angle light scattering (green), low angle light scattering (black), viscometer (blue), ultra violet (purple). The molecular weight of species is shown in olive.

Table 2: Summary of the results from multi-detector SEC

	Human IgG				Sheep IgG		
	Peak 1	Peak 2	Peak 3	Peak 4	Peak 1	Peak 2	Peak 3
Mw (kDa)	722.8	459.0	299.5	147.5	551.7	306.7	152.0
Mw/Mn	1.0334	1.0008	1.0002	1.0002	1.0697	1.0010	1.0002
Rh (nm)	11.0	8.2	6.7	4.8	8.8	6.8	5.0
IV (dL/g)	0.159	0.086	0.068	0.049	0.097	0.064	0.051
Identity	higher order aggregate	trimer	dimer	monomer	higher order aggregate	dimer	monomer
% Composition	1.3	2.4	11.4	84.9	2.8	11.6	85.6

The absolute Mw values obtained for Human IgG Peaks 1-3 are in line with the expected values for the trimer, dimer and monomer species, respectively. Similarly, the respective Mw values determined for sheep IgG Peaks 1 and 2 are in good agreement with dimer and monomer species. Additional information provided by the viscometer allows the determination of the hydrodynamic radii of both IgG monomers and a comparison of these values rationalizes the respective elution order of the two molecules i.e. human IgG monomer is smaller (R_h 4.8 nm) and thus elutes slightly later than the sheep IgG monomer. Signals at points upstream of the dimer and trimer peaks (R_V < 14.0 mL) relate to molecules of progressively higher molecular weights and most likely correspond to randomly associated higher order aggregates (Figure 3). This idea is supported by the relatively high polydispersity (Mw/Mn) associated with these molecules i.e. 1.0334 and 1.0697 for human and sheep mixtures. Clearly, there are differences in aggregate composition between the two IgG samples and the presence of a significant trimer population in the human but not sheep samples may indicate differences in the stability or routes of aggregation for this sample and may warrant further investigation.

Shape-dependent inaccuracies in column calibration SEC

Overall, the Mw values determined by column calibration SEC are larger than those determined by multi-detector SEC analyses. For peaks corresponding to the monomer (in each case) the Mw values reasonably allow identification of the monomer species; however, for aggregate peaks – such as the dimer – the Mw values deviate from the expected values by as much as 28% and thus identification cannot be made with any certainty. These errors – to which multi-detection analysis is not susceptible – can be attributed to the influence of shape on the elution of antibody molecules and their aggregates through the column matrix. According to size exclusion theory, the column permeation characteristics of a protein molecule are governed by hydrodynamic volume. Since hydrodynamic volume is a function of both mass and structure, it is possible for a protein of a given molecular weight to have an equivalent or smaller hydrodynamic volume as that of a protein with a lower molecular weight whose atoms are less densely packed or whose structure is elongated. In practice, if calibration curves were constructed with a series of these two differently shaped molecules they would yield different slopes. The use of globular proteins in our column calibration – as is the standard method – means that we are imparting an assumption of globular shape onto our samples. Given that the antibody structure is not globular but Y-shaped, this assumption manifests itself in increasingly overestimated and erroneous Mw values with increasing molecular size.

Conclusion

Analyses of two different IgG samples were undertaken by column calibration and multi-detector SEC. Unlike column calibration SEC, the data collected by multi-detector SEC allows conclusive assignment of all discrete peaks to particular oligomeric states and allows differences in the aggregation states between the samples to be discerned and quantified.

Using this information can enable biopharmaceutical manufacturers to identify and understand the behavioral characteristics of different candidates in different formulations or under different conditions. This in turn allows better understanding and control of subsequent production and efficacy of such drugs. The sensitivity of Malvern’s OMNISEC system enables measurements of a few micrograms or less of sample. Its autosampler can make injections with no sample waste making it a particularly frugal system and it can keep samples cool in the autosampler prior to measurement to prevent aggregation while the sample is waiting to be measured. In combinations, these capabilities make it an ideal system for the characterization of proteins and biopharmaceuticals.

Malvern Instruments Limited

Grovewood Road, Malvern,
Worcestershire, UK. WR14
1XZ

Tel: +44 1684 892456
Fax: +44 1684 892789
www.malvern.com

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