The Benefits of SEC-MALLS in Biopharmaceutical Applications

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Size exclusion chromatography (SEC) is a standard technique for the analysis of monoclonal antibodies (mAbs) and proteins. Since aggregates and fragments can influence the efficacy and safety of the final product, low and high molecular species have to be closely monitored during quality control. SEC separates compounds according to their hydrodynamic radius, with larger molecules such as antibody aggregates having lower elution volumes since they are excluded from the silica pores and therefore have shorter pathways through the column. Smaller molecules including antibody fragments elute later since they are able to diffuse into the pores resulting in them having longer pathways through the column. Therefore, SEC is a valuable technique for characterising these biomolecules as they are differing in size.

Limitations of SEC-UV/RI analyses

A calibration with known molecular weight standards and ultraviolet (UV) or refractive index (RI) detection gives information on the molecular weight of the analyte. However, in SEC it is assumed that the target analyte has the same molecular conformation as the compounds in the calibration standard which is not always true. A typical example would be two structurally complex proteins with different conformations and therefore different hydrodynamic radii but the same molecular weight. Using SEC, different retention times for the proteins will be obtained: the more globular shaped protein will elute later as it can diffuse into the pores and has a longer pathway through the column, whereas the more extended shaped protein will elute earlier as it cannot diffuse or only to a limited degree into the pores. Calculating the molecular weight based on the previous calibration will therefore lead to significant differences in the apparent molecular weight, but in fact they have the same molecular weight.

Overcoming obstacles with light scattering detection

As SEC-UV (or -RI) can lead to incorrect molecular weight determination, more precise detection techniques are required in order to obtain a more reliable analysis. Hyphenating SEC with light scattering (LS) detection helps to overcome these limitations.

LS can be measured by static or dynamic light scattering detectors. Static light scattering (SLS) detectors measure time-averaged scattering intensities, while dynamic light scattering (DLS) detectors record light intensity fluctuations. Both detection options can be coupled with SEC, but typically a multi-angle (laser) light scattering detector (MALLS or MALS), which is a type of SLS detector, is combined with SEC.

In MALLS detection, a laser beam shines through a sample solution containing the target molecule. The light is scattered in multiple directions and the intensity is measured at fixed angles e.g. 50° or 90° *(see Figure 1)*. For molecules <100 kDa, the scattering pattern is isotropic, which means that there is no difference in the intensity of the scattered light between different angles which is why detectors at different angles are not needed. However, for analytes >100 kDa the scattering is anisotropic making different detectors a necessity as the scattering profile is not uniform.

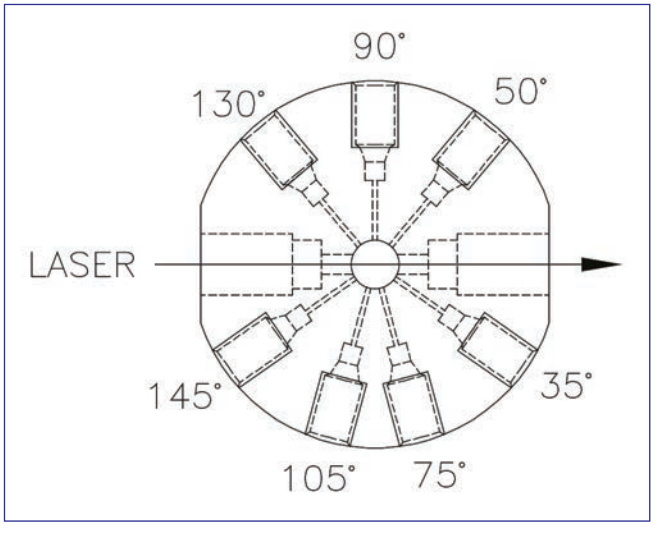

Figure 1. Cell design of a MALLS detector.

Advantages of MALLS detection

The most valuable benefit of MALLS detection over other techniques is that there is no column calibration needed at all. Since the light intensity is proportional to the molecular weight the true value can be directly calculated.

In addition, MALLS gives information on the size and conformation of the macromolecule and also on the molecular weight distribution. Since the sample is already separated by the SEC column coupled to the detector, it is perfectly fractionated for the following MALLS detection. SEC-UV can only provide information on the average concentration, whereas MALLS reveals the purity of the sample solution according to the molecular weight distribution.

Increased sensitivity for aggregates

Coupling SEC with MALLS detection is a powerful tool for the characterisation of biomolecules. In general, LS shows high signals for higher molecular weight species such as aggregates of antibodies compared to UV or RI detectors. *Figure 2* shows a comparison of the analysis of bovine serum albumin (BSA, 66 kDa) using UV and MALLS detection highlighting the improved sensitivity of the LS detection of higher molecular weight species. Also, the direct dependency of the molecular weight and the intensity is demonstrated as the relative proportion between UV and LS signal gets bigger the larger the aggregate becomes.

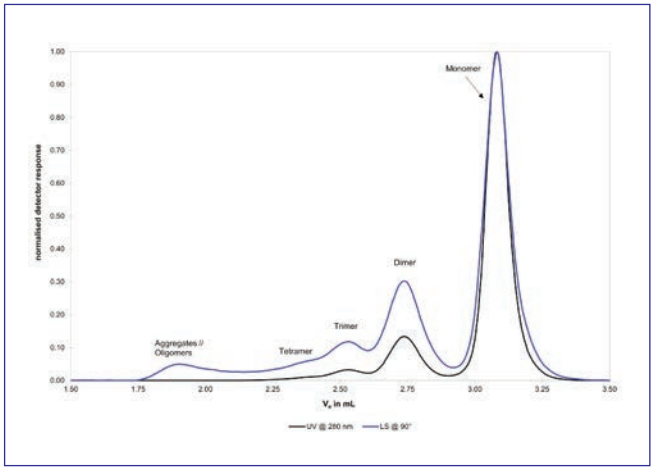

Figure 2. Chromatogram of BSA recorded with UV at 280 nm (black) and MALLS at 90°C (blue). The separation was performed using a YMC-SEC MAB column (3 µm, 300 x 4.6 mm ID).

Choosing the right buffer and salt concentration

Different buffer systems have been tested for the analysis of Bevacizumab (Avastin®) using MALLS and UV detection. As typically phosphate buffers with non-denaturing conditions (pH 6-8) containing NaCl are used in SEC, the following buffers have been tested: a phosphate buffered saline pH 7.4 (PBS; containing 0.138 M NaCl, 0.027 M KCl) and phosphate buffers pH 6.6 (Sigma-Aldrich, 0.034 M) with varying concentrations of NaCl (0.1, 0.3 and 0.5 M).

It turned out that a defined minimum ionic strength is necessary to achieve a robust method with good resolution which is not achieved by the use of phosphate buffer with 0.1 M NaCl. No significant difference could be determined between the chromatograms obtained using PBS buffer and phosphate buffer with 0.3 or 0.5 M NaCl *(Figure 3)*. However, compared to the UV signal, the MALLS signal shows 2 higher molar mass species which are aggregates of Bevacizumab at about 2.0 and 2.3 mL elution volume. With PBS buffer a further signal could be detected at about 4.0 mL.

A YMC-SEC MAB column was used which was designed for the SEC analysis of antibodies. This column has a pore size of 25 nm which shows good resolution for low and high molecular species of antibodies. In order to prevent spikes in the chromatogram caused by particles from the column itself stationary phases of the highest purity are required. YMC SEC columns are highly inert making them the ideal choice for coupling with MALLS detection as they provide signals unaffected by spikes.

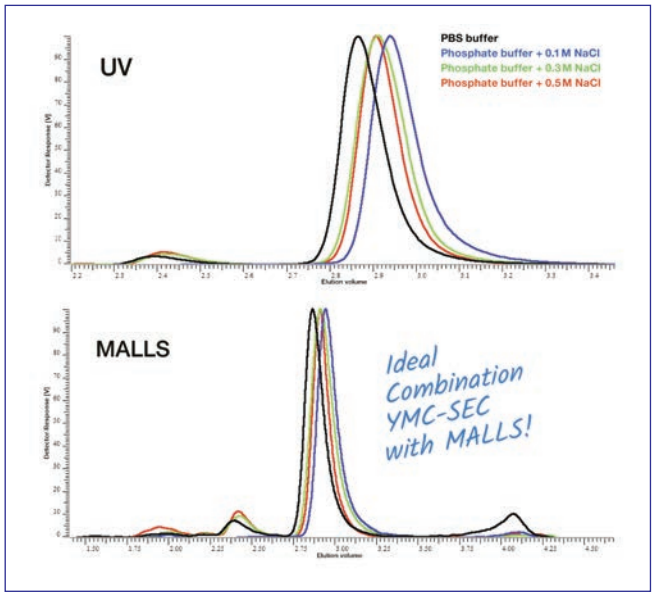

Figure 3. Chromatograms of Bevacizumab using different eluents. Top: UV at 280 nm. Bottom: MALLS detection trace (90° angle). Black: PBS buffer; blue: phosphate buffer + 0.1 M NaCl; green: phosphate buffer + 0.3 M NaCl; red: phosphate buffer + 0.5 M NaCl.

Influence of injection volume and flow rate

For further investigation on the influence of the injection volume and flow rate, phosphate buffer with 0.3 M NaCl was chosen. Increasing the injection volume from 10 µL through 25 µL and up to 50 µL did not show any influence on the resolution. Also decreasing the flow rate from 0.33 mL/min to 0.165 ml/min did not improve the resolution *(Figure 4)* which is why the higher flow rate was preferred for a higher sample throughput.

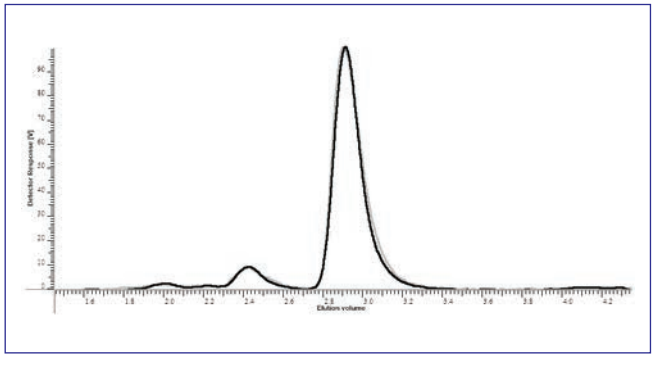

Figure 4. MALLS detection chromatograms (90° angle) at 0.33 mL/min (black) and 0.165 mL/min (grey).

Table 1. Chromatographic conditions.

Column:	YMC-SEC MAB (3 µm, 25 nm), 300 x 4.6 mm ID
Part No.:	DLM25S03-3046WT
Eluent:	Phosphate buffer pH 6.6 containing 0.3 M NaCl
Flow rate:	0.33 mL/min
Temperature:	25°C
Detection:	MALLS at 90° angle (PSS SLD7100)
Injection volume:	10 µL
Sample:	Bevacizumab (Avastin®) dosage form (10 mg/mL, diluted to 1 mg/mL)
System:	PSS-SECcurity GPC systems, 1260 Infinity II
Software:	WinGPC Unichrom

Conclusion

Size exclusion chromatography coupled to UV or RI detection alone is often not sufficient to obtain comprehensive information on a sample. Coupling SEC to MALLS provides additional information making it a powerful tool in biopharmaceutical separations. As high purity columns are required for MALLS detection, YMC SEC columns are an ideal choice due to their highly inert particles. For the antibody Bevacizumab the influence of different buffers, injection volumes and flow rates on the analysis has been shown.

Chromatograms courtesy of PSS Polymer Standards Service GmbH, Mainz, Germany.