YMC SEC columns and light scattering detection

Size exclusion chromatography (SEC) is not only used to separate compounds according to molar mass (MW) but also to gain information on the relative distribution of the MW of a sample. In combination with light scattering detection, SEC delivers absolute molar mass data for the characterisation of macromolecules such as proteins and antibodies. The greatest advantage is that there is no need for column calibration. Further, the signal intensity directly depends on the molar mass, injected mass and refractive index increment squared. This results in high signal intensities for higher MW materials, such as larger aggregates, which provides increased sensitivities which cannot be obtained by using a concentration detector (e.g. UV, RI).
In contrast, light scattering (LS) is a technique that is suitable to generate absolute molar mass data for the characterisation of macromolecules such as proteins or antibodies.

Multi angle laser light scattering detection

Light scattering 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 size exclusion chromatography.
SEC is typically combined with multi-angle (laser) light scattering (MALLS or MALS) detection, which belongs to the SLS systems. The light scattering caused by a substance in a solution is detected at various fixed angles (e.g. 15°, 90°) to screen the particle form factor. The angular dependence is required to obtain the z-average of the radius of gyration. Further results are the weight average molar mass and second virial coefficient.
MALLS as an absolute measurement technique provides direct information about biopolymer properties such as molar mass and size of the macromolecule, but also about aggregation, conformation, and chain branching.

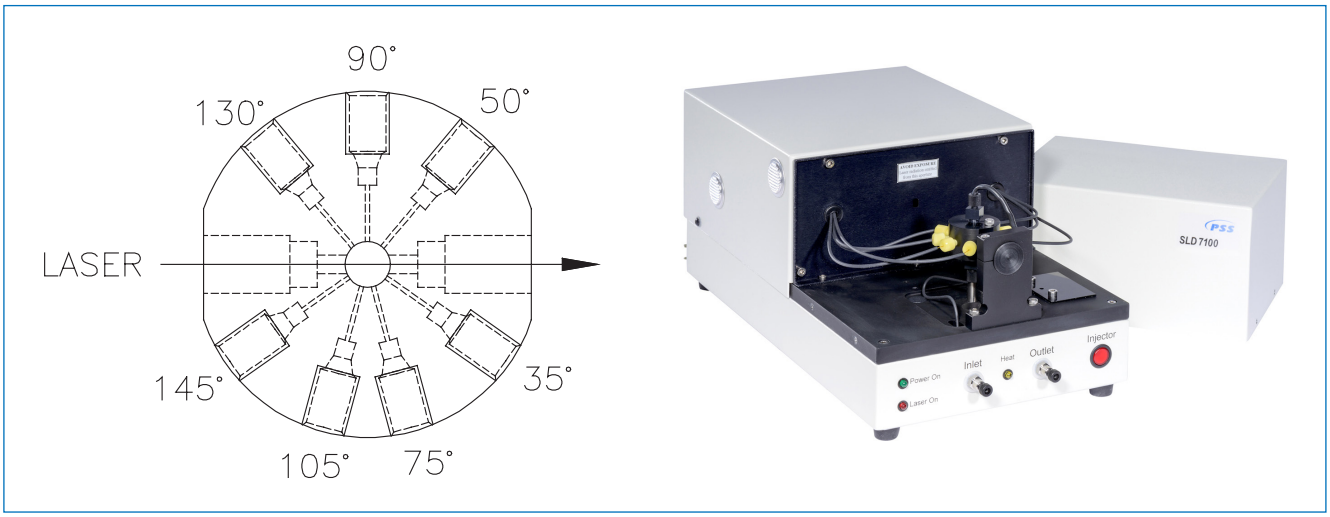

Figure 1: Cell design of a MALLS detector (left) used e.g. in the PSS MALLS detector SLD7100 (right).

MALLS detectors consist of several detectors placed at different angles to an incoming laser beam, such as 7 detectors in the PSS detector SLD7100 (figure 1). The intensities of the scattered light can differ between the angles depending on the scattering properties of the sample, depending on whether it consists of anisotropic or isotropic scattering particles. For isotropic scattering substances (Rayleigh, $d < \lambda/20$), which are usually <10–12 nm (<100 kDa), detectors at different angles are not needed, as there is no difference in the intensity of the scattered light between the angles. It is a necessity to have different detectors for anisotropic scattering compounds (Debye, $\lambda/20 < d < \lambda$), as an angular extrapolation is required. The scattered light amount of a solution from a certain angle θ is defined as the excess Rayleigh ratio $R(\theta)$. This ratio is corrected for angular or rather distance dependency and incident light intensity levelling out instrumental specifications. [Further information about light scattering detection can be found **here**].

SEC-MALLS for structural and dimensional characterisation of biomolecules

MALLS online detection is ideally combined with prior separation by SEC to obtain information about globular proteins or MAbs, their monomers and associates.
Molar mass sensitive detectors such as MALLS are more sensitive towards high molecular weights and molecules of large hydrodynamic radii, and less sensitive for smaller molecules, which make them valuable for biopolymer evaluation.

The coupling of SEC-UV/RI with a MALLS detector is a powerful tool for structure determination, when analysing fractionated samples. Figure 2 shows the chromatograms of bovine serum albumin (BSA, 66 kDa) recorded by UV and MALLS detection highlighting the higher sensitivity of the LS detection of species of higher molecular weight. Also, the direct dependency of the LS signal on the MW is demonstrated compared to the UV signal.

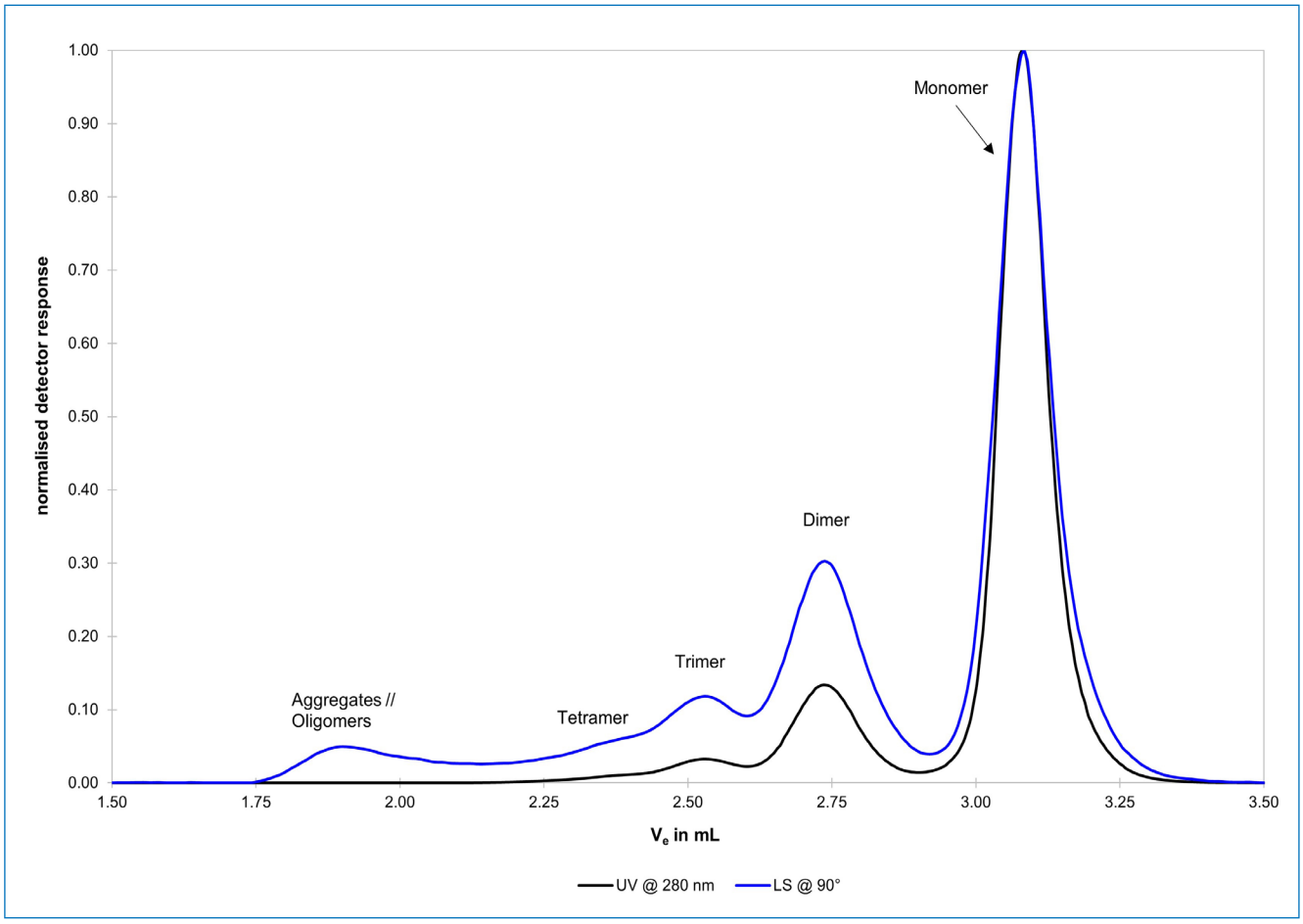

Figure 2: Chromatogram of BSA recorded with UV at 280 nm (black) and MALLS at 90° (blue). The separation was performed using a YMC-SEC MAB (3 µm, 300 x 4.6 mm ID) column.

Compatibility of YMC SEC columns with light scattering

In order to combine SEC with MALLS, the SEC column has to be compatible with LS detection. Therefore, very high column purity is required to prevent spikes in the chromatogram caused by particles from the column itself (figure 3). Such spikes cannot be seen in UV or RI detection.

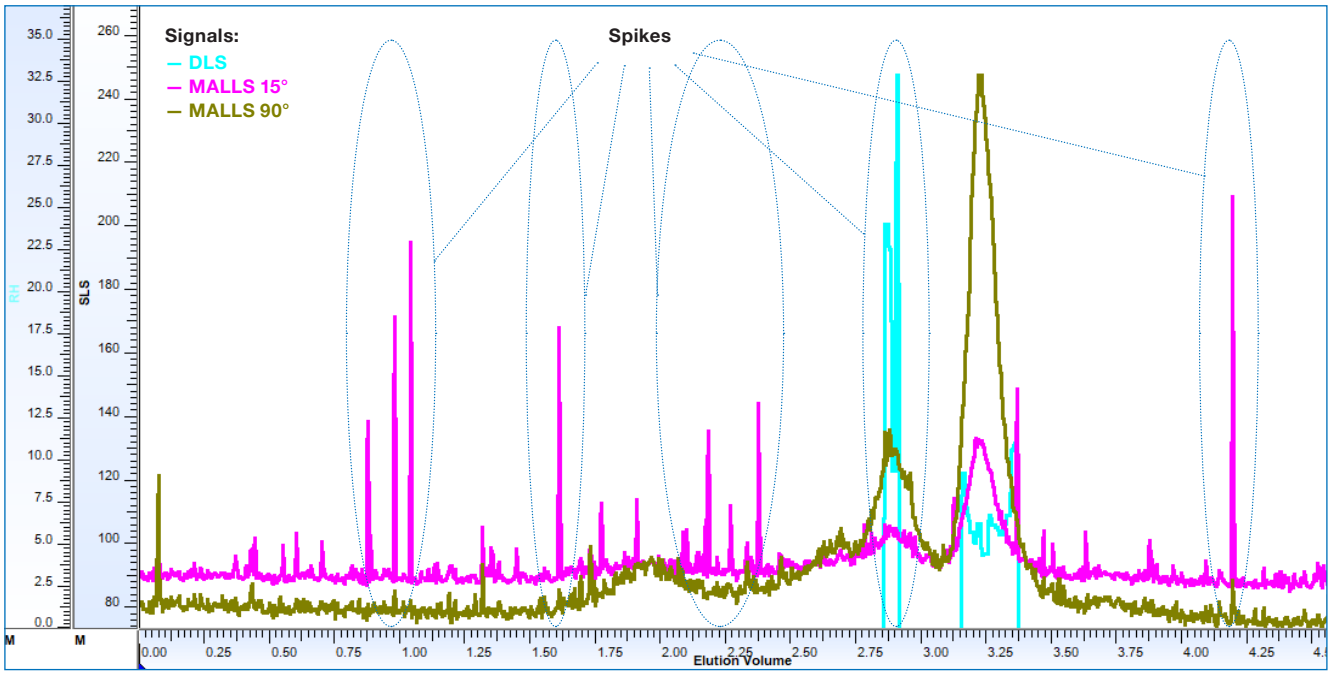

Figure 3: An example of spikes which can occur during MALLS detection.

YMC offers different SEC columns, with several pore and particle sizes. YMC-Pack Diol columns are available with 60 Å, 120 Å, 200 Å and 300 Å and with 3 µm and 5 µm for HPLC, while 2 µm columns for UHPLC proposes are available in combination with the 200 Å and 300 Å pore sizes. YMC-SEC MAB columns which are dedicated for monomer, fragment and aggregate analysis of antibodies are available with 250 Å and 3 µm particles.

All YMC SEC columns show a high compatibility with MALLS detection using typical eluents in BioLC such as phosphate buffers, as their noise levels are very low as required for such detection modes.

Using a YMC-Pack Diol-120 column, aggregates of γ-globulin can be determined by SLS-, DLS- as well as UV detection (figure 4). As expected, SLS and DLS are more sensitive for aggregation than UV and show high signal intensities for higher molecular weight species compared to the UV signal. Further, the hydrodynamic radii (RH) are directly observed by DLS, while no additional particle scattering can be detected.

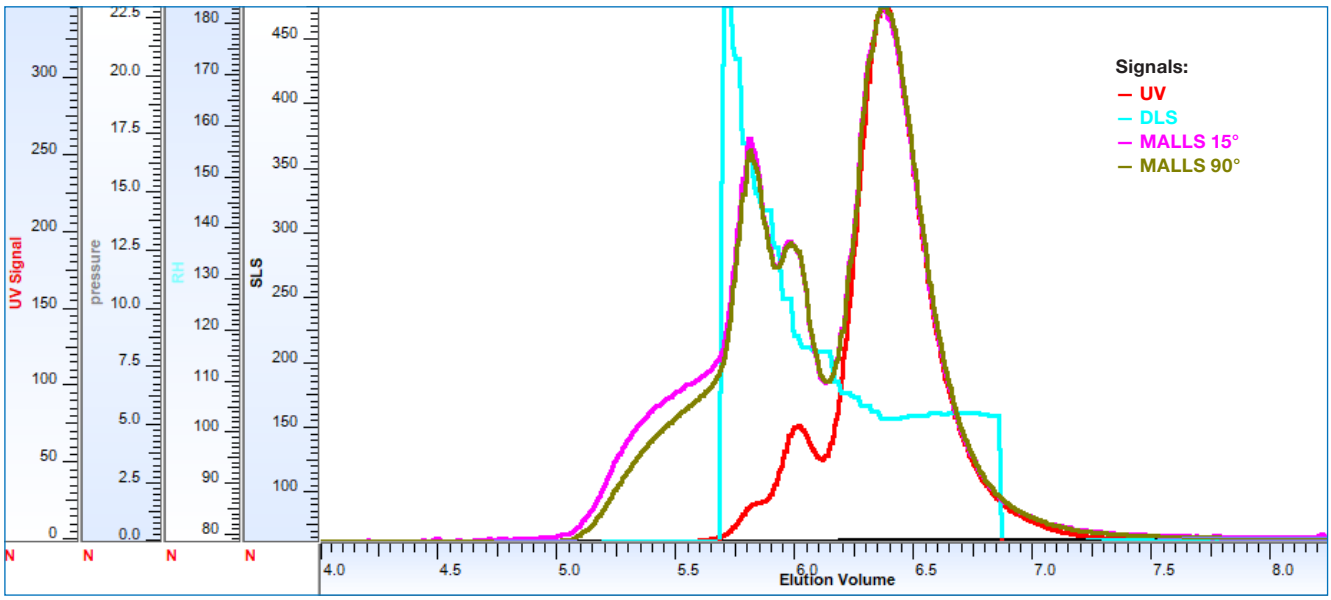

Figure 4: Measurement of γ-globulin using a YMC-Pack Diol-120 (3 µm, 300 x 8 mm ID) column and a phosphate buffer (34 mM + 0.5 M NaCl; pH 6.6) as eluent.

Only slight particle scattering is observed (MALLS signal at 15°) in the measurement of an IgG solution (figure 5). Again, all three detection modes can be used to determine aggregates to monitor aggregation as a function of the protein treatment, while LS signal intensities are more pronounced. The DLS shows the size depending of the separation. Beside the radius of gyration Rg, the hydrodynamic radius RH is a further feature of the size of the protein sample. It is the radius of a hypothetical sphere with its hydration layer. From the ratio of both one can estimate the topology of the molecule. The primary information is the diffusion coefficient given by unhindered Brownian motion. The hydrodynamic radius is calculated from the diffusion coefficient by Stokes-Einstein-equation.

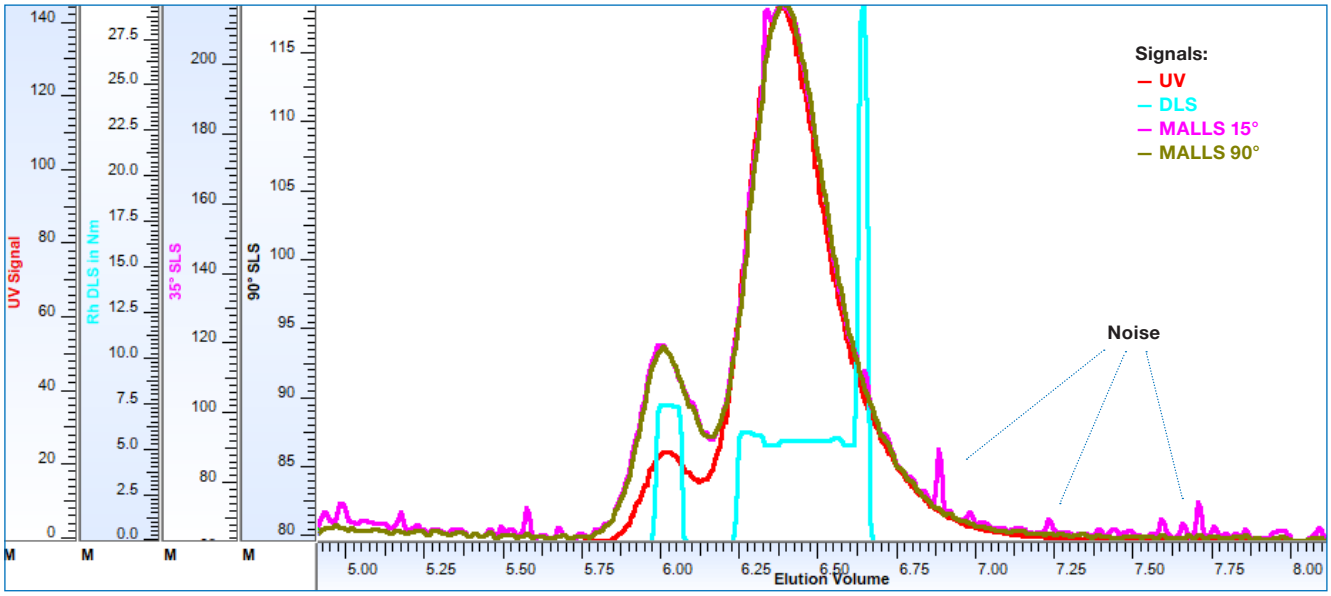

Figure 5: Measurement of IgG using a YMC-Pack Diol-120 column and UV, DLS as well as MALLS detection.

If the columns show some particle scattering initially, their noise level and particle spikes are decreasing after initial equilibration at first use and remain at a very low level (figure 6). This applies to all LS detection options.

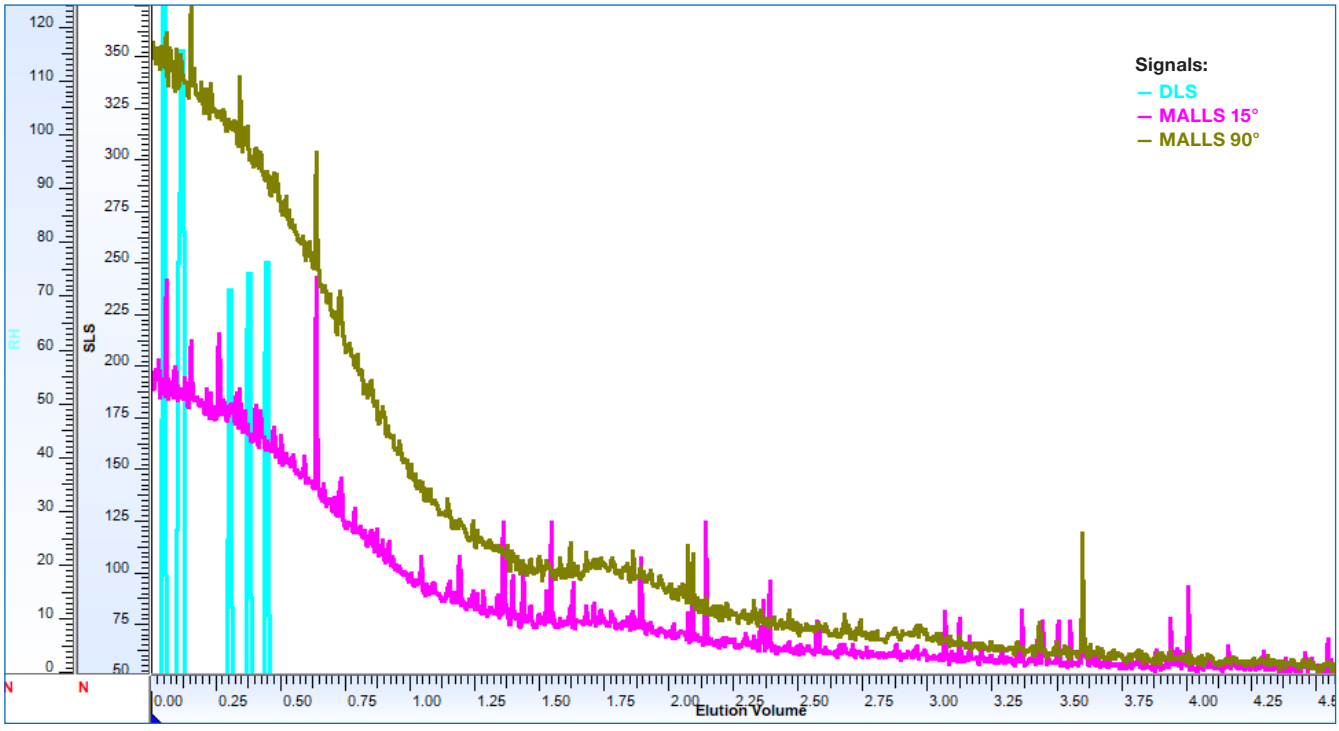

Figure 6: Monitoring by LS of initial rinsing of a YMC-SEC MAB column, showing the rapid decrease of noise signal.

Conclusion

The combination of size exclusion chromatography and light scattering detection is the method of choice for the evaluation of molecular weight, especially for higher molecular species such as aggregates or oligomers. It is a powerful technique as not only is information about the MW determined, but also information about the sample size and topology can be obtained. Therefore, ageing of MAb solutions, where aggregation is typically taking place, can be precisely monitored.

In order to combine SEC with MALLS detection, the columns have to fulfil criteria towards their inertness, in order to provide unaffected signals. These criteria are completely fulfilled by YMC's SEC columns such as YMC-Pack Diol with their different pore sizes as well as the specialised YMC-SEC MAB column. This makes YMC SEC columns fully compatible with MALLS detection so that they can be applied without any restrictions.

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