SEC-Light Scattering for Biomolecular Characterisation

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The area of biomolecular characterisation for molecular weight determination has increased rapidly with the requirements for a complete picture of the behaviour of biomolecules in solution being both critical not only for the academic understanding, but also from a regulatory viewpoint for safety and efficacy. For example with bioconjugates such as pegylated interferon, used in the treatment of chronic hepatitis, it is essential for the molar masses to be above that of the kidney cutoff to increase half-life and therefore efficacy.

A simply yet powerful technique in the analyst's toolbox is Size Exclusion Chromatography, SEC, in which the molecules of interest are separated on their size in solution. This technique in a less analytical form has been used for isolation and purification of proteins for many years, often being referred to as Gel Filtration Chromatography. Although SEC provides unique information, there is a limitation to the utility of SEC for protein analysis. Typically, the molecular weights are generated in SEC by reference to a calibration curve, created with protein standards. This can be an important limitation as the elution behaviour of certain proteins is not purely determined by size exclusion but also by interactions with the column or an extreme shape in solution, leading to erroneous results. However, by the simple addition of a Light Scattering Detector to the SEC system this limitation is overcome, as the molecular weight determined by such a detector is independent of retention time and therefore of any column calibration. The light scattering response is directly proportional to the molar mass, as can be seen in equation 1, so even proteins that elute by mechanisms other than pure SEC will give accurate molecular weight values.

Equation 1: Simplified Light Scattering

$$R\theta = c\,M\,(dn/dc)^2\,P\theta\,K\theta$$

Where Rθ = Light scattering response
c = concentration
M = Molar Mass
dn/dc = specific refractive index increment
Pθ = Scattering Function
Kθ = Light Scattering Constant

This is classical, static light scattering, and is most commonly employed to determine absolute molar mass. The detector is attached in series with the concentration detector and a laser is directed into the flow cell, with measurement of the resultant scattering being performed at one or more angles. Pθ is the scattering factor, a function of the angular dependence of light scattering with molecular size, which for the majority of globular proteins equates to one. However, for some biomolecules of larger molar mass such as bioconjugates or membrane proteins this value can drop below one indicating that angular dependence does exist. By employing a light scattering detector of more than one angle, Pθ can be determined for these large molecules and from this a measure of molecular size, Rg, the radius of gyration, can be ascertained.

In another form of the light scattering measurement, the field of detection is reduced so that the scattering of photons by individual biomolecules passing through the detection volume can be measured. Larger particles move more slowly through the solvent and thus the fluctuations in intensity caused by them are slower in frequency than those caused by smaller particles. Measuring these small intensity variations allows calculation of the diffusion coefficient which is related using the Stokes-Einstein equation to R_H, the hydrodynamic radius, and this technique is known as Dynamic Light Scattering, DLS. This is an alternate measure of size compared to that from static light scattering, and the ratio between the two is the shape factor, ρ, which can be related to protein conformation. By combining the two light scattering techniques around a single flow cell accurate molecular weight and molecular size information can be obtained, while minimising band-broadening.

Within the Pharma industry a large area of interest is in therapeutic and diagnostic proteins, such as monoclonal antibodies, mAb. These can be used to target specific sites, and have great success and future potential in alleviating many diseases and ailments, from cancer to arthritis. The use of a Light Scattering device not only provides an accurate molecular weight and size, but also identifies and quantifies oligomers and aggregation, crucial factors when studying biomolecules. The presence and ratios of these can change the efficacy of mAbs in clinical research applications with severe implications, with adverse reactions or even risk of death. Aggregation or denaturing can occur throughout all phases of the drug development process due to improper production, formulation, storage, or handling conditions, with SEC-light scattering being an ideal and invaluable technique at every step to ensure the highest quality of product for the regulatory agencies.

The chromatogram in Figure 1 highlights the benefits of light scattering. With UV or RI detection, the response is proportional to the concentration across the molecular weight range. As the light scattering signal also responds to molar mass, the detector is much more sensitive at high molecular weight, and is therefore able to better detect oligomers and the presence of aggregates.

From the data in Figure 1, the absolute molar mass and molecular size of the dimer, trimer and tetramer were determined, with the

relative ratios to each other.

The increasing inaccuracy of the oligomers is due to coelution, which would be countered with increasing SEC resolution at high molar mass.

Proteins are utilised across industries including throughout the cosmetic industry in skin and hair products, and are sourced from plants, animals and organisms such as fungi and algae. The importance of ensuring that the supplied material is fit for purpose is undeniable, and light scattering detection can highlight material that has aggregated or degraded due to improper collection or storage. This complements the use of SEC which is regularly employed to ensure batch to batch reproducibility.

SEC- Light Scattering provides complementary information for other techniques. X-ray crystallographic studies are often a key requirement and important area of interest, with aggregates adversely influencing the likelihood of proteins forming suitable single crystals. During the SEC-LS experiment the aggregates are detected and precious time is not wasted trying to form a crystal.

Through light scattering, both static and dynamic, absolute molecular weights are achieved, independent of elution volume or the standards used to calibrate. Molecular sizes are determined and the conformation of the biomolecule can be resolved. Light Scattering is very sensitive to high molecular weight material, providing facile detection of aggregates and oligomers even at low concentrations. By empowering the SEC experiment with the addition of a light scattering detector, a deeper understanding of your biomolecule is attained.

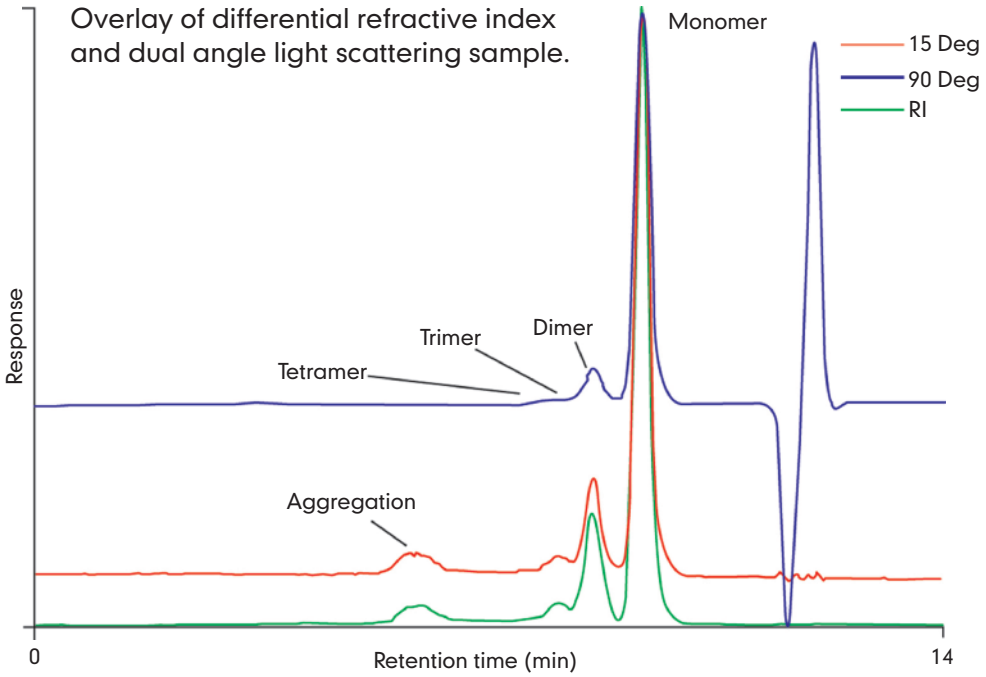

Figure 1: Overlay of all detector traces in the SEC-Light Scattering analysis of BSA

	Molar Mass (Da)	Abundance (%)	Relative to monomer	RH (nm)
Monomer	669,000	88.5		3.4
Dimer	1,349,000	9.8	2.02	5.0
Trimer	1,970,000	1.2	2.94	6.1
Tetramer	2,793,000	0.5	5.17	

Table 1: Results of SEC-Light Scattering analysis of BSA