Expanding the Boundaries of Light Scattering for Macromolecules

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In the past four decades, light scattering detection (LS) has become a widely used technique to obtain the supposed "absolute" or true molecular weight of macromolecules such as synthetic polymers, biopolymers, proteins, and antibodies. When coupled with size exclusion chromatography (SEC), molecular weight and size, as well as aggregation or branching can be investigated to provide analytical scientists with a powerful characterisation tool for these large molecules.

Whether new biopharmaceutical products or polymers with tailored properties, the macromolecules' complexity increases, requiring even more extensive and in-depth characterisation for a full understanding of the material. With limited amounts of costly samples available for analysis, analytical scientists are facing the challenge of finding new technologies that help them improve and optimise their analytical characterisation.

The latest light scattering technology introduced by Tosoh Bioscience features an entirely new detector design that addresses the limitations and shortcomings of the current, state-of-the-art LS instruments.

Principles and theory of light scattering

When a beam of light illuminates molecules in a solvent, oscillating dipoles are generated within the molecules, re-emitting a small amount of light in all directions. This physical phenomenon shows two main properties. Firstly, the intensity of the scattered light is directly related to the molecular weight of the molecules: higher molecular weight molecules scatter more light. Secondly, the scattered light's intensity is not identical in all directions, and this dissymmetric scattering pattern is related to the size and shape of the molecules.

Consequently, molecular weight information is obtained from the intensity of the scattered light, while molecular size is obtained by examining intensity changes with the angle of observation.

Rayleigh defined the theory and equations of light scattering by sub-micron particles. He established the relationship of the intensity of scattered light to the molecular weight and size of the molecules, the concentration of the solution, and the measurement angle [1].

Rayleigh determined the molecular weight of a molecule can be obtained directly from the intensity of the scattered light in the same direction as the incident beam (0° angle), regardless of the molecule's size and shape.

However, due to the incident beam, measuring the amount of scattered light at 0° angle is technically impossible. Light scattering instruments nevertheless have to work around this technical challenge to provide the molecular weight information.

Traditional light scattering instruments and their limitations

The first LS instruments, in the 1970's, used a low angle light scattering approach (LALS) in which the angle of measurement is close enough to 0° to assume safely that the measurement at such a low angle is the same as the theoretical 0° measurement. This is still considered the most accurate molecular weight measurement method, as it requires no additional assumptions or extrapolations. However, those early LALS instruments were not easy to use and a low angle measurement alone does not provide size information since it does not measure the angular dependence of the scattered light intensity.

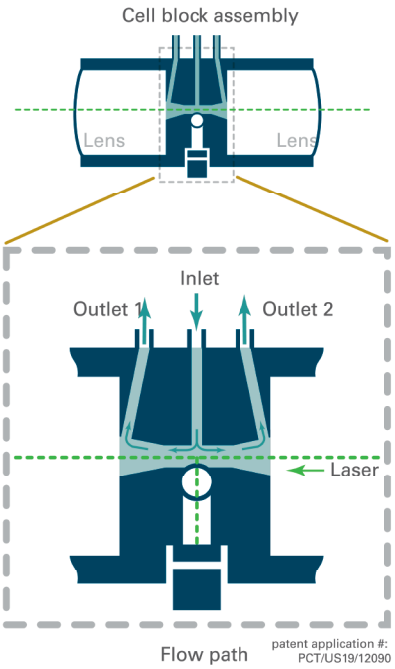


Figure 1: LenS₃ flow channel.

To achieve both easier molecular weight and size measurements simultaneously, multi-angle light scattering (MALS) detectors were developed to collect light at multiple angles. These measurements are extrapolated back to 0° for molecular weight (MW) determination, while the scattering pattern is mapped to obtain size information described as the radius of gyration (R_g).

MALS instruments typically consist of multiple photodiodes (detectors) arranged in the same plane, around a circular or cylindrical flow cell. There are several technical limitations to this design since the geometry of this design offers limited space available for the physical location

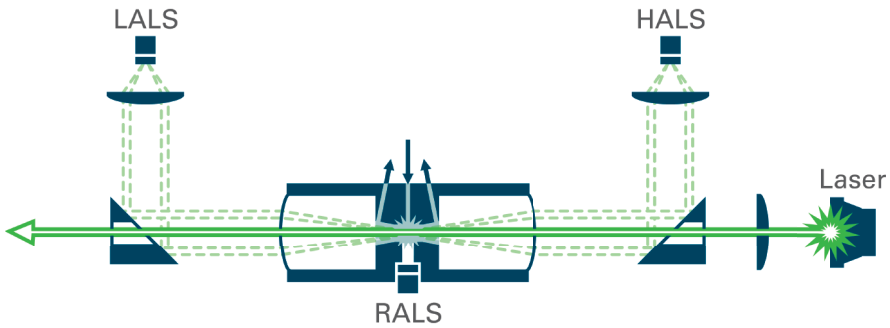


Figure 2: LenS3 MALS enhanced optics, showing the low angle (LALS), right angle (RALS) and high angle (HALS) light scattering detectors.

of the detectors around the cell. As a result, the lowest and highest measurable angles are some distance from 0° and 180°. Furthermore, due to the type of glass employed for the optical flow cell, reflection of the scattered light inevitably occurs on the cell walls, thus creating stray light that interferes with the measurement. The resulting generated noise affects the lower and higher angles to a greater extent compared to the 90° angle. Consequently, it is common for those to be excluded from use in data processing.

What do these technical limitations mean for SEC-MALS users?

- Firstly, the noise from stray light limits the overall sensitivity of traditional MALS detectors. For example, this affects their capability to detect low amounts of protein aggregates, increasing the amount of sample required for each analysis.
- Secondly, the lowest angle is critical to obtain an accurate molecular weight since the Rayleigh equation says that MW is proportional to the scattered light intensity only at 0°. The extrapolation may cause significant errors in molecular weight determination when low angles are unusable.
- Thirdly, having the lowest and highest possible angles is necessary for the size determination of smaller molecules by detecting the small differences in scattered intensity. Practically, traditional MALS detectors cannot provide angular dissymmetry for molecules smaller than 10-12nm (R_g).

Innovative LS instrument design

To address the highlighted limitations, a completely new approach to MALS instrument design has been developed. The LenS3 MALS detector (Tosoh Bioscience, King of Prussia, PA, USA) combines a novel flow channel concept with improved optics to provide more signal intensity while reducing the core noise at each angle of measurement.

New flow channel configuration

The traditional flow cell is replaced with an elongated flow path with a dual-cone shape, as shown in Figure 1.

This cell block consists of a black, non-refractive, inert polymeric material (PEEK) assembled with two optical lenses that seal the flow chamber and let the incident beam go through the chamber. The inlet flow splits in half at the centre of the flow path and exits from two outlets. The laser beam illuminates the sample in the entire flow path, maximising the scattering volume, hence the number of molecules that interact with the incident light and ultimately increasing the scattering intensities. The chamber's non-refractive material prevents the scattered light hitting the wall from bouncing back, leading to stray light and subsequent noise. The conical shape defines the forward (10°) and backward (170°) angles of collection of scattered light, while the perpendicular (90°) measurement is made at the centre of the channel through a separate observation window equipped with a spherical lens.

Improved optics

The LenS3 MALS detector also features improved optics to provide even more intensity of scattered light and cleaner signals (Figure 2).

Using a green laser (λ_0=505nm), the scattered intensity increases by a factor of three, compared to the typical red laser (λ_0=660nm) used in most LS instruments, as scattering is proportional to $1/\lambda_0 4$.

The optical bench includes mirrors in both the backscattering and forward scattering positions, with a hole where the incident beam can travel through the mirrors so that the incident beam is effectively eliminated. Only the annulus of light at the desired angles 10° and 170° is collected.

Overall, the gain in performance and sensitivity of the LenS3 MALS detectors, as compared to traditional MALS instruments, comes from the combination of the following elements:

- The wider angles of measurement:
  - True, usable ultra-low angle (LALS at 10°) for accurate and direct MW determination without extrapolation
  - Ultra-high angle (HALS at 170°) used in conjunction with the LALS and the 90° angle (RALS) to detect the smallest difference in scattered intensity for R_g measurements of smaller molecules.
- The novel flow channel:
  - Elongated conical shape to maximise scattering volume and thus signal intensity.
  - Black, inert polymeric material eliminates stray light to reduce noise.
- The advanced optics:
  - A green laser to increase the intensity of scattering by a factor of three.

Table 1: Experimental chromatographic conditions for the applications

	Monoclonal Antibody	Oligonucleotide	Polystyrene
Columns	TSKgel UP-SW3000 (2 µm, 4.6 mm x 30 cm)	TSKgel UP-SW2000 (2 µm 4.6 mm x 30 cm)	TSKgel GMHHR-N (5 µm, 7.8 mm x 30 cm)
Mobile phase	100 mmol/L NaH_2PO_4, pH 6.8 + 100 mmol/L Na_2SO_4	0.5 mol/L NaCl, 0.1 mol/L EDTA, pH 7.5 0.1 mol/L Na_2SO_4, 0.03% NaN_3 in 0.1 mol/L phosphate buffer	Toluene
Flow Rate	0.35 mL /min	0.3mL/min	1 mL/min
Temperature	25°C	30°C	40°C
Detection	MALS	UV@260 nm; MALS	MALS
Sample	Herceptin biosimilar	20 bases custom oligonucleotide with MW= 6141 Da (1 mg/mL)	Polystyrene standards

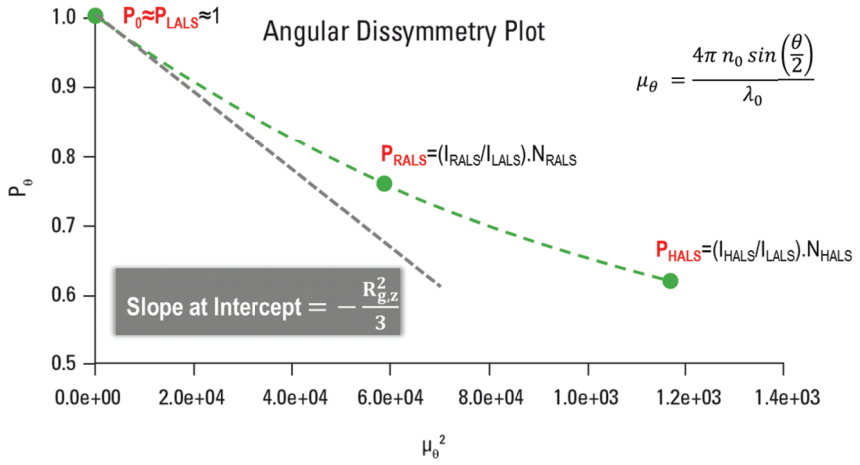


Figure 3: Angular dissymmetry plot for direct R_g determination.

- Elimination of the incident beam to obtain a cleaner signal at low and high angles.

Molecular weight and R_g determination

Thanks to the presence of a low angle measurement with an excellent signal-to-noise ratio, MW can be measured directly on the LenS3 MALS without the need for an extrapolation procedure such as the complex Zimm plot. The only underlying assumption is that if the molecules are within the size range that SEC columns can separate, the scattering intensity at 10° equals the intensity at 0° [1]. The molecular weight of the molecules is simply proportional to LALS intensity.

A new method for determining R_g was developed to take advantage of the detector design. From Rayleigh's equation for sufficiently dilute solutions, one can define the angular dependence as the ratio of the intensity of scattered light at a given angle to that at 0° angle. A plot of this ratio for the LALS, RALS and HALS against the angle of observation is used to map the angular dependence pattern and provide a direct R_g determination from the slope of the obtained curve (Figure 3).

Benefits for the characterisation of macromolecules

With the higher sensitivity provided by the LenS3 MALS detector smaller quantities of material are required to characterise samples accurately, which is critical in applications where the quantity of sample available may be a limiting factor. For protein applications, the presence of aggregates and fragments of an antibody can also be detected down to a much lower level when compared to traditional MALS designs.

Samples with a low molecular weight or a low refractive index increment (dn/dc) inherently scatter a lower amount of light. A MALS detector such as the LenS3 allows the MW of those types of molecules to be measured with greater accuracy.

Additionally, the position of the extreme angles and their higher signal-to-noise ratio opens new areas of applications for the size measurement of macromolecules with an R_g below 10nm.

Examples of applications

Experimental Conditions

Experimental conditions for the three application examples are listed in Table 1.

Monoclonal Antibody

A biosimilar of Herceptin® (trastuzumab), was analysed by UHPLC-SEC-MALS using the LenS3 MALS detector. Decreasing amounts of sample were injected to determine the limit of detection in these conditions. Aggregates, monomer, and monoclonal antibody fragments were readily observed down to 50 ng of loading. Figure 4 (A and B) shows that its monomer was easily detected at only 2ng loading.

Oligonucleotide

The LenS3 MALS was also used to investigate low MW oligonucleotides at extremely low concentrations. Accurate molecular weight profiling of an unpurified oligonucleotide sample was successfully achieved. The results in Figure 5 demonstrate excellent sensitivity and reproducibility of retention time and MW.

Polystyrene

Analysis of polystyrene standards was conducted in toluene for size determination. Table 2 shows R_g values below 10 nm for the first time ever reported by light scattering. The values observed by the LenS3 MALS are validated by small-angle X-ray scattering (SAXS) measurements reported in the literature [2].

Conclusions

The novel configuration of the LenS3 MALS detector features an elongated flow chamber made of non-refractive inert material, associated with an improved optical bench and a green laser. This new design increases signal intensity while minimising noise, resulting in exceptional detection sensitivity.

Furthermore, with extreme angles at 10° and 170° in addition to the 90° angle, the instrument can detect angular dependence to an extremely low level to measure the lowest R_g ever reported by light scattering. The detector also takes advantage of the low angle to measure MW directly and accurately without extrapolation.

This innovative approach represents the first significant advancement in light scattering technologies in four decades. This will benefit the analysis of all types of macromolecules, from biomolecules to synthetic polymers.

Table 2: R_g of polystyrene standards in toluene measured by LenS3 MALS.

Sample ID	MW [Da]	Conc. [mg/mL]	R_g by LenS3 [nm]	R_g by SAXS [nm]	Difference [%]
A5000	5,796	4.81	2.11	2.04	3.37%
F-1	10,650	4.27	3.04	2.93	3.69%
F-2	18,554	3.22	4.34	4.32	0.46%
F-4	40,510	2.79	6.59	6.69	1.51%
F-10	100,432	1.97	10.48	N/A	N/A
F-20	195,787	1.02	15.78	16.2	2.63%

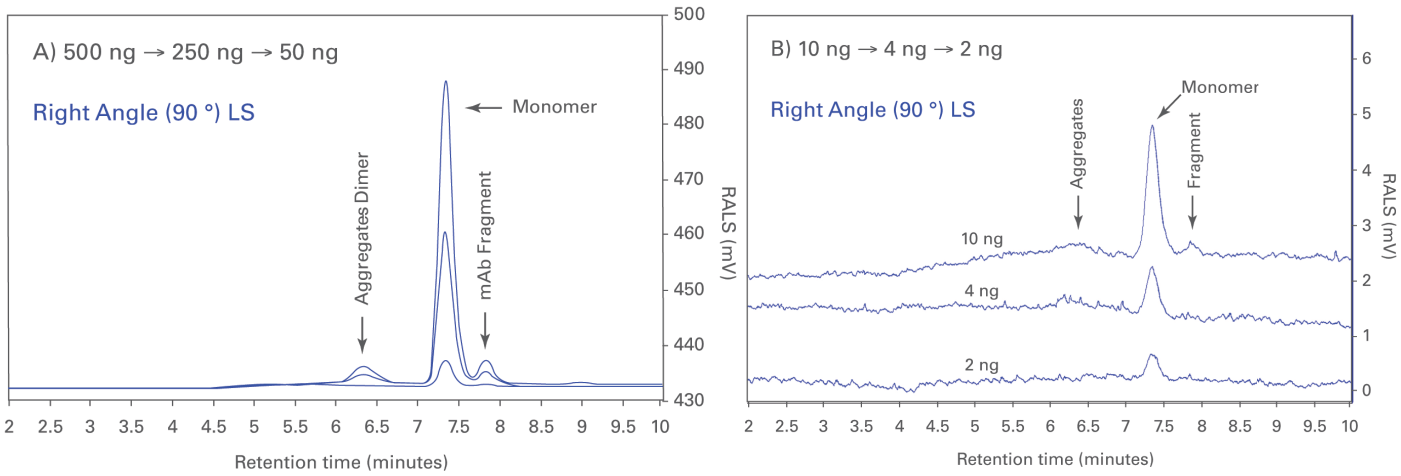
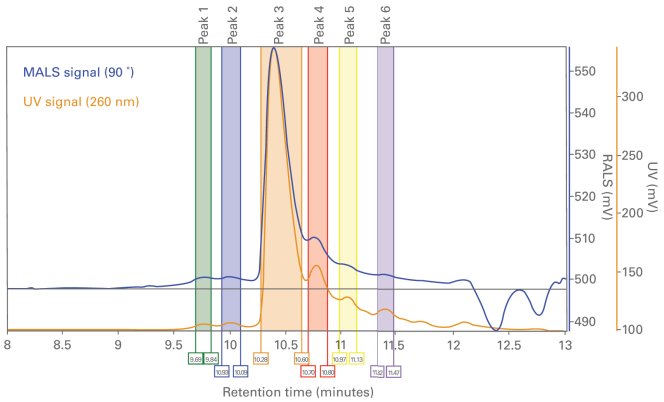


Figure 4 (A and B): Light scattering signal of Herceptin biosimilar..



Peak	Retention time (min)	% RSD	MW (Da)	% RSD
1	9.774	0.1%	13,599	2.1%
2	10.012	0.0%	11,550	1.9%
3	10.398	0.1%	6,398	0.7%
4	10.776	0.1%	5,751	1.5%
5	11.053	0.1%	5,177	2.3%
6	11.422	0.2%	4,446	5.5%

Figure 5: Molecular weight analysis of oligonucleotides (triplicate injection).

References

[1.]: Kratochvil, P. Classical Light Scattering from Polymer Solutions; Elsevier: New York, 1987.

[2.] Abe, Fumiaki, et al., Macromolecules Vol 26 Issue 8 (1993) Pages 1884-1890