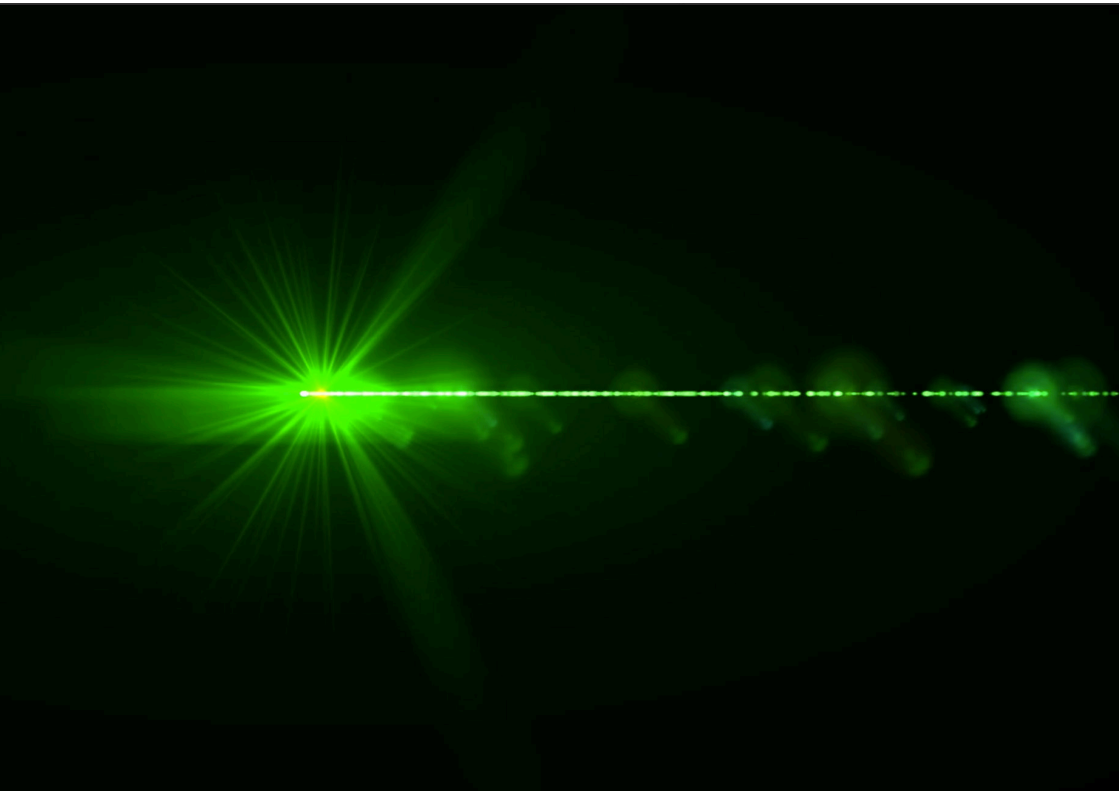
ANALYTICAL SCIENCE

TOSOH

# THE TEXTBOOK OF LIGHT SCATTERING



## TOSOH BIOSCIENCE

## Why light scattering?

**A direct measurement of molecular weight and size of macromolecules**
When coupled with gel permeation/size exclusion chromatography (GPC/SEC), light scattering (LS) detection allows the measurement of true – or "absolute" – molecular weight (MW) of macromolecules, as opposed to the relative values obtained from conventional column calibration methods. In addition to MW, LS can provide the radius of gyration ($R_g$), which describes the size of macromolecules. By measuring the scattered light at each elution fraction, the full MW and size distribution of a polymer or a biomolecule can thus be mapped accurately.

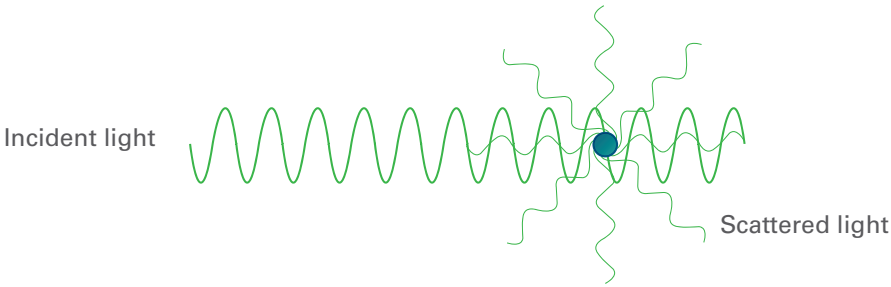
## What is light scattering?

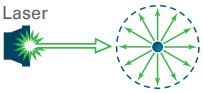
**A natural phenomenon turned into an analytical technique**
Light scattering is a natural phenomenon that occurs every time a beam of light hits atoms, molecules, or particles. For example, light scattering in the atmosphere is responsible for the blue color of the sky and the warm, red-orange color of the sunrise and sunset[1].

Light can be physically described as a propagating electromagnetic field. When a beam of light hits a molecule, the incident oscillating electric field creates an oscillating dipole within the molecule. In turn, these oscillating electrons generate new electromagnetic radiation – the scattered light – in all directions of space.

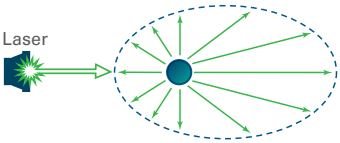
With macromolecule sizes typically encountered in GPC/SEC analyses, light is scattered by multiple points within the molecule. As a result, interferences between the scattered light from different sources occur. In the direction of the incident beam, all scattered radiations interfere in an additive manner, thus increasing the intensity of the scattered light as a function of the number of scattering points. In all other directions, due to phase shift, interferences may locally be additive or subtractive, resulting in an overall lower scattered intensity.

In practice, the intensity of the scattered light decreases with increasing scattering angle. This phenomenon is called angular dependence or angular dissymmetry.






The scattering pattern of a point object is considered uniform




Non-point molecules scatter more light in the forward direction

## How is light scattering used in GPC/SEC?

**The two principles of light scattering**

1. Higher molecular weight molecules scatter more light.
   *The amount of scattered light primarily depends on the molecular weight of the molecule.*
2. The intensity of the scattered light differs in all directions.
   *The angular dissymmetry is related to the size and shape ($R_g$) of the molecules.*

In light scattering experiments, molecular weight and size are obtained from respectively the intensity and the angular dissymmetry of the scattered light.

**The Rayleigh equation**

Lord Rayleigh developed the theory of light scattering[2] in the late 19th century. It expresses the intensity of the scattered light as a function of the angle of observation, the molecular weight of the sample, and the concentration of the solution, yielding the equation below:

Rayleigh Equation $$\frac{k_{opt}.c}{R_\theta} = \frac{1}{M.P_\theta} + 2A_2.c$$ Eq. 1

And

$R_\Theta$ is the excess Rayleigh scattering ratio – directly related to the intensity of the light scattered at angle Θ
M is the molecular weight of the sample
C is the concentration of the sample
$A_2$ is the second virial coefficient – relating to sample/solvent interactions
$P_\Theta$ is the particle scattering (or angular dissymmetry) function, with $P_\Theta = R_\Theta/R_0$

Where $k_{opt}$ is an optical constant: $$k_{opt} = \frac{4\pi^2.n_o^2.\left(\frac{dn}{dc}\right)^2}{N_A.\lambda_0^4}$$ Eq. 2

And

$N_A$, Avogadro's number
$n_0$, the refractive index of the solvent
λ0, the wavelength of the laser in vacuum
dn/dc, the refractive index increment of the sample in the solvent

At zero angle, $P_\Theta = P_0 = 1$ and the intensity of the scattered light *only depends on molecular weight and concentration.*

Rayleigh Equation at Θ=0 $$\frac{k_{opt}.c}{R_0} = \frac{1}{M} + 2A_2.c$$ Eq. 3

In chromatography mode, due to the very low concentration of each eluted fraction flowing through the detector cell at a given time, it is safe to assume that the impact of the second virial coefficient, $A_2$, in the Rayleigh equation (Eq. 1) is insignificant and therefore, negligible.

Therefore, Equation 3 can be simplified further to:

$$R_0 = k_{opt} \cdot C \cdot M \qquad \text{Eq. 4}$$

*As a result, it is possible to obtain the molecular weight of the sample if $R_0$ can be measured and $k_{opt}$ and concentration are known.*

At all angles different from zero, the particle scattering function, $P_\Theta$, describes the angular dependence of the scattered light, which is how the intensity of the scattered light changes with the angle of observation.

Equation 4 becomes: $$R_\Theta = k_{opt} \cdot c \cdot M \cdot P_\Theta \qquad \text{Eq. 5}$$

From Debye's work[2] on the relationship between the particle scattering function and the structure of the particle, the following equation can be derived:

$$\lim_{\theta \to 0} P_\theta = 1 - \frac{{R_g}^2}{3} {\mu_\theta}^2 \qquad \text{Eq. 6}$$

Where $$\mu_\theta = \frac{4\pi n_0 Sin\left(\frac{\theta}{2}\right)}{\lambda_0}$$

The radius of gyration of the sample is obtained by measuring and comparing the intensity of the scattered light at multiple angles to study the angular dependence.

## How do light scattering detectors work?

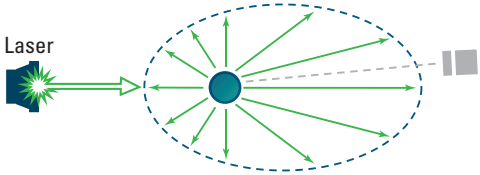
**A challenging measurement**

Since the 1970s, different light scattering detector technologies have been developed as commercial instruments for coupling with GPC/SEC systems[3] to complement the historical "batch measurements" performed on optical benches.

From Equation 4, one should measure the Rayleigh ratio $R_0$ (at 0°-angle) to obtain a direct measurement of the molecular weight of the molecules in the solution. However, measuring the intensity of the scattered light in the direction of the incident beam is not practical. It is technically impossible to differentiate the amount of scattered light from the way more intense incident beam. The Rayleigh theory also dictates that one should map the angular dissymmetry to determine the size ($R_g$) of the molecules.

The following detection technologies originate from the possible practical approaches to work around these two challenges.

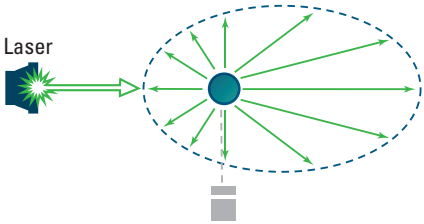
**Low angle light scattering (LALS)**




LALS detectors measure the scattered light at a *single angle extremely close to 0°* (typically < 15°), where the particle scattering function (Eq. 6) is $P_{LALS} \approx P_0 = 1$ for macromolecules of all sizes. Equation 4 thus holds true, and *molecular weight can be obtained directly* without any assumptions or extrapolations, and regardless of the angular dissymmetry.

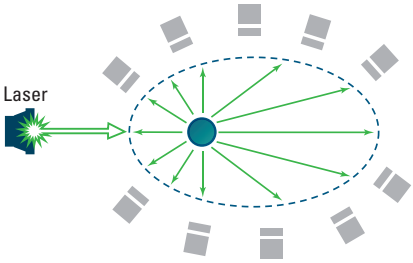
LALS is the purest and most accurate form of light scattering for the measurement of molecular weight. Obviously, measurements at a low angle are more difficult due to the proximity of the incident beam. $R_g$ cannot be obtained from this single angle measurement.

**Right angle light scattering (RALS)**



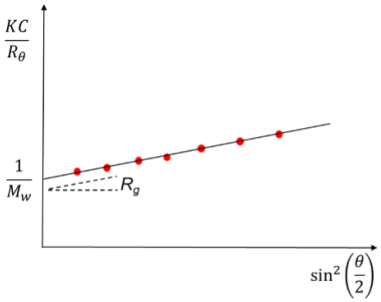

RALS detectors measure the scattered light at a *single 90°-angle* without consideration for angular dissymmetry. This offers a direct measurement of *molecular weight only for smaller macromolecules*, typically below 15 nm in radius, for which the angular dissymmetry can be ignored or neglected (i.e. when $P_{RALS} > 0.95$, hence the error on MW is less than 5% if one assumes $P_{RALS} \approx 1$). $R_g$ cannot be obtained from this single angle measurement.

**Multi-angle light scattering (MALS)**



MALS detectors measure the scattered light at *multiple angles* (3 or more) to map the scattering pattern of the molecule. The *molecular weight is obtained by extrapolating* the scattered intensity back to angle Θ=0. If angular dissymmetry is detectable, it is used to determine the radius of gyration, $R_g$ of the molecule.

A traditional graphical representation used in GPC/SEC-MALS measurements is the Zimm plot




*Traditional Zimm plot to obtain MW and $R_g$ from MALS measurements*

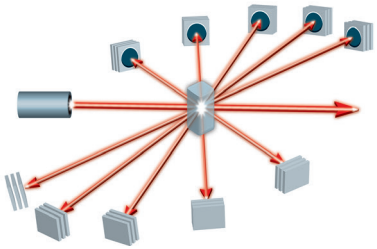
Molecular weight is obtained from the intercept of the extrapolation curve with the Y-axis, whereas the slope gives the radius of gyration of the molecule.

## How the latest technology expands the boundaries of light scattering detection

**Traditional MALS design**

While single-angle detectors (LALS or RALS) can provide MW, only multi-angle detectors (MALS) can provide both MW and size ($R_g$).

MALS instruments typically consist of multiple photodiodes (detectors) arranged in the same plane around a circular or cylindrical flow cell.



*Traditional MALS setup*

There are several technical limitations to this design:

- the lowest and highest measurable angles are at a distance from 0° and 180° (interference by the incident beam and limited space available for the physical location of the detectors around the cell),

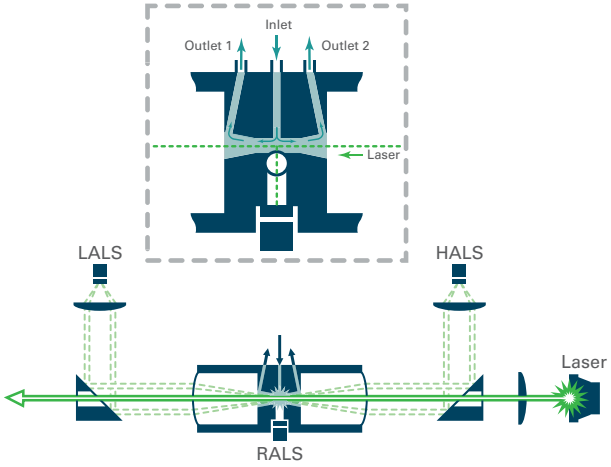
- reflection of the scattered light inevitably occurs on the optical flow cell walls (glass), creating stray light that interferes with the measurement (especially at lower and higher angles)

The resulting noise in this design *reduces the overall sensitivity* of the instrument, causes potentially significant *errors in MW determination*, and limits $R_g$ measurements from the angular dissymmetry to *sizes larger than 10-12 nm*.

**Latest LS instrument design**

A completely new approach to MALS instrument design has been developed by Tosoh[4, 5, 6] to address the highlighted limitations by combining a novel flow channel concept with improved optics to provide more signal intensity while reducing the core noise at each angle of measurement.

The traditional flow cell is replaced with an elongated flow path with a dual-cone shape.




*LenS3 MALS detector design*

Consisting of a black, non-refractive, and inert polymeric material (PEEK), the flow cell is sealed with two optical lenses, and the sample is introduced directly into its center.

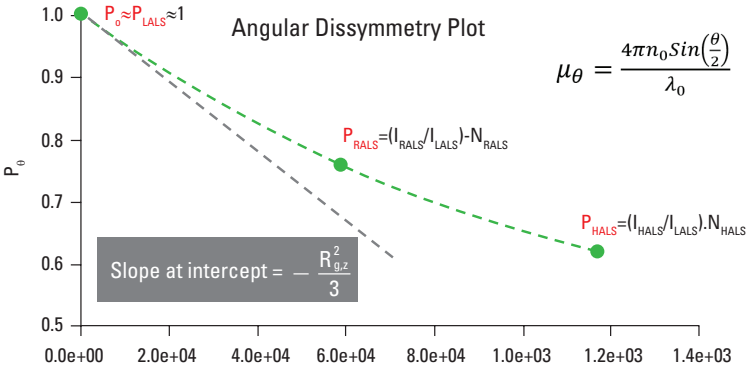
- The elongated flow path maximizes the scattering volume, hence *increasing the scattering intensities*.
- The chamber's non-refractive material *reduces noise* from undesired stray light.
- The conical shape and optical path define the forward (10°) and backward (170°) angles of collection of scattered light, while the third measurement is made at the center of the channel at 90°.
- The incident beam is effectively eliminated to collect the scattered light at 10° and 170° with *high-quality signals*.
- The *green laser* ($\lambda_0$=505 nm) provides about three times higher scattering intensity over typical red lasers ($\lambda_0$=660 nm), as per Eq. 1 and 2.

**Molecular weight and $R_g$ determination**
Thanks to the presence of a LALS measurement with an excellent signal-to-noise ratio, *MW is measured directly on the LenS3 MALS without the need for an extrapolation* procedure such as the 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°. The molecular weight of the molecules is simply proportional to LALS intensity.

A new method for determining $R_g$ is also used to take advantage of the detector design. From Rayleigh's equation for sufficiently dilute solutions, one can define the angular dependence as follows:

$$P_\Theta = I_\Theta / I_0$$

A plot of this ratio for the LALS (10°), RALS (90°), and HALS (170°) 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 (angular dissymmetry plot).



*Angular dissymmetry plot for $R_g$ determination*

To summarize, the new approach to MALS developed by Tosoh is the only method that allows direct determination of both MW and $R_g$ without extrapolation.

## Which applications of SEC-MALS will benefit from the extended capabilities of the LenS3 MALS detector?

**More complex macromolecules require more in-depth characterization**
Whether new biopharmaceutical products or polymers with tailored properties for conventional or biomedical usage, the macromolecules' complexity increases, requiring even more extensive and in-depth characterization for a complete understanding of the material.

The higher sensitivity and broader measurement range provided by the LenS3 MALS detector are beneficial for all applications, especially in the following situations:

- Samples with *low concentration* or available only in *very small quantities* for characterization
- *Low molecular weight and/or low dn/dc* samples, which scatter less light by definition
- Presence of impurities, aggregates, or degradation products and fragments in *trace amounts*
- Full molecular weight, size, and conformation analysis of macromolecules all the way f*rom ultra-small to ultra-high MW and* $R_g$ (even below 10nm).

Tosoh's experienced applications team has been working on improving SEC-MALS characterizations of multiple types of polymers and biomolecules, together with our academic and industrial partners. Examples include:

- Branched polymers and copolymers,
- Polymer nanoparticles
- Drug delivery vehicles
- Oligonucleotides
- Biotherapeutic proteins and monoclonal antibodies

To learn more about what the LenS3 MALS detector can do to enhance your SEC-MALS analysis, visit www.tosohbioscience.com or email us at sales-marketing.tbg@tosoh.com.

## Practical tips and tricks for good light scattering measurements

**Light scattering is very sensitive to the presence of undesired particles**

Simple precautions should be applied to avoid high noise or spikes on the LS detector signal:

- Make sure your *chromatography system is clean* and in good working conditions. Performing routine maintenance and thorough cleaning on a regular basis is generally helpful.
- Use high purity solvents, water, and salts (HPLC grade) to prepare your mobile phase.
- Always *filter your mobile phase and use a post-pump filter* to remove any particles from the chromatography system.
- *Flush your columns separately* to waste with a large volume of mobile phase at a low flow rate before connecting your light scattering detector. Increase your flow rate gradually.
- Whenever possible, filter your samples to remove insoluble material and larger aggregates or particulates.
- A post-column filter may also be added to eliminate fine particles released by the column packing material if needed. Make sure you carefully select your filter membrane porosity and material: it should be compatible with your mobile phase, sample chemistry, and expected size range.

**Molecular weight from light scattering is only as "absolute" as can be**

To obtain as reliable and accurate as possible results, the following is recommended:

- Prepare accurate concentrations for your samples and enter the value in the sample sequence. This input concentration value may be used when processing the SEC-

MALS data to obtain either sample recovery (from a known sample dn/dc value) or an estimated dn/dc value (assuming 100% recovery).

- *Use an accurate dn/dc value*, either from previous measurements, from literature, or by measuring it.
- *Verify your system calibration* on a regular basis with a known standard (or internal reference sample) by processing it like any sample and checking the calculated results (MW and concentration). It is common practice to add a standard in every sample sequence.
- Perform a full system calibration every time a significant change occurs (different solvent, change in tubing and connections, system cleaning or maintenance). This system calibration includes detectors' calibration, flow path correction (detector offsets and band broadening correction), and MALS normalization.
- Multiple injections of each sample or standard (duplicate or triplicate) are recommended to assess reproducibility and detect any chromatographic issue.

**Preserve your light scattering detector**

Light scattering detectors' cells are not more prone to getting dirty or contaminated than other types of detectors. Still, by definition, light scattering detectors will "see" dirt or contamination occur much sooner than other detectors.

Some simple tricks can help avoid such problems:

- Monitor your light scattering signals baselines and noise level over time. If increasing, perform your routine system rinsing or cleaning procedure.
- If using mobile phases containing salts (like aqueous eluents or buffers), switch the complete system back to pure water or pure solvent once your sample analyses are completed.
- *If possible, never stop the flow*. Let the pump run at a very low flow rate (0.1 mL/min or lower) at all times and refill your solvent reservoir with fresh filtered solvent regularly. This greatly helps prevent contamination or LS cell from getting dirty.
- If the system is to be unused for a longer period, switch it to a safe storage solvent (e.g., THF, water with 20% ethanol, or 10% acetone).

## References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2. Kratochvil P., Classical Light Scattering from Polymer Solutions; Elsevier: New York, 1987
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5. Patent application #: PCT/US19/12090: Light Scattering Detectors and Methods for the Same
6. Patent application #: PCT/US19/12095: Light Scattering Detectors and Methods for the Same
7. Patent application #: PCT/US21/49552: Light Scattering Detectors and Methods for the Same


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