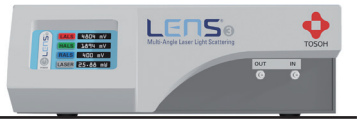
TOSOH

White Paper



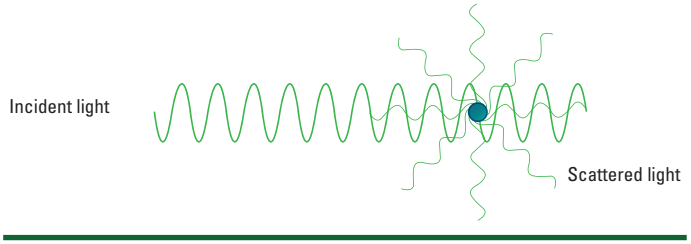
Light Scattering for Determination of Molecular Weight and Radius of Gyration

Light scattering of macromolecules in solution has seen increasing interest over the past decades from scientists studying polymers and proteins. Since the mid 1950's and its inception, size exclusion (or gel permeation) chromatography (GPC/SEC) has been the standard technique for the characterization of molecular weight distributions by separating macromolecules according to their size. The addition of light scattering detection online with GPC/SEC systems allows the measurement of true – or absolute – molecular weight of macromolecules, as opposed to the relative values obtained from conventional column calibration techniques. Moreover, light scattering can also provide size and conformation of macromolecules without any assumption on shape.

Principles of Light Scattering

In general, 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. These oscillating electrons generate a new electromagnetic radiation – which is called scattered light – in all directions of space *(Figure 1)*.

Figure 1. Schematic of light scattering principle



If the molecules are small enough in comparison with the wavelength of the incident light, then they can be considered point scatterers and scatter light equally in all directions. This is called isotropic scattering.

For bigger molecules, light is scattered by multiple points within the molecule. Interferences between the scattered light from different sources then occur, due to phase shift, in all directions except that of the incident beam. The further away from the incident light, the more interferences. As a result, the intensity of the scattered light decreases with increasing angle of observation. This phenomenon is called angular dependence.

The Rayleigh equation

The theory of light scattering that applies to macromolecules in solution with sizes in the range studied in GPC/SEC was developed by Lord Rayleigh 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:

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

And

R_θ is the excess Rayleigh scattering ratio – directly related to the intensity of the light scattered by the sample solution above that of the pure solvent at angle θ, divided by the intensity of the incident beam.

M is the molecular weight of the sample

c is the concentration of the sample

A_2 is the second virial coefficient – relates to sample/solvent interactions

P_θ is the particle scattering (or angular dissymmetry) function, with $P_\theta = R_\theta/R_0$

Where k_{opt} is an optical constant for a vertically polarized light:

$$k_{opt} = \frac{4\pi^2.n_o^2.\left(\frac{dn}{dc}\right)^2}{N_A.\lambda_0^4} \qquad \text{Eq. 2}$$

And

N_A, Avogadro's number

n_0, the refractive index of the solvent

λ_0, the wavelength of the laser in vacuum

dn/dc, the refractive index increment of the sample in the solvent

Measuring Molecular Weight

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

Rayleigh Equation at $\theta = 0$:

$$\frac{k_{opt}.c}{R_0} = \frac{1}{M} + 2A_2.c \qquad \text{Eq. 3}$$

At sufficiently low concentrations, the second virial coefficient in Eq. 3 is negligible, and the Rayleigh equation then simplifies further to:

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

From Eq. 4 one can obtain the molecular weight of the sample if R_0 can be measured, and k_{opt} and solution concentration values are known.

However, measuring the intensity of the scattered light in the direction of the incident beam is not practical. It is technically impossible to detect the amount of the scattered light from the incident beam, which has significantly higher intensity.

Various technical approaches can be used to obtain R_0, and are detailed in the next section.

Measuring Radius of Gyration (R_g)

The particle scattering function, P_θ, describes the angular dependence of the scattered light, which is how the intensity of the scattered light changes with the angle of observation.

From Debye's work on the relation between the particle scattering function and the structure of the particle, the following equations can be derived [Ref. 1]:

$$\lim_{\theta \to 0} P_\theta = 1 - \frac{{R_g}^2}{3}{\mu_\theta}^2 \qquad \text{Eq. 5}$$

Or

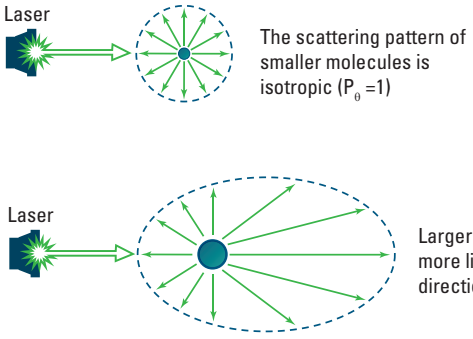
$$\lim_{\theta \to 0} \frac{1}{P_\theta} = 1 + \frac{{R_g}^2}{3}{\mu_\theta}^2 \qquad \text{Eq. 6}$$

Where

$$\mu_\theta = \frac{4\pi n_0 Sin\left(\frac{\theta}{2}\right)}{\lambda_0} \qquad \text{Eq. 7}$$

The larger the size of the sample, the more angular dependence is observed ($P_\theta < 1$). Conversely, samples with small radii show very low angular dependence and exhibit isotropic scattering ($P_\theta \approx 1$) *(Figure 2)*.

Figure 2. Illustration of the angular dependence of scattered light with the size of the molecule



Note: It is noticeable that Eq. 5 and 6 do not rely on any assumption on particle shape, therefore they are valid for all types of macromolecules in solution, regardless of their conformation (sphere, random coil or rod-like) and size.

In order to obtain the radius of gyration from a light scattering measurement, it is thus necessary to study the angular dependence by measuring and comparing the intensity of the scattered light at multiple angles of observation.

Just like for molecular weight, different approaches are available to get the R_g value from multi-angle light scattering measurements (see details in the next section).

Practical Methods of Light Scattering

This section describes the most common and the latest approaches that are currently used to analyze macromolecules by light scattering.

Batch light scattering

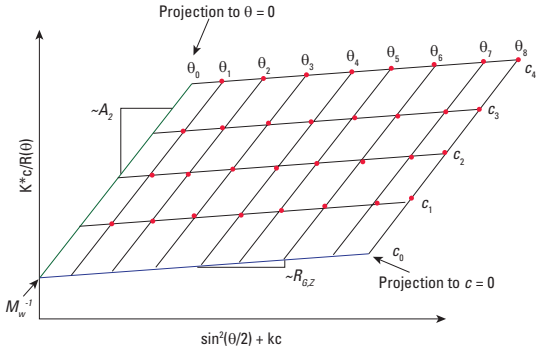
Historically, light scattering measurements were performed exclusively on optical benches in "batch mode". In contrast to "flow mode", where a Multi-Angle Light Scattering (MALS) detector is connected to a GPC/SEC system and the measurement is performed as the solution flows through the detector cell, "batch mode" measurements are performed in a static cell or cuvette without any separation.

The Rayleigh equation (Eq. 1) shows that the intensity of the scattered light for a given macromolecule with molecular weight M, depends on two variables: (1) the angle of observation, θ, and (2) the concentration of the solution, c.

The Zimm plot is a graphical representation of how the scattered intensity varies with those two parameters ($k_{opt} \cdot c/R_\theta$ is plotted against $\sin^2(\frac{\theta}{2})+k.c$, where k is an arbitrary coefficient). Measurements of R_θ are made at various angles and concentrations, and extrapolations are made to θ=0 and c=0 to obtain the weight average molecular weight (M_w), the z-average radius of gyration ($R_{g,z}$) and the second virial coefficient (A_2) of the sample.

Figure 3 below is an example of a Zimm plot.

Figure 3. Typical Zimm plot obtained by MALS in batch mode



The intercept on the Y-axis gives M_w and the slopes at intercept of the extrapolations for c=0 and θ=0 provide $R_{g,z}$ and A_2, respectively.

Although tedious, these types of measurements work well for large macromolecules that exhibit significant angular dependence, thus allowing the measurement of all three parameters: M_w, R_g and A_2.

It is noteworthy to mention that the batch mode light scattering technique can be challenging, especially when the sample solution contains impurities or other components that may interfere with the measurement. For example, the presence of high molecular weight or large size species (e.g. aggregates, microgels, dust, or bacteria) can distort the data by adding extra scattering, disproportional to the samples of interest. Hence, coupling light scattering with a separation technique such as GPC/SEC can be a more effective characterization tool and can provide additional information on the polydispersity or distribution of sample of interest.

Light Scattering coupled with GPC/SEC

For smaller soluble macromolecules, i.e. below ≈100 nm in radius, light scattering measurements can be performed by coupling the detector with a GPC/SEC system. Once the molecules are separated based on their size in the GPC/SEC column(s), each eluted fraction is then analyzed by the light scattering detector, and the resulting profile or distribution of molecular size and weight is obtained.

In general, 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 1 can be simplified further to:

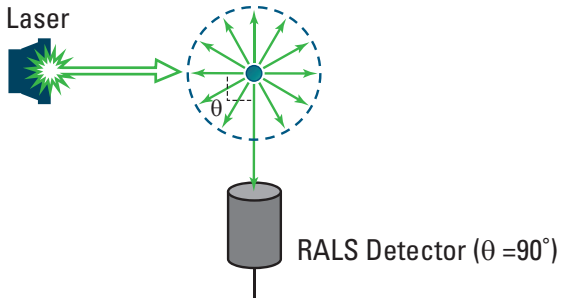
$$R_\theta = k_{opt} \cdot c \cdot M \cdot P_\theta \qquad \text{Eq. 8}$$

Several types of commercial light scattering measurements are commonly used in-line with GPC/SEC separation.

Right Angle Light Scattering (RALS) *(Figure 4)*

RALS detectors measure light scattering at 90°-angle without consideration to angular dependence. Although this technique offers a direct measurement of molecular weight using a single angle, it is limited to smaller macromolecules, typically below 15 nm in radius, for which the angular dependence can be ignored ($P_{RALS} \approx P_0 = 1$). RALS measurements are thus perfectly suitable for small molecules like proteins and peptides, monoclonal antibodies, antibody drug conjugates, as well as most random-coil polymers with molecular weight below 200 KDa (including oligomers, pre-polymers, resins, and oligosaccharides…). The radius of gyration cannot be measured by a single RALS instrument.

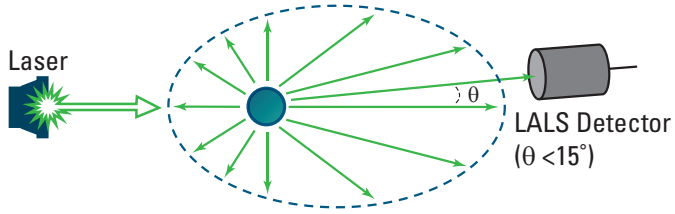
Figure 4. Schematic of a right angle light scattering (RALS) detector



Low Angle Light Scattering (LALS) *(Figure 5)*

LALS detectors are the purest and most accurate form of light scattering for the measurement of molecular weight. Typically, the angle of measurement for such detectors tend to be below 15°, which makes the measurement more difficult due to the proximity to the incident beam. LALS detectors directly measure molecular weight without any assumptions or additional Zimm extrapolation procedure. The scattered light is measured at a single angle close to 0° where $P_{LALS} \approx P_0 = 1$ for macromolecules of all sizes. Like the RALS detector, the radius of gyration cannot be measured by LALS alone.

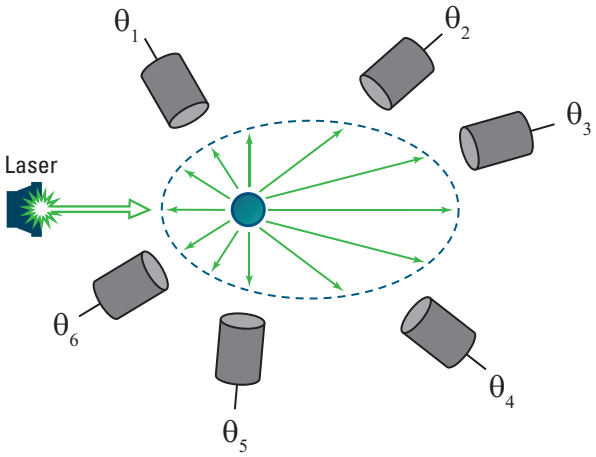
Figure 5. Schematic of a low angle light scattering (LALS) detector



Multi-Angle Light Scattering (MALS) *(Figure 6)*

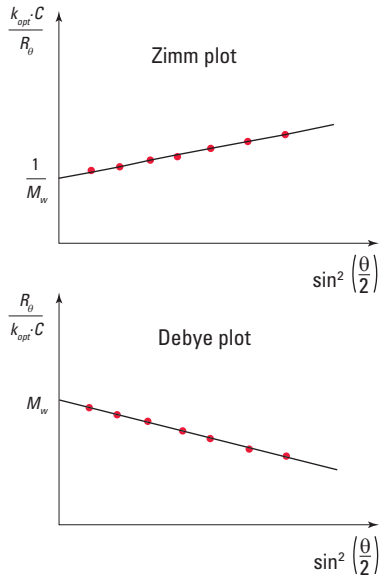
MALS detectors offer multiple angles, typically 3 or more ranging from 20° to 150°, to measure the scattered light. The geometry and the cell design of existing MALS detectors make it impractical to measure at lower and higher angles. Hence, the molecular weight for each slice of the GPC/SEC chromatogram is obtained by the Zimm plot method, i.e. extrapolation back to angle θ=0. The extrapolation method can be challenging at times, especially when dealing with larger molecules that require higher order fits. However, the same extrapolation enables the measurement of angular dependence, where it is detectable, to determine radius of gyration, $R_{g,z}$, for the eluted fractions.

Figure 6. Schematic of a multi-angle light scattering (MALS) detector



It is important to mention that in GPC/SEC-MALS measurements, aside from the Zimm plot, the Debye plot is also commonly used for the extrapolation method, which is a simple variation of the Zimm plot *(Figures 7a and 7b)*. The choice comes down to the best linearity and fit, especially for the lower angles, to improve the precision and accuracy of the y-axis intercept.

Figure 7a and b. Zimm (a) and Debye (b) plots obtained from multi-angle light scattering (MALS) measurements



Regardless of the extrapolation choice, the slope of the fit at the y-axis intercept is directly proportional to the radius of gyration. The smaller the molecules, the shallower the slope, and inversely, the larger the molecules, the steeper the slope will be.

In practice, for small molecules below 10-12 nm, no significant difference in the intensity of the scattered light with the angle of observation can be reliably measured using the excisting commercial detectors. As a result, for such a molecular size range, the extrapolation is almost flat, and R_g cannot be obtained.

Note: As an indication, a 100 kDa (or kg/mol) random coil polymer has a radius of about 12 nm.

LALS and MALS combined [Ref 2]

The most recent approach is to combine the best of the existing methods into a single light scattering instrument.

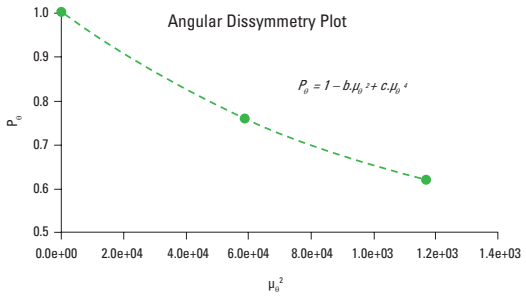
This groundbreaking approach is essentially a MALS concept incorporating an extreme low angle (LALS – 10°) and an extreme high angle (HALS – 170°) with a right angle (RALS – 90°) to form a 3-Angle MALS detector (10° + 90° + 170°).

There are multiple benefits to combining these three angles:

- LALS directly measures absolute molecular weight for all sizes of molecules, regardless of the angular dependence.
- The RALS detector can be used to measure small samples (with no significant angular dependence) in situations where the LALS can be limiting (GPC/SEC system contamination, column shedding etc.)
- The MALS approach allows the measurement of the radius of gyration.
- The new approach is also able to detect angular dependence to considerably lower size range, simply because the smallest differences in Particle Scattering Function values, P_θ, can be substantiated using the extreme angles. As a result, R_g determination can be extended to levels significantly lower than the historical 10-12 nm limitation.

The new approach also uses a novel method for the determination of R_g. Instead of the traditional Zimm or Debye plot, the particle scattering function, P_θ, itself is directly fitted at each of the three angles on the Angular Dissymmetry Plot. Assuming $I_{10}\approx I_0$ (thus $P_{10}\approx P_0=1$), the P_θ values for RALS and HALS can easily be calculated using the Normalization Factor for each angle ($P_\theta=N_\theta \cdot I_\theta/I_0$). The P_θ values are then plotted against μ_θ^2 (where, $\mu_\theta=[4.\pi.n_0.\mathrm{Sin}(\frac{\theta}{2})]/\lambda_0$) to generate the Angular Dissymmetry Plot *(Figure 8)*.

Figure 8. Angular dissymmetry plot for the determination of R_g



Using Eq. 5:

$$\lim_{\theta\to 0} P_\theta = 1 - \frac{{R_g}^2}{3}{\mu_\theta}^2$$

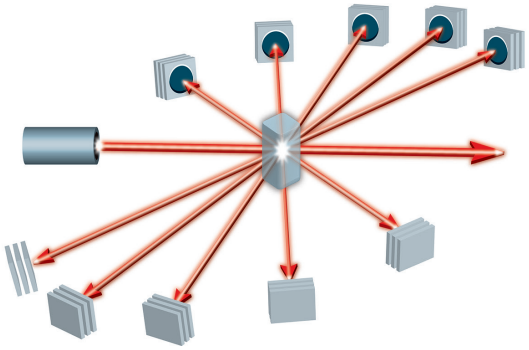
the Radius of Gyration, R_g, is directly proportional to the slope of the fit at the y-axis intercept (θ=0):

$$R_{g,z} = \sqrt{3b}$$

It is important to point out that the new method for R_g determination does not require concentration and refractive index increment (*dn/dc*) values of the sample solution or any assumption on the shape of the particle. This significantly simplifies the process and make R_g determination more practical.

Normalization

In all MALS instruments, the detectors are placed in the same plane around the sample cell.



Each angle measures the scattered light emitted by a different portion of the sample solution in the cell, called the scattering volume. This scattering volume is the cross section between the incident beam and the beam of scattered light collected by the photo-detector, hence highly dependent on the geometry of the setup. The intensity of the scattered light is related to the number of molecules in the scattering volume, therefore the larger the scattering volume, the higher the intensity or response of the detector.

In order to use MALS to measure and compare the intensities of scattered light at various angles, the instrument must undergo a normalization process that corrects for the geometric differences between the detectors, or the varying scattering volumes for different angles. This normalization procedure also corrects for differences in the actual distance between the photo-detectors at each angle and the scattering volume, as well as potential optical effects (refraction or absorption at cell walls) and electronic response differences.

The following methods can be employed to obtain the normalization factors for MALS detectors.

Single-point normalization using an iso-scatter reference

Single-point normalization is the most common commercially-available method. It involves using a low molecular weight polymer standard or a small globular protein as a so-called "iso-scatter reference". The assumption here is that the iso-scatter reference will have no angular dependence and the intensity of the scattered light at all directions will be the same. As such, the detector responses are collected at every angle and then normalized to a reference angle, so all detectors at all angles give the same response. Once the normalization factors are obtained ($N_\theta=I_\theta/I_{ref}$), they are applied to all subsequent measurements.

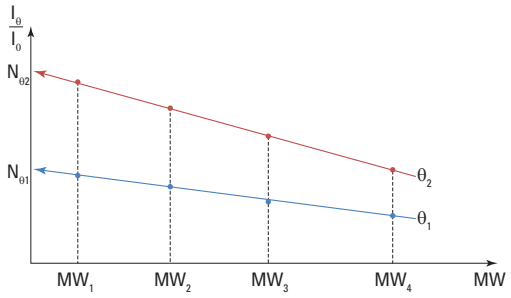
Note 1: Depending on the cell design and detectors' arrangements, the scattering volume and thus, the normalization factors, may vary with the refractive index of the solvent used.

Note 2: When the radius of gyration of the reference used for normalization is known, the value can be used to correct for the small, yet actual, angular dependence in the normalization process, resulting in more accurate determination of R_g.

Multiple references of known molecular weight [Ref 3]

This approach uses multiple known polymer or protein references with varying molecular weights to determine the "true" relationship between angular dissymmetry and molecular weight/size for all angles. The procedure involves light scattering measurements of the reference samples to obtain Angular Dissymmetry Ratio, I_θ/I_0, for each angle at a given reference molecular weight/size. By extrapolating the results to MW→0, the normalization factor for each angle can be obtained. Therefore, for each angle, the y-axis intercept of the extrapolation fit is its normalization factor *(Figure 9)*. Similar to the Single-Point technique, the normalization procedure is independent of the type of reference(s), but the accuracy of the molecular weight values is instrumental in obtaining reliable normalization factors.

Figure 9. Normalization plot using a series of references of known molecular weights



It is important to point out that this method is only possible if one of the measuring angles is low enough (i.e. below 15°) to assume $I_{LALS} \approx I_0$. The low angle then becomes the reference detector for the normalization factors.

Conclusions

Light scattering is the technique of choice for the determination of molecular weight and size of polymers and proteins in solution, and even a more powerful tool when coupled with size exclusion chromatography. In the past four decades, only two different types of light scattering detector technologies have been commercially available (MALS vs. LALS+RALS), forcing users to choose one over the other. While low angle light scattering detectors provide a direct measurement of molecular weight without extrapolation or assumptions, the technique offers no additional information on size or conformation. On the other hand, multi-angle light scattering detectors employ Zimm or Debye plots to provide additional size and conformation information, but require tedious extrapolation procedures to make the measurements. In any case, none of the existing detector designs allow measurement of size below 10-12 nm radius, which constitute a large group of today's GPC/SEC applications. Featuring a novel cell geometry, an improved normalization process and a new method for utilizing the angular dependence, the latest technology combines and extends the benefits of both LALS and MALS approaches, allowing direct measurement of molecular weight and determination of radius of gyration down to a few nanometers.

References

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

[Ref. 2]: Patent application #: PCT/US19/12090: Light Scattering Detectors and Sample Cells for the Same

[Ref. 3]: Patent application #: PCT/US19/12095: Light Scattering Detectors and Methods for the Same