STATIC LIGHT SCATTERING TECHNOLOGIES FOR **GPC/SEC** EXPLAINED

Abstract:

The aim of this guide is to provide the reader with a clear understanding of the different technological approaches used to measure molecular weight by static light scattering in a GPC/SEC experiment. It will explain and differentiate between the techniques and technologies used in SLS, MALS, RALS, and LALS. It assumes no prior knowledge of the light scattering theory or instrumentation and should be ideal for those new to light scattering and those looking to increase their knowledge in the area. The guide covers an introduction to the theory and background of molecular weight measurements by static light scattering. It is hoped that the information contained in here will help users to make an informed decision about the most appropriate light scattering technology to use.

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SUMMARY

Static light scattering is a technique to measure the molecular weight using the relationship between the intensity of light scattered by a molecule and its molecular weight and size. These relationships are described by Rayleigh theory which states that the molecular weight of a molecule is proportional to the Rayleigh ratio of scattered light i.e. the ratio of scattered light intensity to incident light intensity.

All static light scattering instruments detect the amount of light scattered by a sample to measure its molecular weight; however, as molecules grow in size, a second factor called angular dependence becomes significant. Angular dependence affects the intensity of scattered light and hence the calculated molecular weight. It must therefore be accounted for.

- A RALS detector collects scattered light at 90°. It can measure molecular weight with high sensitivity for samples that scatter light isotropically i.e. equally in all directions; however it cannot measure molecular weight for anisotropic scatterers (those affected by angular dependence).

- A LALS detector collects scattered light at 7°. It can measure molecular weight for all molecules but has a lower signal-to-noise.

- By combining RALS and LALS detectors into a hybrid system the molecular weight of all samples can be measured while maximising signal-to-noise where required. It therefore offers the strengths of both LALS and RALS with none of their weaknesses.

- A MALS detector collects scattered light at many angles. This data is used to model the angular dependence to account for it in the molecular weight calculation. It can measure molecular weight for both isotropic and anisotropic scatterers and for anisotropic scatterers can also measure the radius of gyration.

- All static light scattered instruments must be calibrated before use. Calibration can either be performed using a molecular weight standard or a scattering standard and each has advantages and disadvantages that are described in the calibration section.

GLOSSARY OF ACRONYMS

GPC	Gel permeation chromatography
SEC	Size exclusion chromatography
LALS	Low angle light scattering
RALS	Right angle light scattering (90° light scattering)
MALS	Multi angle light scattering
SLS	Static light scattering
DLS	Dynamic light scattering
R_g	Radius of gyration
R_H	Hydrodynamic radius
MW	Molecular weight (Molar Mass)
Mn	Number-average molecular weight
Mw	Weight-average molecular weight
Mz	Z-average molecular weight
A_2	Second virial coefficient (B_{22})

INTRODUCTION

Light scattering can be a confusing topic. There are a number of different techniques that come under the general heading of light scattering and a range of parameters that can be measured. The lines between these can become blurred, as some systems use more than one technique in a single instrument, so it is very easy to lose sight of the most important factors when considering which light scattering technology is right for your application.

Static light scattering, also called classical light scattering, is a technique used to measure molecular weight and molecular radius of gyration. Within static light scattering, there are a number of different technologies with acronyms such as SLS, MALS, LALS and RALS and others. Each of these is subtly different and each has advantages and disadvantages.

This white paper describes, in some detail, the principles and theories behind static light scattering. The theory, technique and the different technologies are described and compared. By reading this white paper, you should be able to understand the background to these measurements and make an informed choice on the most appropriate solution for your application.

Each section provides a great deal of detail, but begins with a few key points to summarise the information contained so you can decide whether you need to read the detail in that section.

What is molecular weight?

Key Points

- Molecular weight is the molar mass of the molecule under study.
- There are a number of moments that can be calculated from a molecular weight distribution including Mn, Mw and Mz.
- Polydispersity describes the broadness of the molecular weight distribution.

Details

Molecular weight is a property that describes the mass of an individual molecule. Scientifically, it is the mass of a material required to make 1 mole of the sample. For example, carbon has a molecular weight 12 g/mol. The units of molecular weight are grams per mole (g/mol). However these are often referred to as Daltons (Da), or sometimes more conveniently kilo Daltons (kDa)

For a pure protein sample, it is expected that it would have a fixed molecular weight and that all of the protein molecules within the sample should have the same molecular weight. For a natural or synthetic polymer sample, the molecular weight of the molecules

within a particular sample will have a distribution that could have many forms. The term that describes the overall width of the distribution is 'polydispersity'. A sample with a very narrow range of molecular weights is said to have a low polydispersity or to be very monodisperse. A sample with a wider range of molecular weights is said to have a high polydispersity.

The majority of samples contain a range of molecular weights, particularly natural and synthetic polymers. These distributions of molecular weights can be described in a number of ways, and one common way is to use the molecular weight moments Mn, Mw and Mz, which are the number-averaged, weight-averaged and z-averaged molecular weights respectively. These are defined by equations shown below, where c_i and M_i are the concentration and molecular weight at each data slice:

$$\overline{Mn} = \frac{\sum c_i}{\sum c_i / M_i} \qquad \overline{Mw} = \frac{\sum M_i c_i}{\sum c_i} \qquad \overline{Mz} = \frac{\sum M_i^2 c_i}{\sum M_i c_i}$$

Intuitively, the number-averaged molecular weight is the total mass of material divided by the total number of molecules and is therefore a numerical average. It is weighted towards the lower mass molecules which will be more numerous. Properties such as vapour pressure lowering, freezing point depression and osmotic pressure depend on the number of molecules and not on their size. The weight average molecular weight is calculated by multiplying by the molecules mass, so the average is biased towards the larger molecules in the distribution. Properties like diffusion, sedimentation, light scattering depend both on the size and the mass of the molecules. The z-average molecular weight includes a further multiplication of the molecules' molecular weight. It is therefore heavily weighted to the largest molecules in the sample and can be determined directly by the ultracentrifugation technique.

Using these three values, it is possible to get an idea of the entire molecular weight distribution. Clearly, the closer these values are to each other, the lower the polydispersity and the further apart they are, the higher the polydispersity. Polydispersity is defined according to the equation

$$Pd = \frac{M_w}{M_n}$$

Since Mw is always equal to or larger than Mn, the lowest possible value for polydispersity (PD) is 1 but there is no theoretical upper limit. Natural and synthetic polymers have a wide range of polydispersities but since proteins have fixed molecular weights, we can expect the polydispersity of a protein to be very low (close to 1) for well-behaved proteins.

What is molecular size?

Key Points

- Molecular size is the physical size of the molecule.
- When discussing size from static light scattering alone, we are discussing R_g, the radius of gyration.
- The units of size are in nanometers.

Details

Molecular size is the physical size of a molecule. Typically, a single value is used to describe the size. This value is the radius of a sphere of an equivalent size to the molecule being measured. The two most commonly used values of molecular size are R_H (hydrodynamic radius) and R_g (radius of gyration). The hydrodynamic radius is the radius of an equivalent sphere that diffuses at the same speed as the molecule under study. It is calculated either from dynamic light scattering or from intrinsic viscosity and so will not be considered further here. (For more information on hydrodynamic radius see the technical notes, "Dynamic Light Scattering: An Introduction in 30 Minutes" and "Protein and polymer molecular size by GPC/SEC" – see references).

The radius of gyration is the root-mean-square of the radii from the centre of mass to the different mass cores within the molecule. It is sometimes called R_{rms} (root-mean-square). The units for a molecular size are nanometers (nm). R_g is calculated using static light scattering so will be considered in more detail later in the document.

What is light scattering?

Key Point

- When light hits a molecule or particle, some of that light is absorbed and re-emitted in all directions.

Details

When a photon collides with a molecule, some of the energy from the photon is used to initiate what is called an oscillating dipole within the molecule. This energy is subsequently re-emitted by the molecule in all directions as light. We see this phenomenon every day in white clouds, sunsets or in dust passing through a beam of sunlight or light from a projector. The principles behind light scattering can be used to measure a number of properties related to the molecule.

STATIC LIGHT SCATTERING THEORY

How is molecular weight related to light scattering?

Key Point

- Rayleigh theory describes the relationship between the intensity of the light scattered by a sample and its size and molecular weight.

Details

The properties of the light scattered by molecules and particles vary depending on the object doing the scattering as defined in the Rayleigh equation.

$$\frac{KC}{R_\theta} = \left(\frac{1}{Mw} + 2A_2C\right)\frac{1}{P_\theta}$$

Where:

- C = the sample concentration,
- θ is the measurement angle,
- R_θ = the Rayleigh ratio (the ratio of scattered light intensity to incident light intensity) at the measurement angle θ
- Mw = Weight average molecular weight,
- A_2 = the second virial coefficient.

K and P_θ are more complex terms defined as:

$$K = \frac{4\pi^2}{\lambda_0{}^4 N_A}\left(n_0\frac{dn}{dc}\right)^2$$

Where:

- λ_0 is the laser wavelength in a vacuum,
- N_A is Avogadro's number,
- n_0 is the refractive index of the solvent,
- dn/dc is the refractive index increment of the sample.

$$\frac{1}{P_\theta} = 1 + \frac{16\pi^2 n_0^2 R_g^2}{3\lambda_0^2} Sin^2\left(\frac{\theta}{2}\right)$$

Where:

- R_g is the molecule's radius of gyration

In simpler terms, the Rayleigh equation tells us that the intensity of scattered light at a given angle is dependent on a number of factors including both molecular weight and molecular size of the sample under study. As can be seen in the Rayleigh equation, molecules with higher molecular weight and larger sizes will scatter more light. While the increase in the intensity of light scattered is linear with molecular weight, it is non-linear with respect to size.

So, if we know all of the other factors in the Rayleigh equation, we can measure the intensity of the scattered light (related to R_θ) and calculate the sample's molecular weight.

What is static light scattering?

Key Point

- Static light scattering is the technique we use to measure molecular weight using light scattering.

Details

Static light scattering (SLS) uses an optical arrangement such that the signal detected is 'static' or stable. By measuring the intensity of light scattered by a sample in situations where the other constants are known, the molecular weight of the sample can be calculated.

The term static light scattering is used to differentiate the technique from dynamic light scattering which is a separate, though not unrelated, technique for measuring particle size. For more information on dynamic light scattering, see the Malvern technical note "Dynamic Light Scattering: An Introduction in 30 Minutes" or the 'inform' white paper "A basic guide to particle characterization" on the Malvern website.

Static light scattering in GPC/SEC

Key Points

- SLS measurements are most commonly performed as part of a GPC/SEC system but can be made in cuvettes as well.
- To perform SLS measurements, a concentration detector is also required which is typically RI but can also be UV.

Details

Static light scattering measurements can be made in a cuvette or in a gel-permeation chromatography (GPC)/size-exclusion chromatography system (SEC). Using a chromatography system removes a number of problems to do with preparing and purifying the sample and so is the most common implementation of SLS. Additionally, GPC/SEC allows us to easily combine the light scattering data with data from a concentration detector to measure the sample's concentration at the same time and thus use both sets of information in our calculations. The most common concentration detector is a refractive index detector (RI) but an ultraviolet (UV) absorbance detector can also be used.

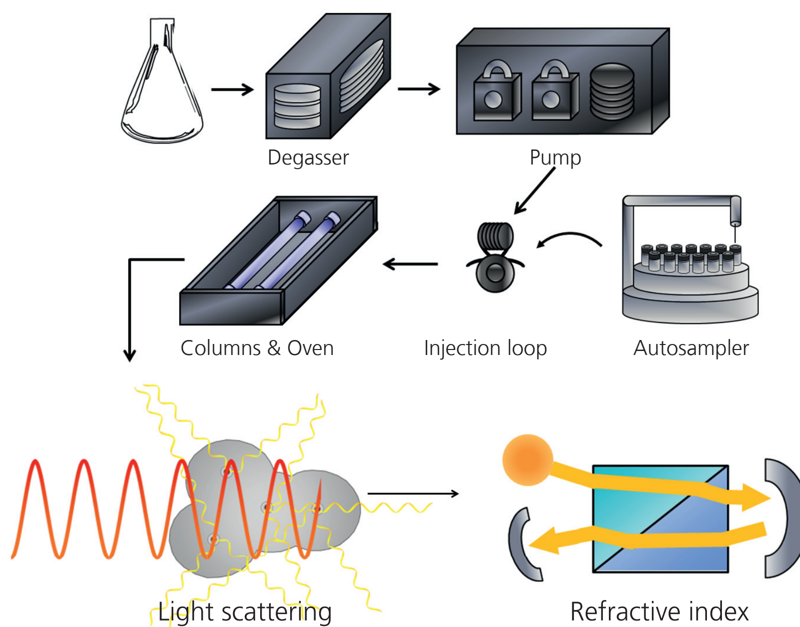


Figure 1: Schematic of a GPC/SEC system. The degasser, pump, injection loop and columns are standard with the optional autosampler. After separation, the molecules are measured by one or more detectors in sequence, in this case, a light scattering detector followed by a refractive index detector.

DIFFERENT LIGHT SCATTERING TECHNOLOGIES

An introduction to angular dependence

Key Points

- At higher molecular size, the intensity of light molecules scatter will start to vary with measurement angle and we must account for this phenomenon. This is called ‘angular dependence’
- Isotropic scatterers are small (R_g < ≈15 nm) and have little or no angular dependence in the intensity of the light they scatter.
- Anisotropic scatterers are larger (R_g > ≈15 nm) and the intensity of the light they scatter varies with angle.
- To accurately measure molecular weight, we must account for this and this is what different instruments do in different ways.
- If we account for the angular dependence by measuring it (i.e. MALS), we can use the data to calculate R_g

Details

The Rayleigh equation shown previously includes the term $1/P_\theta$, which so far we have assumed we know the values for. However, this is actually not true and it is here that all of the differences between different light scattering instruments come into play.

P_θ, or form factor, is related to the size of the molecule and the angle at which the scattering is determined. This term is defined as:

$$\frac{1}{P_\theta} = 1 + \frac{16\pi^2 {n_0}^2 {R_g}^2}{3{\lambda_0}^2} Sin^2\left(\frac{\theta}{2}\right)$$

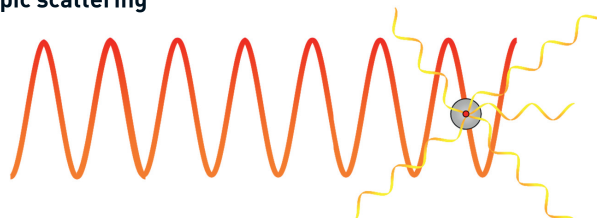
Where:

- n_0 is the refractive index of the solvent,
- R_g is the molecule’s radius of gyration,
- λ_0 is the laser wavelength in a vacuum,
- θ is the measurement angle.

It can be seen from the equation that $1/P_\theta$ is dependent on a number of factors to do with both the sample and the measurement. These include the solvent refractive index (n_0), the laser wavelength in a vacuum (λ_o), the measurement angle (θ) and the size of the molecule we are measuring (R_g).

This means that for larger molecules, the amount of light scattered will depend on the measurement angle. This is called 'angular dependence' and the reason for this phenomenon is that as the molecular size increases the scattered photons no longer scatter independently, but constructively and destructively interfere with each other as shown in the figure 2.

A: Isotropic scattering



B: Anisotropic scattering

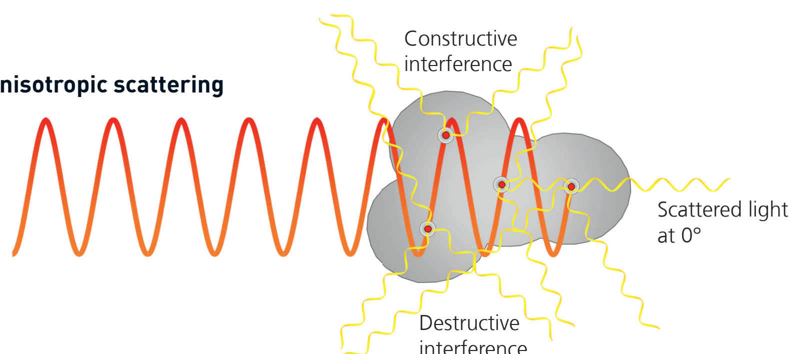


Figure 2: A. An isotropic scatterer is small relative to the wavelength of the light and scatters light evenly in all directions. B. An anisotropic scatterer has significant size compared with the wavelength of the incident light and scatters light in different directions with different intensities.

When the molecule is small with respect to the laser, as in figure 2A, it acts as a point scatterer. In this situation the light is scattered by the molecule with even intensity in all directions. These molecules are called 'isotropic scatterers'.

However, as the molecule grows in size with respect to the laser, the molecule's size and structure starts to become important. Individual photons from the laser are scattered by different points within the molecule. This is dependent on R_g. These scattered photons vary in phase so will interfere with each other and the resulting measured intensity will depend on the observation position. These molecules are called 'anisotropic scatterers'. At the sizes we are interested in, all of the interference is destructive which means that the intensity will always appear to be lower than if we assumed it was an isotropic scatterer. If we were to use this intensity to calculate the molecular weight using the Rayleigh equation, we would therefore underestimate it.

In order to remove this effect, the Rayleigh equation tells us that if we were able to make a measurement of the intensity of the scattered light at a θ of 0°, then $\sin^2(\theta/2)$ would be 0 and $1/P_\theta$ would be 1. So at 0° the intensity of scattered light would be unaffected by the interference and

we could relate the intensity to the molecular weight in the same way we can with smaller molecules, measuring at a 90° angle. Unfortunately, we cannot do this as the intensity of the illuminating laser at 0° is so much stronger than the scattered light that we cannot separate out the purely scattered light. As we cannot measure directly at 0° we need to find an alternative technique.

The rule of thumb to use when deciding where angular dependence starts is to say that, at a diameter below 1/20th of the laser wavelength, molecules scatter isotropically, and above this level you have anisotropic scattering. Most instruments usually have lasers with wavelengths between 633 and 670 nm (red) so the limit of isotropic scattering is between 15.8 and 16.8 nm radius (Because of the dependence on laser wavelength, the same calculation for a laser with a wavelength of 532 nm (green) will give a value of 13 nm).

This nominal value of around 15 nm radius for the onset of measurable angular dependence will also be the lower limit at which you can still reliably measure the molecular size, R_g . (See "How do we get the molecular size, R_g, from the data?" below)

The different types of light scattering instrument are designed to overcome the angular dependence issue in different ways and these are described in the next section.

How does angular dependence affect our calculations?

Key Points

- We must infer the scattering at 0° based on the scattering at other angles
- A Zimm plot of KC/R_θ vs $\sin^2(\theta/2)$ can be used to do this.
- The molecular size, R_g, can also be determined from the plot

Details

In order to overcome the angular dependence, we must decide what the intensity of the scattered light is at 0° based on data from other angles. The best way to calculate and represent this is in a 'Guinier Plot', which is a simplification of a Zimm plot where the second virial coefficient is not required.

A Guinnier plot is a plot of KC/R_θ as a function of angle ($\sin^2(\theta/2)$). Other plots and models are available but this is the most common. An example is shown in the figure 3.

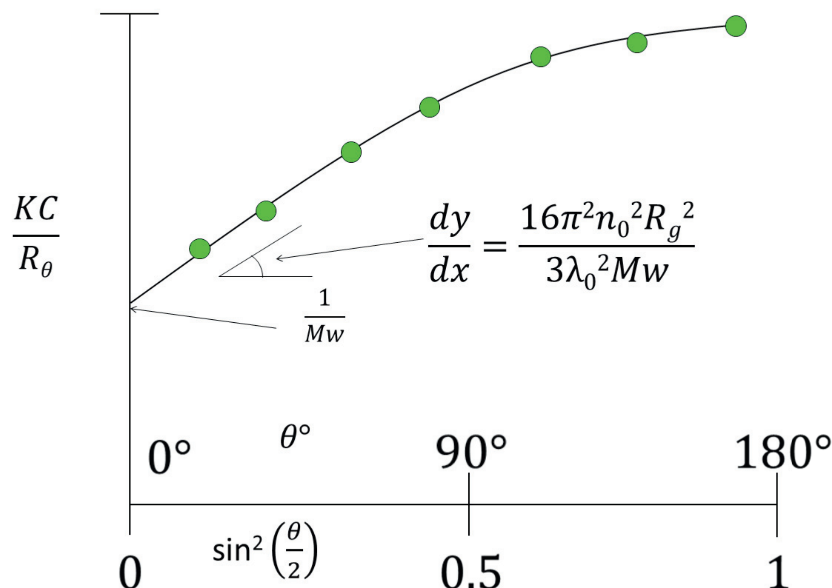


Figure 3: A Guinnier plot shows KC/R_θ as a function of $\sin^2(\theta/2)$. The intercept of the line is $1/Mw$ and the initial slope of the line is related to R_g.

Measurements of light scattering intensity are made at the desired angle. With knowledge of the sample's concentration and other factors, KC/R_θ can then be calculated and is plotted in the graph as a function of $\sin^2(\theta/2)$. The y-intercept (which equates to an angle of 0°) is 1/Mw from which the molecular weight can be calculated easily.

How do we get the molecular size, R_g, from the data?

Key Points

- The molecular size, R_g, can be determined from the initial slope of the angular dependence plot
- There must be sufficient angular dependence to see a slope above the noise

Details

The initial slope of the line is:

$$\frac{dy}{dx} = \frac{16\pi^2 n_0^2 R_g^2}{3\lambda_0^2 Mw}$$

R_g can then be calculated from this equation with relative ease. However, at small molecular sizes, the slope will be very small and hidden in the noise in the data. Only when the molecule gets to an appreciable size compared to the wavelength of the laser light will the slope be large enough to obtain a reliable R_g value. As described above, the nominal

threshold for a 633nm laser is an R_g of ≈15 nm. However, the absolute limit for measuring R_g may be lower or higher than this nominal 15 nm, depending on the sample, solvent and the conditions of measurement. Many MALS instrument manufacturers put a limit of 10 nm (or even lower) on their instrument specifications. This is often for standard polymers of high dn/dc values and at high concentrations run under ideal conditions. In practice, this limit will not be achievable on all samples. At the other extreme, with samples of poor dn/dc run under non-ideal conditions, the smallest R_g that can be measured may be much higher than the nominal 15nm value.

LIGHT SCATTERING INSTRUMENTS

There are 4 different types of SLS instruments available. RALS, LALS, Hybrid RALS/LALS and MALS.

Right-angle light scattering (RALS)

Key points

- Measure only at 90°,
- Have the best signal-to-noise ratio and sensitivity,
- Have the smallest flow cells,
- Can only measure molecular weight accurately for molecules with $R_g < \approx 15$ nm,
- Are excellent for measuring protein molecular weight,
- Cannot measure R_g.

Details

A RALS device is the simplest light scattering instrument available. It measures the intensity of the light scattered at 90° to the incident beam as in figure 4A. The molecular weight of the sample is then calculated directly from the intensity measured and the sample concentration. In the Zimm plot in figure 4B the intensity of the scattered light is measured far from the y-axis and it is assumed that the sample scattering is isotropic (i.e. equal at all angles).

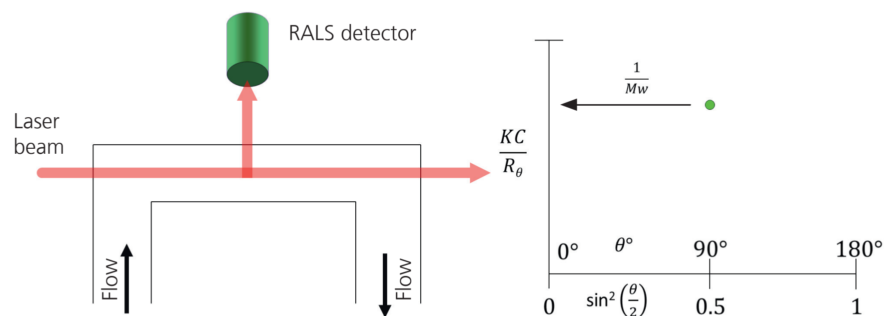


Figure 4: A. Schematic of a RALS detector showing the flow passing through the flow cell. Light from the laser enters at the end of the cell and is collected at 90°. B. When using RALS, the Debye plot is reduced to a single point which is assumed to be equal at every angle and therefore equal 1/Mw.

A RALS system has a number of advantages:

- It is a very straightforward instrument to build without any complicated optics. All that is required is a flow cell with a window on the side which allows the cell to have a low volume.
- Since the light is passing through the window/liquid interface at 90°, any flare or noise created by the change in refractive index is minimised.
- As a consequence, a RALS detector is the simplest form of SLS detector and, thanks to the low noise, it has the best signal-to-noise ratio and the best sensitivity.

However, RALS detectors have some limitations:

- Since the only measurement is at 90°, the assumption is that the intensity of the scattered light is the same at this angle as it is as 0°. This is clearly not true for any molecule large enough to display any form of significant angular dependence in the light that it scatters. Therefore a RALS detector cannot accurately measure the molecular weight of any molecule with an $R_g > \approx 15$nm, as discussed in the earlier section.
- As the measurement is only being made at one angle, the slope of the line on the Zimm plot cannot be measured so it is not possible to calculate R_g.

Since proteins are almost always smaller than 15nm radius, and scatter light only weakly, they require a sensitive detector. Hence RALS detectors are excellent for measuring protein molecular weight.

Low-angle light scattering (LALS)

Key points

- Measure at the lowest angle possible,
- Measure the molecular weight of all molecules with the highest accuracy,
- Have low volume flow cells,
- Cannot measure R_g.

Details

A LALS detector measures the intensity of light scattered at an angle that is as close as possible to 0° as shown in figure 5A. This has the advantage that the intensity will be very close to the intensity at 0° and so the calculated molecular weight will be very close to the actual molecular weight of the molecule. To be considered a LALS detector, the angle must be less than 10° and the most common LALS detectors have a measurement angle of 7° but the closer the measurement to 0°, the more accurate the measurement will be. This will be true irrespective of the size of the molecule that we are measuring. Looking at the Zimm plot (figure 5B), a LALS measurement is very close to the axis and therefore very close to the y-intercept. This means that the error by measuring at 7° is very low. With a LALS measurement at 7°, $\sin^2(\theta/2)$ is just 0.0037, which equates to less than 1% error in the MW even for the largest molecules.

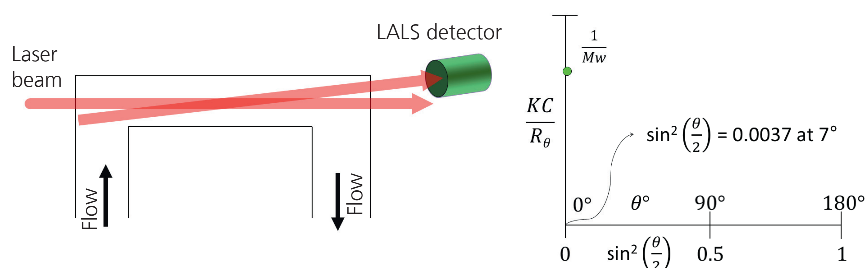


Figure 5: A. Schematic of a LALS detector showing the flow passing through the flow cell. Light from the laser enters at the end of the cell and is collected through the same exit window at a low angle e.g. 7°. B. When using LALS, the Debye plot is reduced to a single point which is very close to the y-axis and therefore equal 1/Mw for all molecules.

A LALS system has a number of advantages:

- LALS measures the intensity of light scattering very close to the axis of the Zimm plot so the calculated molecular weight has the highest accuracy.
- The LALS principle applies to the measurement of the molecular weight of any molecule.
- A dedicated LALS detector can have a small flow cell since it only has to accommodate one measurement angle.

LALS detectors also have a number of limitations:

- Since the scattered light is only being measured at a single angle, a LALS system cannot measure R_g.
- LALS detectors do not quite have the sensitivity of RALS detectors making them less sensitive for weakly scattering samples. This is not usually an issue as LALS detectors are most often used for larger molecules.
- It is more difficult to build a LALS detector as the scattered light and the laser light are very close to each other so the scattered light must be measured without collecting the incident laser light as well. In addition, LALS detectors are sensitive to contamination because contaminating particles are usually large and large particles scatter predominantly in the forward direction. As a consequence of both of these factors, early LALS detectors were very noisy and difficult to work with. Similarly, the lower angles on MALS detectors are often noisy for the same reason. However, modern dedicated LALS instruments are able to isolate the scattered light from the incident light thereby minimising the noise and achieving much better sensitivity.

Overall, LALS measurements offer an accurate way to measure molecular weight using static light scattering. LALS is particularly useful for measuring the molecular weight of large anisotropic scatterers such as synthetic and natural polymers.

RALS/LALS hybrid detectors

Key points

- RALS/LALS hybrids combine RALS and LALS detectors into a single unit,
- Use RALS to maximise sensitivity for weak scatterers,
- Use LALS to maximise the accuracy for anistropic scatterers,
- Maintain the small flow cells of RALS and LALS detectors,
- Enable R_g to be estimated.

Details

Based on the discussion above, it is clear that RALS and LALS detectors are complementary RALS detectors have the sensitivity to measure small isotropic scatterers and LALS detectors can measure the molecular weight of large molecules with unrivalled accuracy. A RALS/LALS hybrid therefore combines these two measurements into a single cell as shown in figure 6A. On the Zimm plot, the RALS detector is used to measure molecules whose size is below the ≈15 nm threshold of isotropic scattering. The LALS detector is used to accurately measure molecules whose size is above the ≈15 nm threshold of isotropic scattering. Software switches automatically from LALS to RALS according to the detector signals. For samples that scatter anisotropically, the ratio of the two calculated molecular weights can be used to estimate P_θ. This in turn can be used to estimate a value for R_g assuming a structural model such as a random coil or a hard sphere.

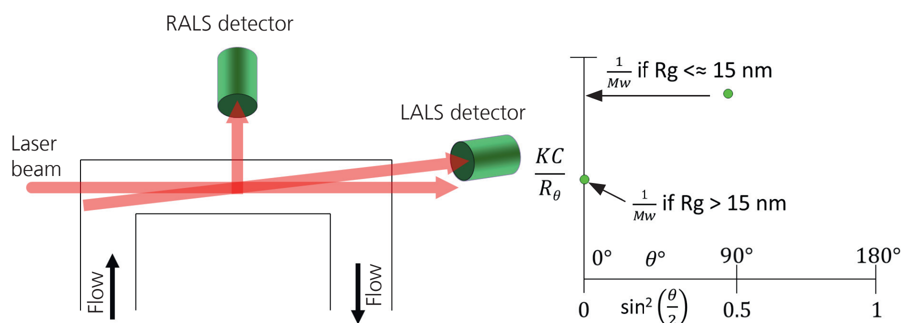


Figure 6: A. Schematic of a RALS/LALS hybrid detector showing the flow passing through the flow cell. Light from the laser enters at the end of the cell and is collected at 90° and through the same exit window at a low angle e.g. 7°. B. When using RALS/LALS, the Debye plot is reduced to two points where the RALS value is used to maximise sensitivity for isotropic scatterers and the LALS value is used for the most accurate molecular weight for anisotropic scatterers.

The RALS/LALS combination offers the advantages of both:

- The RALS detector offers the best sensitivity for smaller molecules.
- The LALS detector offers the best sensitivity for larger molecules.
- The two angles can be compared simultaneously in software to get the best data at every point on the chromatogram.
- A RALS/LALS hybrid can maintain the small flow cell associated with both detectors.
- By combining the data from both angles, it is possible to measure the slope of the line on the Zimm plot and calculate a good estimate of R_g.

Limitations of the RALS/LALS hybrid include:

- LALS data can be noisy if the GPC/SEC system is not clean
- The R_g calculation has a limited accuracy.

Overall, a RALS/LALS hybrid offers the advantages of RALS and LALS and suffers none of the drawbacks of either. This makes a RALS/LALS hybrid system excellent for measuring the molecular weight of any sample.

Multi-angle light scattering (MALS)

Key points

- Measures the intensity of light scattered at multiple angles and extrapolates back to 0°.
- Measurements at multiple angles can increase confidence in the data.
- Angles can be removed if required.
- Measures the molecular weight of molecules of all sizes.
- Measures the R_g of molecules > ≈ 15 nm.
- It is difficult to know how to select the fit model.
- The design of the optics means MALS systems have larger flow cells.
- The complexity of the optical design can introduce noise and reduce accuracy
- The optical complexity increases the cost

Details

As the name suggests, MALS detectors measure the intensity of the scattered light at many angles as can be seen in figure 7A. By plotting these points on a Zimm plot (figure 7B), a best fit line can be extrapolated back to 0° from where the molecular weight can be calculated. The initial slope of this line enables an accurate calculation of molecular size, R_g.

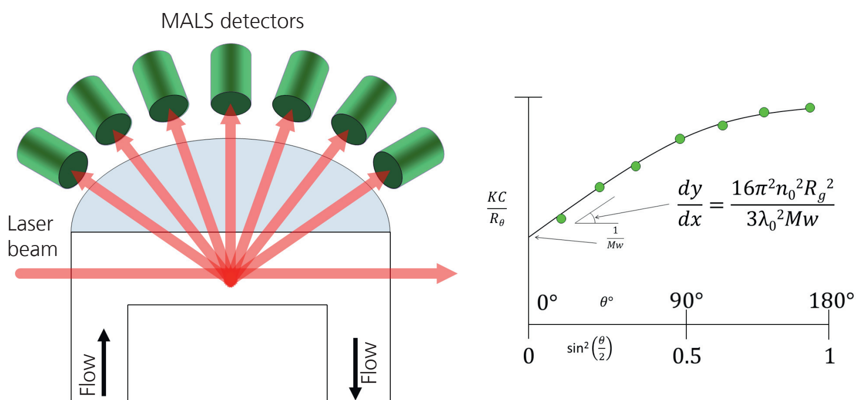


Figure 7: A. Schematic of a MALS detector showing the flow passing through the flow cell. Light from the laser enters at the end of the cell and scattered light exits at different angles. The scattered light is collected by multiple detectors. B. When using MALS, the Debye plot is completed and extrapolated back to 0°. The molecular weight is calculated from the intercept and R_g is calculated from the initial slop of the line.

MALS detectors have a number of their own advantages:

- By measuring the intensity of the scattered light at many angles, the user can be more confident in the results from any given angle by comparing it to its neighbouring angles.
- MALS measurements can measure the molecular weight of all molecules from small to large since any angular dependence in the light scattering is always accounted for.

- By measuring at many angles, it is possible to make an accurate measurement of R_g.
- By studying the shape of the line in the Zimm plot, MALS offers insights into the angular dependence of the scattered light.
- If any angle fails or includes too much noise, it can be removed without seriously affecting the result

However, MALS systems also have some limitations:

- Since the exact shape and structure of the molecule are unknown, it is difficult to know which extrapolation fit and model will give the correct answer.
- In order to incorporate more angles, MALS flow cells must have a larger volume resulting in increased peak broadening
- The complexity of the cell means lower angles are often noisier than a RALS or LALS measurement which can reduce the measurement accuracy.

When making measurements using MALS, the most important factor is that the form of the extrapolation is unknown and accurate fitting is particularly dependent on the number of low angles and the accuracy of the points. It is therefore important that a MALS instrument should have as many low angles as possible as this will provide the most accurate extrapolation back to 0°. In general, in order to maximise the accuracy of the extrapolation, having more angles is beneficial.

In summary, MALS offers a universal solution for measuring molecular weight and R_g of all different types of sample but the nature of the design forces some compromises on the measurement.

Summary of the benefits of MALS, LALS, and RALS systems

- LALS is the best measurement technique for accurate molecular weight measurements of large ($R_g > \approx$10-15 nm radius) molecules because it minimises the effect of the angular dependence of the scattered light and eliminates the need for extrapolation.
- Small molecules scatter light isotropically, meaning that there is no angular dependence. In this situation, a single measurement at 90° (RALS) offers the most sensitive and accurate measurement for these weakly scattering molecules. This includes almost all protein applications.
- MALS measures molecular weight over the whole size range.
- MALS offers insights into the angular dependence of the scattered light for large molecules (>10-15 nm radius) enabling the highest quality and most accurate measurement of R_g when compared with a RALS/LALS hybrid. R_g can be used to characterise structure for these large molecules.

CALIBRATION

Are static light scattering measurements 'absolute'?

Light scattering techniques are often called 'absolute' but this is often misunderstood.

- The addition of a light scattering detector to an SEC system frees us from all forms of column calibration and allows us to measure the molecular weight of the sample directly without generating a calibration curve. In this respect we often call the technique absolute.
- From a more fundamental perspective, the theory described above comes from first principles with no assumptions and so, again, in this respect it is often described as an 'absolute' technique. However, even though the method derives from first principles, at some point in the process, we still have to relate the magnitude of the signal from a photo-detector (i.e. the intensity of the scattered light) to molecular weight of the molecule doing the scattering. This will always require some form of instrument calibration or the generation of response factors etc. so the technique cannot really be said to be truly 'absolute'.

Principle of instrument calibration

Although the column doesn't need calibrating, all light scattering devices require calibration in some form. The only question is how the device should be calibrated and this can be done in a number of ways:

The principle of light scattering calibration, as with all calibrations, is to reference the size of the response signal to the parameter being measured. In static light scattering, we relate the magnitude of the signal from the photo-detector to the intensity of the scattered light and then to the molecular weight of the sample. As well as all the sample dependent factors described in the Rayleigh equation, the measured intensity of the scattered light will depend on a large number of instrument dependent variables including:

- Incident laser power,
- Detector sensitivity,
- Scattering volume (the intersection volume of the laser beam and the detection optics),
- The distance from the scattering volume to the detector,
- Flare and refraction effects at the interface between the cell and the windows.

It is not possible to characterise all of these components in a system to the accuracy required of the measurement; so instead they are all accounted for by a calibration step.

There are two methods by which a system can be calibrated but they are functionally identical.

Molecular weight standard-based calibration

Key points

- Calibration with a molecular weight standard calibrates all light scattering detectors at the same time.
- Calibration in this way is done under the conditions of the measurement.
- This method also allows other detectors calibration constants, inter-detector volumes, and peak broadening corrections to be calculated simultaneously.
- This method allows the calibration to be checked and repeated at any time.

Details

The principle behind a molecular weight standard-based calibration is to run a sample of known molecular weight through the SLS detector while it is attached to a GPC/SEC system. The magnitude of the response of the light scattering detector and the known sample concentration, molecular weight and dn/dc are used to calculate an instrument response factor or calibration constant for the light scattering detector. Discounting other values such as the solvent refractive index and laser wavelength, the light scattering detector calibration constant is defined as:

$$k_{ls} = \frac{\delta LS_{std}}{Mw_{std} \cdot \left(\frac{dn}{dc}\right)_{std}^{2} \cdot mass_{std}}$$

Where:

- k_{ls} = the light scattering calibration constant,
- δLS_{std} = measured intensity of light scattered by the standard,
- Mw_{std} = the molecular weight of the standard,
- dn/dc = is the standard refractive index increment,
- $mass_{std}$ = the injected mass of the standard.

With a standard, the concentration and injection volume (and therefore mass) are known, as is the standard molecular weight and dn/dc.

This allows the calibration constant to be calculated and can be performed on every angle in a light scattering detector simultaneously.

Once the calibration constant has been determined, the molecular weight for any sample can be measured:

$$Mw = \frac{\delta LS}{\left(\frac{dn}{dc}\right)^2 . mass. k_{ls}}$$

Where:

- Mw = the molecular weight of the sample,
- δLS = measured intensity of light scattered by the sample,
- dn/dc = is the sample refractive index increment,
- mass = the injected mass of the sample,
- k_{ls} = the light scattering calibration constant.

In a GPC/SEC system containing multiple detectors, all of them can be calibrated at the same time using a standard that is well characterised and traceable. A further benefit of calibrating the detectors in this way is that the inter-detector volumes between the different detector flow cells and the band broadening and tailing that occur as the sample travels between detectors can all be accounted for in the same step.

The final benefit of this method is that the system calibration can be checked and/or re-calibrated at any time by the user. This is particularly useful as instrument constants will drift over time due to a number of factors such as degradation of light sources, cleanliness of flow cells, changing detector tubing etc.

A molecular weight standard-based calibration therefore allows all of the instrument response factors/calibration constants to be derived at the same time as the inter-detector volumes and peak broadening parameters are all calculated. It offers the most effective way of calibrating a system and, by its very nature, it also offers a clear way to check the calibration of a system by comparing the responses of the standard to that at the time of calibration.

Scattering standard-based calibration

Key points

- Calibration with a scattering standard compares the scattering from the sample to that of a standard with a known Rayleigh ratio.
- This calibration only calibrates the 90° detector. Other detectors must be 'normalised' using a second step with another standard.
- Inter-detector volumes, and peak broadening and tailing calculations must all be performed separately

Details

The principle behind a scattering standard-based calibration is to fill the light scattering flow cell with a solvent of known scattering such as toluene. Toluene has a well characterised Rayleigh ratio (the ratio of scattered light intensity to incident light intensity) so by measuring the scattering signal observed from toluene it is possible to calculate the scattering signal from the sample. R_θ for the sample is calculated according to the equation:

$$R_\theta = \frac{I_A {n_0}^2}{I_T {n_T}^2} R_T$$

Where:

- R_θ = the sample Rayleigh ratio,
- I_A = the scattered light intensity from sample,
- n_0 = the refractive index of the sample solvent,
- I_T = the intensity of light scattered by toluene,
- n_T = the refractive index of toluene,
- R_T = the Rayleigh ratio of toluene.

The sample molecular weight is then calculated by inputting this value of R_θ into the Rayleigh equation. This means that the calibration constant in this situation is essentially:

$$k_{ls} = \frac{1}{I_T {n_T}^2} R_T$$

Although you do not need a molecular weight value to calibrate a detector, the principle of calibrating with a scattering standard is identical in that you are using a standard to measure the response of the light scattering detector to a known sample.

This method has a number of other significant drawbacks:

- Since the scattering standard is usually a different solvent to that used to make measurements, and therefore has a different refractive index, it can only be used to calibrate the 90° detector. All of the other detector responses in a system containing more than one angle must subsequently be normalised against the 90° detector. This requires running a standard through the system and acquiring normalisation factors for the non-90° detectors. A normalisation factor is simply a multiplier of the 90° calibration constant, so is functionally just another calibration constant.
- Secondly, a scattering standard-based calibration does not allow you to correct for inter-detector volumes or peak broadening or tailing so this must also be done separately, either with the normalisation or as another step.
- Since a scattering standard-based calibration requires taking the light scattering detector completely off-line, it is a much more labour intensive process, often requiring the unit to be returned to the manufacturer for calibration. This also means that it is very difficult to check that the calibration is still valid.

Overall then, a scattering standard-based calibration is a more labour intensive process that does not improve the calibration and only serves to add more hurdles to achieving an accurate calibration of a system.

USEFUL REFERENCES

Dynamic Light Scattering: An Introduction in 30 Minutes

http://www.malvern.com/malvern/kbase.nsf/allbyno/KB000792/$file/MRK656-01_An_Introduction_to_DLS.pdf

A basic guide to particle characterization

http://www.malvern.com/malvern/kbase.nsf/allbyno/KB003174/$file/MRK1806-01.pdf

Protein and polymer molecular weight by GPC/SEC

http://www.malvern.com/malvern/kbase.nsf/allbyno/KB002319/$file/MRK1348-01.pdf

Kratochvil, P. (1987) Classical Light Scattering From Polymer Solutions. Elsevier

Striegel, A.M. et al. (2009) Modern Size Exclusion Chromatography. John Wiley & Sons

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