

## Aggregation analysis of biopharmaceutical products – use of light scattering technologies for gel permeation chromatography – size exclusion chromatography

### Abstract

The aim of drug development is to design a formulation and a manufacturing process that ensures the product is able to withstand stresses, while also meeting the shelf life stipulated in the Quality Target Product Profile.

Understanding degradation pathways, detecting and characterizing aggregate formation and detecting changes in structure and conformation are of great value during (bio)pharmaceutical development, and are central to the QbD (Quality by Design) and Design Space approaches currently being promoted by regulatory agencies.

The range of (bio)pharmaceutical products has expanded greatly and now includes such products as hormones, enzymes, peptides, and antibodies.

While proteins and peptides are a highly valuable source of potential therapeutic agents, their dynamic and complex nature means the development, manufacture and commercialisation of biopharmaceutical products remains a challenging process.

Increasing attention is paid to aggregation of biopharmaceuticals by regulatory authorities and relevant industry guidelines (International Conference on Harmonisation (ICH) Q6B) recommend monitoring of changes of the product as a result of manufacture and/or storage, including aggregate characterization.

Static Light Scattering (SLS) is a new tool that can be applied to synthetic polymers, proteins, peptides, finished pharmaceuticals, and particles such as liposomes, micelles and encapsulated proteins. With measurements being made in a continuous flow mode from High Performance Liquid Chromatography (HPLC) separation and the ability to be connected with traditional detectors such as Ultra Violet (UV) or Refractive Index (RI) in series, SLS technology helps development and formulation scientists to focus on the primary question of 'What happens to my product?'

### Regulatory expectations

Guideline ICH Q5C 'Stability Testing of Biotechnological/Biological Products' states that 'The use of relevant physicochemical, biochemical and immunochemical analytical methodologies should permit a comprehensive characterisation of the drug substance and/or drug product (e.g., molecular size, charge, hydrophobicity) and the accurate detection of degradation changes that may result from deamidation, oxidation, sulfoxidation, aggregation or fragmentation during storage. As examples, methods that may contribute to this include electrophoresis (SDS-PAGE, immunoelectrophoresis, Western blot, isoelectrofocusing), high-resolution chromatography (e.g., reversed-phase chromatography, gel filtration, ion exchange, affinity chromatography), and peptide mapping'.<sup>1</sup>

<sup>1</sup> ICH harmonised tripartite guideline Q5C, "Stability Testing of Biotechnological/Biological Products", dated 30 November 1995.

Increasing attention is paid to aggregation of biopharmaceuticals by regulatory authorities and relevant industry guidelines (ICH Q6B) recommend monitoring of changes of the product as a result of manufacture and/or storage, including aggregate characterization.<sup>2</sup>

The European Medicines Agency (EMA) guideline on development, production, characterization and specifications for monoclonal antibodies and related products states: "Multimers and aggregates

*should also be appropriately characterized using a combination of methods. The formation of aggregates, subvisible and visible particulates in the drug product is important and should be investigated and closely monitored on batch release and during stability studies. In addition to the pharmacopoeial test for particulate matter, other orthogonal analytical methods may be necessary to determine levels and the nature of particulates."*

In addition to that instruction; Guideline ICH Q6B 'Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products' states for product-related impurities including degradation products, the following represents the most frequently encountered molecular variants of the desired product and lists relevant technology for their assessment. Such variants may need considerable effort in isolation and characterisation in order to identify the type of modification(s). Degradation products arising during manufacture and/or storage in significant amounts should be tested for and monitored against appropriately established acceptance criteria including but not necessarily limited to;

- a) **Truncated forms:** Hydrolytic enzymes or chemicals may catalyse the cleavage of peptide bonds. These may be detected by HPLC or SDS-PAGE. Peptide mapping may be useful, depending on the property of the variant.
- b) **Other modified forms:** Deamidated, isomerised, mismatched S-S linked, oxidised or altered conjugated forms (e.g., glycosylation, phosphorylation) may be detected and characterised by chromatographic, electrophoretic and/or other relevant analytical methods (e.g., HPLC, capillary electrophoresis, mass spectroscopy, circular dichroism).
- c) **Aggregates:** The category of aggregates includes dimers and higher multiples of the desired product. These are generally resolved from the desired product and product related substances, and quantitated by appropriate analytical procedures (e.g., size exclusion chromatography (SEC), capillary electrophoresis).

**Tepnel Pharma Services is now able to offer analytical services that can support you in characterising aggregate formation. The introduction of SLS detection to our service portfolio will complement our many years of chromatographic experience in the field of peptides and proteins.**

### SLS detector technology

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.

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.

<sup>2</sup> ICH HARMONISED TRIPARTITE GUIDELINE Q6B, "SPECIFICATIONS: TEST PROCEDURES AND ACCEPTANCE CRITERIA FOR BIOTECHNOLOGICAL/BIOLOGICAL PRODUCTS, dated 10 March 1999.

<sup>3</sup> EMA/CHMP/BWP/532517/2008, Guideline on development, production, characterisation and specification for monoclonal antibodies and related products, 21 July 2016.

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, (Static Light Scattering) MALS (Multi Angle Light Scattering), LALS (Low-Angle Light Scattering) and RALS (Right Angle Light Scattering) and others.

- 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.

Each of these detection techniques are subtly different and each has advantages and disadvantages. The advantages and disadvantages of each are highlighted as follows:

#### Right Angle Light Scattering (RALS) system:

Advantages	Disadvantages
<ul style="list-style-type: none"> <li>• 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.</li> <li>• 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.</li> <li>• 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.</li> </ul>	<ul style="list-style-type: none"> <li>• 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 <math>R_g &gt; \approx 15\text{nm}</math>, as discussed in the earlier section.</li> <li>• 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 <math>R_g</math>.</li> </ul>

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) system

Advantages	Disadvantages
<ul style="list-style-type: none"> <li>• 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.</li> <li>• The LALS principle applies to the measurement of the molecular weight of any molecule.</li> <li>• A dedicated LALS detector can have a small flow cell since it only has to accommodate one measurement angle.</li> </ul>	<ul style="list-style-type: none"> <li>• Since the scattered light is only being measured at a single angle, a LALS system cannot measure <math>R_g</math>.</li> <li>• 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.</li> <li>• 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.</li> </ul>

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 detector system:

Advantages	Disadvantages
<ul style="list-style-type: none"> <li>• The RALS detector offers the best sensitivity for smaller molecules.</li> <li>• The LALS detector offers the best sensitivity for larger molecules.</li> <li>• The two angles can be compared simultaneously in software to get the best data at every point on the chromatogram.</li> <li>• A RALS/LALS hybrid can maintain the small flow cell associated with both detectors.</li> <li>• 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 <math>R_g</math>.</li> </ul>	<ul style="list-style-type: none"> <li>• LALS data can be noisy if the GPC/SEC system is not clean</li> <li>• The <math>R_g</math> calculation has a limited accuracy.</li> </ul>

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) system:

Advantages	Disadvantages
<ul style="list-style-type: none"><li>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.</li><li>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.</li><li>By measuring at many angles, it is possible to make an accurate measurement of <math>R_g</math>.</li><li>By studying the shape of the line in the Zimm plot, MALS offers insights into the angular dependence of the scattered light.</li><li>If any angle fails or includes too much noise, it can be removed without seriously affecting the result.</li></ul>	<ul style="list-style-type: none"><li>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.</li><li>In order to incorporate more angles, MALS flow cells must have a larger volume resulting in increased peak broadening.</li><li>The complexity of the cell means lower angles are often noisier than a RALS or LALS measurement which can reduce the measurement accuracy.</li></ul>

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  $0^\circ$ . 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.

### Practical Applications

SEC is the usual technique employed for separating different sizes of molecular masses. SEC is based on the principle that a sample is separated according to hydrodynamic volume of the individual molecules, essentially 'the big ones come out first and the smaller ones elute later'. By choosing a suitable SEC column packing the resolution of the system can be defined.

Having produced a mode of separation, the next step is to convert the time at which samples eluted into a measurement of molecular mass. If the molar mass of a standard were known, then the time at which this standard eluted should be equal to a specific molar mass. Using multiple standards, a calibration curve of time versus molar mass could be constructed. The drawback of this is that unknown samples are measured in relation to known standards and these standards may or may not have any relation to the sample of interest. If the relationship between molar mass and hydrodynamic volume of the standard to sample is not the same then the calibration will be nulled. The vast majority of this work is performed based on the habit of using calibration standards and the hope that they mimic the actual molecules of interest.

Next is the decision on the most appropriate detection technique to utilise. 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.

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.

SLS can be applied to synthetic polymers, proteins, peptides, pharmaceuticals, and particles such as liposomes, micelles and encapsulated proteins. Measurement is made in a continuous flow mode from HPLC separation and can be connected in series with traditional detectors such as UV or RI.

### Conclusion

At Tepnel Pharma Services, we understand that a successful (formulation) development of (bio)pharmaceutical products requires sensitive and specific analytical techniques for the detection of chemical and physical instabilities. Tepnel Pharma Services offers contract laboratory services with respect to analytical characterization, method development, validation and routine testing of proteins, vaccines, oligonucleotides, and other biopharmaceuticals in a cGMP environment.

We can focus on the primary question 'What happens to my product?'

SLS detection added to our service portfolio will complement our many years of chromatographic experience in the field of peptides and proteins to help you answer that question.

### Useful References

- 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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