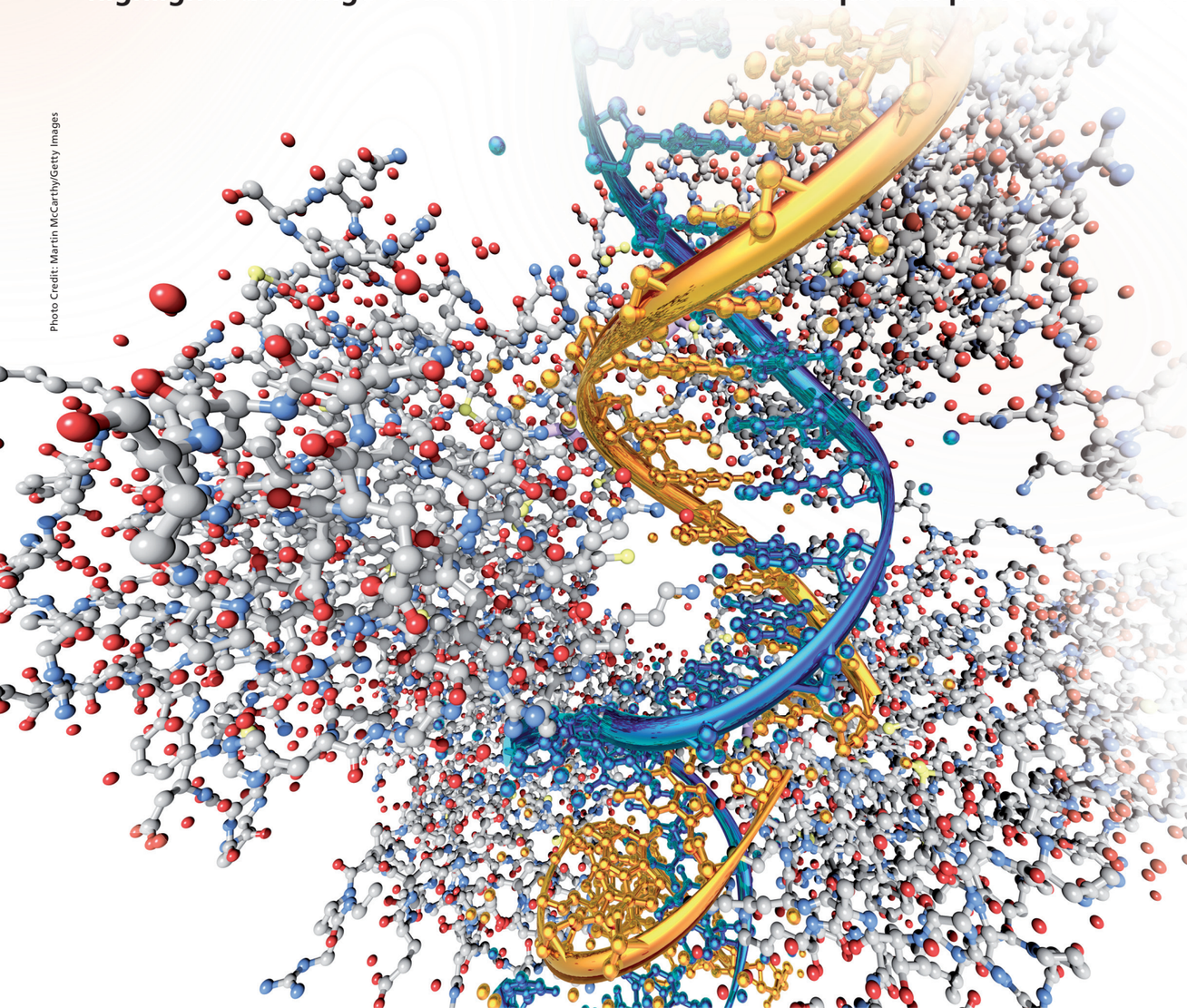
“Critical to Quality” Measurements of Protein Aggregation in Biopharmaceuticals

Mark Pothecary and Lisa Newey-Keane, Malvern Instruments, Malvern, Worcestershire, UK.

In biopharmaceutical development the stability of biological molecules in drug formulations is important. One of the key parameters in assessing formulation stability is the detection and measurement of protein aggregates; the immunogenicity of which can significantly affect product safety. Aggregates above a few microns in size are characterized using visual techniques, while those less than 100 nm are typically studied using size exclusion chromatography (SEC). However, there remains a range of intermediately sized aggregates that are not so well characterized. Comprehensive characterization of aggregates in this region requires the adoption of multiple technologies: SEC coupled with light scattering (LS) detectors can play an important role. This article describes three different approaches to the measurement and quantification of aggregates in protein solutions, and highlights the range of information that each technique can provide about the sample.



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The pharmaceutical industry has recently undergone a significant shift from the development of small molecule drugs to the development of biopharmaceuticals; drugs where the active ingredient is derived from biological molecules, such as proteins. Biopharmaceuticals are expected to represent approximately 50% of all licensed drugs by 2020. Drivers for this change include diminishing returns on small molecule development, better targeting of biotherapeutic drugs and the difficulty in “genericizing” biological drugs.

Protein aggregation is a result of numerous sample treatments, prolonged storage or freeze-thaw action. The structure of a protein is determined by Van der Waals forces, hydrogen bonds, disulphide linkages and hydrophobic interactions. These intramolecular forces can be disrupted by changing conditions to reveal buried regions of the protein. These regions can interact with other proteins to form larger complexes of misfolded proteins, aggregates. Hydrophobic regions within proteins are especially susceptible to this effect. Aggregates are often strongly held together by these forces meaning that their formation is irreversible, continuing to grow up to and beyond the point where they become insoluble.

As aggregates form, the activity of the component proteins is likely to be lost, thereby reducing the efficacy of the product. Aggregates have been shown in numerous studies to be immunogenic. Reduced efficacy and increased immunogenicity of a drug formulation are highly undesirable, so understanding and monitoring protein aggregation in drug formulations is crucial to product quality. This article describes three different approaches to the measurement and quantification of aggregates in protein

Figure 1: Chromatogram of pepsin run on a SEC-Triple Detection (SEC-TD) system, showing refractive index (RI) signal in red, light scattering (LS) signal in green and measured molecular weight in black.

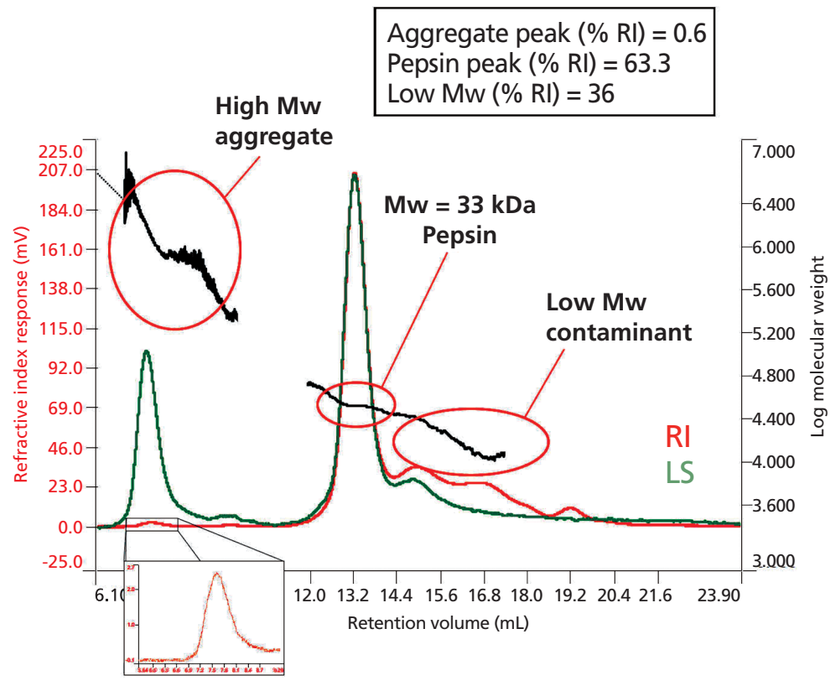
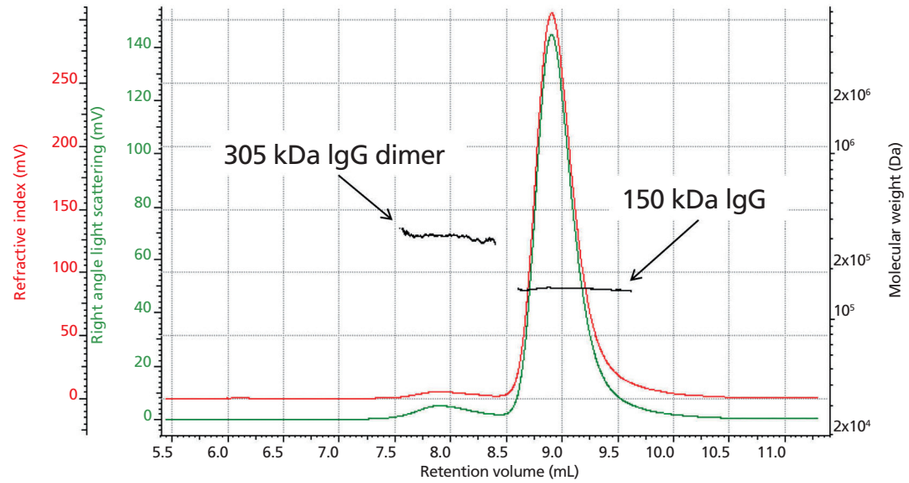


Figure 2: Chromatogram of IgG run on a SEC-Triple Detection (SEC-TD) system, showing RI signal in red, light scattering signal in green and measured molecular weight in black.



solutions with a focus on size exclusion chromatography (SEC), but also discussing dynamic light scattering (DLS) and resonant mass measurement (RMM).

Size Exclusion Chromatography With Triple Detection (SEC-TD)

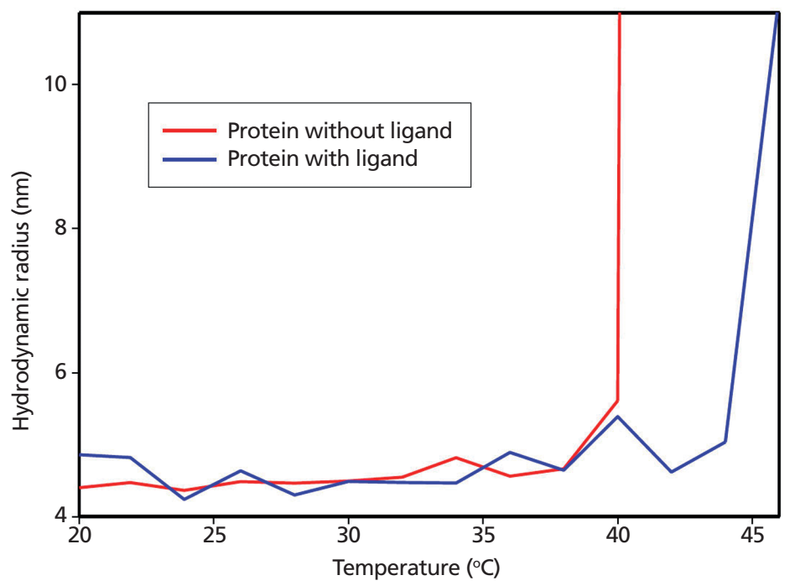
SEC, also known as gel permeation chromatography (GPC), is routinely used in bioscience laboratories to characterize purified and recombinant proteins. SEC is a chromatographic technique used to measure molecular weight or resolve oligomer mixtures. It separates molecules in solution on the basis of size by pumping them through specialized columns containing a microporous packing material.

Modern analytical systems use multiple detectors such as refractive index (RI), ultraviolet (UV), light scattering (LS) and viscometry (IV) to give extensive characterization of protein samples, non-reliant on column calibration to determine the protein's molecular weight. RI and UV allow accurate concentration measurement; light scattering is utilized for molecular weight determination; and viscometry measures the intrinsic viscosity of a sample, an indicator of structural changes

SEC combined with a single detector, such as a UV detector, remains the gold standard by which all protein samples are tested for aggregation and those aggregates are quantified. However, with the addition of extra detectors, it is possible to more thoroughly characterize those samples than just assessing their component quantities. The addition of light scattering allows the molecular weight of proteins and their aggregates to be measured directly, independent of their elution volume, thereby allowing differentiation between oligomers of the original protein and denatured aggregates. These will have different activities and different levels of immunogenicity so it is crucial to understand what the individual peaks represent. For example, Figure 1 shows a chromatogram from some commercially available pepsin. The largest peak has a stable (monodisperse) molecular weight of 33 kDa indicating that this is the monomeric pepsin. Eluting earlier is a small peak with a high and very variable (polydisperse) molecular weight. This clearly indicates that this material is comprised of denatured aggregates as opposed to a stable oligomer. Eluting after the monomer peak is some material with a variable and low molecular weight. This peak represents a degradation product and is noteworthy because it indicates instability. Pepsin is a digestive protein, and so may have undergone a process of partial self-digestion to produce these low-molecular-weight species.

Figure 2 shows the GPC/SEC chromatograph of a sample of immunoglobulin G (IgG). This trace contains

Figure 3: Trend graph showing hydrodynamic size from dynamic light scattering (DLS) as a function of incubation temperature. The line in red is the protein alone and the line in blue shows the protein incubated with its ligand.



two peaks but the IgG chromatogram is different to that of the pepsin in Figure 1. In this case, the molecular weight is stable across both peaks. The larger peak has a molecular weight of 150 kDa, clearly identifying it as IgG, while the second peak has a molecular weight of 306 kDa. This appears to be a stable dimeric form opposed to a larger denatured aggregate.

SEC is therefore a useful quality control tool for quantifying the presence of aggregates in a sample. SEC with light scattering can, however, provide more information, in the form of molecular weight, to a single SEC run. This information can distinguish between monomers, oligomers and more polydisperse aggregates to help more strongly identify individual populations, thereby improving its functionality within the biopharmaceutical workflow.

A further refinement to couple SEC measurements is to add the capability of dynamic light scattering (DLS), which will be discussed in the next section.

Dynamic Light Scattering

DLS uses the intensity fluctuations of scattered light to measure the size and size distribution of proteins in solution. These intensity fluctuations are induced by the movement of proteins as a result of collisions with solvent molecules, known as Brownian motion. Specifically, light scattered by molecules undergoing Brownian motion interferes either constructively or destructively, depending on the relative positions of the protein molecules in the scattering volume. The rate of the detected intensity fluctuations is used to calculate a diffusion coefficient, which in turn is converted into a hydrodynamic size using the Stokes Einstein equation.

DLS is more commonly used in the earlier stages of formulation development because it is ideal as a screening tool to study a range of different buffers. DLS is a rapid measurement, as well as being non-invasive (meaning that the sample can be measured *in situ*), which means that it can be used to monitor a protein's behaviour with respect to treatment (for example, incubation time or temperature). This can be performed across a range of formulation conditions. Furthermore, DLS is extremely sensitive to the presence of the earliest aggregates within a sample. This makes it a very powerful tool for qualitatively identifying aggregation at the earliest stages and to rapidly identify conditions where a protein is more likely to aggregate.

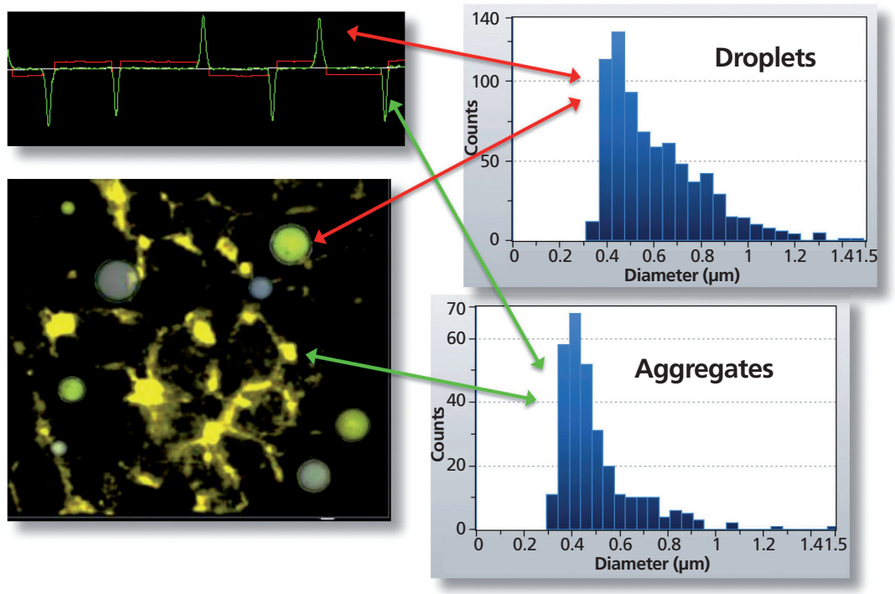
As an example, Figure 3 shows a DLS measurement where the size has been measured as a function of the incubation temperature in the presence and absence of a particular ligand. It can be seen that in the presence of the ligand, the temperature can be raised 5 °C higher (from 40 °C to 45 °C) before the protein starts to denature and aggregate (as indicated by the increase in size). By detecting samples with the highest aggregation temperature, DLS can identify the most nominally stable sample and thus the conditions least likely to induce protein aggregation.

Resonant Mass Measurement

Resonant mass measurement (RMM) is a relatively new technique used to detect and count subvisible and submicron particles in a sample, and to measure their size and mass distributions. Since RMM is a counting technique, it can produce number-based distributions of these particles. A micro electro-mechanical systems (MEMS) sensor, which contains a resonating cantilever with a microfluidic channel embedded in its surface, is used to make the measurement. When a particle of between 50 nm to 5 µm flows through the fluidic channel, it alters the resonating frequency of the cantilever, indicating the particle's buoyant mass. The dry mass and size of the particle is extrapolated from this value.

The sensor is also able to provide information on sample concentration, viscosity, density and volume; it is able to detect and measure both negatively and positively buoyant particles within a sample. The detection of positively buoyant particles is extremely useful, for example, to distinguish between protein aggregates and contaminating silicone oil droplets in a biopharmaceutical formulation. In the late stages of development and production, this is incredibly useful. Currently there is a

Figure 4: Two size distributions from the same resonant mass measurement analysis on a single protein sample. One shows the positively buoyant silicone oil droplets and the other, the negatively buoyant protein aggregate particles.



gap between the visible techniques and the solution-based techniques (for example, SEC) where it is difficult to measure the level of aggregation. This is of growing concern to regulators because they want to know the level of aggregation in a sample and the identity of any particles found. Therefore, in quality control, RMM can be used to measure the level of aggregates in this size range and also to distinguish between protein aggregates and silicone oil, a common contaminant originating from syringes used to deliver drugs.

Figure 4 shows two size distributions from a single protein sample. One shows positively buoyant particles and the other, negatively buoyant particles.

Conclusion

The shift of the pharmaceutical industry from its dependence on small molecules to large molecules, including proteins, has raised many new challenges in drug development. Among them, developing stable formulations has shown itself to be crucial to successful delivery, efficacy and lack of immunogenicity. This challenge requires a number of technologies to completely understand and monitor the behaviour of biopharmaceuticals.

This article has discussed a number of techniques and individually these techniques are useful, each providing valuable insight into proteins' behaviour in formulation, but together, they deliver a powerful array of techniques invaluable to the development and manufacturing of biopharmaceuticals.

Mark Pothecary studied biochemistry at Bath University, UK, before moving to London to do a Masters and PhD also in biochemistry. His PhD project investigated the cardiovascular benefits of wine polyphenols. Mark joined Malvern in 2008 as a product technical specialist for the Zetasizer Nano and Viscotek products concentrating in bioscience SEC applications. In 2010, he became Product Manager where he is responsible for new products and quality issues. Mark currently works in the Houston offices.

Lisa Newey-Keane is Biopharmaceutical Product Manager at Malvern Instruments. She holds a PhD in microbiology, pathology and protein biochemistry from the University of Birmingham, UK. After graduating, Lisa moved to the product characterization team at Lonza Biologics in Slough and specialized in the use of HPLC and MS for the analysis of therapeutic antibodies, and transferred these skills to her later work for Shimadzu. Lisa has also worked within bioanalysis both at Novozymes in Nottingham and most recently at Quotient Bioresearch in Cambridgeshire, where she specialized in antimicrobial drug development.

E-mail: **salesinfo@malvern.com**
Website: **www.malvern.com**

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