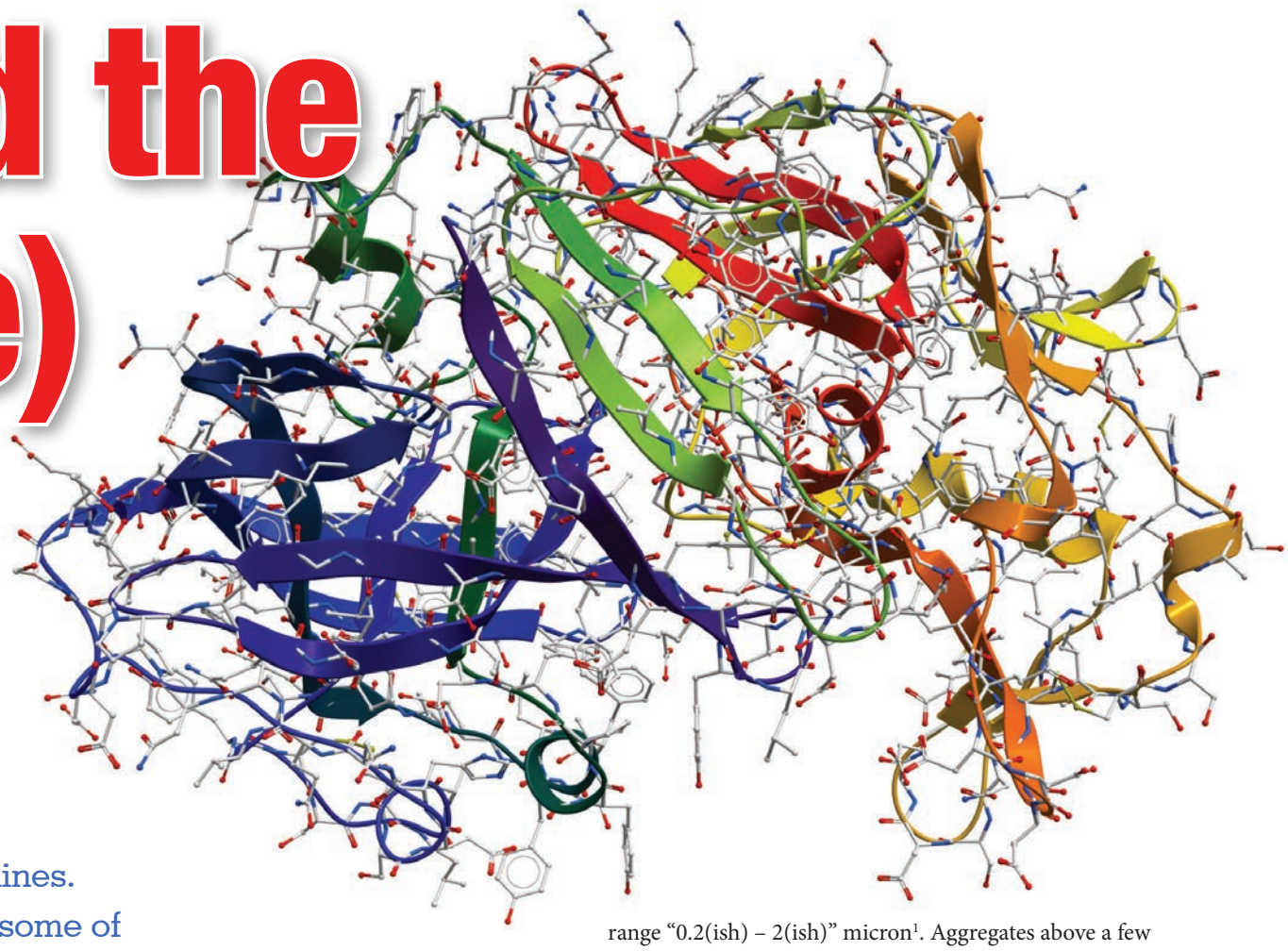
Mind the (size) gap



Large biological molecules continue to be strong candidates in drug development pipelines. Stephen Ball describes some of the techniques being used to measure protein aggregation in these biopharmaceuticals

As biological molecules continue to occupy an increasing proportion of many pharmaceutical companies' drug development pipelines, much attention is being given to addressing the associated development, manufacturing and regulatory challenges. The definition of purity and potency for biopharmaceuticals is substantially more complex than for small molecule drugs, with the potential for immunogenicity being a key concern to manufacturers and regulators alike. This in turn is highlighting the need for analytical tools that help develop a complete picture of particles and aggregates in biopharmaceutical materials and there is a growing emphasis on physicochemical characterisation for both intrinsic particles and contaminants.

Moving a molecule from discovery into pre-formulation is a critical step. The physicochemical properties of the molecule are significant determining factors for downstream success in formulation and delivery, and the earlier these can be profiled the greater the economic impact, whether this is in identifying potentials for success or in discarding likely failures. At this point the ideal is to be able to conduct a battery of tests, non-destructively, on very small volumes of sample, and much effort is going into focusing and refining the testing processes available.

Here and further down the development path, right through to end product testing, detecting and measuring protein aggregation is one of the most important functions, since understanding this aspect of behaviour is critical to product formulation, stability and safety. A protein's structure is maintained through a combination of Van der Waals forces, hydrogen bonds, disulphide linkages and hydrophobic interactions, some or all of which can be disrupted with changes to environmental conditions. Consequences may include misfolding or the formation of aggregates that have the capacity to grow to the point of insolubility. Along the way protein activity is often lost and many aggregates have the potential to become immunogenic, with clear implications for both the efficacy and the safety of the final therapeutic product.

Current regulatory expectations are that there should be characterisation of aggregates and subvisible particles in the range "0.2(ish) – 2(ish)" micron[1]. Aggregates above a few microns in size can be characterised using visual methods. Below this, dynamic light scattering (DLS) and size exclusion chromatography (SEC) are both established analytical techniques used in characterising protein aggregates. Figure 1 illustrates the technologies and measurement ranges they cover. The relatively new technique of resonant mass measurement (RMM) is now also being used to detect and count subvisible particles in the critically important size range 50 nm to 5 µm, and to reliably measure their buoyant mass, dry mass and size. Resonant Mass Measurement provides a window into sub-visible and sub-micron aggregates.

DLS is rapid and non-invasive, and lends itself especially to measuring proteins in solution for screening applications in the early stages of formulation development. The technique measures the intensity fluctuations of scattered light resulting from proteins undergoing Brownian motion, and converts this to size and a size distribution. One of its great benefits is the ability to detect aggregation at the earliest stages, making it a potent technique in determining the conditions most likely to encourage or deter aggregation.

Selecting optimal DLS technology for protein applications relies, in part, on choosing a system with a suitable detector set-up. When the size of the protein in solution is very small, compared with the wavelength of light, the intensity of the scattered light has no angular dependence, which means that measurement at any angle will give the same results. However as size increases, to more than ≈λ/10, scattered intensity in the forward direction begins to increase making it important to gather data at suitable angles. Instruments that incorporate back scattering technology, for example, measure in both the backward and forward direction (see data below) to address this issue and are, as a result, able to efficiently monitor progressive protein aggregation.

Size exclusion chromatography (SEC), as its name suggests, separates dissolved molecules on the basis of hydrodynamic size, and is routinely used in bioscience laboratories to characterize purified and recombinant proteins. When using SEC as an analytical technique, productivity and information flow are governed by the detection technologies applied to the eluting, separated fractions.

Single detector SEC, using an ultraviolet (UV) detector to monitor concentration, remains the traditional choice for testing all proteins for aggregation, but more modern multi-detector systems are increasingly finding favour in this application space because of the complementary information and understanding they provide. For example, systems that combine refractive index (RI), UV, light

THE AUTHOR

Stephen Ball is Product Marketing Manager for Malvern Instruments.
+44 (0) 1684 892456
salesinfo@malvern.com

REFERENCES

1. Presentation given by Susan Kirshner from the FDA Office of Biotechnology. Download at: http://www.aaps.org/uploadedFiles/Content/Sections_and_Groups/Focus_Groups/Protein_Aggregation_and_Biological_Consequences/PABCFG_Kirshner2012.pdf

scattering and viscosity detection comprehensively characterise protein samples, providing molecular weight data as well as details of structural changes and features, while simultaneously avoiding the need for column calibration.

Figure 3 shows the GPC/SEC chromatograph of a sample of BSA, showing the response obtained on a system consisting of RI and light scattering detectors. The RI signal, in red, shows a large response at around 25ml, which the molecular weight, calculated from light scattering (in green), allows us to identify as the monomer. The smaller peaks, eluting slightly earlier are identified from their molecular weight as the dimer and trimer. The large light scattering response in the earliest peak indicates that there is a large amount of aggregated material present which can be seen to have a large and polydisperse molecular weight. Categorising these peaks so definitively would not have been possible in a single detector system.

A relative newcomer to the analytical portfolio, resonant mass measurement (RMM) detects and counts sub visible and submicron particles in a sample, and measures size and mass distributions. Since RMM is a counting technique, these distributions are generated on the basis of number, giving the technique sensitivity to sparse particle populations.

At the heart of a RMM instrument lies a Micro Electro-Mechanical Systems (MEMS) sensor which contains a resonating cantilever with a microfluidic channel embedded in its surface. When individual particles in the size range 50 nm – 5 µm flow through the channel, the resonating frequency of the cantilever is altered, to a degree that correlates with the particle's buoyant mass. The dry mass and size of the particle can therefore be extrapolated from a detected change.

The MEMS sensor simultaneously provides information about sample concentration, viscosity, density, and volume, and is able to detect and measure both negatively and positively buoyant particles. The resulting capability to differentiate particles on the basis of buoyancy has real and practical value, for example, when trying to measure protein aggregates in a sample contaminated with silicone oil droplets (figure 4). This can be a common problem in the late stages of development and into production, where silicone oil is a frequently encountered contaminant introduced by the syringes used for drug delivery.

An additional benefit of RMM is that it bridges a recognised gap between visible and solution based techniques, such as SEC, where it can be difficult to measure the level of aggregation. Regulators are concerned to have all aspects of aggregation reliably quantified and understood so this gives the technique a useful function in answering to analytical requirements, right through to quality control, even in the presence of common contaminants.

The complexity and variability inherent in biological molecules is presenting many new challenges for the rapidly evolving biopharmaceutical sector. Applying existing technologies to the measurement of protein particles or aggregates in finished products is extremely important but there is a growing need for measurements that can help understand the physical and chemical mechanisms that drive such interactions, and will underpin the development of robust and stable formulations. Analytical solutions providers have to match this pace of change, and anticipate and respond to the needs of the industry with agile product development that focuses on measuring a new and still emerging set of 'quality attributes'. LN

> The definition of purity and potency for biopharmaceuticals is substantially more complex than for small molecule drugs, with the potential for immunogenicity being a key concern to manufacturers and regulators alike

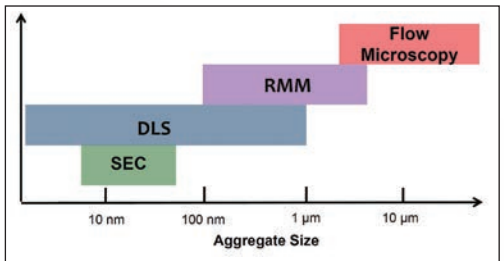


Figure 1: A range of analytical techniques is needed to ensure accurate measurement across all possible aggregates sizes.

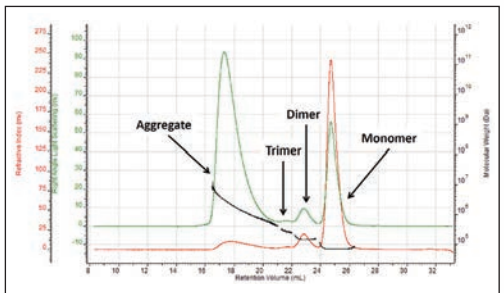


Figure 3: BSA measured on a GPC/SEC system consisting of RI and LS detectors.

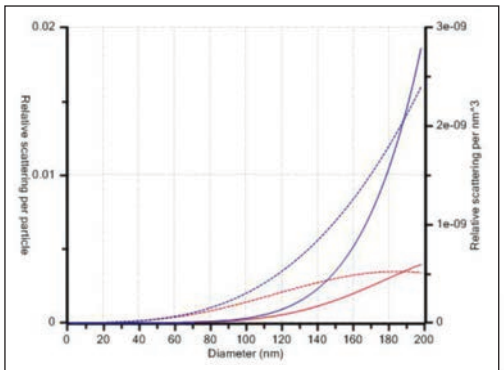


Figure 2: Measuring both backward (173 degrees) and forward (12.8 degrees) scattering ensures accurate size measurement throughout an aggregation process.

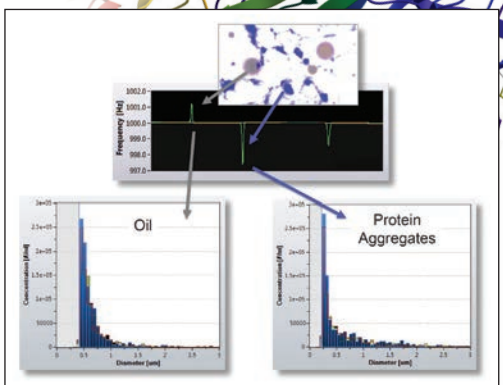


Figure 4: Resonant mass measurement (Archimedes, Malvern Instruments) detects two size distributions in this single protein sample, positively buoyant silicone oil droplets and negatively buoyant protein aggregate particles.