New technique to detect protein aggregates in biological actives

Jean-Luc Brousseau puts the case for a new analytical approach – a coupling of dynamic light scattering and size exclusion chromatography known as absolute size exclusion chromatography (ASEC).

Jean-Luc Brousseau préconise de réunir la dispersion lumineuse dynamique et la chromatographie d'exclusion en une nouvelle approche analytique appelée chromatographie d'exclusion de taille absolue (ASEC).

Jean-Luc Brousseau beleuchtet eine neue Analysemethode - eine Kombination aus dynamischer Lichtstreuung und Größen-Ausschluss-Chromatographie, die unter der Bezeichnung ASEC (Absolute Size Exclusion Chromatography) bekannt ist.

There are a great many biological actives under development or in clinical trials. These are being produced by the biotechnology sector for the treatment or prevention of a wide variety of conditions. Unfortunately some biological actives will trigger an immune response when the body identifies the material as foreign. A number of factors can prompt such an immune response, the most common being: certain components in the formulation, the concentration of the active, the extent of glycosylation/pegylation, contaminants, and degradation or aggregation of the active.

Chromatography plays an integral role in the preparation of these materials. In the quest for safe and effective biological actives, the technique of light scattering works in tandem with chromatography, providing innovative ways of characterising these molecules.

Taking precautions

Formulation and glycosylation/pegylation protocols are developed when the actives are at the research stage. Steps are taken and methods are developed to avoid contamination, and are coupled with the use of sterile disposables and working in low dust environments. Concentration should be adjusted for maximum efficacy with the minimum of negative effects. Degradation and aggregation of the active usually occurs after the formulation is prepared and tested. It is especially important to investigate and control this transformation of the product to ensure shelf life and proper storage conditions.

Immunogenicity is increased when an active is transformed by aggregation or degradation. Fortunately, the immune response tends to be benign, resulting only in the loss of efficacy of the active. However, in some rare cases, the immune response can be life threatening. Care should be taken and guidelines developed in relation to the factors that trigger immunoactivity.

The first step in resolving the aggregation and degradation problem is to understand the sources and develop metrics to predict the response. Careful measurement of the active's conformation, degradation and aggregation should be made for full characterisation and to be able to predict the response.

Aggregation is defined as the direct mutual attraction between particles or molecules through van der Waals forces or chemical bonding. The aggregate can be reversible or irreversible, soluble or insoluble – the latter is usually the case with a high molecular weight protein aggregate. The best method for studying the onset of aggregation is light scattering, because of its high sensitivity to large particle/molecules. The scattering intensity is proportional to size to the sixth power for small molecules such as proteins. So a molecule twice as big as the original monomer will scatter 64 times more light. This means that light scattering techniques are highly sensitive to aggregation.

Since it is known that large particles often trigger immunoactive responses, the US Food and Drug Administration (FDA) has put together some draft guidance recommending light scattering analysis and particle size measurements.

When aggregates have a size of around a micrometre they will usually precipitate within a few minutes to a few days. The problem of sampling now becomes important. However, before the aggregates reach this size, the onset of their formation can be monitored using light scattering techniques.

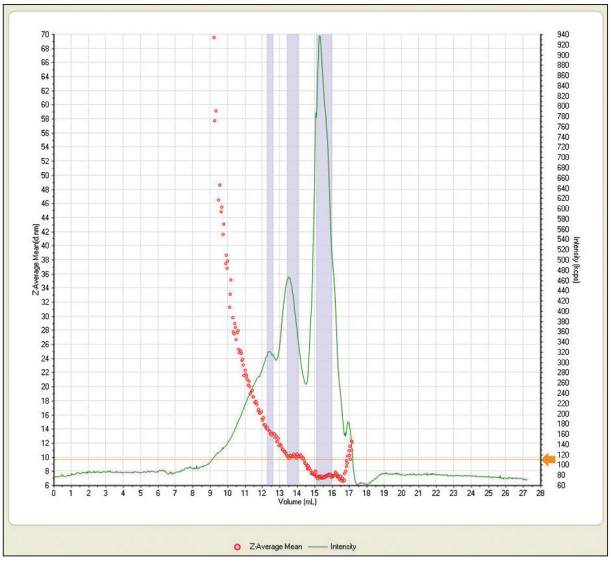

Fig. 1. Absolute size exclusion chromatography (ASEC) of bovine serum albumin (BSA).

Commercial instruments such as the Zetasizer Nano light scattering system from Malvern Instruments can be used to characterise samples to determine levels of aggregation.

Practical approaches

Bioactives – often proteins – are usually purified by fractionation. A chromatography method is used to purify and the therapeutic protein for use in initial trials. Fraction collection of the protein is repeated until sufficient material is collected for use.

With size exclusion chromatography (SEC)/gel permeation chromatography (GPC) pores in the separation column can become plugged with impurities resulting in elution times becoming shorter and shorter.

Therefore, careful monitoring of the fraction collection over time and over repeated runs is critical. Also, once a column becomes plugged, it must be regenerated since the build up of impurities means a loss of resolving power.

Once the above is complete, the active therapeutic protein collected can be used for trial. A number of methods will be used to test these proteins for purity.

Size measurement

One quick way to assess the state of the active is to measure its size. Size and size distribution will be indicative of the purity of the active collected. Furthermore, the size will indicate any degradation or aggregation in the sample.

Dynamic light scattering (DLS) measurements on an active require only a few microlitres of sample. Measurements can be made in less than five minutes and yield information about the size and the polydispersity of the active.

A low polydispersity indicates a good fractionation efficiency while a high polydispersity would indicate either a problem with the fraction collected or a change in the active composition since the fractionation. These changes are usually the result of aggregation, or are due to degradation.

Furthermore, DLS analysis can be used to assess or predict the stability of the protein as a function of time or temperature. These studies are often used to optimise solution conditions in order to stabilise the active ahead of clinical trials.

The recent development of 'flow-mode' for one of the leading DLS systems (Zetasizer Nano) now makes it possible to use the same instrument to monitor the fractionation online.

"Dynamic light scattering analysis can be used to assess or predict the stability of the protein as a function of time or temperature."

Jean-Luc Brousseau, Business Development Specialist, Malvern Instruments

When therapeutic proteins are eluting from the column, this system can be used to measure the size of the protein online and in real-time, allowing evaluation of the best separation method, or for use in quality control of the separation.

An example of flow-mode output is shown in Fig. 1. This rapid assessment of different solution conditions means it is possible to directly assess their effects on protein stability.

This recent use of DLS coupled with size exclusion chromatography is called absolute size exclusion chromatography (ASEC). A method is said to be absolute when no calibration is needed.

In ASEC, the size of molecules is measured as they elute, no matter when they elute. The fluctuation of the scattering intensity due to Brownian motion of the molecules in solution is measured by directing a laser through the sample as it passes through a flow cell.

The flow cell can be quickly inserted into the DLS detector and so is easily added to the existing chromatography setup. Changing between flow and batch cells takes only a few seconds. The DLS measurement then yields the size and polydispersity of the molecules. The size can be used to estimate a molecular weight for globular proteins, since it has been shown that every protein monomer has a single conformation.

ASEC can measure protein monomer, dimer and trimers accurately while they are eluting from the column. It is simple, requiring only a single instrument for the estimation of the molecular weight, and requires no calibration.

Size measurement is a quick way of assessing the stability and distribution of biological actives in solution. Size can be measured after fractionation or in real-time during fractionation. With ASEC, no column calibration is needed and the role of different eluents can be tested really quickly. Measurements are quick and accurate with an easy change in between batch and flow-mode measurements.

Light scattering, being highly sensitive to large sizes, is the best method for detecting aggregation, one of the main factors in causing immune response when biological actives are used in patients. ◆

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This article was first published in *EUROPEAN LABORATORY SCIENTIST, JUNE 2008* www.scientistlive.com