

Assessing the quality of proteins using light scattering techniques

Ulf Nobbmann uses two case studies to illustrate the practical application of different light scattering techniques.

Ulf Nobbmann utilise deux études de cas pour illustrer l'application pratique de différentes techniques de diffusion de la lumière.

Ulf Nobbmann illustriert anhand von zwei Fallstudien die praktische Anwendung von unterschiedlichen Lichtstreuungstechniken.

Proteins consist of polypeptide chains that are sensitive to a wide range of parameters such as temperature and chemical environment. Preparation method, storage conditions and/or buffer choice can all influence the size and quality of proteins in a sample.

Understanding the impact of different variables on a given protein is essential both for the production and maintenance of high quality materials.

Light scattering techniques are proving ideal for protein analysis as they deliver data on both size and molecular weight. Dynamic light scattering (DLS) exploits the link between hydrodynamic particle size and diffusion rate, calculated from measurements of time-dependent light fluctuations.

Static light scattering (SLS) involves the determination of molecular weight from measurements of time-averaged intensity at a range of sample concentrations. Together the techniques provide complementary insights into the factors influencing protein quality.

Here, two case studies illustrate the practical application of light scattering techniques. The first compares the properties of two therapeutic antibody samples, one a treated analogue of the other.

The second study is a controlled investigation of the impact of storage conditions on immunoglobulin G (IgG). Both cases reference data generated using instruments from the Zetasizer family of particle characterisation systems (Malvern Instruments), the first commercially available instruments to combine

dynamic, static and electrophoretic light scattering measurement capabilities.

Fig. 1. shows DLS data for treated and untreated samples of a therapeutic antibody supplied in an ammonium phosphate buffer. Details of the associated treatment process are unknown.

The samples are clearly different. The treated sample contains particles with a diameter of more than 50 nm whereas the z-average size of the untreated sample is around 11 nm, the expected value for the antibody.

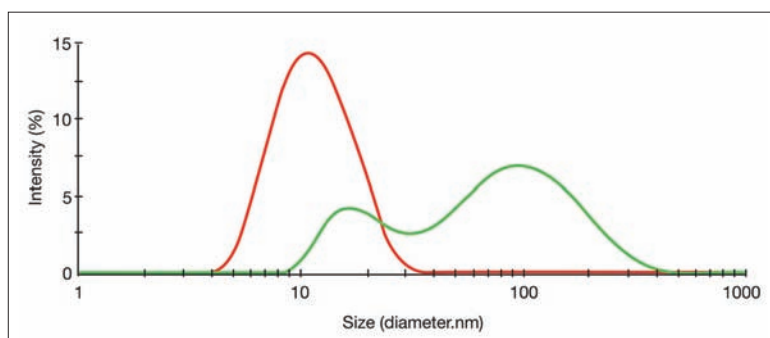


Fig. 1. Size distribution from pure (red) and treated (green) antibody sample.

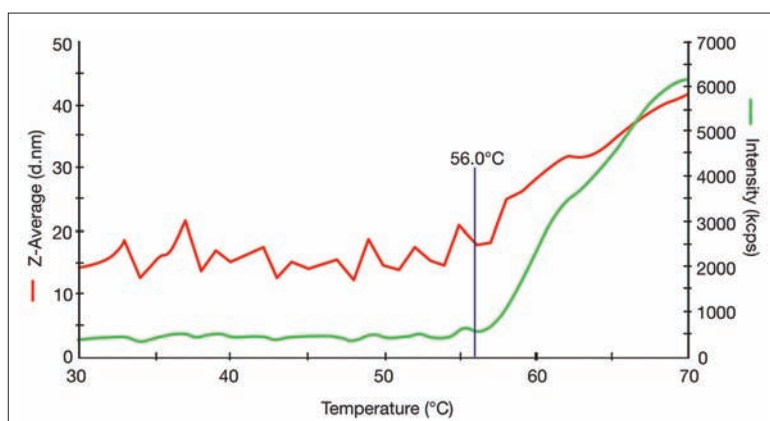


Fig. 2. Melting point determination of the untreated sample. $T_M = 56.0^\circ\text{C}$.

Visually, the broad peak shape of the treated sample suggests the presence of oligomeric assemblies and/or aggregates. The associated polydispersity index is greater than 0.1, indicating the presence of more than one species. These results suggest that the treatment process has promoted aggregation of the antibody.



SLS measurements of molecular weight, known to be in the region of 145 kDa, support this view. The measured molecular weight for duplicate untreated samples is slightly low (129 ± 21 and 139 ± 8 kDa), a result attributable to the presence of antibody fragments not removed in the purification process. The results from duplicate treated samples on the other hand have a much higher molecular weight of 1210 ± 125 and 1270 ± 65 kDa, providing further evidence of aggregation.

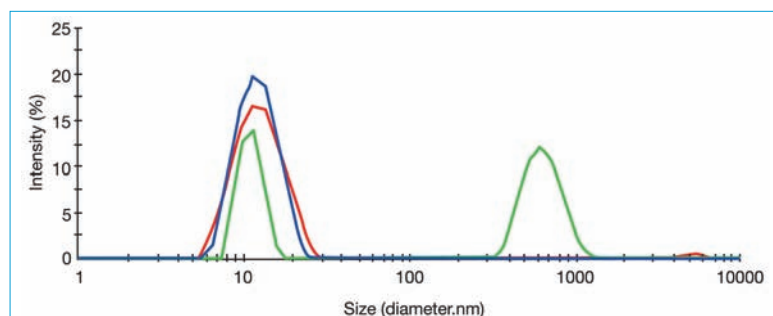


Fig. 3. Size distribution (by percent intensity) from antibody samples stored under different conditions: 4 °C (blue), 25 °C then 4 °C (red) and 5x frozen/thawed (green).

A temperature scanning routine embedded in the Zetasizer software permits the study of size and scattering intensity as a function of temperature. A protein 'melts' under the influence of heat when the molecules denature – leading to massive aggregation. This transition is visible in light scattering studies as a significant increase in both size and scattering intensity. The untreated samples exhibit this behaviour at a temperature of around 56 °C (Fig. 2), the melting point for the antibody. With the treated sample no transition is observed; the treatment process has already permanently denatured the antibody.

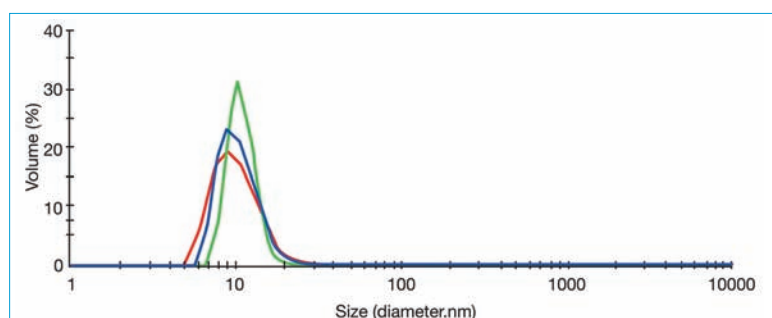


Fig. 4. Size distribution (by percent volume) from antibody samples stored under different conditions: 4 °C (blue), 25 °C then 4 °C (red) and 5x frozen/thawed (green).

To investigate the effect of storage conditions on IgG, freshly prepared antibody samples were treated in the following ways: stored for 35 days at 4 °C; stored for 31 days at 25 °C, then for four days at 4 °C; and subjected to five freeze/thaw cycles then stored at 4 °C.

All samples were prepared at a concentration of 0.8 mg/ml in an ammonium hydrocarbonate buffer, at a pH of 6.8.

All three samples show a peak at around 10 nm (Fig. 3), the expected diameter of the antibody, but the relative width of this peak and the size distribution is different for each sample. With those samples stored under non-ideal conditions, there is evidence of large aggregates, particularly where subjected to freeze/thaw cycles. The 'quality' of each sample is evident from z-average diameter and polydispersity index data, which show that freeze/thaw cycling is particularly detrimental.

Displaying size data in terms of a volume distribution highlights the sensitivity of DLS. Even for the sample subjected to the worst conditions, the volume distribution shows no evidence of aggregation (Fig. 4) because the percentage volume contribution of the main protein peak is still 99.6 per cent. This means that the distribution by intensity is extremely sensitive to changes in the size and aggregation state. Because of this DLS is a powerful technique for detecting the presence of very small numbers of relatively large particles, which can give an early indication of stability issues during protein storage or during a treatment process.

Static light scattering results for the sample stored exclusively at 4 °C reflect the expected molecular weight for IgG, with a measured value of 150 ± 13 kDa. The sample stored under warmer conditions has a higher molecular weight, 176 ± 6 kDa, indicating a degree of aggregation or oligomerisation. The third sample however also has a measured molecular weight close to the expected value, 156 ± 12 kDa. In this case aggregates are present at a level at which the Zetasizer's advanced dust rejection algorithm can be used to remove the contribution of the large particles. This algorithm allows the measurement of molecular weight of the protein, despite the presence of some large aggregates.

Table 1. Size and polydispersity index are influenced by storage conditions.

Storage Condition	Z average (diameter, nm)	Pdi
35 days at 4 °C	11.4	0.075
31 days at 25 °C, then 4 °C	11.7	0.172
5 freeze/thaw cycles, then 4 °C	152	0.646

In conclusion, then, light scattering techniques provide rapid access to accurate molecular weight and size information for the characterisation of proteins. With the Zetasizer, measurements of these and other sample properties are straightforward, even with small sample volumes and low concentrations. These are extremely important considerations for the large majority of pharmaceutical and biomolecular applications. ♦

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