PFQ
Pharmaceutical
Formulation & Quality

CAPSULE

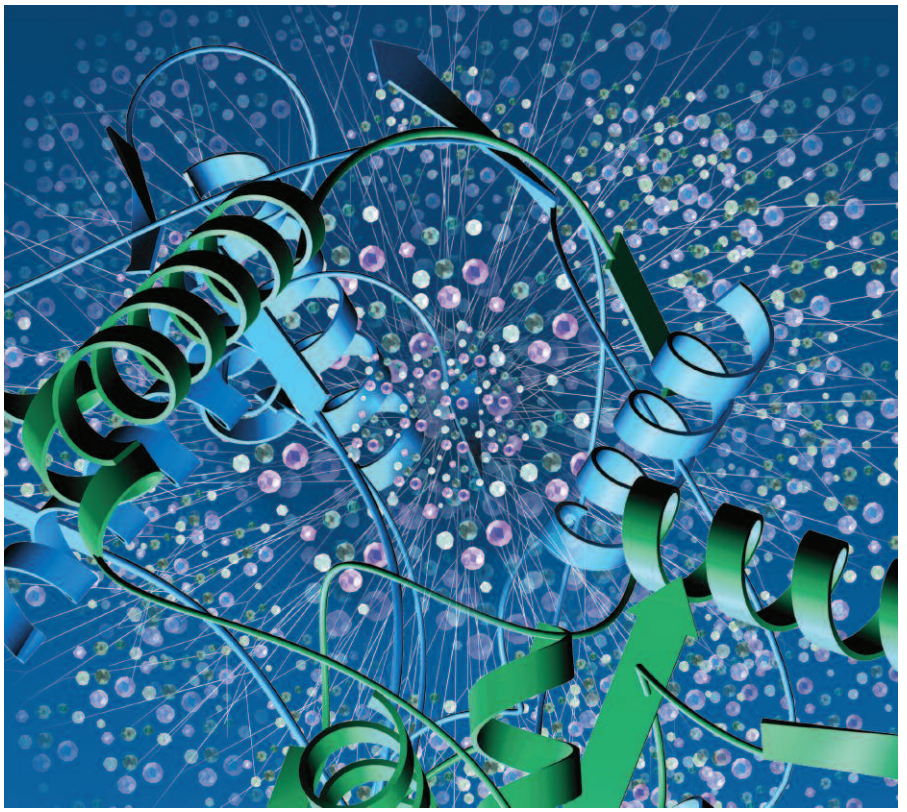
High quality, animal-free recombinant human albumins (rAlbumins) provide pharmaceutical manufacturers with the fully characterized and regulatory-compliant multipurpose excipients needed to optimize process development timelines and product quality, and hasten regulatory acceptance of the final drug product.

STABILITY

Tools for Evaluating the Stability of Human Recombinant Albumins Used in Human Therapeutics

Dynamic light scattering and size exclusion chromatography with light-scattering detection

› **By Karl Nicholls, Neil Dodsworth, Phil Morton, Dr. Mark Pothecary, Dr. Oksana Leszczyszyn, and Dr. Hanna Jankevics**



Aggregation and misfolding during long-term storage is a major challenge in the development of structurally altered proteins for therapeutic applications. While a liquid formulation is both easy to handle and economical during manufacture, many proteins are difficult to formulate as stable solutions. Storage stresses such as temperature changes, shear strain, surface absorption, and high protein concentration can cause conformational changes and precipitation.

Implicated in adverse immunogenic side effects, aggregation must be minimized during handling, shipping, and long-term storage. Formulations must be optimized to ensure the efficacious delivery of clinical applications and rigorously tested to provide a detailed understanding and confirmation of their stability under a variety of conditions.

Here we use dynamic light scattering in cuvette mode and size exclusion chromatography with light-scattering detection to demonstrate both the short- and long-term stability of novel recombinant human albumins (rAlbumins) that have been specifically developed and optimized to deliver a stable, safe, and regulatory-compliant product for the formulation markets.

Novel Recombinant Human Albumins

Excipients incorporated into the formulation process not only stabilize the drug product but also assist in the administration and release of the active pharmaceutical ingredient. However, strict regulatory limitations in the use of blood- and plasma-derived materials have arisen due to safety concerns regarding the potential risk of transmitting infectious agents such as HIV, hepatitis, variant Creutzfeld-Jacob disease, and West Nile virus. This challenge has given rise to the development of high-quality, animal-free, recombinant human albumins for use in a range of applications, including the formulation of pharmaceutical drugs and vaccines and medical device manufacturing.

Albucult and Recombumin (Novozymes Biopharma, UK) are examples of fully characterized, regulatory-compliant multipurpose excipients that are optimized to prevent or minimize physical, as well as chemical, degradation of drug substances. Providing a tertiary level of animal-free status and lowering the risk of potential contamination, recombinant supplements are suitable for any stage of the clinical pathway.

Both Albucult and Recombumin are derived from proprietary *Saccharomyces cerevisiae* yeast expression technology. Formulated at pH 7, Recombumin is structurally identical to human serum albumin.

Recombumin is the world's first commercially available rAlbumin approved for use in the manufacture of human therapeutics and has been shown to have a shelf life of greater than five years at 5°C.[1] Albucult was further developed to deliver process and performance consistency for applications such as drug and vaccine manufacture, device coating, IVF media, specialized cell culture, and cell therapy applications. The GMP formulation is stable for at least four years to date.[2]

Typical storage times for both protein and non-protein formulations depend on a number of factors. Storage times are generally between one and three years. However, shelf life for protein-based formulations is typically shorter than that of non-protein based formulations.

Testing Protein Stability with Light-Scattering Techniques

The effects of pH, temperature, and concentration on oligomerization and aggregation behaviors can be studied in order to demonstrate short-term stability and predict shelf life. Dynamic light scattering provides a rapid measurement—about two minutes per sample—that allows the comparison of a number of sample conditions within a short time and without the need to alter or dilute the sample. As the following case studies show, DLS can provide much information about the stability and relative composition of different protein formulations. Pre-screening by DLS can therefore minimize total analysis time, directing the experimenter to undertake more time-consuming measurements, such as size exclusion chromatography—typically 15-20 minutes per sample—on only the most promising formulations.

CASE STUDY

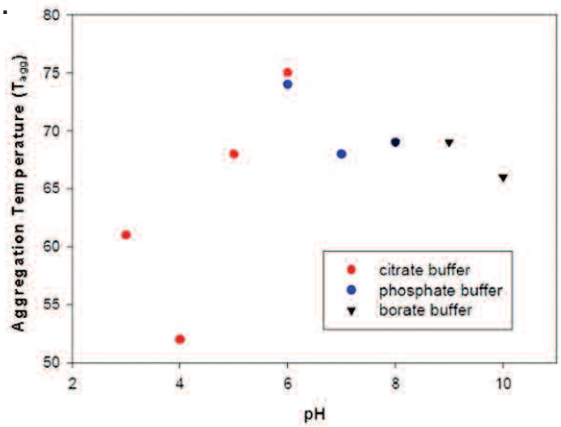
Understanding Protein Thermal Stability with DLS

DLS was used to study the thermal stability of Recombumin over the range of pH conditions in which APIs are commonly formulated. In this technique, the mean size of the molecule was monitored as a function of temperature, and the stability of a given formulation was indicated by the delayed onset of unfolding and aggregation. For Recombumin, the knowledge of the aggregation temperature allows determination of the most appropriate pH conditions required for a stable liquid formulation.

The samples were measured with the Zetasizer APS (Malvern Instruments, UK), an automated plate sampler system that allows unattended dynamic light scattering measurements of multiple samples. An algorithm is applied to the DLS data, combining a number of factors to detect where unfolding and aggregation begins. The aggregation temperatures determined for Recombumin samples at various pH conditions are shown in Figure 1.

The lowest aggregation temperatures are observed for buffers at and below pH 4, which suggests that these conditions are less favorable for a stable formulation. In more basic formulations—those at and above pH 5—the aggregation temperatures are all greater, with the maximum aggregation temperature observed at pH 6. Therefore, under the conditions studied here and at least over the times taken for these measurements, citrate and phosphate buffers with a pH of 6 provide the most stable formulations.

Figure 1.



Recombumin: DLS-determined aggregation temperature plotted as a function of pH.

To understand how these aggregation temperatures correlate with observations from long-term stability studies, DLS measurements were carried out on the same Recombumin formulations at six- and nine-month intervals after storage at 2-8°C (Figures 2and 2b, respectively). Shifts in the mean hydrodynamic size to larger values are indicative of aggregation, and on this premise the formulations at and below pH 4 are deemed the least stable. This observation is in agreement with the indications from aggregations temperature studies.

From these comparisons, it is clear that the determined aggregation point is related to the low temperature stability of the sample and can be used as an indicator of longer-term stability. However, at higher pHs, other factors, such as ionic strength, sample concentration, and the presence of additives, may become more significant players in contributing to longer-term formulation stability.

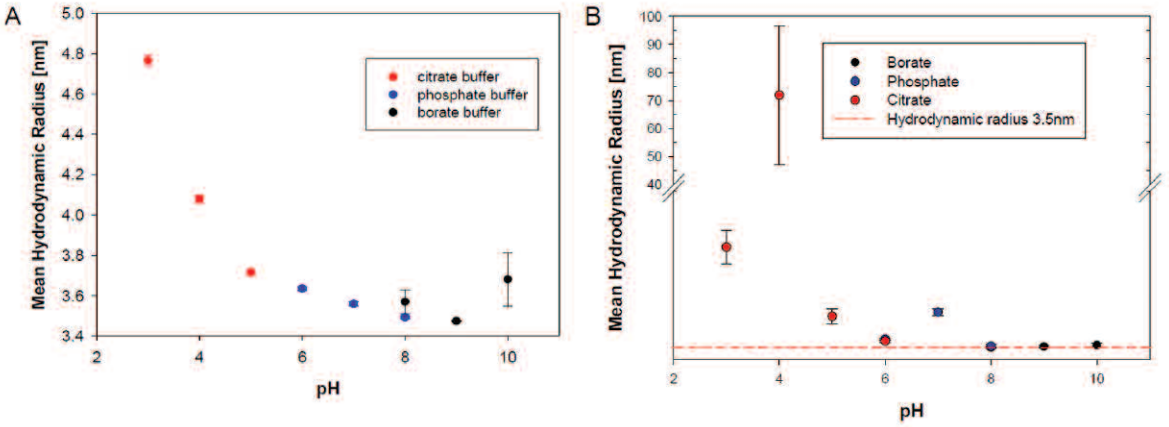
CASE STUDY

Monitoring Protein Structural Stability

DLS gives a clear indication of not only large aggregates but also small oligomers or aggregates present in the sample. In comparison, SEC can provide even more detailed insight and, when combined with light-scattering detection, is an excellent tool for characterizing the type of oligomers or aggregates. This example demonstrates the resolving and detection capabilities of size exclusion chromatography light scattering and dynamic light scattering.

To mimic the appearance of small oligomers, mixtures of two different proteins were used. An Albucult sample (66 kDa recombinant human albumin) was used for the monomer protein. This protein shows very good stability in its formu-

Figure 2.



Z-average size for Recombumin formulated at different pHs. A: 6 months and B: 9 months after storage at 2-8°C.

lation buffer and is therefore a useful DLS reference sample. Alcohol dehydrogenase was used for the second sample. At about 150 kDa, ADH is similar in molecular weight to a dimer of Albucult.

Stock solutions of both proteins were prepared in phosphate buffered saline and filtered to ensure that no aggregates or dust particles were present in either sample. The Albucult sample was spiked with increasing amounts of ADH. The level of ADH is given in %ADH (in terms of moles of ADH compared to total moles of protein in the sample). The samples were first measured by DLS on a Zetasizer Nano ZS (Malvern Instruments, UK). Duplicate measurements of 100 µl of each sample were injected for analysis by SEC-LS on a Viscotek TDAmax (Malvern Instruments, UK).

SEC-LS measurements show partial separation of the Albucult and ADH peaks as shown in the refractive index chromatograms (Figure 3). In samples in which the concentration of the ADH was below 1.5%, neither the UV nor the RI detectors were sensitive enough to allow accurate determination of the molecular weight. However, at concentrations of ADH above 7.7%, the detection of both light-scattering and RI responses was sufficient to allow determination of the molecular weight. As seen in Table 1, the values determined for Albucult and ADH are close to the known molecular weight values. In this case, the measured %ADH value is slightly below the input value due to the incomplete resolution between the two peaks and could be improved by additional columns, although at the cost of a longer measurement time.

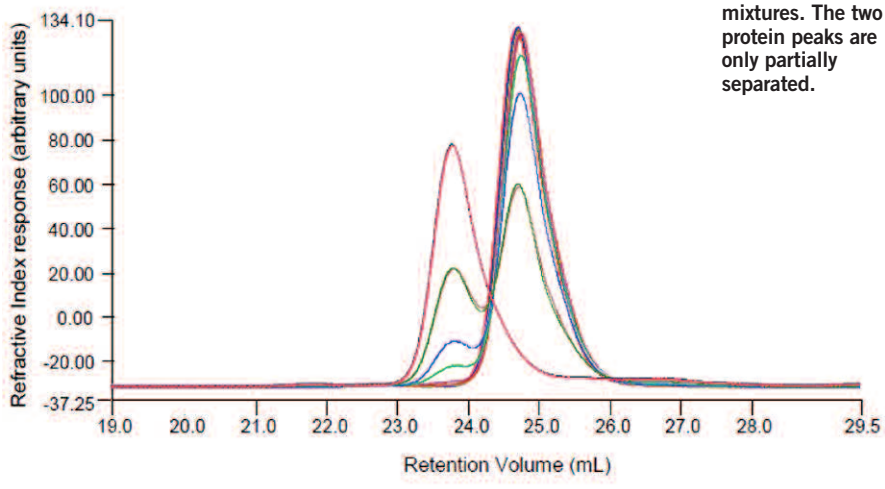
In contrast to SEC-LS measurements, DLS in batch mode is not able to resolve these two proteins, because the resolution in DLS requires a minimum threefold difference in population sizes. In the case of these proteins, this would be equivalent to a molecule of RH ~10.8 nm (where Albucult RH =3.6 nm), which corresponds to a globular protein complex with a molecular weight of around 880 kDa, a value far larger than the 150 kDa ADH.

Nonetheless, DLS is extremely sensitive to changes in the sample, and although it will not discern these two populations, the resulting distribution will change in its mean size and width, allowing comparisons between samples and identification of samples in which substantial oligomerization or aggregation is occurring. This comparison can be used to monitor the same sample over time, for example, or to determine the difference between samples, as in this case study.

In Figure 4, the change in scattering intensity (derived count rate) and the intensity weighted mean radius (Z average) are plotted as a function of the %ADH in the total sample. It is clear that, just as in the SEC-LS measurements, there is a substantial change detected at 7.7% ADH and above, but the mean size of the sample is already increasing at 1.5% of ADH, indicating the presence of oligomers.

Light scattering is capable of detecting the ADH at very low levels, both in SEC-LS and DLS measurements. The limiting fac-

Figure 3.

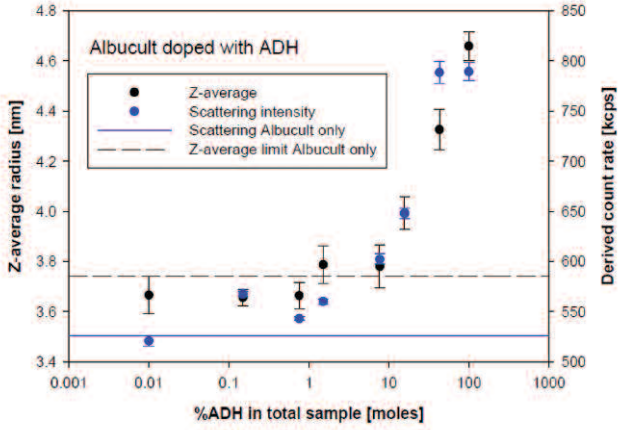


Chromatogram of Albucult and ADH mixtures. The two protein peaks are only partially separated.

Table 1. SEC-LS results for the range of ADH-spiked Albucult sample.

% ADH in Stock Solution	Measured % ADH		Albumin Mw (kDa)		ADH Mw (kDa)	
	Run 1	Run 2	Run 1	Run 2	Run 1	Run 2
0.00	0	0	67	67	N/A	N/A
0.15	0	0	67	67	N/A	N/A
0.76	0	0	66.8	66.8	N/A	N/A
1.51	0	0	66.5	67.1	N/A	N/A
7.72	4.1	4.1	69.3	69.5	152	151.7
15.85	10.4	10.5	73	71.8	149.5	151.5
42.97	32.3	32.1	76.3	75.8	146.7	145.5
100	100	100	N/A	N/A	147.7	148

Figure 4.



DLS Z-average radius and derived count rates (in kilo counts per second) for the ADH-spiked Albucult samples.

tors determining the molecular weight in the SEC-LS measurements are the concentration signal and the resolving power of the SEC column used.

High-quality, animal-free recombinant human albumins provide pharmaceutical manufacturers with the fully characterized and regulatory-compliant multipurpose excipients needed to optimize process development timelines and product quality, and hasten regulatory acceptance of the final drug product.

SEC-LS and DLS are useful tools in the rigorous testing required to provide a detailed understanding and confirmation of protein stability under a variety of conditions. High-quality rAlbumins provide useful reference samples when evaluating the effects of such parameters as pH, temperature, and concentration on oligomerization and aggregation behaviors.

Whether applied to formulation optimization, quality monitoring, or testing for long-term stability, SEC-LS is an excellent tool for the detailed characterization of the type of oligomers or aggregates present in samples of therapeutic proteins. In comparison, DLS provides a rapid measurement that allows the comparison of a number of sample conditions within a short time and gives a clear indication of the presence of large aggregates and small oligomers or aggregates. In this case, these tools provide detailed stability data supporting the confident inclusion of Recombumin and Albucult in pharmaceutical formulations. ■

REFERENCES

1. Novozymes Biopharma US Inc. Recombumin Product Specification sheet. Novozymes Biopharma website. Available at: www.biopharma.novozymes.com/en/information-centre/brochures-and-datasheets/Documents/Recombumin_prod_spec_FINAL.pdf. Accessed June 3, 2012.
2. Novozymes Biopharma US Inc. Albucult Product Specification sheet. Novozymes Biopharma website. Available at: www.biopharma.novozymes.com/en/information-centre/brochures-and-datasheets/Documents/Abucult_prod_spec_FINAL.pdf. Accessed June 3, 2012.

Karl Nicholls is a research scientist, **Neil Dodsworth** a protein chemistry science manager, and **Phil Morton** a process development manager with Novozymes Biopharma. **Dr. Mark Pothecary** is a product manager, **Dr. Oksana Leszczyszyn** a product technical specialist for separations, and **Dr. Hanna Jankevics** a product technical specialist for biophysical characterization for Malvern.

Editor's Choice

1. Nicholls K. Investigating the stability of recombinant human serum albumin under a range of pH conditions with both time and temperature. Poster presented at: BioProcess International Europe 2010; May 2010; Vienna, Austria.
2. Novozymes Biopharma US Inc. Malvern Instruments. Using light scattering to study the thermal stability of pH stressed Recombumin, and predict shelf life. Application Note MRK1617-01. Available at: www.malvern.com/malvern/kbase.nsf/allbyno/KB002893/$file/MRK1617-01.pdf. Accessed June 3, 2012.
3. Novozymes Biopharma US Inc. Malvern Instruments. SEC-LS vs DLS: A Case study in detecting and resolving small protein oligomers. Application Note MRK1615-01. Available at: www.malvern.com/malvern/kbase.nsf/allbyno/KB002894/$file/MRK1615-01.pdf. Accessed June 3, 2012.

Reprinted with permission from PFQ Magazine June/July 2012.