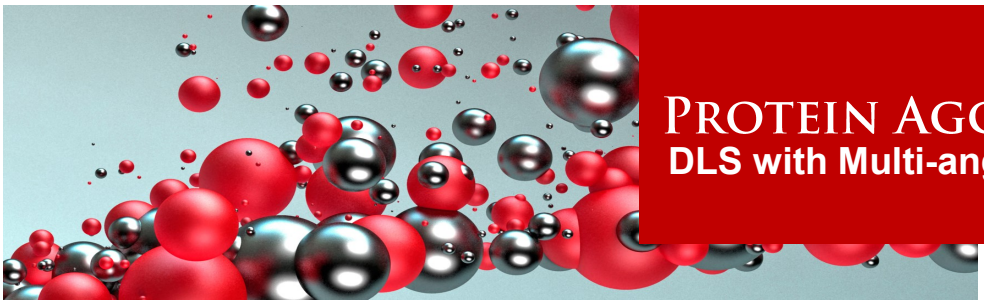

PROTEIN AGGREGATION
DLS with Multi-angle

AN–158

Application Note

Multi-angle Sizing of Proteins and Protein Aggregation

Proteins prepared using hot and cold methods were sized at 90, 60, and 20 degrees using the Nicomp 380 Dynamic Light Scattering (DLS) Particle Size Analyzer equipped with a multiangle goniometer. This capability was coupled with a high powered 15 mW laser diode and a high gain avalanche photo-diode detector providing a gain increase of 12 over the standard Nicomp 380. Since larger particles scatter relatively more light in the forward direction, low angle DLS has the potential to provide important information on the aggregation of proteins and other macromolecules.

Dynamic Light Scattering (DLS) is a common particle sizing technique for sizing sub-micron particles. The intensity of light scattered in a particular direction by dispersed particles tends to periodically change with time. These fluctuations in the intensity versus time profile are caused by the constant changing of particle positions brought on by Brownian motion. DLS instruments obtain from the intensity versus time profile a correlation function. This exponentially decaying correlation function is analyzed for characteristic decay times which are related to diffusion coefficients and then by the Stokes-Einstein equation, to particle radius.

DLS has several advantages over laser diffraction. Firstly, DLS is an absolute measurement, where knowledge of the composition and optical properties of the suspended particles is not needed. This can be helpful if the optical properties of the suspended particles are not known or if the suspension is made up of particles with different optical properties. Secondly, the patented Nicomp analysis algorithm allows skewed unimodal or close bi-modals to be measured with a relatively high resolution and accuracy. DLS also has the ability to accurately size particles smaller than 50 nm. This capability is illustrated by the following data.

Proteins are macromolecules essential to human health. Many medical drug therapies involve the replacement of missing or defective proteins. The study of protein synthesis is an area important to the successful implementation of protein drug therapies. It is important to establish the most efficient way to manufacture proteins which lessens the possibility of aggregation, often the result of protein denaturation, and increases stability. DLS can provide a quick and easy way to determine the size of small protein molecules. Furthermore, by utilizing multi-angle DLS, quantitative information can be obtained about protein aggregation.

In this study, BSA, a common protein, was prepared by two methods. Figure 1 contains the Intensity-weighted PSDs of each protein obtained at 90 degrees to the incident beam.

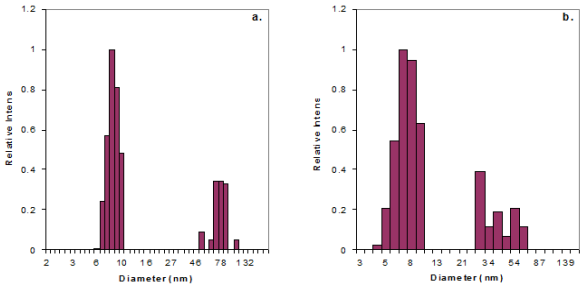

Figure 1: DLS Size Distribution of Proteins at 90 degrees. a. Cold Prep; b. Hot Prep.

The Multi-Angle Nicomp 380, used in the following measurements, was equipped with a high powered 15 mW laser diode and a high gain avalanche photo-diode detector (APD). Each PSD consists of two peaks, one at 8 nm and the other around 50 nm. The first peak can be identified with the native protein. The mean diameter is in good agreement with previous measurements made on the same protein. The second peak most likely represents the aggregates which are present. This secondary peak is not well defined owing to the fact that only a small number of these particles are in the view volume.

To increase the amount of signal coming from the aggregate particles, the angle of detection for the Nicomp 380 was moved to 20 degrees relative to the incident light. The reason for going to lower angles is that larger particles tend to scatter light mostly in the forward direction (Fraunhofer diffraction), in this way a more accurate accounting of the aggregates can be obtained.

The aggregate peak was poorly defined at 90 degrees, suggesting that few of these particles were present. Figure 2 contains the Intensity-weighted PSDs made at 20 degrees for each preparation.

Again, the resulting distributions consist of two peaks with the native peak at 8 nm. The main difference is that the aggregate peak comes in strong and well defined and is the largest of the two.

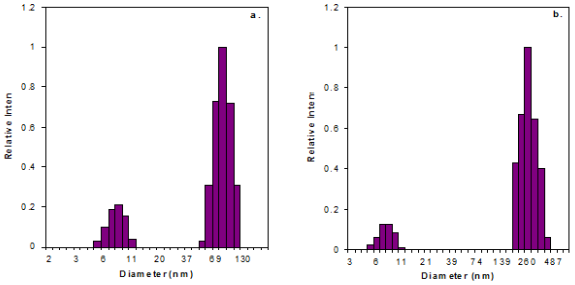

Figure 2: DLS Distribution of Proteins at 20 degrees. a. Cold Prep; b. Hot Prep

This peak has a mean diameter of 80 nm for the cold preparation and 265 nm for the warm preparation. This data, in and of itself is extraordinary for several reasons. One, the Nicomp 380 has exhibited excellent resolution, both at 90 and 20 degrees, being able to separate the native peak from the aggregates in each case. Secondly, the mean diameter of the native peak is unchanged at each angle demonstrating a multi-angle capability not present in other light scattering instruments.

But the story is not finished. The cold preparation was expected to have more aggregation but from the results in Figure 2, it would appear that the opposite was true. Figure 3 contains the 20 degree DLS data from the warm prep sample after filtration, separately, through two different filters, one which filters 100,000 MW unit particles and the other which filtered 1 million MW unit particles. Surprisingly, the aggregate peak dominated the PSDs for each filtered sample. Consider the 100K filtered sample data. The PSD consists of a single peak at 440 nm, much larger than the pore size of the filter. There was no signal from the native peak. The same held for the 1 Meg filtered sample, although the aggregate peak was much smaller. Several new aliquots of sample verified these results.

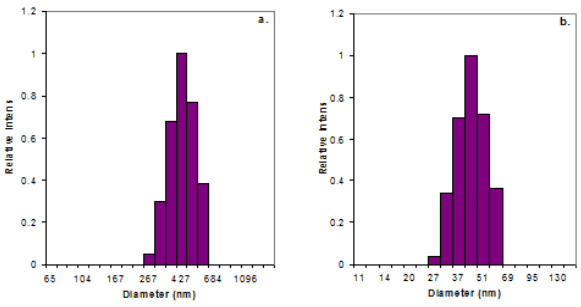

Figure 3: DLS at 20 degrees of Warm Prep. After Filtration. a: 100K MWU; b. 1 Meg MWU.

We were able to unravel this mystery by testing the water. An aliquot of pure water was passed through the 100 K filter. Figure 4 contains those results. Again, the measurements were made at 20 degrees. Large scattering intensities were observed at 20 degrees and the analysis produced a peak at 330 nm, quite similar to the mean obtained from the 100 K filtered protein sample. Was it the filter? We tested an aliquot of water without filtering.

These results are also in Figure 4. The result was a peak at 35 nm and the largest scattering intensity seen so far at 20 degrees. How is this all explained? Nanopure systems (as well as other filtering equipment) tend to aerate water. As a matter of fact, one leading manufacturer of pure water systems is selling a new improved filtering system, which fixes this problem.

It is clear from Figure 4a that the microbubbles in the water were about 35 nm in size. Filtering the water caused the bubbles to "aggregate", leading to the previous results, in Figure 4b. It is know that applying shear forces to colloidal systems does cause this to happen. The smaller diameter peak seen in the 1 Meg filtered results of Figure 3b can be explained because the larger pore size in this filter meant that less shear was applied to the bubbles so they didn't aggregate as much. As a matter of fact, the mean diameter is very similar to that of the microbubbles.

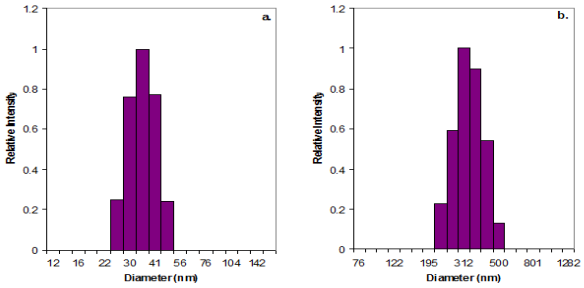

Figure 4: DLS at 20 degrees of water used to dilute proteins. a. Unfiltered; b. Filtered 100K MWU

We did one final test on these protein samples. One way to de-aerate water is to sonicate it. We applied light sonication the warm prep sample, which was passed through the 100 K filter. These results can be seen in Figure 5. The aggregate peak still remains but the amount of micro bubbles decreased enough that the native protein peak could now be seen. This seems to further support the bubble theory.

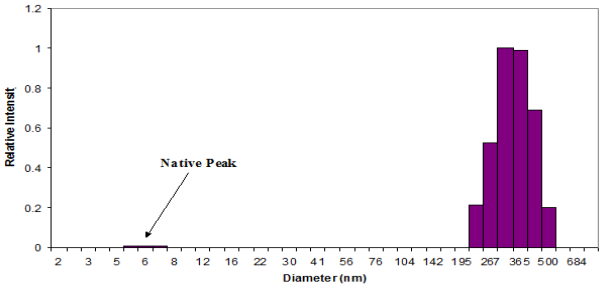

Figure 5: DLS at 20 degrees of Hot Prep Protein after Filtering 100K MWU and Sonication

In conclusion, the data presented in this paper demonstrates clearly the multiangle capability of the Nicomp 380 and how it can be brought to bear on protein and other macromolecule applications. This data also shows how powerful the cost-effective 15mW/APD additions are.

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