application note

The Effect of Concentration on Dynamic Light Scattering Measurements of Proteins

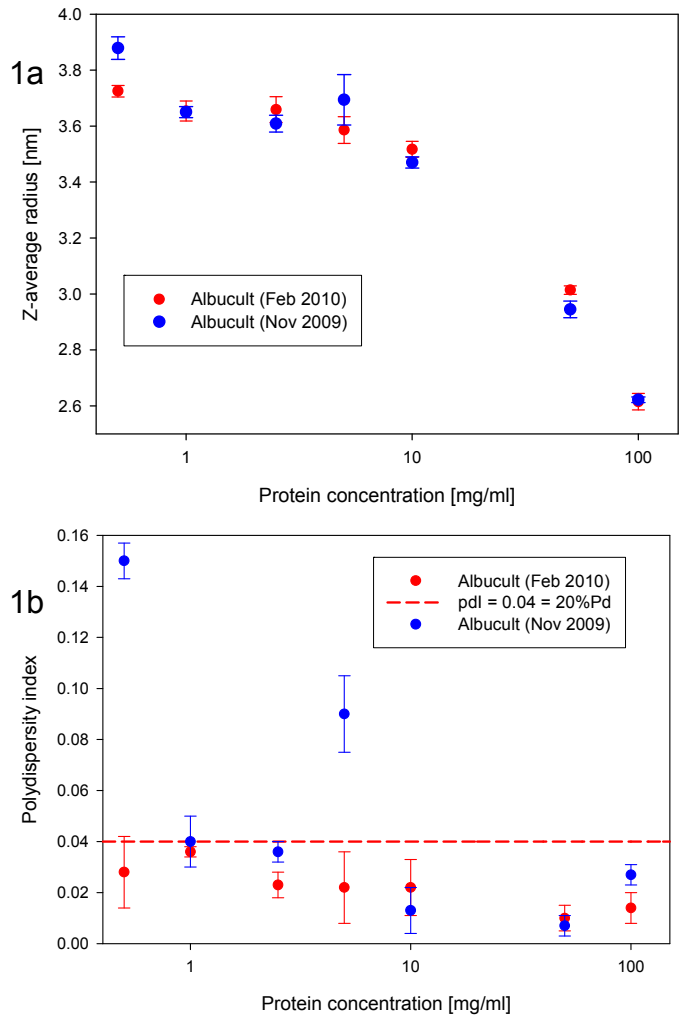

Figure 1(a): z-average radii and figure **1(b):** polydispersity index values, for Albucult® at different concentrations. Stock concentration 100mg/ml and diluted in formulation buffer down to 0.5mg/ml. ● represent samples measured just after preparation in November 2009, ● represent measurements taken after 2 months storage (February 2010) at 25°C.

Introduction

Protein samples can display inter-particle and particle-solvent interactions that can cause substantial distortion in the apparent diffusion coefficients determined by dynamic light scattering (DLS) and hence affect the calculated hydrodynamic size [1]. These inter-protein and protein-solvent interactions are electrostatic interactions which are sometimes referred to as virial effects.

The effects are typically displayed at high concentrations, although what is considered to be a high concentration is dependent on the protein in question as well as the buffer it is suspended in. For many proteins these effects are often observed at concentrations of 5 to 10mg/ml and upwards.

In general, so called molecular crowding effects will cause the apparent size of the sample to grow even though the sample remains 100% monomeric. However, in some cases, especially for many protein samples, the apparent size appears unrealistically small. All proteins are electrically charged and at high concentrations their diffusion can couple to the rapid diffusion of small counter-ions, producing a decrease in the apparent size of the proteins. These electrostatic effects are very strong when the ionic strength of a solution is low and the protein electric charge is high, reducing the apparent size three-fold. For example, an antibody can yield a size as low as 2nm in radius, giving unrealistic values of the estimated molecular weight.

In this application note, the behavior of Albucult® protein samples at high concentrations will be presented. Albucult® is a recombinant human serum albumin from Novozymes Biopharma UK. The results obtained suggest that molecular crowding in the relatively low ionic formulation buffer is the cause of the effects seen and the way in which this conclusion was reached is discussed.

Experimental

Albucult® was kindly provided by Novozymes Biopharma UK Ltd and is a recombinant human serum albumin (HSA) manufactured to provide a stable HSA sample to ensure good performance for drug, vaccine and device manufacturing.

The Albucult® sample was measured at its stock concentration, 100mg/ml and then further diluted stepwise down to 0.5mg/ml in the formulation buffer which contains 145mM NaCl and 8mM sodium octanoate. The buffer was filtered with a 0.22µm sterile filter before use.

In a second experiment, an NaCl titration, increasing amounts of NaCl were used to monitor the behaviour of the Albucult. Two aliquots of 50mg/ml Albucult were prepared. To one of them, NaCl was added up to a concentration of 1M. The two samples were then mixed in different proportions so that the concentration of the Albucult remained constant but with an increasing concentration of NaCl up to a maximum of 1M. The size of the samples was then measured by DLS.

The samples were measured in a Zetasizer Nano using 100µl plastic disposable cuvettes. The samples were measured at 25°C after a 2 minute equilibration time to ensure that the sample was thermally equilibrated. Each sample was measured using 3 repeats; the standard deviations from these repeats are shown as the error bars on the figures in this application note. The measurement length was automatically determined by the software to ensure optimal data quality.

Results

Protein Concentration Effect

As seen in figure 1(a), the Albucult® samples show concentration dependence in their hydrodynamic size. At low concentrations, up to

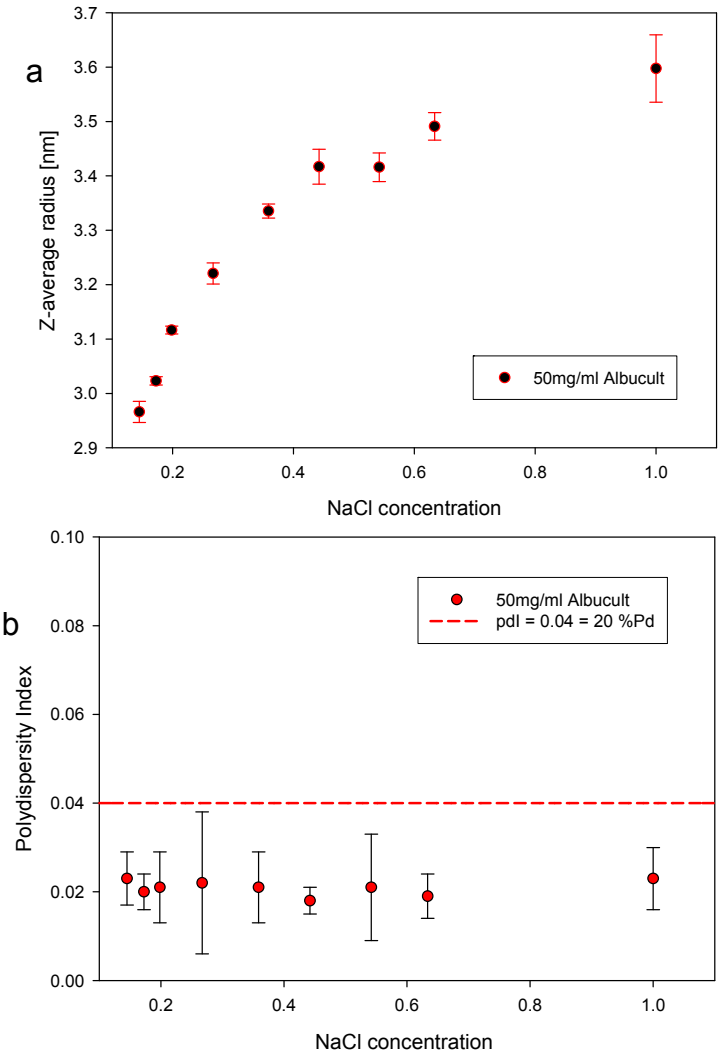

Figure 2: NaCl titration into a 50mg/ml Albucult® sample (a) the change in z-average radius with increasing NaCl concentration from the starting concentration of 145mM NaCl in the formulation buffer up to 1M NaCl, (b) the polydispersity index of the sample does not change as the NaCl concentration is increased.

10mg/ml, a shallow gradient is seen, but as the concentration increases the effect becomes more pronounced with decreasing apparent hydrodynamic size.

Figure 1 summarises the results from two separate measurements of the same samples. The samples were left out on the laboratory bench over a time period of two months and measured at the start and end of this period. The samples show a remarkable reproducibility for the two measurements, indicating that the formulation is successful ensuring the stability of the protein over this time frame.

The polydispersity index values for these samples indicate that the majority of them are monodisperse (below a value of 0.04, or 20%Pd). This further demonstrates the high stability of the sample as there are no detectable aggregates.

When the results obtained from DLS are concentration dependent, it is recommended that the size obtained is extrapolated to infinite dilution. Such a plot is called a dynamic Debye plot [2] and yields the true hydrodynamic radii of Albucult® at infinite dilution of 3.72nm and 3.76nm respectively for the November 2009 and February 2010 measurements.

The Effect of Adding Salt to the Apparent Hydrodynamic Radius

At high protein concentrations, a smaller size than expected is measured for Albucult®, a 66kDa protein, as seen in figure 1. This is due to so called electrostatic/virial effects, and these effects can be suppressed by addition of salt to the solution to screen the protein-protein interactions.

In figure 2(a), the change in the apparent hydrodynamic size is shown for a sample of 50mg/ml of Albucult® as the concentration of NaCl was increased. The protein concentration was kept constant during the titration.

The size obtained for 50mg/ml Albucult® at 1M NaCl is 3.6+/-0.12nm, which is very close to the values obtained from the dynamic Debye plots (3.72 & 3.76nm).

Interestingly, there is no change in the polydispersity of the sample as the salt concentration is increased, (figure 2(b)), the sample shows a PdI of around 0.02 over the full salt concentration range. This is what would be expected if the small size is due to electrostatic repulsion between molecules in the sample as discussed in the introduction section.

Summary

These measurements show how repeatable hydrodynamic size measurements by DLS can be obtained for stable protein samples. The dilution series allow the calculation of the size at infinite dilution. However, the addition of salt will ensure the measurement of the expected hydrodynamic size without any sample dilution. These two experiments provide an explanation for the apparently low size measured at the stock concentration of Albucult and suggests that it is due to molecular crowding effects. There does not seem to be any effect on the sample stability.

These results also show that, although the correct size is not measured for a highly concentrated protein in the absence of salt, the size measured can be used for relative comparisons between formulations to ensure that the sample is not changing over time or storage conditions (figure 1).

References:

1. Andreis C, Clauwaert J. Photon Correlation Spectroscopy and Light Scattering of Eye Lens Proteins at High Concentrations. Biophys J. 47 (5) 1985:591-605

2. International Organization for Standardization. 1996 ISO13321 Methods for determination of particle size distribution part 8: photon correlation spectroscopy.

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