A Taylor dispersion analysis method for the sizing of therapeutic proteins and their aggregates using nanolitre sample quantities

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Abstract

The escalating number of new therapeutic biopharmaceuticals being developed and their high value increases the need for the development of novel analytical technologies. Faster analysis time, high accuracy, low sample consumption and the ability to monitor process flow are all essential prerequisites. In this paper we explore the use of a novel analytical instrument using UV area imaging and Taylor dispersion analysis (TDA) to determine the hydrodynamic radius of BSA in an aggregated state and monitor it with time. Protein aggregation and its reversibility over time has been measured for a number of BSA samples (stressed and unstressed) by TDA with the results obtained being compared to those obtained from dynamic light scattering (DLS) and microcalorimetry. Correlations between the techniques for investigating protein aggregation behaviour were explored. The reproducibility of TDA measurements enabled the stability and reversibility of BSA aggregates to be more readily monitored than by using the other techniques.

Keywords *Taylor dispersion analysis, aggregation, dynamic light scattering, microcalorimetry, bovine serum albumin.*

1. Introduction

The presence of aggregates in biopharmaceutical products is undesirable for many reasons. A major concern is the potential immunogeneic response that aggregates can induce on administration. The detection of low levels of aggregated proteins in solution may only be determined by a limited number of techniques, many of which require in-depth method development, multi-stage sample preparation and lengthy time of analysis.

Dynamic light scattering (DLS) has been widely utilised in the area of protein characterisation. Limited sample preparation is required and it provides a relatively quick analysis time compared to other methods. However, the use of DLS however often has to be combined with other analytical techniques as the higher sensitivity of the instrument to larger particles can make analysis of some solutions problematic. This is particularly so if larger particles or dust are present in the solution.

Size exclusion chromatography (SEC) can also be used to determine the hydrodynamic volume of molecules and is widely used in industrial applications. Here, however the position of the eluted peak does not only depend on protein size but also on its shape. A second effect that can change the peak elution position is if the protein interacts with the column matrix. The presence of certain excipients, i.e. carbohydrates in formulations has been reported lead to inaccurate results in SEC. A calibration curve is therefore required and a set of relative standards is used resulting in greater analytical time and effort. The coupling of SEC with light scattering techniques is being utilised as a method of overcoming many of the problems associated with SEC analysis.

Analytical ultracentrifugation (AUC) represents the gold standard for determining the hydrodynamic properties of proteins. However, its high cost and large size along with a time consuming procedure make this method of analysis impractical for routine analysis.

The ActiPix TDA200 combines UV area imaging and Taylor dispersion analysis (TDA) for determining diffusion coefficients and hydrodynamic radii of proteins in solution. The detector monitors broadening of a band of a therapeutic protein or small molecule solution injected into a stream of buffer solution and driven through a fused-silica capillary. The band is imaged at two windows, the first on entry to and the second on exit from a loop in the capillary. The hydrodynamic radius follows from the measured differences between peak times (first moments) and variances (second moments) at the two windows. This technique was used to analyse various native, heat stressed and mixed BSA samples over periods of time. The results were compared to those obtained from DLS and microcalorimetry.

2. Materials and Methods

2.1 Materials

Bovine serum albumin (A2153, batch 18K0663) and Phosphate buffered saline tablets were purchased from Sigma-Aldrich, Dorset, UK.

Fused silica capillary was purchased from Composite metal services, Shipley, UK.

2.2 Methods

2.2.1 Taylor Dispersion analysis

TDA was performed on a TDA200 HT nano-sizing system (Paraytec Ltd., York, UK). Samples (56nl) were injected into fused silica capillary under a continuous flow of buffer (2mm/s) using a CE injection system (PrinCE, Prince Technologies B.V., Netherlands). The detector head is placed inside the CE which allows for temperature

control. The total capillary length, with ID:OD dimensions of 75:360 µm, was 143.3cm with length to first window being 46cm and length between windows 48.5cm. The capillary was cleaned between samples using a sodium hydroxide wash (1M). The UV Detection wavelength used was 214nm.

2.2.2 Dynamic light scattering

Dynamic light scattering results were obtained using a Malvern zetasizer nano-S system (Malvern, UK). Samples were placed in a semi-micro disposable cuvette and held at 25^0C during analysis. Each Sample was recorded three times with 7 sub-runs of ten seconds using the multimodal mode. The Z average diameter and polydispersity index were calculated from the correlation function using the dispersion technology software.

2.2.3 High sensitivity DSC

HSDSC analysis was performed using a Microcal VP-DSC (Microcal Inc. USA).Prior to running any samples a reference profile (baseline) was obtained using phosphate buffered saline (PBS), which was then subtracted from the sample profile. All scans were carried out at a rate of one degree per minute. The unfolding transition temperature, T_m was taken as the peak maxima and was measured by the calorimeter upon linear connection of the baseline and integration from it using ORIGIN DSC data analysis software.

2.2.4 Protein preparation

BSA was dissolved to the required concentration in Phosphate buffered saline (PBS). PBS (10 mM, pH 7.4) was prepared by dissolving the required number of tablets in distilled, de-ionised water.

Aggregation was induced by heating 2ml of a 10mg/ml BSA solution to 66^0C in a thermovac (Microcal, UK) for 20 minutes before being cooled rapidly to 4^0C.

3. Results and discussion

Figure 1 shows a typical TDA trace from the TDA200 of BSA (10mg/ml)

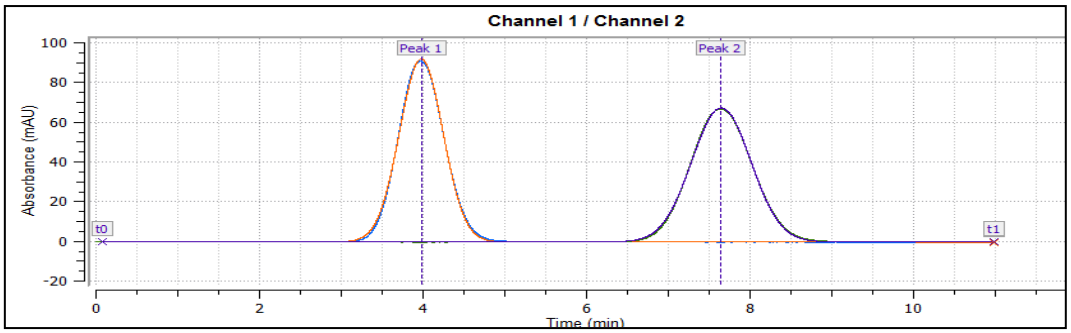

Figure 1. TDA analysis of BSA (10 mg/ml)

The hydrodynamic radius results obtained for the unstressed BSA sample using TDA were in very good agreement to those obtained using DLS as shown in Table 1.

Sample	TDA hydrodynamic radius (nm) ± SD (n=5)	DLS hydrodynamic radius (main peak value) (nm) ± SD (n=3)
BSA 10 mg/ml	4.18 ± 0.01 (RSD 0.24%)	4.17 ± 0.09 (RSD 2.23%)
Heat shocked sample A	6.57 ± 0.03 (RSD 0.50%)	9.85 ± 0.13 (RSD 1.29%)
Heat shocked sample A + 24 hours	6.45 ± 0.04 (RSD 0.60%)	10.08 ± 0.71 (RSD 7.09%)
Heat shocked sample A + 48 hours	6.44 ± 0.02 (RSD 0.25%)	9.72 ± 0.01 (RSD 0.15%)
Heat shocked sample A + 1 week	6.25 ± 0.05 (RSD 0.81%)	9.85 ± 0.59 (RSD 6.03%)
Heat shocked sample B	6.75 ± 0.06 (RSD 0.82%)	11.19 ± 0.04 (RSD 0.35%)
Heat shocked sample B + 24 hours	6.66 ± 0.05 (RSD 0.79%)	10.09 ± 0.31 (RSD 3.09%)
Heat shocked sample B + 48 hours	6.62 ± 0.01 (RSD 0.20%)	10.13 ± 0.23 (RSD 2.30%)
Heat shocked sample B + 1 week	6.45 ± 0.04 (RSD 0.63%)	10.13 ± 1.22 (RSD 1.22%)

TDA and DLS both indicated that over time some aggregates were disaggregating and that the aggregate level had decreased, however DLS was less sensitive than TDA to these subtle changes and was found not to be as helpful in drawing such conclusions. The basis of the TDA measurement is less biased to the larger sized particles when averaging, hence the difference in the average absolute size between DLS and TDA in

the presence of large numbers of aggregates. Figure 2 shows a typical DLS result for BSA (10 mg/ml)

which indicates that large particulates are present. The presence of these particulates gives rise to the higher RSD values seen when compared to the TDA results.

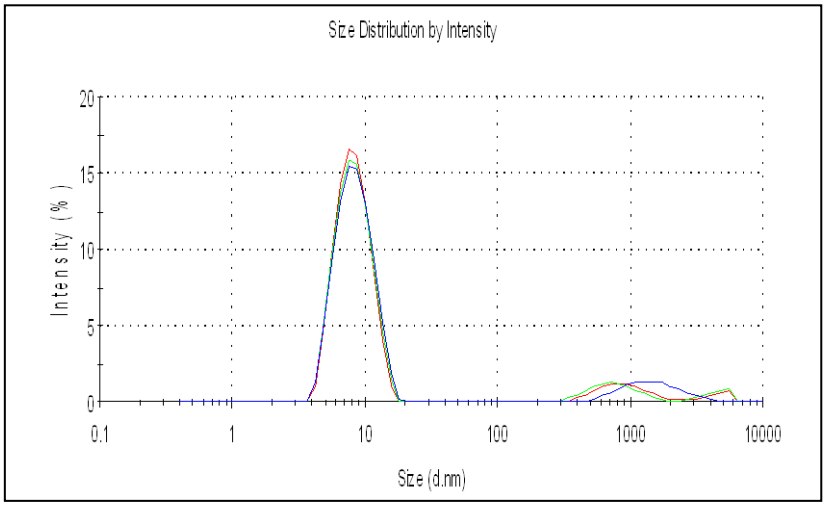

Figure 2. Typical DLS result for BSA (10mg/ml)

Figure 3 shows an overlay of TDA results of BSA before and after heating. There is a notable difference between the two traces with a reduced peak height and an increase in peak width for the heat treated sample indicating that the sample has physically changed.

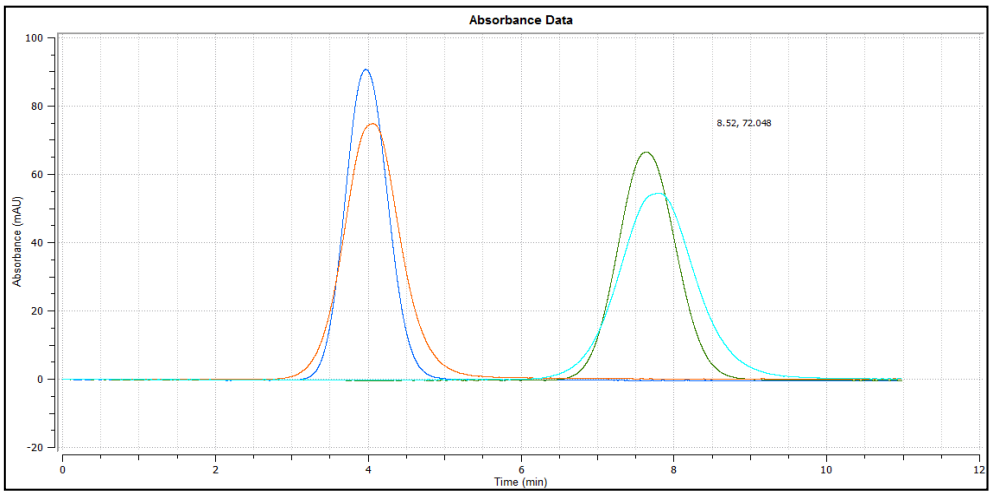

Figure 3. TDA analysis overlay of BSA (10mg/ml) and heat shocked BSA. Aggregated material analysis trace is lower in intensity at both windows.

The corresponding DLS results for the heat treated BSA samples are shown in Figure 4. There is a notable reduction in intensity of the most intense peak (compared to Figure 2) which has shifted to the right indicating an increase in particle size. The average hydrodynamic radius indicated by the DLS results therefore indicates that little monomer remains in the sample after heat treatment.

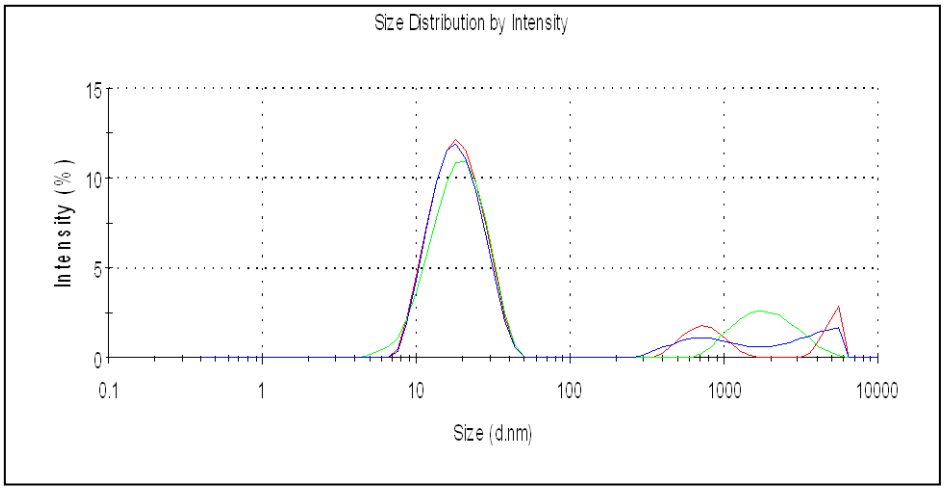

Figure 4. Representative DLS results of heat treated BSA (10 mg/ml)

The results in Table 1 show that after storage TDA detected a decrease in hydrodynamic radius of the heat stressed BSA which is indicative that the aggregates were disaggregating with time. In our hands, DLS was not as repeatable as TDA and therefore with DLS we were not able to statistically discriminate small decreases in hydrodynamic radius. In order to obtain DLS results with a lower RSD it may be necessary to filter samples, however this can induce a change in the aggregation of the samples.

Figure 5 shows a typical heating/reheating profile of BSA (10 mg/ml).

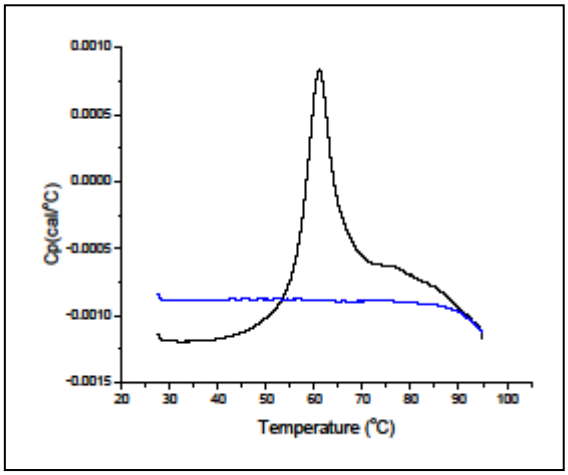

Figure 5. Thermal heat/reheat profile of BSA (10 mg/ml)

In the initial heating cycle the thermal unfolding of the sample is evident as a broad exothermic peak. In the second heating cycle there are no peaks present indicating that the thermal unfolding is irreversible.

4. Conclusions

Good agreement between the average hydrodynamic radius of native BSA samples as determined by DLS and TDA techniques was obtained. A higher level of repeatability was obtained with TDA indicated by low RSD values (less than 1% in all cases). TDA repeatability had the advantage over DLS to enable more discriminatory monitoring of the disaggregation of the stressed BSA with time.

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