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A nanolitre method to determine the hydrodynamic radius of proteins and small molecules by Taylor dispersion analysis

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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. We evaluate a novel analytical instrument that combines UV area imaging and Taylor dispersion analysis (TDA) to determine the hydrodynamic radius of proteins and small molecules in solution. Benchmarking the results against dynamic light scattering, we report the influence of injection system, injection volume, flow rates, analyte concentration and highlight the importance of washing procedures. Issues arising from the manual injection valve in the alpha laboratory system that led to high standard deviations were eliminated by incorporating an automated injector in a beta system. The hydrodynamic radii obtained show good correlation with literature values and in most cases a relative standard deviation of less than 5%.

The system is fully automated after coupling to the CE which allows for multiple injections and sample/buffer changes without operator intervention. The small sample size (approx. 60 nL), the lack of sample preparation required, and the speed of analysis (approx. 2–3 mins) makes this instrument highly applicable to the real-time analysis of inherently unstable, high cost biopharmaceutical materials where understanding their aggregation state and size is important.

1. Introduction

With increasing numbers of therapeutic proteins being produced due to the advancements in recombinant DNA technologies, the development of fast, highly sensitive analytical tools has become essential. The high value of these biopharmaceutical products lends the need to develop not only a fast and accurate method of analysis but also one that with development can be used in-line. Methods predictive of longer-term stability/quality issues are desirable, as is an ultimate goal of the intelligent switching of process flow to avoid any unnecessary loss of main component material.

The ability to size proteins in solution is limited to a number of techniques, many of which require in-depth method development, multi-stage sample preparation and required lengthy time of analysis. Dynamic light scattering (DLS) has been widely utilised in the area of protein formulation and characterisation. Limited sample preparation is required and its relatively quick analysis time makes it an essential instrument in the protein analysis laboratory. The scattering intensity of a protein in solution is proportional to the square of the molecular weight. For this reason DLS is extremely sensitive to the onset of protein aggregation caused by processing, induced stresses and storage. The use of DLS for the monitoring and detection of aggregation of several proteins has been reported in the literature. Here we mention only a few of the numerous proteins whose aggregation behaviour has been published; Lysozyme (Poznanski et al., 2005) human immunoglobulin G (Ahrer et al., 2006; Bermudez and Forciniti, 2004) β-lactoglobulin (Elofsson et al., 1996), bovine serum albumin (Adel et al., 2008). The crystallization of proteins has also been widely studied using DLS. Ovalbumin (Szymanski et al., 2005) and Lysozyme (Onuma and Kanzaki, 2007) are two model proteins whose crystallization behaviour has been studied in-depth using this technique. The use of DLS is often complemented with other analytical techniques because the high sensitivity of the instrument can make analysis of some solutions problematic in-particularly if larger particle/s are present in the solution. DLS is ineffective in the analysis of opaque systems, as light cannot be transmitted through the sample. A major problem in DLS analysis is the phenomena of multiple scattering. Multiple scattering occurs in concentrated samples due to the close proximity of particles which can lead to underestimation of hydrodynamic radius values. This leads to DLS

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generally being accepted as a method for analysis of dilute solutions only. A more in-depth and general description of the application of light scattering of proteins has been published by Murphy (1997).

Size exclusion chromatography (SEC), often also referred to as gel filtration chromatography (GFC) is used to determine the hydrodynamic volume of molecules and is widely used in industrial applications. Its high precision does not, unfortunately, come without drawbacks. 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 differently with the column matrix as it ages. The presence of certain excipients, e.g. carbohydrates, in formulations has been reported lead to inaccurate results in SEC (Ye, 2006) due to the shifting of elution position. A calibration curve is therefore required and a set of relative standards is used. Such method development increases the length of overall analytical time. Careful selection of standards is critical if UV–vis detectors are used, where the protein and the standard have different conformations, since the results obtained may be unreliable (Oliva et al., 2001). The coupling of SEC with light scattering techniques is being utilised as a method of overcoming many of the problems associated with SEC analysis (Beaullieu et al., 2005; Yi et al., 2009; Wang and Lucey, 2003).

Analytical ultracentrifugation (AUC) represents the gold standard for determining the hydrodynamic properties of proteins. The utility of the AUC for analysing monoclonal antibodies has been demonstrated by Lu et al. (2007) and Pekar and Sukumar (2007). However, its high cost and bulky size along with a time consuming procedure make this method of analysis impractical for routine analysis.

The theory of Taylor dispersion analysis (TDA) for determining diffusion coefficients and hydrodynamic radii was first described by Taylor in 1953 (Taylor, 1953) and developed further by Aris in 1956 to incorporate molecular axial diffusion. The principal of the method is based on the broadening of a solute plug under laminar flow conditions in a straight tube which has a diameter much smaller than its length. The use of silica capillary to replace glass tubes was reported in 1994 (Bello et al.) who analysed a series of small and large molecules. A limited number of research groups have reported the use of TDA for the analysis of metal nanoparticles (Wuelfing et al., 1999), polymers (Le Saux and Cottet, 2008) and amino acids (Sharma et al., 2005; Min et al., 2007). It is not known why TDA has not become more widely used and further development of any instrumentation is not presented in the literature. A likely factor is the time and cost involved in developing new technologies and the lack of literature detailing TDA as a viable technique.

The purpose of this work was to evaluate and develop the use of a novel analytical instrument based on Taylor dispersion analysis (Actipix TDA200, Paraytec Ltd., UK) to measure the hydrodynamic radius of proteins and small molecules in solution. The effects of sample size, sample concentration, flow rate, capillary dimensions, syringe driver type, system automation, data analysis software and cleaning protocols were all investigated. Day to day variability was also evaluated.

The novel analytical instrument uses 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 by a syringe pump 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 (Fig. 1).

Band broadening due to Taylor dispersion is calculated from absorbance versus time data using the peak centre times at the first and second window, t_1 and t_2 respectively, and the corresponding standard deviations, t_1 and t_2 (band broadening) using Eq. (1).

$$R_h = \frac{4k_B T(\tau_2^2 - \tau_1^2)}{\pi\eta r^2(t_2 - t_1)} \quad (1)$$

where R_h is hydrodynamic radius; K_B is the Boltzman constant; T is the temperature; η is the viscosity; and r is the radius of capillary.

The opportunity to size proteins at nano scale, at high concentration and without the use of a stationary phase is timely and would offer advantage over existing techniques. The results obtained using the TDA technique will thus be compared with those from the conventional size measuring technique of dynamic light scattering.

2. Materials and methods

2.1. Materials

Caffeine (batch 88F7708), Lysozyme (batch 90K1922), Ovalbumin (batch 126K7009), BSA (A2153, batch 18K0663), BSA (A2058, batch 097k0741), and PBS tablets were all purchased from Sigma–Aldrich, Dorset, UK.

IgG4 and hGH and other test materials were donated by Eli Lilly, Avecia and Lonza Biologics.

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

2.2. Methods

2.2.1. Sample preparation

IgG was analysed as supplied without any further preparation.

BSA, Ovalbumin and caffeine were 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.

Lysozyme was dissolved in 0.1 M sodium acetate buffer, pH adjusted to 4.6 using concentrated HCL.

Filtered samples were passed through a 13 mm, 0.2 μm PTFE syringe filter (BGB Analytik, Target, lot 311200007052).

2.2.2. Alpha laboratory instrument

Two versions of a TDA instrument were evaluated. Firstly a manual valve based injection system (the alpha system) and secondly an automated injector version (the beta system).

For the alpha system, buffer is pumped in a continuous flow (0.5 μl/min) into fused silica capillary through the injection valve (CN4 cheminert valve, Thames Restek UK, Ltd.) using a syringe driver (KR analytical, UK). Capillary then passes into the sensor head from the injection valve. The buffer passes across the first window in the capillary (which is situated directly below the sensor), it then flows through a loop of capillary before returning back through the sensor head and passing the second window (which is parallel to the first window under the sensor). Sample (10 nL) is injected into the flow of buffer via the manual injection valve.

Typical operating conditions were; 0.1–100 mg/ml solutions, 214 nm filter, Flowrate 0.5 μl/min, Capillary size 75:200 μm ID:OD. Such conditions resulted in analysis times of approximately 2–5 min per injection.

2.2.3. Beta laboratory instrument

The beta laboratory instrument is an automated version of the alpha instrument and works on the same principal. In the beta instrument however, the manual injection valve and syringe driver are replaced by a CE system (Agilent 3D CE, Agilent technologies, Basel, Switzerland), or (PrinCE, Prince technologies B.V., Netherlands). The detector head is placed inside the CE

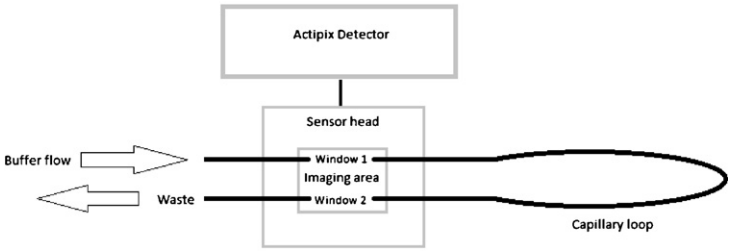

Fig. 1. Schematic representation of Actipix TDA200 instrumentation.

which allows for temperature control. Typical operating conditions were; Sample concentration 0.1–100 mg/ml, detection wavelength 214 nm, capillary size 75:200 μm ID:OD, injection volume 60 nL (50 mbar, 18 s), flow rate during run 3–4 μl/min. Under the above conditions a 30-min run time for 10 injections and analysis was possible. A method was developed for capillary conditioning/cleaning and was optimised to lower sample sticking to the internal walls and reducing the standard deviation between runs and involved alkali washing as per CE methodologies.

2.2.4. DLS

Dynamic light scattering results were obtained using a Malvern nano-S system (Malvern, UK). Samples were placed in a semi-micro disposable cuvette and held at 25 °C 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.

3. Results and discussion

The development and validation of a new instrument consisting of several different component parts presents multiple challenges. The re-designing and upgrading of software and hardware and determining the correct consumables have taken many months of collaboration. The initial problems associated with the alpha laboratory system were attributed to the manual nature of the sample injection. The valve was prone to blockage and leaks and it had no temperature control. Even though the actual injected volume of sample was only 10 nL, this procedure was sample intensive because more was consumed to ensure that the injection loop was filled completely. If not performed in this way, air bubbles produced an erroneous response that was a much larger UV response than the sample of interest. The shape of the peaks was affected by the delivery of the sample via this method. For example, a compression effect was seen as the stepper motor of the syringe pump was rotated, this lead to a cyclic trend in the radius measurements. Despite the problems incurred a number of samples were analysed and results gave hydrodynamic radius values that correlated well with those reported in the literature.

Results for a series of samples analysed on the alpha laboratory instrument are shown in Table 1.

Caffeine is included as it was used as a small molecule reference standard for the Actipix; however its small hydrodynamic radius is at the lower limit of the sizing range for DLS which was calibrated using glass standard spheres. Caffeine was selected as a small molecule standard as its analysis by TDA is reported in the literature by several authors (Leaist and Hui, 1990). The Actipix TDA200 is advantageous over these earlier methods as only nanolitre volumes are required for analysis where previously micro-litre volumes were required. Fused silica capillary allows for coupling to an autosampler permitting multiple samples to be analysed with no need for manual analyst input, thus considerably reducing experimental time and increasing data output.

DLS results (Table 1) produced high standard deviations. Since some of the samples tested were not of clinical grade, the samples were filtered and re-analysed to remove any larger impurities. After filtering the standard deviation was reduced, however values expressed as relative standard deviation were greater than 100 percent. DLS is very susceptible to the presence of large particles (scattering is proportional to sixth power of radius). Inspection of the data from these early experiments revealed that the TDA approach best correlated with those results from DLS for the highest purity samples.

The initial data analysis from the alpha system showed that some peaks were not Gaussian (required for accurate analysis) and many displayed tailing which led to high deviation between runs. The injections, however, show good reproducibility in area and height. There were small peaks seen in the second pass window immediately beneath the first pass peak. The observation was attributed to cross talk interference between the two windows. Such behaviour can be eliminated by elongation of the capillary length between the two window passes.

Brownsey et al. (2003) report the hydrodynamic radius of the native form of BSA in the pH range of 4–8 to be 3.7 nm. The results obtained for the Actipix BSA analysis are in close correlation to the

Table 1
Hydrodynamic sizing results for DLS and Actipix alpha laboratory system.

Sample	DLS Un-Filtered *Z* average diameter (nm) ± SD (*n* = 10)	DLS filtered *Z* average diameter (nm) ± SD (*n* = 10)	Actipix un-filtered radius (nm) ± SD (*n* = 10)	Actipix filtered radius (nm) ± SD (*n* = 10)
Caffeine 500 ppm	NA	NA	0.43 ± 0.04	NA
Caffeine 1000 ppm	NA	NA	0.39 ± 0.04	NA
Caffeine 2000 ppm	NA	NA	0.39 ± 0.04	NA
BSA (A2153-10G) 1 mg/ml	165 ± 190	62.5 ± 91.5	3.5 ± 0.9	2.8 ± 0.6
BSA (A2153-10G) 10 mg/ml	5.0 ± 0.8	7.8 ± 0.2	3.8 ± 0.4	3.6 ± 0.3
BSA (A2058-5G) 1 mg/ml	58.6 ± 77.3	18.3 ± 3.4	3.5 ± 0.6	3.3 ± 0.6
BSA (A2058-5G) 10 mg/ml	15.2 ± 13.5	16.8 ± 0.7	3.7 ± 0.4	4.1 ± 0.8
Ovalbumin 1 mg/ml	351 ± 551	112 ± 162	2.9 ± 0.4	2.9 ± 0.4
Ovalbumin 10 mg/ml	32.2 ± 42.1	11.7 ± 21.2	3.1 ± 0.5	3.1 ± 0.3
Lysozyme 10 mg/ml	372 ± 569	2.1 ± 0.1	1.5 ± 0.3	1.6 ± 0.3
IgG_4 25 mg/ml	6.88 ± 0.07	NA	5.56 ± 0.17	NA

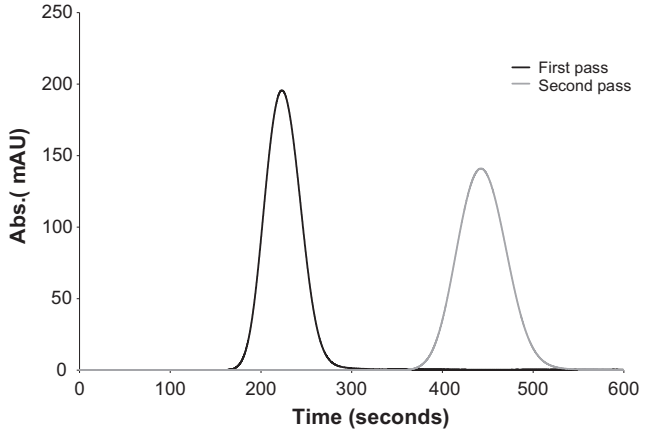

Fig. 2. Taylor dispersion analysis profile for IgG (25 mg/ml) in PBS showing first (black) and second (grey) passes through the actipix detector.

reported value. The Actipix results for the Ovalbumin, Lysozyme and IgG4 also closely correlate well to those reported in the literature (Croguennec et al., 2007; Bonincontro et al., 2001; Fujita et al., 1999, respectively). All samples gave relatively high standard deviations. The ability to discriminate between populations of heterogeneous samples would require much lower standard deviation. The high standard deviations were attributed to the issues listed previously with the manual injection valve.

An automated injection system was proposed as a solution to eliminate the problems caused by the manual valve, and the Actipix detector was coupled to an autosampler of the type used in standard Capillary electrophoresis (CE). This configuration became the beta laboratory instrument.

Method development to reduce deviation and increase reproducibility between runs was carried out using caffeine on the beta instrument. A concentration dependent increase in radius was observed at higher caffeine concentrations, therefore the concentration of caffeine was limited to 1000 ppm. Reproducibility was unaffected by change in the concentration of caffeine over the range used in the study. The capillary length was then varied in order to minimise deviations between runs. The alpha instrument results indicated that overlapping peaks would be more likely obtained if there was not enough distance between the two analysis windows, this was therefore increased. The total length of the capillary was also increased with the initial and final portions (before and after analysis windows) being doubled in length to reduce pressure effects. Increase in capillary length as described above resulted in an overall reduction of RSD from 25% to 9–10%. Flow rates of the buffer were then optimised to give a further reduction in RSD to 8%. Automation of the instrument by coupling to an autosampler reduced the RSD yet further to approximately 3%. Due to the nature of the injection method on the automated system it was then possible to manipulate sample size injected which had been fixed previously due to the manual valve having a defined sample loop volumes. Sample size injection was evaluated between 30 and 65 nL, with optimal reproducibility was seen in samples between 55 and 60 nL. Finally a cleaning protocol was developed utilising sodium hydroxide solutions. This enabled capillary cleaning along with conditioning of the inner wall of the capillary. Reproducibility of the system to analyse caffeine had been optimised to give RSD values of less than 1%. This optimised method was therefore adopted for all further analysis of samples using the beta instrument. Several software improvements to this point enabled more accurate peak fitting which yielded better reproducibility much more advanced and fitting of the peaks. Fig. 2 shows a typical Taylor dispersion profile for IgG (25 mg/ml in PBS) obtained using the beta laboratory instrument after method optimisation.

Table 2
Hydrodynamic radius results using Actipix beta laboratory instrument.

Sample	Radius (nm) (n = 10)
Caffeine (1000 ppm)	0.342 ± 0.007 (RSD 2%)
IgG_4 (mg/ml)	5.56 ± 0.15 (RSD 2.7%)
BSA (A2153-10G) 10 mg/ml	3.91 ± 0.03 (RSD 0.8%)
BSA (A2153-10G) 100 mg/ml	4.84 ± 0.03 (RSD 0.6%)
Ovalbumin 1 mg/ml	3.24 ± 0.07 (RSD 2.2%)
Ovalbumin 10 mg/ml	3.24 ± 0.04 (RSD 1.2%)
Ovalbumin 100 mg/ml	4.15 ± 0.02 (RSD 0.5%)
hGH 1 mg/ml	2.59 ± 0.15 (5.8% RSD)

Table 3
Hydrodynamic radius results for a series of caffeine samples.

Sample	Radius (nm)	Stdev (nm)	RSD (%)
0.50 mg/ml (n = 30)	0.351	0.007	2.09
0.75 mg/ml (n = 30)	0.343	0.005	1.55
1.00 mg/ml (n = 90)	0.347	0.005	1.40
1.25 mg/ml (n = 30)	0.357	0.007	1.96
1.50 mg/ml (n = 30)	0.356	0.004	1.07
3.00 mg/ml (n = 30)	0.368	0.004	1.00
10 mg/ml (n = 89)	0.425	0.009	2.05
20 mg/ml (n = 30)	0.491	0.008	1.55

Table 2 shows results for the Actipix beta laboratory instrument.

The results for all of the samples show a notably reduced standard deviation with relative standard deviations (RSD) in all the samples with the exception of hGH being less than 3%.

Further experimental work involved limited validation of the system to determine the precision and linearity of the beta laboratory instrument. Initial work involved the analysis of caffeine as the chosen standard. Multiple solutions were made, a selection in triplicate, and the results for these samples are shown in Table 3.

The low RSD values obtained are comparable to traditional sizing techniques. The instrument automatically calculates the hydrodynamic radius in the software, therefore a 30 sample injection set can be obtained in only 60 min and expend less than 0.002 ml of material. This gives the Actipix beta laboratory system multiple advantages over other sizing techniques. It is apparent from the results that there is an increase in hydrodynamic radius with caffeine concentration; this is attributed to the reported tendency of caffeine to self associate into dimers (Danilov and Shestopalova, 2004; Origlia-Luster et al., 2002; Poltev et al., 2004).

The same series of dilutions were carried out for Ovalbumin; the results are shown in Table 4.

The results for the ovalbumin dilution series show that there is again a low standard deviation for the samples. None of the samples were filtered and/or dialysed prior to analysis which indicates that even without sample preparation the Actipix gives very reproducible results. The results also show that the concentration of ovalbumin has little effect on the hydrodynamic radius within the concentration range studied and indicates protein–protein interactions that are less concentration dependent. This is an indication that ovalbumin has a lower tendency to self-associate than caffeine under the conditions used.

Table 4
Hydrodynamic radius results for a series of ovalbumin samples.

Sample	R_h (nm)	Standard deviation (nm)	RSD (%)
0.50 mg/ml (n = 30)	3.123	0.173	5.55
0.75 mg/ml (n = 29)	3.161	0.089	2.79
1.00 mg/ml (n = 88)	3.195	0.120	3.75
1.50 mg/ml (n = 27)	3.232	0.077	2.39
3.00 mg/ml (n = 27)	3.165	0.037	1.19
10 mg/ml (n = 89)	3.211	0.135	4.19
20 mg/ml (n = 30)	3.259	0.274	8.40

Table 5
Hydrodynamic radius results for a series of IgG_4 samples.

Sample	R_h (nm)	Standard deviation (nm)	RSD (%)
1.25 mg/ml (n = 25)	5.54	0.17	2.98
6.25 mg/ml (n = 30)	5.59	0.16	2.95
12.5 mg/ml (n = 90)	5.96	0.07	1.17
25 mg/ml (n = 30)	5.80	0.22	3.77

A limited number of IgG dilutions were analysed (Table 5) and a general trend of increased protein–protein interaction with concentration is observable in the hydrodynamic radius values but this is not statistically significant.

The results show that the analysis of a larger molecular weight and potentially commercial antibody product is as readily obtainable and accurate on the instrument as small molecule and protein standards. The RSD values are well within accepted industrial standards and the potential application of an online version of this instrument make it extremely interesting as a commercially applicable technique. Full temperature control of the detector was achieved through the thermostat compartment of the CE and this was thought to contribute to the improved standard deviations over the manual loop injector. In addition, the auto sampler's reproducibility of sample delivery was also thought to be a contributory factor.

We anticipate the system to have potential in the detection of aggregates and the likelihood of their formation. We are exploring the utility of the system to screen formulation and purification buffer scenarios.

4. Conclusions

The actipix detector is able to size proteins accurately over a wide range of conditions without the need for dilution or any stationary phase interaction. In this paper we have shown that coupling the TDA analysis to an automated injection system resulted in excellent reproducibility between runs and that by optimising the method, RSD values typically less than 3% were obtained. Hydrodynamic radii have been determined at different concentrations and have been shown to increase with concentration for caffeine indicating self-association. Ovalbumin did not show this self-association behaviour to the same extent. Determined hydrodynamic radius was shown to be most repeatable under the conditions employed for samples with injection volumes of the range 55 and to 60 nL.

The small sample size required (60 nL) and the speed of analysis (approx. 3 mins) makes this instrument extremely useful for high cost biopharmaceutical materials.

Other advantages include the limited method development required and ability to handle a wide range of sample concentrations and buffer types without prior manipulation being required or exposure to a stationary gel phase.

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