A novel approach to measurement of hydrodynamic radius for a standard protein using UV area imaging detection

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This article describes a novel approach to measurement of hydrodynamic radius for a standard protein using UV area imaging detection. The method allows proteins to be used in their native form, without any labelling or denaturation. A plug of protein solution is injected into a fused silica capillary, driven through the capillary by application of pressure, and detected using UV area imaging as it passes windows at entrance to and exit from a loop in the capillary. The radius of the protein is determined by analysis of band broadening due to Taylor dispersion. The method is applicable over a wide concentration range and uses only nanolitres of sample.

Overview of UV area imaging

UV area imaging has been developed on a commercial basis by Paraytec Ltd through their patented ActiPix™ technology. The ActiPix is the world's first UV area imaging detector, designed for on-line monitoring at a single UV wavelength, particularly for use with separation methods such as liquid chromatography (LC) and capillary electrophoresis (CE).

The miniature size allows the detector to be used as a 'plug and play' accessory linked to existing separations instrumentation and in line with a mass spectrometer. Detection is performed at a selected wavelength by means of exchangeable filters. When light is shone through a liquid-filled capillary, the liquid inside the capillary and the capillary vessel combine to act as a cylindrical lens. With a fused silica capillary, there is excellent light transmission in the UV down to 190 nm.

A key innovation of this technology is use of an area imaging array instead of the more usual linear photodiode array. This allows light passing through the sample in the capillary to be readily referenced against light from the same source. This process is illustrated using the ray diagram in Figure 1. The light focused through the centre of the capillary is represented in blue, the reference light is represented as yellow, and the boundary between reference and sample is represented by the purple lines. The major benefit of using this self-referencing process is that it is independent of any light fluctuations, resulting in a very stable absorbance signal output.

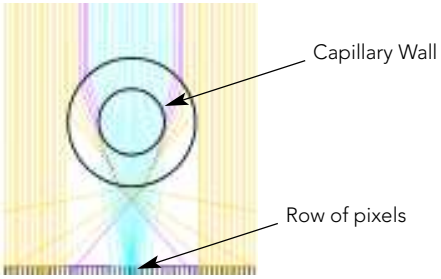

Figure 1. Ray diagram illustrating the basis of self-referencing system used in the ActiPix

An additional benefit is use of the area detector with multiplexed separations: up to 8 capillaries can be imaged side by side using the ActiPix area detector. Since each capillary acts as a combined UV lens and sample vessel, no external optics or expensive UV lenses are required.

Principles of measuring hydrodynamic radius

In this method a looped capillary is used, essentially taking the space of two capillaries. A plug of the sample (typically a few nanolitres) is injected at the capillary inlet and driven by application of external pressure along the capillary. UV absorption of the protein zone is recorded during the 1st and 2nd passes through the imaging area (Figure 2). Whilst the area of the peak is the same, the widths of both peaks are different: the signal from the second window has a greater width and lower amplitude due to Taylor dispersion. Taylor dispersion analysis (TDA) provides an absolute, simple and rapid method for determining values of diffusion coefficients and hydrodynamic radii, as exemplified in work by Cottet et al..[1,2] Previous work[1,2] was carried out using capillaries with a single detection window, and experiments run over a range of pressures to allow contributions to variance due to Taylor dispersion to be separated from those due to other sources such as peak injection. Our approach using area imaging and two windows allows all data to be collected extremely quickly in a single experiment. The peaks are fitted with an appropriate peak fitting function using

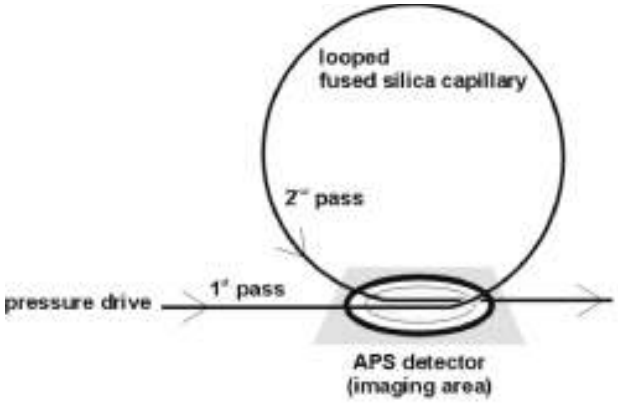

Figure 2: Schematic representation of method for sizing using looped capillary passing over the ActiPix

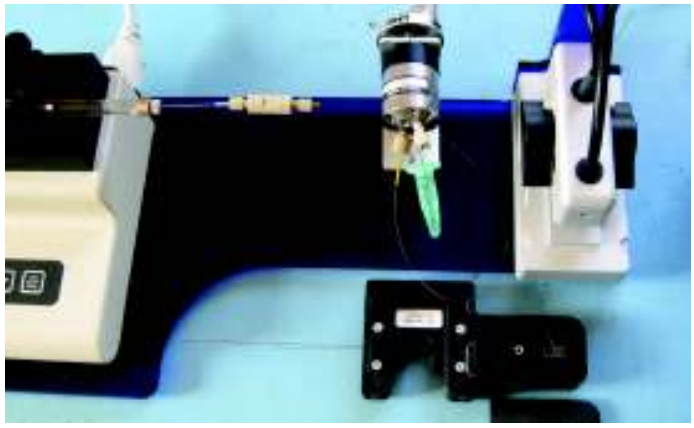

Figure 3. Integrated system for sizing with syringe pump, injection valve and ActiPix detector. Sizing cartridge shown disassembled.

commercially available software. The area under the peak corresponds to the amount of the protein injected. The standard deviations are used to calculate the hydrodynamic radius of the protein.

Experimental

Instrumentation and materials

The ActiPix D100 UV imaging system was used in conjunction with a Sizing cartridge and a 214 nm wavelength filter. Commercial CE systems provide convenient arrangements for injection and pressure drive through capillaries. Note that the method will work with most CE instruments, provided that a cartridge is available (e.g. that for MS) bringing the capillary outside the instrument. A 75 µm I.D., 200 µm O.D. fused silica capillary (Polymicro Technologies) of length 90 cm was used in sizing measurements with the ActiPix detector. Appropriate lengths of the various capillary sections for a PrinCE CE system are 30 cm to first window, 30 cm loop, 30 cm to outlet, and for an Agilent HP3D CE system 45 cm to first window, 30 cm loop, 15 cm to outlet.

Bovine serum albumin (BSA) was obtained from Sigma Aldrich (A7906). 50 mM borate at pH 8.92 was prepared by adjusting the pH of 100 mM boric acid to pH 8.92 with 100 mM LiOH and adding the appropriate amount of HPLC grade water to bring to volume. Sample solutions comprised BSA dissolved in buffer, with concentrations in the range 0.05 - 5.0 mg ml^{-1}.

The sequence for use with a PrinCE instrument was: rinse out the capillary with buffer for 1 min at 1000 mBar; inject sample for 6 s at 100 mBar; inject buffer for 6 s at 100 mBar to prevent any leakage of sample; start run; apply pressure (250 mBar) for 2.5 min.

For rapid size determination of pure proteins, an integrated system (Figure 3) available from Paraytec uses a syringe pump to drive the flow, an injection valve to introduce a nanolitre volume of sample into the capillary flow line, and a sizing cartridge in which the capillary is looped to provide the 2nd pass in the reverse direction to the 1st pass over the ActiPix UV area imager.

Results and data analysis

Figure 4 shows the absorbance versus time plot of a single injection of BSA (1.0 mg ml^{-1}) making two passes over the detection area. The band broadening in this pressure driven flow is clearly evident in comparing the peak profiles on 1st and 2nd pass.

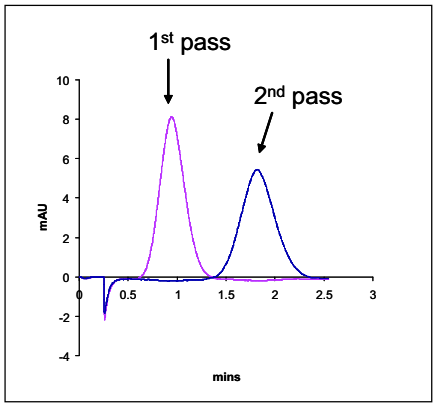

Figure 4: Absorbance vs time plot of single injection of BSA (1.0 mg ml^{-1}) in two passes over the ActiPix imaging area. PrinCE capillary electrophoresis system used to provide injection and pressure drive.

Data is processed to obtain the peak centre times at the first and second capillary windows, t_1 and t_2 respectively, and the corresponding standard deviations, τ_1 and τ_2. An example of suitable peak fitting software is PeakFit, available from Systat Software. Symmetric peaks such as those in Figure 4 are analysed with a Gaussian function. The difference in variance between the two peaks due to Taylor dispersion is given by

$$\tau_2^2 - \tau_1^2 = \frac{r^2 (t_2 - t_1)}{24D} \qquad (1)$$

where r is the radius of the tubing and D is the diffusion coefficient of the protein.

The diffusion coefficient is related to the hydrodynamic radius, R_h, via the Stokes equation

$$D = \frac{k_B T}{6\pi\eta R_h} \qquad (2)$$

where k_B is the Boltzmann constant, T the absolute temperature and η the viscosity of the solution. For dilute solutions used in these experiments, the viscosity of the solution may be assumed to be that of water at that temperature. Combination of Equations 1 and 2 gives the equation used to calculate R_h directly from the times and standard deviations of the protein peak measured at the first and second passes over the imaging area.

$$R_h = \frac{4k_B T (\tau_2^2 - \tau_1^2)}{\pi r^2 (t_2 - t_1)} \qquad (3)$$

Values determined using Equation 3 for the radius of BSA over a range of concentrations are given in Table 1. Three repeat

measurements were made at each concentration. The standard deviations in the values of the radius are in all cases seen to be 5% or less.

Table 1: Measured hydrodynamic radius for BSA across a wide concentration range*

Concentration (mg mL^{-1})	Hydrodynamic radius (nm)
0.05	3.26 ± 0.16
0.25	3.22 ± 0.08
0.50	3.64 ± 0.11
1.00	3.35 ± 0.05
2.00	3.39 ± 0.06

** PrinCE CE instrument, temperature 25 °C, 50 mM borate buffer pH 8.92; data analysis using Gaussian peak fitting model.*

Conclusions

Measurements of hydrodynamic radius have been carried out for bovine serum albumin over the range 0.05 – 2 mg mL^{-1}, in a 50 mM borate buffer at pH 8.9. The experimental setup with a single capillary looped through the same imaging zone provides for continuous flow of the sample plug, allowing measurement of plug profile change during pressure-driven traversal of the loop. The ActiPix D100 UV area detector uniquely images the same capillary in two locations, allowing measurement of time-dependent band broadening and hydrodynamic radius. Each run takes less than 3 minutes.

Further applications

This method provides a quick, easily integrated technique to determine hydrodynamic radius of proteins. An important benefit is that proteins do not require denaturation and can be analyzed in standard buffer solutions. Whilst borate was used here, other measurements using Taylor dispersion analysis have been reported in phosphate buffers[1] and there should be no problems with use of the ActiPix detector in any protein solution media. The setup is easily converted to other experimental configurations within minutes, for example to make change of UV monitoring wavelength from 214 to 280 nm. This is a label-free technique applicable to proteins in their native conformational states, and there is no requirement for protein derivatisation or staining. Another benefit is the potential to obtain the concentration of the protein in the sample solution at the same time as its radius. Concentration may be calculated from the absorbance integrated across the peak, the volume injected, and the molar absorption coefficient.[3] Finally, the method using Taylor Dispersion Analysis to determine hydrodynamic radii is applicable over a wide range of sizes and molar masses (e.g. pharmaceuticals, peptides, protein aggregates).

Acknowledgement

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References

1. *Cottet, H; Biron, J-P; Martin, M. Anal. Chem. 2007, 79, 9066-9073.*
2. *Cottet, H; Martin, M; Papillaud, A; Souaïd, E; Collet, H; Commeyras, A. Biomacromolecules 2007, 8, 3235-3243.*
3. *Pace, C N; Vajdos, F; Fee, L; Grimsley, G; Gray, T. Protein Sci. 1995, 4, 2411-2423.*