

The Diffusion Barrier Technique, Practical Aspects and Data interpretation



Introduction

The diffusion barrier technique is a method for reliably measuring the electrophoretic mobility of proteins by minimising the impact of the measurement process [1,2].

Electrophoretic mobility is the rate of movement of a molecule or particle to an electrode in response to an applied electric field. Laser Doppler electrophoresis is commonly used to measure electrophoretic mobility. However, contact of the protein with the electrodes required for the application of the electric field is known to damage proteins and can cause them to denature and aggregate [3]. It is often erroneously thought that it is the electric field itself of a few volts per centimetre that causes the protein aggregation. In fact, it is contact of the proteins with the electrodes that causes the aggregation. Furthermore, capillary zone electrophoresis, a common technique used for the separation and characterisation of proteins, uses field strengths of hundreds of volts per centimetre and does not suffer a similar problem.

The diffusion barrier technique separates the sample molecules from the electrodes, so the effect on the protein caused by the measurement is dramatically reduced. The diffusion barrier technique manages this by introducing a small plug of sample e.g. 20 μL , separated from the electrodes by the same buffer that the protein is dissolved in. The physical distance between the sample and the electrodes means that the sample is protected from damage by contact with the electrodes. Since the protection is afforded simply by the distance of the sample from the electrodes, the sample is protected for as long as it takes for it to diffuse to the electrodes. This can be many hours, which makes measurement of the sample much more reliable, as the mobility of only the soluble native protein is measured [3].

This application note describes the practical considerations of using the diffusion barrier technique in order to achieve the best results.

Size measurements

It is recommended that a size measurement of the sample is made before and after the zeta potential measurement.

Ideally, the sample should be monodisperse i.e. contain no aggregates. The size distribution should consist of a single peak and the polydispersity index (PDI) should be as low as possible, ideally below 0.1. An increase in the PDI after a



Figure 1: A. Cell entirely filled with blue dextran in 10 mM NaCl. B. Cell filled with 10 mM NaCl and loaded with 50 μL blue dextran for the diffusion barrier

measurement is an excellent indicator that aggregates have begun to form. The protein mobility measurement type in the Zetasizer software version 7 and above will automate these size and mobility measurements.

Loading the sample

The method is to first fill the folded capillary cell with the buffer that the protein is dissolved in using the usual Luer fitting syringe. It is then helpful if the cell is put into the Zetasizer for 2 to 3 minutes to allow the temperature to equilibrate. This will reduce any temperature related fluid motion such as convection. The cell should then be removed and between 20 and 100 μL of the sample pipetted directly into the bottom of the cell.

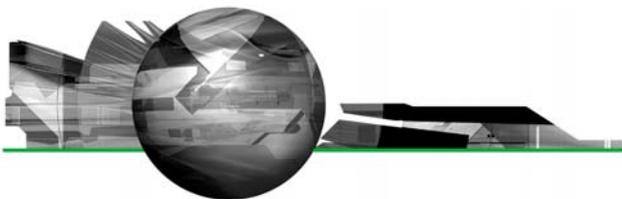


Figure 2: 200 µl gel loading tips ideal for loading a cell for the diffusion barrier method.

This is best achieved using a gel loading tip such as the one shown in Figure 2. Using this tip, it is possible to reach to the bottom of the inside of the folded capillary cell and load the sample directly into the position in the cell where the mobility measurement is made (Figure 3). In order to produce the best data, it is important to make sure that any mixing of the sample with the buffer is minimised during loading. In the folded capillary cell, the measurement window is at the very bottom of the U-bend so the sample must be loaded into this region. In order to minimise the diffusion of the sample within the cell, the sample buffer must be the same as the buffer making up the diffusion barrier.



Figure 3: Gel loading tip in folded capillary cell for loading sample plug for the diffusion barrier technique.

Figure 1 shows two folded capillary cells loaded with 4 mg/mL blue dextran dissolved in 10 mM NaCl to illustrate this. The cell on the left has been filled completely with blue dextran and the sample is clearly visible throughout the cell. The cell on the right has been loaded using the diffusion barrier technique. This cell was prefilled with 10 mM NaCl and the temperature allowed to equilibrate. 50 µL of the 4 mg/mL blue dextran solution was then loaded into the measurement volume using a pipette and gel loading tip. The cell was then carefully capped. It is clear that the blue plug of the sample is sitting in the measurement volume of the cell and is a considerable distance from the electrodes at the top of the cell.

If the cell is kept upright and handled carefully, the sample will not diffuse around the cell.

Before the measurement is started the temperature should be allowed to equilibrate.

Making a mobility measurement

In principle, a mobility measurement using the diffusion barrier technique is exactly the same as when making a conventional measurement in the Zetasizer Nano. Light scattered by a molecule or particle undergoing electrophoresis is combined with a reference beam and the rate of change of phase of the combined beam is measured and used to calculate the electrophoretic mobility and thus the zeta potential.

In practise, a couple of changes are required to improve the data quality of the measurements made using the diffusion barrier technique.

In order to avoid significant Joule heating, a voltage should be selected based on the conductivity of the buffer used. A buffer with a higher ionic strength will have higher conductivity resulting in increased Joule heating at a given voltage. An appropriate voltage is selected automatically in the software, but for these measurements, improvements can be made by setting a lower voltage.

Table 1: Suggested voltages and maximum number of sub-runs for given ionic strength buffers.

Salt concentration, mM	Voltage, V	Runs
200	20	20
100	40	20
10	150	40

Table 1 shows which voltage values are most appropriate for a solution of given ionic strength and the maximum number of sub-runs that should be performed at these ionic strengths. The voltage used can be set in the 'Advanced'

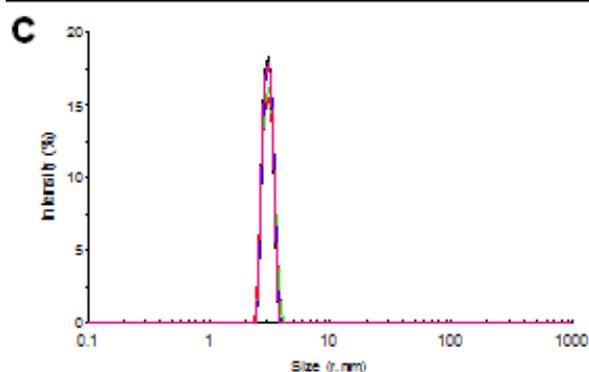
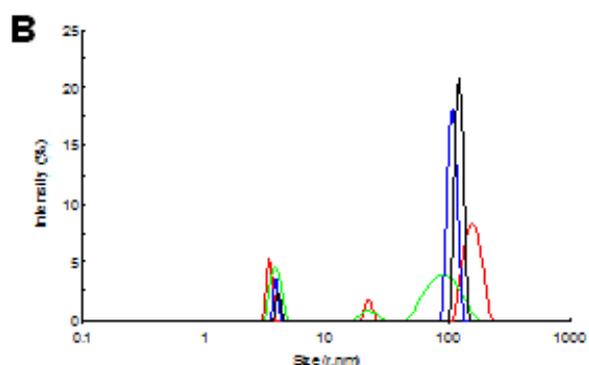
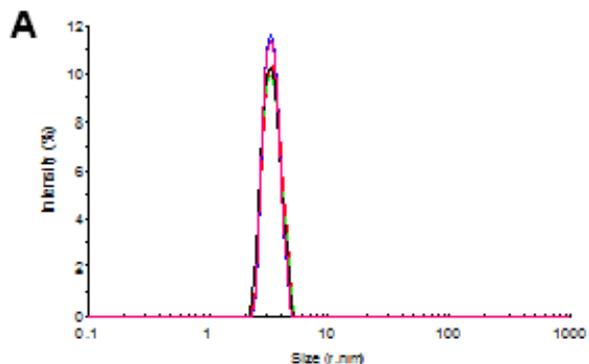
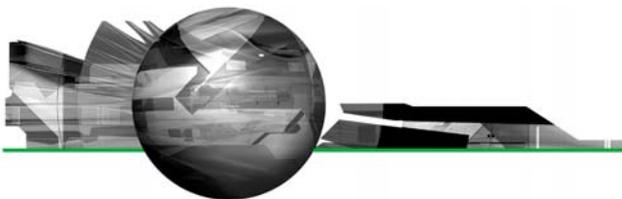


Figure 4. A. Overlaid size distributions of HSA before mobility measurements. B. Overlaid size distributions of HSA after conventional mobility measurements. C. Overlaid size distributions after mobility measurements using the diffusion barrier technique.

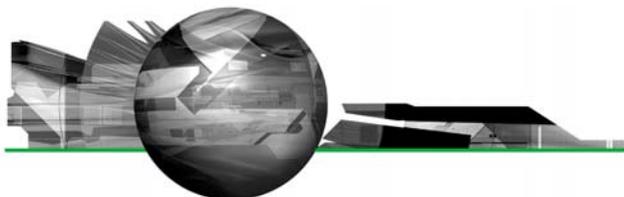
branch of the SOP option tree. In addition, the 'measurement duration' (set in the 'measurement' section of the SOP) should be set to manual and the number of runs should be set to a maximum of 20, again to minimise Joule heating.

Finally, at least 5 measurements should be made to ensure that the measured values are repeatable. It is recommended that a 180 second delay is used between measurements, again to ensure that the temperature is equilibrated between measurements.

The protein mobility measurement type in the Zetasizer software version 7 and above will automate all of these settings making the measurement as simple as possible.

Assessing Data Quality

It is recommended that size measurements are made both before and after the zeta potential measurements to check the sample has not changed as a result of the measurement. If the mobility measurement has affected the sample and caused it to aggregate, these aggregates should be present in the data from the size measurement following the mobility measurements. Figure 4A shows examples of size measurements before any mobility measurements. This sample is aggregate free and has a PDI <0.05. After a conventional zeta potential measurement, aggregates have formed and the PDI is close to 0.2 (Figure 4B). However, when the diffusion barrier technique is used, no aggregates form and the PDI after the mobility measurement remains below 0.05 (Figure 4C).



Additionally, the frequency plot can be used to monitor the presence of aggregates in the sample. The frequency plot available in the software shows the distribution of frequencies in the scattered light. For proteins, which have a significant Brownian component in their overall mobility, the peak in the frequency plot is expected to be broad. Aggregates in the sample will have lower Brownian motion, resulting in a sharp peak in the frequency plot. The presence of a broad peak in the frequency plot is therefore an excellent way to identify high quality data. A frequency plot containing a sharp peak is highly likely to contain information derived from aggregates and as these will have a different mobility to the native protein, the wrong answer will be obtained. Figure 5 shows an example of two frequency plots of protein mobility measurements. Figure 5A shows a broad peak where the diffusion barrier technique was used. This is repeatable with the plots from consecutive measurements overlaying well and indicating that there are no aggregates present. Figure 5B shows an overlay of consecutive measurements where the diffusion barrier technique was not used. The first measurement is the red line which shows a broad distribution and a good measurement. However, as the measurements progress indicated by the green blue and then black lines, it is clear that a large and sharp peak is forming, which represents the sample as it is aggregating during the measurement. This plot is not consistent and hence repeatable measurements of mobility will not be obtained.

One of the effects that will reduce the repeatability of the measurement is Joule heating, which is the heat that is generated when an electric field is applied to a conductive solution. The conductivity measurement is an excellent way to identify the presence of any Joule heating that occurs as Joule heating causes the conductivity of the sample to increase. In order to minimise this effect, the conductivity should be limited to an increase of no more than 5-10% between consecutive measurements. If it has increased more than this, it is an indication that significant heating has occurred, which may affect the sample and will reduce the repeatability of the measurement. In this case a longer

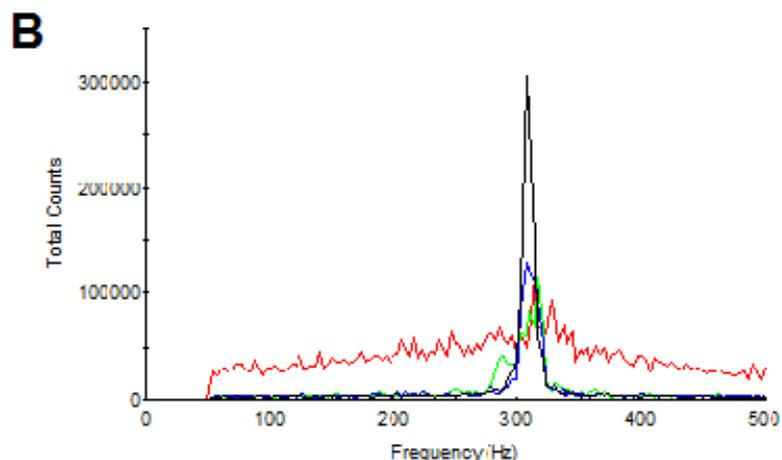
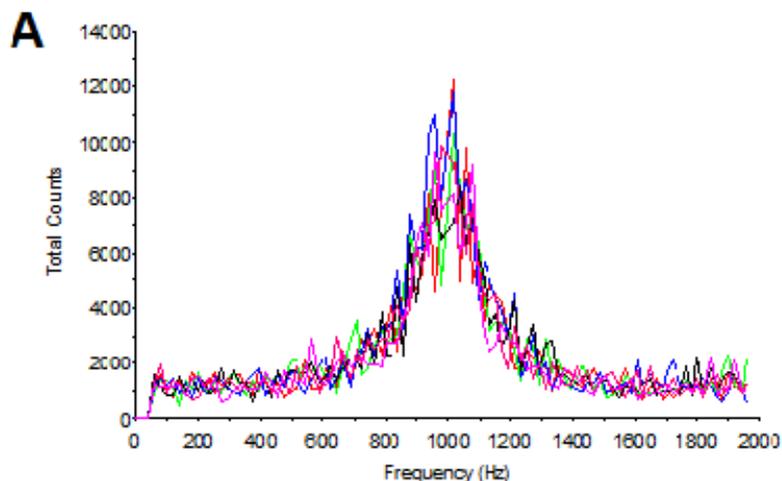
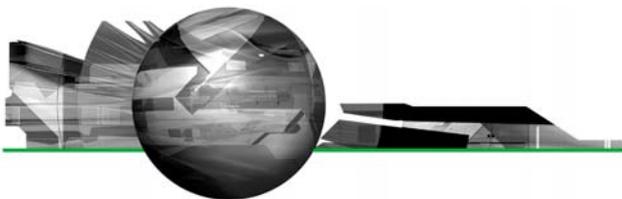


Figure 5: A. Overlaid frequency plots from consecutive measurements made using the diffusion barrier technique.
B. Overlaid frequency plots from conventional mobility measurements.

equilibration time between measurements should be allowed.

Finally, it is always recommended that repeat measurements are made. At least 5 measurements should be performed to see that the data is repeatable, and during method development, it is recommended that a cheap substitute protein is tested in order to optimise the methodology.



Analysis

If the tests of data quality all suggest that the results are reliable, that aggregates have not been formed, and that repeatable measurements relate to the native form of the protein, then the most appropriate way to report the data is the next consideration.

Laser Doppler electrophoresis measures the electrophoretic mobility of molecules and particles. Typically, this is related to the sample zeta potential using Henry's equation and in aqueous systems, the Smoluchowski model is often used for simplicity. However, the Smoluchowski model is not always appropriate for proteins so it is generally recommended that the measured electrophoretic mobility is quoted along with the temperature, rather than the calculated zeta potential.

The protein charge calculator in version 7 and above of the Zetasizer software will allow the overall protein charge to be calculated based on the results of the mobility measurement.

Conclusions

Using the diffusion barrier technique, highly repeatable measurements of the mobility of proteins can be made without any damage to the protein itself.

This application note has described a protocol using a diffusion barrier technique that protects the protein from denaturation and aggregation during the measurement by keeping the sample away from the electrodes.

A number of tests have been described to monitor data quality to ensure that the mobility measured is that of the native protein and not of aggregates that may have formed during a measurement using inappropriate settings.

References

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[3] Corbett & Jack, *Colloids & Surfaces A: Physicochemical and Engineering Aspects*. (2010) 376 pp31-41

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