Off and On: Advances in Nanoparticle and Protein Electrophoretic Mobility Measurements by MP-PALS in Batch and Online Modes

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Nanoparticle formulations are ubiquitous throughout modern pharmaceutical development; from protein biotherapeutics and vaccine adjuvants through to virus-like particles and emerging novel drug delivery systems. However, developing these complex formulations and suspensions is a challenging process. Proteins and nanoparticles are often unstable in suspension, with aggregation a particularly pressing concern. Unchecked aggregation can reduce the bioavailability of a nanotherapeutic or, for proteins, even induce a dangerous immunogenic response in the patient. Developing a stable formulation is therefore critical to meeting performance, safety and commercial targets.

Characterisation of the net molecular charge of proteins in solution is a key component in optimising formulation stability. Likewise, the zeta potential of nanoparticles is an essential predictor of stability. While conventional techniques for determining electrophoretic mobility (and hence net molecular charge or zeta potential) are generally able to analyse electrophoretic mobility of nanoparticles reliably in many formulations, they may fail under certain conditions such high-salt buffers, and are prone to damaging the sample when measuring proteins or other biomacromolecules. Massively Parallel Phase Analysis Light Scattering (MP-PALS) overcomes these challenges to provide reliable, reproducible and nondestructive electrophoretic mobility measurements of fragile biomolecules and other particles in practically all formulation buffers.

This article explores the technological advantages of MP-PALS and its role across bio/nanopharmaceutical development. Case studies illustrate how automated MP-PALS enables rapid, high throughout analysis of electrophoretic mobility and particle size in formulation. Fluorescent particles that are usually unsuited to standard zeta potential analysis are readily characterised by MP-PALS. Data are also presented on new investigations into online coupling of MP-PALS with Size Exclusion Chromatography (SEC). This novel technique performs electrophoretic mobility measurements in real-time as samples are eluted from the SEC column, providing mobility measurements across the entire size distribution.

The Challenges of Mobility Analysis

Electrophoretic mobility is the measure of particle movement in solution as a result of its charge. Zeta potential (defined as the magnitude of charge surrounding the molecule in solution) and molecular net charge are determined by combining mobility measurements with particle size, typically measured as hydrodynamic radius (R_h) via dynamic light scattering (DLS). Systems with a strong positive or negative zeta potential, around ±30 mV or more, are considered inherently stable as particles repel one another in formulation. The closer zeta potential and electrophoretic mobility values come to 0, the isoelectric point, the greater the chance of flocculation and aggregation.

The method of determining electrophoretic mobility is relatively straightforward. An electric field of several V/cm is applied to charged particles in solution. If the particles are positively charged, the mobility will be positive, if they carry no charge mobility will be 0, and if they are negatively charged particles will move in the opposite direction to the field and mobility will be negative. Particle velocity is then determined using a laser Doppler shift analysis (phase analysis light scattering, or PALS) and the mobility is calculated as the ratio of the terminal velocity to the applied field *(Figure 1)*.

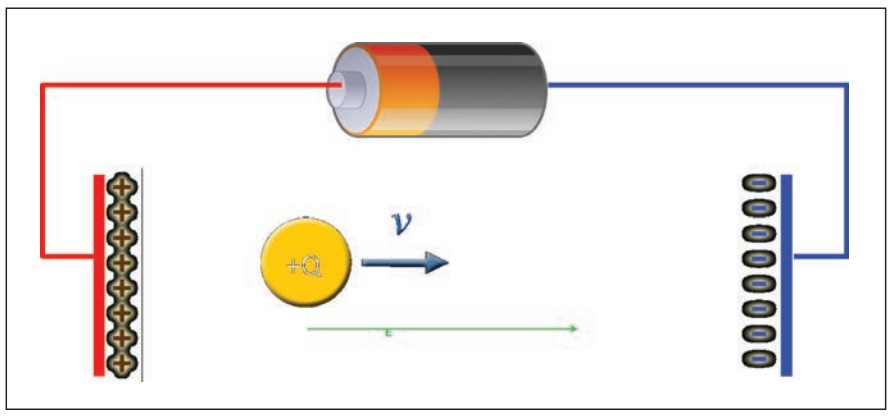

Figure 1. Applying an electric field to protein particles in formulation enables the measurement of electrophoretic mobility, a key stability predicting parameter.

Measuring protein and nanoparticle mobility presents a unique set of challenges, particularly for aqueous samples with conductivities near or above physiological saline conditions. Particles in the nano size region do not scatter much light and instruments traditionally used for zeta potential analysis are not designed to measure particles below ~ 10 nm in size. Moreover, Brownian motion dominates movement in this nano region, making it difficult to extract electrophoretic drift from the noisy background of random motion. Integrating many scans to pinpoint electrophoretic mobility is one way of overcoming this limitation. This approach entailing prolonged exposure to electrical fields may damage the sample, leading to diminishing returns on data reliability as the experiment progresses.

Another common solution to improving signal-to-noise is increasing the applied voltage to enhance electrophoretic drift. However, high voltages often produce redox reactions and electrolysis on the surface of the electrodes, which in turn produces gas bubbles. The resultant higher electrical current also heats the solution, producing thermal convection. Interference from the gas bubbles and convection currents distorts the driving electric field and decreases the reliability of the mobility data; bubbles may also interfere with light scattering signals. These effects are particularly prevalent at higher salt conditions where currents are higher and electrochemical effects more prevalent. Finally, high voltages also damage delicate samples, literally 'burning' them to the electrodes. Within biopharmaceutical development, where fragile, high cost and low volume samples are commonplace, there is a clear demand for a nondestructive technique that measures electrophoretic mobility rapidly with low electric fields.

Massively Parallel Phase Analysis Light Scattering (MP-PALS) meets these challenges by affording much higher sensitivity, extending robust particle size and electrophoretic mobility measurements to nanoparticles and proteins in native buffer solutions. The combination of MP-PALS with additional unique capabilities such as automation and coupling zeta potential analysis online with size-based separation substantially improves the productivity of biopharmaceutical screening while delivering greater insight into the charge properties of bio/nanoparticles.

Introducing the Innovative MP-PALS

Like standard PALS, MP-PALS measures electrophoretic mobility using two laser beams: one sent through the sample solution and another reference beam diverted around the sample cell. The light from the beams is then interfered at the detector *(Figure 2)*. When the particles move inside the cell they pick up a slight Doppler shift which imposes a periodic phase difference on the scattered signal relative to the reference beam, quantified by measuring interference beats between the two beams. These data are then used to determine the velocity of the suspended particles, while an oscillating mirror varies the path length periodically in order to determine the direction of motion and establish the sign of the mobility.

The innovation of MP-PALS is the implementation of an array of low-noise, high-dynamic-range p-i-n photodiode detectors (photodetector array, or PDA) replacing the standard avalanche photodiode (APD). The high-performance detector array incorporates 31 parallel detection channels, equivalent to having 31 instruments measuring one sample at the same time. Smart data handling software uses these results to average out Brownian motion and determine the contribution of just electrophoretic drift to particle motion over short acquisition times. As a result of the greatly enhanced sensitivity, applied voltage and exposure time to the electric field may be reduced, overcoming the key challenges of conventional PALS. Crucially, measurements are very fast, 1-30 seconds for nanoparticles and even proteins despite applying low fields, meaning analysis is complete before convection can develop.

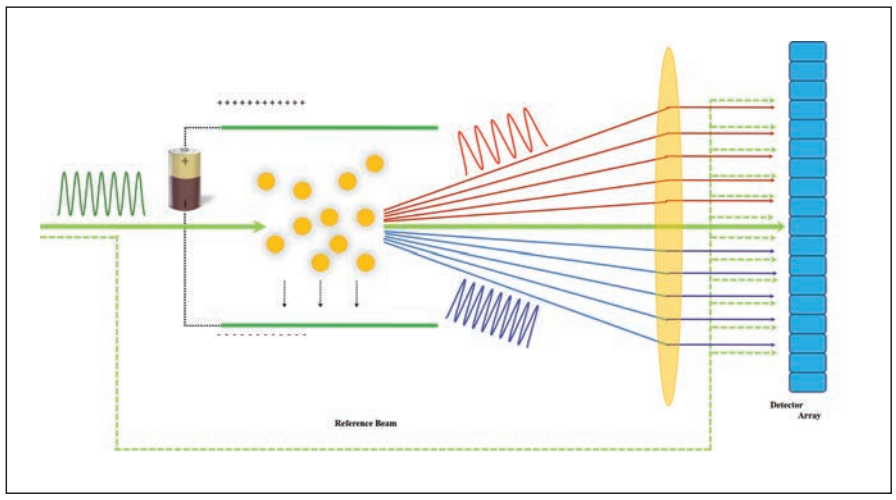

Figure 2. Modern MP-PALS systems allow users to measure the electrophoretic mobility of delicate nanoparticle and protein formulations quickly and non-destructively.

Like most PALS instruments, MP-PALS utilises an APD detector for particle size analysis by DLS. Unlike most PALS instruments which use the same APD for PALS and DLS, and hence must alternate between size and mobility measurements, the dedicated MP-PALS diode array detector means that size is measured simultaneously with mobility. Sample degradation due to e.g. aggregation under the applied field is readily monitored in real time by simultaneous MP-PALS and DLS measurements. Moreover, the high dynamic range of the PDA permits characterisation of fluorescing samples without the saturation effect commonly encountered when an APD is used for PALS.

Modern electrophoretic mobility instruments employing MP-PALS incorporate a flow cell rather than the conventional cuvette. One advantage of this design is the option for coupling to an external pressurising unit to prevent bubble generation, and hence enable analysing even small samples, such as proteins, at high ionic strength equivalent to hundreds of mM NaCl. Pressurisation also removes any microbubbles that may be inadvertently injected during sampling.

MP-PALS systems incorporating flow cells are compatible with standard HPLC autosampling units for high-throughput protein screening. In this way, dozens of runs can be programmed automatically, drastically reducing the intensity of operation and freeing labour for more expert tasks. As a closed system, the flow cell configuration is well suited to volatile organic solvents as well as aqueous buffers.

In normal cuvette, manual flow cell or autosampler-based operation, the flow is stopped after each injection to ensure that the only motion detected is from particle movement within the system, rather than movement of the entire solution. However in a properly designed flow cell the sample flows in a direction perpendicular to the electric field, and so the component of motion due to flow can be decoupled from the electrophoretic drift via an appropriate analysis algorithm. Potentially, this means advanced MP-PALS systems can be coupled to Size Exclusion Chromatography (SEC) or Field Flow Fractionation (FFF) separation techniques in order to generate a size-mobility distribution without having to perform lengthy separations and batch measurements.

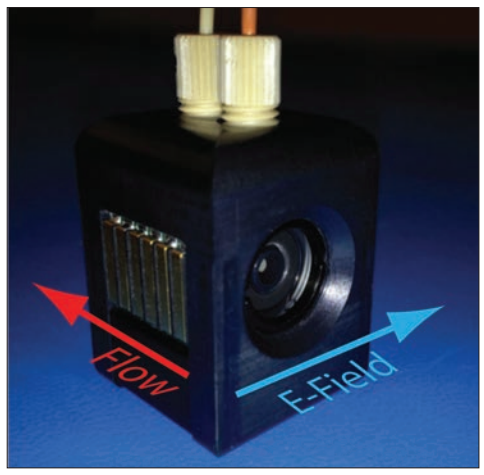

Figure 3. The sample flow through the cell is perpendicular to the electric field allowing for online coupling of MP-PALS to separation techniques.

The following case studies illustrate how innovations in automated and online MP-PALS technology deliver greater efficiency and depth of knowledge for the optimisation of nanoparticle formulations.

Case Study 1: Using Automated MP-PALS to Monitor Aluminium Adjuvant Particles

Aluminum particle adjuvants are commonly added to vaccine formulations to enhance their immunogenicity. The addition of such additives may impact the stability of the formulation and thorough studies are required to assess their effect on biopharmaceutical performance. However, aluminium has a very low redox potential, around -1.661V and applying too high a voltage will damage the sample during analysis.

To determine whether an adjuvant is damaged during analysis an automated protocol was developed using the Möbiuζ® MP-PALS instrument (Wyatt Technology Corp.). The Möbiuζ makes reliable, reproducible and non-destructive electrophoretic mobility measurements of macromolecules as small as 2nm in radius in addition to simultaneous DLS measurements. The set of voltages selected for investigation were 1.5V, 2.0V, 2.5V and 6.0V. Five consecutive samples of an aluminium adjuvant formulation were injected into the flow cell and analysed at a set voltage for 5 seconds.

This process was then repeated twice more with 1 minute gaps in between runs. Afterwards the voltage was increased and the process repeated. *Figure 4* shows how the mobility of the samples varied over time at different voltages.

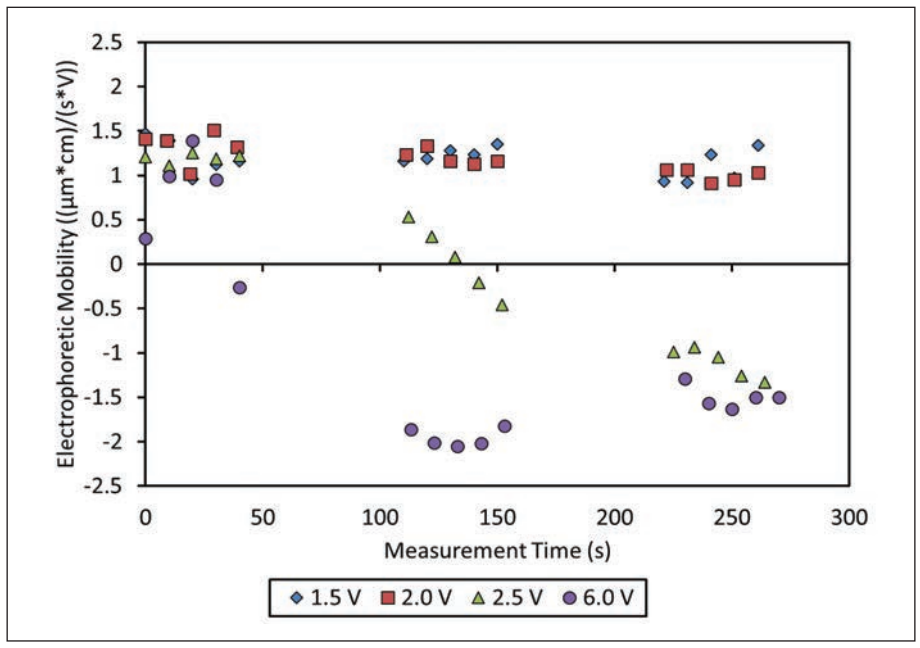

Figure 4. Automated mobility measurements reveal that aluminium adjuvant mobility turns negative at higher voltage, indicating that reduction has occurred.

As long as a small voltage is maintained the mobility of the system stays in the region of +1.3 mobility units (MBU) throughout the experiment. However at higher voltages mobility becomes highly negative as the aluminium is reduced. The impact of this reduction process on overall stability and performance may be significant, highlighting the benefit of performing mobility studies with low voltage MP-PALS rather than alternative techniques. The ability to automate measurements accelerates high volume trend studies, a further advantage of MP-PALS in biotherapeutic screening.

Case Study 2: Moving MP-PALS Online with SEC and FFF

To demonstrate the performance of online MP-PALS three different proteins, Thryoglobulin Bovine, Serum Albumen and Carbonic Anhydrase, were analysed first in batch mode with the Möbiuζ MP-PALS instrument. The proteins were then mixed together and fractionated using SEC coupled to the Möbiuζ MP-PALS. Electrophoretic mobility measurements were made in real-time as they eluted from the column. An additional separation of three different sizes of latex spheres, 50, 100 and 200 nm in diameter, was carried out by means of an Eclipse Dualtec FFF system (Wyatt Technology Corp.) with a Möbiuζ downstream.

The batch measurements of the three proteins revealed the Mw values to be 660 kDa, 66.4 kDa and 30-50 kDa respectively. The samples were then mixed together and separated using SEC. *Figure 5* shows the mobility data overlaid with a continuous phase plot, derived from a Refractive Index detector (RI) measuring the concentration of the samples as they elute.

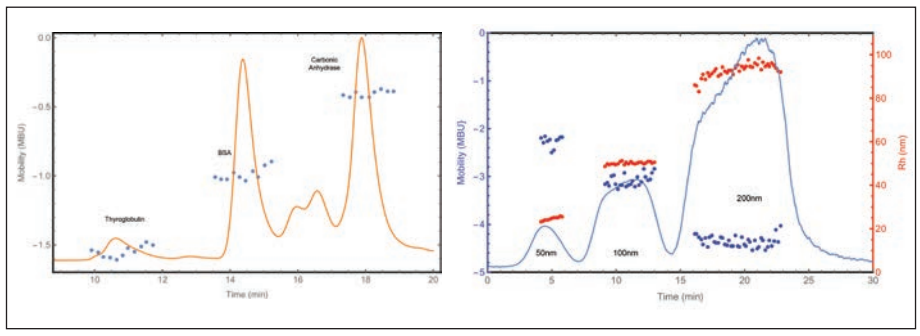

Figure 5. Combining concentration and mobility data for each point allows users to generate a high-resolution size-mobility distribution plot. Left: proteins separated by SEC; right: latex spheres separated by FFF.

From these data it is clear that the smaller carbonic anhydrase molecule has a mobility close to the isoelectric point, suggesting it is the least stable of the proteins. By combining mobility and concentration data at every single point in time it is possible to generate a high-resolution mobility spectrum for the formulation, something that cannot be achieved with batch measurements alone. This new innovation allows users to easily separate out and compare the behaviour of protein fractions and to secure a detailed understanding of formulation behaviour.

Reliable, Reproducible and Non-Destructive

With recent advances in nanomedicine, biopharmaceuticals and other nanomaterials, the demands on formulation development are greater than ever before. Detailed understanding of factors affecting stability is essential to ensure products meet and surpass regulatory and safety targets. MP-PALS is emerging as a key technique for the low voltage, nondestructive measurement of protein and nanoparticle size and electrophoretic mobility. With 31 parallel channels in the PDA, MP-PALS combines maximum sensitivity and robust measurements with short acquisition times. Alongside automation for high-throughput applications, and with emerging online mobility measurement capabilities, MP-PALS delivers fast and reliable results for practically all aqueous buffer formulations as well as organic solvents.