



KEY CONSIDERATIONS FOR ACCURATE QUANTIFICATION OF SUB-MICRON PARTICLES IN PHARMACEUTICALS

In this article, Jean-Luc Fraikin, PhD, Chief Executive Officer of Spectradyne, discusses the use of resistive pulse sensing as a method to characterise nanoparticles in biologic formulations. Spectradyne has developed a new implementation of this technique, MPRS, that makes it a practical analytical method for industrial pharmaceutical applications.

INTRODUCTION

The therapeutic and diagnostic applications of biological materials are proliferating, including antibodies and antibody drug conjugates for direct therapeutic delivery, viruses for delivery of novel gene therapies, and extracellular vesicles for delivering complex therapeutic signalling agents. Significantly, each of these classes of material includes a key component with dimensions in the nanometre scale, in some cases including the delivery vehicles themselves, and in many cases unwanted aggregates. Quantification of both desired and spurious nanoparticle components in these materials is critical, at all stages of development, to properly evaluate efficacy and ensure product safety. Accurate physical characterisation of biological nanoparticles in the sub-micron size range is therefore an increasingly important requirement for the drug delivery industry.

The intent of this article is to clarify the key considerations for the proper measurement of sub-micron particles

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near an instrument’s limit of detection, shed light (pun intended) on certain inherent limitations of optical particle characterisation technologies, and provide a reference for researchers facing these kinds of measurement challenges in the drug delivery industry.

ACCURATE MEASUREMENTS OF BIOLOGICAL NANOPARTICLES

The accurate measurement of sub-micron particles, in general, becomes increasingly difficult as they decrease in size and their measurement signals are reduced to the detection limits of the measurement instrument. While cryo-transmission electron microscopy (CryoTEM) remains the gold standard for sizing nanoscale particles, and this technology’s sub-nanometre sizing resolution makes it very powerful as an occasional analytical tool, the cost and slow speed of this technique render it unsuitable for routine use. Historically, the most commonly used particle measurement technologies for the sub-micron size range have been optical techniques such as dynamic light scattering (DLS), nanoparticle tracking analysis (NTA) and flow cytometry (FC).

Biological nanoparticles however have three common characteristics that make them exceptionally difficult to measure using optical techniques, primarily because these measurements rely on detecting light scattered from these very small particles.



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First, the intensity of the scattered light decreases dramatically as the particles get smaller, scaling with a sixth-power dependence on diameter.¹ A 100 nm particle therefore scatters one million times less light than a 1 µm particle. This dependence, coupled with the finite dynamic range of optical sensors, limits the practical range of sizes that can be measured in a single sample and makes detection of small particles significantly more challenging.

Secondly, the physical composition of most biological materials results in a particle index of refraction that is similar to that of the surrounding, typically aqueous, media, in some cases with indices that differ by only a few percent. The scattered light intensity is proportional to this index difference (also called contrast), so can result in another reduction of the signal by a factor of 10 to 100. The combination of low index-contrast and small size significantly weakens the intensity of light that scatters from biological nanoparticles, thereby limiting the sensitivity of optical measurement techniques to these sub-micron particles.

Finally, the complex origins of biological nanoparticles (e.g. aggregation processes or shedding from cells) yield real-world samples with diverse material composition and a broad range of particle sizes. The high degree of size polydispersity in these samples places a significant burden on sizing resolution in the case of NTA, which measures scattered light from single particles, and presents an insurmountable obstacle for DLS, which performs an ensemble measurement of all particles in the incident light path and cannot tolerate significant polydispersity.

Non-optical techniques for measuring nanoparticle size and concentration provide a powerful alternative to these optical techniques. The most common is based on an electrical measurement technique known as resistive pulse sensing (RPS), historically referred to as the Coulter principle after the inventor of the technique, Wallace Coulter.² RPS is a well-proven technique for measuring the size and concentration

of large particles (>1 µm) and has been the gold standard for decades for whole-blood cell counting.

RPS is ideally suited to the measurement of biological nanoparticles for three reasons:

1. The detection signal scales linearly with particle volume, so the dynamic range of sizes that can be measured in a single sample is much larger.
2. RPS measurements are independent of the material composition of the particles, and therefore do not suffer from the same loss of sensitivity as optical techniques.
3. Because particles are measured individually with high precision in RPS, high polydispersity is not a significant issue. It is possible to perform particle measurements directly in complex media, such as serum, urine and other biological fluids whose polydispersity would otherwise confound light scattering techniques.

So why has the use of RPS historically been limited to large particles alone? RPS requires that all particles to be measured pass through a physical constriction for detection, and the size of this constriction must be decreased in order to detect smaller particles. Real-world samples contain a significant concentration of particles that are larger than the small constriction size required for nanoparticle measurements. Therefore, in the simplest implementations of RPS, the large particles in the sample cause frequent blockages of the constriction and prohibit practical use of the technique.

In 2009, in an early attempt to circumvent this obstacle, Izon Science (Christchurch, New Zealand) developed the qNano for RPS measurements. Izon's implementation set the constriction in a deformable membrane (“Tuneable” RPS) that could be adjusted to allow blockages to pass through before resuming measurements. While the technology has been cited in a number of academic publications, its deployment has been limited in industrial applications that demand high throughput and turnkey operation.

More recently, Spectradyne has commercialised a different approach to RPS that significantly improves the practicality of the technique for routine nanoparticle analysis of real-world samples in an industrial context.³ Spectradyne's nCS1 instrument is a microfluidic implementation of RPS (MRPS) and leverages manufacturing techniques from the semiconductor industry to incorporate a number of fluidic features in a disposable analysis cartridge that permits nanoparticle analysis while significantly reducing blockage events. MRPS enables straightforward measurements of highly polydisperse biological nanoparticle samples such as protein aggregates, serum, urine and crude preparations of extracellular vesicles, and is seeing adoption by prominent researchers in the pharmaceutical industry.⁴

Regardless of an instrument's underlying principle of operation, all instruments are eventually limited by their intrinsic sensitivity and noise. But as biological nanoparticles increase in importance in the drug delivery industry, regulators such as the US FDA increasingly recognise the importance of using a complete set of characterisation methods, as stated by Susan Kirshner, PhD, in a 2012 talk entitled, “Regulatory expectations for analysis of aggregates and particles”, including methods that are orthogonal to conventional light-based techniques. MRPS represents a practical and easy-to-use alternative that satisfies these regulatory expectations.

MEASUREMENT EXAMPLES

Three measurement examples are presented below that illustrate the importance of the above considerations in real-world drug delivery industry applications. First, measurements of a simple extracellular vesicle preparation using three different particle analysis methods (NTA, CryoTEM and MRPS) are compared to show the importance of using orthogonal measurement techniques. Second, an aggregated protein sample is measured by NTA and MRPS and illustrates how the instrument limitations described above apply to a different class of sample. Finally, measurements of a series of stressed protein samples are used to demonstrate how the smaller particle detection limit of MRPS enables earlier detection of protein aggregation.

Example 1: Extracellular Vesicles Three Ways

Unless they are carefully purified, samples of extracellular vesicles (EVs) naturally exhibit an approximate power-law distribution of particle concentration versus particle size.⁵ Such a broad particle size distribution provides an excellent opportunity to evaluate the sensitivity of different measurement techniques over a comprehensive size range in a relevant sample type. For this measurement example, a simple sample of EVs from human cell-free urine was analysed using MRPS, NTA, and CryoTEM (Figure 1).

MRPS shows a clear power-law dependence of concentration on particle size that extends down to 50 nm, the limit of detection for the analysis cartridge used in this measurement. Importantly, the MRPS measurements are in excellent agreement with those of CryoTEM, the gold standard for measuring size and relative concentration of particles in this type of sample.

The NTA measurement results highlight the limitation of the NTA technique for detecting small particles in this type of sample. NTA significantly under-reports the concentration of smaller particles, with a divergence from the CryoTEM result becoming apparent, starting at 200 nm and increasing dramatically below about 150 nm. The discrepancy between NTA and CryoTEM expands to several orders of magnitude below 150 nm diameter.

Researchers must therefore take care when interpreting NTA data such as these, especially as the resulting profile appears to indicate the presence of a peak in the size distribution around 150 nm, which is not a real feature of the particle size distribution. The peak can be accurately identified as an artefact of the measurement technique only when orthogonal methods such as MRPS or CryoTEM are used for comparison, as in this example. Unfortunately, inaccurate optically-based measurements of EV size distributions, such as this NTA measurement, appear often in the literature and are not generally supported by orthogonal techniques such as MRPS or CryoTEM.

Example 2: Protein Aggregation

In the process of aggregation, protein monomers a few nanometres in diameter aggregate into dimers, trimers and subsequently into larger particles that can grow to be as large as tens of microns in diameter. This process generates a highly polydisperse mixture that contains particles

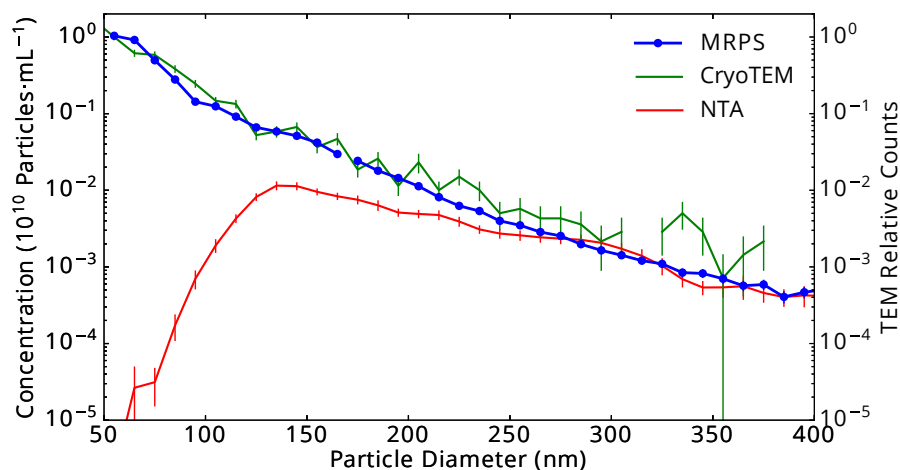


Figure 1: Extracellular vesicles isolated from human serum measured using three orthogonal techniques: MRPS, CryoTEM and NTA. The loss of sensitivity of the optical technique to small, weakly-scattering biological nanoparticles becomes apparent when compared with orthogonal techniques such as MRPS and the gold standard, CryoTEM.

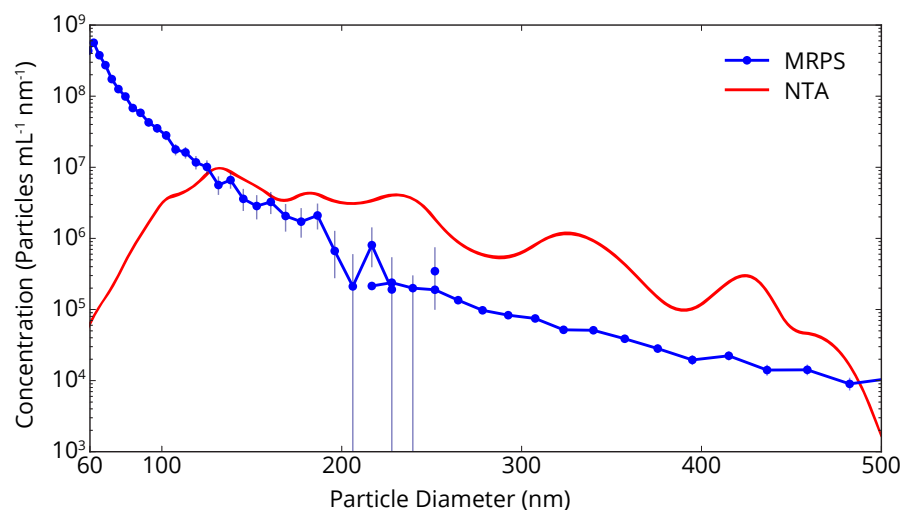


Figure 2: Protein aggregates in a stressed sample show the expected power-law dependence of concentration on size when measured by MRPS. The size range of sensitivity of the NTA is apparent as the method underreports concentration outside approximately 150–450 nm diameter.

“Accurate and precise measurements of smaller biological nanoparticles, such as those obtained with MRPS, enable significant time savings for real-world drug delivery applications.”

spanning many orders of magnitude in diameter and concentration, with an approximate power-law distribution of aggregate concentration versus particle size.

In this specific example, a proprietary protein sample was prepared at 5 mg/mL in phosphate-buffered saline and was stressed

at elevated temperature for 24 hours to accelerate the aggregation process. The particle size distribution in the sample was measured by NTA and MRPS (Figure 2). The MRPS measurements show the expected power-law dependence of concentration on size, and further indicate that the power-law dependence extends down to at least 60 nm, which is the small size limit of detection of the analysis cartridge used for this measurement.

The sensitivity limitations of the optical technique are clearly illustrated in this example as well. While NTA readily detects particles in the sample between about 150 nm and 450 nm, below 150 nm the concentration reported by NTA decreases sharply (note the log scale on the vertical axis), likely because of the very dramatic

reduction in scattering intensity for these smaller protein aggregates. Due to this spurious apparent decrease in concentration versus size, the data could be incorrectly interpreted as indicating a peak in the size distribution. Without using an orthogonal technique such as MRPS to verify the true nature of the particle size distribution, the unwary researcher may be led astray.

Example 3: MRPS Enables Earlier Detection of Protein Aggregation

Accurate and precise measurements of smaller biological nanoparticles, such as those obtained with MRPS, enable significant time savings for real-world drug delivery applications. In this example, MRPS was used to quantify protein aggregates in stressed drug formulations. The results show that stress-induced aggregation can be detected significantly earlier in the process by detecting and analysing concentrations of smaller particles.

Aliquots of a proprietary biologic drug formulation were stressed for times varying from 0 minutes (control) to 60 minutes, with MRPS subsequently used to quantify aggregates in each sample (Figure 3). The data show the expected trend very clearly: as the amount of stress applied to the samples increases, the concentration of aggregates in the sample increases across all particle sizes.

Importantly however, aggregate concentration increases first at small particle sizes and later at large sizes. This effect is apparent in the data, as shown in the

inset to Figure 3. When the concentration is measured on the subranges R1 to R3 (small particles to large, respectively), the effect of stress on the sample is apparent in R1 at the first time point, taken after just 10 min. This behaviour is consistent with expectations, since protein aggregates must start small before growing to larger sizes, and presents a significant opportunity to save time in formulation development.

Using accurate methods for quantifying smaller nanoparticles such as MRPS, formulation stress tests can be performed in a fraction of the time that would be required using optical techniques that lack sufficient sensitivity to detect the smaller, earlier-stage, aggregates. In an industrial environment, the ability to obtain this data both faster and earlier in the process can lead to a significant increase in process efficiency.

CONCLUSION

As therapeutic and diagnostic applications of nanoscale biological materials proliferate, their accurate quantification becomes increasingly important. This article demonstrates that, while ubiquitous, optical methods for nanoparticle analysis must be used with a complete understanding of their limitations, and conclusions should be supported by results from orthogonal measurement techniques. The cited examples illustrate how artefacts resulting from the sensitivity limitations of optical techniques

can misrepresent the true composition of complex biological nanoparticle samples. The examples also show that practical techniques, such as MRPS, are capable of accurate quantitative measurements of sub-micron biological particles and offer an opportunity for significant savings of time and money in real-world pharmaceutical industry applications.

ABOUT THE COMPANY

Spectradyne develops innovative analytical technologies with a focus on delivering accurate measurements of particles in the submicron size range. The company operates across a broad set of industries, including pharmaceuticals and nanomedicine, cosmetics and semiconductor processing.

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Jean-Luc Fraikin is Chief Executive Officer and a co-founder of Spectradyne. Dr Fraikin obtained his PhD in physics from the University of California, Santa Barbara, where he studied electrical sensing in fluidic systems and particle analysis. He also received postdoctoral training in biochemistry and cancer cell biology and developed microfluidics-based diagnostics in industry before co-founding Spectradyne.

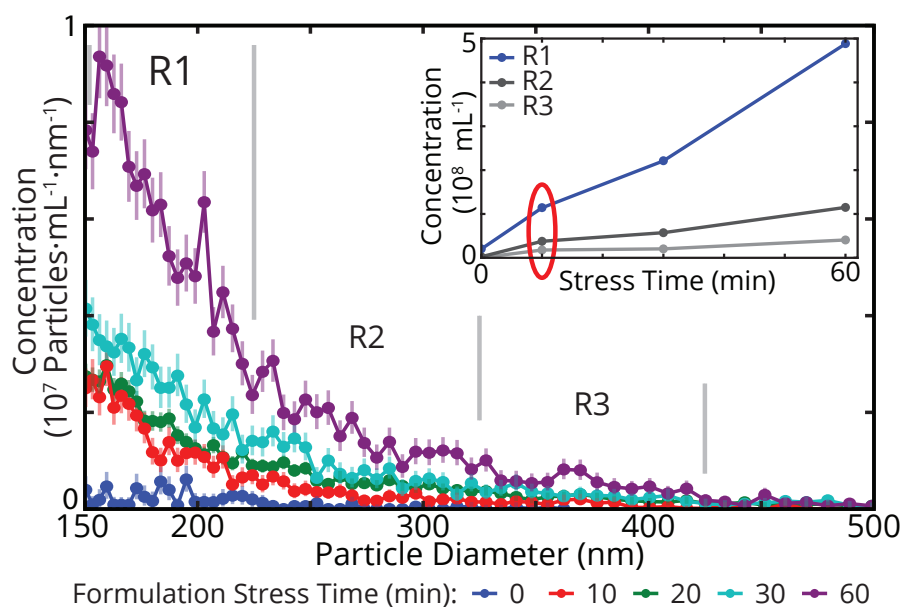


Figure 3: Protein aggregates in a stressed sample show the expected trend of increasing concentration with increasing stress. Concentration measurements on three subranges of particle size (inset) demonstrate that the effect of stress on the sample are detected earlier by quantifying smaller aggregates.