Accurate Measurements of Biological Nanoparticles

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Over the past few years there has been an increasing amount of research and development of nanoparticles and biologics as potential vehicles for drug delivery. The need to be able to accurately measure the size and concentration of these particles has put demands on instrumentation which they have struggled to meet. 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).

Dynamic Light Scattering (DLS) has been used for a number of years providing a practical means of sizing submicron particles providing a mean diameter of the particle measured together with some form of polydispersity measurement. The first commercially available instrument specifically designed for sub-micron sizing, the Coulter Nanosizer was released in 1979.In the early instruments information was limited to just a 'polydispersity index' with a numerical value. As computing power became more readily available and cheaper, various mathematical algorithms such as Contin, and Pearson V were incorporated to provide a calculated size distribution from the basic autocorrelation function they generate, however the basic measurement is still a single signal from numerous particles simultaneously from which the distribution is calculated.

Flow Cytometry has also been available for many years and has been used for the biological materials we are investigating however, its use is not widespread for this application and its sensitivity and practicality in the area of interest is limited.

More recently (2003) Nanoparticle Tracking Analysis (NTA) has taken the same basic measurement of particle Brownian motion that is used by DLS measurements but the scattered light from individual particles are captured by a camera enabling the individual particles to be tracked from which size and size distribution can be determined. As it measures individual tracking events within an optical region of known size/volume it can also report particle concentration. Original NTA's used on a single light source for measurement limiting the dynamic range of size determined in a single measurement, however in a more recent development (2016) multiple light sources are used to improve the dynamic range and improving measurements of polydisperse samples.

Another recent commercially available technique is that of dynamic imaging where particles are passed in front of a camera which images them and then using proprietary software can generate a number of image parameters on each individual particle. This type of technique is becoming commonplace when looking for protein aggregates in therapeutic protein solutions where the imaging enables the protein aggregates to be distinguished from other particles which may be present such as silicon oil droplets. Recent developments in this field have enabled measurements down to 0.3µm (300nm) to be made however for most applications in the biological nanoparticle application, it's lower limit still seems to be too high.

Optical based techniques provide meaningful data for what might be termed 'industrial' applications however 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.

First, the intensity of the scattered light decreases dramatically as the particles get smaller, scaling with a sixth-power dependence on diameter, thus a 100 nm particle therefore scatters one million times less light than a 1 µm particle making detection of small particles in the same sample a larger one significantly more challenging.

In addition the physical composition of most biological materials results particle refractive index that is similar to that of the suspending fluid. The scattered light intensity is proportional to this index difference, and 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 for these sub- micron particles.

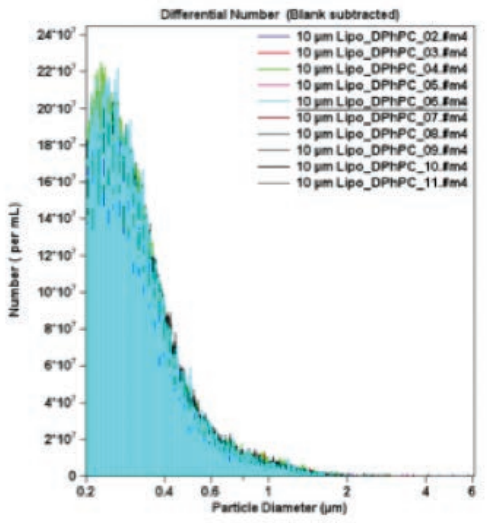
Finally, the complex origins of biological nanoparticles (e.g. aggregation processes or shedding from cells) yield real-world samples with diverse material composition and a broad range of particle sizes. The high degree of size polydispersity in these samples places a significant burden on sizing resolution 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 Electrical Sensing Zone (ESZ) or Resistive Pulse Sensing (RPS) and is historically referred to as the Coulter principle after the inventor of the technique, Wallace Coulter. It 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 blood cell counting.

It's ideally suited to the measurement of biological nanoparticles for three reasons:

Because the detection signal scales linearly with particle volume, the dynamic range of sizes that can be measured in a single sample is much larger.

The measurements are independent of the colour, refractive index or shape composition of the particles (and therefore do not suffer from the same loss of sensitivity as optical techniques.



Finally because particles are measured individually with high precision, high polydispersity is not a significant issue. It is possible to perform particle measurements directly resolving particle peaks in complex media, such as serum, urine and other biological fluids whose polydispersity cannot be measured by light scattering techniques.

The latest ESZ instrument can measure particles down to 0.2µm (200nm) and has been used to measure biologicals such as liposomes.

Figure 1: Ten runs on a liposome sample on a Beckman Coulter Multisizer 4e

This method had been limited to measurement of particles to just under 1 micron until recently, because of the need for the particles to pass through a physical constriction or aperture for detection, and the size of the aperture must be decreased to detect smaller particles and 200nm represents the lower limit of the current technology. Also of relevance to researchers in particular is that a minimum volume of 4ml of particle suspension is required to make a measurement which may not be available.

Real-world samples contain a significant concentration of particles that are larger than the size of aperture size required for nanoparticle measurements. Therefore, in the simplest implementations of ESZ/RPS, the large particles in the sample cause frequent blockages of the aperture and prohibit practical use of the technique. Several years ago one attempt was made to overcome this obstacle by setting the aperture in a deformable membrane that could be adjusted to allow blockages to pass through before resuming measurement. This approach has been cited in numerous academic papers but its deployment has been limited in industrial applications that demand high throughput and simple operation.

A recent development has commercialised a different approach which improves the practicality of the technique for routine measurements of real world samples which only requires 3µl of sample.

The Spectradyne 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 biopharma industry.

Different cartridges are available for the nNCS1 in the same way that different apertures are available are available on conventional ESZ system to optimise resolution and size of the sample being measured. Similarly where the size distribution is very polydisperse two cartridges or apertures can be used one after the other on the sample to make measurements with the data from both being overlaid to produce a final size distribution.

An example of the instruments capability of resolving particle distributions compared to other techniques.

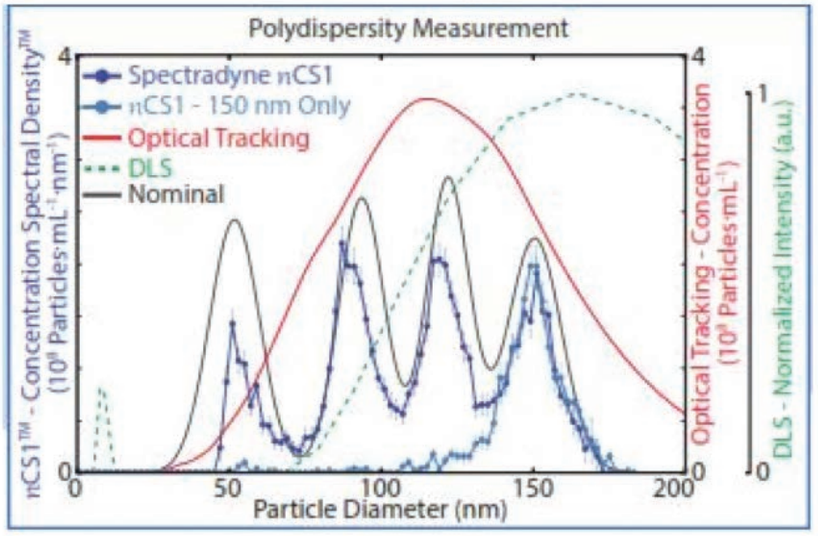


Figure 2: Comparison of resolution of multi modal populations for MRPS, DLS and NTA

Due to the limitations of optical techniques as previously described, what amount to false 'peaks' in the data can be generated.

This has caused users of such techniques to expect to see a peak in their size distribution where in reality there is no distinct population of particles.

The MRPS technique has been applied to a number of more 'real world' sample such as vesicles, where the origin of the vesicles can be quantified.

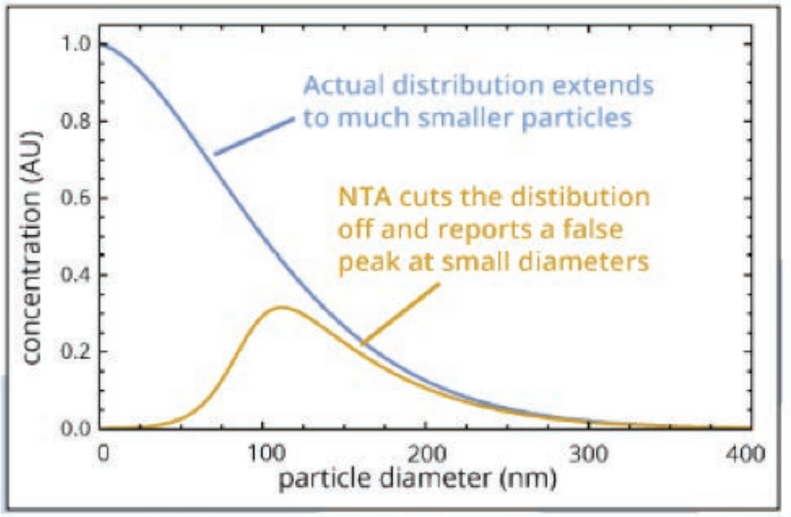


Figure 3: Example of false peak generated by NTA data

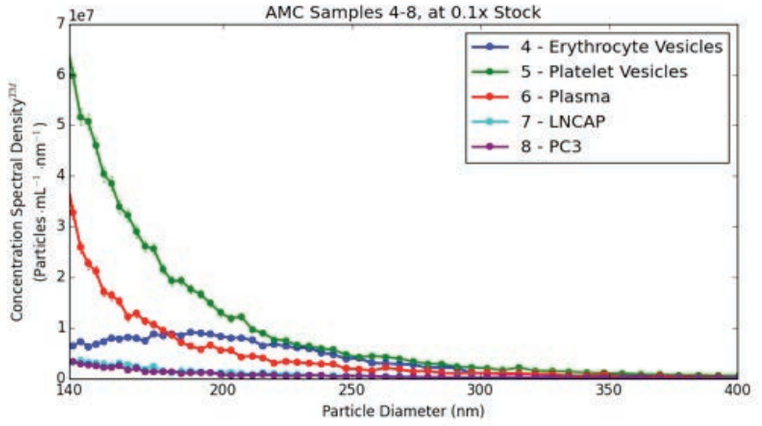


Figure 4: nCS1 data showing vesicles from different sources

Using the nNCS1 can enable the differences between vesicles from different sources to be quantified. The advantages of the method for measuring biological nanoparticles are becoming recognized and national bodies such as the FDA.

Conclusion

Whilst optical techniques such as DLS and NTA provide some useful data, the MRPS development of the Coulter Counter gold standard technique for cell counting and sizing provides an alternative method for measuring biological nanoparticles and offers advantages of currently used methods.