

See What's Really in Your Formulation with Backgrounded Membrane Imaging

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Experiments in this application brief were performed on the HORIZON® system, an earlier generation of Waters BMI technology. The Aura™ platform now replaces HORIZON as the current standard for BMI, offering improved image resolution and more accurate quantitative particle analysis.

Introduction

Protein aggregation in therapeutic protein products can induce adverse immunogenic responses in patients¹⁻⁴. Per the FDA's recommendations, "strategies to minimize aggregate formation should be developed as early as feasible in product development"⁵ and "an assessment should be made of the range and levels of subvisible particles (2–10 µm) present in therapeutic protein products initially and over the course of the shelf life"⁵. Yet, accurate subvisible protein aggregation analysis has remained elusive to protein drug formulators since current subvisible analyzers are not sensitive enough to measure protein aggregates effectively.

In this application note, we explore how backgrounded membrane imaging (BMI), a high refractive index contrast technique used by the HORIZON particle analysis system, performs highly sensitive measurements of subvisible protein aggregates in the 2 µm–4 mm size range, enabling 30% higher reproducibility compared to flow imaging.

Results and Discussion

Increased Refractive Index Contrast for Improved Measurement Sensitivity

Refractive index contrast (Δn) plays a key role in how accurately protein aggregates are measured by a particle analysis system. Protein aggregates can have a significant water content and therefore a refractive index (n) that very closely resembles that of water (Figure 1).⁶⁻⁸

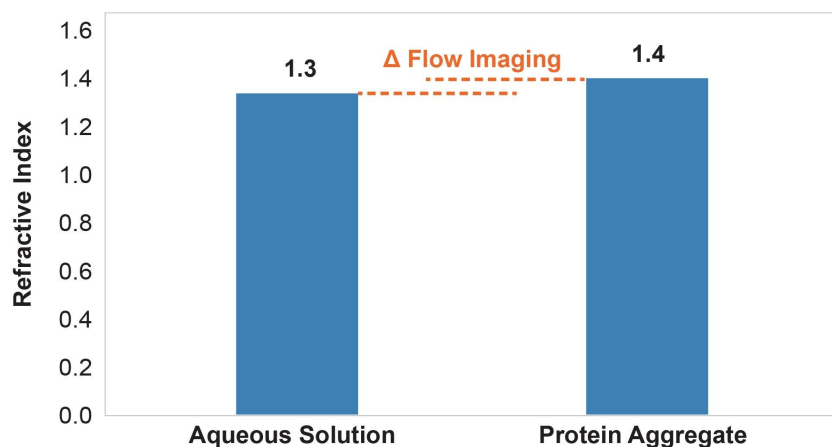


Figure 1. The similar refractive index between protein aggregates and the surrounding aqueous solution ($\Delta n < 0.1$) results in low data quality, causing FI and LO systems to undercount and undersize subvisible particle aggregates.

This is a basic optical limitation of translucent particles, which are difficult to analyze using fluidics-based particle analyzers like flow imagers (FI) and light obscuration (LO) due to poor image and poor signal contrast. It is now well established that a low Δn causes FI and LO systems to dramatically undercount and undersize subvisible protein aggregates^{7,9} to the point that some of these particles are essentially invisible to these techniques.^{8,10} This is because all image analysis systems work on intensity thresholding (essentially establishing the contrast) that determines where a particle edge begins and where it ends (Figure 2). If measured in air (high refractive index contrast) particles are properly thresholded and sized. But as the refractive index contrast between the particles and the solution

decreases, the particles will appear smaller and dimmer, as shown in Figures 2 b,c.

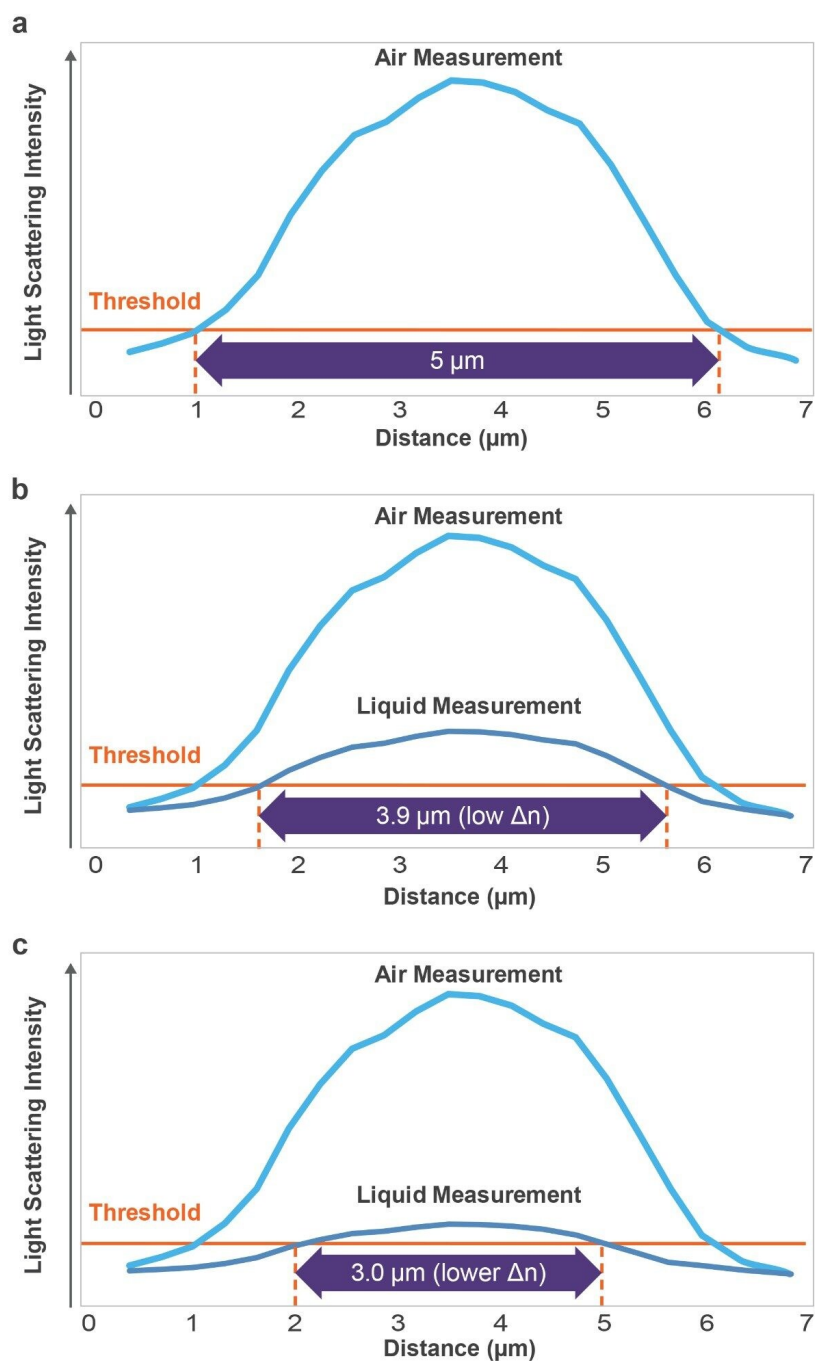


Figure 2. Intensity thresholding in image analysis systems and the impact of low refractive index contrast (Δn). Air particles have a large Δn (a). As the refractive index contrast between the solution and the particle drops,

the particle is measured as a dimmer and smaller particle (b–c).

To add to the difficulty of using fluidics-based systems, excipients commonly used to decrease aggregate formation, like sucrose and polysorbate, can increase the refractive index of a formulation¹⁰, further reducing an already small Δn to negatively impact protein aggregate counts and size distribution. To overcome the basic optical limitations of fluidic-based subvisible particle analyzers, Waters developed BMI, the technique that powers the HORIZON[®] system. BMI enables high contrast measurements by measuring subvisible particles in air instead of aqueous solution, significantly increasing the refractive index contrast for improved count accuracy and particle size determination (Figure 3).

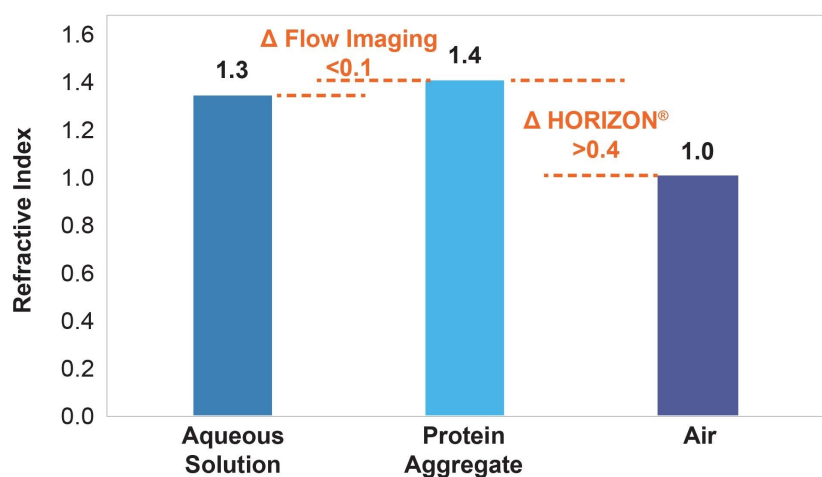


Figure 3. Refractive index differences between protein aggregates in aqueous solutions (FI) and air (HORIZON[®]). The higher refractive index contrast with the HORIZON[®] system improves data accuracy, robustness, and sensitivity.

Here, we compare the particle counting sensitivity of the HORIZON[®] instrument with flow imagers via two studies. The first investigates the impact of particle refractive index by looking at protein aggregate count accuracy across a wide particle concentration range in a pharmaceutical solution. The second

investigates the impact of solution refractive index by looking at subvisible particle counting accuracy in the presence of a common excipient at varying concentrations.

Study 1 — Understanding the Impact of Protein Aggregate Refractive Index

IgG Protein Aggregate Study

IgG protein aggregates were made using an alternative stir-agitation method based on Kiese *et. al.*¹¹ and Mahler *et. al.*¹² Briefly, anti-streptavidin IgG1 was dialyzed using 10 kDa MWCO Spectra/Por 7 tubing (Spectrum Laboratories). A concentration of 1 mg/mL was prepared with distilled, deionized water Milli-Q (Millipore) in 20 mM acetic acid (Fisher) adjusted to pH 5.0. The solution was stirred at 650 rpm for 24 hrs at room temperature.

The Stir Agitated protein aggregates were then serially diluted three-fold into filtered phosphate buffered saline (PBS). Each sample was run on both a HORIZON[®] system (N=4 wells at 50 µL per well) and a flow imager (N=3 at 100 µL measured per sample). For each 100 µL measured in the flow imager, an additional 200 µL was needed to purge the flow cell.

Particle counts for the HORIZON[®] system and the flow imager showed a linear response with respect to each other. However, the total particle counts measured with BMI using the HORIZON[®] instrument were roughly 3.26X higher than the total counts measured with the flow imager over the entire concentration range (Figure 4). Additionally, the HORIZON[®] system produced average CVs of 14.4% using 50 µL samples while the flow imager produced average CVs of 20.1% in 300 µL samples with increasing variability as the particle size decreased. This demonstrates that the HORIZON[®] instrument produced more robust results with 6X less sample volume per measurement. While results from both systems exhibited the expected particle size exponential decay, the flow imager counted significantly less particles in size bins <10 µm, suggesting that the refractive index is a bigger factor in these measurements (Figure 5).

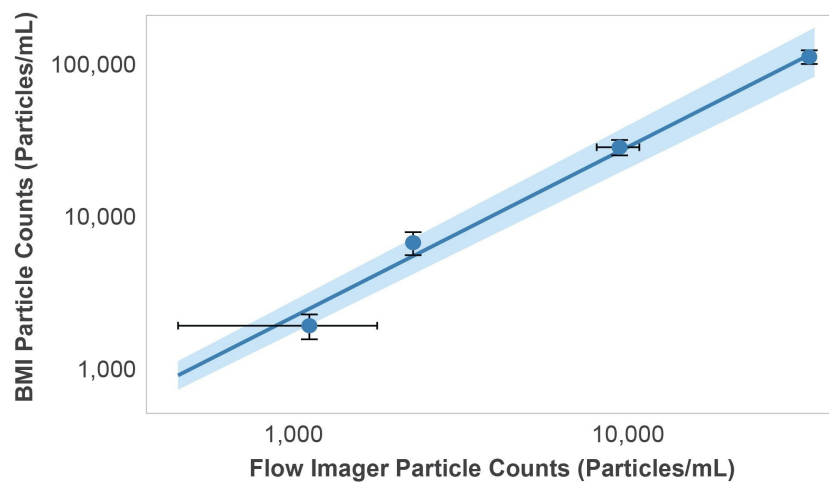


Figure 4. Comparison of HORIZON® and flow imager IgG particle counts. Both systems showed a linear response, but the HORIZON® system demonstrated increased sensitivity, detecting 3.26X more particles compared to flow imager for this formulation. 95% confidence interval of the linear fit (light blue).

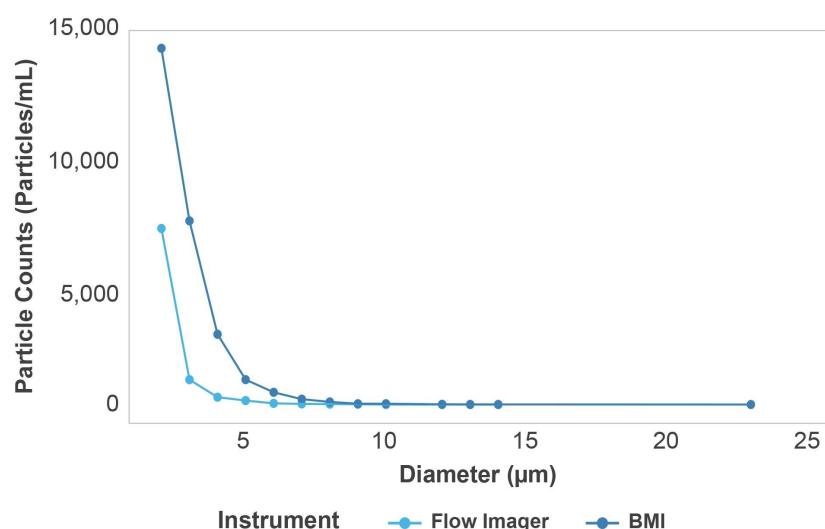


Figure 5. Particle size distributions as a function of counts for HORIZON® and flow imager. The HORIZON® system consistently detected more particles in every size bin compared to flow imager, with differences becoming more dramatic as particle size decreases.

Study 2 — Understanding the Impact of Solution Refractive Index

Excipient Effect Study

Protein aggregates made of immunoglobulin G (IgG) antibody were generated using the periodic interfacial compression method described in Bee *et al.*¹³ Briefly, IgG protein was diluted into pH 7.4 phosphate buffer saline then filtered using 0.2 µm pore size sterile filters. Samples were then rotated at 15 rpm for 3 hrs at 22 °C room temperature.

Solutions of interfacially compressed IgG aggregates were each formulated with varying sucrose concentrations from 0 to 20% w/v. Sucrose solutions were prepared using crystalline sucrose (Fisher, catalog #S2-500GM) dissolved into distilled deionized water. A 100% stock sucrose solution was prepared by mixing 100 mg sucrose into 100 mL ddH₂O. The 100% stock sucrose solution was then diluted into 50% (50:50 stock sucrose:particles) and a 20% sucrose solution + particles (20:30:50 sucrose:water:particles) and the 10% sucrose solution (10:40:50 stock sucrose:water:particles). Three wells were assayed on the HORIZON® system for each sucrose concentration (N=4), at a volume of 50 µL

each. Sucrose concentrations had no impact on the IgG aggregates particle count measured with the HORIZON® system. All results gave expected measurements with the average CV for IgG aggregates=5.4% (Figure 6).

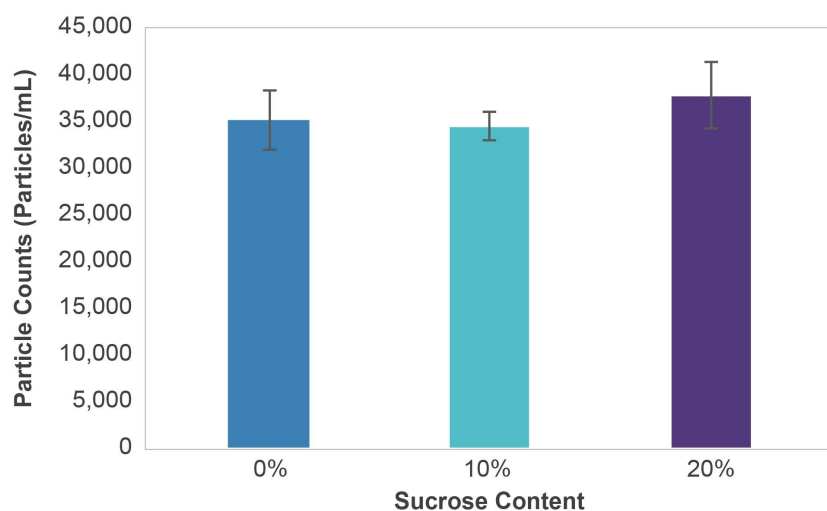


Figure 6. IgG particle counts in varying sucrose concentrations.

The HORIZON® system generates accurate particle counts even in the presence of increasing concentrations of sucrose.

The use of IgG, a low refractive index particle, demonstrated that changing the refractive index of the solution had no effect on the particles counted by the HORIZON® system.

Conclusion

When studying the impact of protein aggregate refractive index, IgG protein aggregates analyzed on both the HORIZON® instrument and flow imager systems produced equivalent particle count trends. However, the HORIZON® system was more sensitive (measured more overall counts) and had overall lower variability with average CVs of 14.4% compared to the flow imager which produced average CVs of 20.1%. The differences in variability and counts were most pronounced at lower concentrations and size

range respectively. Given that most particles in a therapeutic drug formulation are small, the inability of flow imagers to accurately count the smallest of protein aggregates has a dramatic impact on the total number of particles measured. This can mean an undercounting by as much as 3–10X depending on the protein and formulation conditions.

High variability at low concentrations in flow imagers can pose a hurdle for early stage analysis where sample is scarce. In low volume analysis (<250 µL), the possibility of measuring fewer particles increases, therefore it is imperative to have high sensitivity measurements when already challenged by low sampling statistics.

In the excipient effect study, sucrose did not have an impact on the overall particle counts when measured using the HORIZON[®] system. This contrasts with previous studies comparing excipient effects on three flow imagers.¹⁰ In each case, the particle counts decreased up to 30% or more using standard sucrose formulation concentrations of 0–20%. The difference between the data generated by BMI and FI can be attributed to the fact that the HORIZON[®] instrument is not affected by particle or solution refractive index.

Flow imagers are deeply vulnerable to the impact of refractive index when measuring protein aggregates due to their translucency and the negative impact of surfactants. Due to this basic optical phenomenon, flow imagers can dramatically undersize and undercount particles. Finding the right tool for the job will have a critical impact on data accuracy and sensitivity. Powered with BMI, the HORIZON[®] system provides high refractive index contrast analysis to enable accurate, robust, and sensitive subvisible particle counting from 2 µm–4 mm in as little as 25 µL of sample and 96 samples in under 2 hours.

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