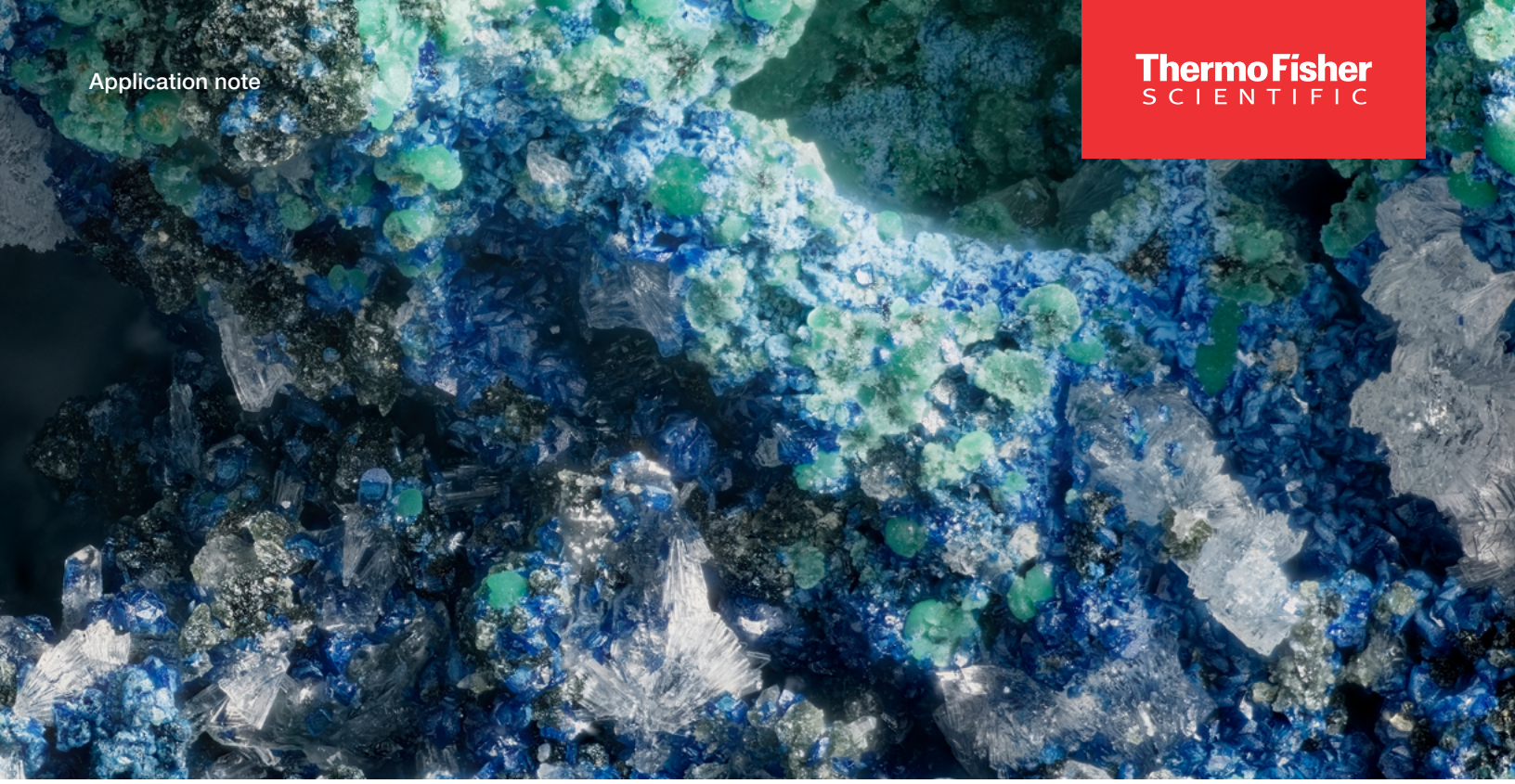
Application note



Simplifying microcrystal analysis for droplet-based serial crystallography

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Overview

SURFACtoBioTech GmbH (Surfacto) is a Hamburg-based biotech startup developing proprietary droplet injection systems for use on beamlines at particle accelerator research infrastructures. Protein microcrystals are of primary interest for Surfacto's droplet-based serial crystallography products; however, standardized batch microcrystal production approaches are still being understood. The quality and standardization of microcrystals, especially in serial crystallography, is fundamental to producing reliable diffraction measurements during beamtime. Current widely used methodologies for batch crystallization do not directly measure the fraction of starting protein that enters the crystalline phase.

This application note presents a microvolume, ratiometric workflow in which the Thermo Scientific™ NanoDrop™ Ultra^{C} FL Microvolume UV-Vis Spectrophotometer and Fluorometer quantifies protein concentration at every stage of a lysozyme crystallization workflow to achieve improved microcrystal standardization. This note establishes the NanoDrop Ultra^{C} FL instrument as a simple, reagent-free, and sample-efficient tool for protein concentration analysis across complete crystallization workflows.

Introduction

Serial crystallography beamlines convert micron-sized protein crystals into three-dimensional structural models by collecting diffraction data from thousands of individually evaluated microcrystals. Synchrotron sources run serial synchrotron crystallography (SSX), and X-ray free-electron lasers run serial femtosecond crystallography (SFX); both techniques produce room-temperature structural data one frame at a time. At least 10,000 indexed frames are needed to approximate a full structure.[1,2]

Surfacto's droplet-based sample delivery synchronization systems inject microcrystal droplet-suspensions directly into the beamline for analysis. We observed that the quality of microcrystal suspensions can contribute to whether a beamtime session succeeds.

Hen egg white lysozyme (HEWL) is one of the most widely used model proteins for developing crystallization workflows and contributes to the development of standardized sample preparation.[3] The batch crystallization method for HEWL is established; however, we experienced wide variabilities in developing standardized microcrystal characterization approaches for use in our startup. Several steps in a commonly used workflow are not consistently verified, and user error can result in several issues regarding microcrystals:

1. Inconsistent size distributions
2. Different crystal lattice shapes
3. Ineffective formation of microcrystals

These issues can lead to low-resolution indexed frames, beam downtime, and increased costs during measurements. To maximize the benefits of microcrystal suspensions in SSX and SFX experiments, the accurate determination of microcrystal concentration is crucial, because concentration has a direct correlation to the indexed hit rate.[4]

The concentration of microcrystals in suspension is often estimated using a settled volume fraction (v/v) of 18% as the benchmark. However, the volume fraction describes the space the crystals occupy rather than the protein they contain, and batch crystallization converts a fraction of the dissolved protein into microcrystals in a manner that is not well characterized. It is therefore necessary to develop standardization strategies that quantify concentration across the crystallization workflow. One common method for determining crystal concentration is microscopy and counting using a hemocytometer and image analysis. Unfortunately, this approach is time- and sample-consuming and it is also user-dependent.

We solved the challenges mentioned regarding concentration determination by integrating the NanoDrop Ultra^{C} FL bench-top instrument into our startup lab workflows, as illustrated in Figure 1. This application note investigates the potential of using absorbance at 280 nm (A280) measurements for the quantitative analysis of HEWL microcrystal batch suspension production. Because suspended crystals scatter light and increase the apparent absorbance, the standard Protein A280 application is compared with a Custom Method created using the NanoDrop Ultra^{C} FL software that enables us to characterize and standardize our samples for droplet injections. The dissolved protein stock and crystal supernatants are measured as references and enable a ratiometric approach. The results indicate that the NanoDrop Ultra^{C} FL instrument serves as a simple, reagent-free alternative to traditional methods for determining protein and crystal concentration, establishing a new analysis approach for batch crystallization.

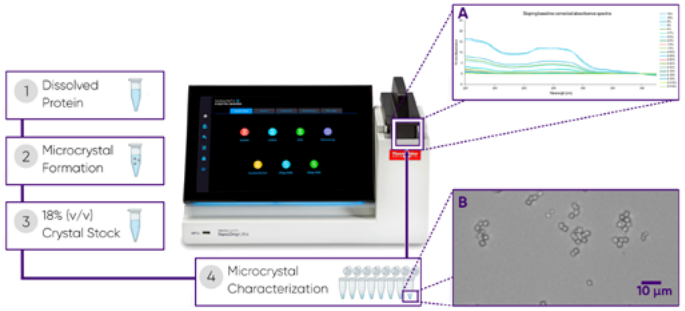


Figure 1. Workflow for protein microcrystal stock preparation and characterization. Lyophilized lysozyme dissolves in buffer (1); the NanoDrop Ultra^{C} FL instrument measures protein concentration before crystallization buffer induces microcrystal formation (2), yielding an 18% (v/v) crystal stock (3). Serial dilution from 0.018% to 18% (v/v) (4) follows, and the NanoDrop Ultra^{C} FL instrument measures both the supernatant and the resuspended crystals at each dilution step for characterization (Figure 1A). Microscopic analysis traditionally determines crystal size (Figure 1B). This illustration was created with BioRender and adjusted with Affinity Studio.

Experimental procedures

Buffers

HEWL microcrystal batch production followed previously described methods.[3] These are the three main buffers used:

1. Dissolving buffer containing 50 mM Sodium Acetate (NaOAc) at pH 3.5
2. Crystallization buffer containing 0.1 M NaOAc at pH 3.5, 5 % PEG 6000, 3.2 M Sodium Chloride (NaCl)
3. Storage buffer containing 50 mM NaOAc at pH 3.5, 1.7 M NaCl

Buffer characterization and blanking

Each buffer served as its own blank on the NanoDrop Ultra^{C} FL instrument before measuring the corresponding protein or crystal sample. Triplicate, 2 µL aliquots of each buffer established background and scattering controls at 280 nm using the standard Protein A280 application.

Protein stock preparation

Lyophilized lysozyme (Carl Roth GmbH, CAS No.: 12650-88-3, batch 515379519) was suspended into dissolving buffer at a target concentration of 10 mg/mL. The standard approach was to dissolve 100 mg in 800 µL and use the NanoDrop Ultra^{C} FL instrument to adjust the concentration with dissolving buffer until the measurement indicated the target concentration had been achieved. The stock dissolved protein was stored on ice during measurements. The NanoDrop Ultra^{C} FL instrument measured the stock concentration from a 2 µL aliquot using the standard Protein A280 application with an E1% (L/gm-cm) value of 26.4, and the stock was adjusted with additional dissolving buffer if the measured concentration deviated from target.

Batch crystallization

Lysozyme solution and crystallization buffer were combined at a 1:1 ratio by volume at 18 °C to 20 °C, with both solutions pre-equilibrated on a heated thermoblock. Vortexing the lysozyme solution while pipetting in crystallization buffer drove rapid crystal formation, and microscopy with image-based sizing analysis confirmed an average crystal size of 2.24 µm ± 0.25 µm. The suspension was washed by the following procedure:

1. Centrifugation at 200 x g for 1.5 minutes
2. Removal of supernatant
3. Resuspension of the pellet in storage buffer

Two additional buffer exchanges followed at 200 x g for up to 3 minutes with gentle resuspension between steps. Gravity filtration through a 20 µm nylon mesh removed larger particles, and overnight sedimentation allowed the packed crystal volume to be adjusted to 18% of total suspension volume with storage buffer.

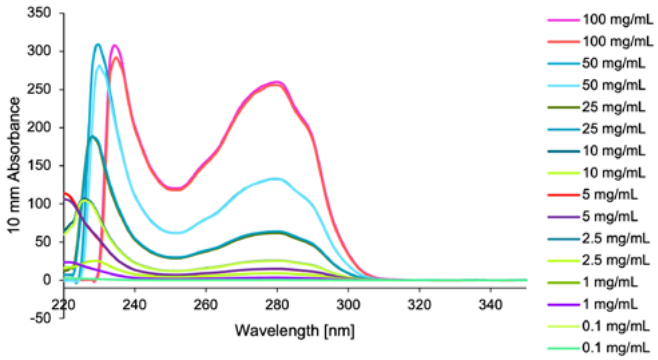
Dilution series and absorbance

A dilution series of microcrystal suspensions spanning 0.018% to 18% packed crystal volume used a combination of logarithmic and twofold scaling, followed by overnight sedimentation. Supernatants, crystal suspensions, and protein dilutions were each measured in triplicate using the standard Protein A280 application, and again using a Custom Method with 280 nm as the analysis wavelength and with sloping baseline correction enabled from 330-350 nm. Ratiometric analysis and plotting of recorded values was used to understand the reproducibility of the diluted microcrystals.

Results

The dissolved lysozyme protein dilution series revealed three distinct absorbance regimes (Figure 2). Recognizing them underscores the ability of the NanoDrop Ultra^{C} FL instrument to span a wide dynamic range, including high protein concentrations, with a reliable calibration curve. Because the A280 application optimizes pathlength based on A280 absorbance, any spectra below ~240 nm is attributed to pathlength differences. We also observed a spectral shift in the highest concentration (100 mg/mL), which was most likely due to shortening of the pathlength by the instrument upon receiving very high absorbance signals from the measured sample. The fact that simple dilution restored the canonical spectrum further illustrates that the observed changes in spectrum predominantly reflect an instrument-selected pathlength effect and colloidal effects associated with high absorbance, rather than irreversible structural changes in lysozyme samples.

A



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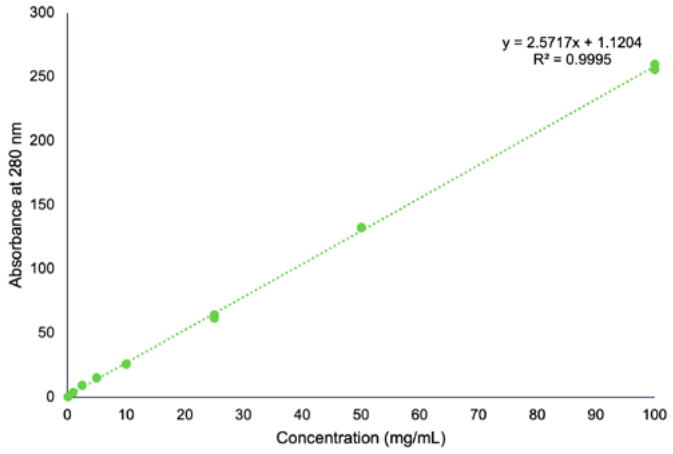


Figure 2. Absorbance spectrum of dissolved lysozyme protein measured using the Protein A280 application over 220-350 nm with baseline correction at 340 nm. The dissolved protein absorbance spectra are presented in A. In B, the peak absorbance at 280 nm is plotted, demonstrating that the trend reported on the NanoDrop Ultra^{C} FL instrument represents the decrease in absorbance values by diluting the protein. Each concentration was measured at least twice (n=3).

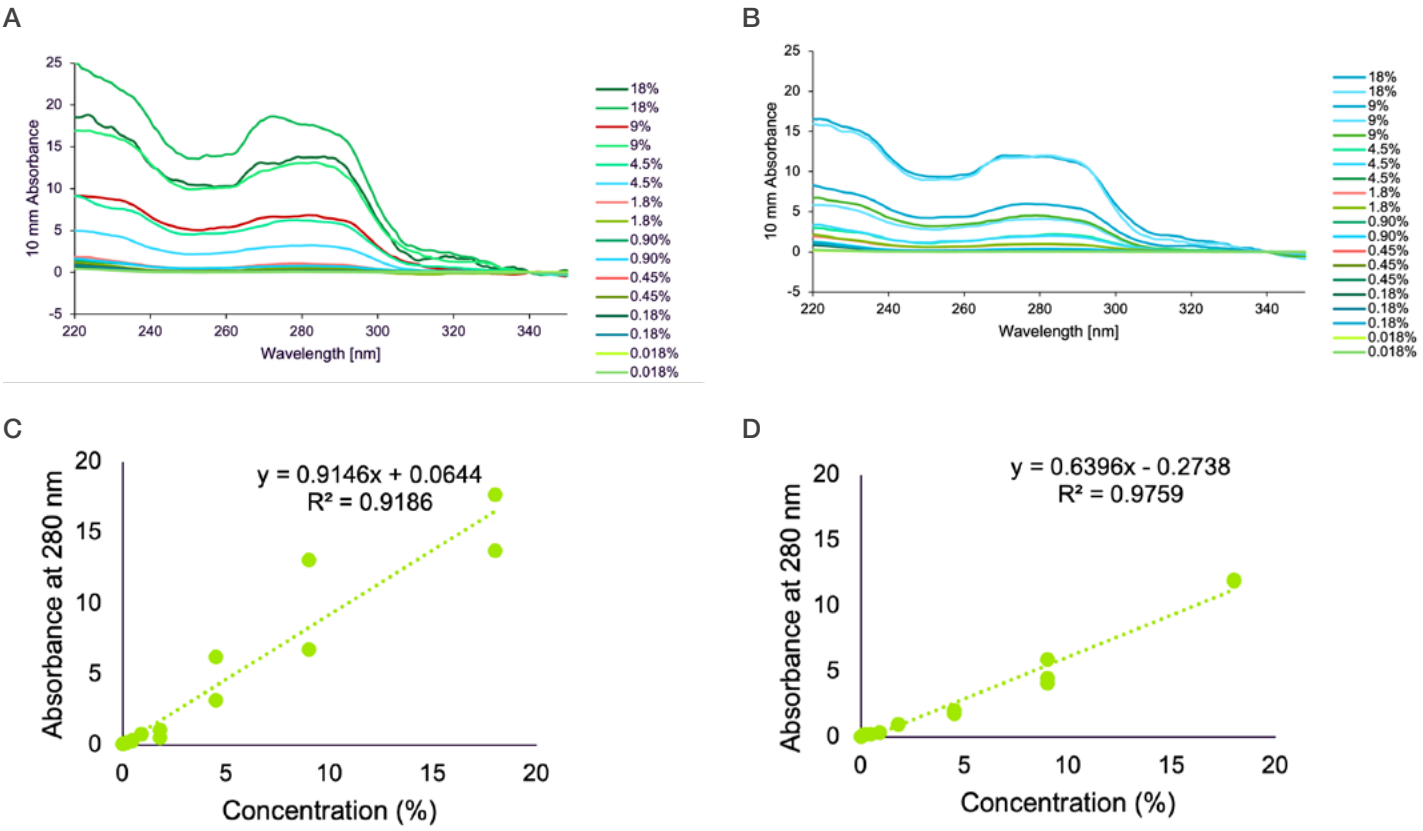
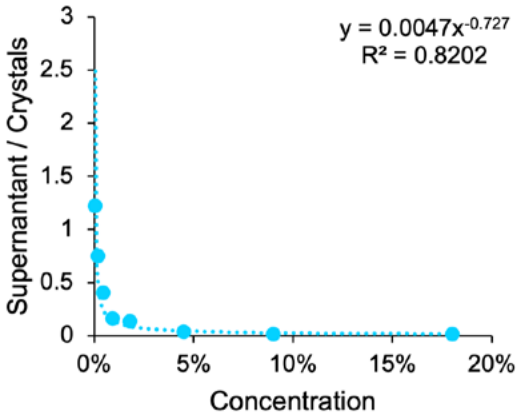


Figure 3. Comparison of point vs sloping baseline correction for microcrystal dilution calibration. (A) The absorbance spectrum of lysozyme crystals measured using the Protein A280 application over 220-350nm with baseline point correction at 340 nm is presented. The trend reported on the NanoDrop Ultra^{C} FL instrument represents the decrease in absorbance values with reduction in crystal concentration. Each concentration was measured at least twice (n=3). (B) The absorbance spectrum of lysozyme crystals measured using the Custom Method with sloping baseline correction (330-350nm) is demonstrated. The optimized trend corrects for the scattering observed in A with higher concentration of crystals on the Protein A280 application with only a single baseline correction. Each concentration was measured at least twice (n=3). (C), (D) These two graphs depict the peak absorbances processed from the spectral data represented in A and B, respectively.

Absorbance measurements of the microcrystal dilutions showed scatter concentrated at higher wavelengths, motivating a sloping baseline correction to isolate true protein signal from light scattering. Figure 3 shows the absorbances recorded for the lysozyme microcrystal dilutions derived from 100 mg/mL starting protein concentration using the Protein A280 application. The absorbance trend with a single-point correction shows likely scatter around the higher wavelength range (Figure 3A). Instead of the single point baseline correction, we decided to test the sloping baseline correction regime to account for the likely observed scatter. As demonstrated in Figure 3B, the measured absorbances of the calibrated microcrystal standards indicates a decrease in absorbance with dilution of the microcrystals. The Custom Method with sloping baseline correction across a regime allows the user to correct for the scattering in the sample by deriving the baseline correction region appropriate for their sample types and consequently to record true absorbance of the analyte from turbid solutions.

The absorbance ratio between supernatant and crystal suspension estimates what fraction of total protein signal comes from free, uncrystallized protein, and by extension how much protein is incorporated into the crystal lattice. Figure 4A shows the steady decline in uncrystallized protein fraction with increasing concentrations of protein standards flatlines after 4.5%, indicating the efficiency threshold of the crystallization protocol. This trend suggests gradual supersaturation occurs as more protein is incorporated into crystals, because the crystal solution has substantially higher A280 values than the supernatant alone. We also implemented a similar analysis with the sloping baseline correction in Figure 4B and noticed an improvement in calibration trend which yielded a higher coefficient of determination (single point R^2 = 0.8202 vs. sloping R^2 = 0.9332) consistent with an improved fitting of the curve.

A



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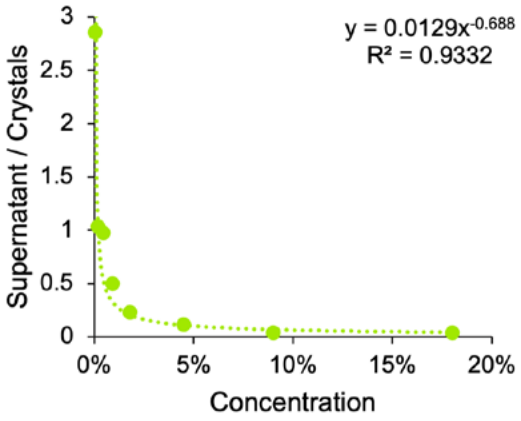

Figure 4. Calibration curves demonstrating the ratiometric distribution of uncrystallized fraction of lysozyme upon dilution. The plot in (A) demonstrates the single-point baseline corrected calibration while (B) implements the sloping baseline approach instead. This approach overall enables an improvement in estimation of the uncrystallized fraction to improve the fidelity. (n=3)

Discussion

Standardized characterization of microcrystal samples is important for the development of batch and scaled production approaches that are needed in serial crystallography workflows. The integration of simple checkpoints enabled by NanoDrop Ultra^C FL spectrophotometry measurements, summarized in Figure 1 in this application note, improve overall microcrystal production workflows, reduce errors, and provide real-time, low-sample-consumption quality evaluations. In a real-world scenario, the A280 methods that we described can be implemented during microcrystal batch formation so that beamline scientists can make an informed “go” or “no-go” decision on a batch before committing it to the beamline.

The instrument’s extended photometric range for the A280 method, as evaluated in Figure 2, accommodates the undiluted lysozyme stock and is able to resolve its full dilution series without requiring separate protocols for concentrated and diluted samples. This provides the first quality check that starting stock concentrations of dissolved protein are correct, and increases confidence in downstream microcrystal formation results.

The NanoDrop Ultra^C FL spectrophotometry approach for validating the dilution of microcrystal samples can be used for cross-validation during downstream measurements. The approach provides a quantitative readout of crystallization characterization which can be used in lieu of the current status quo of a semi-qualitative visual evaluation. In Figure 3 we illustrate the second quality check for microcrystal dilution, which is useful when calibrating beamline parameters in real time. For example, it is common practice to inject different microcrystal % (v/v) to evaluate the limit of detection for different diffractometer detectors. Having improved confidence in microcrystal dilution and quality thereof enhances real-time beamtime calibrations and can enable beamline scientists to move more rapidly to protein microcrystals other than lysozyme. The NanoDrop Ultra^C FL instrument enables quick adjustments for sample control, even for sample with low volumes.

In Figure 4, it can be seen that a lower supernatant-to-suspension ratio signals low to no soluble protein in the suspension, meaning it is a crystal-dense suspension that is ready for injection. A higher ratio flags a batch with a soluble protein pool that could underperform at the beamline and propagate forward into poor signal-to-noise-ratios in diffraction measurements. While these quality controls are important for lysozyme batch microcrystal characterization, we believe it will be especially useful in future batch and scaling approaches for other more precious proteins.

Conclusion

Integrating the NanoDrop Ultra^{C} FL spectrophotometer and fluorometer into our batch crystallization standardization approaches enabled us to efficiently evaluate our workflows. We found that the use of the Protein A280 application and the enablement of sloping baseline corrections in a Custom Method improved our checkpoints, with minimal sample consumption and improved confidence in the use of our microcrystal suspensions for droplet injection. OD600-based microcrystal quantification provides a rapid, reproducible estimate of crystal concentration that can be implemented directly during beamtime. Its short measurement time and reduced variability relative to manual hemocytometer counting make it well-suited for real-time sample-quality assessment and for standardizing crystal loading across serial crystallography experiments. Because serial crystallography experiments often operate under severe sample constraints, even modest gains in crystal enrichment translate directly into more efficient use of beamtime. We estimate this standardization will contribute to continued developments and optimization in batch microcrystal formation approaches as well as improved microcrystal quality control during beamtime.

AI Declaration: Artificial intelligence was used as a language-support tool during preparation of this application note. It assisted with translation and grammar of the manuscript text. All scientific content, experimental design, data generation, data analysis, interpretation, conclusions, and final editorial decisions were performed and verified by the authors. The authors take full responsibility for the accuracy and integrity of the final document.

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