ABSTRACT: Crystallization is a potential cost-effective alternative to chromatography for the purification of biotherapeutic proteins. Crystallization kinetics are required for the design and control of such processes, but only a limited quantity of proteins is available during the initial stage of process development. This article describes the design of a droplet-based evaporative system for the evaluation of candidate crystallization conditions and the estimation of kinetics using only a droplet (on the order of μL) of protein solution. The temperature and humidity of air fed to a flow cell containing the droplet are controlled for evaporation and rehydration of the droplet, which are used for manipulating supersaturation. Dual-angle images of the droplet are taken and analyzed on-line to obtain the droplet volume and crystal sizes. Crystallization kinetics are estimated based on a first-principles process model and experimental data. Tight control of temperature and humidity of the air, fast and accurate image analysis, and accurate estimation of crystallization kinetics are experimentally demonstrated for a model protein lysozyme. The estimated kinetics are suitable for the model-based design and control of protein crystallization processes.

1. INTRODUCTION

Packed-bed chromatography is the most common bioseparation process for biopharmaceutical manufacturing because of its high resolution. However, even with recent technologies for operational improvements including periodic counter-current chromatography, simulated moving bed chromatography, and multi-column counter-current solvent gradient purification,¹ chromatographic purification scales linearly with production rate and is a substantial proportion of the manufacturing costs. Chromatography has become more than half of the cost of goods for some biotherapeutic proteins, which will increase as titers and product demands continue to increase.³—⁶

Among alternative non-chromatography separation methods, crystallization is already widely used for the purification of small-molecule pharmaceutical compounds due to its cost-effectiveness. Crystallization has operating costs that scale sub-linearly with throughput.³,⁸ Furthermore, crystallization is conducive for the application of process analytical technology during operation. On-line real-time measurements enable model identification, real-time process monitoring, and advanced control implementation. This capability would enable a digital manufacturing approach to be applied in bioseparation in which manufacturing is integrated around a computer system using modern system engineering tools.³,⁸

Crystallization technology for large-molecule therapeutic proteins is much less mature than for small molecules and is mostly still in research and development other than a few isolated products.¹,³—⁶ Protein crystallization is mainly studied by micro-batch scale protein crystallization experiments focused on high-throughput protein crystallization screening for creating single protein crystals large enough for protein structure identification. These studies include the use of robotic systems⁵,¹⁰ that perform automated mixing and operation, microfluidic systems¹¹—¹⁴ for mixing and encapsulating reagents, evaporation-based systems¹⁵—¹⁷ with a precipitant reservoir or pumps for adding precipitant solution.
and water, and dialysis systems\textsuperscript{18} with a flowing precipitant reservoir.

Few studies consider operation at the production scale or characterize the crystallization kinetics needed for systematic scale-up to that scale. A limited quantity of protein is available during the initial stage of process development, which motivates the development of small-scale experimental systems with the capability of collecting experimental data of sufficient quantity and quality for the estimation of crystallization kinetics. This article describes the design of a droplet-based evaporative system for the evaluation of candidate crystallization conditions and the estimation of kinetics using only a minimum quantity of protein. In the developed system, air of controlled temperature and humidity is fed to a flow cell containing the droplet of protein solution to control evaporation and rehydration of the droplet, which are used to manipulate supersaturation. Dual-angle in situ images of the droplet are taken and analyzed to provide on-line measurements of droplet volume and crystal sizes. These measurements are used to estimate crystallization kinetics based on a first-principles model developed for the droplet system. The developed droplet-based evaporative crystallization system is experimentally demonstrated for the model protein lysozyme, which has well-characterized solubility and crystallization kinetics.

2. MATERIALS AND METHODS

2.1. Materials. Protein stock solutions (4, 10, 15, and 35 g/L) were prepared by dissolving hen egg white lysozyme (Hampton Research) in 50 mM citrate buffer, obtained from citric acid and sodium hydroxide (Sigma-Aldrich), at pH 4.5. Citrate buffer was used instead of acetate buffer, which is the most commonly used buffer for lysozyme crystallization because the volatile acetate buffer can evaporate during the operation. The concentration of the protein stock solution was determined by absorbance measurements at 280 nm (Thermo Scientific, NanoDrop One). Precipitant stock solutions (0.5 and 2.5 M) were prepared by dissolving sodium chloride (Sigma-Aldrich) in the citrate buffer. Both solutions were sterile-filtered through 0.22 μm polyethersulfone membranes (Corning). Solutions with target protein and precipitant concentration were prepared by mixing the protein and precipitant stock solutions.

2.2. Solubility Test. The solubility test was performed similarly as a study in the literature.\textsuperscript{19} The solubility of lysozyme in 50 mM citrate buffer at pH 4.5 was measured with respect to the concentration of sodium chloride. Supersaturated solutions for solubility measurements with sodium chloride concentrations from 0.25 to 1.25 M were prepared by mixing the protein and precipitant stock solutions. The supersaturated solutions were then stored at 4 °C for 1–3 days to initiate nucleation and grow crystals. The resulting solutions with crystals were placed in an incubating/cooling shaker (VWR) at 25 °C under extensive shaking with 500 rpm. Increasing the concentration of lysozyme in the solutions due to redissolving crystals was monitored periodically by absorbance measurements at 280 nm until equilibrium was reached (3–5 days). The standard deviations were calculated from three independent experiments for each concentration of sodium chloride.

2.3. System Prototype. The process flow diagram of the droplet-based evaporative crystallization system is shown in Figure 1a. A droplet containing aqueous protein solution was hung on the siliconized glass cover slide (Hampton Research) inside an optically visible flow cell for the real-time imaging using microscopes. The flow cell was designed in SolidWorks (Figure 1b) (available for download\textsuperscript{20}) and 3D-printed with a HP Multi Jet Fusion PA12 (92 × 29 × 34 mm, Sculpteo). Air of controlled temperature and humidity in a chamber was continuously fed to the flow cell for controlled evaporation and rehydration of the droplet. The humidity in the chamber was controlled by manipulating the ratio of the flow rates of wet air from a designed humidifier and dry air from a separate supply by using a three-way solenoid pinch valve (Masterflex). The temperature in the chamber was controlled by manipulating the set-point temperature of water in a programmable circulating water bath.

Figure 1. (a) Schematic diagram of the droplet-based evaporation crystallization system. Black lines indicate air flow, blue lines indicate fluid flow, and red lines indicate information flow. (b) 3D drawing view of the designed flow cell in SolidWorks. (c) Schematic diagram of the designed dual-angle in situ imaging system. (d) Photo of the flow cell and the dual-angle in situ imaging system.
(VWR, model 1197P) that was fed to the heat sink (McMaster, Copper tube) integrated into the chamber. The upstream chamber and line to the flow cell were strongly insulated to minimize thermal losses, which would allow the temperature of the air to the flow cell to be set as low as a few degrees above freezing. Evaporation and rehydration were used as handles for dynamically increasing and decreasing the supersaturation, which is the driving force for crystallization.

A programmable logic controller (PLC, Koyo Click CCO-12DRE-1-D) was used for low-level control of the system. The programmable circulating water bath was connected and actuated using the RS-232 port, while the three-way solenoid pinch valve was connected and actuated using the discrete relay output. The relative humidity/temperature transmitter (Omega, model HX15) was connected using the analogue current input. Proportional integral control was designed using the internal model control method21 and programmed in the PLC for control of the humidity and temperature. The PLC was connected via MODBUS TCP to a computer for data collection and human-machine interface.

A schematic diagram of the designed dual-angle in situ imaging system is shown in Figure 1c. The horizontal-view images were obtained using a microscope (OMAX, model V331A) with a camera (The Imaging Source, model DFK 22BU03). The vertical-view images were obtained using a microscope (Leica, model DM2500) with a camera (Sony, model ILCE-5100). The cameras were connected via USB to the computer for data collection. The images were then analyzed on-line to obtain the droplet volume and crystal sizes.

2.4. Crystallization Experiment. A 5 μL droplet of solution with a protein concentration of 2.0 g/L and a precipitant concentration of 0.25 M was pipetted on a siliconized glass cover slide, immediately inverted, and placed in the flow cell. Then, the flow cell was sealed with glass slides, o-rings, and rubber bands (Figure 1d). Evaporation and rehydration of the droplet were carried out at a controlled temperature and relative humidity. The droplet was monitored periodically with the imaging system for the droplet volume and crystal sizes.

2.5. Image Analysis Algorithms. The key steps of an on-line algorithm for analyzing the horizontal view images are schematized in Figure 2. The raw image (Figure 2a) is first filtered with a high-pass 2D spatial Gaussian filter to de-background and thresholded to obtain a black-and-white image (Figure 2b). Then, morphological closing and opening are performed to close any openings in the area around the droplet boundary (Figure 2c). Finally, the 2D convex hull is constructed from the detected boundary, and the top boundary is flattened for a more realistic shape (Figure 2d). The volume of the droplet is calculated with the detected boundary assuming rotational symmetry

\[ V = \frac{\pi}{4} \int_{z_0}^{z_c} d(z) \, dz \approx \frac{\pi}{4} \sum_{i=1}^{n_z} d_i^2 \Delta z \]

where \( z_0 \) is the height of the top of the boundary, \( z_c \) is the height at the bottom of the boundary, \( d(z) \) is the diameter at height \( z \), and \( n_z \) is the number of discretization points. The image analysis software is available for download.

A schematic of the key steps of the on-line algorithm for analyzing the vertical-view images is shown in Figure 3. The raw image (Figure 3a) is again filtered and thresholded (Figure 3b), and the detected areas around the droplet boundary are removed (Figure 3c). Morphological closing is performed to close any openings in the boundaries, and then holes in the boundaries are filled (Figure 3d). Finally, morphological opening is performed to remove areas smaller than the threshold size (Figure 3e) to obtain the areas of the detected crystals (Figure 3f). The image analysis software is available for download.20 The threshold size is chosen as 3 pixels to remove the remaining background noise. The pixel size of the vertical-view images is 1.955 μm/pixel, which results in a minimal crystal size detectable of 5.865 μm.

3. MODEL DEVELOPMENT

3.1. Solubility. The protein solubility is changed during evaporation/rehydration due to change in the concentration of precipitants in the droplet. At low ionic strength, salting-in based on the electrostatic interactions increases the solubility when the ionic strength increases. At high ionic strength, salting-out based on the hydrophobic interactions decreases the solubility when ionic strength increases. These relationships are typically modeled as

\[ \ln \frac{C_{P,\text{sat}}}{C_{P,\text{sat},0}} = k_i \sqrt{I} - k_o I \]

where the ionic strength is given by

\[ I = \frac{1}{2} \sum_{S \in S} S_{\text{c}} C_{S_{\text{c}}} \]

\( C_{P,\text{sat}} \) is the protein solubility in the solution, \( C_{P,\text{sat},0} \) is the protein solubility in pure water, \( k_i \) is the salting-in constant, \( k_o \) is the salting-out constant, \( S_{\text{c}} \) is the charge, and \( S_{\text{c}} \) is the concentration of the salt ion \( S_{\text{c}} \) and \( S \) is the set of salt ions in the solution.

For lysozyme, salting-in is not observed at a low ionic strength over a large range of pH due to a predominant electrostatic screening of the positively charged protein and adsorption of salt ions by the protein. In addition, the salting-out behavior of lysozyme observed in the experiments and reported in the literature is not consistent with eq. The observed behavior of lysozyme can be described by the phase equilibrium condition of the protein between the solution and solid in terms of chemical potentials. Then, the protein solubility is expressed as

\[ \ln C_{P,\text{sat}} = a - b \ln I - c I \]

where \( a, b, c \) and \( c \) are solubility constants. This expression does not account for the change of the chemical potential of the protein in the solid phase but has been shown to correlate well with lysozyme solubility data.

3.2. Crystal Growth. Lysozyme is observed to crystallize in the tetragonal structure, which has two characteristic lengths:

![Figure 2. Summary of the designed image analysis algorithm for the horizontal view images. (a) Raw images are (b) filtered with a high-pass 2D spatial Gaussian filter and thresholded. (c) Morphological closing and opening are performed. (d) The 2D convex hull is constructed and the top boundary is flattened.](image-url)
L and W (Figure 4a). With the given crystal structure, the volume of the crystal is

\[ V_C = W^2L \]  

The projected area of the crystal measured from the vertical-view images depends on observation angles \( \theta \) and \( \phi \) (Figure 4b)

\[ A_C = WL(\cos \theta + \sin \theta)\sin \phi + \frac{W^2}{4}(2\cos \phi - 2\sin \theta)\sin \phi \tan \alpha + |\cos \phi - (\cos \theta - \sin \theta)\sin \phi \tan \alpha| \]  

where \( \alpha \) is the angle in the hexagon (see the Supporting Information for details). From molecular orientations of the crystal structure, \( \tan \alpha = (37.9 \text{ Å})/(4 \times 28 \text{ Å}) \). The observation angles are within the ranges \( 0 \leq \theta \leq \pi/4 \) and \( 0 \leq \phi \leq \pi/2 \) due to symmetry of the crystal structure.

The protein crystal growth in the droplet is described by a deterministic model for crystal sizes and solution mass balances

\[ \frac{\text{d}L_n}{\text{d}t} = \begin{cases} \text{0}, & \text{if } t < t_n, \\ G_{L_n}, & \text{otherwise}, \end{cases} \quad n = 1, ..., N \]  

\[ \frac{\text{d}W_n}{\text{d}t} = \begin{cases} \text{0}, & \text{if } t < t_n, \\ G_{W_n}, & \text{otherwise}, \end{cases} \quad n = 1, ..., N \]  

\[ \frac{\text{d}C_p}{\text{d}t} = \frac{1}{V} \left[ J_{C_p} - \rho_{C_p} \sum_{n=1}^{N} (W_{nL_nG_W}) \right] \]
\[
\frac{dC_s}{dt} = \frac{J C_s}{V} \tag{10}
\]
\[
\frac{dV}{dt} = -J \tag{11}
\]
where \(C_p\) is the protein concentration, \(C_s\) is the salt concentration, \(V\) is the droplet volume, \(G_L\) and \(G_W\) are crystal growth rates, \(t_n\) is the time when at least \(n\) crystals have nucleated, \(N\) is the number of crystals, \(\rho_{cp}\) is the density of protein in a protein crystal, and \(J\) is the evaporation rate. The overall density of a protein crystal is the sum of the density of its components (protein \(p\), bound water \(w\), and free solvent \(s\)).27

\[
\rho_i = \sum_{i=p,w,s} \rho_{is} = \sum_{i=p,w,s} \phi_i \rho_i \tag{12}
\]
where \(\phi_i\) is the volume fraction, \(\rho_i\) is the apparent density, and the subscripts \(i\) refer to the components. For lysozyme, \(\rho_{cp} \approx 0.73\ g/\text{mL}\) was determined from \(\rho_{cp} + \rho_{cw} \approx 0.88\ g/\text{mL}, \phi_i \approx 0.34,\) and \(1/\rho_p \approx 0.70\ \text{mL}/g.28\)

The crystal growth is observed to be dominated by a continuous spiral growth from screw dislocation at low supersaturation and by two-dimensional nucleation and subsequent growth at high supersaturation.29,30 Because twodimensional nucleation was observed to remain as a viable growth mechanism even at low supersaturation for lysozyme,31,32 the crystal growth rate is modeled by the birth-andspread model33,34

\[
G_L = k_{L,i}(S - 1)^{2/3}(\ln S)^{\frac{1}{6}}\exp\left(-\frac{k_{L,2}}{\ln S}\right) \tag{13}
\]
\[
G_W = k_{W,i}(S - 1)^{2/3}(\ln S)^{\frac{1}{6}}\exp\left(-\frac{k_{W,2}}{\ln S}\right) \tag{14}
\]
where \(S = C_p/C_{p, sat}\) is the supersaturation and \(k_L\) and \(k_W\) are growth rate constants.

### 3.3. Nucleation

The nucleation of the first protein crystal in the droplet is described by the stochastic model7,35

\[
\frac{dP_0}{dt} = -B_0(C_{p,0})VP_0, \quad P_0(0) = 0 \tag{15}
\]
\[
\frac{dC_{p,0}(t)}{dt} = \frac{1}{V} C_{p,0}, \quad C_{p,0}(0) = C_p(0) \tag{16}
\]
where \(P_0\) is the time evolution of the probability that the droplet contains no crystals and \(B_0(C_{p,0})\) is the nucleation rate at the protein concentration \(C_{p,0}\) when the droplet contains no crystals. Then, the cumulative distribution function (CDF), the probability distribution function (PDF), and the mean time when at least one crystal has nucleated are \(7,35\)

\[
F(t) = P(T_1 \leq t) = 1 - P_0(t) \tag{17}
\]
\[
f(t) = \frac{dF(t)}{dt} = B_0(C_{p,0})VP_0 \tag{18}
\]
\[
\bar{T}_i = \int_0^\infty f_i(t) \, dt = \int_0^\infty t B_0(C_{p,0})VP_0 \, dt \tag{19}
\]
where \(T_i\) is the random variable for the time when at least \(i\) crystals have nucleated.

For the nucleation of subsequent crystals, the protein concentration decreases as already nucleated crystals grow to deplete the protein from the solution. The nucleation of the subsequent crystals can be described by

\[
\frac{dP}{dt} = -B_0(C_{p,n})VP_s, \quad P_i(t_i) = 1 \tag{20}
\]
\[
\frac{dC_{p,n}(t)}{dt} = \frac{1}{V} \left[J C_{p,n} - \rho_{cp} \sum_{i=1}^{n} (W_i^2 G_L + 2W_i L_i G_W)\right], \quad C_{p,n}(t_n) = C_{p,n-1}(t_n) \tag{21}
\]
where \(P_i\) is the time evolution of the probability that the droplet contains \(n\) crystals, and \(C_{p,n}\) is the protein concentration when the droplet contains \(n\) crystals given that \(T_i = t_i, i = 1, ..., n\). Then, the CDF, PDF, and mean time when at least \(n + 1\) crystals have nucleated, given that the \((n + 1)\)th crystal has nucleated and \(T_i = t_i, i = 1, ..., n\), are

\[
F_{n+1}(t) = P(T_{n+1} \leq t | T_{n+1} \in \mathbb{R}, T_i = t_i, i = 1, ..., n) \tag{22}
\]
\[
F_{n+1}(t) = \frac{P_{n+1}(t)}{P_{n+1}(\infty)} \tag{23}
\]
\[
\bar{T}_{n+1} = \int_0^\infty f_{n+1}(t) dt = \frac{\int_0^\infty tB_0(C_{p,0})VP_s(t) \, dt}{1 - P_s(\infty)} \tag{24}
\]
The primary nucleation rate is modeled by the expression from the classical theory of nucleation7,36

\[
B_0(C_{p,0}) = \frac{k_{h,1}C_{p,0}}{(\ln S)^{\frac{1}{2}}} \tag{25}
\]
where \(k_{h,1}\) and \(k_{h,2}\) are nucleation parameters and \(S_0 = C_p/C_{p, sat}\) is the supersaturation. Although primary nucleation for some systems follows an alternative mechanism known as two-step nucleation,37 the above expression has been observed to closely fit the experimental nucleation data for most systems.38

### 4. RESULTS AND DISCUSSION

#### 4.1. Solubility

The solubility of lysozyme in acetate buffer is extensively studied in the literature. For droplet-based evaporative crystallization, the volatility of the acetate buffer is not appropriate as it would evaporate from the droplet during operation. In this study, a non-volatile citrate buffer is used instead. The solubility of lysozyme was measured for a sodium chloride concentration from 0.25 to 1.25 M (Table 1). The solubility constants were estimated with least squares \((a = 2.02, b = 1.19, c = 1.42)\) and the solubility of lysozyme at

| Table 1. Solubility Measurements of Lysozyme in 50 mM Citrate Buffer at pH 4.5 and 25°C |
|---|
| \(C_s\) [M] | 0.25 | 0.50 | 0.75 | 1.00 | 1.25 |
| \(C_{p, sat}\) [g/L] | 27.46 ± 0.16 | 8.53 ± 0.03 | 3.41 ± 0.03 | 1.80 ± 0.02 | 1.20 ± 0.02 |

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different precipitant concentrations was well described by the model prediction, with a root-mean-squared error (rmse) of 0.15 g/L (Figure 5). Figure 5b shows that the solubility of lysozyme is not described by the commonly used linear salting-out term.

4.2. System Demonstration. Droplet-based evaporative crystallization experimentation was performed with an initial droplet condition of lysozyme concentration $C_P(t=0) = 2.0$ g/L and sodium chloride concentration $C_S(t=0) = 0.25$ M. Air flowing into the flow cell was controlled with set-points of temperature $T_{air,SP} = 25^\circ C$ and relative humidity $H_{air,SP} = 93\%$.

During operation, the set-point of the relative humidity was changed to $H_{air,SP} = 94\%$ at $\sim$45 h and $H_{air,SP} = 95\%$ at $\sim$70 h. The controllers designed for the system responded quickly and accurately with no instability for tracking the set-points of both temperature and relative humidity of the air fed to the flow cell (Figure 6a,b). The system manipulated the set-point temperature of the water circulated to the heat sink in the chamber to be slightly lower than the set-point temperature of air ($\sim$0.6 $^\circ C$) to cool the warm wet air from the humidifier (Figure 6c). The wet air ratio manipulated by the three-way solenoid valve was similar to the set-point relative humidity of the air (difference within 1%), which means that the absolute humidity of the warm wet air from the humidifier was almost same as that of the saturated air at the set-point temperature (Figure 6d).

The system took horizontal-view images every 12 s during the operation, which were analyzed on-line to obtain the droplet volume as described in Section 2.5 (Figure 7a). First, the droplet evaporates until reaching equilibrium with the air at
93% relative humidity (Figure 8a,b). Then, as the relative humidity was increased to 94 and 95%, the droplet rehydrates to equilibrate with the air at increased relative humidity (Figure 8c,d). The equilibrated volume of the droplet measured at each relative humidity is larger than the value calculated from the water activity of sodium chloride solutions in the literature (Figure 9a), which means that the equilibrated concentration of sodium chloride is lower than expected (Figure 9b). These differences are due to a slight decrease of the air temperature as it flows through the flow cell, resulting in an increase in the relative humidity. Because the water activity of sodium chloride solution is nearly independent of temperature of around 25 °C, this slight decrease in air temperature does not affect the expected values derived from the water activity of sodium chloride solutions. The equilibrated volume and concentration of sodium chloride agree well with the expected values when the relative humidity is adjusted with a 0.5 °C decrease in the air temperature (Figure 9a,b), which demonstrates that the rotational symmetry for calculating the volume of the droplet is a reasonable assumption.

Vertical-view images are taken every 5 min during the operation in the system and analyzed on-line to obtain the projected areas of the crystals (Section 2.5). The first crystal was detected at ∼6.5 h, subsequently resulting in a total of six crystals in the droplet (Figure 7b). Accurate detection of crystals from the images at various time points demonstrates the generality of the image analysis algorithm (Figure 10). Although all crystals have the same tetragonal structure, they appear as different shapes in the vertical-view images due to different observation angles (Section 3.2). Different growth trends are observed for some projected area of crystals because the crystal growth rates for the two characteristic lengths $L$ and $W$ have different supersaturation dependences.

Figure 7. (a) On-line volume measurements of the droplet in the flow cell and (b) projected area measurements of the crystals in the droplet from the image analysis algorithm.

Figure 8. Analyzed horizontal view images at (a) 0 h, (b) 40 h, (c) 65 h, and (d) 80 h.

to equilibrate with the air at increased relative humidity (Figure 8c,d). The equilibrated volume of the droplet measured at each relative humidity is larger than the value calculated from the water activity of sodium chloride solutions in the literature (Figure 9a), which means that the

equilibrated concentration of sodium chloride is lower than expected (Figure 9b). These differences are due to a slight decrease of the air temperature as it flows through the flow cell, resulting in an increase in the relative humidity. Because the water activity of sodium chloride solution is nearly independent of temperature of around 25 °C, this slight decrease in air temperature does not affect the expected values derived from the water activity of sodium chloride solutions. The equilibrated volume and concentration of sodium chloride agree well with the expected values when the relative humidity is adjusted with a 0.5 °C decrease in the air temperature (Figure 9a,b), which demonstrates that the rotational symmetry for calculating the volume of the droplet is a reasonable assumption.

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Figure 9. (a) Equilibrium volume of the droplet and (b) concentration of sodium chloride in the droplet as a function of relative humidity. Solid lines are expected values derived from water activities of sodium chloride solutions. Circles are experimental data and diamonds are experimental data adjusted with a 0.5 °C decrease in the air temperature.
4.3. Crystallization Kinetics Estimation. Measurements of the volume of the droplet and the projected areas of the crystals were used to estimate the crystal growth kinetics. The model predictions with estimated crystal growth kinetics are compared with the crystal projected area measurements in Figure 11. The evaporation rates during the operation were calculated from the droplet volume measurements. The parameters for the crystal growth were estimated by least squares (Table 2). The covariance of the parameter estimates were computed by:

\[ V_\theta \approx \left( \frac{\partial x(\theta)^T}{\partial \theta} V_c^{-1} \frac{\partial x(\theta)}{\partial \theta} \right)^{-1}, \]

\[ V_c = \left( \frac{(y - x(\theta))^T (y - x(\theta))}{n - 1} \right) I_n \]  

where \( x(\theta) \) is the vector of estimated states that depend on the vector of parameters \( \theta \), \( y \) is the vector of measured states, and \( n \) is number of experimental data points. The sensitivities of the states with respect to the parameters were computed via sensitivity equations. The correlation of parameter estimates are given by (Table 3):

\[ R_{\theta_i,\theta_j} = \frac{V_{\theta_i,\theta_j}}{\sqrt{V_{\theta_i,\theta_i} V_{\theta_j,\theta_j}}} \]  

The projected area measurement data of the crystals at different supersaturation conditions are well described by the model, with an rmse of 170 \( \mu \text{m}^2 \), which is within 0.5–2.5% of the measured crystal projected areas. The estimated crystal growth kinetics capture the different dependencies of the two characteristic lengths \( L \) and \( W \) on supersaturation, as has been reported in the literature (Figure 12a). These different dependencies are also consistent with the on-line crystal images. Vertical-view images for the second crystal during the

Figure 10. Analyzed vertical view images at (a) 5 h, (b) 10 h, (c) 20 h, and (d) 40 h.

Figure 11. Model predictions (solid lines) and experimental data (dotted lines) for (a) protein concentration, (b) salt concentration, (c) supersaturation, and (d) projected crystal areas.
Table 2. Estimated Values of Parameters for Crystallization Kinetics

| Parameter | Value              |
|-----------|--------------------|
| $k_{L1}$  | 0.752 ± 0.009      |
| $k_{L2}$  | $(7.05 ± 1.77) \times 10^{-2}$ |
| $k_{W1}$  | $(1.50 ± 0.34) \times 10^1$ |
| $k_{W2}$  | 10.5 ± 0.1         |

Table 3. Correlations of the Estimated Parameters for the Crystal Growth Kinetics

| Parameter | Value |
|-----------|-------|
| $k_{L1}$  | 1     |
| $k_{L2}$  | 0.985 |
| $k_{W1}$  | -0.855|
| $k_{W2}$  | -0.860|

5. CONCLUSIONS

A droplet-based evaporative system is described for evaluating crystallization conditions and estimating kinetics using only a droplet of protein solution (on the order of microliters). The system feeds air of controlled temperature and humidity to a flow cell containing the droplet to control evaporation and rehydration of the droplet and consequently the supersaturation. The horizontal- and vertical-view images of the droplet are collected by a dual-angle in situ imaging system and analyzed on-line to obtain the volume of the droplet and the projected area of the crystals. These on-line measurement data are used to estimate the crystallization kinetics based on a process model for the stochastic crystal nucleation and deterministic crystal growth in the droplet.

Experimentation with the model protein lysozyme demonstrates tight control of the temperature and relative humidity of the air fed to the droplet and fast and accurate image analysis by image analysis algorithms. The equilibrated droplet volume is related by a thermodynamic model to the relative humidity by the water activity of the solution. Although not a focus of this work, this model could be incorporated in a higher-level controller to control the droplet volume by manipulating the set-point of the relative humidity of the air. Projected crystal area measurements and corresponding nucleation induction time measurements show good agreement with a mathematical model that incorporates the crystallization kinetics estimated from data collected by the droplet-based evaporative crystallization system. The system can be used to study the effects of crystallization additives on the solubility and crystallization kinetics.

The capability of the droplet-based evaporative crystallization system to estimate crystallization kinetics using only a
minimum quantity of protein enables a systematic approach to
design and control protein crystallization at the early stage of
process development. The estimated crystallization kinetics
provide information required for the model-based optimal
scale-up to production scale. The crystallization kinetics
estimated from the droplet-based system would remain
applicable to process models for larger-scale crystallization
systems. These population balance models would be
augmented with secondary nucleation, aggregation, and
breakage rate expressions, where significant, for which
physics-based models are available (e.g., ref 47). The
population balance model would be combined with computa-
tional fluid dynamics if the mixing at the larger scale deviates
significantly from ideal conditions (e.g., refs 48 and 49).

■ ASSOCIATED CONTENT

Supporting Information
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Detailed derivation of the projected area of the crystal
measured from the vertical-view images (PDF)

Figure 13. Analyzed vertical-view images of the second crystal at (a) 10 h, (b) 15 h, (c) 20 h, (d) 30 h, and (e) 40 h.

Figure 14. Model predictions (solid lines) of the (a) time evolution of the probability that the droplet contains n crystals, (b) cumulative
distribution function, and (c) probability distribution function of the time when at least n crystals have nucleated. The circles denote mean values
from the model predictions and the crosses denote experimental data. (d) Model predictions (circles and bars) of mean and 75% prediction interval
of the time and experimental data (crosses) of the time when at least n crystals have nucleated.
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Notes
The authors declare no competing financial interest.

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