Supporting Information

Controlled protein crystal nucleation in microreactors: The effect of the droplet volume *versus* high supersaturation levels

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S1. Droplet curvature

For the studied droplet volume range, any effect of the droplet size on the surface tension is not expected ¹, while the temperature effect due to Marangoni effects needs further discussion ². This effect can be correlated to a buoyancy force, opposed by viscous drag and by heat diffusion, and, consequently, flow ³. The successive cooling and heating steps result in a thermal gradient effect ⁴ responsible for the thermocapillary motion ^{5,6} within a radial surface tension gradient ⁷ (from the highest to the lowest surface energy) ⁸. This is observed during the experiments since the droplets contact the channel wall due to the film drainage, where the film disappears in less than 60 ms. This value comes from a calculation assuming isothermal conditions and spherical droplets, and even neglecting van der Waals forces (see Table S1). The drainage time (t_{drain}) [s] is calculated by

$$t_{\text{drain}} = 20 d_d \sqrt{\frac{\mu_c}{u_d \sigma}}, \quad (\text{S1})$$

where d_d [m] is the droplet diameter, μ_c [Pa·s] the dynamic viscosity of the continuous phase, u_d [m·s⁻¹] the superficial velocity of the dispersed phase, and σ [N·m⁻¹] the interfacial tension.

Finally, the hypothesis of immobile interface is also assumed since the condition $t_{\text{drop}} \ll t_{\text{film}}$ is not satisfied for the cases under study ⁹ (see Table S1). The values for the diffusive time scale (t_{drop}) [s] and the film time scale (t_{film}) [s] are indicated in Table S1, which can be calculated by

$$t_{\text{drop}} = \frac{L_d}{u_d}, \quad (\text{S2.1})$$

and

$$t_{\text{film}} = \frac{e}{u_d}, \quad (\text{S2.2})$$

respectively; where L_d [m] is the droplet length, and e [m] the film thickness calculated using *Bretherton's law* and described by ¹⁰

$$e = 1.34 \frac{d_d}{2} \left(\frac{u_d \mu_c}{\sigma} \right)^{2/3}. \quad (\text{S2.3})$$

During the cooling step, from *i.e.* 25 °C to 15 °C, the tail curvature changes, and the front remains unchanged. During the heating step, from *i.e.* 15 °C to 25 °C, the tail curvature slowly recovers and the front changes its curvature. As soon as the final temperature is reached, the receding and advancing contact angles recover their initial values ⁵. This is generated from a non-uniform surface gradient along the flow direction ⁴. Since the carrier film disappears in less than 60 ms and the droplet generation occurs within the metastable region, the hypothesis of promoting nucleation by increasing the channel wall wettability and droplet curvature changes can be excluded. For the crystallization points belonging to the nucleation zone, this hypothesis might also be excluded as the crystals do not preferentially appear at the droplet interface. Finally, an extensive revision on the thermocapillary motion in several microfluidics' applications can be found in ¹¹.

Table S1 Overview of the hydrodynamic points under study.

Hydrodynamic condition	#1	#2	#3
Q_c [ml·min ⁻¹]	0.6	0.6	0.4
Q_d [ml·min ⁻¹]	0.2	0.7	1
α_c [-]	0.75	0.5	0.3
L_d [mm]	1.1	2.1	4.0
V_d [μl]	0.7	3.2	12.1
t_{drop} [s]	0.26	0.14	0.18
e [μm]	1.5	3.4	4.3
t_{film} [ms]	0.35	0.23	0.20
t_{drain} [ms]	48	26	22

[Note: Q_c – Flow rate of the continuous phase, Q_d – flow rate of the dispersed phase, α_c – volumetric flow rate of the continuous phase ($\alpha_c = Q_c / (Q_c + Q_d)$), V_d – droplet volume.]

S2. Protein self-assembly theory

Different authors have been reporting experimental approaches to measure concentration changes in a protein solution along time during the crystallization steps, nucleation and crystal growth. This includes the works reported by Ataka & Asai (1988)¹², Vekilov & Vorontsova (2014)¹³, Ferreira *et al.* (2017)¹⁴, Yang *et al.* (2018)¹⁵, and Yang *et al.* (2019)¹⁶. Ataka & Asai (1988) tracked the experiments using optical microscopy (OM) and UV absorption, while Vekilov & Vorontsova (2014) reported different techniques to experimentally understand the two-steps nucleation mechanism, with Atomic Force Microscopy (AFM), Dynamic Light Scattering (DLS) and Brownian Microscopy (BM). Ferreira *et al.* (2017) used DLS and OM to explain the influence of protein oligomerization and metastable clusters during the nucleation step. Finally, Yang and co-workers measured UV absorbance in both batch and continuous oscillatory flow crystallization processes.

The cases under study involve an additional level of complexity since the crystallization takes place inside confined droplets surrounded by an immiscible carrier fluid. Therefore, this makes any online analysis technique difficult to implement. For this reason, the protein

concentration on the bulk phase along time will be estimated using the analytical solution derived from the protein self-assembly theory.

General theory

This theory explains protein crystallization following an analogy with polymerization from a thermodynamic and kinetic point-of-view^{17,18}. Nucleation and crystal growth kinetics are represented by Equations (S3.1) and (S3.2), respectively^{18,19}. Nuclei is formed as a result of the interaction between 3 or 4 monomers and the crystal, while growth proceeds via attachment of monomers. The initial-conditions, expressed by Equation (S3.3), imply that at the initial time, nuclei formation is spontaneous, without adding seeds^{18,19}.

$$\frac{dC_n}{dt} = k_1 C_m^{i_0}, \quad (\text{S3.1})$$

$$-\frac{dC_m}{dt} = k_2 C_n C_m, \quad (\text{S3.2})$$

and

$$\text{I.C.: } t = 0 \rightarrow \begin{cases} C_n = 0 \\ C_m = C_0 \end{cases}, \quad (\text{S3.3})$$

where C_n [mol·m⁻³] is the nuclei concentration, C_m [mol·m⁻³] the monomer concentration, i_0 [-] the unit cells number (number of molecules that constitute the nucleus) (usually $i_0 = 2$ for tetragonal and $i_0 = 4$ for orthorhombic crystals)²⁰, k_1 and k_2 the rate constants for nucleation and crystal growth, respectively, and C_0 [mol·m⁻³] the initial protein concentration. With some algebraic manipulation, a single first-order ordinary differential equation is obtained, which is in fact a Riccati equation^{18,19}

$$\frac{dC_n}{dt} = -\frac{1}{2} k_2 i_0 C_n^2 + k_1 C_0^{i_0}. \quad (\text{S4})$$

Integrating Equation (S4) and attending to the initial-conditions of the problem [Equation (S3.3)], nuclei concentration along time is expressed by^{18,19}

$$\ln \frac{1 + C_n \sqrt{\frac{1}{2} \frac{k_2}{k_1} \frac{i_0}{C_0^{i_0}}}}{1 - C_n \sqrt{\frac{1}{2} \frac{k_2}{k_1} \frac{i_0}{C_0^{i_0}}}} = \sqrt{k_1 k_2} \sqrt{2 i_0 C_0^{i_0}} t. \quad (S5)$$

Simplifying Equation (S5) by using the equality expressed in Equation (S3.1), a dependence of nuclei concentration along time can be obtained using Equation (S6.1)^{18,19}. Following a similar approach, the monomeric concentration along time can also be calculated as the concentration along time expressed by Equation (S6.2)²⁰.

$$C_n(t) = \sqrt{2 \frac{k_1}{k_2} \frac{1}{i_0} (C_0^{i_0} - C_m^{i_0})}, \quad (S6.1)$$

and

$$C_m(t) = C_0 \operatorname{sech}^{2/i_0} \left(\sqrt{k_1 k_2} \sqrt{\frac{1}{2} i_0 C_0^{i_0}} t \right). \quad (S6.2)$$

Theory validation

The derived equations were validated using data reported in the literature, both in batch and continuous microreactors^{15,16,19–21}. The obtained data and the model fitting and parameters are presented in Figures S1-S4 and Table S2 for both tetragonal and orthorhombic crystals. Furthermore, the resulting conclusions will contribute to a generic modeling framework for the analysis and design of crystallization experiments. The fitting parameters are determined through the minimization of the sum of quadratic errors between the theoretically calculated and experimentally obtained concentrations $\left[\sum (C_{p,\text{exp}}^b - C_{p,\text{analyt}}^b)^2 \right]$, where the kinetic constant value ($k_1 k_2$) is a single calculated parameter.

Fig. S1 Protein concentration remaining in the bulk phase (C_p^b) normalized with the initial protein concentration (C_p^0) along time (t) for different: **(a.1)** Initial protein concentrations (C_p^0), **(a.2)** Oscillation frequencies (f), and **(a.3)** Oscillation amplitudes (x_0) reported by Yang *et al.* (2018)¹⁵ for a batch oscillatory flow crystallizer [Markers represent experimental points and lines the fitting model].

Fig. S2 Protein concentration remaining in the bulk phase (C_p^b) normalized with the initial protein concentration (C_p^0) along time (t) for different: **(a.1)** Shaking rates (r), and **(a.2)** Initial protein concentrations (C_p^0) reported by Yang *et al.* (2019)¹⁶ for a batch shaking crystallizer [Markers represent experimental points and lines the fitting model].

Fig. S3 Protein concentration remaining in the bulk phase (C_p^b) normalized with the initial protein concentration (C_p^0) along time (t) for: **(a.1)** Shorter time periods, and **(a.2)** Longer time periods reported by Ataka and co-workers^{19,20} for a batch crystallizer [Markers represent experimental points and lines the fitting model].

Fig. S4 Protein concentration remaining in the bulk phase (C_p^b) normalized with the initial protein concentration (C_p^0) along time (t) for: **(a.1)** Orthorhombic crystals at pH = 4.6 ($T = 35\text{ }^\circ\text{C}$), **(a.2)** Orthorhombic crystals at pH = 6.0 ($T = 35\text{ }^\circ\text{C}$), and **(a.3)** Tetragonal crystals at pH = 6.0 ($T = 5\text{ }^\circ\text{C}$) reported by Bessho *et al.* (1994)²¹ for a batch crystallizer [Markers represent experimental points and lines the fitting model].

Table S2 Kinetic constant values for the different studies reported in the literature.

Case study	C_p^0	i_0	$k_1 k_2$	Additional conditions
Yang <i>et al.</i> (2018) ¹⁵ Batch oscillatory flow crystallizer	65 mg·ml ⁻¹	2	$4.8 \times 10^{-8} \text{ (mg·ml}^{-1}\text{)}^{-4} \cdot \text{min}^{-2}$	$T = 20 \text{ }^\circ\text{C} \mid \text{pH} = 4.9$ $x_0 = 20 \text{ mm} \mid f = 0.1 \text{ Hz}$
	55 mg·ml ⁻¹		$4.7 \times 10^{-8} \text{ (mg·ml}^{-1}\text{)}^{-4} \cdot \text{min}^{-2}$	
	35 mg·ml ⁻¹		$5.9 \times 10^{-8} \text{ (mg·ml}^{-1}\text{)}^{-4} \cdot \text{min}^{-2}$	
	30 mg·ml ⁻¹		$6.1 \times 10^{-8} \text{ (mg·ml}^{-1}\text{)}^{-4} \cdot \text{min}^{-2}$	
	25 mg·ml ⁻¹		$3.8 \times 10^{-8} \text{ (mg·ml}^{-1}\text{)}^{-4} \cdot \text{min}^{-2}$	
	25 mg·ml ⁻¹	2	$3.5 \times 10^{-6} \text{ (mg·ml}^{-1}\text{)}^{-4} \cdot \text{min}^{-2}$	$x_0 = 20 \text{ mm} \mid f = 0.5 \text{ Hz}$
			$2.7 \times 10^{-8} \text{ (mg·ml}^{-1}\text{)}^{-4} \cdot \text{min}^{-2}$	$x_0 = 20 \text{ mm} \mid f = 0.2\text{-}0.25 \text{ Hz}$
			$3.0 \times 10^{-8} \text{ (mg·ml}^{-1}\text{)}^{-4} \cdot \text{min}^{-2}$	$x_0 = 20 \text{ mm} \mid f = 0.1\text{-}0.15 \text{ Hz}$
			$7.1 \times 10^{-7} \text{ (mg·ml}^{-1}\text{)}^{-4} \cdot \text{min}^{-2}$	$x_0 = 20 \text{ mm} \mid f = 0.05 \text{ Hz}$
			$1.0 \times 10^{-7} \text{ (mg·ml}^{-1}\text{)}^{-4} \cdot \text{min}^{-2}$	$f = 0.15 \text{ Hz} \mid x_0 = 20\text{-}25 \text{ mm}$
Yang <i>et al.</i> (2019) ¹⁶ Batch shaking crystallizer	25 mg·ml ⁻¹	2	$3.4 \times 10^{-8} \text{ (mg·ml}^{-1}\text{)}^{-4} \cdot \text{min}^{-2}$	$f = 0.15 \text{ Hz} \mid x_0 = 10\text{-}15 \text{ mm}$
			$9.0 \times 10^{-8} \text{ (mg·ml}^{-1}\text{)}^{-4} \cdot \text{min}^{-2}$	$T = 20 \text{ }^\circ\text{C} \mid \text{pH} = 4.8$
				$r = 100 \text{ rpm}$
	45 mg·ml ⁻¹	2	$1.2 \times 10^{-7} \text{ (mg·ml}^{-1}\text{)}^{-4} \cdot \text{min}^{-2}$	$r = 150 \text{ rpm}$
			$2.3 \times 10^{-7} \text{ (mg·ml}^{-1}\text{)}^{-4} \cdot \text{min}^{-2}$	$r = 200 \text{ rpm}$
			$1.6 \times 10^{-7} \text{ (mg·ml}^{-1}\text{)}^{-4} \cdot \text{min}^{-2}$	$r = 100 \text{ rpm}$
			$2.3 \times 10^{-7} \text{ (mg·ml}^{-1}\text{)}^{-4} \cdot \text{min}^{-2}$	$r = 150 \text{ rpm}$
Yang <i>et al.</i> (2019) ¹⁶ Batch oscillatory flow crystallizer	50 mg·ml ⁻¹	2	$8.7 \times 10^{-7} \text{ (mg·ml}^{-1}\text{)}^{-4} \cdot \text{min}^{-2}$	$r = 200 \text{ rpm}$
	45 mg·ml ⁻¹		$4.1 \times 10^{-7} \text{ (mg·ml}^{-1}\text{)}^{-4} \cdot \text{min}^{-2}$	$T = 20 \text{ }^\circ\text{C} \mid \text{pH} = 4.8$ $x_0 = 20 \text{ mm} \mid f = 0.5 \text{ Hz}$
	35 mg·ml ⁻¹		$1.8 \times 10^{-7} \text{ (mg·ml}^{-1}\text{)}^{-4} \cdot \text{min}^{-2}$	
	25 mg·ml ⁻¹		$1.4 \times 10^{-7} \text{ (mg·ml}^{-1}\text{)}^{-4} \cdot \text{min}^{-2}$	
	25 mg·ml ⁻¹		$8.2 \times 10^{-5} \text{ (mg·ml}^{-1}\text{)}^{-4} \cdot \text{min}^{-2}$	
	15 mg·ml ⁻¹		$5.1 \times 10^{-8} \text{ (mg·ml}^{-1}\text{)}^{-4} \cdot \text{min}^{-2}$	
Ataka & Asai (1990) ¹⁹ & Ataka (1995) ²⁰ Batch crystallizer	10.2%	4	$2.0 \times 10^{-4} \text{ \%}^{-4} \cdot \text{days}^{-2}$	$T = 35 \text{ }^\circ\text{C} \mid \text{pH} = 4.6$ $C_{\text{NaCl}} = 3\%$
	9.7%		$1.7 \times 10^{-4} \text{ \%}^{-4} \cdot \text{days}^{-2}$	
	7.7%		$9.0 \times 10^{-5} \text{ \%}^{-4} \cdot \text{days}^{-2}$	
	4.7%		$6.0 \times 10^{-5} \text{ \%}^{-4} \cdot \text{days}^{-2}$	
	3.2%		$5.5 \times 10^{-5} \text{ \%}^{-4} \cdot \text{days}^{-2}$	
	2.1%		$7.5 \times 10^{-5} \text{ \%}^{-4} \cdot \text{days}^{-2}$	
Ataka & Asai (1990) ¹⁹ & Ataka (1995) ²⁰ Batch crystallizer	10.2%	4	$6.2 \times 10^{-4} \text{ \%}^{-4} \cdot \text{days}^{-2}$	$T = 35 \text{ }^\circ\text{C} \mid \text{pH} = 4.6$ $C_{\text{NaCl}} = 3\%$
	9.7%		$4.1 \times 10^{-4} \text{ \%}^{-4} \cdot \text{days}^{-2}$	
	7.7%		$2.0 \times 10^{-4} \text{ \%}^{-4} \cdot \text{days}^{-2}$	
	4.7%		$1.4 \times 10^{-5} \text{ \%}^{-4} \cdot \text{days}^{-2}$	
	3.2%		$6.4 \times 10^{-5} \text{ \%}^{-4} \cdot \text{days}^{-2}$	
	2.1%		$1.4 \times 10^{-5} \text{ \%}^{-4} \cdot \text{days}^{-2}$	
Bessho <i>et al.</i> (1994) ²¹ Batch crystallizer	9.71%	4	$1.6 \times 10^{-4} \text{ \%}^{-4} \cdot \text{days}^{-2}$	$T = 35 \text{ }^\circ\text{C} \mid \text{pH} = 4.6$ $C_{\text{NaCl}} = 3\%$
	7.73%		$3.3 \times 10^{-5} \text{ \%}^{-4} \cdot \text{days}^{-2}$	
	4.66%		$2.4 \times 10^{-6} \text{ \%}^{-4} \cdot \text{days}^{-2}$	
	3.19%		$1.7 \times 10^{-7} \text{ \%}^{-4} \cdot \text{days}^{-2}$	
	2.07%		$1.5 \times 10^{-8} \text{ \%}^{-4} \cdot \text{days}^{-2}$	
	6.57%	4	$1.0 \times 10^{-5} \text{ \%}^{-4} \cdot \text{days}^{-2}$	$T = 35 \text{ }^\circ\text{C} \mid \text{pH} = 6.0$ $C_{\text{NaCl}} = 3\%$
	5.71%		$4.6 \times 10^{-6} \text{ \%}^{-4} \cdot \text{days}^{-2}$	
	4.69%		$2.0 \times 10^{-6} \text{ \%}^{-4} \cdot \text{days}^{-2}$	
	3.79%		$5.8 \times 10^{-7} \text{ \%}^{-4} \cdot \text{days}^{-2}$	
	2.85%		$2.6 \times 10^{-7} \text{ \%}^{-4} \cdot \text{days}^{-2}$	
	1.95%	2	$5.0 \times 10^{-8} \text{ \%}^{-4} \cdot \text{days}^{-2}$	$T = 5 \text{ }^\circ\text{C} \mid \text{pH} = 6.0$ $C_{\text{NaCl}} = 3\%$
	2.10%		$1.8 \times 10^{-2} \text{ \%}^{-4} \cdot \text{days}^{-2}$	
	1.47%		$4.3 \times 10^{-3} \text{ \%}^{-4} \cdot \text{days}^{-2}$	
	1.12%		$8.4 \times 10^{-4} \text{ \%}^{-4} \cdot \text{days}^{-2}$	
	0.88%		$1.7 \times 10^{-4} \text{ \%}^{-4} \cdot \text{days}^{-2}$	
	0.63%		$1.6 \times 10^{-5} \text{ \%}^{-4} \cdot \text{days}^{-2}$	

From the cases under study, it seems reasonable to assume a value for the kinetic constant based on the works reported by Yang and co-workers^{15,16}. The theory cannot be used to draw any conclusion about the double-pulse temperature experiments, it only can be used to give a rough estimation of the protein concentration in the bulk phase along time. The temperature, pH, and protein and precipitant agent concentrations are among the range reported in those studies, besides the fact that the crystal morphology is identical (tetragonal crystals, $i_0 = 2$). The values do not seem to indicate a clear tendency for the effect of the oscillation and amplitude frequencies on the kinetic constant values. However, higher shaking rates seem to contribute to higher kinetic constant values. Thereby, it is necessary to investigate the results obtained with batch crystallizers for observing an effect of the initial protein concentration on the kinetic constant. Based on the previous points, it will be assumed a kinetic constant value of $1.0 \times 10^{-9} (\text{mg} \cdot \text{ml}^{-1})^{-4} \cdot \text{min}^{-2}$, which results in a protein concentration in the bulk phase of $39.9 \text{ mg} \cdot \text{ml}^{-1}$ and $16.7 \text{ mg} \cdot \text{ml}^{-1}$ after 1 h and 20 h, respectively ($T = 20 \text{ }^\circ\text{C}$, $S_0 = 5.4$). The last value explains the reason for the appearance of large crystals during the isothermal experiments. While the experiment starts inside the nucleation zone, due to the protein consumption, the crystals will start growing after roughly 12 h, which corresponds to the moment at which the metastability concentration is reached ($27.5 \text{ mg} \cdot \text{ml}^{-1}$). Even though the available data at different temperature values is limited, it is possible to induce from the results on Table S2 that higher supersaturations contribute to higher kinetic constant values, which results in steeper protein concentration decays. This can explain the reason for the crystal growing during the double-pulse temperature experiments. For the experiments conducted at $10 \text{ }^\circ\text{C}$ (nucleation temperature, $S_0 = 17.9$) and $20 \text{ }^\circ\text{C}$ (crystal growth temperature, $S_0 = 5.4$), even though these supersaturation values belong to the nucleation zone, the concentration decay will make the protein solution to surpass the metastability limit and promote the crystal growth inside the metastable zone. However, for the experiments conducted at $15 \text{ }^\circ\text{C}$ (nucleation temperature, $S_0 = 9.6$) and $25 \text{ }^\circ\text{C}$ (crystal growth temperature, $S_0 = 3.4$), the concentration decay during the nucleation time (1 h) seems to not be enough to generate crystals with a detectable size.

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