Kinetics of HSA crystallization and its relationship with the phase diagram

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A B S T R A C T

Using the protein human serum albumin (HSA) and the trivalent salt cerium chloride (CeCl₃) to tune the effective interactions, we induce crystallization and connect the crystallization kinetics to different regions of the phase diagram. The nucleation density, the nucleation rate, as well as the crystal size show maxima slightly above the specific salt concentration c* for aggregation. The comparison with data of the reduced second virial coefficient suggests that 𝐵_2/𝐵_2^∞ alone is insufficient to explain the observed trends. We thus discuss further aspects impacting the crystallization behavior. We are able to link and distinguish the kinetic regimes regarding their relative distance to phase boundaries, namely c* as well as the liquid–liquid phase separation (LLPS) border.

1. Introduction

A major challenge in understanding the structure of proteins, and thus their function, is acquiring high quality crystals suitable for diffraction [1]. This is of key importance in various areas ranging from pharmacy and medicine to structural biology. Yet, crystallization remains difficult to predict and control due to the complex interactions that proteins exhibit in solution and the associated non-trivial phase behavior [2–5]. A particular challenge for the fundamental understanding is the relationship of the phase diagram with the crystallization kinetics and resulting density and size of crystals [5–15].

Human serum albumin (HSA) is the most abundant protein in the human blood stream and has many important physiological functions, e.g., maintaining the osmotic pressure to carrying small molecules [16, 17]. Since HSA is a globular and net-negatively charged protein at neutral pH, it is an ideal object to study cation-induced crystallization. Specifically multivalent ions offer an intriguing way of inducing crystallization, as they can trigger effective attractive interactions, which can be controlled by the salt concentration c₂ [18–23].

Trivalent ions can give rise to reentrant condensation (RC) phase behavior of the protein solutions [20]. At neutral pH, the proteins exhibit a net repulsive force due to their charge and the aqueous solution is clear (regime I). For c₂ above the first specific salt concentration c*, the ions bind to the proteins, diminishing their net charge, and may even facilitate the bridging of proteins, causing the proteins to aggregate and the solution to become turbid. This second regime (regime II) can also consist of different condensed phases, namely a dense and a dilute protein solution caused by a metastable liquid–liquid phase separation (LLPS) [20]. As c₂ is further increased above a second specific concentration (c**), more ions bind to the protein, causing the proteins to undergo an effective charge inversion (regime III) and the solution to be clear again [18,24].

Previous work on the HSA-CeCl₃ system focused on the crystallization pathway and the role of LLPS [25]. Nucleation mostly occurs in regime II of the phase diagram, near the LLPS loop [26]. Apparently, the dense liquid phase (DLP) acts as a reservoir for nucleation [25].

In the present paper, we shed light on the crystallization process by studying the kinetics. In fact, connecting the kinetics to the phase behavior is a key ingredient for the overall understanding of the crystallization and the fine-tuning of, e.g., nucleation probability, crystal size and quality.

This paper is organized as follows. In Section 2, the materials and the experimental methods are explained. Section 3 is dedicated to the results, where we first explain in detail the phase diagram with its peculiarities and then the kinetic behavior in different regions of the phase diagram. The conclusions are presented in Section 4. A number of complementary plots of the large body of data is provided in the Supplementary Information.

2. Materials and experimental methods

2.1. Materials

HSA and CeCl₃ were purchased from Sigma-Aldrich, now Merck, with guaranteed purities of 97% for HSA (product no.A9511) and...
99.99% for CeCl₃ (product no. 429406). Protein and salt were used as received without further purification. The stock solutions were prepared by dissolving weighed amounts of protein or salt powder in deionized (18.2 MΩ) and degassed Millipore water, respectively, at room temperature. A freshly prepared protein solution was incubated overnight to ensure the complete dissolution of the protein powder. Subsequently, the protein concentration was determined by UV–Vis-spectroscopy, using an extinction coefficient of HSA of ε = 0.531 ml mg⁻¹ cm⁻¹ at a wavelength of λ = 278 nm [27]. The HSA stock solutions were kept in the fridge for up to three weeks. The CeCl₃ solution was stored at room temperature and was used for all experiments in this study.

We note that for protein work, generally there is a certain batch-to-batch variation. The results presented here are consistent within one batch, and general statements apply to other batches as well.

2.2. Methods

2.2.1. Determination of phase boundaries and crystallization conditions

Since in regime II the effective intermolecular interactions are mostly attractive and crystallization is facilitated by the multivalent cations, crystallization is expected to occur predominantly here. Thus, conditions within regime II were chosen for the crystallization experiments. To ensure that the chosen salt concentrations are located within the second regime, the phase boundaries of the second regime (c⁺ and c∗∗) were determined by visual inspection of a dilution series (similar to Ref. [25]). For all experiments, the temperature was kept constant at 23 ± 0.5 °C.

2.2.2. Optical microscopy

For optical microscopy experiments, fresh sample solutions were prepared. After adding the salt solution, the required amount of volume was transferred into spacers, which were previously mounted on a glass slide and subsequently covered with a cover slide. These spacers composed of double-sided adhesive film were purchased from Thermo Scientific™ with an area of 1 cm², a height of 0.25 mm and 25 μl volume.

The samples were investigated using a bright-field optical microscope (Axio Scope.A1, Carl Zeiss AG). Images were recorded by a microscope-included camera (AxioCam Icc5, Carl Zeiss AG) in combination with the software ZEISS ZEN 3.2. The same software was also used for length and area measurements of the crystals. The diameter of a crystal could be determined with an error of ± 5 μm.

Crystallization was observed at fixed time intervals from a few hours after preparation to a maximum of 14 days. At these times, images were recorded, and the number of crystals grown inside the spacer was counted. For the crystal counting, a 10x objective was used, which translates into the smallest detectable crystal size of about 25 μm. The crystal counting comes with a certain inherent error, because the nuclei only become visible with a certain time delay, which has to be considered when discussing the temporal onset of nucleation. Nevertheless, the nucleation rate can be obtained by counting the visible crystals under the assumption that every critical nucleus grows at the same rate into a crystal of detectable size [28,29].

To acquire three-dimensional (3D) complementary images of the crystals, images of selected samples were taken with a confocal fluorescence microscope (Leica SP8). For this, the samples had to be inverted, since the microscope was mounted on an inverted microscope body. HSA shows an intrinsic fluorescence, which originates from the tryptophan (Trp) 214 residue of the protein [30]. The excitation laser employed had a wavelength of 488 nm, and a 10x objective (air) was used. Three-dimensional images were generated from an acquired z-stack of confocal images. For this purpose, the software Fiji (version ImageJ 1.53c) [31] and the plugins Volume Viewer and 3D Viewer [32] were used. Contrast and brightness were adjusted in Fiji [31].

Fig. 1. Phase diagram with the phase boundaries c⁺ and c∗∗. The number of crystals per cm² (i.e., the nucleation density) after 14 days is represented by differently colored filled circles. The nucleation density for the respective conditions within the HSA/CeCl₃ phase diagram is indicated according to the legend. Samples at c⁺ = 20, 35, 50, 80 and 100 mg/ml with varying c𝑝 were investigated in spacers with a volume of 25 μl. The error of the counted crystal numbers is estimated to be about 10%. The vertical box around c⁺ = 50 mg/ml indicates the conditions employed in Fig. 2. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3. Results and discussion

To establish a connection between the crystallization kinetics and the phase diagram, the crystallization conditions were determined by systematically varying the protein and salt concentration. Fig. 1 can be regarded as a multidimensional map of the HSA-CeCl₃ crystallization regime that is used as a guide for the subsequent kinetic analysis (Section 3.2). The studied observables along a fixed protein concentration of c𝑝 = 50 mg/ml, namely nucleation density, kinetic growth rate k, and maximum crystal size are compiled in Fig. 2.

3.1. Phase diagram and crystallization conditions

Fig. 1 shows the experimental phase diagram with the phase boundaries c⁺ and c∗∗ and the number of crystals (14 days after preparation in spacers with 25 μl volume) found at different conditions across the phase diagram.

This already leads to several important qualitative observations. First of all, crystallization was observed only in the lower part of the second regime. In general, this is in good agreement with previous experimental results that crystallization is only observed in regime II. Here, the value of the reduced second virial coefficient B₂ = B₂⁺ of the bulk solution is clearly negative, indicating a net attraction between proteins (see Ref. [26]). The model of ion-activated attractive patches can successfully describe the underlying mechanism of attraction and phase behavior [24]. The absence of HSA crystallization at high c₁ may be caused by the high occupation of binding sites by metal ions, which could block the protein–protein contacts or change the crystallization pathway [18,19,26].

Second, with increasing c𝑝, a broadening of the second regime can be observed. However, the conditions at which crystals were found do not reach significantly higher c₁ values with increasing c𝑝 (Fig. 1). This means that the width of the second regime in terms of absolute salt concentration is not reflected in a broader c⁺-window for crystallization. Reasons for this observation might be the strong aggregation of proteins, which is observed at high c𝑝. This might drive proteins to aggregate too quickly, leading to unfavorable binding and blocking of surface sites of more productive crystal growth [33]. In addition, the aggregates formed might be rather stable, which would drastically lower the supersaturation of the remaining solution and inhibit crystal nucleation.
Last, as can be seen from Figs. 1 and 2b, the nucleation density exhibits a sharp maximum shortly above $c^*$ and then decays rapidly upon increasing $c_s$. Data of the crystal number density for a fixed $c_p$ of 50 mg/ml is given in Fig. 2b. In this region, the value of the reduced second virial coefficient $B_2/B_2^{HS}$ of the bulk solution corresponds to strong attractive interactions (Ref. [26]); however, the pronounced nucleation density maximum from Fig. 2b is not reflected in a sharp minimum of the $B_2/B_2^{HS}$ values. Instead, the minimum extends to far higher $c_s$. This suggests that the values of the second virial coefficient indeed indicate the window of conditions suitable for crystallization for our system as phenomenologically predicted by George and Wilson [34], but are not sufficient to predict the details of nucleation densities and the corresponding kinetics of our system. The steep increase in nucleation density crossing $c^*$ is indeed reflected in a sharp decrease in the $B_2/B_2^{HS}$ values, however, the nucleation density shows a much more dramatic decrease after reaching maximum values than $B_2/B_2^{HS}$ [26]. In particular, for directional bonds and localized attractive sites as in our system (see also Ref. [24]), it is clear that there will be subtleties (anisotropic interactions) that will not be captured in $B_2/B_2^{HS}$. We also note that it is expected that for a given crystal structure, a certain stoichiometry will be optimal [18,24,25]. Therefore, even if there are still attractive interactions when moving above a certain salt concentration, the stoichiometry might no longer be ideal for supporting the growth of the crystal.

### 3.2. Nucleation kinetics

The detailed characterization of the crystallization regime of the HSA-CeCl$_3$ of the previous section permits a kinetic study of the crystal nucleation and growth. Here we investigate the effect of the protein and salt concentration on the kinetic parameters (Sections 3.2.2 and 3.2.3), as well as on size and shape of the crystals (Section 3.3).

#### 3.2.1. Shape of the kinetic curve

Fig. 3 shows nucleation densities as a function of time for different conditions at $c_p = 50 \text{ mg/ml}$. We find that the number of crystals increases exponentially after an initial lag time corresponding to $t_0$ and then saturates (see also Figure A.7 in the SI for other $c_p$). To extract quantitative kinetic parameters, such as the characteristic growth rate $k$ and the incubation parameter $t_0$, the data are fitted with a sigmoid function [35]:

$$n(t) = \frac{n_s}{(1 + \exp(-k \cdot (t - t_0)))}$$  

where $n_s$ is the saturated nuclei number density. We note that for very small $n_s$, a fit with the sigmoidal curve is only reasonable in limits due to the high relative error in the quantification of the crystal numbers. Still, for all conditions across the phase diagram, the sigmoidal function can be considered a good fit. The fit qualities $R^2$ of the curves in Fig. 3 and A.7 lie between 0.9138 and 0.9971.

From the fitted curves, the incubation parameter $t_0$, as well as the characteristic nucleation rate $k$ could be obtained. The time $t_0$ is marked with a red star for each curve in Fig. 3. The following sections investigate how the kinetic rate $k$ is controlled by the protein concentration and the salt concentration.

#### 3.2.2. Kinetic analysis

In order to rationalize the kinetic parameters and put them into a context, we will now discuss a model for their dependence on $c_p$ and $c_s$. We emphasize that this scaling (inspired by the dynamics study in Ref. [36]) is certainly oversimplified, but serves to organize the data. Both the protein and the relative salt concentration are expected to affect the kinetics of the HSA-CeCl$_3$ crystallization. Regarding the effect of $c_p$, we expect that the number of proteins in solution is proportional to the flux, i.e., the number of molecules that reach a surface in a given time. In the present system, proteins from the bulk solution reach the nuclei’s surface and are incorporated into the crystal lattice. For the
sake of the next step in the analysis, we assume that the higher the flux, the faster are the nucleation kinetics. This can be applied to both, a fast onset of the nucleation (small \( t_\text{n} \)) and a high nucleation rate (high \( k \)). With this, we obtain the relation
\[
c_p \propto 1/t_\text{n} \propto k. \tag{2}
\]
The scalability of both, \( t_\text{n} \) and \( k \) with the protein concentration can be tested in the kinetic analysis. This means that for otherwise comparable conditions, calculated values of \( k / c_p \) and likewise \( t_\text{n} \cdot c_p \) should be constant within this simple picture.

The term “comparable conditions” here refers to a consistent driving force, which is mostly controlled by \( c_i \) in this system, as it dominantly determines the strength of the effective interactions. Within the framework of our simplified analysis, the following considerations are made. In the present system, with its rich phase behavior, we expect strong differences in the interactions at different locations in the phase diagram \([37,38]\). We can speculate that the distance to the phase boundaries is an important measure, as it can impact the nucleation mechanism and its kinetics \([37,38]\). In the spirit of Sorarufl et al. \([36]\), we can employ the ratio between salt and protein concentration \((c_s / c_p)\) for comparing different samples, since this corresponds to approximately constant distances to the phase transition boundaries of \( c^* \) and the LLPS region and with that comparable driving forces, at least within a certain window of conditions.

Eq. \((2)\) is supported by Barlow et al. as they assumed the characteristic rate \( k \) to be proportional to the initial protein concentration:
\[
k = \text{const} \cdot c_i. \tag{39}
\]
Furthermore, Schmit et al. developed a theory that predicts the nucleation rates (as well as crystal growth speed) to increase with the protein concentration \([33]\). This will be tested in the following.

\subsection*{3.2.3. Dependence of kinetics on the protein concentration}
For the study of the kinetic dependence on protein concentration, the \((c_i / c_p)\) ratio, as a measure of the position in the phase diagram and with that a measure of the effective interactions, is kept constant. For all \((c_i / c_p)\), most values of \( k / c_p \) lie between 0.002 and 0.008 \(\text{ml mg}^{-1} \text{h}^{-1}\). As an example, Fig. 4a displays the values of \( k / c_p \) as a function of \( c_p \) for \( c_i/c_p = 6.8 \) (see Figures A.8 and A.9 for the corresponding plots at other \( c_i/c_p \) values). Fig. 4b illustrates the corresponding conditions in the phase diagram of the present system. Taking the error bars into account, it can be further observed that most data points show indeed little variation in the \( c_p \) range investigated, which is consistent with the model assumptions.

It has to be noted that at all \( c_i / c_p \) (apart from \( c_i / c_p = 5.8 \)), there are deviations from \( k / c_p = \text{const} \) at high protein concentrations (see Fig. 4, A.8 and A.9), i.e., indeed the range of the validity of the model is limited, as expected. In essence, at \( c_p \geq 65 \text{mg/ml} \), the values of \( k / c_p \) are significantly smaller than at lower \( c_p \). This deviation might be caused by the fast aggregation at high \( c_p \), which can lead to non-productive binding of protein molecules and hence might impact the nucleation process \([33]\). Another conceivable reason would be that the (amorphous) aggregates are too large at high \( c_p \) and take longer to solubilize again (or some might even be insoluble), which would lower the supersaturation of the solution and thus could slow down the nucleation rate. Galkin and Vekilov reported similar deviations in the nucleation rates at high protein concentrations (high supersaturations) for lysozyme \([40,41]\).

Nonetheless, we find that the values of \( k / c_p \) are roughly in the same range, confirming the assumption that \( k \) is proportional to the initial \( c_i \) for similar positions in the phase diagram (constant \( c_i / c_p \)), corresponding to comparable strengths of salt-induced attractive forces. This suggests that a similar mechanism underlies the nucleation process under these conditions and its kinetic rate \( k \) is limited by the flux of molecules to the surface of nuclei (which is proportional to \( c_p \)), meaning the more material is in solution, the higher the characteristic rate of the nucleation process. Thus, nucleation and crystal growth seem to be diffusion limited. Hence, the protein molecules are expected to orient themselves quickly and attach to the crystal lattice in a proper orientation faster than the diffusion towards the crystal surface takes place. Similar observations were already made for protein and small molecule crystals \([42]\). In contrast, for lysozyme, the kinetic incorporation of proteins was reported to be the rate-limiting step \([43]\).

\subsection*{3.2.4. Dependence of kinetics on the salt concentration}
To study the influence of the salt concentration on the nucleation kinetics, we analyze the nucleation rate at a constant protein concentration (see Fig. 2d) of \( c_p = 50 \text{mg/ml} \). As \( c_i \) is increased, two phase transitions, the \( c^* \) border and the LLPS are found in the phase diagram not far away from each other in the present system (see Fig. 1).

It was demonstrated for various systems that close to (or inside) the LLPS regime, the kinetics of the crystallization speed up \([4,37,44,45]\). This may have several reasons. First, simulations, theoretical studies and experiments showed an enhanced crystallization behavior close to the critical point due to density fluctuations \([4,37,46]\). Second, the interfacial energy between dense phase and crystal can be lower than between initial solution and crystal, resulting in a decreased activation energy barrier for the crystallization process \([7]\). Last, the surface of the dense droplets can act as heterogeneous nucleation site \([47]\).

For the present system, unfortunately only an approximate LLPS boundary is available for the conditions \( c_p = 50 \text{mg/ml} \) (see extrapolated LLPS loop in Fig. 1). A macroscopic observation (in the bulk) of the phase separation, such as the one performed for a similar system (bovine serum albumin with CeCl\(_3\), Ref. \([48]\)) was not possible,
because the amount of dense phase was either too small for detection or the liquid dense phase was accompanied by aggregates. Alternatively, we employed a microscopic investigation of LLPS at certain salt concentrations at $c_p = 50$ mg/mL. From light microscopy images, it becomes apparent that phase separation occurs over a broad $c_i$ range at $c_p = 50$ mg/mL. Note that due to possible confinement effects of the spacers, the phase boundaries might be shifted slightly compared to the bulk. Phase separation on the microscopy slides is observed in the form of dense droplets at almost all salt concentrations in the second regime, i.e., even at lower and higher $c_i$ values than crystallization is observed.

Interestingly, variations in the amount of droplets (phase separation) are noticeable when $c_i$ is varied. In the $c_i$-range where the kinetic parameters and the nucleation density decrease, we see an increase in the amount of microscopic dense phase droplets. One possible explanation for this is given by the fact that the nucleation process and the resulting crystallization is observed to appear in the dilute phase [25]. Consequently, where a new metastable dense phase is formed, the resulting concentration in the dilute phase is lower. Thus, if the rate of crystallization is dependent on the number of proteins in the dilute phase, the formation of a dense phase leads to slower kinetics of the crystallization channel due to the existence of a competing kinetic channel, i.e., the conversion to the dense phase [4].

There is some analogy with the simulations performed by Lutsko of macromolecules in solution, which are in agreement with the slower nucleation rates [49]. He observed a dense-solution (liquid-like) layer forming on the surface of the crystal so that the energy barrier is associated with the freezing transition and multiple barriers must be overcome leading to slower nucleation rates. Concerning a potential effect of the LLPS on the nucleation and growth of the crystals, it remains unclear whether the existence of LLPS has an influence or possible effects compete, since we find microscopic LLPS for all crystallizing conditions in the present system.

Apart from the decrease in protein concentration within the dilute phase compared to the initial solution due to LLPS, a possible explanation could be the higher occupation of protein binding sites (by Ce$^{3+}$ ions) and associated changes in the interactions with increasing $c_i$, which potentially causes the slowing down of the characteristic rate.

In addition to the occurrence of LLPS and possible related effects on the crystallization behavior, the salt concentration is key in the process. Importantly, the multivalent ions are not only a way to control the interactions, but they are also incorporated into the crystal [25], for which it is likely that certain ratios/stoichiometries are preferred. With these two mechanisms at play (interactions and stoichiometry), it appears rather likely that crystallization (apart from being a nonlinear process that obviously does not exactly have to follow $B_2/B^{H5}$) depends very sensitively on $c_i$, and that there may be a rather narrow “sweet spot” of nucleation.

It has to be noted here that we find the systematic behavior for each given batch of protein. The absolute nucleation rate appears to be highly sensitive to impurities in the protein solution (see comment in Section 2.1). Nevertheless, the observation that the kinetic rate exhibits a sharp maximum above $c^*$ upon increasing $c_i$ is a robust finding, reflecting the clear salt-specific trend already observed in nucleation density.

### 3.2.5. Evolution of size and shape

Having explained where in our phase diagram crystals are able to nucleate, we have a closer look at the size and the shape of the individual crystals. Interestingly, in contrast to other systems we have studied, only one type of morphology was observed for the present system [18, 19, 48, 50, 51]. The crystals of HSA crystallized in the presence of CeCl$_3$ show a lentil-like morphology for all $c_p$ and $c_i$ as revealed by light and fluorescence microscopy (Fig. 5).

In addition to observing ensemble-averaged kinetics, also the growth of individual crystals was tracked over time. Typical growth curves of individual crystals are depicted in Figure A.12. Since the growth curves were found to exhibit a similar monotonically increasing trend with time, we conclude that it is sufficient to compare only the “final” size of the crystals (after 14 days), for comparing crystal sizes and the implied growth kinetics. The crystal sizes after 14 days of the largest crystal for each sample are given in Fig. 2c as a function of $c_i$. We find that the crystal size (after 14 days) reaches a maximum shortly above $c^*$, then decreases again. This supports the notion that the most favorable conditions for crystallization, are found close to the phase boundary. Even though the maximum of the nucleation density peak is slightly shifted compared to the maximum of crystal size, we still observe a remarkably similar sharp trend favoring salt concentrations shortly after exceeding $c^*$ for maximum growth.

### 4. IV. Conclusions

Using HSA with CeCl$_3$ as a model system, this study aimed at connecting protein crystallization kinetics to the phase diagram.

The nucleation density data reveal that for this system, crystals are only found in the lower part of regime II. Intriguingly, the nucleation density peaks slightly above $c^*$ and then decays rapidly, potentially due to the increased occupation of binding sites at the HSA molecules with higher $c_i$ and the associated changes in interaction [18, 19, 26].

In the analysis of nucleation kinetics, the model of a sigmoidal dependence of the nucleation density on time was successfully applied to the HSA-CeCl$_3$ system, similar to what has been reported for various other systems [35, 39, 50]. The analysis of the kinetic data showed that the onset ($t_*$) decreases and the characteristic rate of the nucleation ($k$) increases with the protein concentration $c_p$ when conditions with constant distances from the phase boundaries are compared. This suggests that a similar mechanism underlies the nucleation process, the speed of which (in terms of $k$ and $t_*$) is determined by $c_p$ (i.e., diffusion-limited), with variations only at high $c_p$.

All crystallization conditions studied here lie in the vicinity of the extrapolated bulk LLPS loop. We note that the occurrence of LLPS does not appear to be the sole mechanism for the rapid decrease in nucleation density and rate. Possible effects such as density fluctuations or a decreased activation energy barrier on the one hand and a reduced protein concentration due to dense phase formation on the other hand...
appear to work in opposite directions or do not significantly impact the crystallization process under the present conditions.

Similar to the nucleation density and rate, the maximum crystal size was found to peak slightly above $c^*$. Thus, all parameters studied show a strong, sharp peak in the lower regime II: Based on these results, it is reasonable to conclude that a particular location in the phase diagram, namely shortly after $c_s$ surpasses $c^*$, is favorable for nucleation and crystal growth.

Furthermore, the comparison with $B_2/V^{2.5}$ data suggests that the reduced second virial coefficient alone fails to explain the intricacies of the observed trends, as it does not reflect a sharp attractive minimum. This emphasizes the importance of $c_s$, which goes beyond controlling the interactions, as the salt cations are physically incorporated into the crystal lattice. We speculate that, in addition to direct protein–protein contacts, the observed trends are related to the specific and local interactions induced by ion-activated patches, for which there may be favorable conditions at a specific stoichiometry.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found online at https://doi.org/10.1016/j.jcrysgro.2022.126959.

References
