Real-Time Observation of Protein Dense Liquid Cluster Evolution during Nucleation in Protein Crystallization

*Robin Schubert[a], Arne Meyer[b], Daniela Baitan[a,b], Karsten Dierks[b], Markus Perbandt[a] and Christian Betzel[a]**

[a] Institute for Biochemistry and Molecular Biology, The Hamburg Center for Ultrafast Imaging, University of Hamburg, Notkestr. 85, 22607 Hamburg (Germany)

[b] XtalConcepts, Marlowring 19, 22525 Hamburg (Germany)

Abstract: Controlled navigation in the phase diagram of protein crystallization and probing by advanced Dynamic Light Scattering (DLS) technology provided new information and more insights about the early processes during the nucleation process. The observed hydrodynamic radius distribution pattern clearly reveals a two-step mechanism of nucleation and the occurrence of liquid dense protein clusters, which were verified by transmission electron microscopy. The growth kinetics of these protein clusters, forming distinct radii fractions, is analyzed in real-time. Further, the data confirmed that critical nuclei show a distinct different radius distribution than the liquid dense clusters. The data and results provide experimental evidence that during nucleation, a formation of distinct liquid clusters with high protein concentration occur prior to a transition to crystal nuclei by increasing the internal structural order of these clusters, subsequently.

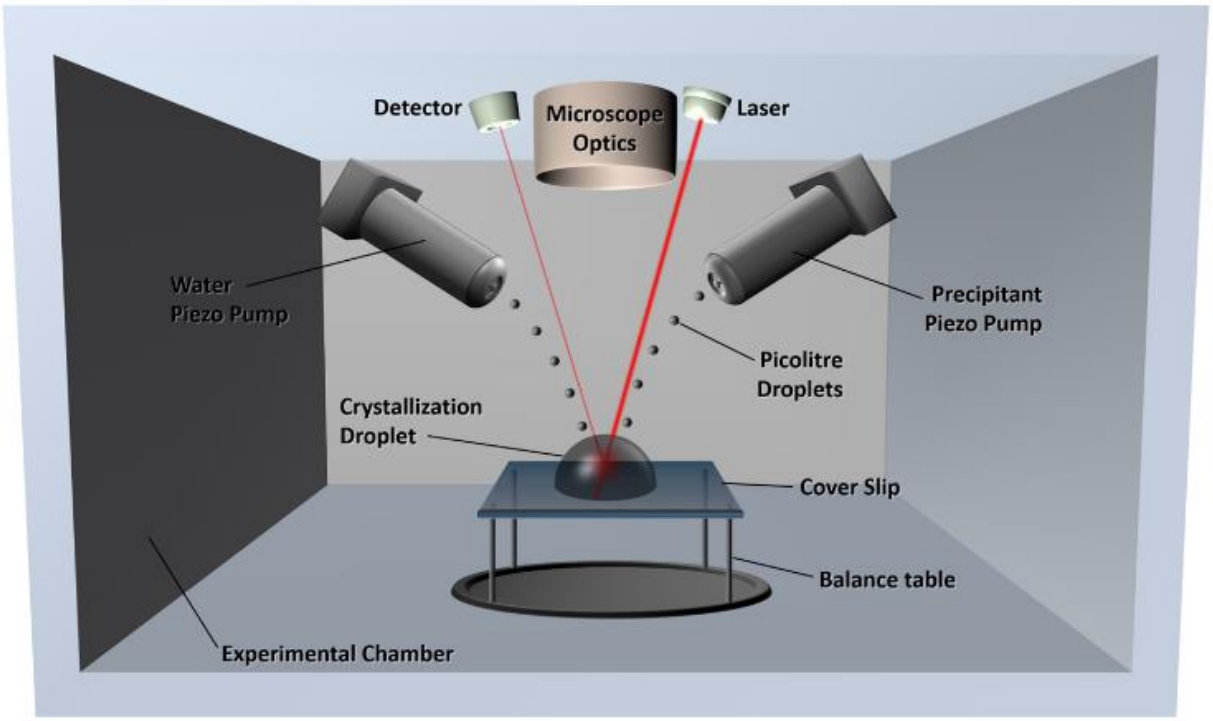
* Corresponding author: Christian Betzel, Phone: +49 4089984744, Fax: +4989984747, christian.betzel@uni-hamburg.de

In protein crystallization, a protein solution is typically brought into supersaturation to induce the thermodynamically driven crystal formation. In classical crystallization the formation of a few crystal nuclei, which can then grow to individual large protein crystals by addition of free protein from the bulk, is favored.[1] For the preparation of protein nanocrystals, to be used for diffraction data collection applying serial crystallography (SFX),[2] this conventional approach needs to be modified, in order to increase the number of nucleation events in the crystallization droplet.[3,4] Additionally, a better understanding of the nucleation process itself is required to adapt the existing methods to the new requirements in nanocrystallography. Protein crystallography is a well-established method for several decades, however the fundamental understanding of the nucleation process is still incomplete and till now an active area of ongoing research.[5–15] In the classical nucleation theory, the crystal formation is considered to be a first-order phase transition.[15] A simplified version of the phase diagram can be defined by three phases, the dilute solution, a dense liquid and a crystalline state.[15] These states can be described by the two parameters concentration and internal order. According to the classical theory, a nucleation event can be considered as a simultaneous transition of both parameters, from the dilute solution with low concentration and low internal order to a crystalline state with high concentration and high internal order. Based on these fundamentals, the classical theory was extended to a model considering a two-step mechanism of nucleation.[13,16] In this theory it is assumed that for many proteins the nucleation process occurs in two consecutive steps. Firstly, a transition to a higher concentration occurs due to the formation of a dense liquid phase and secondly, a transition to higher internal order of these clusters takes place.[14,17–20] This two-step nucleation model has additional plausibility, because it allows explaining the large discrepancy between the predicted nucleation rates by the classical nucleation theory and the experimentally obtained nucleation rates.[16,20]

Due to the small size of the clusters and low frequency in occurrence in the supersaturated solution, experimental evidence for the hypothesis that the second step during nucleation, a transition to a higher order, occurs inside these clusters is still rare.[21–23] This roots in the fact that following the nucleation process and early crystal growth by optical microscopy is not feasible, because of the optical resolution limit. Therefore, alternative methods need to be applied to study the nucleation process in detail. For several reasons, dynamic light scattering (DLS) depicts to be one of the most suitable methods. The size of the particles that can be investigated by DLS covers a large range from one nanometer up to a few micrometers. Additionally, DLS is an extremely sensitive method to detect a small number of larger particles in solution. Consequently, the occurrence of larger particle clusters during nucleation can be detected by DLS, even if the frequency of their occurrence (volume fraction) in the crystallization solution is rather low ($< 10^{-3}$ %).[24]

In crystallization experiments the protein solution has to be dragged into supersaturation to overcome the energy barrier and to induce the nucleation process as well as crystal growth.[7] In order to allow a feedback controlled navigation in the phase diagram the XtalController technology was developed.[25] In the instrument, a single droplet of protein solution is placed on a highly sensitive microbalance in a precisely temperature and humidity controlled experimental chamber (Scheme 1). In order to add precipitant and water to the sample, two piezoelectric dispensing heads are installed, allowing contact-free addition in nanoliter increments as well as evaporation or dilution of the droplet. This set-up allows following an arbitrary path through the phase diagram. Additionally, DLS measurements can be continuously performed throughout the whole experiment, providing information about the radius distribution of the particles in solution. In the experiments performed in this study it was intended to follow the nucleation process online by

DLS, in order to obtain new experimental insights about the model of the two-step nucleation mechanism. The proteins Aminopeptidase P (APP, from *Plasmodium falciparum*), Thioredoxin (from *Wuchereria bancrofti*), 5-(hydroxyethyl)-4-methylthiazole kinase (ThiM, from *Staphylococcus aureus*), thaumatin (from *Thaumatococcus daniellii*) and Mistletoe lectin I (MLI, from *Viscum album*) were investigated to enhance the general validity of drawn conclusions about the nucleation mechanism. Exemplarily, the experiments and data obtained for MLI are described and summarized in the following section, while the experimental data and results obtained for the other proteins are shown in the supplementary section.



Scheme 1. Schematic presentation of the temperature- and humidity-controlled experimental chamber of the XtalController instrument.

Mistletoe lectin I (MLI) from *Viscum album* with a concentration of 3.2 mg mL^{-1} was tested by placing 3.5 µL of the protein solution on the cover slip fixed on a microbalance. The radius

distribution, the recorded weight, as well as the calculated concentration of protein and precipitant over time are shown in Figure 1 (A and B). At the beginning of the experiment the initial hydrodynamic radius of the protein heterodimer can be seen. After a few initial DLS measurements the injection of precipitant was started and continued until a change in the radius distribution was observed. A second radius fraction with particles of a size around 200 nm became visible after approximately 15 minutes, indicating supersaturation of the protein solution. The particles continued to grow in size, while more precipitant was added, until a final precipitant concentration of 0.75 M ammonium sulfate was reached. From this time point onwards the condition of the droplet was kept constant by compensating evaporation via corresponding water injection. The hydrodynamic radius of the particles obtained in a second radius fraction was determined to be approximately 400 nm. The size of the particles grew further over time and three hours after initiation of the experiment particles with a hydrodynamic radius of approximately 1 µm were detected. Additionally, a third fraction with a radius of approx. 200 - 300 nm formed between the large particles and the heterodimer-fraction, which remained constant.

In order to visualize the transition of the heterodimeric MLI, in the beginning of the experiment, to the complex radius distribution after injection of precipitant, the recorded autocorrelation function (ACF) is plotted over time for the first 1.5 hours of the experiment and shown in figure 1 C. It can be seen, that the ACF changed from a monomodal decay with a decay time constant of 21 µs to a multimodal decay, from which three decay time constants have been fitted by the CONTIN algorithm (35.5 µs, 2.2 ms and 9.8 ms).[26] Although the increase in viscosity during precipitant injection has been taken into account for the hydrodynamic radius calculation (supporting information), a slight increase in the radius of the MLI heterodimer fraction is observed. This indicates that electrostatic interactions, induced by the altered chemical

environment upon precipitant injection, might have a minor influence on the calculated absolute hydrodynamic radius.

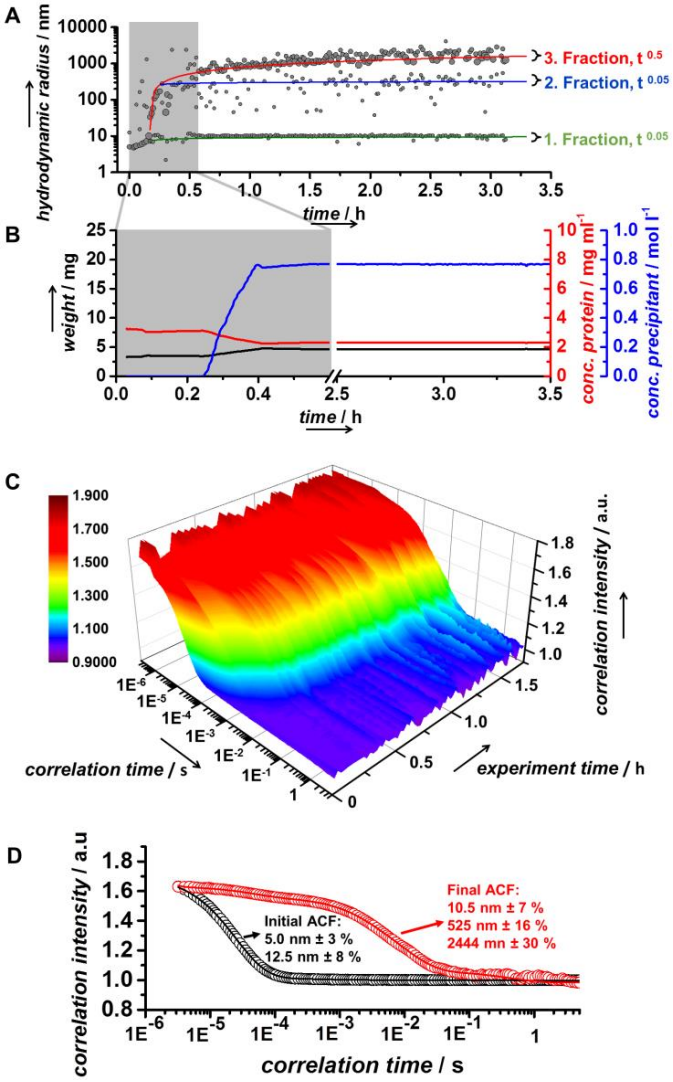


Figure 1. Controlled induction of MLI crystal nucleation, applying the XtalController. [A] The graph shows the evolution of the hydrodynamic particle radii in the crystallization droplet over time. Precipitant injection up to 0.75 M induces nucleation and results in the formation of a complex radius distribution pattern with three distinct radius fractions. The size of the particles in the largest fraction grows proportionally to $t^{0.5}$, while the other two fractions remain constant in size. [B] The change in the recorded weight (black curve) of the sample over time and the hence

calculated changes in protein (red curve) and precipitant concentration (blue curve) is presented. Please note the break in the x-axis in the lower graph for a better visualization of the precipitant injection. [C] The autocorrelation function (ACF) for the first 1.5 hours of the experiment is plotted in a surface presentation. A transition from a monomodal decay in the beginning of the experiment to a multimodal decay after addition of precipitant can be seen. [D] The ACF of the first (black) and last (red) DLS measurement as well as the fit from the CONTIN algorithm is shown. The calculated hydrodynamic radius of all identified radius fractions with relative error (polydispersity index) is listed. All graphs have been prepared using the software Origin (OriginPro 9.1, OriginLab, Northampton, USA).

It has been proposed by P. Vekilov that the nucleation process of crystallization for many proteins is a two-step mechanism, where the transition to higher order occurs after a transition to higher concentration.[16] He proposed that liquid-liquid dense clusters represent stable intermediates in supersaturated protein solutions and that the nucleation of crystals occurs within these clusters.[20] This model is supported by a study where the nucleation within protein clusters could be followed by depolarized oblique illumination dark-field microscopy.[23]

A detailed analysis of the particles detected by DLS applying visualizing methods is challenging, because the labile samples need to be chemically cross-linked before, in order to stabilize the particles in the high vacuum applied on samples for electron microscopy.

The recorded transmission electron microscopy (TEM) images of the MLI sample, negative stained with uranyl acetate, are shown in figure 2. TEM microscopy images indicate protein clusters with a size between 200 nm and around 1 µm have formed, corresponding to the larger radius fractions in the DLS radius pattern. Most of the particles have an irregular spherical shape, comparable to macroscopic phase separation, commonly seen in crystallization experiments.

However, in the same sample also particles with a defined triangular shape are visible (Figure 2 B). A similar tetrahedral morphology was observed in a TEM image of a MLI nanocrystal produced by a crystallization experiment applying the XtalController and published by Meyer et al.[25] Therefore, it can be assumed that the shaping structure in these particles are a result of first nuclei of MLI crystals, which have formed inside a protein dense liquid cluster and have grown to nanocrystals over time. These images demonstrate the first microscopic observation of the transition from a cluster with high protein concentration to a crystal with higher order, as postulated by the two-step nucleation model.

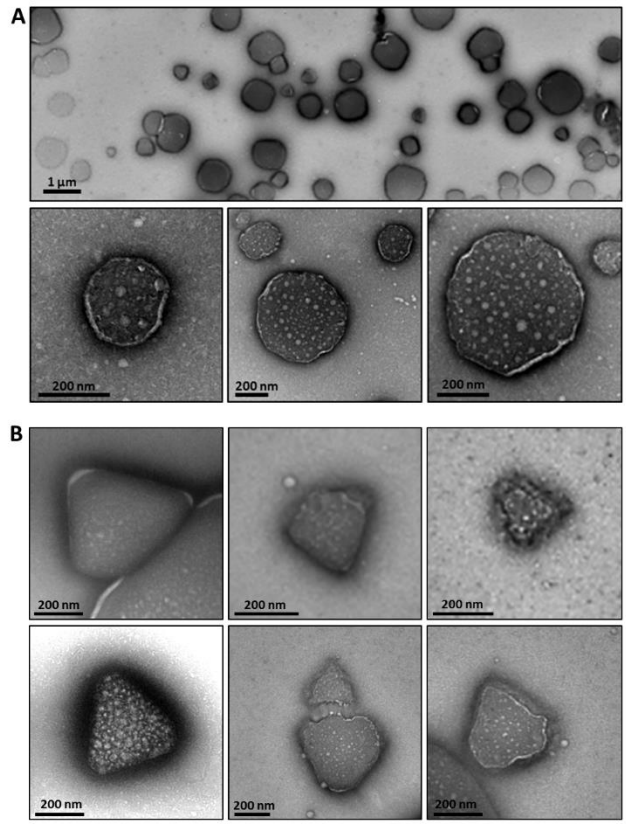


Figure 2. Transmission electron microscopy images of a MLI sample obtained via the XtalController experiment. [A] The sample is negative stained with uranyl acetate and shows protein dense liquid clusters with a size of 200 nm to 1 µm, as identified in the radius distribution pattern from the DLS measurements. The particles mostly possess an irregular spherical shape. [B]

Additionally, geometrically ordered particles with triangular shape and surrounded by amorphous protein are visible. This provides additional indication that the nucleation occurs in protein dense-liquid clusters during the two-step mechanism of nucleation.

Similar hydrodynamic radius distribution patterns over time have been observed for the proteins ThiM, APP, Thaumatin and Thioredoxin and all corresponding data and results are shown and summarized in the supplementary information. The dimensions of the observed particle clusters obtained via the XtalController experiments are shown in Figure 1 (and Figure S2-5). They are in good agreement with so far reported cluster sizes detected within nucleation and reported in other studies.[24,27] The growth kinetics of the protein clusters, forming the different radius fractions, was analyzed to obtain more information about their composition. It has been shown by Lifshitz & Slezov that the particle size of clusters increases asymptotically with the cube root of time when diffusion-limited growth in supersaturated solutions is present ($t^{0.33}$).[28] This theory was extended by Wagner, who described that the size evolves with $t^{0.5}$ in case of interface-limited growth.[29] In the experiment shown in Figure 1, the size of the largest radius fraction (fraction 3) increases proportionally to $t^{0.5}$. This demonstrates that the rate of mass increase during cluster evolution is constant and reveals that the cluster aggregation follows interface-limited growth. In contrast, the growth kinetics of the cluster size in the experiments with the proteins thioredoxin, APP and thaumatin revealed diffusion-limited growth (supporting information, Figure S1, S3 and S4).

The XtalController technology allows a feedback controlled navigation in the phase diagram and was applied to gain new information about the nucleation behavior of proteins during crystallization. After initial formation of particle clusters with a hydrodynamic radius of approximately 100 nm a complex radius distribution pattern evolved over time. The growth

kinetics of protein dense clusters, forming a distinct radius fraction, reveal that the rate of mass increase during cluster evolution is constant and provides evidence that the cluster growth is either mainly diffusion-limited or interface-limited.

Characterization of the sample suspensions by TEM confirms that the observed radius distribution pattern is a result of the two-step nucleation mechanism. It can be seen that protein dense liquid clusters represent stable intermediates in supersaturated protein solutions. Further, the postulation that crystal nucleation occurs within these clusters can be supported by the presence of geometrically ordered particles. Therefore, the presented results demonstrate a so far unique microscopic observation of the transition from a cluster with high protein concentration to a crystal with higher structural order.

ASSOCIATED CONTENT

The following files are available free of charge.

Supporting information contains experimental details about protein sample preparation, XtalController Setup, experimental procedure as well as the results of experiments with the proteins APP, Thioredoxin, ThiM, and Thaumatin (Supporting information in pdf format).

AUTHOR INFORMATION

Corresponding Author

*Corresponding author: Christian Betzel, Institute for Biochemistry and Molecular Biology, The Hamburg Center for Ultrafast Imaging, University of Hamburg, Notkestr. 85, 22607 Hamburg (Germany), E-mail: christian.betzel@uni-hamburg.de

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Funding Sources

This work was supported by the excellence cluster 'The Hamburg Centre for Ultrafast Imaging - Structure, Dynamics and Control of Matter at the Atomic Scale', by the Deutsche Luft und Raumfahrt Agentur (DLR) via grant 50WB1423, by the EU via the ITN network X-Probe, by Bundesministerium für Bildung und Forschung (BMBF) grants referring to the project numbers 05K13GUA, 05K13GU2 and 01DN13037 as well as by the Hamburg Ministry of Science and Research via the graduate school DELIGRAH.

ACKNOWLEDGMENT

The authors would like to thank Dr. Rudolph Reimer from the Heinrich-Pette-Institute for experimental virology (Hamburg, Germany) for his great support with electron microscopy imaging.

ABBREVIATIONS

DLS, dynamic light scattering; SFX, serial femtosecond crystallography; APP, AminopeptidaseP; ThiM, 5-(hydroxyethyl)-4-methylthiazole kinase; MLI, mistletoe lectin I; ACF, autocorrelation function; TEM, transmission electron microscopy

References

(1) McPherson, A. *Crystallization of biological macromolecules*; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 1999.

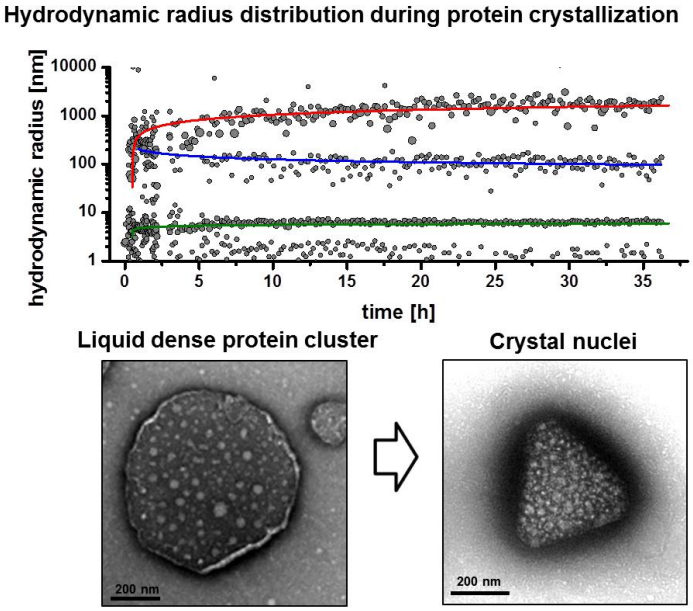
(2) Martin-Garcia, J. M.; Conrad, C. E.; Coe, J.; Roy-Chowdhury, S.; Fromme, P. Review: Serial femtosecond crystallography: A revolution in structural biology. *Archives of biochemistry and biophysics* **2016,** DOI: 10.1016/j.abb.2016.03.036.
(3) Kupitz, C.; Grotjohann, I.; Conrad, C. E.; Roy-Chowdhury, S.; Fromme, R.; Fromme, P. Microcrystallization techniques for serial femtosecond crystallography using photosystem II from Thermosynechococcus elongatus as a model system. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences* **2014**, *369*, 20130316, DOI: 10.1098/rstb.2013.0316.
(4) McPherson, A.; Cudney, B. Optimization of crystallization conditions for biological macromolecules. *Acta crystallographica. Section F, Structural biology communications* **2014**, *70*, 1445–1467, DOI: 10.1107/S2053230X14019670.
(5) Nicolis, G.; Maes, D. *Kinetics and Thermodynamics of Multistep Nucleation and Self-Assembly in Nanoscale Materials*; John Wiley & Sons, Inc: Hoboken, NJ, USA, 2012.
(6) Malkin, A. J.; McPherson, A. Light-scattering investigations of nucleation processes and kinetics of crystallization in macromolecular systems. *Acta crystallographica. Section D, Biological crystallography* **1994**, *50*, 385–395, DOI: 10.1107/S0907444993013319.
(7) Penkova, A.; Pan, W.; Hodjaoglu, F.; Vekilov, P. G. Nucleation of protein crystals under the influence of solution shear flow. *Annals of the New York Academy of Sciences* **2006**, *1077*, 214–231, DOI: 10.1196/annals.1362.048.
(8) Yau, S.-T.; Vekilov, P. G. Direct Observation of Nucleus Structure and Nucleation Pathways in Apoferritin Crystallization. *J. Am. Chem. Soc.* **2001**, *123*, 1080–1089, DOI: 10.1021/ja003039c.
(9) Haas, C. The Interface between a Protein Crystal and an Aqueous Solution and Its Effects on Nucleation and Crystal Growth. *J. Phys. Chem. B* **2000**, *104*, 368–377, DOI: 10.1021/jp993210a.
(10) Ataka, M.; Asai, M. Analysis of the nucleation and crystal growth kinetics of lysozyme by a theory of self-assembly. *Biophysical journal* **1990**, *58*, 807–811, DOI: 10.1016/S0006-3495(90)82425-5.
(11) Galkin, O.; Vekilov, P. G. Direct Determination of the Nucleation Rates of Protein Crystals. *J. Phys. Chem. B* **1999**, *103*, 10965–10971, DOI: 10.1021/jp992786x.
(12) Feher, G.; Kam, Z. [4]Nucleation and growth of protein crystals: General principles and assays. In *Diffraction methods for biological macromolecules. Part A*; Wyckoff, H. W., Hirs, C H W. Ed, Timasheff, Serge N. Ed, Eds.; Methods in Enzymology; Acedmic Press: Orlando, 1985; pp 77–112.
(13) Wolde, P. R. t. Enhancement of Protein Crystal Nucleation by Critical Density Fluctuations. *Science* **1997**, *277*, 1975–1978, DOI: 10.1126/science.277.5334.1975.
(14) Sleutel, M.; van Driessche, A. Role of clusters in nonclassical nucleation and growth of protein crystals. *Proceedings of the National Academy of Sciences of the United States of America* **2014**, *111*, E546-53, DOI: 10.1073/pnas.1309320111.
(15) Chayen, N. E.; Saridakis, E.; Sear, R. P. Experiment and theory for heterogeneous nucleation of protein crystals in a porous medium. *Proceedings of the National Academy of Sciences of the United States of America* **2006**, *103*, 597–601, DOI: 10.1073/pnas.0504860102.
(16) Vekilov, P. G. Dense Liquid Precursor for the Nucleation of Ordered Solid Phases from Solution. *Crystal Growth & Design* **2004**, *4*, 671–685, DOI: 10.1021/cg049977w.
(17) Pan, W.; Galkin, O.; Filobelo, L.; Nagel, R. L.; Vekilov, P. G. Metastable mesoscopic clusters in solutions of sickle-cell hemoglobin. *Biophysical journal* **2007**, *92*, 267–277, DOI: 10.1529/biophysj.106.094854.

(18) Gebauer, D.; Kellermeier, M.; Gale, J. D.; Bergstrom, L.; Coelfen, H. Pre-nucleation clusters as solute precursors in crystallisation. *CHEMICAL SOCIETY REVIEWS* **2014**, *43*, 2348–2371, DOI: 10.1039/c3cs60451a.
(19) Gliko, O.; Neumaier, N.; Pan, W.; Haase, I.; Fischer, M.; Bacher, A.; Weinkauf, S.; Vekilov, P. G. A metastable prerequisite for the growth of lumazine synthase crystals. *Journal of the American Chemical Society* **2005**, *127*, 3433–3438, DOI: 10.1021/ja043218k.
(20) Vekilov, P. G. The two-step mechanism of nucleation of crystals in solution. *Nanoscale* **2010**, *2*, 2346–2357, DOI: 10.1039/c0nr00628a.
(21) Sauter, A.; Roosen-Runge, F.; Zhang, F.; Lotze, G.; Feoktystov, A.; Jacobs, R. M. J.; Schreiber, F. On the question of two-step nucleation in protein crystallization. *Faraday discussions* **2015**, *179*, 41–58, DOI: 10.1039/c4fd00225c.
(22) Sauter, A.; Roosen-Runge, F.; Zhang, F.; Lotze, G.; Jacobs, R. M. J.; Schreiber, F. Real-time observation of nonclassical protein crystallization kinetics. *Journal of the American Chemical Society* **2015**, *137*, 1485–1491, DOI: 10.1021/ja510533x.
(23) Maes, D.; Vorontsova, M. A.; Potenza, M. A. C.; Sanvito, T.; Sleutel, M.; Giglio, M.; Vekilov, P. G. Do protein crystals nucleate within dense liquid clusters? *Acta crystallographica. Section F, Structural biology communications* **2015**, *71*, 815–822, DOI: 10.1107/S2053230X15008997.
(24) Gliko, O.; Pan, W.; Katsonis, P.; Neumaier, N.; Galkin, O.; Weinkauf, S.; Vekilov, P. G. Metastable liquid clusters in super- and undersaturated protein solutions. *The journal of physical chemistry. B* **2007**, *111*, 3106–3114, DOI: 10.1021/jp068827o.
(25) Meyer, A.; Dierks, K.; Hilterhaus, D.; Klupsch, T.; Muhlig, P.; Kleesiek, J.; Schopflin, R.; Einspahr, H.; Hilgenfeld, R.; Betzel, C. Single-drop optimization of protein crystallization. *Acta crystallographica. Section F, Structural biology and crystallization communications* **2012**, *68*, 994–998, DOI: 10.1107/S1744309112024074.
(26) Provencher, S. W. CONTIN: A general purpose constrained regularization program for inverting noisy linear algebraic and integral equations. *Computer Physics Communications* **1982**, *27*, 229–242, DOI: 10.1016/0010-4655(82)90174-6.
(27) Galkin, O.; Pan, W.; Filobelo, L.; Hirsch, R. E.; Nagel, R. L.; Vekilov, P. G. Two-step mechanism of homogeneous nucleation of sickle cell hemoglobin polymers. *Biophysical journal* **2007**, *93*, 902–913, DOI: 10.1529/biophysj.106.103705.
(28) Lifshitz, M.; Slezov, V. V. Kinetics of Diffusive Decomposition of Supersaturated Solid Solutions. *Journal of Experimental and Theoretical Physics* **1959**, *8*, 331–339.
(29) Wagner, C. Theorie der Alterung von Niderschlagen durch Umlösen (Ostwald Reifung). *Zeitschrift Für Elektrochemie* **1961**, *65*, 581–591.

For Table of Contents Use Only

Real-Time Observation of Protein Dense Liquid Cluster Evolution during Nucleation in Protein Crystallization

Robin Schubert, Arne Meyer, Daniela Baitan, Karsten Dierks, Markus Perbandt and Christian Betzel



Synopsis:

Controlled navigation in the phase diagram of protein crystallization and probing by advanced Dynamic Light Scattering (DLS) technology provided new information and detailed insights about the two-step mechanism of crystal nucleation. The real-time observed hydrodynamic radii distribution reveals the occurrence of liquid dense protein clusters prior to transition to crystal nuclei.