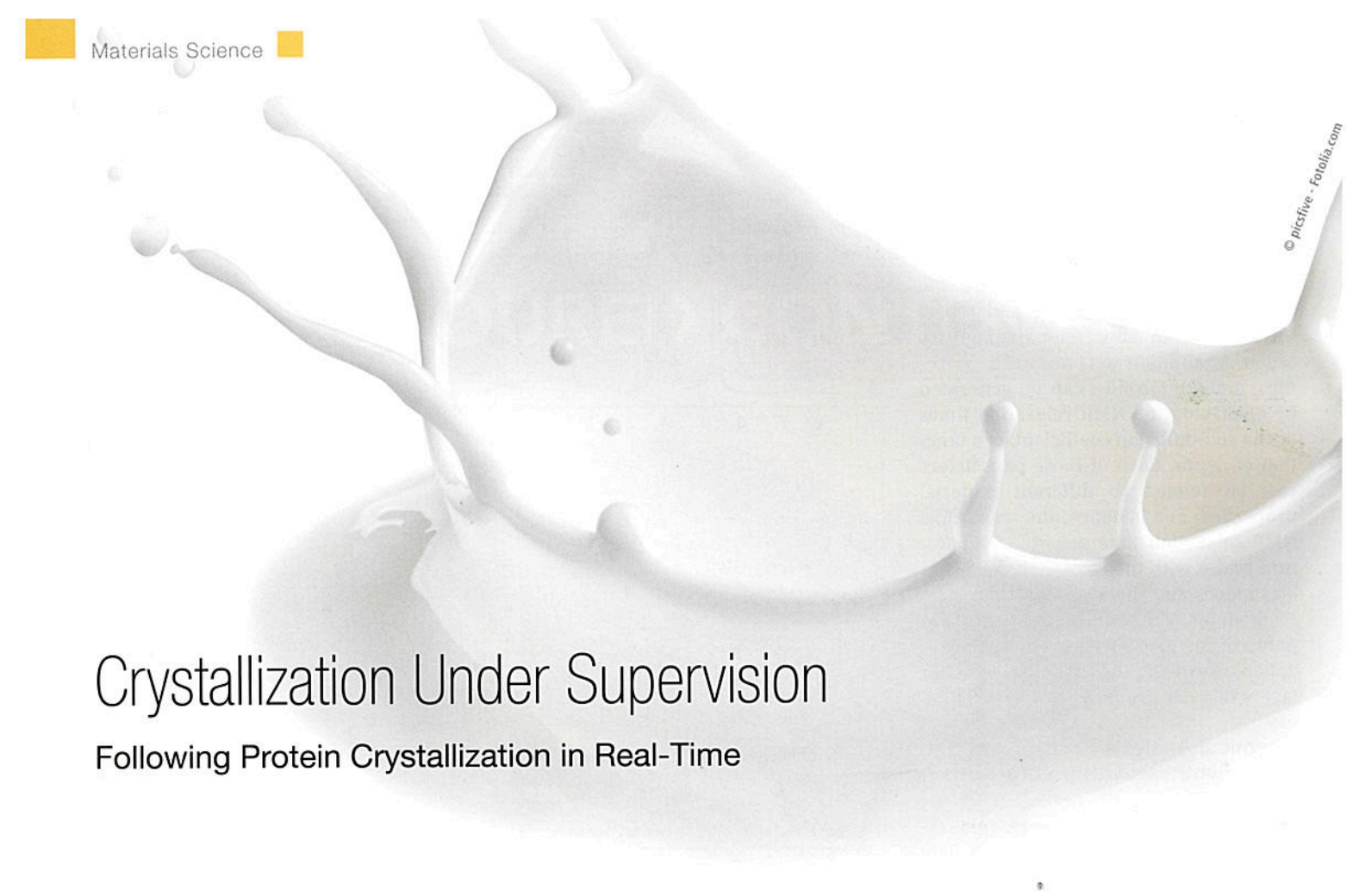

Crystallization Under Supervision

Following Protein Crystallization in Real-Time

The structure of a protein is directly linked to its biological function. Therefore, there is great interest in producing high-quality single crystals of proteins that are needed for structure determination by X-ray diffraction. However, the molecular interactions in protein solutions leading to crystallization are not completely understood, which is why crystal growth is typically performed by trial-and-error approaches in combination with "experience" [1].

Classical nucleation theory (CNT) states that in a supersaturated solution, molecules form a nucleus which has already the density and structure of the final solid [2-4]. Differences between experimental findings and theoretical predictions suggest that crystal nucleation from solution does not necessarily follow the classical route but more complex pathways via an intermediate phase in the crystallization of proteins, colloids, and biomineralization [5-9]. The intermediate can consist of small or large clusters (aggregates) or a dense liquid phase [2,6-7,9-14].

In previous studies, divalent ions have already been shown to be useful for the growth of high quality crystals of insulin and other protein complexes [15,16]. During our own research on globular proteins in solution, we found that a rich phase behavior, including crystallization, can be induced by multivalent metal ions. Upon adding the multivalent salt, samples of many globular proteins show a distinct

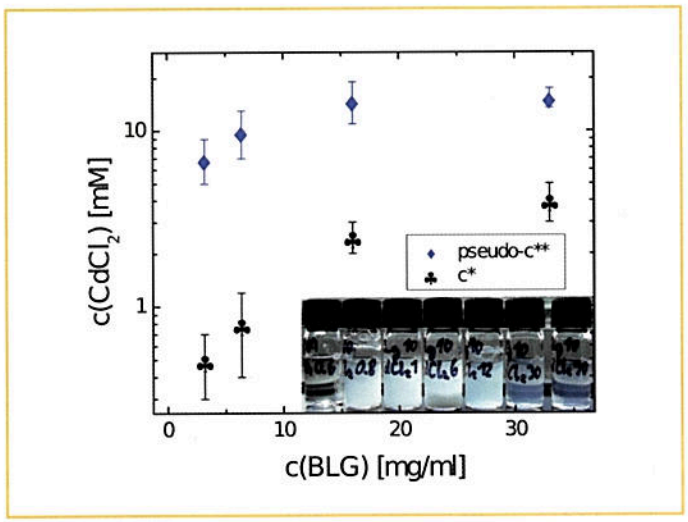

Fig. 1: Reentrant condensation phase diagram for BLG with CdCl2. Inset: Samples with fixed protein and increasing salt concentration. Reprinted with permission from [20]. Copyright 2015 American Chemical Society.

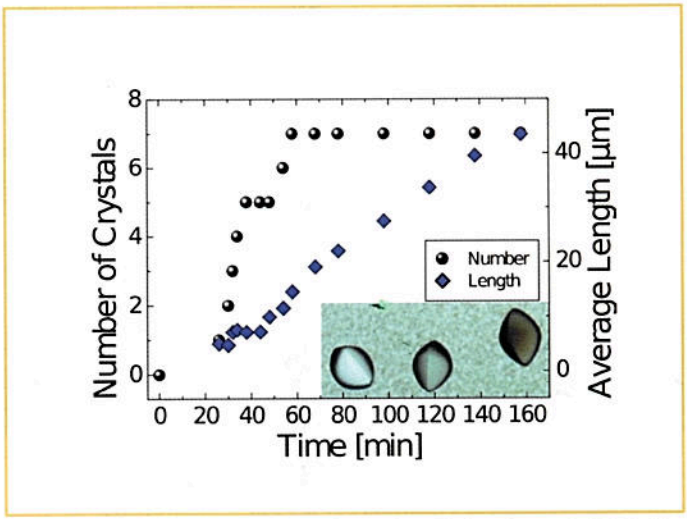

Fig. 2: Number and average length of crystals with time (20 mg/ml BLG, 15 mM CdCl2). Inset: Crystals observed by optical microscopy. Reprinted with permission from [20]. Copyright 2015 American Chemical Society.

transition c* from a clear solution at low salt concentrations (regime I) to a turbid sample with massive precipitates (regime II). At further increased salt concentrations, a second border c** is observed above which samples become completely clear again (reentrant effect, regime III) [17,18]. In the presence of the divalent $ZnCl_2$ or $CdCl_2$, the solution becomes less turbid in regime III, but not completely clear even for very high salt concentrations [19,20]. This partial transition to clearer samples is called pseudo-c**. See also Figure 1 on this behavior.

Materials and Methods

In this work, we dissolved the globular protein ß-lactoglobulin (BLG) from cow milk which was successfully crystallized by our method before [21,27] and the divalent salt $CdCl_2$ in pure water. To gain deeper understanding of the nucleation mechanism, we have performed a real-time study using small angle X-ray scattering (SAXS) and optical microscopy. The temperature was fixed to 20°C. Crystallization for transmission optical microscopy was performed between two narrow, hydrophobically coated glass slides sealed by silicone.

SAXS measurements were performed at the ESRF, Grenoble, France using a vertical capillary holder. SAXS is a powerful method to probe structural features directly. The detected scattered intensity is recorded as a function of q, the momentum transfer, and is dependent on the form factor and the structure factor. The form factor gives information about the shape of the average particle, while the structure factor gives information about the interaction of the particles in solution with each other.

Experiments

Deep in the second regime, small crystals were found, however they were of a poor quality for X-ray diffraction experiments, probably due to their fast growth. In the first and third regime, no crystallization was observed far away from c* and pseudo-c**. Therefore, we focused on conditions close to the boundaries.

Close to c*, samples are clear (below c*) or can be centrifuged, leaving a clear supernatant. Crystals grow directly from this clear solution, without any visible phases other than the initial liquid and the final crystal. Samples in the transition zone of pseudo-c** are slightly turbid after preparation. Under the optical microscope, large aggregates are visible. Removing them by centrifuging significantly reduces the number of crystals.

Initially, the crystals increase strongly in number, leading to saturation, after which crystals still grow significantly for hours, but no new crystals are observed. Both observations suggest that nucleation starts in the aggregates or at their surface, however, lowering the supersaturation by removing protein material by centrifuging or due to crystallization, respectively, also could explain this behavior. Nucleation occurs at higher supersaturations than growth [22]. This fact is occasionally used to artificially separate a nucleation regime from a growth regime in order to obtain crystals with a uniform size and potentially high quality [22]. Figure 2 shows the number of crystals N and the average crystal length $L=\sqrt{(A/N)}$ with the visible area A in the microscopy pictures as a function of time. L was observed to grow slower in the beginning. However, it should be stated that the resolution of optical microscopy is rather low and the method much more error-prone than the later shown scattering results.

SAXS was applied to characterize the structural development of our samples. Close to c*, measurement curves resemble the form factor of the protein (dotted line in Figure 3a). After some time, Bragg peaks appear, revealing crystallization. No indication of structures other than solution and crystal were found.

Close to pseudo-c**, measurement curves deviate heavily from the form factor. This can be explained by the formation of aggregates. With increasing time, a broad peak at intermediate q-values appears (see Fig. 3a). We assign it to a typical length within aggregates. It increases in intensity and Bragg peaks are observed. Their intensity increases while the broad peak shrinks, and finally disappears.

To quantify the relationship between the aggregates (intermediate) and crystals, we evaluate the area under the broad peak and under the Bragg peaks as a function of time (Fig. 3b). We observe two very interesting features: First, the maximum amount of aggregates coincides with a local maximum in the crystallization rate. This is characteristic for a two-step process, where nucleation occurs within the intermedi-

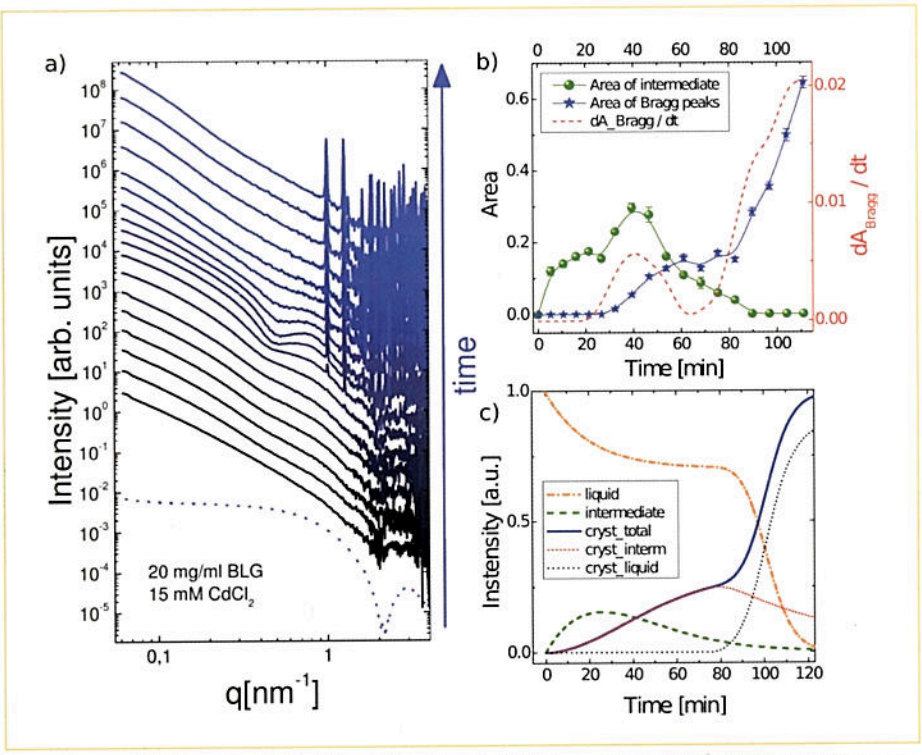

Fig. 3: Fig. modified from Ref [20]. a) Crystallization of BLG followed by real-time SAXS measurements. The form factor of BLG is shown as dotted line. b) Analysis of the area under the Bragg peaks and under the broad peak assigned to the intermediate. c) Rate equation model reproducing the experimental findings. Reprinted with permission from [20]. Copyright 2015 American Chemical Society.

ate phase. Second, when most of the intermediate has been consumed, another strong increase in the area under the Bragg peaks is observable. This can be interpreted as a fast growth process of the crystals in contact with the liquid phase which however is not as suitable for the nucleation of new crystals as the intermediate. The plateau in the area under the Bragg peaks indicates that the initial crystal growth is slower than the final one.

Similar behavior has been observed for other samples close to pseudo-c**.

Model and Discussion

A rate equation model based on processes with a clear physical meaning reproduces the experimental kinetic features. Assuming the formation of aggregates and the formation of crystals as two parallel one-step processes cannot explain the plateau in the crystal fraction. A two-step process of crystal nucleation from a previously formed intermediate however reproduces all features with very good agreement with the data: First, the intermediates form from the solution. Second, crystals nucleate from and grow slowly in the intermediate (possibly due to slower diffusion). Third, once the volume of the intermediate phase per crystal falls below a certain value due to consumption by nucleation and growth, the crystals become exposed to the solution. Finally, they grow in the liquid phase until most of the free proteins are consumed (see Fig. 3c.) We propose a nucleation-dominated regime which is followed by a growth-dominated regime, where the number of crystals becomes nearly constant, in agreement with the results from microscopy.

Our results indicate a decisive role of the protein aggregates on nucleation and a two-step nucleation process for protein crystallization [20,26]. On a more general level, our approach can help to induce crystallization in proteins of unknown structure that are subject of current biochemical research. A similar method was recently used for basic proteins and multivalent anions. In a system of a tyrosinase isoform from Agaricus bisporus, Mauracher *et al.* successfully added $MgCl_2$ or $Na_6[TeW_6O_{24}]*22H_2O$ (TEW) in order to induce crystallization [23]. Hen egg-white lysozyme was likewise crystallized in the presence of TEW. The negatively charged TEW molecules are part of the crystal lattice and bind to positively charged protein sites [24]. We believe that the strategy of manipulating protein interactions with multivalent ions is very promising as a route for crystallization, also for systems which otherwise appear to resist crystallization.

Summary

Our results suggest that multivalent metal salts can help to crystallize proteins. Utilizing reentrant condensation, sign and strength of protein-protein interactions in a large number of systems can be modulated [17,18,21,25]. The optimum conditions for crystallization (weak attraction) can be found close to the boundaries c* and c** or in the vicinity of a liquid-liquid phase separation region [19-21,25,26].

References

[1] Durbin S. and Feher G.: Annu. Rev. Phys. Chem. 47 171-204 (1996)
[2] Gebauer D. und Cölfen H.: Nano Today 6, 564-584 (2011)
[3] Anderson V. J. *et al.*: Nature 416 811-815 (2002)
[4] R Sear: J. Phys.: Condens. Matter 19 466106 (2007)
[5] Erdemir D. *et al.*: Acc. Chem. Res. 42 621-629 (2009)
[6] ten Wolde P. R. and Frenkel D.: Phys. Chem. Chem. Phys. 1 2191-2196 (1997)
[7] Vekilov P. G.: Cryst. Growth Des. 4 671-685 (2004)
[8] Wallace A. F. *et al.*: Science 341 885-889 (2013)
[9] Sear R. P.: International Materials Reviews 57 328-356 (2012)
...
[20] Sauter A. *et al.*: J. Am. Chem. Soc. 137 1485-1491 (2015)

Please contact the authors for more references.

Additional Information

This article is based on the results published in Ref. [20].

Authors
Andrea Sauter[1], Felix Roosen-Runge[2], Fajun Zhang[1], Gudrun Lotze[3], Robert M. J. Jacobs[4], and Frank Schreiber[1]

[1] Institut für Angewandte Physik, Universität Tübingen
[2] Institut Laue-Langevin, Grenoble, France
[3] European Synchrotron Radiation Facility, Grenoble, France
4 Department of Chemistry, Chemistry Research Laboratory, University of Oxford, UK

Contact
Frank Schreiber
Fajun Zhang
University of Tübingen
Tübingen, Germany
frank.schreiber@uni-tuebingen.de
fajun.zhang@uni-tuebingen.de

Related Articles:
http://bit.ly/Crystallization

More Information on the ESRF:
www.esrf.eu