ESRF	**Experiment Title:** The role of cluster formation and metastable liquid-liquid phase separation in protein crystallization	**Experiment number**: SC-3500
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Names and affiliations of applicants (* indicates experimentalists):

SCHREIBER Frank, IAP, Uni-Tuebingen, Germany

*ZHANG Fajun, IAP, Uni-Tuebingen, Germany

*WOLF Marcell, IAP, Uni-Tuebingen, Germany

*JING Bo, IAP, Uni-Tuebingen, Germany

*SKODA Maximilian Willy Anthony / ISIS, RAL, Chilton, Didcot OX11 0OX, UK

*GRIMALDO Macro, ILL, Grenoble

Report:

Crystallization plays a decisive role in many processes in nature and industry [1]. A break through over the last decade has revealed new insights into this step: studies have shown that in colloid and protein solutions, the attractive potential is short ranged compared to their size, which is crucial for their phase behavior and a "two-step" nucleation mechanism has been proposed to explain the crystallization behavior in these systems under suitable conditions, i.e. nucleation events follow a metastable liquid-liquid phase separation (LLPS) [1-3]. The consequence of the formation of protein clusters (transient or equilibrium) and metastable LLPS is that it changes the kinetic pathway of crystal nucleation significantly. However, the role of the protein cluster as well as the dense liquid phase during nucleation and protein crystallization is still not entirely clear [3].

We have recently studied the phase behavior of globular proteins in solution in the presence of multivalent metal ions. It has been shown that solutions of negatively charged globular proteins at neutral pH in the presence of multivalent counterions undergo a "reentrant condensation (RC)" phase behavior [4-8], i.e. a phase-separated regime occurs in between two critical salt concentrations, $c^* < c^{**}$, including a metastable liquid-liquid phase separation (LLPS) [8,9]. Crystallization from the condensed regime follows different mechanisms. Near c^*, crystals grow following a classic nucleation and growth mechanism; near c^{**}, the crystallization follows a two-step mechanism, i.e. crystal growth follows a metastable LLPS [7-9].

The aim of the project is to achieve a deeper understanding on the role of the cluster and liquid-liquid phase separation in protein crystallization. Using beta-lactoglobulin (BLG) and human serum albumin (HSA) as model systems in the presence of YCl_3, we have determined the phase diagrams as a function of protein, and salt concentrations. We have optimized the conditions for the two-step mechanism, i.e, crystal growth that follows a metastable LLPS.

In this beamtime, we have performed the following measurements: (A) Structure evolution of dense liquid phase during a LLPS process in protein (BLG) solutions in the presence of multivalent metal ions.

Figure 1 presents typical SAXS profiles of protein dense phase at different temperatures. The sample contains BLG of 66 mg/ml and 15 mM YCl_3. A hierarchical structure at different length scales is clearly seen at the starting temperature already. Structural evolution of a temperature-dependent process was followed by SAXS. Structural changes at different q ranges were observed. The monomer-monomer correlation peak at 2.2 nm^{-1} decreases its intensity with temperature. The shoulder at 1.8 nm^{-1} corresponds to the dimer form factor. Between, 1.8 and 0.7 nm^{-1}, the intensity increases with decreasing temperature. Similar behavior for q below 0.3 nm^{-1} is observed, indicating the formation of larger objects at low temperature. The characterization of the full hierarchy of structures is promising towards a deep understanding of the two-step pathway of crystallization.

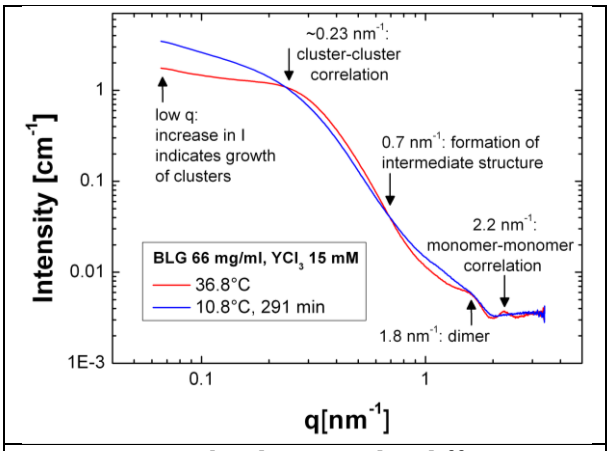

Figure 1. Hierachical structural at different temperatures revealed by SAXS.

(B) Diffraction patterns of protein crystals formed via different growth mechanism. In order to gain structural information of protein crystals, which are not suitable for X-ray diffraction measurements, we measured crystals collected in a capillary using SAXS. In this way, we can compare the structures of crystals with different morphologies. To avoid evaporation of the crystals a small amount of the solution were also filled into the capillary. Figure 2 shows an example of BLG crystals grown from a solution with ZnCl2. The crystal growth follows a two-step procedure.

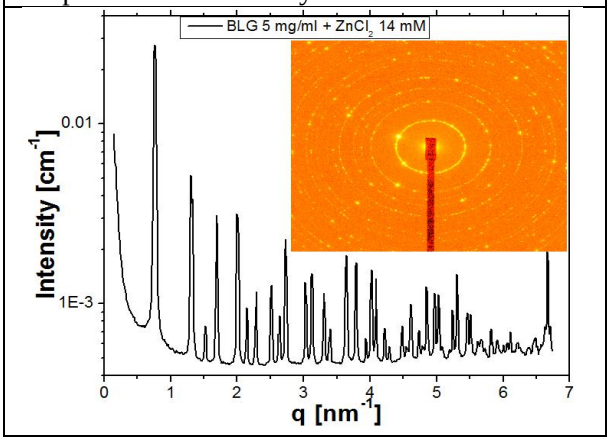

Figure 2. 2D diffraction pattern of BLG crystals formed via a two-step growth.

(C) LLPS and crystallization of proteins induced by PEG addition. Protein solution that exhibit LLPS in the presence of polyethylene glycol (PEG) has been used as a reference system to study the protein interaction and phase transition. PEG provides control over the protein interaction potential in terms of interaction strength and range. The phase diagrams of two protein systems immunoglobulin G (IgG) and glucose isomerase (GI) in the presence of PEG have been established in our lab. In this beamtime, we have performed SAXS measurements for both protein solutions as a function of temperature, protein and PEG concentration and PEG molecular weight, in order to relate it to the macroscopic phase behavior. Data analysis is under way.

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