

Exploring Nucleation Pathways across diverse physicochemical environments: unveiling new strategies to modulate and optimize biomolecular crystallization

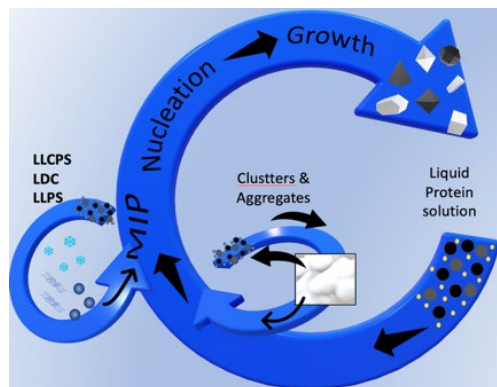
H. Brognaro¹, S. Gevorgyan¹, M. Wang¹, Ch. Betzel¹

¹ University of Hamburg, Institute of Biochemistry and Molecular Biology, Laboratory for Structural Biology of Infection and Inflammation, c/o DESY, Build. 22a, Notkestr. 85, 22603 Hamburg, Germany

christian.betzel@uni-hamburg.de

The crystal nucleation theory has been discussed for nearly a hundred years [1], however the discussion between classical and nonclassical nucleation theories is a still ongoing lively topic [2, 3]. In recent years it has gained again substantial new interest for serial crystallography (SMX, SFX), since tiny crystal in bulk amounts are required for SFX, SMX. The understanding of the individual crystal nucleation process to tune crystallization experiments from obtaining large single crystals towards bulk amounts of small crystals is mandatory [4]. In this context the characteristic of the classical nucleation theory (CNT) assumes a one-step nucleation of solute molecules directly from the supersaturated bulk solution, along with the simultaneous increase in two ordering parameters concentration and structural arrangements guiding the three-dimensional (3D) ordering of a protein crystal [4,5]. However, early proposed simulations have decoupled the development of the two ordering parameters leading towards a multi-step nucleation [6], termed as nonclassical nucleation theory (NCNT), which was supported subsequently by experimental discoveries [7]. In particular, the two-step nucleation theory has been receiving more attention along the last years, since widespread observations of liquid-liquid phase separation (LLPS), as precursor prior to the nucleation process and part of the two-step nucleation process were reported more frequently [4]. In parallel functional LLPS gained also substantial attention in different fields of cellular biology, in cellular fiber formation and for the in vivo formation of crystals [8], covering pathways also involved in diseases [9], as described for cataract, Charcot-Leyden crystals, hemolytic anemia, myopathies and hematin crystals in malaria [10]. Conclusively, the phase separation behaviour observed for distinct protein crystallization experiments is similar and hold homologies to functional intracellular phase separation or condensate formation in cells, creating also membrane-less organelles, forming and dissolving dynamically on demand, as observed by a number of complementary biophysical and bioanalytical investigations [9,10]. Thereby understanding the dynamics of this phenomena holds promise for the development of new therapies to treat for example neurodegenerative diseases and meets the topic of protein crystallization.

We utilized data obtained and published for cellular LLPS to obtain more insights in nucleation pathways in protein crystallization experiments and applied selected proteins, like glucose isomerase (GI), to investigate systematically the early stages of the crystallization process, covering phase separation, condensing and pre-nucleation ordering of protein molecules



in diverse scenarios, including varying ionic and crowding agent conditions, as well as the application of a pulsed electric field (pEF) [11,12]. The main method used to characterize the early events of nucleation was synchronized polarized and depolarized dynamic light scattering (DLS/DDLS), which is capable of collecting the polarized and depolarized component of scattered light from a sample suspension in parallel, thus monitoring the time-resolved evolution of the condensation and initial geometrical ordering of proteins at the early stages of nucleation [12,13]. Data obtained revealed that multiple nucleation pathways, including prior LLPS or formation of clusters and aggregates can be achieved by tuning the specific and non-specific protein interactions. Examples and corresponding data will be presented.

Figure 1. Crystallization pathway scheme from a monomeric protein solution, in presence of precipitants or crowding agents, towards nucleation and further crystal growth. Particles as aggregates, liquid dense clusters, liquid-liquid crystalline phases can be in between dynamically involved and may be led also in between to a metastable intermediate precursor or intermediates before reaching a nucleation and growth.

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The authors acknowledge the support of the Cluster of Excellence ‘Advanced Imaging of Matter’ of the Deutsche Forschungsgemeinschaft (DFG)—EXC 2056—project ID 390715994, and by DFG project BE1443/29-1.