Protein phase separation and determinants of in cell crystallization

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Abstract
Liquid-liquid phase separation (LLPS) in cells is known as a complex physicochemical process causing the formation of membrane-less organelles (MLOs). Cells have well-defined different membrane-surrounded organelles like mitochondria, endoplasmic reticulum, lysosomes, peroxisomes, etc., however, on demand they can create MLOs as stress granules, nucleoli and P bodies to cover vital functions and regulatory activities. However, the mechanism of intracellular molecule assembly into functional compartments within a living cell remains till now not fully understood. In vitro and in vivo investigations unveiled that MLOs emerge after preceding liquid-liquid, liquid-gel, liquid-semi-crystalline, or liquid-crystalline phase separations. Liquid-liquid and liquid-gel MLOs form the majority of cellular phase separation events, while the occurrence of micro-sized crystals in cells was only rarely observed, however can be considered as a result of a preceding protein phase separation event. In vivo, also known and termed as in cellulo crystals, are reported since 1853. In some cases, they have been linked to vital cellular functions, such as storage and detoxification. However, the occurrence of in cellulo crystals is also associated to diseases like cataract, hemoglobin C diseases, etc. Therefore, better knowledge about the involved molecular processes will support drug discovery investigations to cure diseases related to in cellulo crystallization. We summarize physical and chemical determinants known today required for phase separation initiation and formation and in cellulo crystal growth. In recent years it has been demonstrated that LLPS plays a crucial role in cell compartmentalization and formation of MLOs. Here we discuss potential mechanisms and potential crowding agents involved in protein phase separation and in cellulo crystallization.

KEYWORDS
crowding agents, in vivo/in cellulo crystallization, liquid-liquid phase separation, membrane-less and membrane-surrounded organelles
1 | INTRODUCTION

In context of liquid-liquid phase separation (LLPS) of biomolecules into liquid condensates in cells occasionally protein crystals were observed.\(^1\) The observation of crystals in living cells, reported in the literature as in cellulo or in vivo crystallization;\(^1\)\(^-\)\(^6\) biocrystallization of the DNA-binding protein (Dps) and DNA as a response to cellular damage and stress,\(^13\)\(^-\)\(^15\) or β-hematin crystals produced as a detoxification strategy by malaria parasites.\(^16\)\(^-\)\(^19\) Although in cellulo crystallization still is a new exciting area in cell biology, many natively crystallizing proteins in living cells first function as storage such as vitellin yolk protein crystals from bullfrog oocytes,\(^20\) from leopard frogs,\(^21\) from A. aegypti oocytes,\(^22\) lipovitellin from bony fish oocytes,\(^23\) edestin from hemp plant,\(^24\) tobacco seed protein\(^25\) and Cry protein in B. thuringiensis,\(^26\)\(^27\) trichocyst matrix protein in Paramaecium\(^28\)\(^29\) and food milk protein in Diplodota punctata.\(^30\) Secondly crystals can function as rigid encapsulation such as spheroidin from B. mori.\(^31\)

Further functions are compartmentalization, such as reported for intracellular polyhedra bodies in various bacteria (eg, T. neapolitanus)\(^32\) and solid state catalysts, as reported for the urate oxidase from R. novergicus,\(^33\) alcohol oxidase from H. polymorpha\(^34\) and catalase from sunflower.\(^35\) Moreover, crystals occurrence is related to wound sealing in case of Hex-1 from Neurospora crassa, fungal crystals seal the septal core,\(^36\)\(^37\) and P-protein from V. faba.\(^38\) Those abovementioned natively occurring crystals have been detected either by powder diffraction or electron microscopy. In cellulo/in vivo crystallization or crystalline matter in cells has been observed, however the individual precise function remains widely speculative. It may be harmless or harmful and even useful like storage of proteins or peptides, or removal of toxins.

Further, in cellulo crystallization has also been associated with several diseases like cataract,\(^39\)\(^-\)\(^42\) hemoglobin C diseases,\(^43\) formation of Charcot-Leyden crystals (CLCs),\(^44\)\(^-\)\(^47\) Reinke’s crystals\(^48\)\(^-\)\(^50\) or mitochondrial myopathies,\(^51\) and more recently in cellulo crystallization was observed as a result of heterologous overexpression of genes in cell lines of bacteria,\(^26\) insect cells,\(^2\)\(^4\)\(^2\)\(^2\)\(^4\)\(^2\)\(^5\)\(^2\)\(^6\) yeast,\(^12\)\(^2\)\(^4\)\(^7\) CHO (Chinese hamster ovary) or HEK (human embryonic kidney) cells.\(^32\)\(^4\)\(^8\)\(^-\)\(^5\)\(^9\) Mostly, these protein crystals were located in different organelles (mitochondria, peroxisomes, lysosomes, or endoplasmic reticulum), as shown in Table 1.\(^1\)\(^2\)\(^4\)\(^2\)\(^4\)\(^2\)\(^5\)\(^3\)\(^6\)\(^6\) but sometimes also within the cytosol,\(^32\)\(^4\)\(^2\)\(^7\)\(^9\)\(^6\)\(^3\)\(^6\)\(^3\) or even in the nucleus,\(^42\)\(^5\)\(^6\)\(^7\) as shown in Figure 1 and Table 1.

In the context of human diseases called “crystallopathies”,\(^66\) it has been demonstrated that Galectin 10 is the major component of CLCs which plays an essential role in the pathogenicity of several

### Table 1: Examples of selected in vivo crystals observed in different living cell systems

| Protein | Molecular weight | Cell system | Location | Crystal morphology | TEM | References |
|---------|-----------------|-------------|----------|--------------------|-----|------------|
| Calcineurin (HE) | 79 kDa | SF21 insect cells | Cytosol | cubic-rhomboid | Yes | Fan et al\(^59\) |
| Charcot-Leyden crystals (HE & DR) | 15 kDa | HEK293 | Cytosol and nucleus | bipyramidal | No | Hasegawa et al\(^42\) |
| Cypovirus polyhedra (HE) | 29 kDa | SF21 insect cells | Cytosol | cubic | Yes | Coulibaly et al\(^60\) |
| TbcathepsinB (HE) | 37 kDa | SF9 insect cells | rER | Needle-like | Yes | Koopmann et al\(^2\) |
| TblMPDH (HE) | 57 kDa | SF9 insect cells | Cytosol | Needle-like | Yes | Nass, Duszenko et al\(^3\) |
| Firefly luciferase (HE) | 62 kDa | SF9 insect cells | Peroxisomes | Needle-like | Yes | Schönherr et al\(^63\) |
| GFP-μNS (HE) | 45 kDa | SF9 insect cells | Cytosol | Needle-like | No | Schönherr et al\(^63\) |
| Immunoglobulin G (HE) | 150 kDa | CHO cells | ER lumen | Needle-like | No | Hasegawa et al\(^54\) |
| Coral fluorescent Xpa protein (HE) | 26 kDa | HEK293 (rat neuron & mouse fibroblast) | Lysosome, cytosol and nucleus | Needle-like | Yes | Tsutsui et al\(^56\) |
| PAK4-Inka1 complex (HE) | 41 kDa | COS 7 cells (HeLa cells and HEK 293) | Cytosol and nucleus | Needle-like | No | Baskaran et al\(^7\) |
| Neuraminidase (HE & DR) | 36 kDa | COS/CHO/HE K cells | ER | Cubic | No | Hasegawa, Gallat et al\(^58\) |
| γ-Cryallin D (HE & DR) | 19 kDa | CHO/HEK | Cytosol | Hexagonal | Yes | Hasegawa\(^42\) |
| Hydra (NP) | N/A | Hydra | Mitochondria | Orthorhombic | Yes | Davis\(^64\) |
| LAPs/insect sperm (NP) | N/A | Insect sperm | Mitochondria | Crystalline | Yes | Baccetti et al (1977)\(^65\) |

Note: **HE**, in cellulo protein crystallization via heterologous expression; **DR**, disease-related in cellulo protein crystallization; **NP**, natural phenomenon of in cellulo protein crystallization.

*Crystal lattice confirmed by transmission electron microscopy.*
A recent study solved the Galectin 10 structure using CLCs from patients with rhinosinusitis and asthma. Further, CLCs bind and are dissolved by antibodies. This opens new avenues to explore disease related in vivo crystals as drug target. In cellulo protein crystallization has also gained attention as a new and alternative method to produce high amounts of micro- or nano-sized crystals which can be used to determine the 3D structure of the crystallized protein using either high brilliant X-ray free electron laser or highly brilliant micro-focused synchrotron radiation applying serial diffraction data collection. However, despite an increasing number of publications reporting in vivo crystallization, the physicochemical parameters required and the molecular mechanism of in vivo crystallization guiding crystallization in cells are up to date only poorly understood, considering that even conventional, in vitro, protein crystallization till now remains a challenge. In a first assumption, it seems reasonable to consider the process to be analogous to crystal formation in vitro, where a purified protein at relatively high concentration is used for crystallization screening experiments. By mixing the protein solution with different precipitant agents, including salts or organic polymers, the phase diagram of crystallization is systematically screened to achieve supersaturation of the protein and to reach conditions required for nucleation. Additionnally, parameters such as temperature, pH, and others are typically optimized in in vitro protein crystallization experiments. However, the identification of in vitro protein crystal nucleation conditions is till now a trial and error process, and mostly unpredictable. Therefore, today in vitro protein crystallization experiments are routinely performed by screening many conditions applying either vapor diffusion, dialysis, counter diffusion, or batch crystallization techniques. During in vitro crystallization, a protein solution is brought to supersaturation to first induce liquid dense cluster (LDC) formation as a precursor followed by nucleation and further crystal growth, which thermodynamically is a second-order transition. In this context, several in vitro investigations are focused to obtain insights about the LDC formation and nucleation pathways in order to understand protein crystallization in more detail. This data can in principle also support understanding of in vivo crystallization. Likewise, in vivo crystallization at first requires a high local concentration of the protein to render crystallization thermodynamically favorable. Therefore, protein sorting into organelles with limited space seemed originally a prerequisite for in vivo crystallization. However, occurrence of in vivo crystals within the cytosol may also reflect preceding LLPS events. To reach the required protein supersaturation for nucleation and crystal growth, phase separation is supported by crowding agents in the cell, which promote attractive protein-protein interactions and act similar to precipitants in in vitro experiments. In vivo crystallization was observed in different cells and specific organelles. However, the in vivo nucleation mechanism and the molecular components, like crowding agents, essential for phase separation and crystallization are still challenging to address experimentally.

LLPS is a phenomenon denoting the demixing of structurally different molecules in aqueous solution above a certain concentration, considering a distinct physicochemical environment. LLPS is known to be the primary process underlying, for example, the formation of stress granules, the nucleolus, or P bodies. Based on these observations investigations are ongoing, trying to understand the molecular features promoting and regulating the formation of membrane-less organelles (MLOs). For protein crystallographers the concept that proteins or macromolecules can undergo phase
separation is not a new phenomenon, as it is frequently observed during crystallization experiments. LDCs are densely assembled molecules in aqueous solution, appearing in context of LLPS, which can be considered as a mandatory precursor of a nucleation process preceding the in vitro protein crystallization process. As an example, lysozyme undergoes LLPS, gelation, and crystallization depending on certain conditions of temperature, precipitants and protein concentration. More recently it was demonstrated that oligomeric peptides can undergo LLPS when stimulated by low temperature, crowding agents such as polyethylene glycol (PEG), or a pH sometimes close to their isoelectric points. Crowding agents are used for conventional protein crystallization, but also used to investigate the formation of the nucleolus, protein stabilization and folding and formation of other MLOs in vitro. Further, molecular crowding was also noticed to promote amyloid formation. Crowding agents, such as PEGs, dextrans, and even low molecular compounds like trimethylamine N-oxide (TMAO) have been used for in vitro investigations of LLPS.

From these in vitro experiments, it can be concluded that LLPS is obviously the process that governs the formation of membrane-less compartments in cells, which can occur in all different cell organelles or in the cytosol. This assumption is supported by the fact that in vivo grown crystals were observed in different organelles, such as rough endoplasmic reticulum (rER), mitochondria, lysosomes, peroxisomes or the nucleus, as indicated in Figure 1. These facts led us to consider that LLPS might not only be linked to cell compartmentalization or disease-related protein aggregation but may also be a prerequisite for in vivo crystallization.

2 | CELL ORGANELLES, LIQUID-LIQUID PHASE SEPARATION AND IN CELL PROTEIN PHASE STATES

Cells contain membrane-surrounded functional organelles, like the nucleus, mitochondria, lysosomes, endoplasmic reticulum, Golgi apparatus, peroxisomes and MLOs, such as P-bodies, stress granules, Cajal bodies, nucleoli, etc. Organelles represent a specific environment that may differ in solvent content, pH value, ionic strength, internal molecule distribution and other characteristics, to shape them specialized for defined cellular functions. Membrane-surrounded organelles have a well-defined membrane and communicate intensively by different signaling pathways and direct exchange of molecules via specific small vesicle carriers, like COPI-coated vesicles or clathrin-coated vesicles that transport cargo between organelles. In terms of biogenesis many membrane-surrounded organelles are not formed de novo compared to MLOs, which appear at early mitosis. Organelles grow and divide and are distributed between cells during cell division. For example, the membrane of the rER expands during biosynthesis of secretory proteins and becomes part of the Golgi membrane via vesicular transport. From here vesicles are released and by membrane fusion either the plasma membrane or the lysosomal membrane may expand, depending on their protein cargo. The water content of membrane-surrounded organelles is controlled by aquaporins and only to a negligible part by direct perfusion.

In cellulo MLOs are formed by LLPS. They lack a lipid boundary, can contain different types of biomolecules, have specific functions and ensure that distinct cellular functions occur in a spatiotemporally controlled manner. However, our knowledge and understanding of the dynamic assembly, partitioning of molecules and reaction kinetics of MLOs and corresponding LLPS is still limited. Also recent publications highlight the fact that protein complexes, like ribonucleoproteins can be assembled via LLPS, a process involving the concentration of molecules in a confined liquid compartment that stably coexist with the surrounding liquid environment. A number of comparative and complementary in vivo and in vitro studies revealed that phase states of intracellular compartments can be liquid-liquid, liquid-gel, solid-gel, crystalline-solid, semi-crystalline or liquid-crystalline, depending on the surrounding physicochemical conditions and spatial ordering. Also, investigations unveiled that LLPS in vivo and in vitro can particularly involve intrinsically disordered proteins (IDPs)/regions. IDPs need to reach a critical local concentration to undergo LLPS. The presence of crowding molecules, within cells potentiates the conversion of the so-called protein condensates to different transition states, such as gel, liquid-crystalline, crystalline-solid, solid-gel and even to amyloid fibers. Certainly, a more detailed understanding of the underlying control mechanisms on a molecular level and about crowding agents is required to understand phase separation within cells. The already known physicochemical phenomena evaluated from in vitro experiments about LLPS are valuable to design and perform future experiments to obtain more insights about in cellulo LLPS.

3 | IN VIVO CRYSTALS

For more than a century it has been observed that protein crystallization occurs within living cells. In Table 1 we summarize selected examples of in vivo protein crystallization together with their intracellular location inside different organism or cell systems. Further, we grouped them into naturally occurring crystals, like mitochondrial crystals in Hydra and insect sperm, human disease-related crystals (sialidosis, Charcot-Leyden, and cataract) and crystals observed during heterologous expression, such as calcineurin, polyhedra, TbCatB, etc. Interestingly, most in vivo crystals have a needle-like shape, beside a few cubic-rhomboid, hexagonal and bipyramidal morphologies reported till now and they are mainly located within the cytosol or inside membrane surrounded organelles like the endoplasmic reticulum, mitochondria, lysosomes and peroxisomes as shown in Figure 1. Details about in vivo crystallization and crystals observed in cellulo are also reported by Doye and Poon, Duszenko et al, Schönherr et al and Hasegawa.

3.1 | Location of in vivo crystals in cells

Davis, and Baccetti et al reported about naturally formed crystalline or paracrystalline structures within mitochondria in hydra cells and
insect sperm.\textsuperscript{64,65} The intramitochondrial crystals were interpreted as a result of cellular damage or storage material. The crowding agents supporting phase separation and crystal nucleation within the mitochondria are not yet known, but it was recently described that sperm-leucylaminopeptidases (LAPs) are required for male fertility and that they are significant components of mitochondrial paracrystalline material in \textit{Drosophila melanogaster} sperm.\textsuperscript{122} Fan et al described in vivo crystals of calcineurin, a well-known heterodimeric Ser/Thr phosphatase, composed of a catalytic subunit (CNA) of 60 kDa and a regulatory subunit (CNB) of 19 kDa, which crystallized by co-expressing the catalytic subunit (CNA) from \textit{Neurospora crassa} (NorfA) and the human regulatory subunit (CNB) from human using the baculovirus expression systems. Up to three crystals/cell were identified in the cytosol applying transmission electron microscopy (TEM), but till now no X-ray diffraction study has been reported.\textsuperscript{59} Coulibaly et al reported the first atomic structure of cytosolic intracellular cypovirus polyhedra crystals grown in and purified from Sf21 insect cells. Schönherr et al analyzed the in vivo protein crystallization of firefly luciferase and GFP tagged to reovirus \(\mu\)NS (GFP-\(\mu\)NS), applying the Sf9 insect cell system. They demonstrated that growth of in vivo crystals is a highly dynamic process and that these crystals were located either inside of peroxisomes or within the cytosol, respectively. So far, characterization of firefly luciferase using scanning electron microscopy (SEM) and GFP-\(\mu\)NS using X-ray powder diffraction studies have been reported.\textsuperscript{63} Tsutsui et al reported expression of an Xpa Coral protein in HEK293 cells, were crystals were encapsulated by autophagosome/lysosomal membranes (although some crystal-like structures were also found in the nucleus). The authors describe that selective autophagy engulfs the crystals into a cargo within the cells.\textsuperscript{56} Baskaran et al described in vivo crystallization and X-ray structure analysis of human PAK4 in complex with its inhibitor Inka1. PAK4-Inka 1 in vivo crystals grew in the nucleus and cytosol of human cells, respectively.\textsuperscript{57} The crystal structure refined to 2.8 Å resolution revealed details about the PAK4 catalytic domain which binds cellular ATP and the Inka1 inhibitor. Also the authors described that the hexagonal array of PAK4cat subunits can in principle accommodate a variety of other medium or low molecular weight proteins when fused either to the full-length PAK4 or fragments of Inka1, highlighting that PAK4 can act as a molecular flask and chaperon supporting in vivo crystallization of small proteins and facilitating X-ray analysis.\textsuperscript{57}

**FIGURE 2** Light and electron micrographs (TEM and SEM) of in vivo crystals (TbIMP DH and TbCatB). (A) Light micrograph of non-infected High five cells and (B) infected High five cells with TbIMP DH (arrows indicate needle-shaped crystals). (C) SEM image of TbIMP DH crystals (arrows) sticking outside Sf9 cell. (D) TEM image of intersected TbIMP DH crystals in the cytosol. (E) SEM micrograph of a needle-shaped TbCatB crystal (arrows) growing from in to out of a Sf9 cell. (F) TEM image of TbCatB crystal inside rER.

Abbreviations: N, nucleus; NL, nucleolus; NM, nuclear membrane; rER, rough endoplasmic reticulum; V, virus particles
A recent publication by Hasegawa reported that neuraminidase, immunoglobulin G (IgG), γ-crystallin D and CLCs appear during heterologous expression in HEK or CHO cells, respectively, where crystals were identified within ER, cytosol, or nucleus through LLPS. Till now, no structure or X-ray diffraction data were published for these proteins. The author also speculates about potential crowding agents, such as cellular proteins and especially organelle resident proteins that might support in vivo crystallization of neuraminidase, IgG, γ-crystallin D or CLC. They also emphasize the urgent need to identify intracellular crowding agents or external factors to enhance or to predict the possibility to obtain protein crystals in a cell organelle.

In terms of our own investigations, we obtained in vivo grown crystals for Trypanosoma brucei (Tb) CatB and IMPDH using the baculovirus expression system (Sf9 insect cells and High five cells). Initially, we performed a bioanalytical characterization that revealed the identity of the crystallized material and showed the homogeneity of the intracellular crystal lattices by TEM and SEM (Figure 2). Analysis of the TEM micrographs revealed that TbCatB crystals were exclusively located within the rER and TbIMPDH crystals in the cytosol (Figure 2). We applied those crystals for serial diffraction data collection and could solve and refine the structures to 2.4 and 2.8 Å resolution, respectively.

### Table 2: Summary of potential in cellulo crowding agents

| Crowding agents | Chemico-physical properties and functions | References |
|-----------------|-----------------------------------------|------------|
| Ribonucleotides: ATP, GTP, UTP, CTP; Deoxynucleotides: dATP, dGTP, dCTP, dTTP | Source of energy, affecting protein solubility and preventing macromolecular aggregation (ATP hydrotropic activity). Dissolving LLPS droplets and amyloid fibers | Traut,127 Rice and Rosen,128 Frankel et al129 |
| RNAs: 1. snoRNA, snRNA, 2. poly(A)mRNA, 3. rRNA, 4. mRNA, 5. tRNA | RNA species actively contribute to cellular phase separation inside MLOs: Cajal bodies (snoRNA, snRNA), nuclear speckles (poly(A)mRNA), nucleolus (rRNA, snoRNA), para speckles, stress granules (poly(A)mRNA), processing bodies (snRNA) for different cellular function: RNA modification, mRNA maturation and storage, rRNA processing and modulation of gene expression. Induction of phase separation via RNA-protein interactions and RNA-RNA interactions | Weber and Brangwynne,130 Banani et al,91 Langdon and Gladfelter,131 Faya and Anderson,132 Poudyal et al133 |
| Cellular proteins, and oligopeptides (poly-L-arginine, poly-L-lysine, polyallylamine, etc.) | Intracellular protein-protein interactions can lead to different in cellulo phase transitions (liquid-like, aggregation, liquid-crystalline or crystal). Polyallylamine/nucleotides are involved in phase separation. Poly-L-arginine, poly-L-lysine, polyallylamine and related polymers are most abundant among prokaryotes | Poudyal et al,133 Hasegawa42 |
| Chemical chaperones/osmolytes, and other low-molecular-weight metabolites (carbohydrates, fatty acids and sterols, etc.), inorganic ions (Mg2+, H+/OH−, etc.) | Osmolytes or molecular chaperones are known to enhance or reduce the stability of protein molecules. They can trigger non-covalent protein-protein interactions to initiate LLPS. They are used as precipitant agents in vitro protein crystallization. Metabolites and ions are required for folding and catalytic activity of many enzymes. The metabolic enzyme can be regulated through phase separation by interaction with some metabolite | Diamant et al,134 Papp and Csermely,135 Marshall et al,136 Frankel et al,129 Poudyal et al,133 Hasegawa,42 Prouteau et al140 |
3.2 Crowding agents triggering LLPS

To date it remains challenging to investigate in cell phase separation phenomena on a molecular level. Therefore, several investigations on LLPS and biomolecular condensates are currently performed applying well-defined in vitro systems using selected proteins and distinct crowding agents. Synthetic macromolecular crowders, such as PEG, dextran or ficoll are mainly in use to investigate their single or synergy effects to induce or enhance LLPS in vitro. The “self-assembling” of proteins, such as FUS/TDP43, α-synuclein, tau, Aβ and the huntingtin into insoluble fibers that can even further aggregate is under investigation for some years, also in context to nucleic, tau, Aβ nucleotides in early stages during the origin of life. Nucleotides in the cytoplasm of extant biological cells and is discussed to be one relevant electronegative intracellular droplets in the cytoplasm and nucleo-plasm. The observed coacervate droplets were stable over a wide range of pH values and MgCl₂ concentrations.

LLPS is also thought to explain the appearance of polyelectrolyte-rich intracellular droplets in the cytoplasm and nucleoplasm of extant biological cells and is discussed to be one relevant factor in protocellular compartmentalization of nucleic acids and nucleotides in early stages during the origin of life. Nucleotides are typically available in high micromolar or even millimolar quantities in cells, which also qualifies mono- and oligonucleotides to be potential crowding agents. Oligoribonucleotides are also highly abundant as regulatory noncoding RNAs (tRNA, rRNA, miRNA, tmRNA) and can be grouped as polyanions that maintain some chemical similarity as compared to monoribonucleotides. Beside a mostly defined secondary structure the conformational flexibility of RNAs is supporting diverse regulatory protein-RNA interactions and formation of disordered coacervates and MLOs via electrostatic interaction. These interactions are typically enhanced by bivalent cations like Mg²⁺ and are stable over a wide range of physiological pH values. Hence, RNAs, especially RNAs related to the ribosomal translation machinery, may stabilize and/or surround a homogeneous protein phase separation state. Further, RNAs were already identified to be involved in LLPS in vitro. Compared to nucleic acids, the structural diversity of proteins in cells is much higher and many site-specific proteins are available in different quantities in different cellular organelles. Individual proteins were shown to initiate fiber and amyloid formation and surface properties or catalytic activities of a protein may be required to support LLPS formation. This might be either directly or by regulating the water content, the pH value or the abundance of another crowding agent in close proximity. The involvement of proteins in such a process was initially discussed by Hasegawa. Further experimental studies in the field, for example, to identify differences in the proteome during crystal formation, remain to be performed.

Small molecules with diverse chemical and structural properties are known to influence (enhance or reduce) the stability of protein molecules. Resulting changes of the tertiary structure of a protein can trigger and essentially influence noncovalent protein-protein interactions to initiate LLPS and even crystalline lattice order. Thereby, these molecules might also act as in cellulo crowding agents and may be considered as a precipitating agent for in vitro protein crystallization. Typically those compounds are called osmolytes acting as molecular chaperones. This group of structurally diverse molecules includes sugars, amines like betaine, urea and peptides.

Prouteau et al also reviewed that metabolic enzymes can even be regulated through phase separation, which could further regulate the availability of metabolites, acting as crowding agents. Overall, next to other reasons, like local stress-response, pH value or local water content, the availability of certain metabolites most probably influences the location that is suitable to initiate clustering of a protein within a cell.

4 CONCLUSION

Based on the current status of literature and our investigations about in vivo protein crystallization via heterologous expression, we conclude that four requirements need to be fulfilled for intracellular protein crystallization. First, the target protein should be over-expressed under control of a strong promoter to ensure a high translation rate. Second, the protein should accumulate via phase separation and formation of liquid dense protein clusters within cells. Third, UPR (unfolded protein response) should be avoided, as transcriptional regulators inhibit protein biosynthesis of the target gene in the cell expression system. Fourth and most important, specific crowding agents, such as short nucleotides, RNAs or distinct cellular proteins, must be present to support phase separation in context of other factors, like intracellular pH or PTMs inducing or enhancing nucleation. Experimental investigations need to be continued to identify and characterize molecular crowding agents, as a better knowledge about their function holds a significant potential to promote new research directions and to open new avenues to a better understanding of in vivo protein phase separation and crystal nucleation. Finally, in vivo protein crystallization needs to be systematically explored further, to obtain crystals for serial diffraction data collection and in parallel in terms of drug discovery investigations to prevent diseases related to in cellulo protein crystallization.

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CONFLICT OF INTEREST
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AUTHOR CONTRIBUTIONS
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