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Mastering interfaces: advances and challenges in controlling protein nucleation

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Interfaces are ubiquitous in nature and are involved in any physico-chemical process. Crystallizing a protein implies the formation of a new interface between the growing crystalline material and the liquid solution. The nucleation step, which is pivotal to the formation of crystals, is extremely sensitive to the presence of interfaces. Such a feature can be exploited to aid the control of this delicate step, improving the chances of getting protein crystals, the reproducibility of the experiment, and the uniformity of the attributes of the crystals, *i.e.*, size, habit, and form. To do this, several approaches have been proposed in recent years, focusing on the use of controlled functionalization of surfaces and nanoparticles, the establishment of diffusion-dominated systems, and, more in general, the use of heteronucleants and additives. Recent advancements and current challenges in the field are here highlighted, including continuous protein crystallization and micro-crystallization.

1. Introduction

Interest towards complex macromolecules such as proteins is constantly increasing thanks to technological advancements that allow their applications in various fields, including medicine, structural biology, catalysis, and materials science.[1] Biotherapeutics are nowadays a promising treatment approach for many diseases, with many examples already on the market, including monoclonal antibodies (mAbs).[2,3] Determining the structure of these complex molecules not only helps understand their function and how they interact with the processes they are involved in, but also supports the design of new molecules with optimized features. In this context, protein crystals play a central role as they represent the repository of the structural information, which can be unveiled by X-ray scattering.

Nevertheless, the applications of protein crystals are manifold. Crystalline protein materials represent a promising platform for the delivery of biotherapeutics, thanks to the increased stability of the drug in the lattice, the tailored release profile, and the high dose.[4–6] Protein crystals have even been used as biocatalysts: cross-linked enzyme crystals (CLECs) have shown higher activity-to-volume ratios compared to free molecules and are used as catalysts in bioremediation and green chemistry applications.[7]

Protein crystallization can in some cases serve as a purification technique to isolate target proteins from extracts. Downstream processing of biotherapeutics is currently a complex series of delicate and expensive chromatographic steps, leading to very high purification costs, which can be as high as 70% of the total manufacturing costs for mAbs,[8] and limited throughput due to intrinsic limitations in mass transfer.[9] Integrating protein crystallization into the downstream processing pipeline offers a promising approach to improve productivity and reduce overall production costs, preserving protein structure and activity.[10] Unlike chromatography, crystallization can be beneficial as no resins are required, high titres can be more easily managed, and a pure product can be obtained in a single step.

In this scenario, deep understanding of crystallization enables the design of more efficient and robust processes.[11] On the one hand, predictive models based on data mining and machine learning have been developed for protein crystallization.[12] However, the complexity of macromolecular crystallization and the limited data consistency and validity limit the applicability of these models. On the other hand, achieving control over nucleation and crystal attributes is crucial.[13] Several approaches have been applied to enhance protein crystallization, increase throughput and success rates, ranging from protein engineering for site-directed mutagenesis[14] to automated crystallization platforms[15,16] and controlled nucleation.[17] However, obtaining diffraction-quality crystals, or even crystals at all, remains a major obstacle for many proteins.

Interfaces are ubiquitous in any crystallization process. At the screening scale, air/liquid, liquid/liquid, and solid/liquid interfaces are the most common ones, considering micro-batch and hanging drop vapor diffusion (HDVD) crystallization

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techniques. On a larger scale, *i.e.*, inside crystallizers, air/liquid and solid/liquid interfaces are most frequently encountered. Interfaces impact nucleation and crystal growth, as here proteins behave differently compared to the bulk. For example, at the air/water interface, solvent evaporation can occur. This phenomenon, together with the effect of surface tension, can alter protein–protein interactions, favoring molecular alignment for packing into a lattice.[18,19] At the oil/water interface, protein adsorption can occur, leading to increased local supersaturation and facilitated nucleation.[20]

This highlight aims to comment on recent advancements in the field of protein crystallization, with a particular focus on how interfaces impact the nucleation step. An overview of theoretical frameworks concerning protein nucleation is first given. Then, nucleation control by means of tailored interfaces is discussed. Lastly, emerging trends in protein crystallization, *i.e.*, continuous crystallization and micro-crystallization, are briefly presented.

2. What is governing protein nucleation?

2.1 Thermodynamic and kinetic aspects

Protein crystallization is a first-order transition, that is a process initiated by the formation of stable clusters of macromolecules, *i.e.*, critical nuclei, followed by growth into detectable entities, *i.e.*, crystals. The phase behavior of proteins depends on the system supersaturation. Low supersaturation can lead to the formation of nuclei and crystals, whereas excessive supersaturation can trigger the formation of disordered aggregates. A delicate balance between the promotion of nucleation and the formation of good-quality crystals exists.[21]

As for any crystallization process, the phase diagram represents the starting point for understanding and designing protein crystallization. As for small molecules, supersaturation (S) is the driving force for protein crystallization, but the required S values are generally much higher, *i.e.*, 100%. However, the crystallization kinetics of proteins is comparatively slow despite high S because of their complex macromolecular configurations. It has been recently reported that the reason for this might lay in the highly inhomogeneous surface of proteins and the limited number of patches available for the involvement in lattice bonds.[22]

A simplified protein phase diagram is represented in Fig. 1 and can generally be divided into four regions:

- The solubility or undersaturation zone: the area below the solubility curve, crystallization cannot occur as there is no driving force;
- The metastable zone: the area between the solubility and the supersolubility curve, or metastable limit. Here, thermodynamically favorable conditions for crystal growth and kinetically unfavorable conditions for nucleation can be identified. While the solubility curve has an intrinsic thermodynamic nature (*loci* of equilibrium concentrations), the supersolubility curve is a kinetic curve;[23]

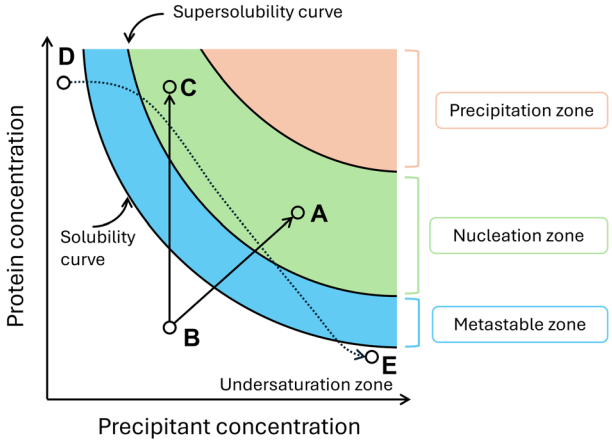

Fig. 1 Protein phase diagram reporting various possible pathways to achieve supersaturation according to batch (A), vapor diffusion (B to A), dialysis (B to C), and counter-diffusion (D to E) crystallization.

- The primary nucleation zone: nucleation occurs in a supersaturated solution;
- The precipitation zone: this area has to be avoided as it leads to amorphous precipitates.

Optimal S results from a complex interplay between ordered aggregation, denaturation, and amorphous precipitation. Many pathways can be followed to achieve desired S and obtain crystals. A successful crystallization path would thus involve S high enough to promote nucleation but low enough to avoid precipitation. Depending on the path followed to generate S, various techniques have been proposed for protein crystallization. The simplest one is batch crystallization, where a single and defined S is investigated (point A in the diagram). Dynamic S conditions are explored with vapour-diffusion based techniques (line from B to A in the diagram), which enable a progressive and continuous change in S levels within the system thanks to the slow removal of water. The slow vapor diffusion rate controls the initial nucleation and crystal growth.[24] Dynamic S conditions can also be explored by means of dialysis crystallization, where protein concentration is constant and precipitant concentration is progressively increased (vertical line from B to C). One last common method is based on counter-diffusion, which involves dynamic S levels thanks to the diffusion of both protein and precipitants. This slow mixing depends on the species diffusivity and results in a complex path on the phase diagram (line D to E).

The nucleation rate, J, determines the probability of nucleation in a given system. The nucleation induction time is defined as the time elapsed between the establishment of supersaturation conditions within a given system and the appearance of critical nuclei. This parameter can be evaluated experimentally, often approximating it to the time when the first detectable crystals appears in solution.[25,26] Other methods, applied to in-line monitoring of nucleation, are based on visual observation, laser, optical signal response, and spectroscopy.[27]

J can be either increased by acting on the surface and volume energies, as stated by the Gibbs–Thompson equation, or by decreasing the energy barrier towards nucleation. The former can be achieved by adding seeds to the system, whereas the latter can be obtained by adding heteronucleants. The presence of a heteronucleant expands the nucleation zone on the phase diagram, including areas of lower supersaturation.[28] The heterogeneous nucleation of proteins has several peculiarities compared to other heterogeneous nucleation processes involving ionic solids. Weak lattice forces stabilize protein crystals, and the interaction between the protein molecule and the surface has to be weak enough, *e.g.*, electrostatic interaction, to allow rotational and translational reorganization of proteins on the surface and the creation of a lattice. When attractive forces are present between the surface and the protein, the protein tends to accumulate at the surface, the local S is increased, and nucleation is favored. When repulsive forces dominate, the protein is repelled and concentrates within a thin layer over the surface. In this case, the surface does not play an active role during nucleation, which happens homogeneously in the bulk, but it is still able to modify the crystallization conditions.[29] The surface can also stabilize pre-nucleation clusters and favor the appearance of ordered arrays of protein molecules, facilitating the formation of stable nuclei thanks to interactions with residues exposed on specific crystal faces.

The metastable zone can also be modified by the presence of external fields, including electric, magnetic, ultrasonic, shear, and light fields, to speed up the nucleation process and reduce required S. For example, it was reported that ultrasounds increased the nucleation probability of lysozyme.[30,31] Nucleation induction within the metastable zone was also achieved by applying electric fields to different protein solutions, which enabled control over the size, number, form, and orientation of protein crystals.[32–36] The electric field affected protein phase behavior because of the alteration of protein–protein interaction potential.[37] Interestingly, the effect of magnetic fields is still to be fully understood, as induction or reduction of protein nucleation has been observed under different conditions.[38,39] Nevertheless, magnetic fields affect crystal growth, and beneficial effects were observed in terms of the obtained crystal quality.[40]

2.2 Nucleation and crystal growth

Nucleation is a stochastic and rare event. A physical interpretation of this process was given by the classical nucleation theory (CNT), proposed by Volmer and Weber in 1920s. CNT provides a well-established overview of homogeneous and heterogeneous nucleation. According to CNT, nucleation occurs within a single step in a supersaturated solution, leading to the ordered clustering of molecules, as shown in Fig. 2a. Surfaces can significantly lower the energetic penalty for nucleation, making heterogeneous nucleation more likely to occur compared to homogeneous nucleation.[41] CNT explains many aspects of protein crystallization, but it can fail when the nucleation rate, the predicted structure of critical clusters, and/or the nuclei size are compared to experimental data. For example, protein critical nuclei consist of few macromolecules, with the nucleus size decreasing with discrete steps from 10/11 to 1/2 molecules when supersaturation increases, which is not in line with CNT.[42] In addition, CNT predictions fails when applied to non-equilibrium systems, which are commonly used to crystallize proteins, *e.g.*, vapor diffusion (VD) or counter-diffusion (CD) crystallization. In these cases, the

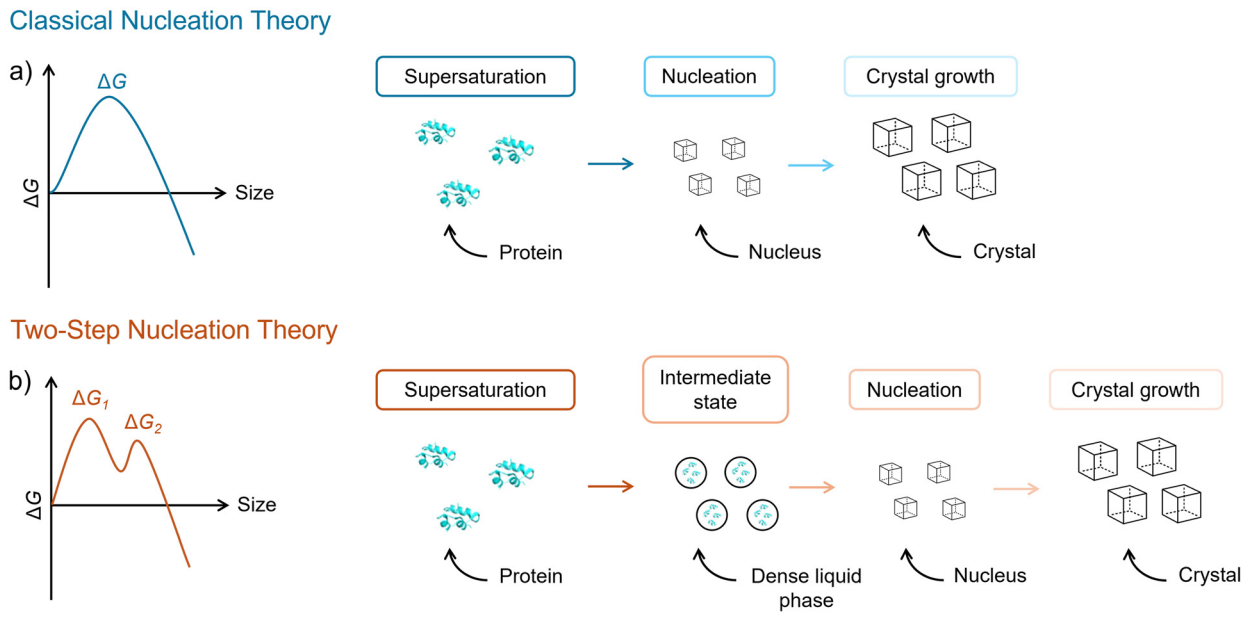

Fig. 2 Comparison between the evolution of the total free energy *vs.* size and the mechanisms of nucleation proposed by (a) Classical Nucleation Theory and (b) Two-step Nucleation Theory.

crystal number density and the crystal size depend not only on the supersaturation level of the system, but also on the path followed to achieve the driving force for crystallization, *i.e.*, the supersaturation rate.[43] CNT also fails to accurately describe even more complex processes than crystallization in solution, as shear-induced crystallization. Ferreira *et al.*[44] coupled rheological analyses to the characterization of insulin behavior in solution to study its nucleation and crystal growth. When insulin crystallization was performed in presence of shear and at intermediate precipitant concentration (2.3 mM $ZnCl_2$) and high temperatures (20 or 40 °C), or low precipitant concentration (1.6 mM $ZnCl_2$) and low temperatures (5 °C), a non-classical description of the process seemed more accurate, as aggregates/agglomerates were first formed and then crystals appeared. Conversely, a description of the process grounded on CNT was accurate when high precipitant concentrations (3.1 or 4.7 mM $ZnCl_2$) were involved, as each critical cluster led to the formation of a crystal.

To better describe the nucleation of macromolecules, several non-classical approaches have been theorized for crystallization processes of inorganics,[45] organics,[46] colloids, and macromolecules,[47] and even for the *in vivo* formation of amyloid fibrils[48] responsible for neurodegenerative diseases. The most successful one is the two-step nucleation theory,[49] proposed almost 80 years after CNT. As schematized in Fig. 2b, this theory is based on density fluctuations happening locally and a difference in the structural order between nuclei and solution. According to this theory, nucleation is described as a two-step process: first, the density of the liquid phase locally increases because of the formation of high protein concentration zones within the solution, then nuclei are formed within these regions.[50,51] The liquid dense phase can be considered as a metastable intermediate, having intermediate thermodynamic stability between the initial solution and the final crystalline phase.[52] Crystals appear within this dense liquid phase, and their growth has also been modeled as a two-step process: crystals initially grow within the dense phase according to a diffusion-limited growth law and then leave it and evolve according to a distinct growth law.[53]

The existence of 100 nm protein-rich clusters has been demonstrated through oblique illumination microscopy, which also confirmed their liquid nature and inhomogeneous structure comprising a viscous core. These clusters would be composed of transient protein oligomers, whose sizes are unaffected by variations in ionic strength and pH. Hydrophobic interactions and partial protein unfolding have been found to be important factors in the clustering mechanism.[54] The formation of dense liquid domains prior to protein nucleation has also been demonstrated by Balogun *et al.*[55] in heavy water *via* nanopore-based resistive pulse sensing. A single lysozyme (HEWL) crystal was grown by controlling local S using a nano-tip. At low protein concentration, transient events were detected and identified as translocation and transformation of protein oligomers and/or further aggregation.

3. Approaches to control protein nucleation by means of interfaces

Protein nucleation is affected by the presence of interfaces, and engineering the solid/liquid or gas/liquid interfaces has proved to be a successful tool to direct the crystallization pathway and tune the features of the obtained crystal population, such as crystal size, form, density, and habit.

The use of functionalized surfaces, particles, gels, porous materials, additives, seeds, or bubbles are just few examples of how interfaces can be tailored to modify the crystallization conditions. Alternatively, nucleation has also been carried out in contactless environments to limit the influence of interfaces on crystallization. A detailed discussion on such aspects is reported in the following.

3.1 Functionalized surfaces

Hanging/sitting drop vapor diffusion (H/SDVD) crystallization is a common protein crystallization technique used for screening purposes. A drop containing an aqueous solution of protein and precipitant is deposited on a solid surface (generally glass) and allowed to equilibrate with a reservoir solution containing a higher concentration of the precipitant. The instauration of a driving force for water vapor transfer progressively increases the supersaturation in the drop, eventually leading to the appearance of crystals. Under these conditions, the surface can be engineered to alter the crystallization environment. Minerals were first found to induce heterogeneous nucleation at reduced supersaturation levels and more rapidly compared to uncontrolled nucleation.[56] A detailed review on the role of different surfaces, including hydrophobic, porous, charged and rough substrates, has been recently published.[57]

The presence of a solid–liquid interface perturbs the crystallization pathway because of protein–surface interactions. For example, the adsorption of human serum albumin (HSA) onto a negatively charged surface (glass) in presence of a trivalent salt under crystallization conditions has been followed *via* quartz crystal microbalance with dissipation monitoring (QCM-D).[58] Results showed that, in few minutes, a layer of irreversibly bound proteins is formed on the surface. Then, enhanced protein adsorption occurs, leading to the formation of a less tightly bound multilayer structure. Within this structure, protein–protein interactions are facilitated and may lead to local reorientation and nucleation, as shown in Fig. 3a. Nucleation therefore happens in the adsorbed thick and soft protein multilayer, and nucleation density directly correlates with higher adsorption. The use of sodium alginate/hyaluronic acid films has also been reported to modulate protein adsorption and supersaturation.[59] In this case, electrostatic interactions and water absorption promoted by the film enhanced crystallization efficiency and controlled crystal size. HEWL was adsorbed onto the surface thanks to the electrostatic interaction between positively charged protein regions and

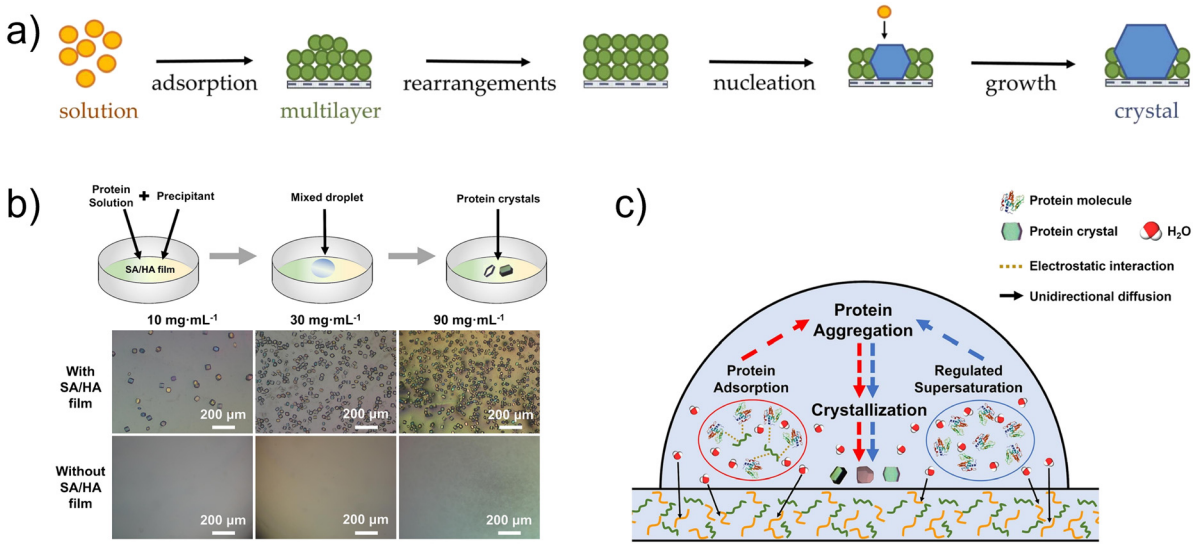

Fig. 3 (a) Schematics of an adsorption-mediated protein crystallization process. Reprinted with permission.[58] Copyright 2024, Elsevier Science. (b) Film-assisted protein crystallization: schematics and optical microscope images of HEWL crystallization with and without films. (c) Mechanism for film-assisted protein crystallization. Reprinted with permission.[59] Copyright 2025, Elsevier Science.

electronegative carboxyl groups exposed by alginate moieties, facilitating cluster formation and subsequent nucleation, as seen in Fig. 3b and c. In general, it was observed that charged surfaces tend to increase the crystallization density and reduce the nucleation time compared to control surfaces.[29] Functionalized mica sheets were employed as heteronucleants thanks to non-specific attractive and local interactions between charged protein residues and ionizable mica groups. These interactions prevailed when large and flexible proteins were used, such as thaumatin and concanavalin A, leading to heterogeneous nucleation.[62]

The exposure of specific chemical groups on surfaces to promote specific interactions with the protein molecules also impacts crystallization kinetics and polymorphism. Glass surfaces functionalized with self-assembled monolayers (SAMs) exposing thiol groups could decrease the nucleation induction time of HEWL from 3 days to 12 h. Depending on the surface chemistry, it was also possible to promote the appearance of up to three different polymorphs of catalase. This effect was attributed to the early stabilization of pre-nucleation clusters corresponding to different crystal forms promoted by the functionalized surface.[60] Monolayers exposing gradient surface properties, *i.e.*, wettability, were also employed to study the adsorption and crystallization behavior of proteins.[61] Results showed that preferential adsorption of HEWL occurred on the more hydrophobic areas of the surface and that more crystals formed in the same region, suggesting a strong correlation between these two phenomena. Recently, Langmuir–Blodgett (LB) templates assisted phycocyanin crystallization. A protein monolayer was formed at the air–water interface and transferred to a solid substrate by controlled dipping. The ordered structure of the LB template could facilitate ordered protein assembly and nucleation, as confirmed by second-order nonlinear imaging of chiral crystal (SONICC) spectroscopy.[63]

3.2 Particles

Particles have been used to mediate protein crystallization *via* electrostatic interactions, adsorption, or specific interactions. Thanks to the large exposed surface area, the probability of binding proteins onto the particles is high. The number of HEWL crystals increased when gold[64] or silica[65] nanoparticles (NPs) were added to the system thanks to increased interactions and higher chances for heterogeneous nucleation to happen. NP surface properties, as wettability, also played a role as hydrophilic NPs led to higher nucleation rates.[66] A layer of protein was created on the surface of gold nanoparticles functionalized with amino acids thanks to specific interactions. Nanoparticles exposing these bioconjugates templated rapid nucleation at low protein concentrations, see Fig. 4a.[67] This strategy has been successfully applied to HEWL in microbatch, and the underlying mechanism was compared to seeding due to the high order of protein molecules immobilized on the NP surface. Surface functionalization was also applied to magnetic particles to rationally design protein crystallization. An affinity molecule towards the protein of interest, such as casein for trypsin crystallization or chitin for HEWL crystallization, was immobilized on the surface of magnetic particles. Faster nucleation and/or removal of undesired compounds, such protease inhibitors, from the crystallization environment were successfully achieved thanks to affinity driven magnetic particles.[68]

Studies performed on nanodiamonds (NDs) also highlighted the impact of NP size and configuration (aggregates or films) on the crystallization of HEWL, ribonuclease A, proteinase K, and catalase.[69] It was found that NDs with smaller size (30 nm) adsorbed proteins more efficiently than those with larger size (100 nm), and that ND films induced the formation of a higher number of crystals compared to aggregates. The former result

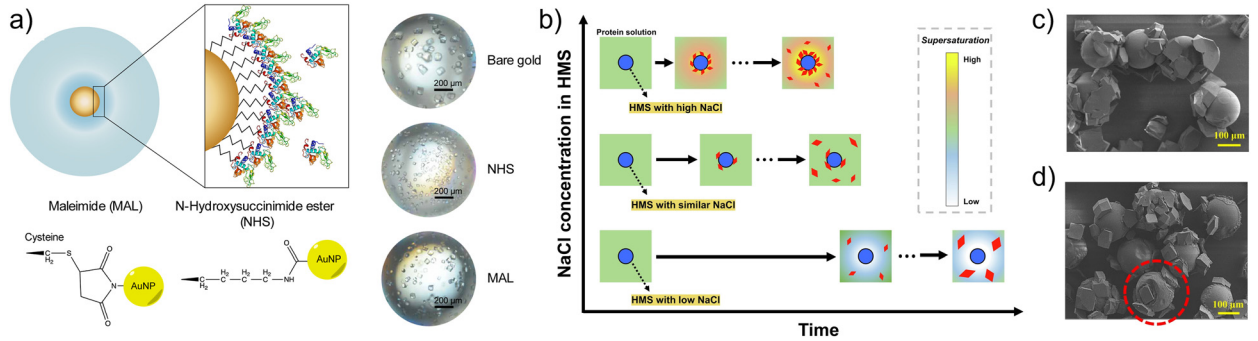

Fig. 4 (a) Schematics of a nanoparticle-mediated protein crystallization process and optical microscope images of crystals obtained from vapor diffusion experiments with bare, NHS, and MAL gold nanoparticles. Reprinted with permission.[67] Copyright 2023, American Chemical Society. (b) Schematics of protein crystallization influenced by microspheres at different salt concentrations. SEM images of crystals on microspheres loaded with (c) 2 and (d) 4 M sodium chloride mixed with 50 mg mL^{-1} HEWL. Highlighted by the red circle is a HEWL crystal embedded in a cavity of the microsphere. Reprinted with permission.[72] Copyright 2024, Elsevier Science.

was attributed to the larger surface-to-volume ratio of 30 nm NDs. The latter result was attributed to a limitation of the motion of the growing crystals offered by the film and to the larger size of the cavities present among NDs in the film.

Particles also impact nucleation kinetics. Graphite templates decreased the nucleation induction time of HEWL by 60% at different protein and precipitant concentrations. Graphene oxides, instead, hindered nucleation at low protein concentration. This behavior was attributed to different electrostatic interactions occurring between the protein and the nucleant: graphite was most effective in modifying the distribution of charge and ions in solution, influencing the local supersaturation.[70] PEGylated graphenes effectively induced the nucleation of five different proteins. Graphene functionalization with PEG is confined to specific spots on its surface and bare patches can act as pores, allowing for protein adsorption on the surface and reorganization into crystals.[71]

Particles have also been recently used to modulate the release of precipitants and serve as heteronucleants thanks to the presence of nanopores and wrinkles on their surface. PEGDA hydrogel microspheres (MS) were loaded with increasing concentration of sodium chloride and aided HEWL crystallization.[72] As shown in Fig. 4b–d, when low salt concentrations were loaded into the microspheres, nucleation in the bulk prevailed. When salt concentration in MS was comparable to the bulk salt concentration, nucleation occurred on MS surface because of protein adsorption and heterogeneous nucleation. When salt concentration in MS exceeded bulk salt concentration, smaller crystals were nucleated on the MS surface.

An interesting application of live cells, *i.e.*, *S. cerevisiae*, as biotemplates for HEWL crystallization has been reported by Sun *et al.*,[73] opening the possibility to employ live cells to support downstream processes. When live cells concentration was small, nucleation was enhanced, whereas the number of crystals was reduced when higher live cells concentrations were employed. The interaction between the cell wall and the protein was demonstrated by visualizing the aggregation of fluorescent HEWL around the cells.

3.3 Gels

(Hydro)gels have been thoroughly exploited to study the crystallization behavior of proteins. Gels are biphasic systems consisting of a liquid phase (a salt and protein solution) and a solid phase (interconnected microporous network). The solid phase may consist of entangled polymeric chains, as in the case of agarose gels, or crosslinked polymeric chains, as in the case of silica or polyacrylamide gels. In the former case, gelling is achieved by means of a variation of one (or more) physical parameters, such as a decrease in the temperature, and the obtained material is generally referred to as "physical gel". The latter material is obtained *via* a polymerization reaction, such as polycondensation, leading to a "chemical gel".[74] Chemical gels may contain reaction by-products, including alcohols or salts, which could interact with proteins and/or alter their solubility. Gel materials possess unique properties as they combine an aqueous environment with absence of convection and buoyancy effects. Mass transport relies on diffusion of protein molecules, simulating micro-gravity conditions and allowing for controlled crystal growth conditions. In gel systems, homogeneous nucleation prevails, probably happening in the larger cavities of the gel, where a higher number of molecules can be encountered and the formation of stable nuclei is more likely to occur. Heterogeneous nucleation happens on foreign particles, but the gel itself acts as a "filter", reducing the probability of interaction with the particles as they are either immobilized in the gel or even incorporated within the gel fibers, being inaccessible to protein macromolecules. Secondary nucleation is almost fully inhibited as secondary nuclei are generated from parent crystals by fluid attrition or solid impact, which are absent in gels.[74] Gel-incorporating protein crystals also show increased mechanical properties, as increased elastic limits and higher fracture stresses.[75]

Agarose gels have been used as nucleation inductors. Tuning the amount of agarose added to the system has been demonstrated to allow control over nucleation density and crystal size, as highlighted in Fig. 5a and b.[76,77] The action of

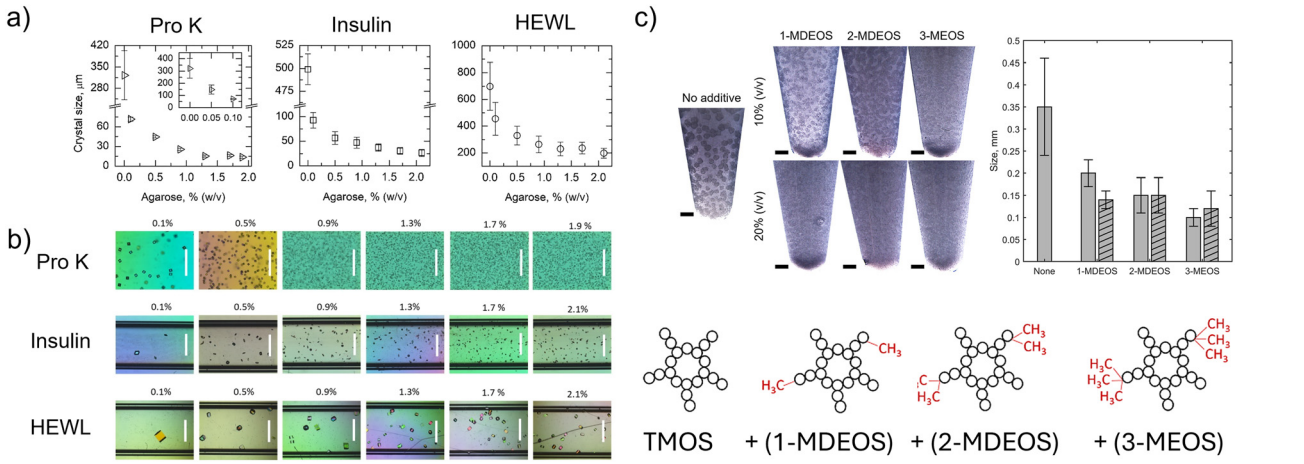

Fig. 5 (a) Crystal size *vs.* agarose gel content for proteinase K (embedded is the enlargement of the 0–0.1% w/v agarose region), insulin, and HEWL. Reprinted with permission.[76] Copyright 2020, American Chemical Society (b) Optical microscope images of crystals grown in agarose gels obtained in flat capillaries. Scale bar is 250 μm for proteinase K and 500 μm for insulin and HEWL. Reprinted with permission.[77] Copyright 2021, MDPI. (c) Optical microscope images of HEWL crystals produced in modified silica gels. Scale bars refer to 1 mm. Crystal size was measured at an additive-to-TMOS (AT) ratio equal to 10 (filled bars) and 20% v/v (single diagonal bars). Below is the structure of tetramethoxysilane (TMOS) gel and gels exposing methyl groups upon addition of methyldiethoxysilane (1-MDEOS), dimethyldiethoxysilane (2-MDEOS), and trimethylethoxysilane (3-MEOS). Reprinted with permission.[81] Copyright 2025, American Chemical Society.

agarose was found to be independent of the protein, as similar trends were observed for lysozyme, insulin, and proteinase K crystallized in batch. Hence, a mechanism based on the retention of water by the agarose fibers was proposed, which resulted in higher actual supersaturation in solution and facilitated nucleation. The diffusion-dominated environment coupled with physical protein-gel interaction resulted in very uniform crystal populations with sizes ranging from hundreds of nanometres to hundreds of micrometres. To overcome the limitations linked to the chemical incompatibility between agarose gelification and certain precipitants commonly used for protein crystallization, *e.g.*, $(NH_4)_2SO_4$ and PEG, counter-diffusion crystallization and free interface diffusion (FID) was used to immobilize the protein within the gel. A strong influence of agarose gel on nucleation was still observed, and a previously unknown crystal form of proteinase K was discovered.[76]

Agarose gels have also been used to sequester nucleants from the crystallization solution in a modified HDVD set-up.[78] Nucleation was promoted by FID of nucleants within the drop, whereas crystal growth was sustained by vapor diffusion. Crystals grew faster compared to control experiments performed in conventional HDVD.

Among the most common chemical gels used to crystallize protein, silica gels can be found. Vidal *et al.*[79] studied the nucleation inhibition effects of silica by small angle neutron scattering (SANS). Results showed that protein adsorption occurred on the gel fibers mediated by electrostatic and hydrogen bonding interactions, decreasing the overall free protein concentration in solution. Silica fibers are incorporated into the crystal lattice, improving its mechanical properties and stability against dehydration and without compromising the crystallographic order. The amount of silica precursor (tetramethoxysilane, TMOS) determines the nucleation density: the higher the gel content, the lower the nucleation density.[80] Recently, the use of additives carrying hydrophobic methyl groups has been proposed to overcome such a limitation.[81] As shown in Fig. 5c, methylated silanes incorporated within the gel network reduce the extent of interaction between the protein macromolecules and the hydroxyl groups on the silica, which is the cause of reduced effective protein concentration in the bulk. Micro-crystallization was successfully achieved in modified silica gels. Nevertheless, the gel content also impacts crystal morphology as increasing silica concentration triggers the transition from well-faceted crystals to rounded ellipsoidal-shaped crystals for lysozyme and thaumatin.[80] This finding was attributed to the decrease in the surface free energy of the crystal faces due to the incorporation of silica fibers, leading to the loss of growth anisotropy.

The use of other types of hydrogels has also been reported. Yan *et al.*[21] proposed hydrogels based on poly (ethylene glycol) diacrylate copolymerized with *N*,*N*-dimethylacrylamide, *i.e.*, *p*(PEGDA-*co*-DMAA), for lysozyme and trypsin crystallization. The synergistic regulation of nucleation sites, provided by the wrinkles on the hydrogel surface, and supersaturation, governed by the diffusion of the precipitant through the hydrogel matrix, accelerated the crystallization process and led to good quality crystals. Alternatively, the self-organization of Fmoc-modified peptides based on hydrophobic and π–π stacking has been exploited to produce supramolecular hydrogels.[82] Short-peptide supramolecular hydrogels can be used to generate composite protein crystals, suitable for biotechnological applications. The influence of the type of peptide (cysteine-based derivatives, fluorenylmethoxycarbonyl-diphenylalanine Fmoc-FF, fluorenylmethoxycarbonyl-dialanine Fmoc-AA) and the type of protein (HEWL, thaumatin, recombinant human insulin, glucose isomerase) on crystallogenesis was studied, with particular attention to the stereochemistry of the gel. It was

found that enantiomeric hydrogels have distinct actions on the nucleation behavior of the same protein.[83] Short-peptide supramolecular hydrogels can be employed to modulate the release profile of insulin crystals and enhance biopharmaceutical stability.[84] Alginate hydrogels can also influence protein diffusion and crystallization thanks to their microstructure, which can be altered by inserting ionic liquids. However, ionic liquids can also act as nucleation agents by direct interaction with the protein and may even direct crystal form selection depending on their ions. For example, nitrate-based ionic liquids triggered the formation of monoclinic HEWL crystals, whereas formate-based ionic liquids induced the formation of tetragonal HEWL crystals.[85]

Finally, lipidic cubic phases (LCP) have been also used as matrices for membrane protein crystallization and proved to be effective in supporting the crystallization of bacteriorhodopsin and other difficult-to-crystallize proteins.[86,87] The lipidic three-dimensional network formed by interconnected channels has large surface area and provide numerous nucleation sites. The protein partitions into the hydrophobic bilayer of LCP and, once nucleation is initiated, crystal growth is sustained thanks to the lateral diffusion of protein molecules in the bilayer. The packing arrangement of the lipids determines the obtained crystal habit as a consequence of protein lateral diffusion kinetics.

3.4 Porous nucleants

A variety of porous nucleants has been proposed over the years. The idea of developing a "universal" nucleant has been pursued by using bioactive glass by Chayen *et al.*[88] first. The mesoporous cavities (2–10 nm) could host protein macromolecules and induce nucleation. The mechanism was confinement-based and was not relying on specific interactions but rather on diffusion–adsorption of protein inside the pores. If the pore was narrow enough, proteins tended to adsorb on its walls and the local protein concentration increased. Crystal nucleation can occur even under conditions that would not lead to heterogeneous nucleation as the energy barrier for nucleation is reduced thanks to the combined effect of confinement and interaction energy with the pore walls.[89] Thanks to the high surface area, mesoporous silica has high protein loading capacity and, when saturated with protein, could effectively induce nucleation in the metastable zone.[90] Molecularly imprinted polymers (MIPs) have also been proposed as effective nucleants, which conjugate molecularly selective sites and confinement effects. Advancements have been reviewed in detail by Zhou *et al.*[91]

3.5 Seeding

Crystals of the same protein or a different one may be used as seeds to promote crystal formation by lowering the supersaturation level needed to trigger nucleation and crystal growth. Single-crystals or micro-crystals may be used as seeds, depending on the crystallization goal.[92,93] Caspy *et al.*[94] have recently studied a mixture of heterogeneous protein crystal fragments. These generic seeds are nanometre-sized templates generated by high-speed mixing that can facilitate heteroepitaxial nucleation of proteins unrelated to the seed composition. Natural seeding materials have also been successfully used to facilitate nucleation, including human and horse hair,[95] and dried seaweed powder.[96] It must be noted that, especially in some industrial processes, seeds-mediated crystallization may not be the preferred choice due to sterility requirements, complex seeds production, and/or purity constraints. Hence, alternative nucleation control technologies have been developed based on ultrasound, gassing, and membranes.[27]

3.6 Additives

Additives can also enhance protein nucleation, but their rational design is not yet streamlined. The use of additives is based on supramolecular chemistry and their design should be guided by the understanding of protein-additive interactions. These molecules are located between neighbouring proteins within the crystal lattice, promoting crystal contact among proteins without interfering with their native folding. Because of their action, they are often named as "molecular glues" as they can directly coordinate, form hydrogen bonds, and/or promote hydrophobic interactions with specific amino-acids. The most common additives used for protein crystallization involve polyoxo–metalates,[97,98] macrocycles,[99,100] and lanthanide complexes.[102] Roux *et al.*[103] showed that the substitution of differently charged pendant arms of a lanthanide complex can strongly impact the crystallization process. A propanol pendant arm was best effective in promoting HEWL, proteinase K, and thaumatin crystallization. Molecular dynamics simulations showed that the modified complex can strongly bind to one HEWL molecule and the flexible pendant arm can form weak bonds with different protein surfaces, acting as a molecular lasso. The binding protein affinity was even more enhanced when additives have been modified to covalently bind proteins. Breibeck *et al.*[98] reported that crystal contacts can be stabilized even more efficiently when polyoxometalates were modified to covalently attach to proteinase K during co-crystallization. Ramberg *et al.*[100] reported the use of a highly anionic sulfonato-calixarene for the cocrystallization of *Ralstonia solanacearum* lectin (RSL). Protein nucleation was successfully tuned acting on the pH or the macrocycle concentration, leading to microcrystalline precipitates or mm-sized crystals, see Fig. 6a and b.

Recently, urea, a common protein denaturant when used at high concentrations, has been used to modulate protein–protein interactions and alter HEWL crystallization in presence of salt.[101] On the one hand, urea acts as a non-specific additive, hindering incorrect protein interactions in non-crystalline orientations. Urea also increases protein solubility by weakening attractive interactions. On the other hand, sodium chloride decreases protein solubility and screens electrostatic interactions. Hence, thanks to the fine-tuning of

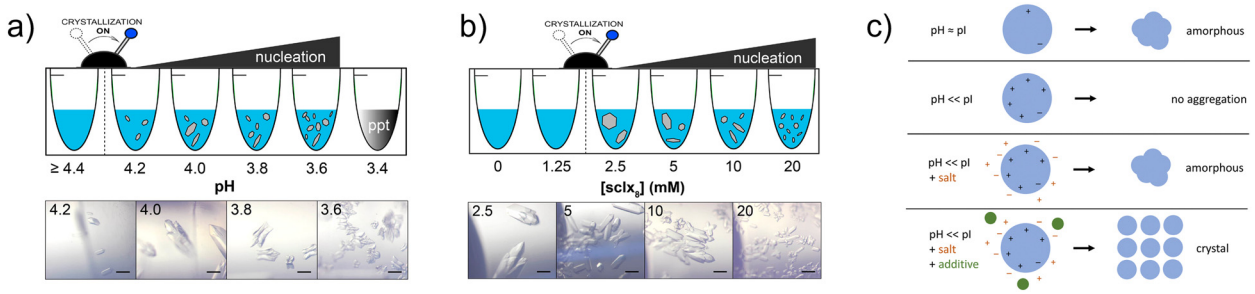

Fig. 6 1 mM 6-bladed β-propeller *Ralstonia solanacearum* lectin (RSL) co-crystallized with sulfonato-calix[8]arene (sclx8) in the absence of precipitant at (a) various pH, 10 mM sclx8 in 20 mM phosphate buffer, and 50 mM NaCl and (b) various sclx8 concentration at pH 4.0. Scale bars are 200 μm. Reprinted with permission.[100] Copyright 2021, American Chemical Society. (c) Schematics of globular protein crystallization in the presence of salts and nonspecific additives. Reprinted with permission.[101] Copyright 2025, Royal Society of Chemistry.

salt and urea concentrations in solution, the balance between attractive and repulsive forces can be modulated in favor of crystallization over amorphous aggregation, as summarized in Fig. 6c.

Additives can also be used to boost protein crystallization after liquid–liquid phase (LLP) separation, following a two-step process.[104] LLP is a metastable phase that, upon addition of 0.1 M HEPES, can rapidly produce tetragonal HEWL crystals even at low salt concentrations (0.1–0.2 M). HEPES can effectively weaken protein–protein interactions, broaden the metastability zone in the protein phase diagram and increase the driving force for nucleation. This ability was independent of the HEPES protonation state, but crystallization was reduced when other molecules sharing functional groups similar to HEPES were used. This suggested that HEPES molecules were bound to HEWL within the lattice.

Protein crystallization can also be promoted by soft templates as amino acids or polymer additives, such as DNA. Single amino acids were used to enhance and control crystallization, as reported for insulin.[105] Their similarity with protein surface chemistry can enhance favorable protein–protein interactions and the formation of nuclei. DNA, instead, has been used because of its highly ordered structure.[106] DNA origami is an effective heteronucleant,[107] and it has been shown that double-stranded DNA favored higher nucleation rates compared to single-stranded DNA. Crystal density was higher when longer sequences were employed.[108]

3.7 Bubbles

The gas–liquid interface represents another opportunity to influence the nucleation process of proteins. This heteronucleant is easy to separate after crystallization is complete and does not introduce impurities in the system. Nevertheless, many processes can simultaneously occur at the gas–liquid interface when a protein solution is involved, *i.e.*, adsorption, unfolding, rearrangements, and their relative contributions are not easily determined. HEWL crystallization was performed *via* HDVD in presence of air bubbles, and the appearance of crystals preferentially occurred on the bubble surface.[109] Successively, gas microbubbles were generated in a microfluidic device to study the impact of different gases on HEWL heterogeneous nucleation.[110] Helium, oxygen, nitrogen, and carbon dioxide were used, and the number of crystals depended on the gas composition, and hence on its solubility and polarizability. The obtained crystals showed curved surfaces due to limitations in crystal growth imposed by the bubbles.

The intentional introduction of bubbles has been implemented to design airlift crystallizers for HEWL crystallization, which allowed for reduced batch time compared to conventional stirred crystallizers.[111] This result was attributed to the reduced induction time for nucleation achieved by the presence of bubbles, which can lower the nucleation energetic barrier.

3.8 Contactless crystallization

Recently, few works investigated protein nucleation limiting the impact of interfaces, such as crystallizer walls or heteronucleants. It was found that externally controlled physical environments can affect the crystallization process of proteins, without the introduction of heteronucleants. Magnetic force boosters have been used to crystallize HEWL while making it levitate in solution without contacting the container.[112] Control over crystal growth was achieved, and the crystals formed a shell-shaped system with a hollow interior. Recently, the movement and the coalescence of nucleation precursors was modified by adding a paramagnetic salt ($MnCl_2$) to the crystallization solution, and the number and location of the nuclei could be controlled in a fully noncontact environment. As shown in Fig. 7, The magnetic field was used to merge all the protein dense liquid phase droplets and form a single nucleus, excluding any influence of interfaces on the process.[113]

4. Emerging trends in protein crystallization

Thanks to tremendous advancements in the understanding of protein crystallization, two major areas in this field have

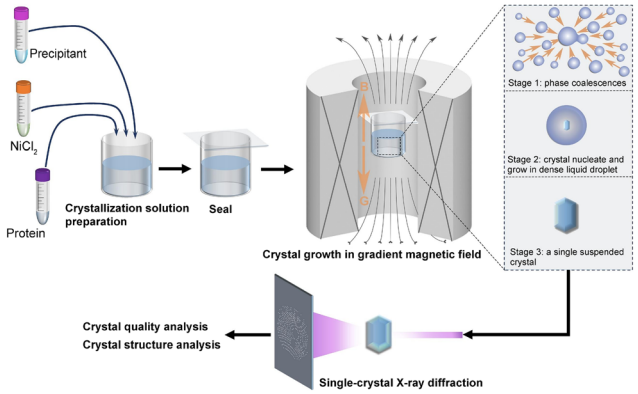

Fig. 7 Schematics of the set-up of contactless crystallization. Reprinted with permission.[113] Copyright 2024, Elsevier Science.

experienced considerable developments in recent years. Emerging trends regarding continuous manufacturing of protein crystals and micro-crystallization to support structural analyses are discussed below.

4.1 Continuous crystallization

Batch crystallization represents the traditional approach applied to proteins. Real-time tracking of the process with focused beam reflectance measurement (FBRM) and thermocouples, coupled to off-line measurement of protein concentration and crystal size, is a valuable tool to follow protein nucleation and crystal growth.[114]

In recent years, interest towards continuous manufacturing has been constantly growing guided by the increased flexibility, robustness, and productivity offered by continuous operations. After reaching steady-state conditions, continuous crystallization yields a product with constant attributes, such as crystal size and habit, facilitating process control and reproducibility. Production capacity has been demonstrated to be higher (g h^{-1}) compared to the batch process.[10] However, shear forces and convective flow significantly impact nucleation and growth. Lack of control over protein nucleation, which is a stochastic process, also complicates the process and its scale-up. At present, nucleation control is still not widely applied to continuous crystallization, but its implementation would be advisable to improve process reproducibility. Only few attempts involving interface-assisted nucleation and external fields have been reported.

Continuous crystallization is generally performed in mixed suspension mixed product removal (MSMPR) crystallizers, or tubular crystallizers. Achieving reproducible product quality attributes (crystal size distribution, crystal quality, crystal number) is critical as many factors may come into play, including mixing efficiency and temperature control.

Depending on the process conditions, *e.g.*, temperature, mixing conditions, presence of interfaces, crystallization may lead to different outcomes. Continuous HEWL crystallization in a MSMPR crystallizer was modelled by kinetic Monte Carlo simulation, in particular as regards nucleation, crystal growth, and dissolution in a fines trap by Kwon *et al.*[115] A model predictive control algorithm was also developed to produce crystals with a target shape distribution by manipulating the crystallizer jacket temperature. The importance of process conditions was also highlighted by Thomas *et al.*,[116] who reported the growth of unusual crystal forms inside MSMPR crystallizers. They obtained a metastable needle-shaped form of HEWL in MSMPR under conditions that would generally lead to the tetragonal form in batch. This was achieved by tuning the operation mode of the crystallizer: reduced attrition and gas–liquid interfaces promoted the metastable form appearance.

Control over nucleation and crystal growth phases is key to ensure reproducible crystallization processes. One common way to achieve this goal is by physically separating nucleation and growth zones. A continuous tubular crystallizer composed of nucleation and crystal growth units was designed by Neugebauer and Khinast.[117] Nucleation and growth were modulated by controlling the local temperature along the crystallizer, and HEWL crystals between 15 and 40 μm were generated. Nevertheless, tubular crystallizers may suffer from long residence times, as laminar flow conditions and low velocity must be guaranteed to minimize protein denaturation, particle attrition, and shear forces, which are detrimental for crystal growth.

Besides traditional crystallizers, emerging approaches based on microfluidics, *i.e.*, slug flow crystallizers (SFC), oscillatory flow crystallizers (OFC), or hybrid membrane crystallization have been proposed for achieving continuous crystallization. For example, continuous OFC has been proposed for the controlled crystallization of HEWL. The Reynolds number, the crystallization time, and the residence time were key parameters in affecting the crystallization outcome. Higher HEWL concentration, together with higher frequency and amplitude of the oscillatory flow, enhanced nucleation, leading to increased number of smaller crystals.[118] SFC have been used to obtain magnetic lysozyme crystallization aided by poly(aspartic acid). The residence time, the process yield, the crystal habit and size have been optimized in SFC set-up by investigating different combinations of gas and liquid velocities.[119]

Lastly, continuous crystallization has also been coupled to external fields. In particular, electric-field assisted protein crystallization has been investigated in continuous flow, offering reduced nucleation induction times. The continuous flow allows high surface-to-volume ratios to design coplanar electrodes featured on millimetre-sized channels. A high number of small-sized crystals could be produced to be used, for example, as seeds.[120]

4.2 Micro-crystallization

X-ray free electron laser (XFEL) technology has guided the development of serial crystallography, which requires small crystals, usually in a few μm-range. Serial synchrotron crystallography (SSX) and serial femto-second crystallography (SFX) are particularly interesting for proteins that cannot be

easily crystallized, as membrane proteins, and allow for room temperature data collection with limited radiation damage.[121–123]

The preparation of a large number of monodisperse microcrystals can equally be as demanding as the growth of a single large protein crystal, and specific techniques must be developed to support their production.[124] Taking into account the limitations on crystal size imposed by the microfluidic delivery system,[125] methodologies based on micro-seeding,[126] FID,[127] or gels[81] have been proposed to fine-tune microcrystal size. Controlled supersaturation in hanging drops was also employed for the rapid formation (30 s–3 min) microcrystals of lysozyme, ferritin, and hemagglutinin.[128] In this case, drops of protein and precipitant solutions were dispensed on coverslips and underwent an initial air-drying step to induce rapid evaporation and then sealed. Single crystals were obtained for short evaporation times, whereas microcrystals appeared at high density for longer evaporation times thanks to the tuning of supersaturation conditions, and hence nucleation.

Nevertheless, scalability must be considered when designing micro-crystallization processes to produce the required volumes of microcrystal suspension for SSX/SFX. In this scenario, batch techniques are the most suitable as they are more easily scalable compared to vapor diffusion-based techniques and crystal density can be adjusted by low-speed centrifugation. Stohrer *et al.*[129] reported that, by fine-tuning the concentration of ammonium sulfate, a large number of monodisperse microcrystals of three different enzymes (*E. coli* l-aspartate α-decarboxylase, *E. coli* copper amine oxidase, *Achromobacter cycloclastes* copper nitrite reductase) could be generated in micro-batch, as shown in Fig. 8a. Microcrystal production was scaled up to a final volume of 2 mL.

Another aspect that is receiving increasing attention is the on-demand generation of microcrystals. Crystals may be freshly prepared just before data collection, eliminating the need of multiple handling steps of protein crystals (crystal freezing, shipping, sample mounting, *etc.*), avoiding crystal ageing, and limiting soaking issues. One way of generating crystals within few seconds is to work with high concentrations of protein and precipitant solutions, as proposed by the JINXED method.[130] The screening of suitable crystallization conditions must be performed upfront and may be time-consuming. Alternatively, additives can be included in the crystallizing system to facilitate nucleation and reduce the precipitant concentration. In this way, microcrystals could be generated not by reducing protein solubility, but rather by enhancing intermolecular contacts among protein molecules. Engilberge *et al.*[102] proposed the use of lanthanide complex (TbXo4 crystallophore) to induce the crystallization of eight model proteins by expanding the nucleation zone in the phase diagram. The crystallophore was also employed for the minute-scale batch production of HEWL nano- or micro-crystals, see Fig. 8b.[131]

As an alternative, fixed-target SX at synchrotrons and XFELs is also a method to determine protein structure. Hit-And-Return (HARE) SX chips do not necessitate a continuous flow of microcrystals, but host randomly oriented microcrystals. Norton-Baker *et al.* reported a crystallization method based on HDVD for the *in situ* production of microcrystals in HARE chips.[132]

5. Conclusions

Protein crystallization is a complex process, where multiple and diverse factors play a role. Among these, interfaces are an intrinsic constituent of the process. Crystallization itself is a process involving the formation of a new solid–liquid interface. Moreover, interfaces can delimit the crystallization environment (air–liquid for HDVD, liquid–liquid for micro-batch under oil, solid–liquid for crystallizers, *etc.*) or they can be intentionally added to the system to guide nucleation (heteronucleants). The understanding of the role of interfaces on protein crystallization guides the rational design of the process and enables control over nucleation phenomena, hence on final crystal size, density, and form. Many different approaches have been proposed to tailor interfaces and guide

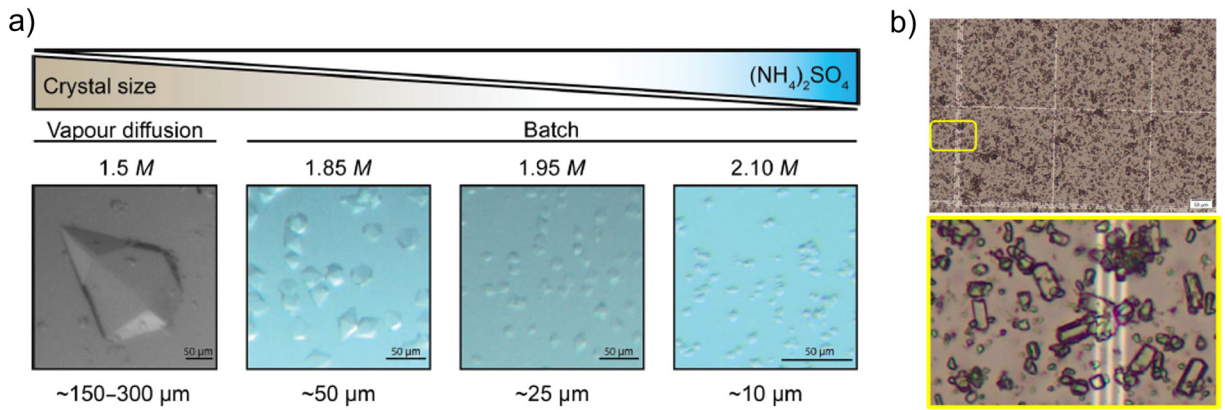

Fig. 8 (a) Crystal size of *E. coli* l-aspartate α-decarboxylase as a function of ammonium sulfate concentration. Reprinted with permission.[129] Copyright 2020, International Union of Crystallography. (b) HEWL microcrystals obtained with lanthanide complexes (TbXo4 crystallophore). Reprinted with permission.[131] Copyright 2024, American Chemical Society.

crystallization, ranging from functionalized surfaces, particles, gels, seeds, porous nucleants, and bubbles. Contactless crystallization has also been studied as an alternative to study unperturbed nucleation. Emerging trends in protein crystallization have also been discussed, focusing on continuous crystallization and micro-crystallization. Interface-assisted nucleation can support further developments in this field, leading to increased understanding of the crystallization phenomenon and more robust and reproducible processes thanks to nucleation control.

Conflicts of interest

There are no conflicts to declare.

Data availability

I declare that no primary research results, software or code have been included and no new data were generated or analysed as part of this Highlight.

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