STRUCTURAL BIOLOGY
COMMUNICATIONS

ISSN 2053-230X

The role of mass transport in protein crystallization

Juan Manuel García-Ruiz,* Fermín Otálora and Alfonso García-Caballero

Laboratorio de Estudios Cristalográficos, Instituto Andaluz de Ciencias de la Tierra, CSIC–Universidad de Granada, Granada, Spain. *Correspondence e-mail: jmgruiz@ugr.es

Received 27 November 2015

Accepted 1 January 2016

Edited by H. M. Einspahr, Lawrenceville, USA

Keywords: mass transport; crystal growth; protein crystallization.

Mass transport takes place within the mesoscopic to macroscopic scale range and plays a key role in crystal growth that may affect the result of the crystallization experiment. The influence of mass transport is different depending on the crystallization technique employed, essentially because each technique reaches supersaturation in its own unique way. In the case of batch experiments, there are some complex phenomena that take place at the interface between solutions upon mixing. These transport instabilities may drastically affect the reproducibility of crystallization experiments, and different outcomes may be obtained depending on whether or not the drop is homogenized. In diffusion experiments with aqueous solutions, evaporation leads to fascinating transport phenomena. When a drop starts to evaporate, there is an increase in concentration near the interface between the drop and the air until a nucleation event eventually takes place. Upon growth, the weight of the floating crystal overcomes the surface tension and the crystal falls to the bottom of the drop. The very growth of the crystal then triggers convective flow and inhomogeneities in supersaturation values in the drop owing to buoyancy of the lighter concentration-depleted solution surrounding the crystal. Finally, the counter-diffusion technique works if, and only if, diffusive mass transport is assured. The technique relies on the propagation of a supersaturation wave that moves across the elongated protein chamber and is the result of the coupling of reaction (crystallization) and diffusion. The goal of this review is to convince protein crystal growers that in spite of the small volume of the typical protein crystallization setup, transport plays a key role in the crystal quality, size and phase in both screening and optimization experiments.

1. Introduction

Mass transport plays a key role in crystal growth. Crystals grow in solution from molecules that must be transported from the bulk of the solution to the interface between the crystal and the solution before they become incorporated into the crystal (Mullin, 1993). This fact is often neglected during laboratory practice either because it is considered to be irrelevant or because the implications for setting up real experiments are not well understood. This paper attempts to provide insights into both misconceptions.

A protein-crystallization project usually involves the screening of a set of crystallization conditions which, if successful, is followed by setting up a number of solutions in an undersaturated state, driving them to a supersaturation state, waiting for nucleation and then growing the crystals to a suitable size and quality. The final target is typically a crystal that is sufficient in size and order to be used for diffraction studies. The fundamental difference among the existing protein-crystallization methods (batch, vapour diffusion, counter-diffusion *etc.*) is the way in which supersaturation is achieved in each, which is caused by differences in terms of mass transport. The relevance of mass-transport processes in protein crystal-growth experiments is introduced in §2, along

© 2016 International Union of Crystallography

with a short discussion on the importance of considering mass-transport issues when designing and setting up actual laboratory experiments. The aspects of mass transport that are relevant to batch experiments and generally to any experiment that involves crystallization-solution preparation are discussed in §3. The homogeneity and behaviour of fluids within crystallizing drops are the subjects of §4. The best-known protein crystal-growth methods with regard to mass transport are counter-diffusion methods. Since there are already several reviews dealing with mass transport using such techniques (Otálora *et al.*, 2009; Biertümpfel *et al.*, 2002; García-Ruiz, 2003; García-Ruiz, Novella *et al.*, 2001; García-Ruiz, Otálora *et al.*, 2001; García-Ruiz *et al.*, 2002; Ng *et al.*, 2003; Otálora & García-Ruiz, 1996, 1997), §5 only summarizes some of the less known features of mass transport in counter-diffusion methods.

2. Why is mass transport important in protein crystallization?

There is no magic in mass transport; it is only a matter of the distribution (space) and evolution (time) of supersaturation within the experimental volume, and this takes place on mesoscopic to macroscopic scales. Consequently, size matters for mass transport. Whereas diffusion is the most efficient mass-transport process at the molecular level, convection is faster on larger scales. All intermolecular interactions are about 32 orders of magnitude stronger than that related to gravity. The Brownian motion velocity of protein molecules is around $3\text{--}30\text{ m s}^{-1}$ at room temperature for molecules with a molecular weight of around $10^4\text{--}10^6\text{ Da}$. These Brownian velocities are orders of magnitude larger than the $0.1\text{--}0.2\text{ mm s}^{-1}$ which may be assumed as a maximum value for bulk gravity-driven terrestrial convection (Fredericks *et al.*, 1994; Kirschhock *et al.*, 2008). Consequently, only (i) the macroscopic mass transport of protein molecules to the growing crystal surface and (ii) the interplay between this transport with their incorporation into the crystal interface provide insight into the influence of mass transport upon growth behaviour or crystal quality.

The incorporation of protein molecules onto the surface of a growing crystal produces a depletion in concentration around the crystal that is replenished by mass transport from the bulk of the solution (Nerad & Shlichta, 1986; McPherson *et al.*, 1999; Vekilov & Chernov, 2003). Consequently, mass transport becomes even more relevant for protein crystallization experiments as the crystal grows larger. The degree of concentration depletion is directly proportional to the growth rate for a fixed mass-transport rate, but the diffusion flow, in turn, is directly proportional to the concentration gradient, as explained by Fick's first law (Vázquez, 2006). Both processes tend to balance each other out since faster incorporation of molecules into the crystal leads immediately to faster transport of molecules to the solution-crystal interface. This results in a given steady-state concentration at the crystal surface that is only dependent on a kinetic coefficient defined by the ratio between the mass-transport rate and the molecular-

attachment kinetics (Chernov, 1984; Otálora *et al.*, 2002). Hence, the supersaturation at which a crystal grows is proportional to this kinetically controlled concentration at the interface.

Crystallization is often used as a method for purification in inorganic and organic chemistry. Impurities are far more frequent in macromolecular solutions than in any other laboratory crystal-growth scenarios and are known to be major contributors to lowering protein crystal quality. It has previously been shown that mass transport plays an important role in the amount and distribution of impurities within the crystal (Carter *et al.*, 1999; Chernov, 2003; Lin *et al.*, 2001). Indeed, impurities may be transported either towards the crystal or away from it. The currently accepted model for impurity transport and incorporation, known as 'diffusional purification' (Lee & Chernov, 2002), proposes the development of an impurity-depletion zone around the growing crystal and describes how this zone can work as a purification step during crystallization.

Once the concentration-depletion zone has been established, the crystal-growth process reaches a quasi-steady state. Both the supersaturation and the concentration of impurities at the crystal surface will remain constant during the growth phase, provided that mass transport is also steady! When there is convective mass transport the solution that is carried to the crystal surface has a different composition to that corresponding to the steady state. In this case, the amount of impurities that become trapped in the crystal can change suddenly (Chernov, 2003), and crystal defects may appear either owing to a large concentration of impurities or because of the accumulated strain caused by changes in the equilibrium lattice parameters as a function of the solution composition. Mass transport is responsible for maintaining or distorting the steady state of crystal growth and in doing so can be the origin of crystal defects in the growing crystal.

On even larger scales, mass transport produces long-range gradients that have been skilfully used in counter-diffusion methods to select and optimize the nucleation and growth conditions. These long-range gradients produce a spatial separation of supersaturation along the elongated reservoir containing the protein solution (typically a capillary), with a moving maximum of supersaturation that runs across the protein chamber with decreasing amplitude and increasing width (García-Ruiz, Otálora *et al.*, 2001). At the same time, as diffusion gradients decrease over time, the point of maximum supersaturation grows at a slower rate, producing nucleation events at supersaturation values that are gradually smaller. This complex, self-organized distribution of nucleation and growth conditions is entirely driven by diffusion and illustrates the importance of controlling mass transport to optimize crystallization experiments.

3. The role of mixing in making a drop: batch experiments

Mixing solutions for crystallization experiments, either to set up a batch experiment or as a preliminary step for vapour-

diffusion experiments, is the first step in most protein crystallization experiments. These solutions usually have different densities, and the compounds in solution have different diffusion coefficients. Depending on the relative solution properties and on the sequence of mixing (denser over lighter or *vice versa*), characteristic complex and defined patterns may develop (Lima & De Wit, 2004; Howard *et al.*, 2009). In most cases the mixing process is assumed to be instantaneous, which would result in uniform values of supersaturation, pH and the concentrations of reactants and additives. However, the mixing of solutions is not immediate, and even microlitre-sized drops cannot be considered to be homogeneous (Savino &

Monti, 1996). Howard *et al.* (2009) studied the mixing of precipitant and protein solutions by optical microscopy and Mach–Zehnder interferometry, and found that complex phenomena occur at the interface between the two solutions upon mixing. These inherently chaotic processes could explain why and how the mixing protocol may affect the reproducibility of crystallization experiments (Newman *et al.*, 2007). Two general situations, illustrated in Fig. 1, can be considered.

(i) When the denser solution is poured over the lighter one (middle row in Fig. 1), the system is gravitationally unstable and Rayleigh–Taylor convection will develop owing to buoyancy forces.

Figure 1

Mixing experiments using two solutions (hen egg-white lysozyme and NaCl) at different concentrations. The left-hand side sketches the concentration (a) and density (b) of the two solutions at different times, from just after dropping the top solution (black lines) to a relatively advanced homogenization state (pink lines). The diagrams in the left column show situations in which the salt (diffusing faster) is on top, while those in the right column show the reverse situation with lysozyme on top. (b) is composed of four diagrams representing solution-density plots for solutions mixed by pouring the denser solution on top of the lighter one (top) and the lighter solution on top of the denser one (bottom). (c) shows three interferograms collected from five selected mixing experiments. The concentrations of salt [in % (w/w)] and lysozyme (Lyso; in mg ml⁻¹) are indicated to the right of each row. The solution composition shown in bold indicates the denser solution. The three columns correspond to three different times of the experiments: immediately after pouring the top solution, 2 s after mixing and 4 s later. The experiment illustrated by the top row of pictures shows the development of a relatively stable diffusion front when the denser and also slower solution is on the bottom. The second row shows the fast, convective (Rayleigh–Taylor) mixing of solutions when the denser solution is poured on top of the lighter one. The third and fourth rows show the development of solution fingering by double-diffusion instability. The mixing is faster for smaller density differences (third row) than for larger differences (fourth row). The fifth row shows a high-supersaturation experiment where the mixing is driven by the combination of two effects: double diffusion and the sedimentation of crystals produced upon mixing.

(ii) When the lighter solution is poured over the denser one (lower row in Fig. 1) the system should be stable. However, the diffusion that takes place at the mixing front triggers the development of physical instabilities that destabilize it because of the large difference in the diffusion coefficients of the precipitant and the protein. The protein diffusivity is typically one order of magnitude smaller than that of salts, so just after mixing, at the interface, the salt molecules travel to the protein solution faster than the protein molecules towards the salt solution (upper row in Fig. 1). This leads to maxima and minima of local density of the salt solution close to the interface, where some droplets of denser solution start falling.

These phenomena affect the homogenization time and the precipitation behaviour, which may influence the result of the crystallization experiment in many practical cases. Under a convective environment there are different local supersaturation values across the solution, which are expected to be particularly high at the interface between the falling and rising droplets. This particularly affects the reproducibility of the experiments, which can yield different results depending on the relative density and diffusivity of the solutions and their components. This is also the case when scaling up the screening experiments, because changing the size of the solution droplets also changes the Reynolds number and therefore the fluid flow regime. The rate of homogenization also depends on the mixing mechanism, being faster when Rayleigh–Taylor convection develops and slower when mixing

is dominated by diffusion or double diffusion. The number of crystals and their size may depend on this rate of homogenization (Fig. 2).

In practical terms, when seeking reproducibility faster homogenization is beneficial and it would then be better to add the denser solution over the lighter one. However, the reverse mixing order may be more advantageous for screening a wider area of the solubility diagram because diffusion or fingering may yield a variable ratio of protein and reactive agent concentrations.

All of the mixing mechanisms observed in these experiments are consequences of natural convection, where the only external force is gravity. Nonetheless, the use of forced convection is also frequently employed in protein crystallization when solutions are mixed using a pipette, aspirating the solution up and down several times (Rupp, 2009). This, however, is very difficult to achieve using automated crystallization robots, so it is practically restricted to manual experiments. The use of forced convection to accelerate mixing is a good option in terms of reproducibility, but even in this case it must be considered that transient, local fluctuations of supersaturation may still develop. Just after injecting the solutions the supersaturation value at the protein–precipitant interface can be several times higher than anticipated after full homogenization. When the overall supersaturation value is sufficiently high nucleation may still take place before the mixing is complete, leading to an irreproducible number of crystals (owing to a high initial nucleation rate) that will be strongly dependent on the velocity of injection, the volume of the two solutions being mixed and even on the geometry of the experimental setup (Chernov, 2003).

At very high supersaturation values, another effect must also be taken into account: the mixing induced by gravity-driven sedimentation of the crystals or amorphous precipitates forming at the interface. This occurs both at the mixing interface and in the moving droplets formed by double diffusion. In both cases the net effect is either distortion or destruction of the depletion gradient around the crystals (Otálora *et al.*, 2001) and acceleration of mixing (Howard *et al.*, 2009).

4. Mass transport in vapour-diffusion experiments

The vapour-diffusion method, an elegant variation of the evaporation method, is the technique preferred by most molecular biologists and crystallographers for the crystallization of macromolecular compounds (McPherson, 1999; Ducruix & Giegé, 1999). In its most classical laboratory implementation, a hanging drop containing the protein and precipitant is equilibrated against a reservoir typically containing double the concentration of precipitant (Bergfors, 2009). Equilibration following Raoult's law provokes the transport of water molecules *via* vapour diffusion from the hanging drop towards the solution placed in the reservoir, thereby increasing the concentration of protein and salt in the drop continuously over time (Shu *et al.*, 1998; Diller & Hol, 1999). By choosing the appropriate starting conditions, the

Figure 2

Density differences between lysozyme and NaCl solutions as a function of their concentration values. The reddish colours on the left indicate areas where the protein solution is denser. Bluish colours indicate regions where the density of the salt solution is higher. White indicates no difference. The colour saturation is proportional to the density difference (*i.e.* the buoyancy force). The diagonal straight line is the isodensity line. The curve to the right is the solubility curve, so solutions to the left of the curve are supersaturated. The dominant mass-transport process is indicated in each region with two alternatives where needed depending on how the experiment has been prepared: the salt (s) on top of the protein (p) or *vice versa*. The position of the denser solution is indicated in grey. The conditions for the experiments shown in Fig. 1(c) are also indicated by white circles (if the lighter solution is put on top) or grey circles (if the denser solution is put on top). The label '1c1' correspond to the top row of Fig. 1(c) and the label '1c5' to the bottom row.

concentration of protein reaches the critical concentration for nucleation and crystalline nuclei start to grow and form crystals ready for X-ray structural studies.

It is currently assumed that the volume of the drop consists of a homogeneous solution because of its small volume, which is typically less than 20 μl (Schwartz & Berglund, 2000). To test this assumption, we have studied the time evolution of a hanging-drop experiment by optical interferometry. Solutions of NaCl at three different concentrations (4.0, 4.4 and 4.8 M) were prepared in distilled water (10 ml). A drop (10 μl) of each of the above solutions was placed in a spectrophotometric cell with an internal depth (in the direction of the laser beam) of 1.0 mm and was allowed to evaporate for 12 h. During this time, interferograms were recorded at periodic intervals (10 s).

Fig. 3 shows four selected frames from the interferometry video illustrating the phenomena occurring during the evaporation of the drop. Before nucleation (Fig. 3*a*), the evaporation of the drop results in an increase in concentration near the drop–air interface (red/yellow rim in the image). Even at this early stage, some degree of buoyancy convection is present in the drop, which produces the asymmetry between

the top and the bottom along the rim. After 5 h of evaporation (Fig. 3*b*) the size of the drop has shrunk quite significantly, with a corresponding increase in supersaturation. At this point, a nucleation event takes place around the rim of the drop on the cover slip and falls to the bottom of the drop by gravity. The consumption of ions by the crystal-growth process produces a depletion in concentration (deep blue regions), leading to density gradients and the incipient formation of a convection plume above the crystal. Further depletion of solute during crystal growth (Fig. 3*c*) produces a clear and relatively steady convection plume in which the lighter, solute-depleted solution rises above the crystal while the denser solution enriched in solute falls over the left and right sides of the drop.

The growth of the crystal has two main effects on the homogenization of the drop. The first and most obvious effect is the formation of a convective flow of solution owing to buoyancy that is present even at the last stages of crystal growth (Fig. 3*d*). The second effect is an inhomogeneous distribution of supersaturation at the crystal surface. Since diffusion in a fluid is an isotropic phenomenon, the depletion gradient around the crystal is almost spherical, while the

Figure 3
Snapshots of the convection plume of a drop with an initial concentration of 4.8 M NaCl.

crystal itself is a polyhedron. This results in a higher concentration (supersaturation) at the corners of the crystal than at the centre of the faces, which can be observed in Fig. 3(d), where the corners are green and yellow whereas the centres of the faces are deep blue. This difference in concentration is quite significant and leads to an increased growth rate at the corners that can eventually produce morphological instabilities, the so-called Berg effect (Berg, 1938), resulting in concave faces, hopper crystals or even dendritic crystals.

Owing to sedimentation by gravity, the crystal tends to grow at the bottom of the drop. This sedimentation, as shown in Fig. 4, is an additional contribution to convective transport in the drop. The crystals that fall (only one is shown in this example for clarity) produce advective fluid flow similar to the effect of tiny stirrers.

One of the drops studied contained a small, insoluble particle that was moving inside it. This particle was used as a marker to determine mean fluid velocities. The velocity was measured both at the bottom of the convection plume (horizontal velocity) and on the side of the plume (vertical velocity). The mean velocity was found to be 0.16 mm s^{-1} in the vertical direction and 0.11 mm s^{-1} in the horizontal direction. This corresponds to Reynolds numbers $Re_v = 0.48$ and $Re_h = 0.33$ (density of water 0.998 g cm^{-3} , diameter of the drop 3.0 mm , dynamic viscosity of water 1002 Pa s). For Reynolds numbers lower than 1, laminar flow takes place. As Figs. 3(c) and 3(d) clearly show, both convection and diffusion operate simultaneously during crystal growth even in micro-litre volumes. The relative development of each of them, convection and diffusion, will define the overall flow regime in

the experiment and the degree of homogenization and steadiness of supersaturation during the growth of the crystals. The relative importance of diffusion and convection is defined in fluid dynamics using dimensionless numbers such as the Grashoff number Gr_N , which gives an account of the ratio between the buoyant forces and the viscous drag forces,

$$Gr_N = L^3 \cdot \alpha \cdot \Delta c \cdot g \cdot \nu^{-2}, \quad (1)$$

where g is the gravity acceleration, ν is the kinematic viscosity, α is the volume expansion coefficient and L is the characteristic dimension of the container. From this equation, it is easily deduced that buoyancy motion can be reduced by lowering the value of g or L , by increasing the viscosity of the solution and by locating the growing crystal in the upper part of the crystallizing solution. It is clear from the Grashoff expression that several techniques can be applied to reduce convection, namely the use of gels (Cudney *et al.*, 1994; Otálora *et al.*, 2009; Lorber *et al.*, 2009; García-Ruiz, 2009; Moreno & Mendoza, 2015), the use of capillaries (Ng *et al.*, 2003; Otálora *et al.*, 2009) or microfluidic chips (Stevens, 2000; Zheng *et al.*, 2003; Squires & Quake, 2005), performing the crystallization in space under low-gravity conditions (Ng, 2002; Lorber, 2002; Snell & Helliwell, 2005; Otálora *et al.*, 2009) and the use of a thickener to increase the viscosity (Lavalette *et al.*, 1999).

5. Mass transport in counter-diffusion methods

The previous sections have referred to mass transport as something that can potentially spoil an experiment in cases where reproducibility, nucleation rate or disorder induced by

Figure 4

Interferograms collected during an NaCl crystallization experiment using the hanging-drop method. The image in (a) was collected right after the nucleation of the crystal close to the drop meniscus. When the crystal reaches a large enough size it falls along the drop surface (b, c) and finally settles at the bottom of the drop (d), developing a convection plume as shown in Fig. 3

growth-rate fluctuations or impurities is an issue. Nonetheless, mass transport, in particular diffusive mass transport, can also be used to design and implement crystal-growth methods with distinct advantages. Counter-diffusion methods are the best-known example of this use of mass transport. There are several reviews of counter-diffusion methods (Otálora *et al.*,

2009; Biertümpfel *et al.*, 2002; García-Ruiz, 2003; García-Ruiz, Novella *et al.*, 2001; García-Ruiz, Otálora *et al.*, 2001; García-Ruiz *et al.*, 2002; Ng *et al.*, 2003; Otálora & García-Ruiz, 1996, 1997). This section will only highlight how mass transport defines the most relevant advantages of the method and how they affect the formation of crystals.

Counter-diffusion methods are based on mixing two solutions by slow diffusion in opposite directions. In the case of protein crystal growth, these solutions are typically a buffered protein solution and a precipitant solution. The concentration of both protein and precipitant must be sufficiently high to yield a supersaturated solution if mixed. Since protein molecules typically diffuse an order of magnitude more slowly than common precipitants, the concentration of precipitant in the volume initially containing the protein increases at a faster rate than the reduction of concentration of protein in the same volume. This produces a maximum of supersaturation at some point within the protein volume, eventually generating a nucleation event (García-Ruiz, Otálora *et al.*, 2001). This first nucleation occurs typically at a very high value of supersaturation and nucleation rate, because both the concentration of protein and the rate of precipitant concentration increase are high. Soon after nucleation, the protein concentration at the points where crystals have formed falls to the equilibrium value (solubility) and the maximum of supersaturation moves towards the protein side and continues to increase until eventually further nucleation events are triggered (García-Ruiz *et al.*, 1999).

The overall behaviour is shown in Fig. 5. The key points of this process are the following.

(i) The maximum value of supersaturation moves along the one-dimensional space owing to the difference in diffusion coefficients and the equilibration of protein concentration around the location where crystals appear. Further nucleation events are most likely to take place at this maximum, so the time evolution of the crystallization sequence is resolved: the later the nucleation, the further from the initial protein–precipitant interface.

(ii) The diffusive gradients diminish over time, as does the diffusive flow proportionally to the gradient. This means that the rate of supersaturation decreases with time. Therefore, further nucleation events occur at lower nucleation rates.

(iii) The protein:precipitant ratio is different on both sides of the supersaturation maximum, and its absolute value is different at various parts of the experiment, so different chemical conditions are screened and automatically optimized. There will be crystals growing at (or close to) the optimal conditions.

(iv) More complex experiments may be designed by including other species that are different from the protein and the precipitant. For instance, allowing a buffer to counter-diffuse could be the most efficient way of performing a pH screening.

There are examples of full screening kits that have been developed for counter-diffusion methods, the main advantage being that screening and optimization are performed in a single experiment. An interesting feature is that the screening

Figure 5

Evolution of a counter-diffusion crystallization experiment of lysozyme using agarose gel over time. The pictures show, from top to bottom, the experiment after $t = 0.00, 1.65, 3.53, 9.60, 21.90$ and 52.65 h. The pictures only show the protein reservoir; the reservoir containing the precipitant (not shown) is on the left and the salt diffuses from the left side. The high supersaturation at the interface between the two solutions produces a massive precipitation of amorphous or microcrystalline protein (left side of the second image). This precipitation and the subsequent relaxation of the diffusive gradients lead to a nucleation front moving from left to right as the maximum supersaturation moves in this direction. The number of crystals nucleated decreases with time and in the right quarter of the plate only a few crystals are present that feature clear concentration-depletion zones around them.

is not limited to the precipitating agent or additives. For instance, one can play with pH too, with allowing a concentrated buffer, an acidic solution or an alkaline solution to counter-diffuse against a protein solution being the most efficient way of performing a pH screening. It has also been demonstrated that crystals grown in capillary tubes can be used to collect X-ray diffraction data without further manipulation by allowing a cryoprotectant to diffuse and then freezing the capillary, which also minimizes crystal handling (Gavira *et al.*, 2002; Ng *et al.*, 2003).

Acknowledgements

We would like to acknowledge Dr Luis David Patiño and Dr Maria Luisa Novella for their assistance in obtaining interferometry data. We also acknowledge the financial support of the Ministerio de Economía y Competitividad of Spain for the Project Consolider-Ingenio CSD2006-00015 'Factoría de Cristalización', as well as Junta de Andalucía for support of the Project RNM 5384 'Tecnología Cristalográfica: contribuyendo al desarrollo socioeconómico en y desde Andalucía'.

References

- Berg, W. F. (1938). *Crystal growth from solutions*. *Proc. R. Soc. Lond. Ser. A*, **164**, 79–95.
- Bergfors, T. M. (2009). *Protein Crystallization*, 2nd ed. La Jolla: International University Line.
- Biertümpfel, C., Basquin, J., Suck, D. & Sauter, C. (2002). *Crystallization of biological macromolecules using agarose gel*. *Acta Cryst.* **D58**, 1657–1659.
- Carter, D. C., Lim, K., Ho, J. X., Wright, B. S., Twigg, P. D., Miller, T. Y., Chapman, J., Keeling, K., Ruble, J., Vekilov, P. G., Thomas, B. R., Rosenberger, F. & Chernov, A. A. (1999). *Lower dimer impurity incorporation may result in higher perfection of HEWL crystals grown in microgravity*. *J. Cryst. Growth*, **196**, 623–637.
- Chernov, A. A. (1984). *Modern Crystallography III – Crystal Growth*. Berlin, Heidelberg: Springer-Verlag.
- Chernov, A. A. (2003). *Protein crystals and their growth*. *J. Struct. Biol.* **142**, 3–21.
- Cudney, R., Patel, S. & McPherson, A. (1994). *Crystallization of macromolecules in silica gels*. *Acta Cryst.* **D50**, 479–483.
- Diller, D. J. & Hol, W. G. J. (1999). *An accurate numerical model for calculating the equilibration rate of a hanging-drop experiment*. *Acta Cryst.* **D55**, 656–663.
- Ducruix, A. & Giegé, R. (1999). *Crystallization of Nucleic Acids and Proteins: A Practical Approach*. Oxford University Press.
- Fredericks, W. J., Hammonds, M. C., Howard, S. B. & Rosenberger, F. (1994). *Density, thermal expansivity, viscosity and refractive index of lysozyme solutions at crystal growth concentrations*. *J. Cryst. Growth*, **141**, 183–192.
- García-Ruiz, J. M. (2003). *Counterdiffusion methods for macromolecular crystallization*. *Methods Enzymol.* **368**, 130–154.
- García-Ruiz, J. M. (2009). *Protein Crystallization*, 2nd ed., edited by T. Bergfors, p. 83. La Jolla: International University Line.
- García-Ruiz, J. M., González-Ramírez, L. A., Gavira, J. A. & Otálora, F. (2002). *Granada crystallisation box: a new device for protein crystallisation by counter-diffusion techniques*. *Acta Cryst.* **D58**, 1638–1642.
- García-Ruiz, J. M., Novella, M. L., Moreno, R. & Gavira, J. A. (2001). *Agarose as crystallization media for proteins*. *J. Cryst. Growth*, **232**, 165–172.
- García-Ruiz, J. M., Novella, M. L. & Otálora, F. (1999). *Supersaturation patterns in counter-diffusion crystallisation methods followed by Mach–Zehnder interferometry*. *J. Cryst. Growth*, **196**, 703–710.
- García-Ruiz, J. M., Otálora, F., Novella, M. L., Gavira, J. A., Sauter, C. & Vidal, O. (2001). *A supersaturation wave of protein crystallization*. *J. Cryst. Growth*, **232**, 149–155.
- Gavira, J. A., Toh, D., López-Jaramillo, J., García-Ruiz, J. M. & Ng, J. D. (2002). *Ab initio crystallographic structure determination of insulin from protein to electron density without crystal handling*. *Acta Cryst.* **D58**, 1147–1154.
- Howard, E. I., Fernandez, J. M. & García-Ruiz, J. M. (2009). *On the mixing of protein crystallization cocktails*. *Cryst. Growth Des.* **9**, 2707–2712.
- Kirschhock, C. E. A., Otálora, F., Piazza, R. & Mazzoni, S. (2008). *Aggregation and crystallisation in space*. *Europhys. News*, **39**, 25–27.
- Lavalette, D., Tétreau, C., Tourbez, M. & Blouquit, Y. (1999). *Microscopic viscosity and rotational diffusion of proteins in a macromolecular environment*. *Biophys. J.* **76**, 2744–2755.
- Lee, C. P. & Chernov, A. A. (2002). *Solutal convection around growing protein crystals and diffusional purification in space*. *J. Cryst. Growth*, **240**, 531–544.
- Lima, D. & De Wit, A. (2004). *Convective instability in protein crystal growth*. *Phys. Rev. E*, **70**, 021603.
- Lin, H., Petsev, D. N., Yau, S.-T., Thomas, B. R. & Vekilov, P. G. (2001). *Lower incorporation of impurities in ferritin crystals by suppression of convection: modeling results*. *Cryst. Growth Des.* **1**, 73–79.
- Lorber, B. (2002). *The crystallization of biological macromolecules under microgravity: a way to more accurate three-dimensional structures?* *Biochim. Biophys. Acta*, **1599**, 1–8.
- Lorber, B., Sauter, C., Théobald-Dietrich, A., Moreno, A., Schellenberger, P., Robert, M.-C., Capelle, B., Sanglier, S., Potier, N. & Giegé, R. (2009). *Crystal growth of proteins, nucleic acids, and viruses in gels*. *Prog. Biophys. Mol. Biol.* **101**, 13–25.
- McPherson, A. (1999). *Crystallization of Biological Macromolecules*. New York: Cold Spring Harbor Laboratory Press.
- McPherson, A., Malkin, A. J., Kuznetsov, Y. G., Koszelak, S., Wells, M., Jenkins, G., Howard, J. & Lawson, G. (1999). *The effects of microgravity on protein crystallization: evidence for concentration gradients around growing crystals*. *J. Cryst. Growth*, **196**, 572–586.
- Moreno, A. & Mendoza, M. E. (2015). *Handbook of Crystal Growth*, Vol. 2, edited by P. Rudolph, pp. 1277–1315. New York: Elsevier.
- Mullin, J. W. (1993). *Crystallization*, 3rd ed. Oxford: Butterworth-Heinemann.
- Nerad, B. A. & Shlichta, P. J. (1986). *Ground-based experiments on the minimization of convection during the growth of crystals from solution*. *J. Cryst. Growth*, **75**, 591–608.
- Newman, J., Xu, J. & Willis, M. C. (2007). *Initial evaluations of the reproducibility of vapor-diffusion crystallization*. *Acta Cryst.* **D63**, 826–832.
- Ng, J. (2002). *Space-grown protein crystals are more useful for structure determination*. *Ann. N. Y. Acad. Sci.* **974**, 598–609.
- Ng, J. D., Gavira, J. A. & García-Ruiz, J. M. (2003). *Protein crystallization by capillary counterdiffusion for applied crystallographic structure determination*. *J. Struct. Biol.* **142**, 218–231.
- Otálora, F. & García-Ruiz, J. M. (1996). *Computer model of the diffusion/reaction interplay in the gel acupuncture method*. *J. Cryst. Growth*, **169**, 361–367.
- Otálora, F. & García-Ruiz, J. M. (1997). *Crystal growth studies in microgravity with the APCF. I. Computer simulation of transport dynamics*. *J. Cryst. Growth*, **182**, 141–154.
- Otálora, F., García-Ruiz, J. M., Carotenuto, L., Castagnolo, D., Novella, M. L. & Chernov, A. A. (2002). *Lysozyme crystal growth kinetics in microgravity*. *Acta Cryst.* **D58**, 1681–1689.
- Otálora, F., Gavira, J. A., Ng, J. D. & García-Ruiz, J. M. (2009). *Counterdiffusion methods applied to protein crystallization*. *Prog. Biophys. Mol. Biol.* **101**, 26–37.
- Otálora, F., Novella, M. L., Gavira, J. A., Thomas, B. R. & García-Ruiz, J. M. (2001). *Experimental evidence for the stability of the*

- depletion zone around a growing protein crystal under microgravity. *Acta Cryst.* **D57**, 412–417.
- Rupp, B. (2009). *Biomolecular Crystallography: Principles, Practice, and Application to Structural Biology*. New York: Garland.
- Savino, R. & Monti, R. (1996). Buoyancy and surface-tension-driven convection in hanging-drop protein crystallizer. *J. Cryst. Growth*, **165**, 308–318.
- Schwartz, M. & Berglund, K. A. (2000). In situ monitoring and control of lysozyme concentration during crystallization in a hanging drop. *J. Cryst. Growth*, **210**, 753–760.
- Shu, Z.-Y., Gong, H.-Y. & Bi, R.-C. (1998). In situ measurement and dynamic control of the evaporation rate in vapor diffusion crystallization of proteins. *J. Cryst. Growth*, **192**, 282–289.
- Snell, E. H. & Helliwell, J. R. (2005). Macromolecular crystallization in microgravity. *Rep. Prog. Phys.* **68**, 799–853.
- Squires, T. M. & Quake, S. R. (2005). Microfluidics: fluid physics at the nanoliter scale. *Rev. Mod. Phys.* **77**, 977–1026.
- Stevens, R. C. (2000). High-throughput protein crystallization. *Curr. Opin. Struct. Biol.* **10**, 558–563.
- Vázquez, J. L. (2006). *The Porous Medium Equation: Mathematical Theory*. Oxford University Press.
- Vekilov, P. G. & Chernov, A. A. (2003). The physics of protein crystallization. *Solid State Phys.* **57**, 1–147.
- Zheng, B., Roach, L.-S. & Ismagilov, R. F. (2003). Screening of protein crystallization conditions on a microfluidic chip using nanoliter-size droplets. *J. Am. Chem. Soc.* **125**, 11170–11171.