4.1.3.5. Free-interface and counter-diffusion methods

In these methods, equilibration occurs by direct diffusion of the crystallant into the protein solution (Salemme, 1972; Garcia-Ruiz & Moreno, 1994). Both methods require minimal convection and, therefore, experiments are conducted in capillaries (Fig. 4.1.3.1*d*,*e*). To avoid too rapid mixing in a free-interface diffusion assay, the less dense solution is poured gently on the densest one. One can also freeze the solution with the crystallant and layer the protein solution on top. The components of each solution in contact will then diffuse into the other compartment.

A more versatile version of this technique is counter-diffusion crystallization (Garcia-Ruiz, 2003; Ng *et al.*, 2003; Otálora *et al.*, 2009), which was referred to as the 'gel acupuncture' method in its first version (Garcia-Ruiz & Moreno, 1994). In a typical experiment, a gel base is formed from agarose or silica in a small container, and a concentrated crystallant solution is poured over its surface. This crystallizing agent permeates the gel by diffusion, forming a gradient. A capillary filled with the protein is inserted into the gel. The crystallant then enters the capillary from the gel and forms an upward gradient in the capillary, promoting crystallization along its length as it rises by pure diffusion. The method operates with a variety of gels and crystallants, with different heights of these agents over the gel and with open or sealed capillaries. It has already been successfully used to crystallize a variety of macromolecules, some of very large size (Garcia-Ruiz *et al.*, 1998; Biertümpfel *et al.*, 2002; Kutá Smatanová *et al.*, 2006).

The main advantage of the counter-diffusion technique is that a wide range of supersaturation conditions is tested in a single experiment. While a precipitate may form at the entrance of the capillary at high supersaturation, monocrystals may grow at the opposite end where supersaturation is lower. A second advantage is that all steps from crystallization to structure solution, including substrate/cryoprotectant/heavy atoms soaking and X-ray analysis can be performed without any crystal handling (Gavira *et al.*, 2002). In recent versions, the diameter and length of counter-diffusion capillaries have been reduced so that assays can be conducted at a 300 nl scale (see Section 4.1.4.1.).

4.1.3.6. Miniaturization, automation and robotics

The first user-friendly attempt to miniaturize and automate crystallization concerned batch crystallization in microdroplets under oil (Chayen *et al.*, 1990). In recent years, robots and other automated instruments, and entire integrated systems, have been developed to accelerate the crystallization process (DeLucas *et al.*, 2003; Hosfield *et al.*, 2003; Luft *et al.*, 2003; Bard *et al.*, 2004; Berry *et al.*, 2006) and the optimization procedures (Newman *et al.*, 2008). They have the capacity to screen thousands of crystallization conditions, and they do so precisely and reliably, with fewer errors and better record keeping than most humans. In many large laboratories, these have become essential pieces of equipment. Using standard, usually commercially available, screening kits, sometimes supplemented by local preferences or family-directed screens, they can often arrive at acceptable crystals in the most expeditious manner possible.

Robotic systems are efficient, tireless and accurate, but they offer another important feature in addition. They can carry out experiments using drop samples of very small volume, drops of 1 µl in most cases, nanolitres in some. This, in turn, produces a requirement for automated, microphotographic visualization instruments, and complex storage and handling systems, and their associated expense. On the other hand, a great advantage emerges in that they can perform enormous numbers of crystallization trials using remarkably little biological sample. This, in turn, relieves the investigator of a significant burden in terms of preparing and purifying macromolecules.

Many of the robotic systems are based on reproducing procedures currently used for manual experiments, such as sitting and hanging drops, and microdrops under oil. They are simply carried out on a much smaller scale. More recently, however, even more miniaturized devices have come on the market. These use what is now commonly called nanotechnology to manipulate small amounts of liquids and fluid streams. These devices are only now seeing rigorous evaluation in laboratories, but they clearly show great promise for the future (see Section 4.1.4). Another effort is underway to develop robotic systems for crystal harvesting and crystallization devices that will allow direct X-ray exposure of crystals where they are grown *in situ* (Viola *et al.*, 2007). These would obviate the need for careful mounting, an often-problematic aspect of data collection.

4.1.4. Advanced crystallization methodologies

In methods that manipulate physical parameters, the effects on crystallization are manifold. Among others, they may influence fluid properties in the crystallization media and movement of molecules (gravity), alter the conformation of the macromolecule (pressure), orient crystals (magnetic field), or influence nucleation (electric field). Thus, initiation of crystallization may be triggered by various mechanisms, growth may be differently influenced and, in favourable cases, crystal quality improved.

4.1.4.1. Crystallization in convection-free media

Theoretical considerations. When a crystal starts to grow, it attracts surrounding molecules and creates a concentration gradient. Since crystallization occurs on earth in the gravity field, this gradient of concentration and density will lead to convective currents in the mother liquor. In addition, as soon as the crystal becomes big enough, it will sink to the bottom of the solution. Convection and sedimentation almost always take place in classical experimental setups and they almost certainly influence crystallization processes. Their contribution would be drastically reduced in the absence of gravity, as occurs in weightlessness, and the theory predicts more regular crystal growth under a microgravity–diffusive regime that should favour enhanced crystal quality. Such considerations have justified space-crystallization programmes and, as a consequence, have contributed to a deeper understanding of the crystallization process of biomacromolecules (Giegé *et al.*, 1995; McPherson, 1996; Kundrot *et al.*, 2001). However, because of limited access to space experimentation, crystallization in weightlessness will never be user friendly. This has stimulated studies for finding easy ways to simulate microgravity conditions in the laboratory. Such methods, where crystal growth is less dependent on convection and more on diffusion, take advantage of gelled media and microfluidic environments.

Use of microgravity. The first observation in microgravity was that the absence of sedimentation permits the growth of individual crystals in suspension, without any perturbation by contact with vessel walls and neighbouring crystals. However, one should bear in mind that even in microgravity small accelerations can occur owing to vehicle movement and crystal displacement has been recorded (*e.g.* Lorber *et al.*, 2000). Microgravity experiments require specific instrumentation with dedicated reactors based on current batch, dialysis and vapour-diffusion methods, or

on more microgravity-relevant approaches, such as free-interface or counter-diffusion, with crystallization vessels often of rather large size (DeLucas *et al.*, 1994; Giegé *et al.*, 1995; McPherson, 1996; Gonzalez-Ramirez, Carrera *et al.*, 2008). Reproducible data, with a substantial number of model proteins (lysozyme, thaumatin, canavalin and several plant viruses), were obtained in the versatile Advanced Protein Crystallization Facility instrument from the European Space Agency (Vergara *et al.*, 2003, 2005). Altogther, an overall positive effect of microgravity on protein crystal growth emerged.

In support of this conclusion are observations of larger sizes for space-grown crystals and improved optical quality, as exemplified by thaumatin crystals (Ng, Lorber *et al.*, 1997). The maximum resolution of diffraction patterns also indicated superiority for microgravity crystals. A striking example is parvalbumin that diffracts to 0.9 Å resolution, while the earth-grown crystals are not suitable for diffraction analysis (Declercq *et al.*, 1999). Also, in several instances, the signal-to-noise ratio of X-ray diffraction data collected from space crystals was greater than for the corresponding earth controls, as for satellite tobacco mosaic virus (McPherson, 1996) and thaumatin (Ng, Lorber *et al.*, 1997; Lorber, Sauter, Robert *et al.*, 1999). An additional criterion is the reduced mosaic spread of reflections recorded from space samples (Snell *et al.*, 1995; Stojanoff *et al.*, 1996; Ng, Lorber *et al.*, 1997; Lorber, Sauter, Robert *et al.*, 1999). Impurity incorporation during growth is another issue and, as shown with lysozyme, the microgravity-grown crystals incorporate fewer impurities than the earth controls (Carter *et al.*, 1999). The best criterion for enhanced crystal quality, however, is the crystallographic structure. In a case study with lysozyme, significant improvement of resolution from 1.6 to 1.35 Å, decreased atomic displacement parameters (ADPs or *B* factors) and a structure of increased clarity have been noted for the space-grown crystals (Carter *et al.*, 1999). In another study on an aspartyl-tRNA synthetase, a strictly comparative analysis showed that crystals grown in microgravity were superior in many respects to controls prepared under otherwise identical conditions on earth, facilitating structure determination at 2.0 Å resolution (Ng *et al.*, 2002).

Nevertheless divergent conclusions were reached concerning the quality of the X-ray structure, which was shown to be improved (Carter *et al.*, 1999) or unaffected (Vaney *et al.*, 1996) by microgravity. This contradiction may originate from different levels of impurities present in the protein samples and/or from non-identical growth conditions in different hardware. Conceivably, alteration by gravity of fluid properties could affect nucleation. Transport is of importance, because the large sizes of proteins imply that they have low diffusivities. Elimination of fluid convection may, however, dramatically affect the movement and distribution of proteins in the fluid and their transport and absorption to crystal surfaces. In addition, many proteins form non-specific aggregates in solution. These may be a major source of the contaminants that are incorporated into crystal lattices. By virtue of their size and low diffusivity, the movement of aggregates and large impurities in solution is even more significantly altered.

On earth there is continuous density-driven convective mixing in the solution due to gradients arising from temperature or from incorporation of molecules by the growing crystal. The effects of diffusive transport in the laboratory are, by comparison with the microgravity case, almost negligible because of the very slow rate of diffusion of large proteins. Because of convective mixing, protein crystals nucleated on earth are continuously exposed to the full concentration of protein nutrient present in the bulk solvent. Convection thus maintains, at the growing crystal interface, excessive supersaturation as growth proceeds. This provides an explanation as to why microgravity may improve the quality of protein crystals. The mechanism for enhanced order and reduction of defects may not be directly due to convective turbulence at growing crystal surfaces, but to reduction of the concentration of nutrient molecules and impurities in the immediate neighbourhood of the growing crystals. As a protein crystal forms in microgravity, a concentration gradient or 'depletion zone' is established around the nucleus. Because protein diffusion is slow and that of impurities may be even slower, the depletion zone is quasi-stable. The net effect is that the surfaces of the growing crystal interface with a local solution phase at a lower concentration of protein nutrient and impurities than exists in the bulk solvent. The crystal, as it grows, experiences a reduction in its local degree of supersaturation and essentially creates for itself an environment equivalent to the metastable region where optimal growth is expected.

Investigations of protein crystallization diverged along two paths. The objective of the first was to produce high-quality crystals for X-ray diffraction analysis. The crystals themselves were the product of the space-bound experiment and biochemical results were secondary. The goal of the second line of investigation was to understand and to control the physics of the process. This second interest was supported by extensive ground-based research. The confluence of results yielded persuasive explanations for the observed improvements in size and quality of protein crystals grown in microgravity and a robust theoretical framework for understanding the phenomena involved. They showed also that protein crystals are more sensitive to the very high degrees of supersaturation at which they are usually grown and to the mass-transport mechanisms responsible for bringing nutrient to their growing surfaces. The self-regulating nature of protein crystallization in microgravity, through the establishment of local concentration gradients of reduced supersaturation, explains why the diffusive transport that predominates produces a significant difference in ultimate crystal quality.

Crystallization in gelled media. Because convection occurs in free solutions, crystallization in gels represents what is essentially a convection-free environment (Henisch, 1988). Thus, the quality of crystals may be improved in gels. Whatever the mechanism of crystallization in gels, the procedure will produce changes in the nucleation and crystal-growth processes, as has been verified with many proteins (Robert & Lefaucheux, 1988; Cudney *et al.*, 1994; Vidal *et al.*, 1999; Biertümpfel *et al.*, 2002; Lorber *et al.*, 2009). Two types of gels have been used, namely, agarose and silica gels. The latter seem to have proven the most adaptable, versatile and useful for proteins. With both agarose and silica gels, it is possible to use a variety of different crystallants, including salts, organic solvents and polymers such as PEG (Gonzalez-Ramirez, Caballero & Garcia-Ruiz, 2008). They also allow the investigator to control pH and temperature. The most successful efforts have involved direct diffusion arrangements, where the crystallant is diffused into a protein-containing gel or *vice versa*. In practice, experiments are conducted in semi-liquid gelled media where the agarose concentration does not exceed 0.6%(*w*/*v*), but crystallization can also take place in 2.0%(*w*/*v*) agarose viscoelastic gels, a condition that does not affect the crystal structures (Sugiyama *et al.*, 2009).

Crystals grown in gels can easily be removed from their soft environment and set up for X-ray analysis. They tend to be robust since, as shown with lysozyme crystals, agarose fibres are incorporated into the crystal lattice (Gavira & Garcia-Ruiz, 2002). Gel

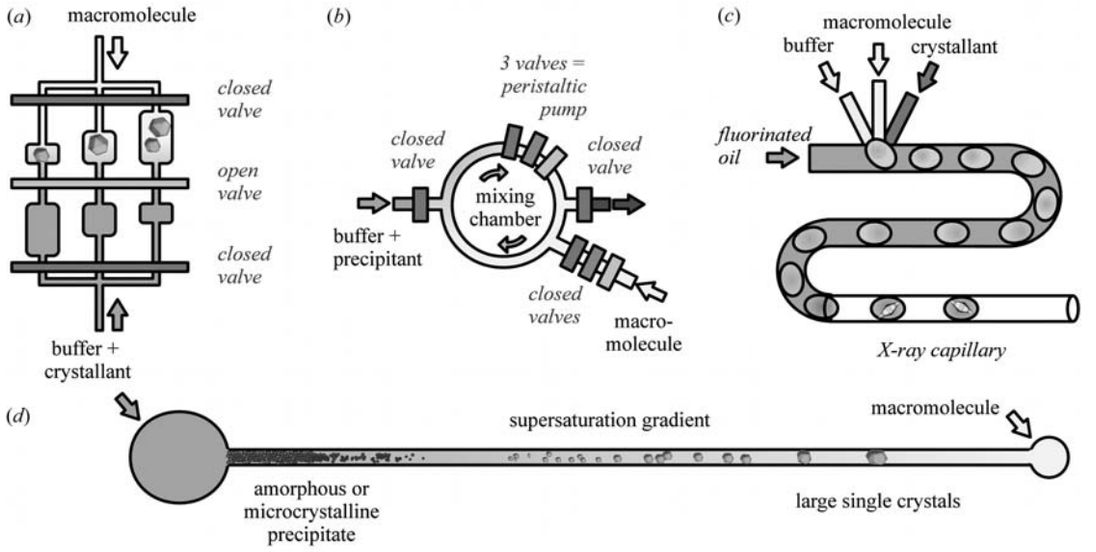

Figure 4.1.4.1
Examples of microfluidic devices designed for biocrystallization. (*a*) Schematic view of one of the 46 modules composing the free-interface diffusion chip. An integrated fluidic circuit dispenses the macromolecule and crystallant solutions into the final chambers. The microchannels are then closed and those connecting the top and bottom chambers are opened with the help of pneumatic valves integrated in the chip. The crystallant diffuses in the protein chamber and triggers crystallization. In this version of the chip, each module is divided into three pairs of chambers with volumes of 5–20 nl to create different protein-to-crystallant concentration ratios. (*b*) The mixing rotor of a formulation chip designed for the high-throughput study of precipitation diagrams. This chip generates protein/buffer/crystallant mixtures at different concentrations in its 5 nl rotor. The three valves in a row constitute a peristaltic pump that homogenizes the mixture. A charge-coupled device (CCD) camera is used to detect the appearance of a precipitate. (*c*) The nanobatch chip. Nanodroplets are produced in a microfluidic channel and displaced by inert oil (the flow rate determines the drop size from 10 to 20 nl). Droplets can be stored in the chip or in capillaries connected to the exit of the chip. They can be inspected and crystals can be characterized by X-ray diffraction. (*d*) Microfluidic chip for counter-diffusion experiments. This method relies on the diffusion of a crystallant into an elongated chamber (the microfluidic channel) containing a macromolecular solution. A concentration gradient is generated that develops along the entire crystallization chamber. The propagating supersaturation wave of gradually decreasing amplitude tests a broad range of nucleation and growth conditions in a single experiment. While a precipitate may form at the entrance of the chamber, monocrystals may grow at the opposite end. Crystals can be observed and analysed by X-ray diffraction directly inside the chip (adapted from Sauter *et al.*, 2007).

growth, because it suppresses convection, has proven to be a useful technique for analysing concentration gradients around growing crystals by interferometric techniques (Robert *et al.*, 1994) and growth mechanisms by differential interference contrast microscopy (Van Driessche, Otalora, Gavira & Sazaki, 2008). In conclusion, gelled media mimic microgravity conditions, preserve crystals once they are grown and, as expected, crystals grown in gels are often of superior quality to controls grown from solutions (Zhu *et al.*, 2001; Moreno *et al.*, 2002; Sauter *et al.*, 2002). Finally, gels can prevent damage during crystal soaking and cryo-cooling (Biertümpfel *et al.*, 2005; Sauter *et al.*, 2009; Lorber *et al.*, 2009).

Crystallization in microfluidic devices. Microfluidic devices were recently introduced in the field of biological crystal growth and represent a new means of crystallizing under diffusive conditions. These systems were primarily intended to miniaturize and to parallelize crystallization assays, thus leading to novel, cost-effective, high-throughput screening approaches. However, because of the small size of their channels and chambers (typically below 100 µm in depth and width), they also provide a diffusive environment comparable to that existing in a capillary tube, in a gel, or under microgravity.

Indeed, the first microfluidic application in biocrystallization was a miniaturized version of the free-interface diffusion technique in which the absence of convection is essential (Hansen *et al.*, 2002). The chip consists of a complex integrated fluidic circuit including two networks of channels, one for liquid handling and a second serving as actuation valves. The chip is dedicated to high-throughput screening and, in its initial version, was designed to test 48 crystallization conditions with less than 10 µl of sample solution. Three parallel sets of chambers are used to bring into contact different proportions of macromolecule and crystallant solutions (Fig. 4.1.4.1*a*). This concept of chip was further modified to combine free-interface diffusion with vapour diffusion (or vapour permeation) for fine tuning the supersaturation achieved in crystallization chambers (Hansen *et al.*, 2006). This technology also led to a 'formulator chip' that can perform hundreds of mixing operations in just a few hours in order to establish precipitation diagrams (Fig. 4.1.4.1*b*). A single assay consumes less than 5 nl sample/buffer/precipitant solution and derived precipitation maps are used to delineate a grid of conditions for crystallization screening (Hansen *et al.*, 2004).

The second crystallization method implemented in microfluidics was 'batch in nanodroplets' (Zheng *et al.*, 2003). The chip design is extremely simple: it consists of inlets for protein, buffer and crystallant solutions, and a microfluidic channel in which 10 nl droplets are prepared by mixing these solutions in various ratios. This device allows a daily formulation of thousands of nanodrops or plugs (Fig. 4.1.4.1*c*), which are carried by a flow of inert fluorocarbon oil. They are stored on the chip or in capillary tubes plugged at the exit of the chip and their content can easily be analysed by X-ray diffraction (Yadav *et al.*, 2005). This method is very well suited for high-throughput screening and, in addition to crystal growth, this technology can be used for many applications in chemistry (Song *et al.*, 2006).

Based on the nanodrop approach, a more complex system has recently been designed for basic research purposes. It is able to formulate droplets and to flow them to storage chambers where they can be concentrated or diluted by water permeation through the chambers' walls. This 'phase chip' is designed to establish

phase diagrams with total control over supersaturation, nucleation and growth kinetics in each individual drop (Shim *et al.*, 2007).

As for free-interface diffusion, the absence of convection in microfluidic channels makes microsystems very appealing for implementing counter-diffusion experiments (Sauter *et al.*, 2007; Ng *et al.*, 2008). Characteristic counter-diffusion features were successfully reproduced in microchannels with a production of crystalline material ranging from microcrystals to large monocrystals along the supersaturation gradient. When made of appropriate polymer material, these counter-diffusion chips also allow a direct on-chip characterization of the crystals by X-ray diffraction, without any further (and potentially deleterious) sample handling (Ng *et al.*, 2008; Dhouib *et al.*, 2009).

These examples illustrate the many advantages of microfluidic chips – low sample consumption, high-throughput screening capabilities, quasi-ideal convectionless growth conditions – and one can anticipate that microfluidic technology will become a popular and affordable tool both for condition screening, optimization and X-ray analysis, and for basic crystallogenesis research.

Simulating other aspects of microgravity crystal growth. Heterogeneous nucleation or crystal growth on the solid surface of crystallization vessels can be avoided under levitation (Rhim & Chung, 1990) and more easily in batch between two oil layers (Chayen, 1996; Lorber & Giegé, 1996). This can also be achieved for the growth of large protein crystals by mild stirring of the solution in two-liquid systems (Adachi *et al.*, 2004).

It was conjectured that other features of weightlessness, such as suppression of convection, could be achieved in the laboratory under hypergravity and when magnetic or electric fields are applied. These possibilities have been tested experimentally. Crystals were grown under forced diffusive transport of the macromolecules in centrifuges (Karpukhina *et al.*, 1975; Lenhoff *et al.*, 1997; Lorber, 2008) and nucleation was shown to be affected by magnetic (Ataka *et al.*, 1997; Sazaki *et al.*, 1997) or external electric (Taleb *et al.*, 1999; Nanev & Penkova, 2001) fields. Interestingly, under these last conditions, growing crystals were shown to have preferential orientations and specific spatial distributions in the crystallization chambers. Magnetic fields produced by small permanent magnets of 1.25 T are sufficient to produce these effects (Astier *et al.*, 1998) and numerical predictions revealed that magnetization forces could damp convection (Qi *et al.*, 2001). For crystallization induced by electric fields, simple devices adapted to vapour-diffusion (Charron *et al.*, 2003) and batch (Al-Haq *et al.*, 2007) methods are available. Crystallization can also be electrochemically assisted by internal electric fields (Frontana-Uribe & Moreno, 2008). In some cases, magnetic and electric fields have been coupled and experiments conducted in gelled media (Sazaki *et al.*, 2004; Moreno *et al.*, 2009).

Although the above methods are not widespread and the underlying physics not completely validated, they can be useful in special cases. For instance, when crystallization attempts systematically yield showers of microcrystals, crystallization inside electric or magnetic fields can be an alternative to obtain monocrystals suitable for X-ray data collection, because the number of nucleation sites is reduced and can be controlled (Moreno & Sazaki, 2004; Hammadi *et al.*, 2009) and crystal quality maintained (Sato *et al.*, 2000; Lübbert *et al.*, 2004).

4.1.4.2. Methods making use of temperature and pressure

Temperature and pressure are familiar thermodynamic parameters. Indeed, many living organisms are thermophiles, even hyperthermophiles, or barophiles/piezophiles and thus have evolved macromolecules stable at temperatures up to 110 °C or pressures up to 100 MPa, *i.e.* 1000-fold atmospheric pressure (Abe & Horikoshi, 2001). Temperature can trigger nucleation, regardless of the crystallization method. This can be done in a controlled manner, but often occurs as an unexpected consequence of accidental temperature variation in the laboratory. Dedicated systems have been designed for temperature-dependent control of nucleation and growth (Astier & Veesler, 2008), and find application for, among other things, the growth of large high-quality protein crystals for neutron crystallography (Budayova-Spano *et al.*, 2007).

Pressure, as anticipated, can trigger nucleation and sustain protein crystal growth (*e.g.* Suzuki *et al.*, 2002). To facilitate analysis of crystallization output, assays under pressure can be done in agarose gel (Kadri *et al.*, 2003). Rather simple equipment is required allowing batch crystallization of ∼12 individual samples of ∼80 µl that can be collectively pressurized up to ∼400 MPa (Lorber *et al.*, 1996). The effects exerted by pressure are multiple and protein dependent, with habit, number, length, shape and solubility of crystals modified under pressure. Further, crystallization volumes and diffraction properties are affected and, interestingly, these physical properties are essentially conserved upon depressurization of the crystals. In particular, differences in the water sites surrounding thaumatin crystals grown at 0.1 and 150 MPa have been observed (Charron *et al.*, 2002). Crystallographic analysis of cowpea mosaic virus crystals compressed at 330 MPa in a diamond-anvil cell demonstrated pressure-induced ordering of the crystals, lower ADPs and a larger number of ordered water molecules (Girard *et al.*, 2005; Lin *et al.*, 2005).

4.1.4.3. Methods making use of crystallization chaperones

Another strategy that has been used for recalcitrant proteins is to combine them in some manner with a second protein, sometimes called a cocrystallization or chaperone protein (Warke & Momany, 2007; Koide, 2009), so that the complex of the two provides an additional chance for success. The idea was first tested with lysozyme complexed with an Fab antibody fragment (Boulot *et al.*, 1988) and has been used particularly with membrane proteins and antibody domains directed against the target protein (Ostermeier *et al.*, 1995). In those cases the antibody fragment enhanced the solubility of the otherwise hydrophobic protein and provided additional lattice contacts in the resultant crystals. There is, in principle, no reason why such 'crystallization chaperones' could not be used with soluble proteins. Likewise the method can be useful for the crystallization of functional RNA fragments (Ye *et al.*, 2008). An alternate possibility with great potential is the recently developed DARPin technology based on the natural ankyrin repeat protein fold with randomized surface residue positions allowing specific binding to virtually any target protein (Sennhauser & Grütter, 2008).

4.1.4.4. Seeding

It is often necessary to reproduce crystals grown previously, where either the formation of nuclei is limiting, or spontaneous nucleation occurs at such a profound level of supersaturation that poor growth results. In such cases, it is desirable to induce growth in a directed fashion at low levels of supersaturation. This can be accomplished by seeding a metastable, supersaturated protein solution with crystals from earlier trials. Seeding also permits one

to uncouple nucleation and growth. Seeding techniques fall into two categories that employ either macroseeds (Thaller *et al.*, 1985) or microcrystals as seeds (Stura *et al.*, 1999; Bergfors, 2003). In both cases, the solution to be seeded should be only slightly supersaturated so that controlled growth can occur.

When seeding with crystals large enough to be manipulated under a microscope, the most important consideration is to eliminate spurious nucleation by transfer of too many seeds. This drawback may be overcome using laser tweezers, a technique that permits non-mechanical, *in situ* manipulation of individual seeds as small as 1 µm (Bancel *et al.*, 1998). Even if a single large crystal is employed, microcrystals adhering to its surface may be carried across to the fresh solution. To avoid this, the macroseed is washed by passing it through a series of intermediate transfer solutions. In doing so, not only are microcrystals removed, but if the wash solutions are chosen properly, some limited dissolution of the seed surface may take place. This has the effect of freshening the seed-crystal surfaces and promoting new growth once it is introduced into the new protein solution. Note that crystals of homologous macromolecules can serve as seeds (Thaller *et al.*, 1985).

In the second approach with microcrystals, the danger is that too many nuclei will be introduced into the fresh supersaturated solution, and masses of crystals will result. To overcome this, a stock solution of microcrystals is serially diluted over a very broad range. Some dilution sample in the series will, on average, have no more than one microseed per ml; others will have several times more, or none at all. An aliquot (∼1 µl) of each sample in the series is then added to fresh crystallization trials. This empirical test, ideally, identifies the correct sample to use for seeding by yielding only one or a small number of single crystals when crystal growth is completed. Microseeds can be introduced into crystallization trials at any stage of microbatch or vapour-diffusion experiments (D'Arcy, MacSweeney & Haber, 2003; D'Arcy *et al.*, 2004) and this process can be automated (D'Arcy *et al.*, 2007; Newman *et al.*, 2008; Khurshid *et al.*, 2010).

4.1.5. From the macromolecule to perfect crystals: the physics view

Each of the four stages in crystallization (prenucleation, nucleation, growth and cessation of growth) can be monitored by specific physical techniques. Although systematic characterization of crystallization is usually not carried out in practice, characterization of individual steps and measurement of the physical properties of crystals obtained under various conditions may help in the design of appropriate experimental conditions to reproducibly obtain crystals of a desired quality (*e.g.* of larger size, improved morphology, increased resolution or greater perfection).

4.1.5.1. Prenucleation and nucleation

DLS relies on the scattering of monochromatic light by aggregates or particles moving in solution. Since diffusivity of the particles is related to their size by the Stokes–Einstein equation, measurement of diffusion coefficients can be translated into hydrodynamic radii. By making measurements as a function of scattering angle, information regarding aggregate shape can also be obtained. For single-component systems, the method for determining the size of macromolecules, viruses and larger particles up to a few µm is straightforward. For polydisperse and concentrated systems, the problem is more complex, but with the use of auto-correlation functions and advances in signal detection, DLS provides good estimates of aggregate size distribution and supplies a diagnostic tool for quality control of proteins and optimization of crystallization conditions. Experimental necessity encouraged the design of dedicated instruments for protein crystallization, combining *e.g.* imaging of crystals and DLS analysis within crystallization droplets (Dierks *et al.*, 2008).

In biocrystallogenesis, investigations based on light scattering have been useful in detecting nucleation prior to the appearance of crystals observable under the light microscope, that is, in understanding prenucleation and nucleation. Many studies have been carried out with lysozyme as the model (Kam *et al.*, 1978; Durbin & Feher, 1996), though not exclusively, and they have been developed with two objectives. One is to analyse the kinetics and the distribution of molecular aggregate sizes as a function of supersaturation. The idea is to understand the nature of prenuclear clusters that form in solution and how they transform into crystal nuclei (Kam *et al.*, 1978; Georgalis *et al.*, 1993; Malkin & McPherson, 1994). Such a quantitative approach has sought to define the underlying kinetic and thermodynamic parameters that govern the nucleation process.

A more practical objective is to use light-scattering methods to predict which combinations of crystallants, additives and physical parameters are most likely to lead to the nucleation and growth of crystals (Mikol, Hirsch & Giegé, 1990; Ferré-D'Amaré & Burley, 1997; Borgstahl, 2007; Wilson, 2003; Niesen *et al.*, 2008). A major goal here is to reduce the number of empirical trials. The analyses depend on the likelihood that precipitates are usually linear, branched and extended in shape, since they represent a kind of random polymerization process (Kam *et al.*, 1978). Aggregates leading to nuclei, on the other hand, tend to be more globular and three dimensional in form. Thus, mother liquors that indicate a nascent precipitate can be identified as a failure, while those that have the character of globular aggregates hold promise for further exploration and refinement. Other analyses have been based on discrimination between polydisperse and monodisperse protein solutions, which suggests that polydispersity hampers crystallization, while monodispersity favours it (Mikol, Hirsch & Giegé, 1990).

A more quantitative approach is based on measurement of the second virial coefficient B2, which serves as a predictor of the type of interaction between macromolecules occurring in solution. Using static light scattering, it was found that mother liquors that invariably yield crystals have second virial coefficients that fall within a narrow range of small negative values. Correlations between the associative properties of proteins in solution, their solubility and the B2 coefficient were highlighted (*e.g.* George *et al.*, 1997; Wilson, 2003), and seem to be a general feature. This is a powerful diagnostic of crystallization conditions.

Related methods, such as fluorescence spectroscopy (Crosio & Jullien, 1992; Forsythe *et al.*, 2006), osmotic pressure (Bonneté *et al.*, 1997; Neal *et al.*, 1999), small-angle X-ray scattering (Finet *et al.*, 1998) and small-angle neutron scattering (Ebel *et al.*, 1999; Gripon *et al.*, 1997; Minezaki *et al.*, 1996; Vidal *et al.*, 1998), were used to investigate specific aspects of protein interactions under precrystallization conditions and produced, in several instances, complementary answers to those from light-scattering studies.

4.1.5.2. Growth and cessation of growth

A number of microscopies and other optical methods can be used for studying the crystal growth of proteins (Van Driessche, Otalora, Sazaki *et al.*, 2008). These are time-lapse video microscopy with polarized light, Schlieren and phase-contrast micro-