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PART 4. CRYSTALLIZATION

Chapter 4.1. General methods

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4.1.1. Introduction

4.1.1.1. Prologue

Macromolecular crystals are much softer than salt crystals. They contain ∼50% of solvent on average, with values ranging from as little as 25 up to 90%. A direct consequence is that, although morphologically indistinguishable, crystals of macromolecules differ in many respects from crystals of low-molecular-mass compounds. While the latter exhibit firm lattice forces, are highly ordered, generally physically hard and brittle, easy to manipulate, can usually be exposed to air, have strong optical properties and diffract X-rays intensely, crystals of macromolecules are, by comparison, smaller in size, they crush easily, disintegrate if allowed to dehydrate, exhibit weak optical properties and diffract X-rays poorly. They are temperature sensitive and undergo extensive damage after prolonged exposure to radiation.

Proteins or nucleic acids build up a crystalline scaffold, which may be imagined as an ordered gel with extensive interstitial spaces through which small molecules can diffuse freely. In proportion to molecular mass, large macromolecules establish far fewer packing interactions than do small molecules inside crystalline lattices. Since these contacts are responsible for the integrity of the crystals, this largely explains the differences in properties between the two types of crystals. Thus, liquid channels and solvent cavities are directly responsible for the generally poor diffraction properties of macromolecular crystals. Owing to the large spaces between adjacent molecules and the related weak lattice forces, every molecule in the crystal may not occupy exactly equivalent orientations and positions. Furthermore, because of their structural complexity and their conformational dynamics, macromolecules in a given crystal form may exhibit slight variations in their folding patterns or dispositions of side chains.

However, high solvent content is not as negative as it might appear at first glance. It allows maintenance of the macromolecular structures virtually unchanged from those in bulk solvent. As a consequence, ligand binding, enzymatic and spectroscopic characteristics, and other biochemical features are essentially the same as for the native molecule in solution. In addition, the dimensions of solvent channels are such that conventional chemical compounds, such as ions and heavy atoms, substrates or other ligands, may be freely diffused into and out of the crystals. Thus, many crystalline enzymes, though immobilized, are completely accessible for experimentation through alteration of the surrounding mother liquor.

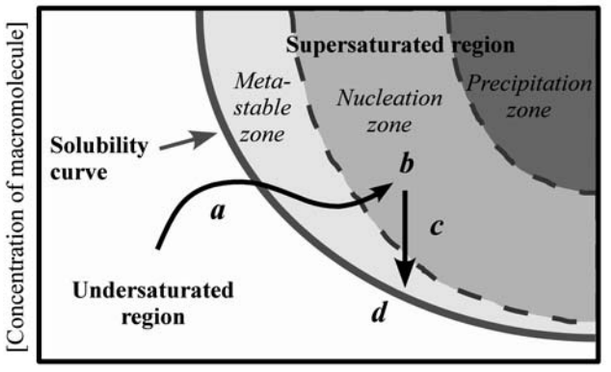
The intrinsic instability of most macromolecules requires that conditions suitable for crystal growth are those that do not perturb their molecular properties. This explains why crystals must be grown from solutions compatible with the target macromolecules, *i.e.* within a narrow range of pH, temperature or ionic strength. Finally, because hydration is essential for the maintenance of structure, crystals of macromolecules must be always bathed in the mother liquor, even during data collection (except in the practice of cryocrystallography).

4.1.1.2. Crystallization principles

The crystallization of biological macromolecules has often been considered unpredictable, although it obeys the same principles as that of small molecules (Giegé *et al.*, 1995; McPherson *et al.*, 1995; Rosenberger, 1996; Chernov, 2003; McPherson & Giegé, 2007). It is, similarly, a multiparametric process. The difference compared with conventional crystal growth arises from the biochemical and biophysical properties of proteins or nucleic acids, and crystallization methods must take into account these features. The methods described below apply for most proteins, large RNAs, multimacromolecular complexes and viruses (for small oligonucleotides or peptides, crystallization by dialysis is not appropriate). For hydrophobic membrane proteins, special techniques are required (see Chapter 4.2).

Crystallization proceeds from macromolecules in solution that 'aggregate' upon entering a supersaturated state and eventually undergo a phase transition. This leads to nuclei formation and ultimately to crystals that grow by different mechanisms. Supersaturation is the driving force of crystallization and is defined as the ratio $[C]/[S]$, where $[C]$ and $[S]$ are the initial concentration of the macromolecule and its final concentration at saturation, *i.e.* its solubility. Nucleation is homogeneous when nuclei form in the bulk of the solution, but heterogeneous when they preferentially form on walls of crystallization vessels, on solid particles (dust, aggregates, seeds), or on the surface of existing crystals. Unlike most conventional crystals, protein crystals are, in general, not initiated from seeds, but are nucleated *ab initio* at high levels of supersaturation that can reach 200 to 1000% (in what follows and for simplicity, the generic name 'protein' is used for macromolecule). It is this high degree of supersaturation that, in large part, distinguishes protein crystal formation from that of conventional crystals. That is, once a stable nucleus has formed, it subsequently grows under very unfavourable conditions of excessive supersaturation. Distant from the metastable zone, where ordered growth could occur, crystals rapidly accumulate nutrient molecules, as well as impurities. They also concomitantly accumulate statistical disorder and a high frequency of defects that exceeds those observed for most conventional crystals.

The different stages of crystallization (*i.e.* pre-nucleation, nucleation, growth, cessation of growth) can be visualized in a phase diagram (Fig. 4.1.1.1). In short, phase diagrams are divided into undersaturated regions (where proteins are soluble) and supersaturated regions (where protein crystals nucleate and grow) delimited by the solubility curve. The supersaturated region is thermodynamically out of equilibrium and can be divided into three kinetically dependent domains: a precipitation domain (at extreme supersaturation) where macromolecules rapidly separate from solution in a solid state either amorphous or microcrystalline, a domain (at lower supersaturation) where



Biochemical parameters
Purity of the macromolecule
Ligands, inhibitors, effectors
Aggregation state of the macromolecule
Post-translational modifications
Source of macromolecule
Proteolysis or hydrolysis
Chemical modifications
Genetic variants
Inherent symmetry of the macromolecule
Stability of the macromolecule
Isoelectric point
History of the sample

Chemical parameters
pH
Crystallant type
Crystallant concentration
Ionic strength
Specific ion effects
Supersaturation
Reductive or oxidative environment
Concentration of the macromolecule
Metal ions
Crosslinkers or polyions
Detergents, surfactants or amphophiles
Non-macromolecular impurities

Physical parameters
Temperature variation
Contact surfaces
Methodology or approach to equilibrium
Gravity
Pressure
Time
Vibrations, sound or mechanical perturbations
Electrostatic or magnetic fields
Dielectric properties of the medium
Viscosity of the medium
Rate of equilibration
Homogeneous or heterogeneous nucleants

Figure 4.1.1.1
Crystallization is a multiparametric process under the control of a great variety of biochemical, chemical and physical parameters. The crystal grower can play with them to drive a macromolecule from the soluble towards the crystalline state. In the top left, a typical two-dimensional phase diagram illustrates how this can be achieved. (*a*) By modifying two parameters (concentrations of macromolecule and crystallant), the system will move from the undersaturated region where the macromolecule is soluble into the supersaturated region beyond the solubility curve where it will try to escape from the solution. The prenucleation trajectory essentially depends on the crystallization method (see Fig. 4.1.3.1). (*b*) As soon as they enter the supersaturation region, macromolecules will tend to aggregate. However, the system needs to cross an energy barrier to produce stable molecular assemblies, the nuclei, and this only happens in the so-called nucleation zone. (*c*) When stable nuclei are formed, they will capture more macromolecular entities from the mother liquor and produce three-dimensional crystals. (*d*) Crystals will grow until the system comes back to the solubility curve, crossing the metastable zone. Growth stops and crystals are in dynamic equilibrium with the mother liquor. When the system is driven to high supersaturation, macromolecules may rather produce amorphous or microcrystalline precipitates than useful monocrystals. Note that the limits between zones of the supersaturated zone (dashed curve) move with time.

nucleation occurs spontaneously and a metastable domain (at low supersaturation) where nucleation does not occur spontaneously but where crystals grow. This domain is favourable for seeding. The wisdom of the crystal grower will be to take advantage of an overall understanding of phase diagrams for designing crystallization strategies and selecting favourable solvent conditions (Sauter, Lorber *et al.*, 1999; Asherie, 2004).

4.1.2. Main parameters that affect crystallization of macromolecules

4.1.2.1. Crystallizing agents

Crystallizing agents (also referred to as crystallants or precipitants) are defined as those chemical species that are able to precipitate a soluble macromolecule and hence to bring it into a supersaturated state needed for its crystallization. They fall into four categories: (i) salts, (ii) organic solvents, (iii) long-chain polymers, and (iv) low-molecular-mass polymers and non-volatile organic compounds (McPherson, 1990). The first two classes are typified by ammonium sulfate and ethanol; higher polymers such as PEGs (polyethylene glycols) of molecular weight (M_r) > 1000, are characteristic of the third. In the fourth are placed compounds such as MPD (2-methyl-2,4-pentanediol) and low-molecular-mass PEGs.

Salts. Salts exert their effects by dehydrating proteins through competition for water molecules (Green & Hughes, 1955). Their ability to do this is roughly proportional to the square of the valences of the ionic species composing the salt. Thus, multivalent ions, particularly anions, are the most efficient. One might think there would be little variation between different salts, so long as their ionic valences were the same, or between two different sulfates, such as lithium and ammonium sulfates. This, however, is often not the case. In addition to salting out, which is a dehydration effect, or lowering of the chemical activity of water, there are specific protein–ion interactions that have other consequences (Riès-Kautt & Ducruix, 1991; Ducruix & Giegé, 1999). This is true because of the polyvalent character of individual proteins, their structural complexity, and the dependence of their physical properties on environmental conditions and interacting molecules. Therefore, restricting the search to one or two salts is never sufficient when attempting to crystallize a protein. Changing the salt may produce crystals of varied quality, morphology and diffraction properties.

It is usually not possible to predict the molarity of a salt required to crystallize a particular protein without some prior knowledge of its behaviour. In general, the concentration is just a small percentage less than that yielding an amorphous precipitate. To determine the precipitation point with a given crystallant, a 10 µl droplet of a 5–15 mg ml^{-1} protein solution is placed in the well of a depression slide and observed under a microscope as increasing amounts of salt solution or organic solvent (in l–2 µl increments) are added. If the well is sealed between additions with a coverslip, the increases can be made over a period of many hours. Indeed, the droplet should equilibrate 10–30 min after each addition, and longer in the neighbourhood of the precipitation point.

Widely used and the most successful salt to grow protein crystals is ammonium sulfate. A variety of other salts included in crystallization screens yield success as well. Sodium malonate, not systematically present in screens, deserves particular attention, since it gave the highest success rate (better than ammonium sulfate) in a comparative study designed to identify the most effective salts for protein crystallization (McPherson, 2001). Following this example, other organic salts have been added to the repertoire (McPherson & Cudney, 2006).

Organic solvents. The most common organic solvents (volatile compounds) utilized are ethanol, methanol, isopropanol, acetone and dioxane. They have been frequently used for crystallizing nucleic acids, particularly tRNAs and duplex oligonucleotides

(Dock *et al.*, 1984). This, in part, stems from the greater tolerance of polynucleotides to organic solvents and their polyanionic character, which appears more sensitive to dielectric effects than proteins. Volatile organic solvents should be used at low temperature and should be added slowly and with good mixing.

Long-chain polymers. PEGs are polymers of various lengths that are useful in crystallogenesis (McPherson, 1976). The larger species with $M_r > 1000$ exist at room temperature as either waxy solids or powders. The M_r's specified by manufacturers are mean values, and the distribution around these means varies. In addition to volume exclusion properties, PEGs share characteristics with salts that compete for water and produce dehydration, and with organic solvents that reduce the dielectric properties of the medium. PEGs also have the advantage of being effective at minimal ionic strength and providing low-electron-density media. The first feature is important because it leads to stronger affinities for ligands than high-ionic-strength media. Thus, there is greater ease in obtaining heavy-atom derivatives and in forming protein–ligand complexes. The second characteristic, their low electron density, implies a lower noise level for structures derived by X-ray diffraction.

The most useful PEGs in crystallogenesis are those in the range 2000–8000. Sizes are generally not completely interchangeable for a given protein, and this parameter has to be optimized by empirical means. An advantage of PEG over other crystallants is that most proteins crystallize within a rather narrow range of PEG concentrations (∼4–18%). In addition, the PEG concentration at which crystals form is rather insensitive, and if one is within 2–3% of the optimal value, some success will be achieved. The advantage is that, when conducting initial trials, one can use a fairly coarse selection of concentrations. This means fewer trials with a corresponding reduction in the amount of material expended. Note that PEGs are often used in conjunction with salts.

Since PEG is not volatile, this agent must be used like salt and equilibrated with the protein by dialysis, slow mixing or vapour diffusion. This latter approach is the most popular. When the reservoir concentration is in the range 5–12%, the protein solution to be equilibrated should initially be at about half that concentration. When the target PEG concentration is higher than 12%, it is advisable to initiate the equilibration at no more than 4–5% below the final value. This reduces lags in time during which the protein might denature. Crystallization of proteins with PEG has proved most successful when ionic strength is low, and more difficult when high. If crystallization proceeds too rapidly, addition of some neutral salt may be used to slow growth. PEG can be used over the entire pH range and a broad temperature range. It should be noted that solutions with PEG may serve as media for microbes, particularly moulds, and if crystallization is attempted at room temperature or over extended periods of time, then retardants, such as azide (∼0.1%), must be included in the protein solutions.

Low-molecular-mass polymers and non-volatile organic compounds. Low-molecular-mass PEG species with $M_r < 1000$, MPD and glycerol are widely used. These are oily liquids that increase the viscosity of the crystallization samples. Glycerol and other polyols have structure-ordering potential and might be useful for crystallizing flexible proteins (Sousa, 1995). MPD has properties midway between PEG and organic solvents, which makes it one of the most successful crystallants for promoting crystallization of biological macromolecules (Anand *et al.*, 2002). Besides efficacy with proteins, ∼50% of oligonucleotides were crystallized with MPD (Li *et al.*, 2007). These low-molecular-mass compounds are often used in combination with salts or long-chain PEGs.

Recent data show that room-temperature ionic liquids have the potential for macromolecular crystallogenesis (Pusey *et al.*, 2007) and can be added to the list of crystallants. They are organic salts that can solubilize proteins while preserving structural stability and enzymatic activity. With regard to crystallization applications, it is worth noticing that imidazolium-based ionic liquids enhance protein folding and suppress aggregation (Lange *et al.*, 2005). As a promising result, several ionic liquids used in the range 3–30%(*v*/*v*) yielded crystals of trypsin and lysozyme (Judge *et al.*, 2009).

4.1.2.2. Physical, physical–chemical and biochemical variables

Many physical, chemical and biological variables influence, to a greater or lesser extent, the crystallization of macromolecules (Fig. 4.1.1.1). The difficulty in assigning the relative importance for each factor is substantial for several reasons. First, each macromolecule has different surface properties and this even applies to molecules that differ by no more than one or a few residues. Therefore one can hardly predict in advance the specific values of a variable or sets of conditions that might be most profitably explored. Furthermore, crystallization parameters are not independent and their interrelations may be difficult to discern. Thus, it is not easy to elaborate firm guidelines related to physical or chemical factors that can increase the success in crystallizing a particular macromolecule. Among them, only temperature and pH have been studied carefully. For pressure or magnetic and electric fields, few investigations have been carried out and virtually nothing is known on the effects of sound, vibrations or viscosity on the growth or final quality of protein crystals (see Section 4.1.4.2).

Current physical–chemical variables. Temperature may be of great importance (Judge *et al.*, 1999; Astier & Veesler, 2008; Lin *et al.*, 2008). In general, it is wise to conduct parallel investigations at 4 and 20 °C. Even if no crystals are observed at either temperature, differences in the solubility behaviour of a protein with various crystallants and effector molecules may give some indication as to whether temperature is likely to play an important role (Christopher *et al.*, 1998). Generally, the solubility of a protein is more sensitive to temperature at low ionic strength than high. One must remember, however, that diffusion rates are less, and equilibration occurs more slowly at colder than higher temperature, so that the time required for crystal formation may be longer at colder temperatures. Although most crystallization trials are done at low (∼4° C) or medium (∼20 °C) temperatures, warmer temperatures in the range 35–40 °C should not be ignored, particularly with molecules that tend to aggregate and with nucleic acids (Dock-Bregeon *et al.*, 1988).

Another important variable is pH (Judge *et al.*, 2009). This follows since the charge character of a macromolecule and all of its consequences are intimately dependent on the ionization state of its components. Not only does its net charge change with pH (and the charge distribution), but also its dipole moment, conformation and often its aggregation state. Thus, an investigation of the behaviour of a specific macromolecule as a function of pH is an essential analysis that should be carried out in performing crystallization assays. Various buffer systems that are compatible with macromolecules are available for that purpose (Newman, 2004). As with temperature, the procedure is, first, to conduct trials at coarse intervals over a broad pH range and then to refine trials in the neighbourhood of those that showed

promise. In refining the pH for optimal growth, it should be recalled that the difference between amorphous precipitate, microcrystals and large single crystals might be due to a ΔpH of less than half a unit.

Other physical variables. Convection and diffusion are two correlated parameters that govern mass transport during the crystallization process, and studies on the physics of macromolecular crystal growth, in particular in relation to microgravity research (Giegé *et al.*, 1995; McPherson, 1996; Kundrot *et al.*, 2001; Helliwell & Chayen, 2007), have evaluated their importance and led to advanced methods of crystallization (see Section 4.1.4). On the other hand, mechanical or other types of physical perturbation of the crystallization media, *e.g.* by external electric or ultrasonic fields (Nanev & Penkova, 2001; Kakinouchi *et al.*, 2006), by continuous light irradiation (Veesler *et al.*, 2006) and by laser pulses (Lee *et al.*, 2008), might be used to induce nucleation. Local increase of protein concentration due to laser-induced cavitation could be a trigger of nucleation (Yoshikawa *et al.*, 2009).

Biochemical variables. It is now generally appreciated that, indeed, the protein is itself the most important and influential parameter in the crystallization endeavour (Dale *et al.*, 2003). When conventional approaches to crystallization have been exhausted there are two remaining options: further purifying the protein, or modifying the protein. The latter may be accomplished by genetic means using recombinant DNA techniques; that is, single or multiple point mutations may be introduced, or truncated forms of the polypeptide generated (see Section 4.1.2.4). Alternatively, traditional chemical reactions may be used to modify existing amino acids, or exposure to modifying enzymes may be employed, for example, the production of truncations by limited proteolysis. There are many examples of truncated proteins being successfully crystallized when the full-length polypeptide could not, *e.g.*, with many aminoacyl-tRNA synthetases (Giegé *et al.*, 2008). With the predictive capability of modern amino-acid sequence analysis and mass spectrometry to identify domains within proteins, designed constructs of predetermined lengths are becoming increasingly used. It has also been suggested that some surface amino acids, such as lysine and glutamic acid, inflict entropic costs when a protein crystallizes (Derewenda, 2004). Modification of such residues by reductive methylation appears to be a useful approach (Kim *et al.*, 2008) that in some cases enhances crystallizability. For instance, the addition of two methyl groups on the side chain of nine lysine residues of a nuclease introduced 44 cohesive CH intramolecular contacts and resulted in crystals diffracting at 1.2 Å resolution (Shaw *et al.*, 2007).

Histidine tags and proteins conjugated with a second protein, such as the maltose binding protein, are frequently produced as a basis for purification procedures for recombinant proteins. There is no reason why these 'tagged' or conjugated proteins should not crystallize and, indeed, many of them do. On the other hand, removal of the tag or conjugate and subsequent crystallization trials provide a further opportunity to obtain crystals of a protein, and should certainly be tried (Carson *et al.*, 2007).

Another possibility to gain access to the structure of proteins recalcitrant to crystallize is to try crystallization of their orthologues. Indeed, proteins are adapted to their cellular environments and orthologues of the same physiological function can exhibit structural and physical–chemical idiosyncrasies leading to modified crystallizability. Thus proteins and macromolecular assemblages from extremophile organisms often crystallize more readily than their cousins from mesophiles or from higher eukaryotes, as shown for instance with aminoacyl-tRNA synthetases (Giegé *et al.*, 2008).

In nucleic acid crystallization, the large size of natural DNA and of many RNA molecules necessitates the design and preparation of adequate structural modules or domains amenable to crystal production. Tailoring an adequate module is often the prerequisite for its crystallization (Ducruix & Giegé, 1999; Holbrook *et al.*, 2001; Golden & Kundrot, 2003). Since RNA architecture is very modular, specific protein-binding domains can be introduced (typically small RNA hairpins) in the target RNA sequence to promote the association with a protein partner (*e.g.* the spliceosomal protein U1A) and hence to increase the possibilities of making packing interactions (Ferré-D'Amaré, 2010). Similarly, this engineering approach can be applied to proteins. A typical example is the production of crystals diffracting to high resolution of a G-protein-coupled receptor. In that case one flexible loop of this membrane protein was replaced by the sequence of T4 lysozyme and this globular exogenic domain created new contacts leading to three-dimensional packing (Cherezov *et al.*, 2007). Another example of engineering is the introduction of a leucine half-zipper into an aminoacyl-tRNA synthetase that has engendered multiple high-quality crystals (Guo *et al.*, 2010).

4.1.2.3. Additives

Certain chemical compounds or small molecules may have dramatic effects on the success of protein crystallization. Additives, as they are often called (McPherson, 1982, 1999; Sauter, Ng *et al.*, 1999), can be decisive in macromolecular crystallization. The most commonly used type of additives, and the only class for which we have any rational basis, are those which may, for physiological reasons, be bound by the protein with consequent favourable changes in its physical–chemical properties or conformation. These include coenzymes and prosthetic groups, inhibitors, enzymatic products, ions and other effector molecules. Often the liganded form is structurally defined and stable, while the unliganded form is not, which will lead to the crystallization of the former and not of the latter. Polyamines and metal ions are useful for nucleic acids (Dock *et al.*, 1984; Ducruix & Giegé, 1999). In terms of crystallization, complexes have to be treated as almost entirely separate problems. This may permit a new opportunity for growing crystals if the native molecule is obstinate. Just as natural substrates or inhibitors are often useful, they can also have the opposite effect of obstructing crystal formation. In such cases, care must be taken to eliminate them from the mother liquor and from the purified protein before crystallization is attempted.

Numerous cases have, however, been reported where molecules were observed to make crucial interactions between macromolecules in the crystal that either helped guide or secure formation of the lattice. Such molecules sometimes had a physiological basis for their unexpected presence, but frequently not. They simply provided essential or at least helpful cross-links within the crystal. Additives that are used in protein crystallization or that might be appropriate for use in crystallization may be classified into nine categories.

Small physiological ligands. Physiologically or biochemically relevant small molecules such as coenzymes, substrate analogues, inhibitors, metal ions, prosthetic groups *etc*. are the first. These bind at the active sites of enzymes, or at specific sites elsewhere on protein molecules, and may promote more stable, homogeneous conformations, or they may induce conformational

changes into alternate states. In any case, the ultimate protein–ligand complex may exhibit a more monodisperse, less dynamic character. The pertinent molecules are, here, specific to the individual protein under study, and their selection for inclusion in mother liquors is amenable to rational analysis informed by the enzymology and biochemistry of the protein under study. That is, one considers all of the possible ligands of the protein and includes them in the screen of potential crystallization conditions.

Chemical protectants. These include reductants, such as 2-mercaptoethanol, gluthathione or dithiothreitol, which prevent oxidation, heavy metal ions, scavengers such as EDTA and EGTA (ethylenediaminetetraacetic acid and ethylene glycol tetraacetic acid), and compounds intended to prevent microbial infection such as sodium azide, pheno- or chlorobutanol. These compounds are generally included for well understood reasons, their effects are predictable and their impact on the crystallization process usually, but not always, of marginal significance. They prevent protein 'ageing', as do protease or nuclease inhibitors (Ducruix & Giegé, 1999), and are desirable when crystallization requires a long period of time to reach completion or when carried out at room temperature in PEG, or in low-ionic-strength solutions (*i.e.* conditions favourable for the growth of contaminating microorganisms).

Solubilizing agents and detergents. These include quaternary ammonium salts (Mirzabekov *et al.*, 1972), sulfobetains (Goldberg *et al.*, 1996), chaotropes like urea (Bolen, 2004), and a range of surfactant and detergent molecules (Neugebauer, 1990; Zulauf, 1990; Wiener, 2004). Because of the interest in membrane proteins, this class of additives was extensively studied and has been broadly applied to many proteins. Remarkably, there is still no consensus on which are most useful, which should be included in screening conditions, or even how they function in the solubilization of macromolecules. Note that ionic liquids at low concentration can enhance protein solubility and were shown to improve crystallization (Chen *et al.*, 2010).

Poisons. Poisons, as they have traditionally been called (McPherson, 1982, 1999), were originally employed to reduce twinning. These are generally low concentrations, 1–5%(*w*/*v*), of common organic solvents. They include compounds such as ethanol, dimethyl sulfoxide, acetone, dioxane, butanol or MPD. Their role in the crystallization process, even after 50 years of use, remains obscure. They probably enhance the solubility of the proteins and slightly reduce the degree of supersaturation in the mother liquor, as well as lower the dielectric constant of the medium, but they may have other effects as well.

Osmolytes, co-solvents and cosmotropes. These are compounds that exert their effects at relatively high concentrations, 1 *M* or more, and include a wide range of molecules such as sucrose, trehalose and other sugars, proline, trimethyl amine *N*-oxide, glycine, betaine, taurine, sarcosine, and a host of others (Washabaugh & Collins, 1986; Jeruzalmi & Steitz, 1997; Bolen, 2004; Collins, 2004). The effect of their inclusion in the mother liquor is usually to stabilize the native conformation of the protein by altering the interaction of the protein's surface with water, or by altering the hydration layer and possibly the structured waters.

Reversible cross-linkers. It has been proposed that the conformations of proteins might be stabilized, and their dynamic character reduced, by providing the proteins with small molecules that could reversibly cross-link charged groups (carboxyl and amino groups) on the protein's surface, or form intramolecular hydrogen-bonding networks using surface polar groups (Maclean *et al.*, 2002). The molecules that have been explored are usually multivalent molecules such as diamino- or dicarboxylic acid-containing molecules, or aliphatic moieties of various lengths carrying some combination of charged groups. It is not known whether the stabilization of proteins by this means is significant enough to affect their crystallization or not. This potential mechanism of altering crystallization behaviour, however, may indeed be pertinent.

Compounds favouring lattice interactions. Classes of compounds useful for stabilizing proteins through non-covalent intramolecular bonds, as described above, may also help create and stabilize protein crystals by interposing themselves between protein molecules and forming intermolecular cross-links (McPherson, 1999; McPherson & Cudney, 2006; Larson *et al.*, 2007, 2008). These cross-bridges may involve purely electrostatic interactions, or they may rely on hydrogen-bonding arrangements as well. The compounds most favourable for forming such 'lattice interactions' are, again, likely to be multivalent charged compounds, but one might expect that their length, or 'reach', would need to be greater, since they would have to extend from one protein molecule to another.

Nucleation and solubility enhancers. A special class of additives would be those materials or compounds that somehow serve to enhance nucleation, including unique surfaces. These may include low concentrations of PEG (Ray & Bracker, 1986), or other polymeric substances such as jeffamine emulsified in solutions of high salt concentration (Kuznetsov *et al.*, 2000, 2001). The microdroplets of the polymeric phase serve to concentrate the protein locally and provide an interface for nucleation to occur. This category should probably also include things like the gel used in cubic lipidic phase crystallization (Nollert, 2004; Caffrey & Cherezov, 2009) and surfaces that promote epitaxy and heterogeneous nucleation (McPherson & Shlichta, 1988*b*; Chayen *et al.*, 2006; Thakur *et al.*, 2008; Saridakis & Chayen, 2009).

Nucleation enhancers when mixed with current crystallants can also enhance protein solubility (at solute/crystal equilibrium). This is the case, *e.g.*, for acetonitrile, dimethyl sulfoxide, glycerol, MPD and PEGs at relatively high concentrations. As shown with lysozyme and xylose isomerase crystallized, respectively, in the presence of glycerol and acetonitrile, the beneficial effects are nucleation at lower supersaturation and crystals of lower mosaicity (Gosavi *et al.*, 2009).

Ionic liquids. These crystallants (see Section 4.1.2.1) can also be considered as additives. Used in the concentration range 0.05–0.15 *M*, several of them (*e.g.* 1-ethyl-3-methylimidazolium tetrafluoroborate) improved the crystallizability of lysozyme, trypsin and an Fab complex, probably by subtle changes in solution conditions since no ionic liquid ions were observed in the crystal structures (Judge *et al.*, 2009; Chen *et al.*, 2010).

4.1.2.4. Purity and homogeneity

The concept of purity is of utmost importance in macromolecular crystallogenesis (Giegé *et al.*, 1986; Rosenberger *et al.*, 1996), even though some macromolecules may crystallize readily from impure solutions (Judge *et al.*, 1998). In practice, macromolecular samples should be cleared of undesired macromolecules and small molecules and, in addition, should be pure in terms of sequence integrity and conformation.

Contaminants can have deleterious effects when they interact with the macromolecule or compete for sites on growing crystals and consequently generate growth disorders (Vekilov & Rosenberger, 1996). Thus, part-per-million amounts of foreign mole-

cules can induce the formation of non-specific aggregates, alter macromolecular solubility, or interfere with nucleation and crystal growth (McPherson *et al.*, 1996, 2004; Moreno *et al.*, 2005; Thomas *et al.*, 1998). These effects are reduced in gel media (Hirschler *et al.*, 1995; Provost & Robert, 1995).

On the other hand, macromolecules that are apparently pure may be microheterogeneous in sequence and/or conformation. Their causes are multiple and can be revealed by analytical tools, such as polyacrylamide gel electrophoresis, isoelectric focusing, nuclear magnetic resonance and mass spectroscopy, but are often overlooked. The most common causes are partial hydrolysis and post-synthetic modifications. Proteolysis represents a major difficulty that must be overcome during protein isolation. In RNAs, hydrolytic cleavages induced by nucleases, metal ions or alkaline pH are common causes of microheterogeneity. These processes can be inhibited by addition of protease or nuclease inhibitors and metal chelators during purification (Ducruix & Giegé, 1999). Heterogeneity in post-synthetic modification patterns in proteins or RNAs can be the result of functional necessity but can also occur when cloned macromolecules are overproduced. Conformational heterogeneity may also originate from ligand binding, intrinsic flexibility of the macromolecule backbones, oxidation of cysteine residues or partial denaturation. Structural homogeneity may be improved by truncation of the flexible parts of the macromolecule under study (see Section 4.1.3). This can also be done *in situ* by addition of trace amounts of protease to crystallization assays (Wernimont & Edwards, 2009). The many reasons that can account for such subtle degradations or modifications explain why altered versions of a macromolecule can be the worst contaminants for its crystallization. Accordingly, the macromolecule itself must be considered as an essential parameter in crystallization (Dale *et al.*, 2003). Control of these phenomena is of crucial importance for the crystallization of macromolecular assemblages, such as the ribosome (Auerbach-Nevo *et al.*, 2005).

Many crystal growers have found a correlation between the outcome of crystallization assays (*i.e.* number of crystals, crystal habit, volume and best diffraction properties) and the quality of macromolecular samples. For this reason, one should never spoil a 'pure' batch by mixing it with another 'pure' one, that may differ as far as microheterogeneities or minute contaminants are concerned. Altogether, purity, good solubility, structural homogeneity and absence of aggregates are good criteria for protein crystallizability (D'Arcy, 1994; Ferré-D'Amaré & Burley, 1997). Dynamic light scattering (DLS) is the appropriate analytical method to verify sample homogeneity, detect aggregates and find solvent conditions that prevent aggregation (Mikol, Hirsch & Giegé, 1990; Borgstahl, 2007; Niesen *et al.*, 2008).

4.1.3. Crystallization arrangements and classical methodologies

Many methods can be used to crystallize macromolecules (Ducruix & Giegé, 1999; McPherson, 1982, 1999). They all aim to bring the macromolecules to an appropriate state of supersaturation (Fig. 4.1.3.1). Although vapour-phase equilibrium and dialysis techniques are favoured, batch and free interface diffusion methods are often used. Besides the physical and chemical variables that affect crystallization (Fig. 4.1.1.1), macromolecular crystal growth is influenced by the crystallization method itself and the geometry of the setup. Generally, in current methods, growth is promoted by the non-equilibrium nature of the crystallization process, which seldom occurs at constant protein concentration. This introduces changes in supersaturation and, hence, may lead to changes in growth mechanism. Crystallization at constant protein concentration, however, can be achieved in special arrangements based on liquid circulation cells (Vekilov & Rosenberger, 1998).

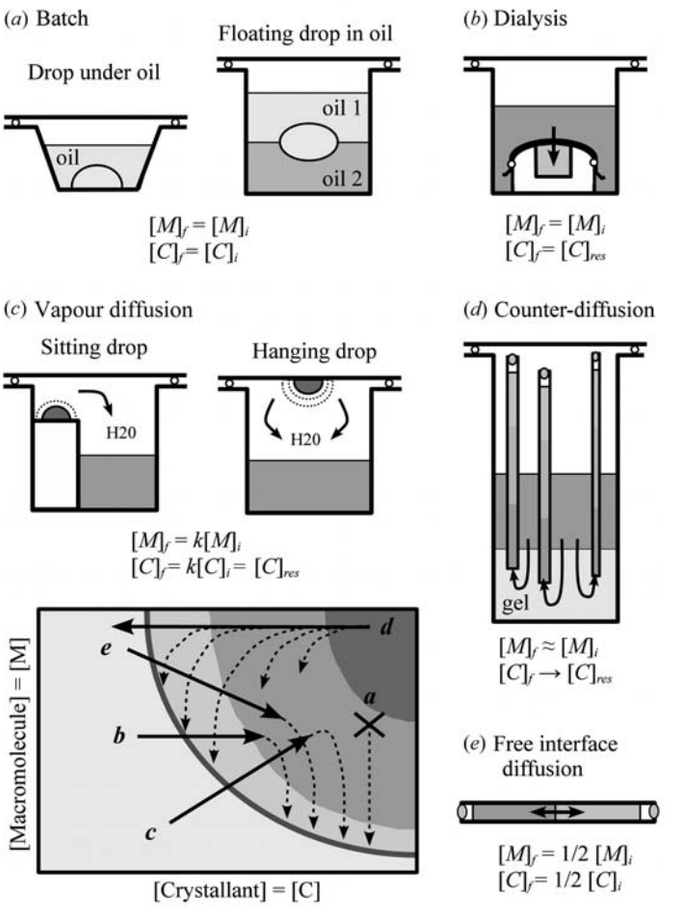


Figure 4.1.3.1
Principles of major methods used to crystallize biological macromolecules. (*a*) Two versions of batch crystallization. (*b*) Dialysis method with Cambridge button. (*c*) Vapour-diffusion crystallization with sitting and hanging drops. (*d*) Counter-diffusion setup in X-ray capillary tubes pinched in a gel layer. (*e*) Crystallization by free-interface diffusion in a capillary where two solutions of equivalent volume are brought into contact. The evolution of the macromolecule concentration, $[M]$, and crystallant concentration, $[C]$, in the different methods is indicated (initial and final concentrations in the crystallization solutions are $[M]_i$, $[M]_f$ and $[C]_i$, $[C]_f$, respectively; $[C]_{res}$ is the concentration of the crystallant in the reservoir, and k is a dilution factor specified by the ratio of the initial concentrations of crystallant in the drop and reservoir. In practice, glass vessels in contact with macromolecules should be silicone-treated in a way to obtain hydrophobic surfaces. Typical equilibration trajectories are illustrated in the phase diagram by black arrows, and the evolution of drops after nucleation and during growth are indicated by dashed arrows.

4.1.3.1. Historical development of methods

Protein crystallization is an old field that started more than 100 years ago (McPherson, 1991). Early methods included protein extractions with salts or organic solvents, or dialysis of salt solutions against water, and they were carried out on the gramme scale. Batch crystallization was the method of choice at that time. A first breakthrough that paralleled the development of X-ray methods occurred in the 1960s with the development of micromethods such as dialysis and vapour diffusion (with protein at the 1–100 mg scale and crystallization assays in the 10–50 µl range). However, it became rapidly apparent that screening of the entire

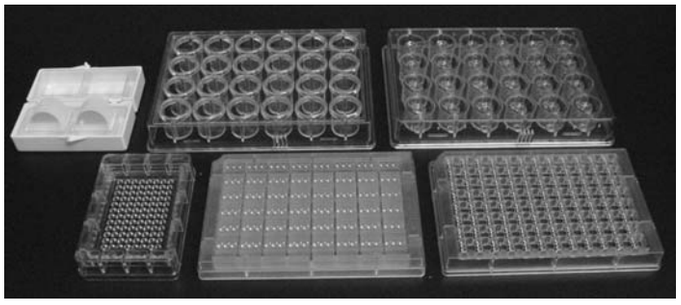


Figure 4.1.3.2
The evolution of crystallization plates from hand-made assays to the high-throughput era. Shown here is an array of crystallization materials and plates. In the upper left is a box of siliconized, glass coverslips used in hanging-drop experiments. Clockwise from the coverslips are a greased VDX plate for hanging-drop experiments, a Cryschem sitting-drop plate, an Intelliplate 96, primarily for use with robotic systems for sitting drops, an Intelliplate 48, and finally, a Vapour–Batch plate that can be used for multiple methods. All of these materials are readily available from commercial sources (photo courtesy of Hampton Research).

crystallization space in searching for appropriate conditions is not feasible in practice. To overcome this difficulty, incomplete factorial methods and other statistical approaches for designing and analysing crystallization experiments were developed (Carter & Carter, 1979; Carter, 1997). A second series of breakthroughs, in the mid-1980s, was stimulated by microgravity research and the advent of structural biology (Giegé *et al.*, 1995). Miniaturization was pursued, molecular biology gave more ready access to samples and more rationality entered the field with, among others, crystallization in gelled media or by counter-diffusion. Moreover, the use of screens was generalized (Jancarik & Kim, 1991) and early robotics facilitated the work of the crystallizers, permitting more extensive screening and yielding improved reproducibility. The last breakthroughs came from structural genomics and have led to further miniaturization (at the nanoscale), more systematic use of robotics and high-throughput screening methods. Finally, advanced methods emerged to produce crystals of better quality (see Section 4.1.4). The size and geometry of crystallization plates recapitulate these methodological developments (Fig. 4.1.3.2).

4.1.3.2. Batch crystallizations

Batch methods are the simplest techniques used to produce protein crystals. They require no more than just mixing the macromolecular solution with crystallants until supersaturation is reached (Fig. 4.1.3.1*a*). Batch crystallization was used to grow crystals in containers of different geometries and samples from the ml to the µl range (McPherson, 1982; Luft *et al.*, 1999*a*). Because one necessarily begins in the supersaturated region of the phase diagram (Fig. 4.1.1.1), nucleation is often excessive. Large crystals, however, can be obtained when the degree of supersaturation is near the metastable region of the crystal/solution phase diagram.

In the early 1990s an automated microbatch crystallization method already allowed the investigation of samples of less than 2 µl (Chayen *et al.*, 1990). In this case, reproducibility is guaranteed. Samples are dispensed and incubated under oil, thus preventing rapid evaporation and uncontrolled concentration changes of the components in the microdroplets. Note, however, that oils are slightly vapour permeable and that the slow evaporation kinetics are dependent on the type of oil (paraffin or silicone oils) used (D'Arcy *et al.*, 1996; Chayen, 1997). The method was adapted for rapid screening of crystallization conditions (D'Arcy, MacSweeney, Stihle & Haber, 2003). It was also adapted for crystallizing proteins in drops suspended between two oil layers (Chayen, 1996; Lorber & Giegé, 1996). Large drops (up to 100 µl) can be deployed allowing easy observation of the crystallization process. Moreover, the absence of contacts between the mother liquor and any solid surfaces results in a lower number of nucleation sites and larger crystals. Note that a hands-free system based on microbatch crystallization in modified X-ray glass capillaries has been developed that allows *in situ* diffraction data collection (Sugahara *et al.*, 2009).

Batch crystallization can also be conducted under high pressure (Lorber *et al.*, 1996) and was adapted for crystallizations on thermal gradients with samples of ∼7 µl accommodated in micropipettes (Luft *et al.*, 1999*b*). This latter method permits rapid screening to delineate optimal temperatures for crystallization and frequently yields crystals of sufficient quality for diffraction analysis. Batch methods also permit crystallizations based on thermonucleation (DeMattei & Feigelson, 1993). In a user-friendly version, transfer of crystallization vessels from one thermostated cabinet to another maintained at a higher or lower temperature, depending on whether the protein has normal or retrograde solubility, can readily be done. A variation of classical batch crystallization is the sequential extraction procedure (Jakoby, 1971), based on the property that the solubility of many proteins in highly concentrated salt solutions exhibits significant, but shallow, temperature dependencies.

4.1.3.3. Dialysis methods

Dialysis readily permits easy variation of many parameters that influence protein crystallization. Different types of systems can be used, but all follow the same general principle. The protein is separated from a large volume of solvent by a semi-permeable membrane that allows passage of small molecules, but prevents that of the macromolecules (Fig. 4.1.3.1*b*). Equilibration kinetics depend on the membrane molecular weight exclusion size, the ratio of the concentrations of crystallant inside and outside the macromolecule chamber, the temperature, and the geometry of the dialysis cell. The simplest technique is to use a dialysis bag (*e.g.* of inner diameter ∼2 mm), but this usually requires at least 100 µl protein solution per trial.

Crystallization by dialysis has been adapted to small volumes (10 µl or less per assay) in microdialysis cells made from capillary tubes closed by dialysis membranes or polyacrylamide gel plugs (Zeppenzauer, 1971). Microdialysis devices exist in a variety of forms; some are derived from the original Zeppenzauer system (Weber & Goodkin, 1970), others are known as the Cambridge button (Reid *et al.*, 1973) or the microdialysis rod (Lee & Cudney, 2004). With such devices, protein solutions are deposited in 10–50 µl depressions in Plexiglass microdialysis buttons, which are then sealed by dialysis membranes fixed by rubber 'O' rings, and subsequently immersed in a solution contained in the wells of Linbro plates (or other vessels). Wells are sealed with glass coverslips and vacuum grease or tape. In the double-dialysis procedure, the equilibration rate is stringently reduced, thereby improving the method as a means of optimizing crystallization conditions (Thomas *et al.*, 1989). Equilibration rates can be manipulated by choosing appropriate membrane molecular weight exclusion limits, distances between dialysis membranes or relative volumes.

4.1.3.4. Vapour-diffusion methods

Crystallization by vapour diffusion was introduced in structural biology for the preparation of tRNA crystals (Hampel *et al.*, 1968). It is well suited for small volumes (as little as 1 µl or less) and has become the favoured method of many experimenters. It is practised in a variety of forms and is the method of choice for robotic applications. In all of its versions, a drop with the protein to be crystallized, together with buffer, crystallant and additives, is equilibrated against a reservoir containing a solution of crystallant at a higher concentration than that in the drop (Fig. 4.1.3.1*c*). Equilibration proceeds by diffusion of the volatile species until the vapour pressure of the drop equals that of the reservoir. If equilibration occurs by water (or organic solvent) exchange from the drop to the reservoir (*e.g.* initial salt concentration in the reservoir higher than in the drop), it leads to a volume decrease of the drop, so that the concentration of all constituents in the drop increases. The situation is reversed if the initial concentration of the crystallizing agent in the reservoir is lower than that in the drop. In that case, water exchange occurs from reservoir to drop. Crystallization of several macromolecules was achieved using this 'reversed' procedure (Giegé *et al.*, 1977; Richard *et al.*, 1995; Jeruzalmi & Steitz, 1997).

Historically, hanging drops have been deployed in Linbro-type tissue culture plates (Fig. 4.1.3.2). These plates contain 24 wells with volumes of ∼2 ml and each well is covered by a glass coverslip sealed with vacuum grease. Drops are composed by mixing 2–10 µl aliquots of the protein with aliquots of the crystallant and additional components, as needed. A variant of the procedure is the HANGMAN method. It utilizes a clear, non-wetting adhesive tape that both supports the protein drops and seals the reservoirs (Luft & De Titta, 1992). Modern arrangements either maintain the 24-well format with more convenient screw-in lids, or adopt the 96-well format with drops deposited on an adhesive sealing tape. The general principle remains the same: a twofold ratio between the concentration of the crystallant in the reservoir and in the drop is most frequently used. This is achieved by mixing a droplet of protein at twice the desired final concentration with an equal volume of the reservoir at the proper concentration (to prevent drops from falling into the reservoir, their final volume should not exceed 15 µl). When no crystals or precipitate are observed in the drops, either sufficient supersaturation has not been reached or only the metastable region has been attained. In the latter case, changing the temperature by a few degrees may be sufficient to initiate nucleation. In the former case, the concentration of precipitant in the reservoir must be increased.

Sitting drops can be dispensed into a variety of different devices. Arrangements consisting of Pyrex plates with a variable number of depressions (up to nine) installed in sealed boxes were used for tRNA crystallization (Dock *et al.*, 1984). Drops of mother liquor are dispensed in the depressions and reservoir solutions with crystallant are poured into the bottom sections of the boxes. These systems are efficient for large drop arrays and can be used for both screening and optimizing crystallization conditions. Multichamber arrangements are suitable for the control of individual assays (Fig. 4.1.3.2). They often consist of polystyrene plates with a variable number of wells (24, 96 and more) that can be individually or collectively sealed. Sitting drops can also be placed on microbridges (Harlos, 1992) or supported by plastic posts in the centres of the wells. Reservoir solutions are contained in the wells in which the microbridges or support posts are placed. Plates with 96 wells, sealed with clear sealing tape, are convenient for large matrix screening. Most of these plates are commercially available and can often be used for a majority of different vapour-diffusion crystallization methodologies (hanging, sitting or sandwich drops, the latter being maintained between two glass plates). A crystallization setup in which drops are deployed in glass tubes, which are maintained vertical and epoxy-sealed on glass coverslips, is known as the plug-drop design (Strickland *et al.*, 1995). Plug-drop units are placed in the wells of Linbro plates surrounded by reservoir solution and then the wells are sealed as usual. With this geometry, crystals do not adhere to glass coverslips as they may with sandwich drops.

Vapour-phase equilibration can be achieved in capillaries (Luft & Cody, 1989) or even directly in X-ray capillaries, as was described for ribosome crystallization (Yonath *et al.*, 1982). This last method may even be essential for fragile crystals, where transferring from crystallization cells to X-ray capillaries or cryo-loops can produce mechanical damage. Vapour-diffusion methods permit easy variations of physical parameters during crystallization, and many successes have been obtained by affecting supersaturation by temperature or pH changes. With ammonium sulfate as the crystallant, it has been shown that the ultimate pH in the drops is imposed by the pH of the reservoir (Mikol *et al.*, 1989). Thus, varying the pH of the reservoir permits adjustment of that in the drops. Sitting drops are also well suited for carrying out epitaxic growth of macromolecule crystals on mineral matrices or other surfaces (McPherson & Shlichta, 1988*a*; Kimble *et al.*, 1998; Stolyarova *et al.*, 2006).

The kinetics of water evaporation (or of any other volatile species) determine the kinetics of supersaturation and, consequently, that of nucleation (Martins, Pëssoa *et al.*, 2008; Martins, Rocha & Damas, 2008). Kinetics measured from hanging drops containing ammonium sulfate, PEG or MPD are significantly influenced by experimental conditions (Mikol, Rodeau & Giegé, 1990; Luft *et al.*, 1996). The parameters that chiefly determine equilibration rates are temperature, initial drop volume (and initial surface-to-volume ratio of the drop and its dilution with respect to the reservoir), water pressure and the chemical nature of the crystallant (Newman, 2005; Newman *et al.*, 2007). The distance separating the drop from the reservoir solution is another critical element and a simple procedure was designed to allow variation of the rate of water equilibration, thereby optimizing crystal-growth conditions (Luft *et al.*, 1996). Evaporation rates can also be monitored and controlled in a weight-sensitive device (Shu *et al.*, 1998). Another method uses oil layered over the reservoir, which permits very slow evaporation of the underlying aqueous solution (Chayen, 1997). The thickness of the oil layer, therefore, dictates evaporation rates and, consequently, crystallization rates (see Section 4.1.3.2).

The period for water equilibration to reach 90% completion can vary from ∼25 h to more than 25 d. The most rapid equilibration occurs with ammonium sulfate, more slowly with MPD, while that with PEG is by far the slowest. An empirical model has been proposed which estimates the minimum duration of equilibration under standard experimental conditions (Mikol, Rodeau & Giegé, 1990). Equilibration, which brings the macromolecules very slowly to a supersaturated state, may explain the crystallization successes with PEG as the crystallant. This explanation is corroborated by experiments showing an increase of the terminal crystal size when equilibration rates are reduced (Chayen, 1997).

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

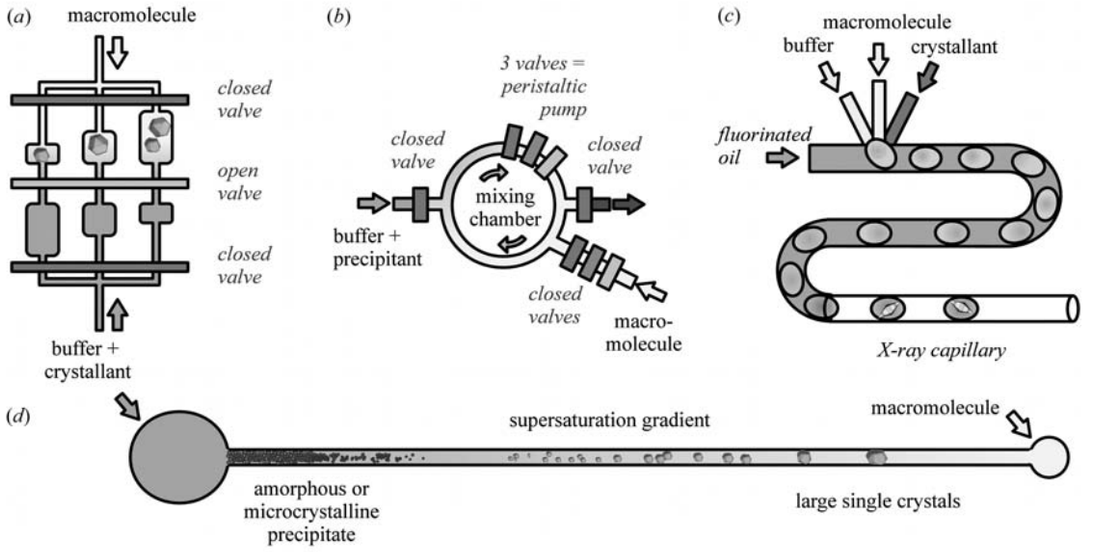


Figure 4.1.4.1
Examples of microfluidic devices designed for biocrystallization. (*a*) Schematic view of one of the 46 modules composing the free-interface diffusion chip. An integrated fluidic circuit dispenses the macromolecule and crystallant solutions into the final chambers. The microchannels are 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-

scopy, Mach–Zehnder and phase-shift Mach–Zehnder interferometry, Michelson interferometry, electron microscopy (EM), atomic force microscopy (AFM) and laser confocal microscopy with differential interference contrast microscopy. Each of these methods provides complementary data which, in combination, have yielded answers to many relevant questions.

Time-lapse video microscopy has been used to measure growth rates (Koszelak & McPherson, 1988; Lorber & Giegé, 1992; Zhu *et al.*, 2001). It was valuable in revealing unexpected phenomena, such as capture and incorporation of microcrystals by larger crystals, contact effects, consequences of sedimentation, flexibility of thin crystals, fluctuations in growth rates and initiation of twinning (Koszelak *et al.*, 1991). Optical microscopy and interferometric methods gave information on concentration gradients that appear as a consequence of incorporation of molecules into the solid state. These methods, however, suffer from rather shallow response dependence with respect to protein concentration. This can be overcome by the introduction of phase-shift methods, as has been successfully achieved in the case of Mach–Zehnder interferometry. With this technique, gradients of protein concentration have been mapped in the mother liquor and around growing crystals. Classical Mach–Zehnder interferometry has been used to monitor diffusion kinetics and supersaturation levels during crystallization, as was done in dialysis setups (Snell *et al.*, 1996) or in counter-diffusion crystal growth cells (Garcia-Ruiz *et al.*, 1999). Using laser confocal microscopy combined with differential interference contrast microscopy it was possible to visualize dislocations in protein crystals during growth (Sazaki *et al.*, 2005). Single-molecule visualization techniques gave access to direct observation of the diffusion of individual fluorescence-labelled protein molecules at an interface between a solution and a protein crystal (Sazaki *et al.*, 2008).

Michelson interferometry can be used for direct growth measurements on crystal surfaces (Komatsu *et al.*, 1993). It depends on the interference of light waves from the bottom surface of a crystal growing from a reflective substrate and the top surface, which is developing and, therefore, changes as a function of time with regard to its topological features. Because growth of a crystal surface is generally dominated by unique growth centres produced by dislocations or two-dimensional nuclei, the surfaces and the resultant interferograms change in a regular and periodic manner. Changes in the interferometric fringes with time provide accurate measures of the tangential and normal growth rates of a crystal (Vekilov *et al.*, 1993; Kuznetsov *et al.*, 1995; Kurihara *et al.*, 1996). From these data, one can determine the surface free energy and the kinetic coefficients that underlie the crystallization process.

EM (Durbin & Feher, 1990) and especially AFM (McPherson *et al.*, 2004) are powerful techniques for the investigation of crystallization mechanisms and their associated kinetics. The power of AFM lies in its ability to investigate crystal surfaces *in situ*, while they are still developing, thus permitting one to directly visualize, over time, the growth and change of a crystal face at near-nanometre resolution. The method is particularly useful in delineating the growth mechanisms involved, identifying dislocations, recording the kinetics of the changes and directly revealing impurity effects on the growth of protein crystals (Konnert *et al.*, 1994; Malkin *et al.*, 1996; Nakada *et al.*, 1999) (Fig. 4.1.5.1). AFM was also applied for the visualization of growth characteristics of crystals made of viruses (Malkin *et al.*, 1995) and RNA (Ng, Kuznetsov *et al.*, 1997). A noteworthy outcome of such studies was the sensitivity of growth to minor temperature changes. A variation of 2–3 °C was sufficient to transform the growth mechanism of yeast $tRNA^{Phe}$ from spiral screw dislocation growth at low supersaturation to two-dimensional island formation at high supersaturation (Ng, Kuznetsov *et al.*, 1997).

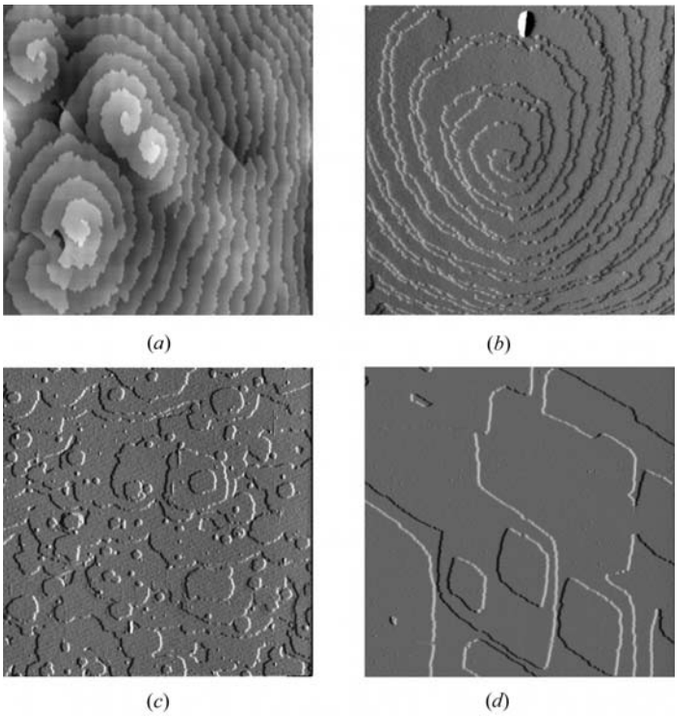


Figure 4.1.5.1
Growth mechanisms and visualization of protein crystal surfaces by AFM. In (*a*) and (*b*) are images of screw dislocations on the surfaces of crystals of the proteins canavalin and trypsin, respectively. The scan areas are 10 μm^2 in (*a*) and 30 μm^2 in (*b*). Screw dislocation growth predominates at low supersaturation. In (*c*) and (*d*) are examples of crystal growth by the formation of two-dimensional islands on the surfaces of crystals of the proteins thaumatin and glucose isomerase, respectively. The scan areas are 20 μm^2 in (*c*) and 11 μm^2 in (*d*). Growth by two-dimensional island formation and spread dominates at higher supersaturation.

4.1.5.3. Uncoupling nucleation and growth, and the constant-growth regime

The preparation of high-quality protein crystals should preferentially occur at lowest supersaturation and under a constant-growth regime. Achieving this aim with conventional crystallization methods is *a priori* not easy since growth of crystals is accompanied by a decrease of supersaturation in the crystallization medium (see the trajectory of crystal/solution equilibration in a phase diagram, Figs. 4.1.1.1 and 4.1.3.1). The implication is that it is possible to change the growth regime during the course of the crystallization process, as could be seen by AFM (Ng, Kuznetsov *et al.*, 1997). Such a change will perturb crystal formation and probably accounts for the frequently observed non-reproducibility in diffraction properties of protein crystals. Using flow cells with a constant supply of fresh protein may help to overcome this difficulty. Separating the nucleation and growth phases is another alternative (Chayen, 2005). This can be straightforwardly done by seeding procedures (see Section 4.1.4.4.).

4.1.5.4. Crystal perfection

The ultimate objective of structural biologists is to analyse crystals of high perfection, in other words, with a minimum of

defects, disorder, impurity incorporation and internal stress. Such imperfections can be visualized using laser confocal microscopy combined with differential interference contrast microscopy (Iimura *et al.*, 2005; Sazaki *et al.*, 2005). They can also be evaluated by the resolution limit of diffraction, which expresses the average disorder of the molecules in the crystal lattice. Wilson plots provide good illustrations of diffraction quality for protein crystals. Other sources of disorder, such as the mosaic structure of the crystal, may strongly influence the quality of the diffraction data. They are responsible for increases in the diffuse background scatter and a broadening of diffraction intensities. These defects are difficult to monitor with precision, and dedicated techniques and instruments are required for accurate analysis.

Mosaicity can be defined experimentally by X-ray rocking width measurements. An overall diagnostic of crystal quality can be obtained by X-ray diffraction topography. Both techniques have been refined with lysozyme as a test case and were used for comparative analysis of crystals grown under different conditions, both on earth and in microgravity. For lysozyme and thaumatin, improvement of the mosaicity, as revealed by decreased rocking widths measured with synchrotron radiation, was observed for the microgravity-grown crystals (Snell *et al.*, 1995; Ng, Lorber *et al.*, 1997; Lorber, Sauter, Robert *et al.*, 1999).

An illustration of mosaic block character in a lysozyme crystal was provided by X-ray topography (Fourme *et al.*, 1995). Comparison of earth- and microgravity-grown lysozyme crystals showed a high density of defects in the earth control crystals, while in the microgravity case, several discrete regions were visible (Stojanoff *et al.*, 1996). X-ray topographs have also been used to compare crystal polymorphs (Izumi *et al.*, 1996), to monitor temperature-controlled growth of tetragonal lysozyme crystals (Stojanoff *et al.*, 1997), to study the effects of solution variations during growth on crystal perfection (Dobrianov *et al.*, 1998; Otálora *et al.*, 1999), to compare crystals grown in solution and in agarose gel (Lorber, Sauter, Ng *et al.*, 1999), and to map defects in the bulk of protein crystals (Hu *et al.*, 2001).

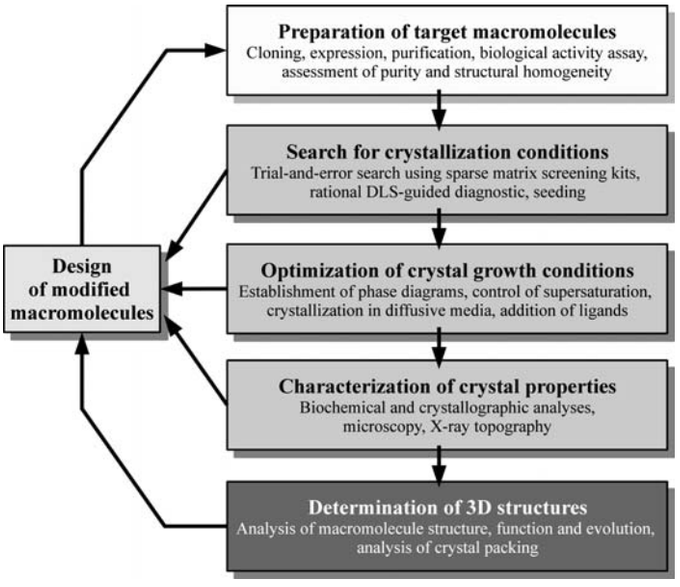


Figure 4.1.6.1
From the target molecule to its three-dimensional structure: a flowchart for a structure detemination. The figure illustrates the different steps the crystal grower will go through to access three-dimensional information. Ideally, the trajectory will be linear, crystallization remaining the major bottleneck in the whole process. If one gets stuck at any stage, the difficulties can often be circumvented by modifying the target molecule, either genetically or (bio)chemically, by improving its purity/homogeneity, or by adding ligands to stabilize its structure.

4.1.6. How to crystallize a new macromolecule: the structural biology view

Fig. 4.1.6.1 schematizes the progress of structural biology projects and highlights the pivotal role of the crystallization step. Despite tremendous progress in macromolecule and crystal preparation, crystallization remains the most serious bottleneck. Various strategies have been proposed to overcome the difficulty (*e.g.* D'Arcy, 1994; Ducruix & Giegé, 1999; Kundrot, 2004; McPherson, 2004; DeLucas *et al.*, 2005; Pusey *et al.*, 2005; Hughes & Ng, 2007; Li *et al.*, 2007; Chayen & Saridakis, 2008). The choice is largely dependent on the type of project [functional biology with well defined protein(s) or structural genomics with large ensembles of known and unknown proteins] and thus on non-exclusive rational, incomplete factorial screening, or empirical approaches.

4.1.6.1. How to start and how to choose what screening kits to start with

In practice, the very first questions posed when one begins to think about crystallization assays are: (i) which crystallization kit to start with, (ii) what to do if no crystals grow and (iii) what happens when one gets crystals. The answer to the first question is in fact simplest. One should start with a screen that does not unreasonably tax the supply of protein but which explores the widest volume of crystallization space, *i.e.* samples the largest number of crystallants and crystallant concentrations over the largest range of pH. There are many kits on the market using 96-sample trials that accomplish this well. If protein is severely limited, then there are 48-sample kits that do the job satisfactorily. In case of no success one should examine the resulting pattern of precipitates, phase separations, clear drops and assorted odd accumulations, and try to divine what might be the best option to try next. Good advice is to check how others have crystallized similar proteins and to try complexes of the protein with its physiological ligands and effectors.

If, on the other hand, one is fortunate and the first kit does indeed yield crystals, but perhaps of insufficient size or quality, or of troubling morphology, then crystallization must be optimized to get better ones. One should also check whether it is really the macromolecule that crystallized and not a ligand or any component of the crystallization mixture. Optimization means varying the chemical and physical parameters of the reagent mix that yielded the crystals and searching the crystallization parameter space by small increments away from the starting point. There is still a component of art and mystery in science, and this is one instance where its appreciation is paramount.

As a practical example, one can consider the 'Silver bullet' concept, which employs a limited set of fundamental crystallization conditions combined with a broad screen of potentially useful small-molecule additives (McPherson & Cudney, 2006), with the hypothesis that some of them might establish intermolecular noncovalent cross-links (Larson *et al.*, 2008). In the 'Uppsala' concept, a crystallization project entails two distinct steps: screening and optimization. The aim of the initial phase is to screen the parameters affecting crystallization as broadly as possible. If promising conditions are found, these are optimized with other protocols including grid and additive screens, seeding, and manipulation of drop kinetics (Bergfors, 2007).

4.1.6.2. Rules and general principles

Rules for sample preparation. The first concern is to obtain a macromolecular sample of the highest quality, second to collate all biochemical and biophysical information characterizing the macromolecule in order to design the best crystallization strategy, and finally, to establish precise protocols that ensure reproducibility of experiments. Solubility screening is an important issue and can be automated (Listwan *et al.*, 2009). Important as well is to clean and sterilize by filtration (over 0.22 µm porosity membranes, or even 0.10 µm) all solutions in contact with pure proteins, to remove dust and other solid particles, and to avoid contamination by microbes (Chayen, 2009). Inclusion of sodium azide in crystallizing solutions may discourage invasive bacteria and fungi. In vapour-diffusion assays, placing a small grain of thymol in the reservoir can prevent contamination. Thymol, however, can occasionally have specific effects on crystal growth (Chayen *et al.*, 1989) and thus may serve as an additive in screenings as well.

The preparation of solutions for crystallization experiments should follow some common rules. Stocks should be prepared with chemicals of the purest grade dissolved in double-distilled water, filtered through 0.22 µm membranes and ultracentrifuged (1 h at 100 000*g*) to remove aggregates. The chemical nature of the buffer is an important parameter, and the pH of buffers, which must be strictly controlled, is often temperature dependent, especially that of Tris buffers. Commercial PEG may contain ionic contaminants (Jurnak, 1986), or contaminants derived from peroxidation, and thus repurification is recommended (Ray & Puvathingal, 1985).

Mother liquors are defined as the solutions that contain all compounds (buffer, crystallant *etc.*) at the final concentration for crystallization, except the protein (to avoid dissolution of the crystals when transferred to a fresh mother liquor, soluble protein should be present). Samples of proteins often contain quantities of salt of unknown composition, and it is therefore wise to dialyse new batches against well characterized buffers. Whatever the crystallization method used, it almost always requires a high concentration of protein. This may imply concentration steps using devices operating under nitrogen pressure, by centrifugation or by lyophilization (note that lyophilization may denature proteins and that non-volatile salts will accumulate). Dialysis against high-molecular-weight PEG may also be used. During concentration, the pH and ionic strength may vary and, if not kept at the appropriate values, denaturation of samples may occur.

How to use phase diagrams and favour reproducibility. Crystallization requires bringing the protein to a supersaturated state that favours nucleation. Use of phase diagrams may be important for that purpose (Haas & Drenth, 1999; Sauter, Lorber *et al.*, 1999; Asherie, 2004; Lorber & Witz, 2008). If solubilities or phase diagrams are unavailable, it is nevertheless important to understand the correlation between solubility and the way supersaturation is reached in the different crystallization methods (Fig. 4.1.3.1). In dialysis, the protein concentration remains constant during equilibration. The initial concentration of the crystallant in the exterior solution leaves the macromolecule in an undersaturated state. With increasing concentration of this agent in the exterior solution, a state of supersaturation can be attained, leading to crystallization or precipitation. In a vapour-diffusion experiment, where the concentration of crystallant in the reservoir exceeds that in the drop, the protein will begin to concentrate from an undersaturated to a supersaturated state, with both protein and crystallant concentrations increasing. Crystals appear in the labile region. For crystals that appear first, the trajectory of equilibration is complex and the remaining concentration of protein in solution will converge towards a point located on the solubility curve. In batch crystallization using a closed vessel, three situations can occur: if the concentration of the protein is undersaturated, crystallization never occurs (unless another parameter such as temperature is varied); if it belongs to the supersaturated region between solubility and precipitation curves, crystals can grow until the remaining concentration of the protein in solution equals its solubility; if supersaturation is too high, the protein precipitates immediately, although in some cases crystals can grow from precipitates by Ostwald ripening (Ng *et al.*, 1996; Lorber & Witz, 2008). Altogether, the crystal habits (*e.g.* Sauter, Lorber *et al.*, 1999; Zhu *et al.*, 2001) and the underlying growth mechanisms (Fig. 4.1.5.1) are dependent on the region in the phase diagram where nucleation occurred and on the equilibration trajectories (Figs. 4.1.1.1 and 4.1.3.1).

Reproducibility should be a strategic concern (Newman *et al.*, 2007), but is often not reached despite the efforts of investigators to work with protein and crystallant solutions of identical chemical composition. Besides the many reasons that could explain this situation, the effect of the mixing protocol of the different components constituting a crystallization drop on the output of the experiment is often overlooked. Since mixing is not instantaneous, it will generate local supersaturation maxima and minima that can affect the crystallization process. Thus, when seeking reproducibility, active mixing of the crystallization drops is recommended, which is easy to perform when working manually but not trivial in automated procedures (Howard *et al.*, 2009).

4.1.6.3. Database mining and statistics

With the number of protein structures solved by X-ray crystallography now approaching 100 000, substantial databases of successful crystallization conditions and procedures have been developed (*e.g.* Peat *et al.*, 2005; Li *et al.*, 2007; Tung & Gallagher, 2009). From these it might be expected that some predictive insight would be obtained regarding the most probable crystallization conditions for proteins in general (*e.g.* Kimber *et al.*, 2003), and for specific families of proteins sharing common physical, chemical or functional properties such as aminoacyl-tRNA synthetases (Giegé *et al.*, 2008). However, the problem is that each protein remains an individual endowed with its own eccentricities, even within a family, and often these dramatically alter its crystallization behaviour. In addition, proteins in general may be exquisitely sensitive to only minor modifications to their properties, further complicating their rational classification in terms of crystal growth.

Nonetheless, statistical analyses are now being widely applied to the expanding databases and some results, both interesting and useful, are beginning to emerge. For example, reduced sets of the most favourable crystallization conditions have been proposed by several groups of investigators based on past successes. These may be useful because they reduce the number of crystallization trials in cases where either the amount of protein is limited or a very large number of constructs are to be screened. Favoured reagents and, particularly, useful additives have been identified by such analyses. Correlations have been sought between the physical or chemical properties of specific proteins and their manner of crystallization, such as between pI and crystallization pH, but this has had only limited success. A promising method

generates relatively accurate predictions from protein composition and collocation of amino acids, pI and hydrophobicity deduced from sequence data, and number of solved structures of similar sequences (Mizianty & Kurgan, 2009). Interestingly, data mining of biophysical properties that control protein crystallization led to the conclusion that crystallization propensity depends primarily on the prevalence of well ordered surface epitopes capable of mediating interprotein interactions rather than on the overall thermodynamic stability of the proteins (Price *et al.*, 2009).

4.1.6.4. Strategic concerns: a summary

Homogeneity and stability. Perhaps the most important property of a system to be crystallized is its purity. Crystallization presupposes that identical units are available for incorporation into a periodic lattice. If crystallization fails, reconsidering purification protocols often helps achieve success. No homogeneous molecular population can remain so if its members alter their form, folding or association state. Hence, it is crucial that proteins in solution not be allowed to denature, aggregate or undergo conformational changes.

Solubility. Before a molecule can be crystallized, it must be solubilized. This means creation of monodisperse solutions free of aggregates and molecular clusters. Solubility and crystallizability strongly depend on substances (organic solvents and PEGs) that reduce the ionic strength of the solution (Papanikolau & Kokkinidis, 1997).

Supersaturation and nucleation. Crystals grow from systems displaced from equilibrium so that restoration requires formation of the solid state. Thus, the first task is to find ways to alter properties of the crystallizing solutions, such as by pH or temperature change, and to create supersaturated states. The number, size and quality of crystals depend on the mechanisms and rates of nuclei formation. For diffraction work in crystallization, one must seek to induce limited nucleation by adjustment of the physical and chemical properties of the system.

Association and variety. In forming crystals, molecules organize themselves through self-association to produce periodically repeating, three-dimensional arrays. Thus, it is necessary to facilitate positive molecular interactions while avoiding formation of precipitate, unspecific aggregates or phase separation. Proteins may crystallize under a wide spectrum of conditions and form many polymorphs. Thus, one should explore as many opportunities for crystallization as possible and explore the widest spectrum of biochemical, chemical and physical parameters.

Control and perfection. The ultimate value of any crystal is dependent on its perfection. Perturbations of the mother liquor are, in general, deleterious. Thus, crystallizing systems have to be maintained at an optimal state, without fluctuations or shock, until the crystals have matured. Crystallization conditions should be such as to favour crystal perfection, to minimize defects and high mosaicity of the growing crystals, and to minimize internal stress and incorporation of impurities. Predictions from crystal-growth theories may help to define such conditions (Chernov, 2003).

Impurities and preservation. Impurities can contribute to a failure to nucleate or to grow quality crystals. Thus, one must discourage their presence in the mother liquor and their incorporation into the lattice. Protein crystals may degrade and lose diffraction quality upon ageing. Thus, once grown, crystals may be stabilized by temperature change, addition of more crystallant or by some other suitable alteration in the mother liquor.

Rational design versus *automation*. When an investigation is focused on a single crystallographic objective, the structure solution of a specific protein or macromolecular complex, then it is wise to put as much time into headwork (thinking) as into handiwork (setting up crystallization samples). Examining and evaluating results, divining insights and setting new directions are certainly as important as actually dispensing samples into crystallization plates. There are, however, instances where it is more efficient to automate the process by which crystallization conditions are identified and optimized. This is true when there is not one, but many, real or potential targets.

4.1.7. The future of protein crystal growth

The fact that high-quality X-ray diffraction data can frequently be obtained from a single crystal of dimensions in the range of 20 to 50 μm has changed the objectives considerably from 40 years ago, when many crystals in the millimetre size range were required for a structure analysis. A consequence of this is that attention is turning increasingly from the systematic growth of large protein crystals (Bailey, 1942) to the nucleation and growth of any crystal. This direction has been further promoted by the development in the last 20 years of methods to use even the data from twinned or disordered crystals. One might be led to believe that the future of crystal growth is shrinking dramatically. Indeed, while smaller, fewer crystals are now the rule (with the exception of those required for neutron diffraction), this has not reduced the value of crystal perfection, nor the requirement that at least some sort of crystal be obtained. Thus, attention is now focused on nucleation, perhaps always the most problematic step in the crystallization process, and enhancing crystal perfection. These continue to remain formidable problems.

Furthermore, the objectives of crystallization, the entities to be crystallized, will continue to become more challenging. In addition to membrane proteins that pose difficult problems because of their solubility (see Chapter 4.2), interest has increasingly turned towards the solutions of the structures of RNA, glycoproteins (Chang *et al.*, 2007), lipoproteins, and larger protein or protein–nucleic acid complexes and assemblies. It is unlikely that crystals with unit cells much above 1200 Å can be solved with current X-ray technologies, but even those assemblies, such as large icosahedral viruses, that do yield crystals amenable to analysis are remarkably fragile in a mechanical sense, and the large unit-cell size requires that the crystals greatly exceed the small sizes of conventional protein crystals. Additional problems will arise from proteins conjugated with other entities of significant size such as lipids and oligosaccharides, which are often disordered, and with proteins that are unstructured, in whole or in part.

Finally, we have come to believe that the structure of a protein in the crystal is the same as the structure of the protein in solution. But when the protein has a spectrum of conformations in solution, as a consequence often of its function, then to visualize it in full one needs to see it in multiple crystal forms. Thus, it will be increasingly necessary to grow crystals not simply of the apo protein, but also of its possible ligand complexes, and possibly in several polymorphs. By studying the protein in a variety of crystal forms, its conformational variety may be appreciated and its dynamic range delineated.

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