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).

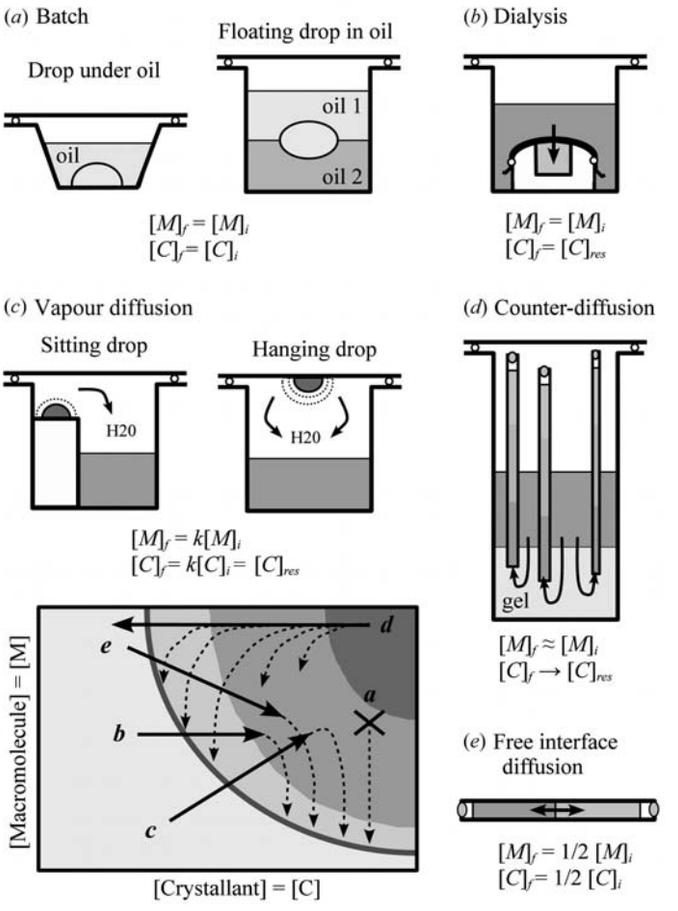

Figure 4.1.3.1
Principles of major methods used to crystallize biological macromolecules. (*a*) Two versions of batch crystallization. (*b*) Dialysis method with Cambridge button. (*c*) Vapour-diffusion crystallization with sitting and hanging drops. (*d*) Counter-diffusion setup in X-ray capillary tubes 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

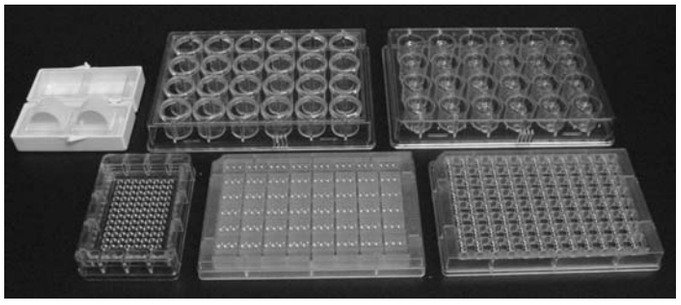

Figure 4.1.3.2
The evolution of crystallization plates from hand-made assays to the high-throughput era. Shown here is an array of crystallization materials and plates. In the upper left is a box of siliconized, glass coverslips used in hanging-drop experiments. Clockwise from the coverslips are a greased 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