TOPICAL REVIEW

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Comparative Studies of Protein Crystallization by Vapour-Diffusion and Microbatch Techniques

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Abstract

Numerous reports have been published in the literature which describe the crystallization of macromolecules by a variety of crystallization methods, including the vapour-diffusion and microbatch techniques. This topical review compares the results of examples of proteins which were crystallized by both vapour-diffusion and microbatch methods. The inherent features of the vapour-diffusion and microbatch methods are discussed and some specific conditions where one method appears more favourable than the other are reported. Guidelines for the conversion of crystallization conditions from vapour diffusion to microbatch (and *vice versa*) are also presented.

Naomi Chayen graduated with a BSc in Pharmacy, followed by an MSc in Pharmaceutical Sciences from the Haddassa Medical Centre, Hebrew University of Jerusalem and worked there as a research assistant and instructor until 1981. She conducted research at the Kennedy Institute of Rheumatology and at Chelsea College, London, and in 1984 obtained a PhD in Biochemistry. In 1984, Chayen joined the Physics Department (Biophysics Section) at Imperial College of Science, Technology and Medicine, London where she has been since, working first with Professor John Squire on muscle structure, and, from 1986, with Professor David Blow FRS on protein crystals. Chayen's scientific interests are focused on the crystallization of proteins and other biological macromolecules, in particular, developing experimental techniques for crystallization. Her work involves the development of novel micromethods and improvement of existing techniques to aid macromolecular crystal growth, as well as analytical studies on the fundamental principles of the crystallization process. Other aspects of her research include experiments on protein crystallization in microgravity; she has been principal investigator on several ESA/NASA missions.

1. Introduction

The choice of method for securing successful results in crystallization trials is an important issue for the crystallization community (Chayen *et al.*, 1996).

Vapour diffusion is the most widely used technique and has produced more crystallized macromolecules than all other methods combined. Batch and dialysis techniques have generated a smaller number of crystals (McPherson, Malkin & Kusnetsov, 1995, based on the data of Gilliland, Tung, Blakeslee & Ladner, 1994), while free-interface diffusion appears to be emerging as the method of choice for crystallization in microgravity (Koszelak, Day, Leja, Cudney & McPherson, 1995; Chayen, Snell, Helliwell & Zagalsky, 1997) rather than for ground experiments. Handling difficulties associated with microdialysis have lead to the increased popularity of batch methods, particularly the microbatch technique and its version which automatically dispenses small trials (1–2 µl final volume) under oil (Chayen, Shaw Stewart, Maeder & Blow, 1990; Chayen, Shaw Stewart & Blow, 1992; Chayen, Shaw Stewart & Baldock, 1994).

All methods of crystallization involve a phase transition in which the protein is in solution at the start of the experiment and comes out of the solution to form crystals when the solution is brought into supersaturation. Once nuclei have formed, the concentration of protein in the solute will drop, thereby leading the system into the metastable zone where growth should occur without the formation of further nuclei (Ducruix & Giegé, 1992; Riès-Kautt & Ducruix, 1992; Mikol & Giegé, 1992; Ataka, 1993; Saridakis, Shaw Stewart, Lloyd & Blow, 1994) (Fig. 1). This paper concentrates on the comparison between the vapour-diffusion and microbatch methods. Other crystallization methods are referred to in detail by McPherson (1982) and by Ducruix & Giegé (1992) and references therein.

1.1. *Vapour diffusion*

Vapour-diffusion methods involve an aqueous drop containing the protein and the crystallization agents in an

amount lower than that required for the formation of crystals. This drop is equilibrated against a reservoir which gradually concentrates the ingredients in the protein drop until equilibrium is reached. During the diffusion process (in which both precipitating agents and the protein become concentrated), a single crystallization trial proceeds through a range of conditions, thereby conducting a self-screening process (dashed curve *B*, Fig. 1). In the hanging-drop technique, a drop up to 10 µl hangs from a cover slip above the reservoir. The sitting-drop and sandwich-drop methods can accommodate a larger drop resting on a surface close to the reservoir.

1.2. *Batch crystallization*

In the batch method, the protein to be crystallized and the crystallizing agents are mixed at their final concentrations at the start of the experiment. Supersaturation is thus achieved upon mixing, and conditions only change as protein comes out of solution into the growing crystals. There is less exploration of the phase diagram, and consequently, several microbatch trials may be required to replace a single vapour-diffusion experiment.

The batch method is associated with the use of large quantities of material, and hence it is not widely adopted. The introduction of the microbatch technique, where the crystallization samples are dispensed as small drops under oil, has overcome the requirement for large quantities of materials, as well as some other difficulties (discussed below) arising from diffusion methods.

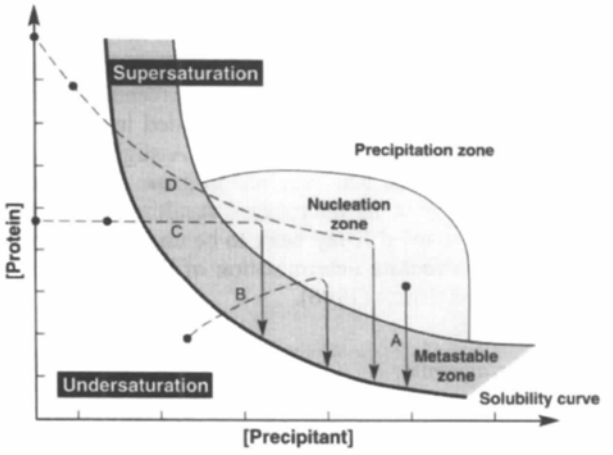

Fig. 1. A schematic drawing of a protein crystallization phase diagram based on two of the most commonly varied parameters, protein and precipitant concentrations. The four major crystallization methods are highlighted showing that in order to produce crystals, all the systems need to reach the same destination, namely the nucleation zone, after which they make their way through the metastable zone and eventually arrive at the solubility curve. Each method achieves this journey *via* a different route. O represents the starting conditions. In the case of dialysis and free-interface diffusion two alternative starting points are shown since the undersaturated protein solution can contain solely protein or alternatively, protein with a low concentration of the precipitating agents. (*A*) Batch crystallization. (*B*) Vapour diffusion. (*C*) Dialysis. (*D*) Free-interface diffusion (also known as liquid/liquid diffusion).

1.3. *The microbatch technique*

The objective of the microbatch technique is to reduce the consumption of sample by generating crystallization trials in very small volumes. It is virtually impossible to accurately compose a drop from all the different components manually. To that end, a computer-controlled micro-dispenser (IMPAX), able to dispense small drops (1–2 µl final volume), ready mixed as batch trials under oil (Chayen, Shaw Stewart, Maeder & Blow, 1990; Chayen, Shaw Stewart & Blow, 1992; Chayen, Shaw Stewart & Baldock, 1994) was designed. The micro-dispenser is used to automatically screen numerous crystallization conditions as 1–2 µl trials. When favourable conditions for crystallization are found, the quality of crystals is optimized by generating a set of conditions throughout the area of interest (Chayen, Shaw Stewart, Maeder & Blow, 1990). The samples, which are dispensed and incubated under the surface of a low-density oil ($\rho = 0.83$–0.92 g cm^{-3}), are protected from evaporation, contamination and physical shock by the oil (Chayen, 1997*a*).

This paper presents some examples of proteins which were crystallized by both vapour-diffusion and microbatch methods and compares the results. The inherent features of the vapour-diffusion and microbatch methods are discussed, and specific conditions where one method appears more favourable than another are reported. Guidelines for the conversion of crystallization conditions from vapour diffusion to microbatch (and *vice versa*) are also presented.

2. Results

Table 1 presents examples of proteins which were crystallized by both the vapour-diffusion and microbatch methods showing the success of each method. The time span of crystallization is normally similar in both methods. A variety of precipitating agents and additives including salts, polyethylene glycol (PEG), Jeffamine, 2-methyl-2,4-pentanediol (MPD), phenol and detergents were tested. All microbatch trials were set up under paraffin oil (paraffin liquid, light colourless, BDH, Poole, Dorset, UK).

2.1. *Glucose isomerase*

Glucose isomerase from *Arthrobacter* B3728 crystallizes in three different forms: a trigonal bipyramidal form is produced when thymol is present in the crystallization medium as an additive, while two orthorhombic forms are attained in its absence (Chayen, Lloyd, Collyer & Blow, 1989).

The original crystallization conditions were reported by Akins, Brick, Jones, Hirayama, Shaw & Blow

Table 1. *Examples of proteins crystallized by vapour-diffusion and microbatch methods showing the success of each method*

✓ Successful crystallization. ✗ Unsuccessful crystallization in spite of repeated trials. The conditions of measurement and details of the crystals are given in the relevant references. In the cases where the crystals were obtained using both methods, crystal size, habitat and X-ray resolution were very similar.

Protein	Precipitant	Vapour diffusion	Microbatch
Hen egg-white lysozyme	NaCl	✓ Ducruix & Giegé (1992)	✓ Chayen *et al.* (1993)
Thaumatin	Na,K tartrate	✓ Cudney (1993)	✓ See Table 2
5S rRNA fragment	MPD	✓ Lorenz *et al.* (1993)	✓ Lorenz (unpublished work)
Lysyl-tRNA synthetase	PEG 4K + LiCl	✓ Onesti *et al.* (1994)	✓ Chayen & Saridakis (Table 2)
Firefly luciferase	PEG 8K + $LiSO_4$ + glycerol	Unstable, Conti *et al.* (1996)	✓ Conti *et al.* (1996)
Apocrustacyanin C_2	NH_4 sulfate + MPD	✓ Wright *et al.* (1992)	✓ See Table 2
Glucose isomerase (orthorhombic forms)	NH_4 sulfate	✓ Akins *et al.* (1986), Chayen *et al.* (1989)	✓ See Table 2
Glucose isomerase (trigonal bipyramids)	NH_4 sulfate + thymol	✓ Akins *et al.* (1986), Chayen *et al.* (1989)	✗ Chayen (unpublished work)
α-Dendrotoxin	NH_4 sulfate + phenol	✓ Skarzynski (1992)	✗ Skarzynski (unpublished work)
β-Crustacyanin	Na,K phosphate	✗ Normile (1995)	✓ Chayen *et al.* (1996)
CP43	PEG + detergents	Very poor quality, Hankamer (unpublished work)	15–20 Å, Hankamer *et al.* (1992)
Xylose isomerase (*Thermogata neapolitana*)	Jeffamine ED 4K	✗ Chayen (unpublished work)	✓ Chayen *et al.* (1997)

(1986) who grew all three crystal forms by the hanging-drop method. Orthorhombic crystals of equal size to those grown in hanging drops were successfully grown in microbatch under oil, by only a slight modification of the concentrations of the protein and precipitating agent (Table 2). However, the trigonal crystals could not be reproduced in microbatch. It was evident that the thymol, being a small organic molecule was dissolved by the oil, thereby disappearing from the drop and preventing the formation of the trigonal crystals.

2.2. *Snake dendrotoxin*

Automated screening of conditions for the crystallization of α-dendrotoxin from green mamba venom was performed in microbatch trials. No crystals were formed, only a transparent glass-like solid phase appeared. Nevertheless, the solubility characteristics for the protein were established. The conditions that produced the appearance of the solid phase were transferred to vapour-diffusion trials in which additives, among them phenol, were tested. In the absence of any additives, the transparent, glass-like solid phase was also produced in the vapour-diffusion trials. Single diffracting crystals were eventually obtained in hanging drops, to which phenol was applied as an additive (Skarzynski, 1992).

2.3. *Apocrustacyanin C_2*

The crystallization in hanging drops of apocrustacyanin C_2, a subunit of the protein α-crustacyanin was reported by Wright *et al.* (1992). The crystallization medium requires 5% MPD. Based on the experience with glucose isomerase and α-dendrotoxin, it was expected that this protein may not crystallize in the presence of oil. Nevertheless crystals of equal quality to those grown in hanging and sitting drops were attained in the microbatch (Table 2).

2.4. *Firefly luciferase*

In the case of the crystallization of firefly luciferase from *Photinus pyralis* reported by Conti, Lloyd, Akins, Franks & Brick (1996), both screening and optimization of conditions were performed in hanging drops using the Jancarick & Kim (1991) sparse-matrix screening protocol. Crystals were obtained but they tended to dissolve after observation under the microscope. A slight adaptation of the conditions was required to grow crystals of equal form and size in microbatch resulting in stable crystals which enabled X-ray work to be undertaken, and leading to the structure determination of this protein by Conti, Franks & Brick (1996).

2.5. *β-Crustacyanin*

β-Crustacyanin is a protein of the lipocalin family, isolated from lobster carapace. Extensive attempts to crystallize this purple-coloured protein by vapour diffusion and liquid/liquid diffusion had failed, when trials using the microbatch method were embarked upon. The project was nearly abandoned when 2 months after setting up the experiments the drops remained totally clear. Modification of the crystallization conditions resulted in precipitation within 24 h but no crystals were seen. Eventually, after a period of four months, crystals measuring 0.5 × 0.1 × 0.1 mm (Fig. 2) were observed from the original conditions which were set up under the paraffin oil (Chayen, Gordon, Phillips, Saridakis & Zagalsky, 1996; Normile, 1995).

Table 2. *Conversion between vapour diffusion and microbatch methods*

Protein	Reference for vapour diffusion	Crystallization compounds	Vapour-diffusion conditions	Microbatch conditions
Thaumatin	Cudney (1993)		100 mg ml^{-1}†	25–30 mg ml^{-1}‡
		PIPES or ADA pH 6.5–6.8	100 m*M*	100 m*M*
		Na,K tartrate	1 *M*	0.5–0.6 *M*
Glucose isomerase from *Arthrobacter* (orthorhombic forms)	Chayen *et al.* (1989)		12 mg ml^{-1}†	8 mg ml^{-1}‡
		Tris–HCl pH 7.0	50 m*M*	50 m*M*
		Ammonium sulfate	1.5 *M*	1.2–1.5 *M*
		$MgCl_2$	10 m*M*	10 m*M*
Lysyl-tRNA synthetase	Onesti *et al.* (1994)		12 mg ml^{-1}†	9.5–12 mg ml^{-1}‡
		PIPES pH 6.8	100 m*M*	100 m*M*
		PEG 4K	20%	16–18%
		LiCl	0.5 *M*	0.5 *M*
Apocrustacyanin C_2	Wright *et al.* (1992)		20 mg ml^{-1}†	12.5 mg ml^{-1}‡
		Tris–HCl pH 9.0	100 m*M*	100 m*M*
		Ammonium sulfate	1.4 *M*	1.0 *M*
		MPD	5%	2.5–5%
		EDTA	1 m*M*	0.5–1 m*M*

† Concentration of the stock solution of protein. ‡ Final concentration of protein in the trial (see text for explanation).

In the quest to obtain crystals within a shorter span of time, attempts were made to convert the conditions to vapour diffusion, nevertheless no crystals were attained.

It transpired that what was taking place was not a true batch experiment but a combination of batch and diffusion. In a typical microbatch experiment, crystallization takes place within a week or two. During this time the drop is well sealed by the paraffin oil. Since water and paraffin oil are essentially immiscible, evaporation during this time is negligible. However, given ample time, slow evaporation can occur (as there is no absolute immiscibility) which can proceed until the drop dries out. It is apparent that the β-crustacyanin solution underwent gradual concentration until it reached the certain point suitable for its nucleation and subsequent growth of crystals.

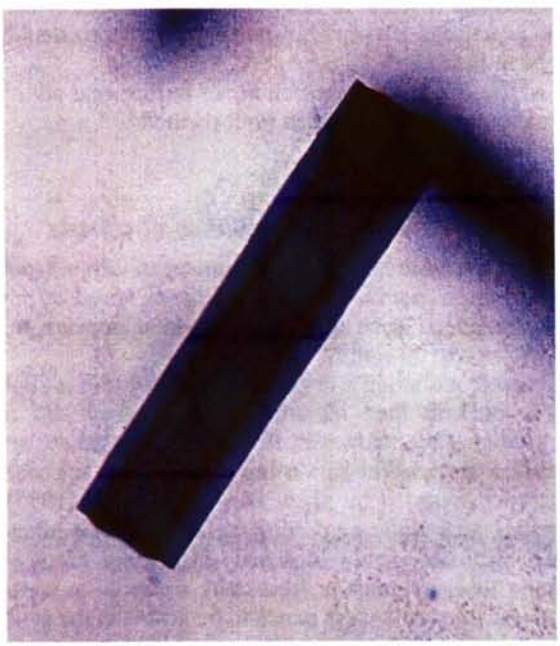

Fig. 2. A Crystal of β-crustacyanin grown by the microbatch method in a 2 µl drop under oil. The photograph shows the natural purple colour of the crystal. Final concentrations of the components in the drop: 7.5 mg ml^{-1} protein, 1.25 *M* phosphate and 0.05 *M* HEPES pH 7.5. Crystal size: 100 × 100 × 500 µm.

2.6. *Chlorophyll binding protein 43 (CP43)*

The chlorophyll binding protein 43 (CP43) of the photosystem II (PSII) membrane protein complex from spinach was prepared in the laboratory of Professor J. Barber, Biochemistry Department, Imperial College, London. It did not seem likely that a membrane protein (which is lipophilic) would lend itself to crystallization in the presence of oil, but as there was no success in obtaining crystals by the vapour-diffusion or dialysis methods, an attempt was made to screen for crystallization conditions in microbatch. Surprisingly, small crystals were observed in solutions containing PEG as precipitant (Hankamer, Chayen, De Las Rivas & Barber, 1992). Optimization of the conditions in 1.7 µl drops under paraffin oil produced crystals measuring 500 × 60 × 50 µm, but their diffraction was no better than 15 Å. Trials to adapt the conditions to vapour diffusion resulted in showers of much thinner and smaller needles than those obtained in the microbatch. It was suggested by Dr B. Hankamer (Biochemistry, Imperial College), that the presence of oil may act as a stimulant

for crystallization by slowly absorbing the detergent from the aqueous drop, thereby encouraging the protein to gradually come out of solution and crystallize. To the best of my knowledge no other attempts have yet been made to crystallize membrane proteins under oil.

2.7. *Experimental summary*

Experimental experience (*e.g.* Chayen, Shaw Stewart, Maeder & Blow, 1990; Chayen, Shaw Stewart & Blow, 1992; Table 1) has indicated that most proteins which crystallize by vapour diffusion also crystallize by microbatch, usually by some modifications of the conditions (Table 2). Exceptions are cases which require the presence of volatile organic compounds which dissolve in oil. Some proteins, as demonstrated above, appear to crystallize solely in microbatch.

3. Discussion

The results in Table 1 demonstrate that in most cases crystals of equal quality (*i.e.* similar in size, crystal habit and X-ray resolution) can be obtained using either vapour-diffusion or microbatch methods. Snake dendrotoxin is an example where although the microbatch method was not successful in production of crystals, the screening phase could be performed automatically using the microbatch technique which was the most rapid means to obtain an indication of the conditions for crystallization using minimal amounts of protein. These conditions could then be adapted to another method of crystallization which proved more successful. β-Crustacyanin, CP43 and firefly luciferase are examples where useful crystals could only be obtained in microbatch, albeit each for a different reason.

Baldock, Mills & Shaw Stewart (1996) compared the outcome of screening experiments performed by vapour diffusion and microbatch, concluding that both methods should be applied to ensure full coverage of all possibilities. The authors also suggest strategies for screening depending on how much protein, time and manpower are available to the experimenter. Similar considerations also apply to the optimization of crystal growth conditions.

4. Conversion of crystallization conditions from vapour diffusion to microbatch

A vapour-diffusion experiment starts with some or all of the components in the drop at a lower concentration than those applied in the reservoir. Through the vapour phase, the concentrations of ingredients in the drop are expected to equilibrate with those in the reservoir. In actual practice it has been shown (Chayen, Akins, Campbell-Smith & Blow, 1988; Kimble, Rousseau & Sambanis, 1995) that the concentration of protein (measured *versus* time) during a vapour-diffusion trial reaches a lower concentration than theoretically expected. This is very likely because protein sequesters some water, lowering its vapour pressure. It is conceivable that the other ingredients in the drop do not quite reach the concentration of those in the reservoir either, especially in cases where crystallization takes place before equilibrium is reached. This has been shown to be true in some cases where PEG was used as the precipitant, although still not in the case of ammonium sulfate and MPD (Mikol, Rodeau & Giegé, 1990). A batch experiment is set up such that the components are at their final concentration at the start of the experiment, and therefore the concentrations applied in batch should be somewhat lower than the final concentrations which the ingredients would be expected to reach at the end of a vapour-diffusion experiment. Table 2 demonstrates several examples in which conditions have been adapted from one method to another.

Rules of thumb may be stated for converting the crystallization conditions from one method to another in order to obtain equal quality crystals.

- In microbatch the concentration of precipitant is normally 10–20% lower than that in the reservoir of a vapour-diffusion trial.
- The **final** concentration of protein in a microbatch trial is generally lower than the starting **stock** solution used in vapour diffusion.
- In cases where crystallization takes place very rapidly, that is before vapour diffusion has reached equilibrium [as in the case of thaumatin (Table 2) which crystallizes within a few hours], significantly lower concentrations of protein and/or precipitant should be used in batch.
- The optimal concentrations of buffers and additives usually remain the same in both methods.

5. Harvesting and mounting of crystals

The issue of harvesting and mounting of crystals out of the oil from microbatch trials has been raised by many experimenters since at a first glance it appears to be mechanically difficult.

Indeed, mounting a crystal from microbatch is slightly more difficult than mounting from a cover slip or a Cryschem plate. However, it is possible to do so, and a detailed protocol for harvesting crystals from microbatch has been reported by Shaw Stewart & Conti (1995). If crystals stick to the supporting surface, which is often the case, they are gently loosened (inside the drop) with micro tools (Hampton Research) or with a whisker. Harvest solution always needs to be added to the crystals before their removal from the oil. This is performed by adding 15–30 µl of harvesting/stabilizing solution to the drop containing the crystals. As for vapour diffusion, in microbatch the harvest solution contains a slightly higher (~5%) concentration of precipitant than that in the drop.

After waiting a short while (up to $\frac{1}{2}$ h) to allow the crystals to equilibrate, a standard micropipette is used to withdraw the enlarged drop from the oil and transfer it to a depression well containing more harvest solution. From that stage onwards, the mounting is continued as it would be performed from a diffusion trial. The presence of the oil can offer the benefit of protecting the drops from physical shock (as they are buoyed by the viscous oil), making unmounted crystals easily transportable.

6. Comparison of the vapour-diffusion and microbatch methods

Vapour diffusion is the most known and well tried method which has proved extremely successful, compared with microbatch which is a relatively new concept. Execution of experiments of both microbatch and vapour diffusion can be conducted manually or automatically as a variety of automated systems are available (*e.g.* Cox & Weber, 1987; Ward, Perozzo & Zuk, 1988; Chayen, Shaw Stewart, Maeder & Blow, 1990; Rubin, Talatous & Larson, 1991; Oldfield, Ceska & Brady, 1991; Chayen, Shaw Stewart & Blow, 1992; Soriano & Fontecilla-Camps, 1993; Sadaoui, Janin & Lewit-Bently, 1994; Chayen, Shaw Stewart & Baldock, 1994).

The fundamental difference between the vapour-diffusion and microbatch methods is that diffusion methods are dynamic systems in which conditions are changing throughout the crystallization process and, hence, there is little control over the experiments once trials have been initiated. In the batch method the precipitant conditions are precisely defined; the samples are mixed at their final concentration at the start of the experiment thus conditions are constant within the normal time (1–3 weeks) of a crystallization experiment.

On the one hand, the gradual change of conditions in a vapour-diffusion trial may be a crucial factor for the formation of crystals which may form during its self-screening process (Fig. 1). On the other hand, the dynamic nature of diffusion methods renders them as unsuitable for conducting diagnostic studies on the process of protein crystal growth, for example, examining the separate phases of nucleation and growth. Such studies are far more reliable and reproducible in a batch system where the volume and composition of a trial remain constant (Ataka & Tanaka, 1986; Ataka, 1993; Saridakis, Shaw Stewart, Lloyd & Blow, 1994). Consequently, finding conditions for seeding is also easier in microbatch (Korkhin, Shaw Stewart & Evdokimov, 1995).

Moreover, in microbatch where the drops are maintained under oil, the samples are never exposed to air and are, therefore, protected from airborne contamination. This makes the microbatch an ideal environment for controlled heterogeneous nucleation experiments (Chayen, Radcliffe & Blow, 1993; Blow, Chayen, Lloyd & Saridakis, 1994; Chayen, 1996). Heterogeneous nucleation, which is often detrimental to the production of suitable crystals for X-ray diffraction, can be induced by the contact of a crystallization sample with the walls of its supporting vessel (Yonath, Müssig & Wittmann, 1982). In the case of vapour diffusion, all trials invariably need to be supported by a surface, whereas by employing microbatch one can significantly reduce the area of contact between a crystallization sample and its supporting vessel simply by altering the dispensing procedure of microbatch trials (Blow, Chayen, Lloyd & Saridakis, 1994). Contact with the supporting vessel can be eliminated entirely by suspending a crystallization drop between two oils of different densitities (Blow, Shaw Stewart & Maeder, 1993; Chayen, 1996; Lorber & Giegé, 1996). Thus, the nucleation can be monitored, and its level can be reduced or increased at will.

Setting up microbatch trials is simpler and speedier than vapour-diffusion trials even when performed manually because it is mechanically less complicated. The microbatch method uses small storage trays compared with vapour-diffusion vessels hence is less space consuming. It also eliminates the need to siliconize cover slips.

Since the volume of microbatch drops is very small, smaller quantities of protein are consumed compared with other methods. 100 trials can be prepared using approximately 1 mg of protein. The accurate dispensing by the syringes controlled by stepping motors, combined with dispensing the samples under oil (which ensures minimal evaporation) allows one to dispense **final trial volumes** of less than 1 µl compared with a minimum of 1 µl of **protein** in the case of standard vapour-diffusion trials. Crystals of diffraction size and quality have been grown in 1 µl drops using the microbatch technique. However, since vapour diffusion has many advantages (McPherson, 1982; Ducruix & Giegé, 1992), further efforts have been made to reduce the amount of protein required for this technique. New developments now facilitate vapour-diffusion trials to be conducted using quantities which are almost as small as those used in microbatch (Chayen, Shaw Stewart & Baldock, 1994).

A major advantage of vapour diffusion is the possibilty of affecting the equilibration rate of the trials and, thus, approach supersaturation more slowly by varying the distance between the reservoir and the crystallization drop (Luft, Arakali, Kiristis, Kalenik, Wawrzak, Cody, Pangborn & DeTitta, 1994; Luft, Allbright, Baird & DeTitta, 1996). A further advantage of vapour diffusion is the ability to alter the composition and/or the concentration of the components in the trial without having to touch the drop. This can be achieved by either concentration or dilution of the reservoir (*e.g.* Yonath, Müssig & Wittmann, 1982; Pryzbylska, 1989). In the case of hanging drops, one can just transfer a cover slip containing the crystallization drop from one reservoir over to another without having to disturb the drop and thus manipulate the conditions (*e.g.* Chayen, Lloyd,

Collyer & Blow, 1989); using sitting drops or a flow cell, the trial drop need not be moved at all. This gives much more flexibility for changing the conditions than in the batch method where any change (other than temperature) involves disturbance of the crystallization drop itself. On the other hand, because vapour diffusion is a dynamic system where conditions are changing throughout the crystallization process, it is not easy to determine which stage of the experiment is optimal for intervention.

Some organic compounds (examples given above and in Table 1) which cannot be applied in microbatch since they interact with the oil, can readily be employed in vapour-diffusion trials. This is an advantage when such substances are required as additives or as precipitants, however the presence of volatile compounds such as ammonia or carbon dioxide may cause problems in the control of pH during vapour diffusion (Mikol, Rodeau & Giegé, 1989).

Using vapour diffusion one often encounters problems concerning changes in drop volume, particularly when precipitants such as polyethylene glycol and volatile solvents are used. The absorbance of a volatile precipitating agent (Yonath, Müssig & Wittmann, 1982) or a slight change in temperature can cause enlargement of the drops, thus diluting the protein and causing dissolution of crystals; this can occur during the short space of time when crystals are being observed under the microscope. Experience has proved that crystals grown in microbatch under oil do not redissolve owing to the above reasons (Chayen, N. E., unpublished work; Conti, Lloyd, Akins, Franks & Brick, 1996), but will of course dissolve in microbatch if the solubility of the protein is temperature dependent (*e.g.* Komatsu, Miyashita & Suzuki, 1993). Enlargement of drops in vapour-diffusion trials can be prevented or reversed by the addition of salt to the reservoir as described by Yonath, Müssig & Wittmann (1982).

As discussed above, many of the difficulties associated with vapour diffusion can be overcome by using the microbatch method, but then the microbatch presents problems of its own. Although microbatch has the advantage of consuming less protein than other methods, not every case (as shown above) is suitable for crystallization under oil, especially not samples containing small volatile organic molecules which are soluble in oil (Table 1) or some organic precipitants which interact with the oil. Another fact that needs to be considered is, that unlike vapour diffusion, where changes of concentrations occur gradually, in the batch method, relatively high concentrations of the ingredients come into contact with each other upon being dispensed simultaneously. This can cause shock nucleation (Saridakis, Shaw Stewart, Lloyd & Blow, 1994) leading to production of crystal showers or even precipitation. Also, to the best of my knowledge, crystallizing complexes and cellular assemblies have not yet been tried in microbatch, and may prove to present unforeseen problems.

7. Further developments

Combinations of the microbatch and diffusion methods are beginning to emerge (Chayen, 1997*a*). The use of different types or combinations of oils which allow faster or slower diffusion through them can regulate the rate of evaporation from microbatch trials, thereby simultaneously retaining the benfits of a microbatch experiment combined with the advantages of a diffusion trial (D'Arcy, Elmore, Stihle & Johnston, 1996). Similar results can be obtained by varying the quantity of oil under which the trials are set (N. E. Chayen, unpublished work).

Another combination is accomplished by placing an oil barrier over a reservoir of a vapour-diffusion trial in order to slow down the equilibration rate and, thus, approach supersaturation more slowly (Chayen, 1997*b*).

The variety of different methods now available offer more sophisticated tools for both searching and for optimizing conditions for crystallization thereby reducing the labour and time required to obtain good crystals of proteins which have not been crystallized previously. Keeping an open mind about utilizing new, and at times unconventional, methods certainly enhances one's chances of success.

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