Modified microbatch and seeding in protein crystallization experiments

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The formation of nuclei in a crystallization experiment requires the interaction of protein molecules until a critical size of aggregate is created. In many crystallization screens sufficiently high levels of saturation are never reached to allow this critical nucleation event to occur. There are at least two possibilities to change this situation. The first is to increase the concentration of the protein and precipitating agent during the experiment to levels where spontaneous nucleation will occur. The second is to influence the nucleation event so that crystals can form at lower concentrations. The use of a modified microbatch method has made the first strategy possible and the use of heterogeneous seeding can be used to influence the second.

Keywords: microbatch; oils; nucleation; protein crystallization; seeding; hairs.

1. Introduction

A great deal of interest has been focussed recently on protein crystallization; this has been highlighted by the activity in the many structural genomics initiatives and in drug discovery programs in the pharmaceutical industry. Major efforts have been directed towards improving ways to screen for crystallization conditions, and new technologies in miniaturisation and automation of the crystallization experiments have now made it possible to set up thousands of crystallization trials in a single experiment (Stevens 2001, Luft 2001). Despite the large number of proteins and screening conditions that have been tested, the success rate is lower than might otherwise have been expected (Dale et al. 2003). In our experience using commercially available crystallization screens typically up to 50% of the drops may remain clear throughout the crystallization experiment, over periods of weeks to months. One reason could be the type of crystallization method used, which is typically the vapour diffusion technique, where the protein and precipitating agents may not reach high enough levels of saturation for nucleation to occur spontaneously.

In the first part of this study we hope to show that a modification of the microbatch method using different types of oils can be used to reach levels of saturation where spontaneous nucleation is more likely to occur. In the second part we will describe experiments demonstrating the use of stable, heterogeneous seeds from natural materials to influence nucleation in or below the metastable phase.

2. Microbatch

The microbatch technique as described by Chayen *et al.*, (1990) was essentially a method suited to the optimization of crystallization conditions rather than screening for initial crystallization conditions. The reason for this was that the paraffin oil does not allow any significant diffusion of water and subsequent concentration of the drop. In our recent study (D'Arcy et al. 2003) we have examined the properties of paraffin oil and a more volatile silicone oil to determine their effect on the crystallization behaviour of a number of test proteins (glucose isomerase, xylanase, trypsin and lysozyme). We compared the rate of appearance of crystals and the total number of crystals obtained using the different oils. In figure 1[*] the total number of crystals obtained for the four different proteins grown under paraffin and silicone oils is shown. There is a clear increase in the number of crystals that could be grown using silicone oil. Our studies have shown that this is in part due to the protein and precipitating agent being concentrated to much higher levels in the silicone compared to the paraffin oil, where, as previously mentioned no significant diffusion of water takes place. The second reason is that the silicone oil itself will evaporate eventually leading to the drops drying out, which must inevitably lead to very elevated concentrations. This concentration effect is seen within approximately 4 days, it is however possible to control the rate of evaporation so that the experiment is stable for much longer periods, by simply placing the crystallization dish in a closed box containing water or reservoir solution. The effect of this modification is shown in figure 2, where a comparison is made for the times of appearance and total number of crystals in paraffin and silicone oil with no reservoir, and silicone with reservoir. Using silicone oil without reservoir, 15 crystals had grown after 4 days, (crystals observed after this time may be salt crystals due to the drops drying out). In an identical experiment where the plate was placed in a box with a reservoir, only 7 crystals were observed after the same period of time, but the total number increased to 15 after 27 days with no drying out. In the control experiment with paraffin oil, only 8 crystals were observed after a period of 27 days

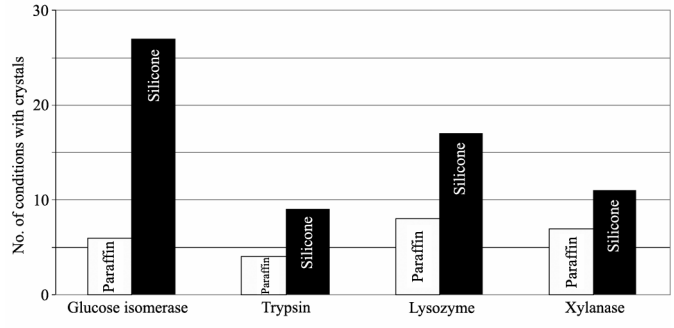


Figure 1

Number of conditions giving crystals with glucose isomerase, trypsin, lysozyme and xylanse using paraffin and silicone oil.

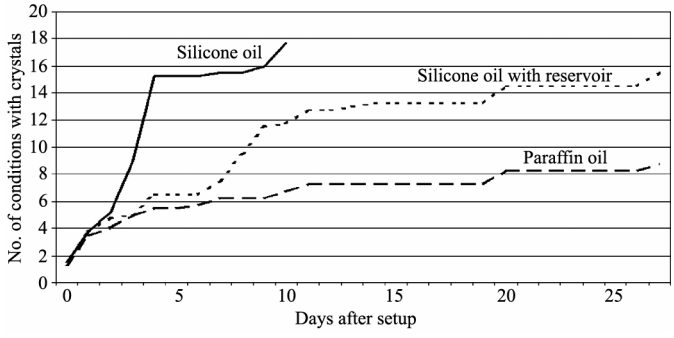


Figure 2

Time of appearance and number of conditions containing crystals during 27 days using paraffin and silicone oil with and without reservoir.

[*] Some of the figures in this article have been previously published in *Acta Cryst.* (2003), D**59**, 396-399 and 1343-1346.

The most common method used for screening crystallization conditions is vapour diffusion. In a typical experiment, the protein and precipitating agent are generally mixed in equal volumes thus reducing the concentrations by half. During the experiment the drop will concentrate until it has reached equilibrium with the reservoir. In many cases these concentrations are not sufficient to allow spontaneous nucleation to occur. It is therefore not surprising, to observe that as many as 50% of the drops may remain clear through-

Table 1

Experiment to show the effect on the number of crystals produced with and without the introduction of hair seeds (each value represents the average number of crystals for at least 3 drops).

Protein	Control (0 seeds)	Seed dilution 1/5	Seed dilution 1/25
Trypsin	0	75	8
Gluc.isom.	1	125	64
Lysozyme	1	9	6
Fab-D	0	8	2

Table 2

Dynamic light scattering characterisation of hair dilutions.

Mass of hair	Volume	nominal concentration	Dilution	Count rate (kcts/s)
0.5 grams	10 ml	50 mg/ml	0	overload
		5 mg/ml	10	4732561
		3.33 mg/ml	15	3211035
		1.66 mg/ml	30	1789973
		1.00 mg/ml	50	1433235

out the crystallization experiment. Using the properties of silicone or silicone/paraffin oil mixtures we have shown that the rate at which the drops concentrate and the end point can be accelerated. Once conditions have been established in this manner they can be repeated in a humid environment to prevent the drops drying out. The silicone oil allows a very rapid concentration of the crystallization drops, which permits a sampling of protein and precipitant concentrations that would not normally be reached in a vapour diffusion experiment. This may permit spontaneous nucleation to occur and increase the number of conditions that will produce crystals in an initial crystallization screen. In addition it is possible to use microbatch at temperatures ranging from 4° C to at least 30°C and set up temperature gradients without the problems that are associated with temperature shifts in vapour diffusion experiments (D'Arcy et al. 2003). The liquid handling systems used for the microbatch method, such as the IMPAX system from Douglas Instruments are simple and easy to operate. The fact that the drops are immediately covered with oil at the beginning of the crystallization experiment could also prevent oxidation of the protein, which might maintain the protein in a more crystallizable state for longer periods. Using this method with proteins determined to be suitable for crystallization by dynamic light scattering (Zulauf and D'Arcy 1992) our success rate has been >70% using screens of 48 conditions. A number of new conditions were found in addition to those reported in the literature and crystals obtained of some of the proteins which had not previously been crystallised. Once conditions have been established in the modified microbatch system it is generally more practical to optimise conditions using vapour diffusion, but in some cases this has not been possible and crystals grown by the microbatch method were used for structure determination.

3. Seeding

In situations where nucleation does not occur spontaneously in a crystallization experiment the introduction of seeds can be used to favour higher local concentrations of macromolecules so that the energy barrier for nucleation is lowered. Seeding is one of the most efficient methods for controlling and improving crystal growth as the seeds are introduced in the metastable zone where growth rather than continued nucleation is favoured. An excellent review on seeding can be found in a special issue of the Journal of Structural Biology (Bergfors 2003) . In our experience seeding is the method of choice in up to 50% of our projects for obtaining X-ray quality crystals once initial conditions have been identified, and introducing seeds into the initial screening could have great potential. Many authors have demonstrated the possibility of using various types of heterogeneous seeding material or surfaces to facilitate nucleation, (McPherson and Schlichta 1987, Chayen et al. 2001, Pechkova et al. 2001, 2002, Fermani et al. 2001, Haushalter and McPherson 2002). Indeed it is by no means uncommon to find crystals growing along a piece of hair or fibre in an otherwise clear crystallization drop (fig 3). In a classical seeding experiment, where micro or macro seed crystals of the same protein are introduced, it is necessary that the concentration of the precipitating agent is high enough to prevent the crystals from dissolving.

A stable, universal nucleation agent that could be introduced at the start of a crystallization experiment would be very attractive and could be expected to dramatically increase the number of "hits" in a typical screen. Animal hair has often been used to transfer seeds because its scaled surface facilitates the trapping of microcrystals (Leung et al. 1989, Stura and Wilson 1991), this property might also serve as a nucleation layer for protein molecules during a crystallization experiment. For this reason, and as already stated, the frequent observation of crystals growing on hairs or fibres, we decided to investigate the feasibility of using animal hair as a heterogeneous seeding material.

The experimental details and preliminary results have been described elsewhere (D'Arcy et al. 2003). Using single pieces of horse hair it was demonstrated that protein crystals had a tendency to form on the surfaces of hairs introduced at the beginning of the crystallization experiment. (Fig 4). A crude suspension of sonicated horse hair was used to demonstrate a nucleation effect on a number of different proteins. By carefully weighing of the starting material and controlling the dilution in aqueous buffer using dynamic light scattering (table 2), it was possible to obtain a seeding stock that could be used reproducibly to induce crystals at levels of saturation where nucleation would not normally occur spontaneously. In table 1 the effect of the introduction of varying amount of hair seeds is shown. For all the proteins tested there was a clear effect on nucleation. In the case of glucose isomerase a 125-fold increase in the number of crystals generated is observed compared to the control drops without seeds under identical conditions. The Fab-D crystallization is an interesting example in this study. In our experience Fab-D from E.coli produced only spherulites under the published conditions for the tetragonal form (Serre et al. 1994). Streak seeding from these microcrystalline aggregates generally produced crystals, which could be used for subsequent seeding experiments to produce large, well-formed bipyramid crystals. This however means that a stock of fresh crystal seeds is continously required and we decided to test if horse hair or homogenate could replace these seeds. The standard conditions for this protein were set up with and without hair seeds at different dilutions. In the control experiment without seeds no crystals were observed, in the trials with hair seeds, crystals could be reproducibly obtained using both the very crude preparation and the sonicated hair suspension. Crystals grown from these seeds had identical morphology to those produced from the homogeneous crystal seeds. In this initial experiment a fairly crude and concentrated hair suspension was used and in some cases once again hairs can be identified as an inclusions within the crystal (Fig. 4a). A subsequent experiment was performed using a dilution of the more homogeneous sonicated hair stock, and a

correlation between seed dilution and crystal nucleation was observed (table 1), A comparison between non seeded drops of Fab-D, crystal seeded drops and diluted horse hair seeds is shown in fig. 5.

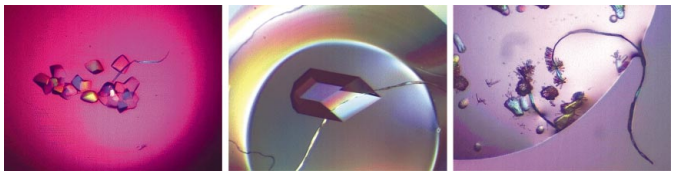
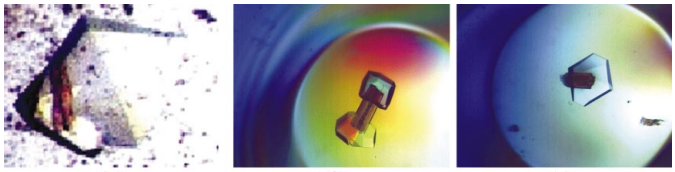


Figure 3

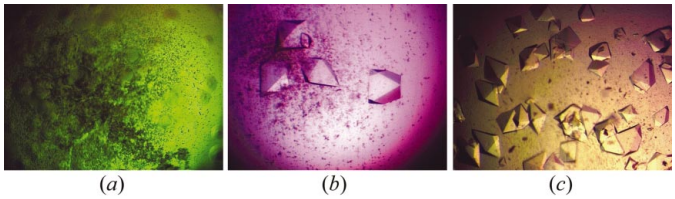
Examples of spontaneous nucleation of protein crystals on fibres.



(*a*) (*b*) (*c*)

Figure 4

Examples of nucleation of protein crystals on horse hair. (a) Fab-D, (b and c) glucose isomerase.



(*a*) (*b*) (*c*)

Figure 5

The effect of introducing the crushed hair seeds Fab-D. (**a**) no seeds, (**b**) crystal seeded, (**c**) 1:10diluted horse hair seeded.

Although the experiments described indicate that a solid component from the hair was causing the nucleation event, we wanted to exclude that it was not some soluble agent extracted from the hair in the aqueous buffers used. We therefore took the hair stock solution used for all the previous experiments and filtered the suspension through 0.22um Millipore spin filters. An experiment was set up using glucose isomerase as described previously with 3 identical drops per condition. The control drops contained no seeds, non diluted hair stock and the filtered hair stock were added to the other drops. An average of 1 crystal per drop was observed in the control experiment, 4 crystals per drop in the filtrate and 120 crystals per drop for the non filtered hair stock. Although this experiment clearly indicates that the main effect comes from the solid pieces of hair, there is also a minor effect from the filtrate. More experiments will be required to explain this effect which could be due to some other biochemical effect such as peptides or metal ions likely to be found in the homogenate in a soluble form.

4. Conclusions

Many efforts have been made in recent years to better understand and control protein crystallization experiments. High throughput crystallization has become a catch phrase and it is certainly possible to perform more and more experiments with less and less protein due to the advances in robotics and nanotechnology. Unfortunately high throughput has not always been translated into high output. Clearly the number of screen conditions is not directly correlated to the number of hits obtained. We have been using a modified microbatch method with relatively small screens containing 48 or 96 conditions such as the "INDEX" screen from Hampton Research for over six years. The success rate is > 70% when the proteins are shown to be suitable for crystallization using dynamic light scattering (Zulauf, M. & D'Arcy A. 1992) . We believe that microbatch is a simple and reliable screening method and the possibilities of using different kinds of oils to influence the kinetics of the crystallization, combined with the facility of employing different temperatures and gradients make it an attractive alternative to standard screening methods. Seeding is a widely used method to improve or reproduce crystals once the original conditions have been found. If however the nucleation event could be aided by the presence of seeds from the beginning of the experiment one would expect to find more hits and possibly a wider range of conditions. Our first experiments to establish if non-crystalline seeds could be used to influence nucleation are encouraging. We have shown this to be the case with a number of different proteins. It has also been possible to replace specific crystals seeds with hair seeds to produce X-ray quality crystals of Fab-D thus eliminating the need to always have fresh seeding stocks requiring more protein.

The seed stocks are easy to prepare, stable in any buffer system and can also be frozen for storage, these types of seeds can be introduced at any time during the experiment at different dilutions. In contrast to using protein crystals as seeds, this method would allow the introduction of seeds during initial screening trials in many different crystallization conditions, which would remain stable throughout the duration of the experiments. We are at present evaluating the validity of this approach for screening, as the experimental procedures are compatible with the modified microbatch system. A number of questions remain to be answered in order to improve, understand and optimise the phenomena we have observed, as the effect of introducing hair seeds seems to vary from protein to protein. Lysozyme for instance requires higher concentrations of hair before an effect is observed. Different types of animal hair from animals of different ages may need to be tested, as well as trying to identify and characterise a possible "nucleation agent" present in the seeding stock and determining the specific interactions (if any) between the seeds and the protein molecules. We feel however that this initial study has shown an effect on more than one protein and that a more detailed study using the combination of the modified microbatch method and heterogeneous seeding in now merited.

References

Chayen, N., Saridakis, E., El-Bahar, R. & Nemirovsky, Y. (2001). J. Mol. Biol. 312, 591-595.
Chayen, N.E., Shaw Stewart, P.D., Meader, D.L. and Blow D.M.. 23 (1990). Appl. Cryst. 297-302.
Bergfors, T. J. (2003).Struct. Biol. 142 (1) 66-76.
Dale, G.E., D'Arcy, A., Oefner, C. (2003). J. Struct. Biol. 142 (1) 88-97.
D'Arcy, A., Mac Sweeney, A., Stihle, M. and Haber, A. (2003). Acta Cryst. D59, 396-399.
Fermani, S., Falini, G., Minnucci, M. & Ripamonti, A. (2001). J. Crystal Growth 224, 327-334.
Haushalter, R., Sun, T. X. and McPherson, A. (2002). American Crystallographic Association meeting.
Leung, C. J., Nall, B. T. and Brayer,G.D (1989) J. Mol. Biol, 206, 785-5.
Luft, J. R., Wolfley, J., Jurisica, I., Glasgow, J., Fortier, S. & DeTitta, G. T. (2001). J. Cryst. Growth, 232, 591-595.
Luft, J. R., Wolfley, J., Jurisica, I., Glasgow, J., Fortier, S. & DeTitta, G. T. (2001). J. Cryst Growth 232, 591-595.
McPherson, A., Shlichta, P. J. (1987). J. Crystal Growth 85, 206-214.
Pechkova, E. & Nicolini, C. (2001). J. Crystal Growth 231, 599-602.
Pechkova, E. & Nicolini, C. (2002). J. Cell Biochemistry 85, 243-251.
Stevens, R.C. (2000). Curr. Opin. Struct. Biol. 5, 558-63.
Zulauf, M. & D'Arcy, A. (1992). J. Crystal Growth, 122, 102-106.