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The advantages of using a modified microbatch method for rapid screening of protein crystallization conditions

Allan D'Arcy,[a]* Aengus Mac Sweeney,[a] Martine Stihle[b] and Alexander Haber[a,c]

[a]Morphochem AG Basel, Switzerland, [b]Hoffmann–La Roche Basel, Switzerland, and [c]University of Freiburg, Germany

Correspondence e-mail: allan.darcy@morphochem.ch

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In this study, characterization and optimization of a modified microbatch crystallization technique has been attempted in order to provide a rapid screening method. Using this method for screening has certain advantages over standard vapour-diffusion methods: no sealing of drops is required, no reservoir solutions are needed and the experiments can easily be performed over a range of temperatures.

1. Introduction

Protein crystallography has seen somewhat of a renaissance in the past few years. The many structural genomics and high-throughput crystallography initiatives have rekindled interest in X-ray crystallography. Not only large pharmaceutical companies but also an increasing number of small biotech companies have established structural biology as a key part of their drug-discovery efforts. An essential part of all these studies, often the rate-limiting step, is finding suitable conditions for growing protein crystals. Structural genomics laboratories are looking for ways to improve the rate at which proteins can be produced, purified and crystallized in a high-throughput manner and have focused on rapid robotic techniques, screening many hundreds of crystallization conditions with very small drops (Stevens, 2000). Luft *et al.* (2001) have automated the microbatch method using paraffin oil to screen as many as 1536 crystallization conditions. Drug-discovery companies need to produce crystals which can be used for structure determination and inhibitor studies, and some have also chosen the high-throughput strategy. We believe that the microbatch method has not been exploited to its full potential and could provide a simple and efficient method to search for initial crystallization conditions using a relatively small screen. The microbatch technique was first described by Chayen *et al.* (1990) as a method that was essentially more suited to optimization than screening, as the paraffin oil used did not allow any significant concentration of the drop. Chayen (1998) also made a comparison between microbatch experiments using paraffin oil and vapour diffusion. However, this study was based on optimized well defined crystallization conditions and did not attempt to determine the efficiency of the microbatch method as a screening procedure.

Our initial experiments comparing microbatch (using paraffin oil) and vapour-diffusion screens showed that fewer crystals were observed in microbatch screens. This led us to believe that the type of oil used had a dramatic effect on the speed and end result of the screening and that the use of more volatile oils gave rise to more rapid crystallization. A 1:1 mixture of silicone and paraffin oils (Al's oil) was shown to be a suitable mixture enabling concentration of both protein and precipitating agent, in which crystals were stable for up to three weeks (D'Arcy *et al.*, 1996). We have subsequently adopted this method using a 1:1 paraffin:silicone oil mixture with a 48 or 96 condition screen (INDEX) for standard screening purposes (D'Arcy *et al.*, 1999; Dale *et al.*, 1999). In the present study, we have examined the possibility of using silicone oil alone under controlled conditions to further increase the number of conditions which give crystals or produce crystals more rapidly. These possibilities combined with the ease of introducing temperature shifts aim to demonstrate some additional advantages of using microbatch methods for screening.

2. Experimental

2.1. Materials and methods

The proteins used in the study were dihydroneopterin aldolase (DHNA, prepared in our own laboratory), lysozyme (Merck catalogue No. 1.05281), glucose isomerase, xylanase (Hampton Research catalogue Nos. HR7-100 and HR7-106), trypsin (Sigma T-8003) and porcine pancreatic elastase (Roche catalogue No. 1 027 891). The protein concentrations were from 10–100 mg ml^{-1}. The oils used were low-viscosity silicone oil (Dow Corning catalogue No. 630024N), highly liquid paraffin oil (Merck catalogue No.

K29717174147) and Al's oil (Hampton Research catalogue No. HR3-413).

A standard IMPAX robot (Douglas Instruments) was used in all experiments, dispensing volumes of 1 µl protein plus 1 µl precipitating agent using Nunc 72-well HLA plates. Crystallization solutions were from the 'INDEX' screen (Hampton Research catalogue No. HR2-134). The temperature-gradient experiments were performed using an M6 mini-incubator (Hampton Research catalogue No. HR3-300).

2.2. Crystallization using different oils

The effect of different oils on the number of conditions giving crystals, the time of appearance and stability of crystals was tested using glucose isomerase, xylanase, trypsin and lysozyme as test proteins, prepared according the manufacturers instructions or in the case of trypsin in the presence of 50 m*M* benzamidine. In a typical experiment using an IMPAX robot, 1 µl of the test protein and 1 µl of screen solution were pipetted under 6 ml of paraffin or silicone oil in a 72-well Nunc HLA plate. The trays were incubated at 297 K. Crystallization trials were observed each day for periods between 5 days and three weeks, depending on the type of experiment.

In order to demonstrate the different properties of silicone or paraffin oil and their influence upon the rate and stability of crystals, glucose isomerase was crystallized using 30%(*w*/*v*) PEG monomethyl ether 550, 100 m*M* HEPES pH 7.0, 50 m*M* $MgCl_2$ with each of the oils. The experiments were observed daily from day one to day five. The results are shown in Fig. 1.

To establish the relative efficacy of using paraffin or silicone oil on the number of screen conditions producing crystals, glucose isomerase, xylanase, trypsin and lysozyme were tested with conditions 49–96 of the INDEX screen. The experiments were observed daily and the number of crystals noted over a period of 5 days (results shown in Fig. 2). Having observed that screens using silicone oil dried out after approximately 4 d, we attempted to determine the effect of reducing the evaporation of the silicone oil by placing the trays in a humid environment. Crystallization trials were set up on the same four test proteins using identical screen conditions to the previous experiment and the crystallization tray with silicone oil was placed in a clear plastic box containing 4 ml of water and sealed with a loose-fitting lid. The control experiment was placed in an identical container with no water and crystal appearance documented from days 1 to 28. The results of this experiment are shown in Fig. 3.

2.3. Crystallization using different temperatures

The modified microbatch method is more suitable than vapour diffusion for the use of temperature as an additional parameter in the search for initial crystallization conditions. Evaporation and condensation cause problems when changing temperature during vapour-diffusion experiments; this is not the case with the microbatch method as the drops are covered with oil. Luft *et al.* (1999) have successfully applied temperature gradients in a vapour-diffusion system. To demonstrate the possibility of using temperature gradients in a simpler manner for screening, an experiment was set up using DHNA as a test protein. 1 µl of the protein at 16 mg ml^{-1} was mixed with 1 µl of

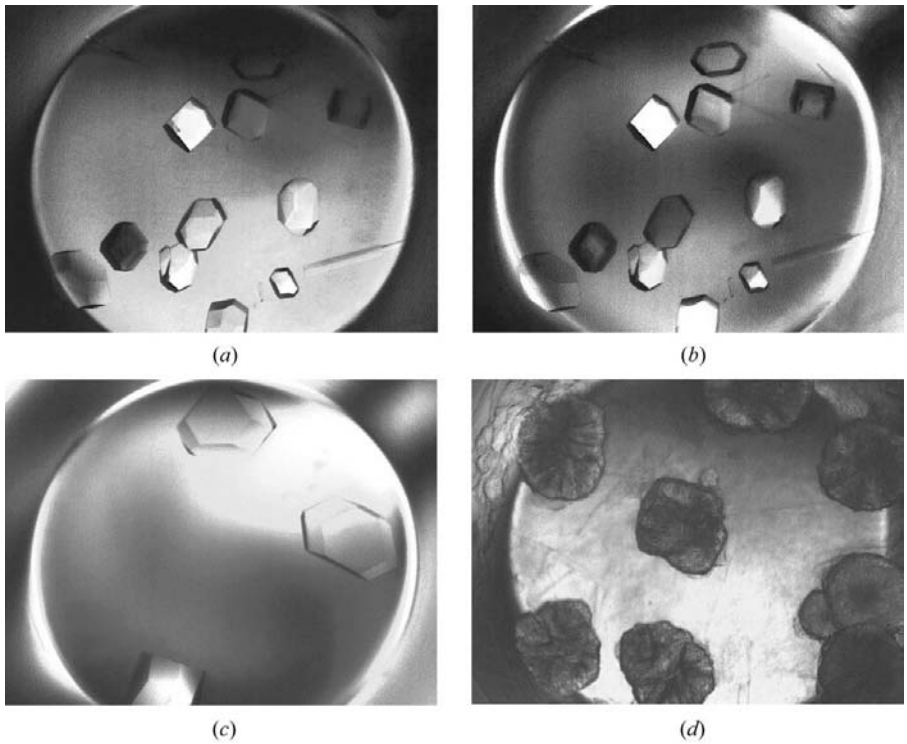

Figure 1
The effect of using paraffin or silicone oil for the crystallization of glucose isomerase. Crystals grown in 30% PEG monomethyl ether 550, 100 m*M* HEPES pH 7.0, 50 m*M* $MgCl_2$ under paraffin or silicone oil (all crystals are at the same magnification). (*a*) Paraffin oil, day 1; (*b*) paraffin oil, day 5; (*c*) silicone oil, day 1; (*d*) silicone oil, day 5.

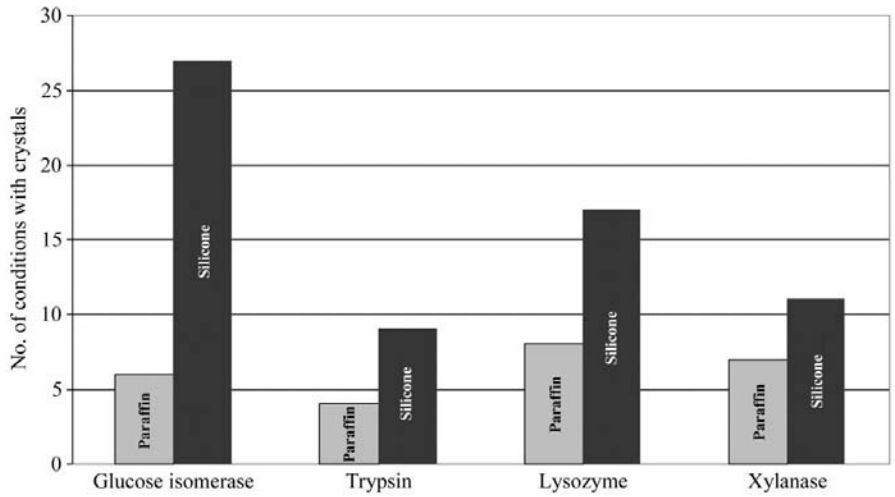

Figure 2
Comparison in the number of conditions that produced crystals of glucose isomerase, trypsin, lysozyme and xylanase using paraffin or silicone oil.

screening solution (conditions 49–96 of the 'INDEX' screen) using the 1:1 paraffin: silicone oil mixture. The trays were placed at 277, 297 and 303 K and the number of conditions in which crystals grew was noted after 4 d. Based on the results of the initial experiment, a second experiment was set up under the same conditions but with a linear temperature gradient from 303 to 277 K over a 16 h period. The results are summarized in Table 1.

3. Results

3.1. Crystallization using different oils

Using paraffin and silicone oils, we were able to influence the rate of crystallization and the stability of crystals grown using the microbatch method. This experiment clearly shows that no substantial concentration of the drops occurs *via* diffusion or evaporation using paraffin oil. This is an important consideration in light of the results of Luft *et al.* (2001), which were based on experiments using paraffin oil and a screen containing 1536 conditions. The published success rate of the 1536-condition screen was surprisingly low, with only 36% of the proteins deemed suitable for crystallization by dynamic light yielding crystals. The situation is dramatically different with silicone oil, which allows a rapid concentration of protein and precipitant within the drop. To demonstrate the difference in the behaviour of the two oils, glucose isomerase was crystallized using 30% PEG monomethyl ether 550, 100 m*M* HEPES pH 7.0, 50 m*M* $MgCl_2$ under paraffin or silicone oil. The crystals grown under paraffin oil did not change in size or appearance from day one to day five, confirming that there is no significant concentration of the drops during this period. Under silicone oil the crystals are larger after 1 d, but eventually the layer of silicone oil no longer covers the drops and they dry out as illustrated in Fig. 1. This is of course inconvenient, but in some cases, *e.g.* where a protein is not stable for extended periods, the investigator may wish to perform an initial screen that could rapidly generate crystals and then select the most promising conditions for optimization. The use of silicone oil may be useful in such cases or in general to increase the number of initial conditions giving crystals over a shorter period of time.

The average number of drops which gave crystals using glucose isomerase, xylanase, trypsin and lysozyme using a 48-condition screen (INDEX conditions 49–96) was increased by a factor of two with all proteins tested when using silicone oil alone compared with paraffin oil. These results are summarized in Fig. 2. As previously mentioned, the disadvantage of using silicone oil alone is that the drops dry out after a period of 3–4 d. To reproduce initial conditions that have rapidly produced crystals in a more controlled manner, the crystallization tray may be placed in a humid environment (a box containing reservoir solution or water) from the beginning of and for the duration of the experiment. Almost the same number of crystals can be obtained over a period of approximately three weeks without the drops drying. A comparison of the number and time of appearance of crystals for paraffin and silicone oil with and without reservoir solution is shown in Fig. 3.

Using silicone oil to screen for initial crystallization conditions generally means that crystals appear more quickly; as a result, the protein is fresher and less likely to undergo proteolysis. In addition, as previously observed by Chayen (1998), it is also possible that the protein is protected from oxidation as the drop is not in contact with air for the duration of the crystallization experiment.

3.2. Crystallization using different temperatures

To examine feasibility and the effects of temperature in a modified microbatch setup, DHNA was screened as a test protein using conditions 48–96 from the 'INDEX' screen. The trays were placed at 277, 293 and 303 K. No crystals were observed at 303 K, while 15 and 25 conditions gave crystals at temperatures of 293 and 277 K, respectively. Based on these results, an experiment was set up in which the tray was placed in an incubator and the temperature was lowered from 303 K (at which the protein appears to be most soluble) to 277 K over a 16 h period. In this gradient experiment, 27 conditions produced crystals and some different crystal morphologies were observed (data not shown). From this simple experiment, it could be shown that the microbatch crystallization was easy to set up, a clear temperature dependence was demonstrated and a temperature gradient was shown to have a marginally positive effect on the number of conditions producing crystals. The results are summarized in Table 1.

Table 1
Experimental results.

Temperature (K)	No. of crystals from 48 conditions
303	0
297	15
277	25
303–277 gradient	27

4. Conclusions

The number of different conditions in a particular screen is not necessarily proportional to the number of successful conditions it will produce and many investigators are reducing their screen sizes based on the statistics of successful solutions. We have used a screen containing between 48 and 96 conditions over the past 6 years in a modified microbatch screen. Using this methodology, our success rate with proteins determined to be 'crystallizable' using

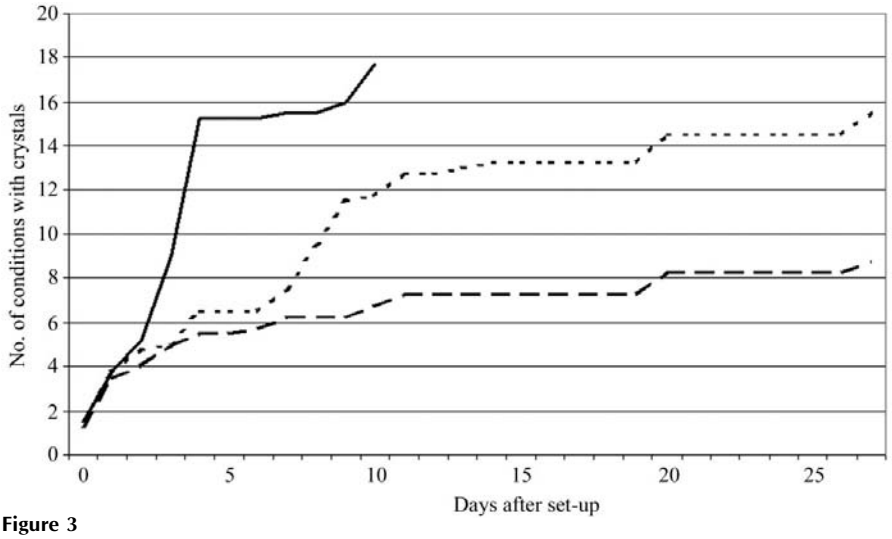

Figure 3
Comparison of time of appearance and number of conditions with crystals during 27 d using paraffin or silicone oil with and without reservoir. Solid lines = silicone oil without reservoir, dotted lines = silicone oil with reservoir, dashed lines = paraffin oil.

dynamic light scattering (D'Arcy, 1994; Ferre-D'Amare & Burley, 1997) is greater than 70%. If one compares this with the results of Luft *et al.* (2001) using microbatch with paraffin oil, it is clear that the type of oil or oils used is of critical importance. In this study, we have explored the possibility of using the properties of silicone oil to increase the success rate while keeping the crystals in a stable environment. In addition, we have demonstrated the ease and effectiveness of using the microbatch method to screen at different temperatures without the condensation problems associated with vapour diffusion.

Relatively simple liquid-handling systems such as the Douglas Instruments crystallization robot are well suited to this method and although it cannot be considered as high throughput or nanocrystallization, it has proved to be an efficient and reliable procedure for screening small amounts of protein in our laboratory.

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