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A Practical kit for Micro-scale Application of the Ceiling Crystallisation Method

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We designed a kit to facilitate the optimisation of protein crystal growth by means of the ceiling crystallisation method. This ceiling kit allows for diffusion-limited crystal growth with a total volume down to 10 μl per trial and air-tight sealing for any type of protein sample. The ceiling crystals grow to sufficient size for diffraction experiments. In this highlight, we fully describe the features of this kit and how easily it can be used to yield protein crystals.

1 Introduction

In macromolecular crystallography, the growth of high quality crystals is a prerequisite for the structure determination of biological macromolecules. There is ample evidence from many studies that diffusion-limited growth conditions can result in the growth of better crystals.[1–4] The merit of diffusion-limited growth is that it allows protein crystals to grow from an initially highly supersaturated solution, which after nucleation undergoes a steady decrease in its supersaturation, so that the incorporation of sediments and impurities is also highly suppressed. Diffusion-limited growth is attained in space microgravity conditions,[5] but can also be accomplished under terrestrial conditions using magnetic fields,[2,6] microcapillaries,[7] and gels.[8] This insight led to the development of commercially available equipment facilitating crystal growth of macromolecules through diffusive mass transport.[9]

Despite the advantages of diffusion-limited growth, most protein crystallisation experiments are performed using the vapour diffusion and micro-batch methods, because they have been automated. Therefore, it is very easy to scan hundreds of different crystallisation condition by means of a robot, which can be set to miniaturize the required volume of the purified protein per single crystallisation trial (1 nl to 10 μl). The ease of the application and the diversity of the trial population increase the possibility of getting the *hit*. Nevertheless, these methods still maintain convection currents, and it would be worthwhile to further optimise crystal quality in a diffusion-limited growth set-up.

We have recently developed an efficient diffusion-limited crystallisation method (ceiling crystallisation) in which protein crystals grow at the very top of a vial overfilled with batch crystallisation solution.[1,10] * Ceiling crystals grow with much less influence of impurities and diffract X-rays to high resolution limits.[1,13] This is mainly due to the development of a depletion zone around a growing crystal, which is not disrupted by convection currents.[14]

Here, we highlight our "ceiling crystallisation kit", which we have recently developed in order to scale the total volume required per experimental trial down to a few μl's, while allowing for tight sealing and convenient handling of the experiments. Our aim is to make the method widely available and further expand its potential, so that it may become an additional tool for optimisation of protein crystal quality.

2 Design considerations

We developed the ceiling method using commercially available micro-centrifuge tubes and micro- and nano-plates, with which we scaled the required solution volume from 1.8 ml down to 10 μl.[1] In these setups, ceiling crystals heterogeneously nucleate on the cover slips.[15] Because solution evaporation must not occur during ceiling crystallisation, tight sealing is required to avoid any solution or air leakage out of or into the growth cell at the cover slip-vial interface. Unlike vapour diffusion, for which vacuum grease or petroleum jelly can be easily used, the ceiling method in its original set-up[1] leads to solution-grease contact. It was shown earlier that

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* We recently discovered that a comparable approach, called *configurational stabilisation*, was developed several decades ago for inorganic crystals ,[11,12] but this did not get a follow-up.

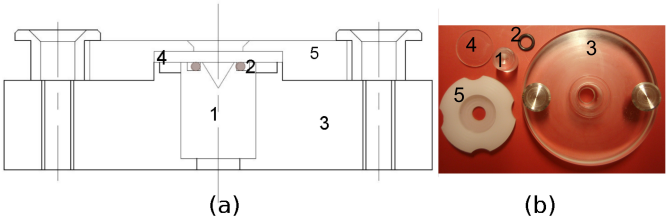

Fig. 1 A cross section (a) of a mounted assembly and a photograph (b) of its components: growth vial (1), rubber ring (2), mounting plate (3), cover slip (4) and Teflon clamping plate (5). The crystallisation solutions overfills the wedge-shaped volume in the growth cell. The ceiling protein crystals should grow underneath the cover slip.

Fig. 2 (a) A Schematic diagram of the growth cell; (b) The mounting plate with an inserted growth cell.

grease does not act as an impurity for proteins,[16] yet we have seen in our experiments a preferential nucleation of protein crystals at the grease side. Although microscopic inspection revealed that the crystals exhibit good optical quality, fishing the crystals and subsequent data collection is too difficult to be accomplished without contaminating the crystal surface with the applied sealant, which in turn adversely affects the final diffraction quality. Moreover, applying grease is not compatible with crystallisation solutions of membrane proteins which almost always contain detergents.

Earlier, screw-capped wells on a plate (EasyXtal 15-Well Tools) have become commercially available for the hanging drop vapour diffusion method.[17] For the ceiling method, this design would provide an alternative for vacuum grease. However, screwing the cap all the way down, is a possible source of air bubbles. Therefore, we have developed a design using a clamped rubber O-ring for an air-tight sealing. The design also facilitates microscopic inspection of the growing crystals.

3 The ceiling crystallisation kit

A ceiling kit consists of a growth cell, mounting plate, cover slip, rubber O-ring and Teflon clamping plate (Fig. 1).

The growth cells are made from optically clear and (preferably) hydrophobic materials (Fig. 2 a). We used Perspex, but

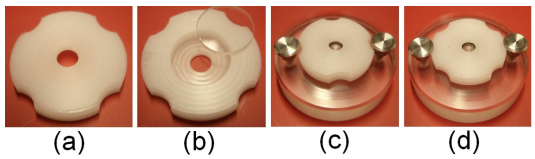

Fig. 3 Teflon clamping plate: outer view (a), inner view showing how a glass cover slip can be inserted into the inner groove (b), the circular notches allows for an easy and tight lock by rotating the Teflon plate (c, d).

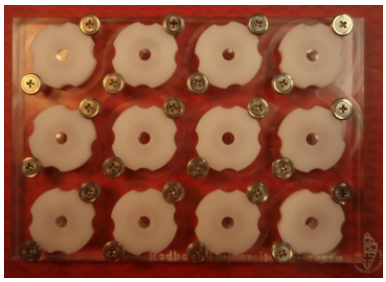

Fig. 4 A plate of 4 × 3 cells for ceiling crystallisation.

polystyrene and polypropylene are even better. For convenient handling, the outer size of these cells (ϕ 6 mm) is the same as that of the cylindrical cavities in the mounting plate (Fig. 2 b). The cells were milled to have cone shaped wells with a total volume of 8 μl, but other shapes and even smaller volumes are also possible. At the upper edge of each well, a groove is made to embed a rubber O-ring (NBR 36624 3.5 × 1, ERIKS) for airtight sealing. The growth cell can then be easily inserted into and removed from the cavity of the optically clear Perspex mounting plate, which can be used repeatedly. On the top of this plate, two stainless steel pins are mounted to hold the Teflon clamping plate (Fig. 3 a, b). The Teflon plate has a hole in its centre to allow for microscopic inspection and a groove to embed a siliconised glass cover slip (ϕ12 mm, 1.2 mm thick, HR8-088, Hampton Research). The clamping plate has 4 circular notches for an easy and tight lock (Fig. 3 c, d). For convenience, 12 growth cells can be mounted on a plate of the standard dimensions (130 × 90 × 8 mm^3) with cylindrical cavities for each complete assembly (Fig. 4).

4 Application

The protein crystallisation solution is prepared using the same protocol for setting (micro-)batch experiments. In case the protein under test has been crystallised by vapour diffusion, brief guidelines for converting vapour diffusion to batch method and vice versa are available elsewhere.[18] The clean growth cell is inserted into the mounting plate and the rubber O-ring is positioned. On the inner side of the clamping plate, a

thick glass cover slip is inserted, which can be surface treated if nucleation needs to be enhanced. The vial is then overfilled with the prepared crystallisation solution and directly covered with the clamping plate embedding a cover slip. During the experiment, the cell should be kept under temperature-controlled and vibration-free conditions. This setup can easily be inspected using a microscope with working distance down to 10 mm. After growth of the protein crystals to their final size, the clamping plate is unlocked and turned upside-down, exposing the ceiling crystals which are attached to the cover slip. The crystals will be wet with a small amount of adhering solution and the addition of a cryoprotectant (if needed) will be easy, as is fishing the crystals on silicon loops for data collection.

5 Results and discussion

Earlier, we have systematically demonstrated the merits of the ceiling crystallisation method using improvised set-ups and several different proteins.[1] So, in this section we only present a few crystals to show that the new ceiling crystallisation kit works well and gives good output.

Protein crystals shown include tetragonal Hen egg white lysozyme (HEWL), bipyramidal thaumatin and orthorhombic bovine pancreatic trypsin as well as crystals of a proprietary enzyme, which was generously offered as a purified sample by NTRC, Oss, the Netherlands. HEWL was crystallised as described elsewhere.[1] Thaumatin crystals grew in a solution containing 20 mg/ml protein and 1.2 M Na/K tartarate in 10 mM bis-TRIS buffer pH 6.5. Trypsin was crystallised from a solution containing 60 mg/ml of the protein, 10 mg/ml benzamidine, 10 mM $CaCl_2$ and 1.87 M $(NH_4)_2SO_4$, buffered with 0.1 m Tris at pH 8.5 and 20^o.

For all four proteins, crystals of good optical quality and with well defined facets were grown using the ceiling kit (Fig. 5). All protein crystals grew to a size suitable for XRD measurements and were comparable to crystals grown in microtubes with 100 $\times$ larger volume. To examine the possible degree of perfection for a ceiling crystal grown in this kit, we performed a pilot X-ray diffraction experiment on ceiling bovine trypsin crystal at beamline ID 23-2 of the European Synchrotron Radiation Facility (ESRF), Grenoble, France. It was grown from commercially available bovine pancreatic trypsin without any further purification. The crystal was freshly fished at the beamline, directly flash-frozen in liquid nitrogen without prior soaking in any cryoprotectant and was cooled in a 100 K nitrogen stream during the measurements. The ceiling trypsin crystal diffracted to atomic resolution (Table 1).

These results show that the ceiling crystallisation method can be scaled down to the micro-litre range and still effectuate the growth of high quality crystals. It was shown earlier by

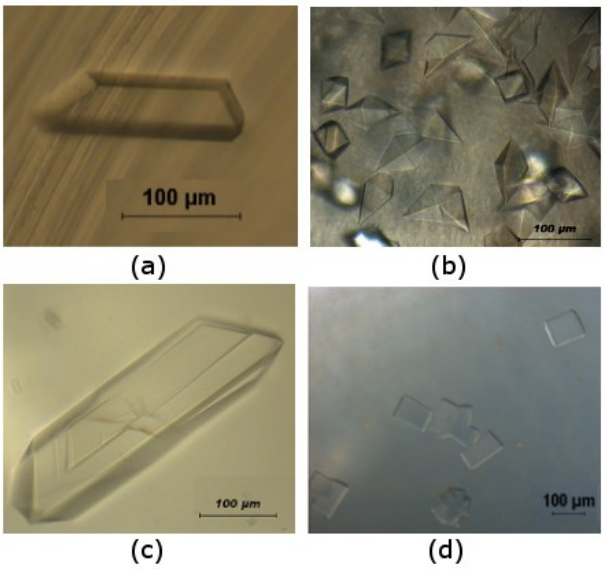

Fig. 5 Micrographs of ceiling tetragonal HEWL(a), bipyramidal thaumatin (b), orthorhombic bovine trypsin (c) and the proprietary enzyme (d) crystals grown in the ceiling crystallisation kit.

numerical simulations that even at this small scale convection can be avoided using the ceiling configuration.[14] †

According to the protein data bank, more than 70% of the structure hits are for crystals grown by using the vapour diffusion method in one of its configurations (hanging, sitting or sandwiched drop). Yet the resolution of more than 90% of determined structures does not exceed 1.5Å. On the other hand, the micro-batch method was shown to yield better crystals and unique crystallisation hits which have not been possible by using the vapour diffusion method.[18] This is because, unlike the micro-batch method, the actual crystallisation conditions which trigger nucleation and crystal growth using the vapour diffusion method are not well defined. This in turn limits the reproducibility of vapour diffusion-grown crystals and render structural studies of most macromolecules to usually rely on a model obtained from a single crystal. Recently, it was also shown that it is very important to determine which details of a macromolecular structure are reproducible or to what extent they may vary.[23] Therefore, optimising crystallisation condi-

† It should be noted that a crystal growing coincidently in contact with the cover slip in a hanging drop vapour diffusion experiment is different from a ceiling crystal. Inherently, a crystallisation droplet in a hanging drop set-up encounters an increase or decrease in its size due to the outward or inward diffusion of vapour to achieve equilibrium with the precipitant in the reservoir, which is usually $0.5-2\times$ the precipitant concentration in the droplet. The surface temperature of this evaporative/condensative drop is non-uniformly decreased/increased by evaporating cooling/condensative heating. Therefore, the surface tension varies along the drop surface.[19,20] This, together with concentration gradients, promotes the so called Marangoni convection, which was reported to be an at least equally strong source of solution stagnancy disturbance as the gravity-induced convection.[21,22]

Table 1 Data collection and processing statistics for a bovine pancreatic trypsin crystal grown in the ceiling kit. Values in parentheses correspond to highest resolution shell.

wavelength	0.8726 Å
Optical resolution	1.11 Å
rotation range	105^o
No. of images	1050
Space group	$P2_12_12_1$
Mosaicity	0.47
Unit cell parameters	62.28, 63.35, 68.25 Å
Resolution limits	50-1.26 (1.35-1.26)Å
Completeness	100 %
Redundancy	4.06
R-factor	4.7% (73.6%)
I/σ	33.4 (1.9)
B-factor	10.83 $Å^2$

tions from this perspective is also very relevant.

In our view, ceiling crystallisation is not intended for high throughput screening,[24] nor is it a replacement for the currently used screening methods. For this, commercial kits are available for efficient and flexible screening of crystallisation conditions for proteins, peptides, nucleic acids, macromolecular complexes and water soluble small molecules. But once the appropriate conditions are determined, preferably using the micro-batch method, the ceiling method is a convenient way to optimise the resultant crystal quality and yield more isomorphous crystals.

6 Conclusions

In conclusion, we designed a dedicated ceiling crystallisation kit, which is easy to use and suitable for any type of protein solution. The designed cell does not really require automation, because mostly few (<30) crystallisation trials are sufficient at this late stage of optimisation. The kit has the following advantages: 1) It allows a total sample volume down to less than 10 μl per trial, but larger volumes are easily implemented, which may be required for growing larger crystals suitable for neutron crystallography; 2) Through the rubber O-ring, it provides mechanical air-tight sealing and there is no need to use vacuum grease or petroleum jelly which are possible sources of contamination, and are, moreover, incompatible with detergent-solubilised membrane proteins;3) It facilitates microscopic inspection, which is required for monitoring experimental progress; 4) It can be used with commercially available cover slips; 5) It facilitates fishing of the resultant protein crystals. We hope that this kit will encourage the crystallisation community to use the ceiling method and we encourage researchers to contact us for discussing the application of this hardware.

7 Acknowledgement

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