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Crystal growth in X-ray-transparent plastic tubing: an alternative for high-throughput applications

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A modified capillary-growth method is described that has substantial advantages for standard and high-throughput protein crystal growth. Protein-containing drops are injected into vapor-permeable flexible X-ray-transparent polyester tubing. The protein concentration in the drop increases over time by water transport through the tubing wall at a rate controlled by the wall thickness and ambient relative humidity. Unlike in conventional vapor-diffusion growth, the evaporation rate from the drop is constant over a longer time period, providing more suitable conditions for nucleation, and can be controlled by varying the tubing thickness and surrounding humidity. *In situ* X-ray diffraction can be performed at room temperature or, by flash-cooling, at low temperatures. Compared with glass capillaries or thick-wall plastic tubing, sealing and handling the tubing and extracting crystals are much easier.

1. Introduction

Capillaries have long been used for protein crystal growth (McPherson, 1999). If X-ray transparent, they can allow X-ray study of crystals at room temperature without additional crystal manipulations. Capillaries are now being explored as a platform for high-throughput crystallization, screening and diffraction. Large numbers of drops with different conditions (isolated from one another using, for example, oil) can be dispensed into long capillary tubes and then observed to identify conditions that yield nucleation and growth (Daoura & Meldrum, 1999; Meldrum *et al.*, 2000; Zheng *et al.*, 2003; Zheng, Tice & Ismagilov, 2004; Zheng, Tice, Roach *et al.*, 2004). Capillary growth requires only small volumes of reagents and protects solutions and crystals from the environment.

Several methods have been used to grow protein crystals inside capillaries. These include vapor diffusion (McPherson, 1999), microdialysis (Zeppezauer *et al.*, 1968), liquid–liquid or free-interface diffusion (Salemme, 1972) and gel acupuncture (García-Ruiz & Moreno, 1994). Of these methods, vapor diffusion is the easiest and the most widely used and has been implemented in an automated system (Meldrum *et al.*, 2000). A small drop containing protein and precipitant is equilibrated against a larger volume of protein-free solution containing a larger precipitant concentration. Water (in vapor form) diffuses from protein drop to the protein-free solution, increasing the protein drop's concentrations of protein and precipitant (Fowlis *et al.*, 1988; Mikol *et al.*, 1990; Sibille *et al.*, 1991; Luft & DeTitta, 1997). In favorable cases, a protein supersaturation is reached where nucleation and growth begin. Protein is then depleted and solution supersaturation reduced until eventually growth stops.

Glass and thick plastic capillaries are the most widely used materials for drop containment (Potter *et al.*, 2004; Luft *et al.*, 1999), but both have major deficiencies in high-throughput applications. Glass scatters X-rays very strongly and so to be X-ray transparent must be made very thin (10 µm). Such thin-walled capillaries are extremely fragile and present a major challenge for automated handling. Plastic tubing is more robust but is thought to require thick walls to prevent evaporation, resulting in inferior optical and X-ray properties. Crystals can in principle be flash-cooled in tubing for low-temperature X-ray diffraction. Thick plastic capillaries have excessive thermal mass and poor thermal conductivity, resulting in much smaller cooling rates, and therefore require much larger cryoprotectant concentrations to prevent crystalline ice formation (Kriminski *et al.*, 2003; Husseini *et al.*, 2005). Extracting crystals from the tubing, which allows removal of excess mother liquor and provides faster flash-cooling and reduced background X-ray scatter, can be very difficult: each piece of tubing contains many growth experiments, opening the segment containing a given crystal is nontrivial and many crystals adhere to the tubing walls.

We recently showed how glass X-ray capillaries can be replaced with thin-wall polyethylene terephthalate (PET) tubing (Kalinin *et al.*, 2005) for crystal mounting and room-temperature X-ray data collection. PET tubing cannot be broken, has excellent mechanical properties, is chemically inert to most substances except strong alkalis (which hydrolyse it) and is highly radiation-resistant (Goodfellow Cambridge Ltd, 2005). Its linear X-ray absorption coefficient is about a quarter of that of the glasses used in X-ray capillaries. It can be easily cut with scissors or a razor blade and easily sealed by thermal impulse compression. It is permeable to water vapor but not to salts, proteins, PEGs and other non-volatile compounds used in crystallization.

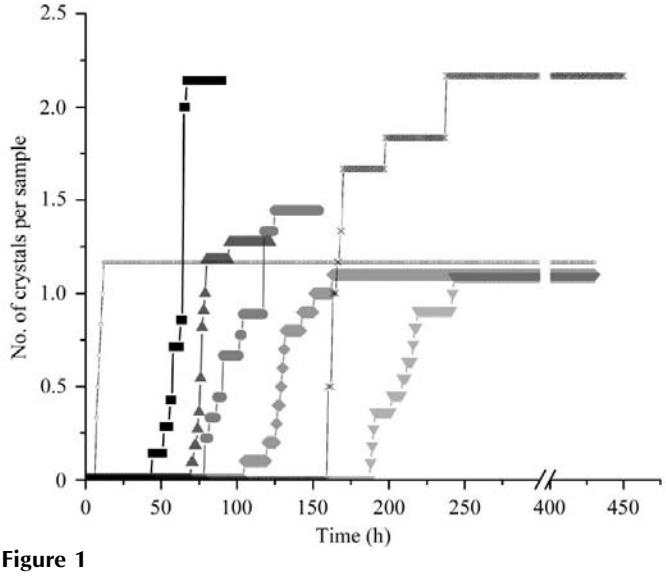

Figure 1
Average number of crystals per sample as a function of time after equilibration with ambient conditions for an initial mother-liquor volume of 1.5 µl and different tubing wall thicknesses and ambient relative humidities. The average was obtained by counting the total number of crystals obtained under given conditions and dividing by the number of samples prepared using those conditions. Squares, 12.7 µm thick tubing at 32% r.h.; triangles, 25.4 µm thick tubing at 10% r.h.; filled circles, 25.4 µm tubing at 32% r.h.; diamonds, 12.7 µm thick tubing at 73% r.h.; inverted triangles, 25.4 µm thick tubing at 73% r.h. Crosses, corresponding results for vapor-diffusion growth in a 1 mm ID X-ray capillary with a separation between protein-containing and protein-free liquids of 10 ± 1 mm. The initial measured rate of vapor transfer between liquids is roughly 6 µg h^{-1}, consistent with the observed time of nucleation. Empty circles, results for equilibration in a 24-well plate of a 2.5 µl hanging drop with a well solution containing 8%(*w*/*v*) NaCl. Each curve in Fig. 1 is an average of results obtained from at least six samples.

Table 1
Evaporation rates and typical initial crystal-growth rates (obtained by measuring the rate of change of the longest crystal dimension when the crystal reached a size of 30–50 µm) for different ambient relative humidities and tubing wall thicknesses.

	25.4 µm wall		12.7 µm wall	
Relative humidity (%)	Evaporation rate (µg h^{-1})	Typical initial rate of crystal growth (µm h^{-1})	Evaporation rate (µg h^{-1})	Typical initial rate of crystal growth (µm h^{-1})
10	12.0 ± 2.5	∼35–45	22.0 ± 5.0	N/A
32	8.0 ± 1.8	∼15–25	12.0 ± 2.5	∼20–30
73	4.5 ± 0.5	∼12–25	7.5 ± 1.0	∼15–25

Here, we describe a variant of capillary-based vapor-diffusion growth in which water transport occurs through the wall of thin plastic tubing rather than between two drops. This approach provides better control of nucleation and growth kinetics, reduced background scatter and rapid flash-cooling for *in situ* X-ray observations and easier crystal retrieval for high-resolution studies.

2. Materials and methods

PET tubing having an inside diameter of 1 mm and manufacturer-specified wall thicknesses of 6.4, 12.7 and 25.4 µm (±20%) was obtained from Advanced Polymers (Salem, NH, USA; catalog Nos. 040025CST, 040050CST and 041100CST, respectively) and cleaned using distilled deionized water. To study protein crystallization, 12.5 mg ml^{-1} hen egg-white lysozyme (Seikagaku America) was dissolved with 2.0%(*w*/*v*) sodium chloride in 0.1 *M* acetate buffer pH 4.8. Under these conditions the protein is fully dissolved and below its saturation concentration (Cacioppo & Pusey, 1991), so that crystal nucleation and growth is controlled entirely by subsequent water transport through the plastic tubing. 1.5 ± 0.1 µl of this solution was injected into 1.0 ± 0.1 cm long pieces of PET tubing. Both ends were then sealed with a thermal impulse sealer.

Samples were placed at room temperature (294 ± 1 K) either in ambient air or else in sealed containers with wells containing saturated solutions of LiCl, $MgCl_2$ and NaCl. The latter produced stable well defined relative humidities of 10 ± 1, 32 ± 2 and 73 ±2%. These hygrometer-measured humidities were reached 3–5 h after the containers were sealed and compare well with published values (Rockland, 1960). Samples were photographed on average once every 4 h to track crystal nucleation and growth. 12 samples for each

humidity/tubing-wall thickness combination were examined. X-ray data were collected from selected samples using a Rigaku rotating-anode diffractometer and MAR 345dtb detector.

3. Results

The thinnest (6.4 µm) tubing proved to be too prone to deformation, making solution injection difficult and the resulting drops irregularly shaped. It was thus excluded from further examination. The 12.7 and 25.4 µm tubing has excellent optical and X-ray properties and good mechanical properties. Table 1 shows crystal-growth rates (measured when crystals were roughly 30–50 µm in size) and water-evaporation rates through the tubing walls for different ambient relative humidities and tubing wall thicknesses. Crystals with odd morphologies arising from problems with the initial nucleus are excluded. Average crystal sizes obtained when growth stopped were 250–400 µm, limited by the starting amount of protein.

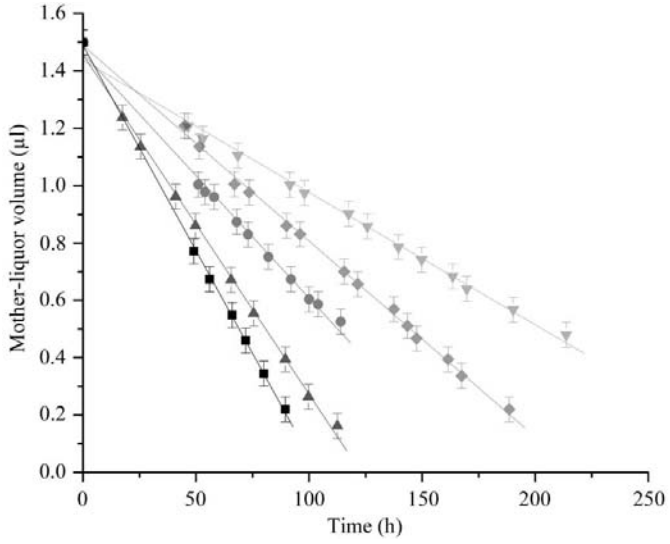

Figure 2
Liquid volume remaining in the tubing as a function of time for different tubing wall thicknesses and ambient relative humidities. Symbols are the same as in Fig. 1.

Fig. 1 shows the average number of well faceted crystals observed per 1.5 µl sample as a function of time after each crystallization experiment was commenced for wall thicknesses of 12.7 and 25.4 µm and various external humidities. The curves extend to the time when the volume of remaining fluid dropped to less than 15% of its initial value. As expected, crystals appear sooner and grow faster when the evaporation rate is higher, owing either to a lower surrounding relative humidity or to a smaller wall thickness.

Fig. 2 shows how the liquid volume within the tubing decreases with time owing to water transport through the tubing wall, measured by optical observation. The initial length of the 1.5 µl sample was about 1.9 mm. For all tubing wall thicknesses and ambient humidities, the decay is linear for volume decreases of up to 80%, which occur over a period of 3–12 d. The volume eventually saturates if the concentrations of salt and protein eventually yield a humidity equal to that of the saturated salt solution in the container; if this is not possible (*e.g.* if the salt in the mother liquor has a higher saturated vapor pressure than that of the container), the water completely evaporates from inside the tubing.

In standard vapor diffusion between protein-containing and protein-free liquids (*e.g.* a drop and well in a plate or two drops in a capillary), the volume approaches an equilibrium value (set by the relative concentrations of precipitant in the two liquids and typically half its initial value) roughly exponentially with a time constant τ (Fowlis *et al.*, 1988; Mikol *et al.*, 1990; Sibille *et al.*, 1991; Luft & DeTitta, 1997). This exponential decay is roughly linear only over a time τ, at which point the volume is roughly 65% of its initial value. Evaporation through thin-walled plastic tubing extends the linear regime to less than 20% of the initial volume. When salts are used as precipitants, equilibration in a standard

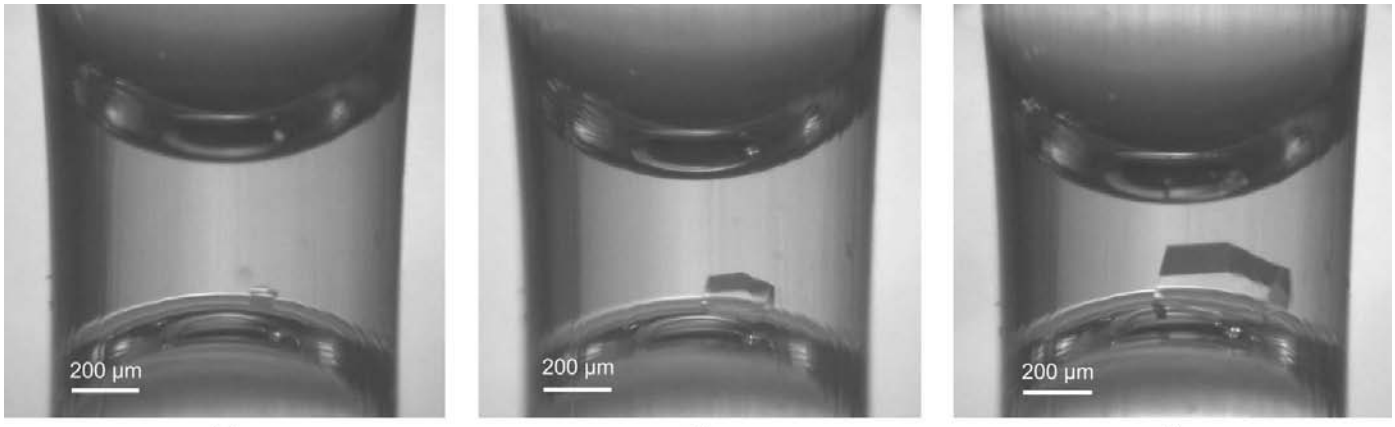

Figure 3
Photographs showing growth of a lysozyme crystal in 25.4 µm thick PET tubing in an ambient humidity of 32% r.h. Images (*a*), (*b*) and (*c*) were acquired 2, 5.5 and 15 h, respectively, after the crystal first became visible, which occurred 77 h after initial setup.

24-well crystallization plate of 1.5 µl drops occurs over a period of only a few hours (assuming there are no apertures or other physical barriers in the vapor path), which can be increased to several hours by addition of PEG. Equilibration between two drops in a glass capillary depends on the capillary diameter and starting concentrations, but is of the order of 100 h. Thus, thin-walled plastic tubing yields much larger fractional volume changes over which the rate of change is linear and much slower volume-decrease rates (especially for small drop volumes) than vapor-diffusion growth in crystallization trays.

Fig. 3 shows a series of images of a crystal growing in a 25.4 µm wall PET tube in a 32% r.h. environment.

4. Discussion

To date, the water permeability of tubing used in capillary growth has been considered to be a problem (Potter *et al.*, 2004), which has been addressed by using (strongly scattering) thick plastic or (fragile) thin glass. Our experiments demonstrate that crystal growth in vapor-permeable polyester tubing is a viable alternative to conventional capillary growth and has some distinct advantages.

(i) The 'well' solution with which the protein-containing drop equilibrates can be eliminated, making it easier to load the tubing and increasing the number of crystallization experiments per unit length.

(ii) The rate at which solvent evaporates from the crystal-growth solution and thus the variation of supersaturation with time can be controlled by varying the wall thickness and the surrounding relative humidity independently of the composition of the growth solution. This allows more quantitative design of growth experiments and a more rational exploration of crystallization phase space. It is well known (*e.g.* Wilson & Suddath, 1992; Gernert *et al.*, 1988; Li *et al.*, 2001) that controlling the evaporation rate has the potential to reduce the number of nuclei per unit volume, increase crystal size and improve crystal quality. In conventional vapor-diffusion growth in crystallization trays, equilibration between the 'well' and protein-containing solution occurs rapidly, so that nuclei form and growth begins at protein supersaturations that can far exceed those required to sustain growth. Equilibration rates can be reduced by lowering the temperature, increasing the drop–reservoir distance or adding PEGs (Fowlis *et al.*, 1988; Mikol *et al.*, 1990; Luft *et al.*, 1996; Luft & DeTitta, 1997). Using thin-walled plastic tubing, the supersaturation can be made to move slowly through the narrow range where nucleation rates drop from months to minutes, resulting in nucleation at lower supersaturations and a smaller number of nuclei.

(iii) Unlike in conventional vapor-diffusion growth, water removal from the drop continues long after nucleation occurs, counteracting the effect of protein depletion by the growing crystal. This may result in more uniform growth rates, larger crystals and a larger fraction of the starting protein being converted to crystalline form.

(iv) Compared with growth in glass capillaries and in conventional sitting- and hanging-drop growth, crystals can be more easily removed from thin-walled plastic tubing, which is required for optimal flash-cooling, minimum background scatter and structure determination to the highest resolutions. In particular, crystals that have adhered to the tubing walls

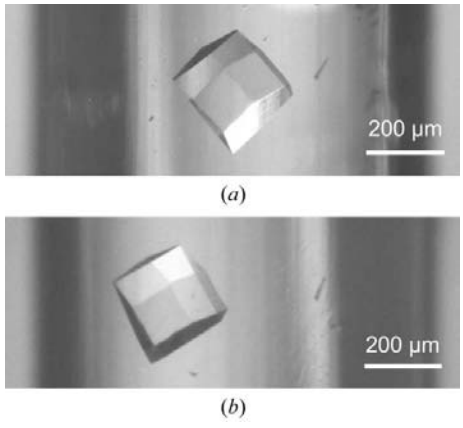

Figure 4
Removal of a HEWL crystal that adhered to the wall during growth. (*a*) After displacing the liquid in the tubing to ensure that the crystal was indeed firmly attached to the wall. (*b*) After gently squeezing the tubing with tweezers. The crystal is now floating freely in the solution.

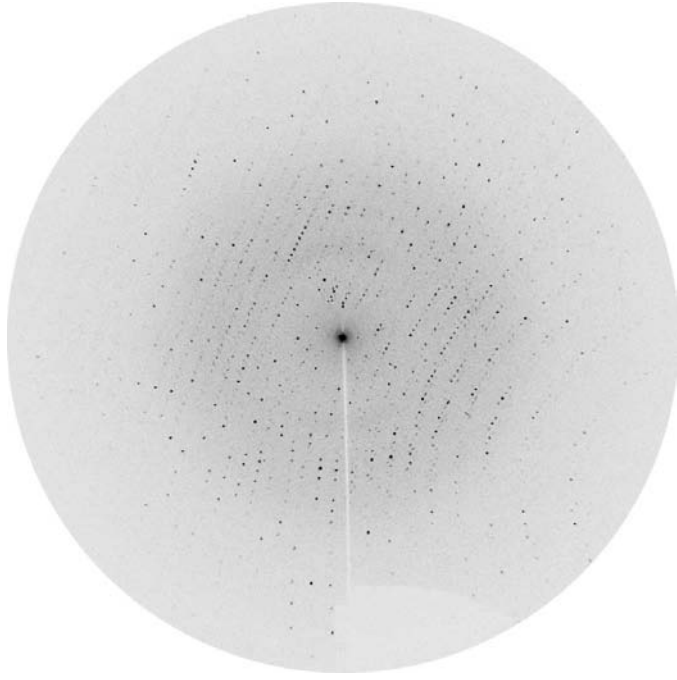

Figure 5
X-ray diffraction from a HEW lysozyme crystal flash-frozen without cryoprotectants in its sealed growth tube by immersion in liquid nitrogen. The crystal was grown in 25.4 µm wall PET tubing in an ambient humidity of 11% r.h. and flash-cooled 120 h after the start of equilibration. In this particular case the liquid receded away from the crystal during growth. The diffraction resolution at the outer radius of the image is around 1.85 Å and no ice rings are visible, presumably owing to the rapid cooling rates and high solute concentrations in the residual liquid surrounding the crystal. In general, cryoprotectants should be added to the growth solution to minimize the chance of hexagonal ice formation.

during growth can usually be released without damage by gently flexing the tubing, as illustrated in Fig. 4.

(v) Because thin-wall PET tubing scatters X-rays even more weakly than standard 10 μm wall glass X-ray capillaries, X-ray analysis can be performed directly on crystals in the tubing, with by far the largest contribution to background scatter coming from liquid surrounding the crystal. As shown in Fig. 5, crystals can also be flash-cooled in sealed tubing and X-ray data collected in a cold gas stream. In this case, the tubing's very thin walls provide little thermal resistance or thermal mass and so flash-cooling rates are comparable to those that would be achieved without surrounding tubing (Husseini, 2005). Provided that it is not too thick, the sealed tubing survives flash-cooling without cracking or other damage. Alternatively, the tubing can be cut on either side of the crystal, the mother liquor removed, and the tubing and crystal mounted for flash cooling and data collection following the method of Nakasako *et al.* (1995).

(vi) Thin-walled plastic tubing has particular advantages over growth in glass capillaries or thick plastic tubing for high-throughput applications. It can be easily sealed by squeezing it together and applying local heat pulses. Consequently, drops in a long tube can be isolated without use of oil or other 'separators' that may contaminate tubing walls when injected. Once individual protein drops have been sealed (like a strip of gum balls) in this way, the tubing can easily be coiled or folded, allowing more flexible storage. It can be easily cut using a sharp blade and so crystals in a particular segment/experiment can be easily excised. The tubing can be cut open and crystals retrieved for high-resolution diffraction studies by inserting a nylon loop or microfabricated X-ray mount. Crystals can also be retrieved by applying pressure at one end of the tubing segment using a pipette tip and pushing its contents out onto, for example, a mesh-type microfabricated X-ray mount (Mitegen, Ithaca, NY, USA).

Crystal growth in thin-walled plastic tubing does have some limitations. The manufacturing process produces slight longitudinal corrugations in the tubing walls that introduce slight optical distortion compared with glass. The tubing cannot be raised to temperatures higher than 343 K without contracting and so cannot be autoclaved, but it can be sterilized using ethylene oxide (EtO) or gamma irradiation. Slower evaporation rates compared with conventional vapor-diffusion growth may be problematic in cases where the protein is unstable and/or subject to degradation. However, the many advantages of thin-walled plastic tubing make it an attractive alternative to glass and thick-walled plastic for both low- and high-throughput capillary growth.

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