

## A cryocooling technique for protein crystals grown by dialysis from volatile solvents

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The transfer of protein crystals from the original crystallization environment to cryoprotectants, heavy-atom solutions, or quartz capillary tubes, exposes crystal droplets to conditions that can cause evaporation of the droplet and damage to the crystal. This problem is particularly acute for crystals grown from volatile solvents or that are otherwise air-sensitive. Here a method that overcomes this problem is applied to crystals of macrophage inflammatory protein II encoded by herpesvirus-8. The method is based on dialysis and makes use of a dialysis adaptor that can be used with 24-well crystallization plates. This method permits the transfer of crystals to cryoprotectant conditions with greater ease and lowers the risk of mechanical damage relative to other available methods.

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### 1. Introduction

A critical step in protein crystallography is overcome when single large protein crystals are obtained. The path from pure protein to single crystals is often tedious, requiring exhaustive searches for the optimal combination of buffer, protein and precipitant concentrations, and temperature that will yield the finest crystals. There is a wide range of buffered solutions that are explored and used for crystallization. Often accompanying these solutions are additives, some of which are volatile compounds like low-molecular-weight aliphatic alcohols and ketones. Handling of crystals grown from solutions with volatile ingredients can prove difficult when crystals are grown by any one of the two common techniques used for screening of crystallization conditions: the hanging-drop and the sitting-drop methods (Ducruix & Giege, 1992). Suitable crystals are commonly mounted in quartz capillary tubes for room-temperature data collection or transferred to a stream of N<sub>2</sub> at 100–110 K for low-temperature data collection. For low-temperature work, often it is necessary first to transfer the crystal from the mother liquor to a suitable cryoprotectant, using either rapid transfer of crystals to the cryoprotectant or gradual exchange of buffer solutions (Rodgers, 1997). During these processes, the hanging/sitting drop is exposed to the surrounding atmosphere. This poses a problem for crystals, particularly those from solutions that contain volatile substances. Since the size of the hanging drop that contains the crystal is usually small, the loss of volatile ingredients by evaporation can be rapid, allowing little time to transfer the crystal to more protective surroundings. This problem can sometimes be addressed by growing crystals directly in quartz

capillary tubes (Phillips, 1985) or under oil (D'Arcy *et al.*, 1996).

The dialysis technique described here addresses the problems indicated above, namely the ability to manipulate crystals without exposure to surrounding atmosphere and the transfer of crystals from crystal growth buffers to stabilizing solutions or cryoprotectants. Traditional dialysis buttons that are fully immersed into cryoprotectants have been successfully applied to solve these problems, but subsequent handling of crystals from the buttons can be difficult. Here we describe a modification of the dialysis method that results in easier manipulation of crystals.

### 2. Experimental

Initial crystallization conditions were first obtained from a sparse-matrix crystal screen. These conditions were refined to obtain better crystals. The above steps were performed by the conventional hanging-drop method, set up in Linbro plates. These crystals could not be transferred to a stream of N<sub>2</sub> or to a cryoprotectant for low-temperature data collection because of the fragility of the crystals once the cover slip was removed from the crystallization well. The dialysis adaptors described below were used for subsequent experiments.

#### 2.1. Materials

Viral macrophage inflammatory protein-II (vMIP-II) was a kind gift from Gryphon Sciences Inc. Crystal Screen I was purchased from Hampton Research Corp. Polyethylene glycol-4000 (PEG-4K) was purchased from Fluka GmbH. Linbro plates were from ICN chemicals, and 22 mm glass coverslips were from Fisher Scientific Co. Dialysis membranes (molecular weight cutoff 3500 Da) were obtained from Spec-

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trum. Paraffin oil and 2-propanol were purchased from J. T. Baker.

## 2.2. Crystallization

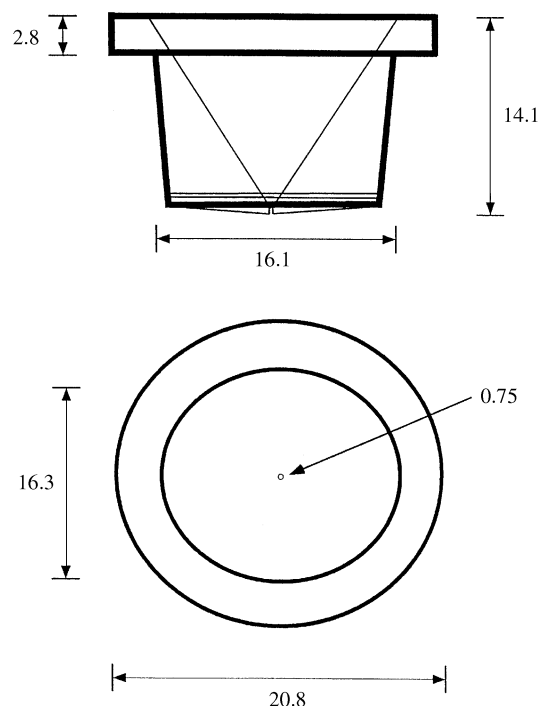
vMIP-II was dissolved in water to a concentration of  $12 \text{ mg ml}^{-1}$  and crystallized as previously described (Shao *et al.*, 1998). Crystallization experiments were performed as vapor-diffusion trials in hanging drops in Linbro plates. Initial crystallization conditions were obtained from a Hampton Sparse Matrix Crystal Screen I. However, these crystals were poorly formed as determined by visual inspection. Better looking crystals were obtained by the vapor-diffusion method by screening precipitant concentrations and pH units around the initial conditions identified from the Hampton Sparse Matrix Crystal Screen I experiment. The best formed crystals were observed in hanging drops that were obtained by mixing  $1 \mu\text{L}$  of the stock vMIP-II solution with an equal volume of reservoir solution that contained 11% PEG-4K, 11% 2-propanol and  $0.1 \text{ M}$  sodium citrate buffer at pH 5.60. Attempts to transfer these crystals to cryoprotectants or directly to a stream of  $\text{N}_2$  for low-temperature data collection were unsuccessful. The crystals began to crack as soon as the cover slip was lifted from the well of the Linbro plate. Attempts to prevent further deterioration of the crystals by adding artificial mother liquor containing 15% glycerol exacerbated the problem.

## 2.3. The dialysis adaptor

The dialysis adaptor is constructed from polystyrene. Earlier acrylic-based prototypes were discarded because of their chemical incompatibility with some organic solvents used in protein crystallization. The dialysis adaptor is conical (Fig. 1). The broad end fits the well of the 24-well Linbro plate comfortably. The tapered end of the dialysis adaptor extends three-quarters deep into the Linbro plate well and has an aperture of approximately  $0.75 \text{ mm}$  in diameter. A schematic of the dialysis adaptor with its dimensions is provided in Fig. 1.

## 2.4. Crystallization from dialysis adaptors

Once initial conditions were obtained for well formed crystals by the hanging-drop method, the dialysis adaptors were used for further experiments, since the transfer of the crystals from the hanging drop proved difficult. The 'dialysis adaptors' were used for crystallization in 24-well Linbro plates (Fig. 2) with approximately  $760 \mu\text{L}$  of reservoir solution in the wells of a Linbro plate. The dialysis adaptor aperture is covered with a piece of moistened semipermeable membrane with a molecular weight cutoff of  $3500 \text{ Da}$  (Spectrum Inc.). The dialysis membrane is held in place with a  $9 \text{ mm}$  O-ring.  $1 \mu\text{L}$  of  $12 \text{ mg ml}^{-1}$  vMIP-II stock solution is pipetted into the dialysis adaptor and mixed with an equal volume of the reservoir solution. The broad 'upper' end of the dialysis adaptor is covered with a coverslip. The dialysis adaptor is then placed in the Linbro plate such that the tapered end that is covered with dialysis membrane is immersed in the reservoir solution in the Linbro plate well. All contacts among the dialysis adaptor,

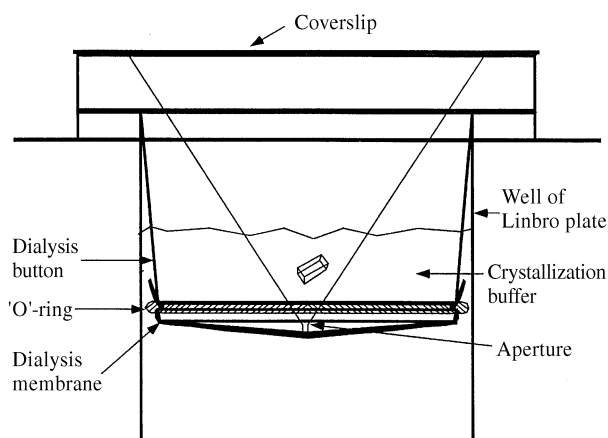


**Figure 1**  
Dimensions of the dialysis adaptor used. All dimensions are in millimeters. (a) View of the vertical cross section of the dialysis adaptor. (b) Horizontal cross section of the tapered end of the adaptor.

glass coverslip and Linbro plate are sealed with silicone grease (Fig. 2). Crystals grow to their full size of  $200\text{--}300 \mu\text{m}$  in 3–4 days.

## 2.5. Transfer to buffered solutions

To prevent ice formation on the crystals during X-ray data collection at  $100\text{--}110 \text{ K}$ , crystals are transferred to a cryoprotectant consisting of 15% PEG-4K, 15% 2-propanol, 15% glycerol and  $0.1 \text{ M}$  sodium citrate at pH 5.6. Dialysis adaptors that contained well formed crystals were removed (with the coverslips in place) from the reservoir solutions used for crystallization and transferred to wells in Linbro plates that had approximately  $760 \mu\text{L}$  of stabilizer with cryoprotectant



**Figure 2**  
A schematic of the dialysis button. The dimensions are not drawn to scale.

solution. The crystals were allowed to stand in the cryoprotectant for not longer than 2 h. This time interval allows sufficient solvent exchange to permit further manipulation of the vMIP-II crystals. The coverslip on the dialysis adaptor was then removed and approximately 15  $\mu\text{L}$  cryoprotectant was pipetted directly into the dialysis adaptor. This was followed by addition of approximately 25  $\mu\text{L}$  paraffin oil of low density ( $\rho = 0.8\text{--}0.9\text{ g ml}^{-1}$ ) into the dialysis adaptor. The paraffin oil forms a protective layer over the cryoprotectant and serves to prevent exposure of the cryoprotectant to the atmosphere, thereby eliminating any evaporation of the 2-propanol.

The above outlined procedure for introducing cryoprotectant was also applied for screening of heavy-atom derivatives. In most cases the heavy atom was dissolved to the desired concentration in the cryoprotectant. When the heavy-atom solutions did not involve the cryoprotectant, the dialysis adaptor containing the crystal was first transferred to the heavy-atom solution aliquoted into a well of a Linbro plate. Prior to data collection, the dialysis adaptor was then transferred, as above, to cryoprotectant.

Selenomethionine-vMIP-II crystals (Se-met vMIP-II) were also grown using the dialysis setup and subjected to the same cryoprotectant buffer exchange protocol described for crystals of native vMIP-II.

## 2.6. Crystal mounting

Crystals of vMIP-II, heavy-atom derivatives and Se-met vMIP-II were all mounted directly on the goniometer head in a stream of  $\text{N}_2$  at 100–110 K. The procedure involved scooping the crystal out of the dialysis adaptor using a Yale needle base in a manner such that the crystal is suspended in a bath of cryoprotectant that is formed within the nylon loop of the needle base. This bath is itself covered by a layer of paraffin oil that is used initially to form a layer over the cryoprotectant in the dialysis adaptor. The presence of the paraffin oil offers the crystal additional protection when mounted directly into the  $\text{N}_2$  stream.

## 3. Results and discussion

Dialysis methods have been used on numerous occasions for protein crystallization (McPherson, 1989; Trakhanov, 1989). We used an adaptor for dialysis designed for 24-well Linbro plates that prevents evaporation of volatile solvent from the crystallization conditions during crystal manipulation. Crystallization and cryoprotectant conditions for vMIP-II were indicated in an earlier report (Shao *et al.*, 1998). However, repeated attempts to transfer crystals from hanging drops directly to cryocooling conditions or to a stream of  $\text{N}_2$  were unsuccessful and prompted us to develop the procedure described here. The crystals cracked within seconds of exposure to atmosphere or during addition of cryoprotectant. Stepwise addition of increasing concentrations of cryoprotectant did not solve the problem. Attempts to grow crystals from sitting drops that were covered with a layer of paraffin or silicone oil, or in the presence of 15% glycerol, were unsuccessful. Crystals either did not grow or did not match the

quality of crystals obtained from hanging drops. The crystals that did grow under paraffin were still unable to withstand the shock of transfer to cryoprotectant. We therefore resorted to the dialysis technique described here.

The advantages of this technique are: (i) the crystal is not exposed to the surrounding atmosphere; (2) the mechanical shock to crystals during transfer to different buffer systems is minimized; (iii) heavy-atom or cryoprotectant solutions can be introduced in a gradual controlled manner, which minimizes shock to crystals due to changing environment. However, there are some disadvantages with this method: (a) the molecular weights of the precipitants (and cryoprotectants) must be smaller than the protein and must be discriminated by a commercially available dialysis membrane to allow entry of the precipitant and other buffer components, but prevent exit of the protein; (b) changes in osmotic pressure can result in a variation in the volume (and concentration) of the protein solution. Hence, the dialysis may be restricted to shorter periods than are sometimes used during heavy-atom soaks. For vMIP-II (MW  $\sim 8$  kDa), we used a dialysis membrane with a molecular weight cutoff of 3500 Da, even though the average molecular weight of the PEG was 4 kDa. No adverse effects were observed during crystallization.

We found the crystals to be extremely sensitive to glycerol concentrations. Concentrations greater than 15% glycerol or incubation for more than 2 h in 15% glycerol (after ceasing dialysis and adding 15  $\mu\text{L}$  of cryoprotectant) resulted in rapid deterioration. Even after appropriate dialysis, vMIP-II crystals did not diffract well when frozen and mounted with cryoprotectant alone. The use of a layer of paraffin oil over the cryoprotectant solution was essential for proper freezing of the crystals. The combination of cryoprotectants with immiscible oils for flash cooling macromolecular crystals has been independently reported (Kwong & Liu, 1999). While these observations may be considered anecdotal, the use of the dialysis adaptor allowed us to screen a large number of cryocooling conditions for these fragile crystals grown from volatile solvents. Since the concentration of glycerol used in the cryoprotectant was relatively low (15%), it is possible that the paraffin oil acted synergistically with the glycerol to provide adequate cryoprotection during flash freezing of the crystals. This technique was critical for solving the three-dimensional structure of vMIP-II (Fernandez *et al.*, manuscript in preparation). It is hoped that the methodology described here will be applicable to other crystallization experiments, particularly with protocols that involve the use of volatile ingredients.

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