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ORIGINAL PAPER

A plate holder for non-destructive testing of mesophase crystallization assays

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Abstract Here, we describe a goniometer holder to mount standard 96-well crystallization plates directly onto the goniometer head of an oscillation camera. This attachment was designed to check crystallization conditions straight from the crystallization plates under X-rays, and was proven to be useful for checking small crystals and solutions that destabilize monoolein-based lipidic cubic phase (LCP) crystallization experiments. A quick procedure for setting up LCP assays employing commercially available instruments is also reported.

Keywords Crystallization · Cubic phase · Membrane proteins · Plate holder

Introduction

When screening crystallization conditions of a soluble protein, all positive results must be further investigated to determine if it is a protein or a salt crystal. A common, but unreliable method to determine this is to crush the crystal and listen for the typical 'click' sound indicating a hard salt grain, or, if nothing is heard, suggesting that the experimental result *was* a protein crystal. Alternatively, the crystal may be stained with a dye, its birefringence tested (only if the protein crystal has not grown in the cubic system) or harvested from the growing medium to test it under X-ray to determine its diffraction pattern. Unfortunately, all these procedures may greatly interfere with the experiment.

The most largely employed strategy for crystallization of membrane proteins is based upon protein detergent-solubilization (Iwata 2002), the so-called in surfo method. Because crystallization methods for solubilized membrane proteins are the same as those employed for soluble proteins (primarily hanging and sitting drop vapor diffusion), checking crystals obtained from in surfo crystallization assays leads to similar difficulties as described above.

High throughput crystallization entails miniaturization of the drop containing the protein and precipitant solutions, which is achieved by dispensing and mixing drops with a final volume of 100 nl or even less. In general, due to aberrations, amorphous precipitations and other negative factors, the optical quality of such experiments is poor. Therefore, it is very difficult to handle crystals under these conditions, and quite often, they become injured when being manipulated with the loop to pick them up for mounting. Also, during this maneuver, the risk of evaporation causing drop precipitation is high. Crystal handling is even more critical, for example, when we obtain a unique specimen and want to test it under X-ray.

A more recent but less popular approach to crystallization of membrane proteins is the so-called in meso crystallization method, which is based on the lipidic cubic phase (LCP) medium or mesophase (Rummel et al. 1998; Caffrey

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2000, 2003). Mesophases are materials composed of lipids and water, arranged in crystallographically well-ordered curved bilayers interwoven by aqueous channels that mimic membrane structures (Cribier et al. 1993). The LCP-based method has been successfully used to crystallize about 50 membrane proteins (Raman et al. 2006), and protein crystals grown from LCP are often superior in quality to detergent-grown crystals (Cherezov et al. 2006).

Throughput in in meso crystallization is limited due to the fact that the current instrumentation is unable to deliver small and highly viscous lipidic dispensions. Recently, a relative inexpensive and versatile robotic system has been developed (Cherezov et al. 2004). This instrument can deliver both precipitant solutions and very small volumes of the protein sample (down to 20 nl), and a trial in a specially designed 96-well glass plates can be setup in about 13 min (Cherezov et al. 2004). Although the development of this robot signifies an important step toward increased throughput in meso crystallization method, due to the technical limitations the instrument is only employed for screening purposes (Cherezov et al. 2004).

In any case, harvesting crystals from an LCP-based medium can be a relatively risky task. In fact, in the LCP-based method, the growing medium is much more viscous, seldom birefringent, and less transparent than the current solutions employed in the traditional methods. To facilitate crystal extraction from the LCP, a procedure based on lipid hydrolysis has been proposed (Nollert and Landau 1998). However, this procedure also involves the interruption of the crystallization experiment, and eventually, the loss of all crystals from the tested drop. Another problem related to working with lipidic cubic phases is the uncertainty regarding the phase type that results from the mixture of the lipid, the protein solution and the additives, such as stabilizing compounds, detergents or precipitants.

As well as homemade solutions, many laboratories have designed a large variety of commercially available screening solutions employed in soluble as well as membrane protein crystallization experiments. Due to their different compositions, not all of these solutions are compatible with the in meso method. Moreover, when solubilized membrane proteins are mixed with lipids to form the LCP, detergents can also have detrimental effects on the cubic phase formation. These facts were previously observed and studied in some limited examples of screen solutions (Cherezov et al. 2001), interactions of surfactants and fatty acids with lipids (Koynova and Tenchov 2001), and one nonionic detergent (Ai and Caffrey 2000). Assuming that the cubic phase plays an essential role in the in meso crystallization method, and since the behavior of complex mixtures of proteins, lipids, water, salts, detergents, etc., at different temperatures, pHs, and concentrations is unpredictable, a simple and reliable testing procedure adapted to the LCP-based method is needed.

In searching for a non-detrimental and non-invasive test to avoid crystal extraction from the LCP crystallization medium and to test the integrity of the cubic phase at any time after the experiment has been set up, we designed and constructed a holder to mount plates directly onto the diffractometer. This holder allows one to test diffraction of crystals individually, avoid extraction from the growing medium, and simultaneously check crystals and the surrounding mesophase. This last feature is a useful application considering that the correct LCP-type must be determined reliably to reproduce mesophase experiments.

Experimental

Holder design and plate assembly

The device described below was specifically designed to be mounted onto a Huber XYZ Goniometric Head 1004 and can be easily adapted to other goniometric heads. The holder was constructed with a rectangular 3.3 mm thick polymethyl methacrylate plate (Fig. 1a) with one of the (short) sides mechanically reinforced by gluing to a methacrylate piece (Fig. 1b). The holder is firmly attached to the top piece of the goniometer head by means of two stainless steel screws (4 mm diameter) and a 7.9 mm thick methacrylate bridge (Fig. 1c). Along the center, the holder has a window or large groove (10×65 mm) that allows the diffracted X-rays to pass through the plate after the X-ray beam reaches the sample. The crystallization plate is attached to the holder with a paper clamp or rubber band, and a row of wells line the holder window (Fig. 1, Isometric view). In order to allow the plate wells to lie on the spindle axis, the top piece of the goniometer head must be remounted 180° out from its normal mounting. This is done by completely unscrewing the top X and Y adjustable screw until the top piece is removed, then turned 180°, and remounted by turning around the adjustable top X and Y screw until the top piece fits into the bottom piece about 10 mm (see Fig. 1, isometric view).

Camera setup

The goniometer head with the plate holder was mounted on a Marresearch image plate system Mar345 camera. To do this, three modifications from the standard camera set-up were needed: first, in order to make enough room for the plate and holder to fit in front of the beam exit, the beamstop was removed; second, an ad hoc beamstop (made of a lead cylinder soldered to the end of a iron wire of about 350 mm long) was mounted on the front of the image plate

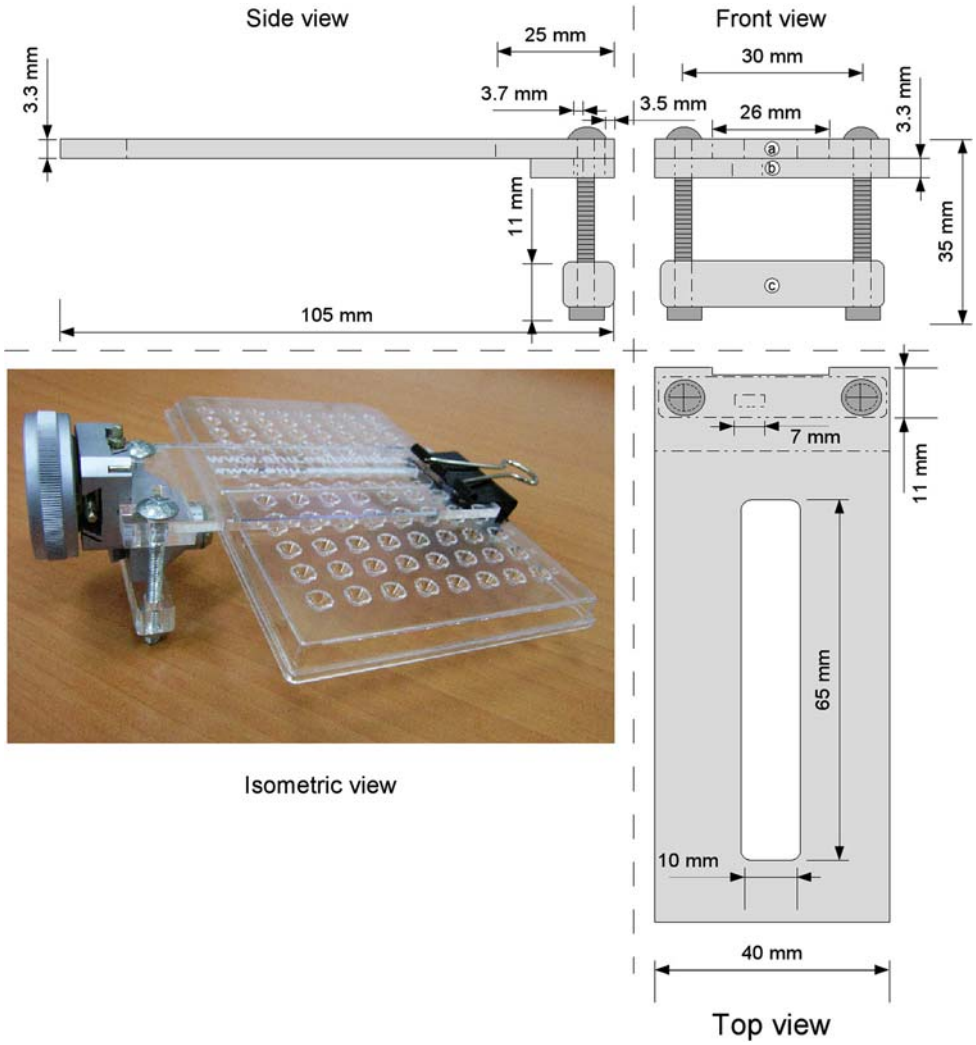

Fig. 1 Construction of the holder. The holder is made of methacrylate with a plate *a* reinforced by a rectangular piece *b*. The holder is attached to the goniometer head by tightening the two stainless steel screws holding the bridge *c*. The plate holder mounted onto the goniometer is shown in the isometric view (see text for more details)

(Fig. 2). This beamstop stops the direct beam, but is small enough to permit the low-angle diffraction to be collected; and third, if a cryo-cooler is also mounted on the equipment, the cryostream nozzle must be removed from the coldhead support stand.

Initially, when the plate is mounted on the goniometer, the psi angle must be 0° in order to approximately set the plate perpendicular to the X-ray beam (Fig. 2). Starting from this position, the image can be focused on the monitor screen by centering a plate region (Fig. 2, inset) in the same way as, for example, a crystal mounted on a cryo-loop is centered. Next, to select the irradiation zone, the coordinates x and y can be adjusted with head screws, the z coordinate adjusted by means of the camera translation screw and the area extended by selecting the aperture of the camera collimator slits. During an oscillation experiment, to avoid clashes between the plate and the camera, the rotation angle around the spindle axis must be adequately selected. A standard crystallization plate (about 12.5 cm long) can be rotated to a maximum angle of about 30° (Fig. 2a). Plate translations by adjusting the z-coordinate parallel to the rotation axis allows irradiating up to four neighboring wells. To irradiate the remaining wells, the plate has to be translated vertically to put another row along the spindle axis, and after completion of one side, the plate rotated and remounted 180° from the initial position to allow the other half plate side to be reached by the X-ray beam.

The optical quality of all plates employed in the experiments described herein was good, and samples were clearly seen through the video camera (Fig. 2b). Therefore, we were able to irradiate crystals individually and subject many areas of a single well to the X-ray beam by simultaneously selecting the appropriate slit apertures and centering the desired area (Fig. 2b). X-ray diffraction measurements were performed using a rotating anode (Bruker-Nonius FR591) operating at 50 kV and 80 mA (unless explicitly noted).

LCP crystallization setups

Volumes of ca. 50 µl monoolein-based LCPs were prepared with a two-50 µl syringe (Hamilton H-R81031) mechanical

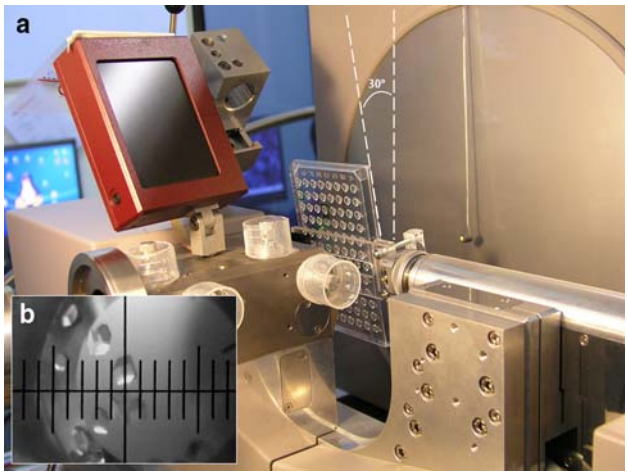

Fig. 2 Experimental set-up for testing plates on the plate holder. **a** The plate holder with an Imp@ct plate mounted on it is placed on the Mar-Research 345 camera. The plate is able to rotate upto a maximum of 30° and can be displaced along the spindle axis to irradiate without dismounting up to four neighboring wells. The handmade lead beamstop is attached to an iron wire and is placed directly at the center of the image plate, hanging from the top of the front cover. The lead cylinder has a circular phosphorus sticker so that the direct beam can be seen on it in order to calibrate the beam stop position. **b** A detailed view of the well can be seen on the screen of the video camera (located in front of the plate by the beam exit), allowing centering of the zone to be irradiated

Fig. 3 Semi-automatic mesophase dispensing. **a** A commercially available PHD Programmable 2000 pump, from Harvard Apparatus, adapted to dispense the sample vertically. When the automatic program is adjusted to dispense aliquots up to 250 nl of cubic phase, two plates can be set-up with only one 50-µl-syringe load. Alongside the pump controller, a humidification system (Honeywell, model V5100N) is set to avoid the dehydration process of the protein–lipid sample. **b** The two syringes (50 µl each) mixer (Cheng et al. 1998) mounted on the homemade mixer holder

mixer (Cheng et al. 1998) mounted on a homemade mixer holder (Fig. 3b). The LCP was prepared by mixing (c.a. 200 times) melted Monoolein (Sigma) at 40°C (60% v/v) with protein solution (40% v/v). Once the LCP was formed, it was transferred to one of the mixer's syringes, the connector dismounted, and a needle (22 gauge, internal diameter 0.41 mm; Hamilton H-R76514) secured to the syringe. The syringe with the LCP was mounted on a syringe pump (Harvard Apparatus, PHD Programmable 2000, Fig. 3) and aliquots of the lipid/protein LCP were dispensed automatically every second. While dispensing, the plate was manually moved sequentially to touch and stick the ejected LCP bolus (with a minimum volume of 100 nl) at the center of each of the plate wells. Immediately after the plate was completely filled with the LCP sample, a precipitant solution was dispensed in each of the 96 plate wells using a Mosquito (MDL) crystallization robot (touching the side of the well and with 'dispense first' operation mode). Plates were sealed with 4 in. sealing tape (Crystal Clear) and stored in an incubator at room temperature (20°C). With this procedure, counting the dispensing of LCP (200 nl aliquots) and precipitant solution (1.2 µl on each well), in order to set-up an Imp@ct (Greiner) 96-well experiment takes less than 5 min to complete. To avoid evaporation during sample dispensing, a humidification system (Honeywell, model V5100N) was placed nearby (see the supplementary material for more information on LCP dehydration), and when storing the crystallization plates, a small dish containing distilled water was permanently placed inside the incubator.

Crystallization experiments of wt KcsA potassium channel were prepared with a 10 mg ml^{-1} protein solution in buffer containing 20 mM Hepes, 1 mM Dodecyl Maltoside and 100 mM KCl. Commercial screening solutions (Wizard I&II, Emerald BioSystems) were layered in each well.

Results

Testing vapor diffusion experiments

Lysozyme (Sigma) at 50 mg ml^{-1} in 100 mM Sodium Acetate pH 4.5 was crystallized in both a 96-well hanging drop Costar plate (Corning) and a sitting drop low profile Greiner CrystalQuick™ plate (low profile, flat bottom), with the well containing 100 mM Sodium acetate pH 4.5 and 5% w/v Sodium chloride (100 nl of sample mixed with 100 nl of precipitant). To prevent liquid spilling during plate oscillation, the mother liquid volume in the Costar and Greiner plate wells was kept at 100 and 50 µl levels, respectively. The crystals obtained can be viewed in Fig. 4a (hanging drop) and Fig. 5a (sitting drop). Figure 4c shows a diffraction image of the hanging drop crystal, using a 175-mm sample to detector distance, a 3-min exposure,

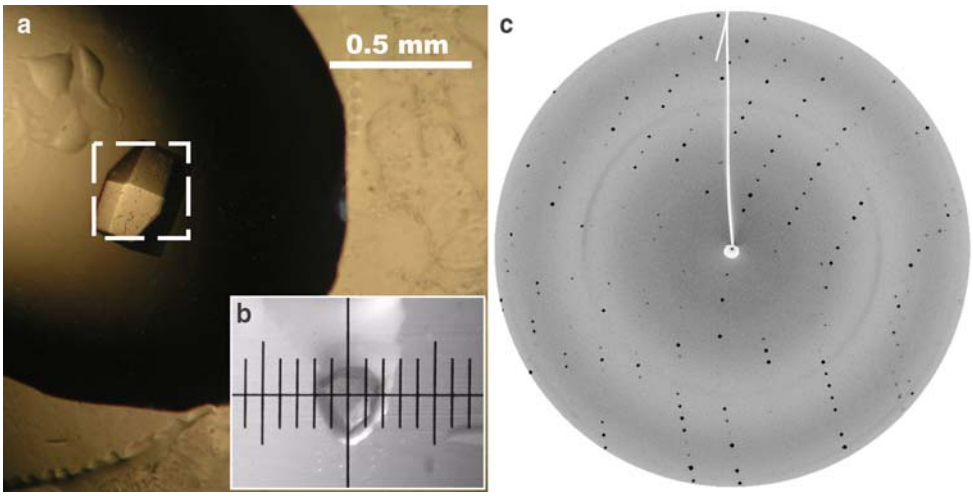

Fig. 4 X-ray test of a crystal grown in a hanging-drop 96-well plate experiment. **a** Lysozyme crystals were obtained in a 96-well hanging drop Costar (Corning) plate (see conditions in the text) and crystals appeared after 2 days. The region inside the *dashed lines* represents the approximate irradiated area (corresponding to the camera second pair slits apertures of 0.3 × 0.3 mm). **b** After the plate is mounted onto the holder, the crystal is centered viewing its image through the Mar345 video camera. Each division on the Vernier represents approximately 0.1 mm. **c** The crystal diffraction image obtained with the plate holder (175 mm sample to detector distance, 3 min exposure and a 3° oscillation) displays straight traits of reflexions lying on planes perpendicular to the spindle axis

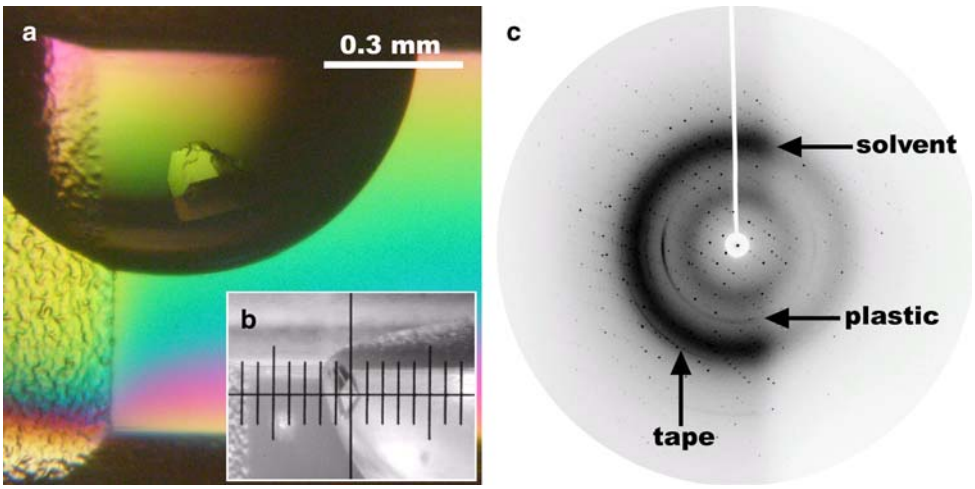

Fig. 5 X-ray test of a crystal obtained in a 96-well plate sitting-drop experiment. **a** View of a Lysozyme crystal under the optical microscope prior to mounting the plate on to the goniometer. Lysozyme crystals were obtained in a 96-well sitting drop CrystalQuick™ (Greiner) plate (see conditions in the text) and crystals appeared after 2 days. **b** Once the plate is mounted onto the holder, the crystal is centered and viewed through the Mar345 video camera. Each division on the Vernier represents approximately 0.1 mm. **c** The crystal diffraction image obtained with the plate holder (150 mm sample to detector distance, 5 min exposure, camera second pair slit apertures of 0.4 × 0.4 mm and a 0.5° oscillation). The shadow appearing at the right part of the diffraction image is due to the shielding caused by the plate, since the crystal slipped to the well's wall after the plate was mounted **c**. The oscillation image shows a typical protein crystal pattern and three haloes coming from the solvent, plastic and sealing tape dispersions (*arrows*)

3° oscillation and the beam collimated by setting the second slits apertures to 0.3 × 0.3 mm. Figure 5c corresponds to the test of a crystal of the sitting drop experiment, obtained using a 150-mm sample to detector distance, a 5-min exposure, 0.5° oscillation and second slit apertures of 0.4 × 0.4 mm. The camera views in Figs. 4b and 5b show how the crystal centering can be done.

On this diffraction image, we can see the crystal diffraction pattern and three concentric haloes: a broad and dark one due to the solvent scattering and two thin discontinuous haloes coming from the plastic plate and sealing tape dispersions.

Testing mesophase crystallization experiments

A typical view of a well containing an LCP-based crystallization experiment is shown in Fig. 6a. The low-angle diffraction pattern of this well shows the characteristic

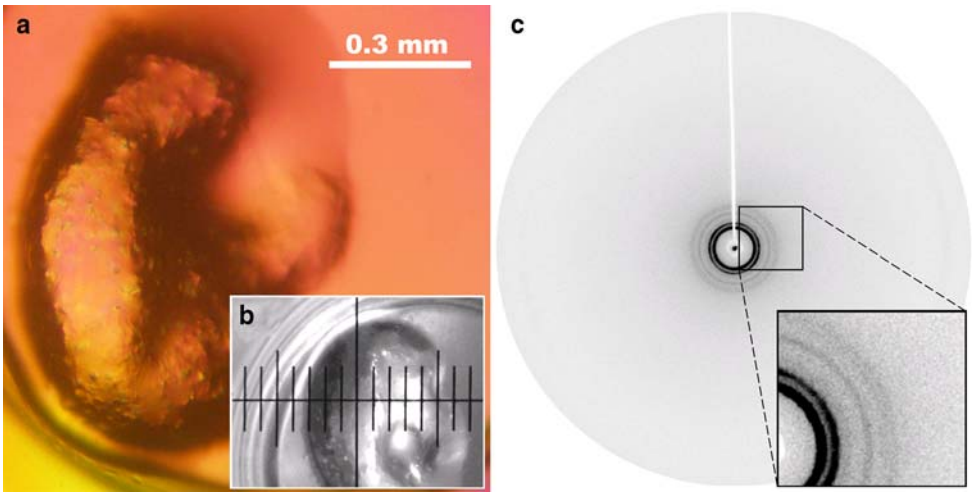

Fig. 6 Testing mesophase sample. **a** After the LCP (250 nl) and the precipitant solution (1.2 µl) were dispensed into each well (Imp@ct plate), the experiment looks like the image shown. **b** The mesophase bolus is centered on the beam and seen through the Mar345 video camera. Each division on the Vernier represents approximately 0.1 mm. **c** The X-ray diffraction pattern of the mesophase (using a second pair of slit apertures of 1.0 × 1.0 mm, 425 mm sample to detector distance, a 20 min exposure and 0.25° oscillation) show 5 reflections (*rings*) having reciprocal *d* spacing in ratios of $1{:}2^{½}{:}3^{½}{:}4^{½}{:}6^{½}$, indicating the presence of Monoolein-based lipidic cubic phase *Pn3m* (Landau and Rosenbusch 1996). The X-ray equipment was running at 50 kV and 100 mA for taking this image

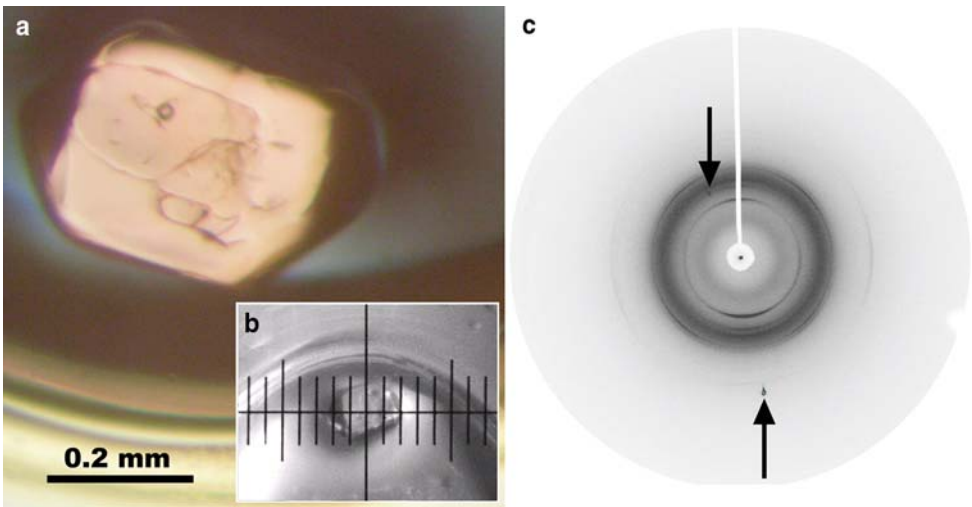

Fig. 7 Testing of a KcsA LCP-based crystallization result. **a** A few days after the experiment was set up, the LCP diffused out extending over the bottom of the well. Incidentally (and most likely due to dehydration), a crystal appears that needs to be tested under X-rays. **b** The crystal is centered viewing its image through the Mar345 video camera. Each division on the Vernier represents approximately 0.1 mm. **c** The diffraction image was obtained with a 175-mm sample to detector distance, 3 min exposure and a 3° oscillation. Here, we observe that the diffracted spots have short reciprocal spacing accounting for the diffraction of a salt crystal (*arrows*). The three diffraction haloes from the precipitant solution and the plastic dispersions appear (see Fig. 5c)

diffraction ring set (Fig. 6c) having reciprocal d-spacing ratios of $1{:}2^{½}{:}3^{½}{:}4^{½}{:}6^{½}$, corresponding to the monoolein–water Pn3m phase (Landau and Rosenbusch 1996). Figure 6b shows the video-camera view.

After maintaining this experimental set-up for few days at 20°C, the initial bolus of LCP diffused out and eventually precipitations, crystals and/or birefringent regions appeared in some wells (Fig. 7a). One such case containing a crystal was tested and the corresponding diffraction image taken at short crystal-to-image plate distance is shown in Fig. 7c. Figure 7b shows the camera view.

The few diffraction spots obtained in this oscillation image (Fig. 7c) corresponds to small cell spacing, suggesting that the tested crystal was salt.

On the other hand, the low-angle diffraction image (taken with a 40-min exposure) tells us that the original cubic phase was destabilized since the Pn3m ring-like pattern disappeared (data not shown), thus showing that there

had been destabilization due to the precipitant solution and/or dehydration. On this diffraction image (Fig. 7c), two low-resolution rings are displayed corresponding to the amorphous lipid and the dispersion from the plate sealing tape (see Fig. 5c).

Conclusions

The X-ray diffraction plate holder reported in this communication can hold standard 96- or 384-well hanging, sitting or micro batch plates. This attachment has proven to be useful for distinguishing protein from salt crystals directly from drops of vapor diffusion setups, as well as crystals immersed in microbatch experiments. Also, although the rotation angle of a mounted plate on the holder is restricted, this angle is large enough to allow the collection of oscillation data in a range of about 30°. Therefore, taking these features into account, the holder can be a useful tool for the current high throughput crystallization experiments of soluble proteins.

Nevertheless, the most useful applications we found for the holder introduced here are those related to the mesophase crystallization experiments. This is due to the ability of the plate holder to allow crystals to be directly irradiated in the growing medium, thus, overcoming the problem of extracting protein crystals from the jelly LCP just for testing.

Also, the holder allows the crystals appearing at early stages of growth to be tested under X-rays without disrupting the experiment. This will, for instance, allow small crystals to be checked and then further to be allowed to grow until they reach a larger handling size.

With this holder, it is also possible to test LCPs and precipitants that may stabilize/destabilize them, saving both screening solutions and lipid.

Additionally, in this work, we describe a rapid semi-automated procedure for dispensing both, small amount of mesophase and precipitant solution in standard crystallization plastic ware. This method is based on commercially available instruments and is useful to perform in meso crystallization experiments.

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