Membrane protein crystallization in lipidic mesophases. A mechanism study using X-ray microdiffraction

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The membrane structural biologist seeks to understand how membrane proteins function at a molecular level. One of the most direct ways of accomplishing this requires knowing the structure of the protein, ideally at atomic resolution. To date, this can only be done by the method of macromolecular crystallography. Integral to the method is the need for three-dimensional crystals of diffraction quality and their production represents a major rate-limiting step in the overall process of structure determination. The \textit{in meso} method is a novel approach for crystallizing membrane proteins. It makes use of lipidic mesophases, the cubic phase in particular. A mechanism for how the method works has been proposed. In this study, we set out to test one aspect of the hypothesis which posits that the protein migrates from the bulk mesophase reservoir to the face of the crystal by way of a lamellar conduit. Using a sub-micrometer-sized X-ray beam the interface between a growing membrane protein crystal and the bulk cubic phase was interrogated with micrometer spatial resolution. Characteristic diffraction from the lamellar phase was observed at the interface as expected. This result supports the proposal that the protein uses a lamellar portal on its way from the bulk mesophase up and into the face of the crystal.

1. Introduction

One of the primary impasses on the route that eventually leads to membrane protein structure through to activity and function is found at the crystal production stage. Diffraction quality crystals, with which structure is determined, are particularly difficult to prepare currently when a membrane source is used. This difficulty reflects our limited ability to manipulate proteins with hydrophobic/amphiphatic surfaces that are normally enveloped with membrane lipid. More often than not, the protein gets trapped as an intractable aggregate in its watery course from membrane to crystal. As a result, access to the structure and thus function of tens of thousands of membrane proteins is limited. In contrast, a veritable cornucopia of soluble proteins have offered up their structure and valuable insight into function, reflecting the relative ease with which they are crystallized. There exists therefore an enormous need for new ways of producing crystals of membrane proteins. One such promising approach makes use of lipidic liquid crystalline phases or mesophases.\textsuperscript{1} To date, this

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so-called in meso method is responsible for 48 of the 563 crystal structures of membrane proteins in the Protein Data Bank (http://www.mpdb.ul.ie). Our working hypothesis concerning the mechanism of membrane protein crystallization from the lipidic mesophase is that crystals grow from a local lamellar phase that is contiguous between the crystal and the bulk cubic phase where the protein is uniformly dispersed initially (Fig. 1). The hypothesis is based on experience gained with growing crystals of the membrane protein bacteriorhodopsin (bR), a light-driven proton pump.

There are two reports in the literature that address the in meso growth of membrane protein crystals by way of a lamellar conduit. The first of these involved freeze–fracture electron microscopic (EM) examinations of microcrystal of the acetylcholine receptor-\(\alpha\)-bungarotoxin complex grown from within a lipid mesophase. EM images showed highly ordered domains of the complex next to lipid lamellae consistent with our working hypothesis. In the second study, atomic force microscopy was used to characterize the in meso crystallogenesis of bR. The authors of that work state that evidence has been obtained for the existence of a lamellar conduit between the protein crystal and the bulk cubic phase.

In the current paper we report on a study designed to test the hypothesis by using a sub-micrometer-sized X-ray beam to profile the immediate environment of the bR

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**Fig. 1** Cartoon representation of the events proposed to take place during the crystallization of an integral membrane protein from the lipidic cubic mesophase. The process begins with the protein reconstituted into the highly curved bilayers of the ‘bicontinuous’ cubic phase (bottom left hand corner of the figure). Added ‘precipitants’ shift the equilibrium away from stability in the cubic membrane. This leads to phase separation wherein protein molecules diffuse from the continuous bilayered reservoir of the cubic phase by way of a sheet-like or lamellar portal (left upper mid-section of figure) to lock into the lattice of the advancing crystal face (right upper mid-section of figure). Salt (positive and negative signs) facilitates crystallization by charge screening. Co-crystallization of the protein and its native lipid is shown in this illustration. As much as possible, the dimensions of the lipid (light brown oval with tail), detergent (pink oval with tail), native membrane lipid (purple oval with tails), protein (blue; outer membrane Vitamin B\(_{12}\) transporter, BtuB; PDB code 1NQE), bilayer and aqueous channels (purple) have been drawn to scale. The lipid bilayer is approximately 40 Å thick.
crystal. Measurements were made at the Advanced Photon Synchrotron Source and a zone plate was used to focus the X-ray beam (1.83 keV) to a spot with a diameter of 400 nm. bR crystals were grown in a specially designed 25 μm thick cell with 100 nm silicon nitride windows. Low-angle diffraction data were recorded with the focused beam while the sample was translated from the bathing cubic phase up and into the bR crystal. The cubic and lamellar mesophases have signature diffraction patterns that were observed to come and go in a predictable manner as the beam interrogated different parts of the sample. The lamellar phase which extends for 1–2 μm from the growing bR crystal may serve as the postulated portal for proteins to pass from the bulk cubic phase to be ratcheted into position at the crystal surface.

Also described in this paper (section 3.9) are the results of an array of important control measurements performed to rule out artifacts that might arise due, in part, to radiation damage.

2. Experimental

2.1. Materials

Monoolein (1-oleoyl-rac-glycerol, lot M239-029-L, 356.54 g mol⁻¹), monopalmito-lein (1-palmitoyl-rac-glycerol, lot M219-A22-E, 328.5 g mol⁻¹) and cholesterol (lot CH-800-N22-K, 386.66 g mol⁻¹) were purchased from Nu Chek Prep, Inc (Elysian, MN). 2,3-Dihydroxypropyl (7Z)-9,10-dibromo-octadecanoate (bromo-monoacylglycerol, lot 180BR-10) was a gift from Avanti Polar Lipids (Alabama, AL). n-Octyl-β-D-glucoside (OG, Anagrade lot OG14, 292.4 g mol⁻¹) was obtained from Anatrace Inc. (Maumee, OH). Chicken egg white lysozyme (lot 71K7032) and salts of the highest quality available were purchased from Sigma-Aldrich (St. Louis, MO). Silver behenate powder was a gift from T. Blanton (Kodak, Rochester, NY).

Bacteriorhodopsin (bR) was solubilized with OG detergent from the purple membrane isolated from Halobacterium salinarum (strain S9) using established protocols. Water (resistivity > 18 MΩ cm) was purified by using a Milli-Q Water System (Millipore Corporation, Bedford, MA) consisting of a carbon filter cartridge, two ion exchange filter cartridges and an organic removal cartridge.

2.2. Microfocus diffraction

Experiments were performed at the Advanced Photon Source (Argonne, IL) on beamline 2-ID-B. Complete details of the experimental setup have been described. Briefly, soft X-rays (1830 eV, 6.755 Å) were focused by a Fresnel zone plate (ZP) to a spot with a minimum diameter of ~400 nm at a focal distance of 11.4 mm from the ZP. The diameter of the beam on the sample was changed by moving the ZP out of focus in the direction of or away from the sample. A 20 μm diameter pinhole was placed 8.6 mm downstream from the ZP to serve as an order sorting aperture (OSA) removing higher diffraction orders from the ZP. A central beamstop, 30 μm in diameter, was glued directly to the exit window of the beampipe upstream of the ZP to block the direct beam from passing through the ZP and the OSA.

A two-circle goniometer (Huber, Model 424) was used to position the sample and the detector in the X-ray beam. The sample cell was mounted on a small manual goniometer head (Huber, Model 1002) fixed to a motorized X–Y translation stage which, in turn, was attached to the inner (theta) circle of the two-circle goniometer. The X–Y stage allowed for sample translations in the direction perpendicular to the beam with a minimum step size of 150 nm. The detector was mounted on a linear Z stage attached to the outer circle (2-theta arm) of the two-circle goniometer. The Z stage was used to move the detector parallel to the X-ray beam and to set the camera (sample-to-detector) distance.

Diffraction patterns were recorded with a liquid nitrogen-cooled CCD detector (Brandeis; 1024 × 1024 pixels; pixel size, 24 μm × 24 μm). The sample-to-detector distance was ~110 mm which was calibrated using silver behenate (d₀₀₁ = 58.4 Å).
To avoid high intensity scatter from the OSA, the detector was repositioned in the beam by adjusting the 2-theta setting. Temperature inside the closed hutch where the sample was housed ranged from 24 to 26 °C and was monitored throughout the study. The hutch was in an air-conditioned room held at ~24 °C. No other temperature control of the sample was implemented.

Diffraction patterns were radially integrated in batch mode using the Fit2D program. Diffraction peaks in intensity versus 2-theta plots were fit using PeakFit (SPSS Inc., Chicago, IL).

2.3. Sample microcell

Details of the design and construction of the wet sample microcell have been reported. The cell was made of two silicon chips with 1 mm × 1 mm and 100 nm thick, low-stress silicon nitride windows that were custom fabricated by Silson Ltd (Northampton, England). One of the chips had a 1 mm wide by 25 μm thick spacer frame made from a photosensitive polymer SU-8 around its perimeter that confined the sample to a 10.5 × 3 mm² area. The sample was sandwiched between the two chips so that the 25 μm spacer defined the thickness of the sample. The maximum sample volume of the microcell was 0.7 μL. Chips were glued to a copper support frame on a 3 mm diameter pin that fit directly into the X-ray goniometer head.

2.4. Samples preparation

All samples of lipid mesophases were prepared using a home-built syringe mixer, as described. Accurate dispensing of the mesophase into the sample cell was done by means of a 50-step repetitive dispenser (Model PB-600, Hamilton Co., Reno, NV) attached to a 10 μL gas-tight syringe (Hamilton Co., Reno, NV), as described. Thus, 200 nL of the mesophase was dispensed onto the front face of the chip with the spacer at a location just outside the window area. On top of the mesophase was placed up to a maximum of 500 nL liquid solution, typically the precipitant. A second chip without the spacer was used to gently squeeze the lipidic mesophase so that it filled the area between the two silicon nitride windows. After the cell was closed, the edges were sealed hermetically with 2-ton epoxy (Devcon, Danver, MA).

Specific sample compositions and preparations used in the study are listed below.

2.4.1. Bacteriorhodopsin (bR) crystals. To grow bR crystals inside the microcells, 60% (w/w) lipid (monoolein, monopalmitolein, or 10 mol% bromo-monoacylglycerol in monoolein) was mixed with 40% (w/w) of a 15 mg bR per mL solution in 25 mM Na–K phosphate buffer pH 5.6 using the syringe mixer. Due to the relatively high OG detergent concentration in the bR samples the lipid/protein solution mixture produces a mixed phase system consisting of the cubic Pn3m and Lα phases. Microcells were loaded with 200 nL of this mixture together with 500 nL of precipitant (2.1–2.4 M Na–K phosphate, pH 5.6) and the samples were incubated at 20 °C. Crystallization was initiated 5–7 days before diffraction measurements were performed. By this time the crystals had grown to ~20–25 μm in the longest dimension and were randomly oriented inside the microcell (Fig. 2(a)–(c)).

2.4.2. Lysozyme crystals. Monoolein was mixed with 50 mg mL⁻¹ lysozyme solution in 0.1 M sodium acetate pH 4.8 buffer in a 3/2 weight ratio which spontaneously formed the cubic phase. Microcells were loaded with 200 nL of the lysozyme-laden mesophase and the bolus was overlain with 500 nL of 7–10% (w/v) NaCl in 0.1 M sodium acetate pH 4.8 as the precipitant. Crystals, grown within the cubic phase for 3–5 days at 20 °C, reached ~100 μm in the maximum dimension (Fig. 2(d)). Two types of lysozyme crystals were observed under these conditions; 3-dimensional tetragonal crystals and needle clusters. The phase behavior in the vicinity of both crystal types was examined in this study.
2.4.3. Cholesterol crystals. Cholesterol and MO at a 1 : 3 mole ratio were co-dissolved in methanol. Solvent was removed under a stream of argon initially and subsequently under vacuum (20 mTorr) for 24 h at room temperature (~20 °C). The dry cholesterol/MO was then homogenized with water in a 3/2 weight ratio using the lipid mixer. The mixture formed a transparent and homogeneous cubic phase. Crystallization of cholesterol was initiated by the addition of 500 nL 0.3 M Na–K

Fig. 2 Photomicrographs of crystals grown by the in meso method in microcells at 20 °C. (a) bR crystals growing in meso in the microcell. In this view, the entire window of the microcell is shown. (b) The same view as in (a) but taken between crossed polarizers to highlight crystal birefringence and the non-birefringent nature of the hosting cubic mesophase (dark background). At certain orientations the bR crystals appear as bluish birefringent objects. Clusters of such dots in the upper left hand corner likely originate from bR microcrystals that are not visible under normal light in (a). (c) A zoomed in view of several bR crystals growing in meso in a microcell. Crystals typically reach a size of 20–25 μm and are randomly oriented with respect to the microcell windows. (d) A crystal of lysozyme growing in meso in a microcell. (e) Plate-like crystals of cholesterol growing in meso in a microcell. The crystals were usually quite large and were aligned parallel to the microcell windows. Often crystals grew to fill the depth (25 μm) of the microcell. (f) X-Ray damage footprints left by the focused 400 nm beam after stepwise scans along orthogonal directions across a bR crystal. This picture illustrates the level of accuracy attained in positioning the sample in the X-ray beam. In this instance, scan lines were supposed to cross at the centre of the crystal. The mismatch of ~6 μm represents the error in beam position determination. Such tracks of radiation damage were used during analysis to improve absolute positional accuracy.
phosphate, pH 5.6. Crystals grew as large plates oriented parallel to the cell windows (Fig. 2(e)) and reached a maximum size within a few days at 20 °C. Often crystals were as thick as the depth of the cell.

2.4.4. Control samples. There were four control sample types prepared for use in this study as follows: (I) Samples of monoolein in water were prepared at a 3/2 ratio by weight, as described above. 200 nL of the corresponding mesophase was loaded into a microcell on top of which 500 nL water was added to guarantee excess water conditions. (II) Control samples of monoolein–OG detergent were prepared by combining monoolein and a 0.4 M OG solution at a 3/2 ratio by weight. 200 nL of the mesophase was added to the microcell followed by 500 nL 0.4 M OG solution. (III) Control samples of monoolein–salt solution were prepared by homogenizing lipid and 4 M Na–K phosphate, pH 5.6, solution at a weight ratio of 3/2. The microcell was loaded with 200 nL of the mesophase followed by 500 nL 4 M Na–K phosphate buffer, pH 5.6. (IV) Samples that mimicked bR crystallization conditions but that did not contain protein were made by homogenizing monoolein with 0.3 M OG in 25 mM Na–K phosphate buffer, pH 5.6, at a weight ratio of 3/2. The microcell was loaded with 200 nL of the mesophase and 500 nL 2.2 M Na–K phosphate buffer, pH 5.6.

3. Results and discussion

3.1. Target crystals

Crystals of a membrane protein (bR) (Fig. 2(a)–(c)), a soluble protein (lysozyme) (Fig. 2(d)) and a lipid (cholesterol) (Fig. 2(e)) were successfully grown in the cubic phase of hydrated monoolein in microcells specially designed for use in this microfocus diffraction study. Under standard conditions, the bR crystals ranged in size from 20–25 μm and the crystals were randomly oriented inside the cell (Fig. 2(c)). Lysozyme produced either three-dimensional tetragonal crystals up to 100 μm in maximum dimension or needle clusters (Fig. 2(d)). Cholesterol crystals grew as plates several hundreds of micrometres long that often filled the depth (25 μm) of the microcell (Fig. 2(e)).

The experimental objective of this study was to scan the interfacial region of the crystals within the bulk mesophase. This was done with either a sub-micrometre-sized focused beam having a diameter of ~400 nm or a defocused beam with a diameter of 5 μm. Typical scanning patterns consisted of 1 μm steps in the X-direction (parallel to the synchrotron orbit) and either 2 or 5 μm steps in the Y-direction (perpendicular to the synchrotron orbit) in the case of the focused beam, and 5 μm steps in the X-direction and 10 μm steps in the Y-direction in the case of the defocused beam.

Accuracy in positioning the crystals with respect to the beam was estimated to be within ~10 μm. The X-ray damage footprint left by the beam on the crystals was used as a ‘landmark’ to relate scanning paths to microphotographs of the crystals. This enabled improved accuracy in defining beam location with respect to the crystal after data collection was complete (Fig. 2(f)).

3.2. Lamellar ‘signatures’

Mesophases have unique small-angle X-ray diffraction patterns. With enough reflections a pattern can be indexed and the phase identified and structurally characterized (see, for example, ref. 14). In this study, we wished to determine the identity of the phase that acts as a conduit between the bulk cubic phase and the crystal. However, the conditions under which these microdiffraction measurements were necessarily made place constraints on the number of reflections that can be recorded at any one time. In most cases, this was limited to a first or second order.
Accordingly, we sought to exploit other characteristics of the assorted phases to assist in their identification. Under the prevailing experimental conditions the bulk cubic phase always gave rise to discrete Bragg reflections or spots (Fig. 3(a)). These arise because the domain size of the cubic phase exceeds the diameter of the beam ($r \sim 5 \text{ mm}$) and the thickness of the sample-holding microcell (25 mm). Accordingly, a spotty pattern plus the corresponding d-spacings of the discrete reflections were used to identify the cubic phase.

In contrast, the lamellar ($L_a$) phase tended to produce powder diffraction rings or arcs (Fig. 3(b)). Such patterns were never observed with the cubic phase in the microcells. Accordingly, powder-like diffraction along with a d-spacing range that is characteristic of the phase were used as hallmarks or signatures of the lamellar phase.

Our working hypothesis posits that a lamellar phase acts as a conduit between the bulk cubic phase and the crystal. Thus, diffraction characteristic of the lamellar phase was looked for in the vicinity of crystals growing in the cubic phase housed in

![Fig. 3](image_url)

**Fig. 3** Microdiffraction patterns recorded with lipidic mesophases and next to bR crystals growing in meso. (a) Pattern from a fully hydrated monoolein sample in the cubic $Pn3m$ phase recorded with a focused beam. Exposure, 1 s. (b) Pattern from a sample of monoolein in excess 0.4 M OG detergent in the $L_a$ phase recorded with a focused beam. Exposure, 1 s. (c) Pattern recorded with a focused beam next to a bR crystal grown in a mesophase prepared with 10% bromo-monoacylglycerol in monoolein. Exposure, 1 s. (d) Pattern recorded with a defocused 5 µm beam in the vicinity of bR microcrystals grown in a mesophase prepared with monoolein. Exposure, 1 s. (e) Pattern recorded with a focused beam next to a bR crystal grown in a mesophase prepared with monoolein. Exposure, 1 s. (f) As in (e) with an exposure of 10 s.
the sample microcell. Indeed, such signatures were seen (Fig. 3(c)–(f)). However, the lamellar diffraction was usually weak and was not always present. For this reason, we considered it necessary to perform a survey involving many crystals and thousands of diffraction patterns with a view to judging the frequency with which the lamellar signature was seen and to determine if this correlated in any way with the orientation of the crystal in the cell.

A total of 84 hexagonal plate-like crystals (see, for example, Fig. 2(c) and (f) and 4(a)–(d)) were included in the survey and all of the crystals were in excess of 10 μm in maximum dimension. Both the focused and defocused beams were used to probe crystal interfaces which was done using step sizes of 1 μm and 5 μm, respectively. The results of the survey are as follows. Crystals that were oriented with the plane of the hexagonal plate perpendicular to the microcell windows had the lamellar signature in all of the nine crystals that were examined, corresponding to a hit score of 100%. In the case of crystals that were tilted, with the plate plane at an angle of greater than 45° to the plane of the window, the hit score dropped to 40% (16 out of 40 crystals). It dropped even further to 11% (4 out of 35 crystals) when the tilt angle went below 45°.

These observations are consistent with our working hypothesis. Thus, proteins are in a sheet-like arrangement in the crystal with the sheet planes parallel to the plane of the hexagonal plate. The expectation is that the bilayers of the lamellar phase in the conduit are continuous and coplanar with the sheets of protein in the crystal (Fig. 1). Thus, since diffraction will occur when the X-ray beam and the plane of the lamellae are parallel, the hit score observed is as expected.

The lamellar phase signature diffraction was usually seen within ~10 μm of a crystal (Fig. 4(a) and (d)). However, in some cases the signature was observed but in the absence of a nearby macroscopic crystal. Careful examination of such regions in the sample using crossed polarized microscopy consistently revealed the presence of dot-like birefringence (Fig. 4(e) and (f)). The frequency of these observations was as follows: 5% of regions with high birefringence had the lamellar signature compared to <0.5% for zones of low birefringence (analysis based on ~1000 patterns). The dot-like birefringence very likely arises from sub-micrometre-sized crystals and is consistent with the hypothesis for a lamellar conduit.

The region in which a continuous lamellar signature was observed depended on the size of the crystal. Thus, for example, with small and sub-micron-sized crystals its maximum extent was 1–2 μm. For big crystals however the zones were generally larger but their extent did not exceed the size of the crystal by more than 1–2 μm in any direction (Fig. 5(a) and (b)).

### 3.3. Lattice parameter of the lamellar ‘signature’ and the cubic phase

The lattice parameter of a mesophase depends on a variety of factors including temperature and sample composition. In this study, we found that the d-spacing of the lamellar signature, which is related to the lattice parameter of the mesophase, also depended on the size of the crystal and that of the X-ray beam. Thus, with large crystals the d-spacing observed with the 400 nm and 5 μm beams was 50–54 Å and 53–54 Å, respectively. In contrast, with small (<10 μm) and sub-micron-sized crystals the corresponding values were 42–43 Å with the focused 400 nm beam, 45–48 Å with a 1 μm beam, and 51–54 Å for the defocused 5 μm beam (Fig. 6(a)). This dramatic change in d-spacing with beam size is likely attributable to X-radiation damage as described below (section 3.4 Radiation damage).

The lattice parameter of the cubic Pn3m phase surrounding scanned crystals varied in the range from 85–97 Å depending on crystallization conditions. The domain size of the cubic phase was typically 50 μm. However, in some samples domains in excess of 200 μm were observed. Further, the cubic phase in crystallization samples displayed some disorder as evidenced by an excess of diffuse scatter in the region between the (110) and (111) reflections, as noted. 7
3.4. Radiation damage

Damage to lipidic mesophases by synchrotron X-radiation has been well documented.\(^{15-19}\) How a particular mesophase responds to a given X-ray exposure depends on the lipid, sample composition including hydration, and temperature. The response also depends on the X-ray dose, dose rate and wavelength.\(^ {19}\)

In the current study, we observed that the d-spacing of the lamellar signature changed depending on the size of the beam used to make the measurement, particularly in the vicinity of small and sub-micron-sized crystals (Fig. 6(a); section 3.3). We attribute this effect to radiation damage. To begin with, the delivered X-ray dose rate depended inversely on beam size. Thus, the 400 nm and 5 \(\mu\)m diameter beams deliver dose rates of 1.4 Grad s\(^{-1}\) and 9 Mrad s\(^{-1}\), respectively. Further, the rate at which the lamellar signature disappeared upon continuous irradiation depended inversely on beam size. Thus, the lamellar ring or arc intensity faded in matters of seconds in the focused 400 nm beam. In contrast, with the defocused 5 \(\mu\)m beam reflection intensity did not change significantly in the first 2 min after which there was a slow loss of intensity. Because intensity did not alter dramatically with time in the latter case, it was possible to track the change in the d-spacing of the lamellar signature throughout the exposure. The initial value was 54 Å and it dropped to 43 Å over a period of about 3 min. It then stabilized in the 42–43 Å range. When mapped to equivalent accumulated dose (compare Fig. 6(a) and (b)), these data support the view that the different d-spacings recorded for the different beam sizes arise due to X-ray damage.

Fig. 4 The occurrence of the lamellar signature in stepwise scans across bR crystals and microcrystals growing in meso. The horizontal and vertical lines represent the regions of the sample interrogated by the X-ray beam in sequential 1 \(\mu\)m steps. Solid blue circles identify locations where the lamellar signature was observed. (a)–(d) bR crystals oriented perpendicular to the microcell windows and to the X-ray beam. The entire area within the box in (c) was scanned with the focused beam in steps of 2 \(\mu\)m. (e), (f) bR microcrystals. The photomicrograph in (f) was taken using crossed polarizers. Dot-like birefringence likely corresponds to sub-micrometer-sized crystals.

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![Image](a)

**Fig. 5** The lamellar signature and its coincidence with a crystal of bR growing in meso. (a) Photomicrograph of the target bR crystal oriented perpendicular to the microcell windows and to the X-ray beam. The entire 50 μm × 50 μm area of the microcell shown was scanned using a focused 400 nm beam with sequential 1 μm steps in the X-(horizontal) direction and sequential 2 μm steps in the Y-(vertical) direction. Exposure time at each location was 1 s. The black horizontal lines mark where the lamellar signature was observed. (b) The intensity of the lamellar signature along one of the horizontal scan lines in (a). The vertical dashed lines represent the edges of the bR crystal. (c) An example of a diffraction pattern recorded during data collection in (a) that includes the lamellar signature. (d) An intensity versus scattering vector plot produced by radially integrating the diffraction pattern in (c). The d-spacing and Miller index of the lamellar reflection are indicated.

Only a limited number of studies on radiation damage to lipidic mesophases have been reported. The above damage-induced rapid and substantial drop in d-spacing, for what we are assuming to be the lamellar phase, has not been documented before.

In most cases, the lamellar signature appears initially as diffraction arcs reflecting a high degree of orientation, as expected. With exposure time and accumulated dose.

![Image](b)

**Fig. 6** d-Spacing of the lamellar signature and its sensitivity to beam size and accumulated X-ray dose. (b) Diffraction patterns were recorded in the vicinity of bR crystals growing in meso using beams of the indicated sizes and giving rise to the corresponding accumulated dose. In all cases, the exposure time was 1 s. (a) Patterns were recorded during continuous irradiation at a fixed location in the sample using a defocused 5 μm diameter beam and 1 s exposures.
the arcs evolve into powder rings. Presumably therefore damage disrupts the oriented multilayers and induces the formation of structures reminiscent of multilamellar vesicles. The results shown in Fig. 7 demonstrate this effect convincingly. The target crystal was oriented parallel to the microcell windows and to the X-ray beam (Fig. 7(a)). Thus, the lamellar signature was not expected to be seen at the crystal/mesophase interface because the lamellae and the beam are orthogonal. Indeed, this was found to be the case (Fig. 7(e) and (f)). However, with time in the beam and thus accumulated dose, the lamellar signature appeared, grew in intensity and developed into a well-defined powder ring (Fig. 7(d) and (e)). At the end of the experiment damage to the crystal was clearly visible (Fig. 7(b)). When viewed between crossed polarizers the damaged zone showed the characteristic extinction cross texture (Fig. 7(c)) of the lamellar phase. What likely happened here is as noted above. Damage induced the lamellar conduit to lose its natural alignment with the crystal and to form what amount to multilamellar vesicles with their characteristic birefringence and powder diffraction. One other possibility not discounted would have the products of crystal damage inducing lamellar phase formation.

3.5. Higher order diffraction

Thus far, we have relied on a single diffraction ring or arc as the hallmark of the lamellar phase. In a separate study, we have shown that under conditions of measurement with the microfocused beam only the lamellar and inverted hexagonal phases produce such diffraction.\(^7\) But a single reflection does not allow for unambiguous phase identification. For proper indexing, higher order reflections are needed. Every effort was made to collect such data in the current study with a view to firming up phase identity. Thus, the detector was repositioned such that data in the vicinity of the (002) reflection of the lamellar phase and the (11) and (20)

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Fig. 7  X-Ray damage-induced expression of the lamellar signature next to a bR crystal growing in meso. (a) The target crystal was oriented parallel to the window of the microcell and to the X-ray beam. (b) The same crystal as in (a) following one hundred exposures of 1 s duration to the defocused 5 μm beam. (c) As in (b) but viewed between crossed polarizers. The site of damage has associated with it the ‘extinction cross’ texture characteristic of the lamellar phase. (d) Powder diffraction ring recorded at the site of damage in (b) characteristic of an unoriented lamellar phase. (e) Diffracted intensity in the lamellar signature during X-irradiation that produced the damage shown in (b)–(d). (f) d-Spacing of the lamellar signature during X-irradiation that produced the damage shown in (b)–(d). Note that in (e) and (f) there is no trace of the lamellar signature in the diffraction pattern during the early stages of the exposure.
reflections of the hexagonal phase could be recorded. Further, the exposure time was increased to 100–200 s and the beam was defocused to 5 μm to minimize and slow radiation damage. Typically, the (002) reflection from the lamellar phase of hydrated monoolein is very weak. Accordingly, additional measurements were made with monopalmitolein and a bromo-monooacylglycerol–monoolein (1 : 9 by mol) mix. Both sample types form the lamellar and cubic Pn3m phases and in both cases the (002) reflection of the lamellar phase is more pronounced. However, of the many crystal-containing samples tested in the course of this study only one showed a trace of the (002) reflection (Fig. 8). In all other cases convincing higher order diffraction was not observed. It is important to appreciate that a failure to see this higher order diffraction does not negate the existence of the lamellar phase.

3.6. Birefringence at the interface

A polarized light micrograph of bR crystals in the cubic phase showed ‘extra’ birefringence in the form of a halo and that spread for ~5 μm around the crystal (see Fig. 3 in ref. 20). This was interpreted as evidence in support of the existence of the proposed lamellar conduit. However, the photomicrograph was taken through a thick-walled and highly curved glass tube in which the crystals grew where light is distorted and where scattering from defects in the cubic phase is possible. We have taken advantage of the microcells used in this study, which have extremely thin and flat windows, to record high quality images of the crystals where optical artifacts are minimized. Photomicrographs of plate-like crystals oriented perpendicularly and parallel to the window plane taken with and without crossed polarizers are presented in Fig. 9. These show no evidence of ‘extra’ birefringence around the crystals to within the resolution of the measurement (~1 μm). This agrees with the
microdiffraction data presented above indicating that the lamellar conduit is likely oriented and that it does not extend for more than 1–2 \( \mu \text{m} \) beyond the crystal.

3.7. Relative orientation of the lipidic cubic phase

The proposal for a lamellar conduit between the bulk cubic phase and the face of the crystal suggests that the two mesophases are contiguous. It was of interest therefore to determine if there was any preferential relative orientation of the lamellar and cubic phases. The data in Fig. 10 would suggest that this is so. In that figure is shown a series of patterns recorded as the sample was moved in the X-ray beam from the bulk cubic phase on one side of a crystal, through the crystal and back into the bulk cubic phase on the other side of the crystal. The crystal was oriented with its hexagonal plane perpendicular to the cell windows. The (200) reflection from the cubic phase was observed in the first frame which arises from the bulk mesophase on one side of the crystal (Fig. 10(a)). In the second frame (Fig. 10(b)) the intensity of the (200) reflection dropped off dramatically and the (001) lamellar signature appeared right next to it. At the mid-point in the scan the lamellar signature dominated and the cubic phase reflection was barely detectable (Fig. 10(c)). Upon continued movement of the sample through the beam the lamellar signature disappeared and the (200) reflection from the cubic phase returned (Fig. 10(d) and (e)).

The near coincidence of the two reflections (Fig. 10(b)) indicates that the layer spacing giving rise to the (001) reflection of the lamellar signature and to the (200) reflection of the cubic phase is very similar. Further, the reflecting planes in the two phases are close to being parallel. In a separate scan, the same observations were made but with the (111) reflection of the cubic \( Pn\bar{3}m \) phase in place of the (200) reflection (data not shown).
These data are also relevant to the lamellar–cubic phase transition mechanism (see ref. 21, for example).

3.8. Displacing the mesophase from the crystal surface

At one point during the course of this study temperature control in the experimental hutch at the synchrotron was lost and the temperature of the sample rose by a few (\(\sim 5 \, ^\circ\text{C}\)) degrees. The sample was subsequently withdrawn and immediately examined microscopically. As revealed in Fig. 11 the temperature rise triggered the formation of small droplets throughout the sample. Some droplets were free in the bulk cubic phase. However, most were associated with bR crystals and almost all of the crystals had a droplet on at least one face.

The droplets are very likely excess aqueous phase that spontaneously forms with increasing temperature. The corresponding temperature–composition phase diagram for the monoolein–water system\(^{13}\) supports this conclusion because the excess water boundary of the cubic phase in that diagram shifts to lower hydration levels with heating. What is particularly interesting about the behaviour recorded in Fig. 11 is that the droplets appeared to be associated with the large flat faces of the crystal with very high frequency. It is seldom that droplets are attached to the other crystal faces. Since the droplets develop presumably by prying the mesophase off the crystal, their noted spatial distribution suggests that the flat hexagonal face of the crystal provides the weakest link. This is consistent with the view that the lamellar phase in the conduit contains bilayer sheets that are coplanar with the layers of protein in the crystal (Fig. 1).

3.9. Control measurements

Given the weakness of the lamellar signature intensity encountered in this study and the relatively low frequency with which it was observed, it was considered important that the proper control studies be performed to lessen the likelihood of artifacts and of misinterpreting the data. Artifacts might arise due to radiation damage, as noted,
or to local inhomogeneities developing in the samples. The control measurements performed and the information they provided are described below.

3.9.1. Monoolein in excess water. This sample was prepared as a reference cubic $Pn\bar{3}m$ phase which is stable at room temperature under conditions of excess water.\(^\text{13}\) It is the most common phase observed during the \textit{in meso} crystallization of membrane proteins. It was necessary to characterize this phase and its behaviour in the microcell under the prevailing conditions. The lattice parameter recorded for this sample was 103 Å which is within the range of values reported in the literature for the bulk cubic $Pn\bar{3}m$ phase.\(^\text{13}\) The average distance over which a continuous cubic $Pn\bar{3}m$ diffraction pattern was recorded in the microcell was 10–15 \SI{}{\mu m}. This then corresponds to the average domain size of the cubic phase. It was also noted that the diffracted intensity and lattice parameter of the cubic $Pn\bar{3}m$ phase were very stable with respect to radiation exposure.

3.9.2. Monoolein and detergent. Our test membrane protein bR brings with it into the lipidic mesophase a certain amount of OG detergent used to solubilize it from the purple membrane of its host cell. A typical bR sample contains 0.3–0.6 M OG.\(^\text{12}\) The detergent can alter mesophase behavior and we have established separately that at high enough concentrations the cubic phase transforms to the lamellar phase. For this control measurement therefore OG was combined with hydrated monoolein at a level that mimics the conditions that prevail during crystallization trials with bR. Here, the sample was found to exist in the lamellar ($L_{\alpha}$) phase and the d-spacing of its (001) reflection recorded in the microdiffraction cell was 48 Å. Because the intensity of the second order (002) reflection from this phase was weak, signal-to-noise was improved by summing intensities over several diffraction patterns (Fig. 3(b)). The lamellar phase was sensitive to radiation damage and this was evidenced by a drop in the diffracted intensity of the (001) reflection (2 times in 100 s, \~{}10 times in 800 s with the focused beam) and a very small reduction in d-spacing (by no more than 0.5–1 Å in 800 s).

3.9.3. MO and salt. High concentrations of salt induce formation of the inverted hexagonal ($H_{\text{II}}$) phase in hydrated monoolein at room temperature.\(^\text{14,22}\) bR crystallization \textit{in meso} is initiated in the presence of 2–2.5 M Na–K phosphate which ordinarily is not enough to trigger $H_{\text{II}}$ phase formation in hydrated monoolein. However, as part of this control study we felt it important to produce the $H_{\text{II}}$ phase and to examine its behaviour in the microcells under prevailing conditions. To this
end, a sample of monoolein with 4 M Na–K phosphate, pH 5.6, was prepared which formed the H_{II} phase as required. The diffraction pattern of the H_{II} phase was powder-like, as was observed with the lamellar phase. The higher order (11) and (20) reflections were just barely detectable. The H_{II} phase was not particularly sensitive to radiation damage as was observed with the cubic phase.

3.9.4. MO, detergent and salt. We have argued that it might be possible for the lamellar signature described above to arise artifactually from microinhomogeneities that develop in the sample during the course of the measurements but that are not related to crystal formation. To test this hypothesis control samples were prepared which included everything in the usual crystallization mix (lipid, detergent, and salt) except the protein. Further, the concentration of detergent and salt was adjusted to match crystallization conditions and samples were prepared, treated and scanned in the same way as the protein-containing samples. Of the 3812 patterns recorded with these control, protein-free samples not one included the lamellar signature. This result supports the view that the lamellar signature is associated with the presence of protein and, by extension, with crystal growth.

3.9.5. Lysozyme crystals. It has been shown that the cubic phase supports the crystallization of water-soluble proteins. A mechanism whereby this occurs has been proposed. Here, the cubic phase is considered to act as an inert gel to slow diffusion in a way that supports crystal growth. However, the proposed mechanism does not involve a lamellar conduit which distinguishes it from that for membrane proteins. An appropriate control measurement therefore was to look for the lamellar signature in the vicinity of a water-soluble protein crystal grown under in meso conditions. This was done using the water-soluble enzyme lysozyme and two different crystal forms of the protein were investigated. A total of 4649 diffraction patterns were recorded in the course of 106 scans performed on four tetragonal crystals and seven needle clusters. In none of the patterns was there a trace of the lamellar signature. This result is consistent with our working hypothesis that the lamellar conduit is integral to the mechanism of membrane protein crystal growth by the in meso method. It essentially rules out the possibility that the lamellar signature arises at the interface between any crystal and the surrounding cubic phase in which it grows.

3.9.2. Cholesterol crystals. We have shown that it is possible to crystallize cholesterol, which is itself a highly apolar lipid, from within the cubic phase. Prior to crystallogenesis, cholesterol presumably resides in the lipid bilayer of the cubic phase and its path into the crystal may be analogous to that of a membrane protein. If so, then the lamellar signature might be expected to show up at the surface of cholesterol crystals. To explore this possibility cholesterol crystals were grown in meso in the microdiffraction cells (Fig. 2(e)). Unfortunately, profiling the crystal interface in search of the lamellar signature was not possible because the hexagonal phase formed almost immediately upon exposing these samples to the X-ray beam. This profound sensitivity to radiation damage was not anticipated.

In the course of these measurements it was noted that the cholesterol-laden cubic phase had an unusually small domain size in the 1–2 μm range. It was expected therefore that the crystals produced in such a system would be equally small if the source cholesterol were to come from a single domain. However, crystals up to 0.2 mm in size grew readily. This finding suggests that, in the case of cholesterol at least, movement of the sterol between domains and up to the crystal face is possible. Whether this reflects bilayer contiguity between domains or some other form of inter-domain transport is not known.
4. Conclusions

Crystallization of membrane proteins by the in meso method has been proposed to involve a lamellar phase-like conduit between the crystal and the bulk lipidic cubic phase. The hypothesis has been tested in the current study by using small-angle X-ray diffraction in combination with a synchrotron-generated microfocused beam to probe the crystal/mesophase interface with micrometre resolution. A hermetically-sealed sample holder with X-ray transmitting windows was built in which crystals could be grown and interrogated by means of the microfocused beam. Signature diffraction from the lamellar phase was observed at the interface between the cubic phase and the surface of suitably oriented crystals consistent with the working hypothesis. Signature diffraction was not observed in the bulk cubic phase unless accompanied by birefringence suggesting the presence of microcrystals. Nor indeed was it seen next to crystals of a water-soluble protein grown in meso as expected. The lamellar signature was found to extend to no more than about 2 µm from the edge of the crystal suggesting that the conduit is of this approximate dimension. Under certain conditions the mesophase pulled away from the crystal surface. It did so in a way that is consistent with our working model for how the lamellar conduit is tethered to the crystal.

Radiation damage was a problem that had to be dealt with in this study. It required many control measurements and the implementation of special sample handling and data collection procedures to minimize its effects.

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