Crystallization of Transmembrane Proteins in cubo: Mechanisms of Crystal Growth and Defect Formation

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Crystallization of membrane proteins is a major stumbling block en route to elucidating their structure and understanding their function. The novel concept of membrane protein crystallization from lipidic cubic phases, “in cubo”, has yielded well-ordered crystals and high-resolution structures of several membrane proteins, yet progress has been slow due to the lack of understanding of the molecular mechanisms of protein transport, crystal nucleation, growth, and defect formation in cubo. Here, we examine at molecular and mesoscopic resolution with atomic force microscopy the morphology of in cubo grown bacteriorhodopsin crystals in inert buffers and during etching by detergent. The results reveal that crystal nucleation occurs following local rearrangement of the highly curved lipidic cubic phase into a lamellar structure, which is akin to that of the native membrane. Crystals grow within the bulk cubic phase surrounded by such lamellar structures, whereby transport towards a growing crystalline layer is constrained to within an individual lamella. This mechanism leads to lack of dislocations, generation of new crystalline layers at numerous locations, and to voids and block boundaries. The characteristic macroscopic lengthscale of these defects suggests that the crystals grow by attachment of single molecules to the nuclei. These insights into the mechanisms of nucleation, growth and transport in cubo provide guidance en route to a rational design of membrane protein crystallization, and promise to further advance the field.

Keywords: transmembrane proteins; lipidic cubic phases; molecular-resolution AFM; crystallization mechanisms; nucleation

Introduction

Genomic sequencing has revealed that about one-third of the human genome codes for proteins having at least one transmembrane segment,1 yet, whereas the protein data bank comprises more than 27,000 entries, fewer than 60 represent distinct membrane protein structures. While several of these were obtained by electron crystallography using two-dimensional crystals or other low-dimensional arrays,2–4 the majority of the structures are solved by X-ray diffraction from three-dimensional crystals.9–14 Preparation of diffraction-quality crystals remains the major bottleneck in the pursuit of high-resolution structures of membrane proteins.15 Therefore, improvements of the existing detergent micelle-based crystallization methods12, as well as entirely new approaches are sought continuously.16–19 The concept of crystallizing membrane proteins in lipidic cubic phases (in cubo)20 is a recent addition to the arsenal of crystallization methods available to crystallographers. Since its introduction, this method has yielded well-ordered crystals and X-ray structures of several membrane proteins.21 While the mechanisms of crystallization of soluble proteins have been studied in considerable detail,22–24 and it has been argued that the crystallization of detergent-solubilized membrane proteins may follow similar mechanisms,12 a hypothetical mechanism for the in cubo crystallization of transmembrane proteins has been proposed only recently,25 and a comprehensive general understanding of the

Abbreviations used: AFM, atomic force microscopy; bR, bacteriorhodopsin; OG, β-octylglucoside.
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mechanisms involved in such crystallization is still missing.

Due to the structure of the lipidic cubic phase, growth conditions in cubo differ in essential ways from those in solution. Indeed, in the lipidic matrix, an integral membrane protein is confined to the three-dimensional network of the curved bilayers, and protein transport occurs along this network. On the other hand, the transport of small molecules involved in crystallization (buffers, precipitants, additives, etc.) is confined to the complementary three-dimensional network of aqueous channels that interpenetrates the lipid network. The transport conditions may be complicated even further in the vicinity of a crystal, where the lipid phase is assumed to convert from a three-dimensional network into a stack of lamellar layers. In contrast, during solution crystallization of both soluble proteins and detergent-solubilized membrane proteins, the building blocks of the crystals, the protein molecules, and the small-molecule precipitants and additives are transported through the same unrestricted isotropic three-dimensional space. Crystals of soluble proteins grow from solution by the attachment of molecules to new crystalline layers spreading on the crystal’s surfaces. These layers are generated either on screw dislocations, two-dimensional nuclei, or three-dimensional nuclei. The errors in these mechanisms that lead to defects have been catalogued, so that the defect-generation sequences are understood at a reasonable level. However, because of the entirely different and more complex nature of transport towards the growing crystal during crystallization in cubo, it is likely that the implementations of these mechanisms of crystallization and defect formation are modified significantly.

Atomic force microscopy (AFM) has become an important tool in the investigations of biological systems. AFM has produced a plethora of novel insights into the crystallization processes of soluble proteins at the macroscopic, mesoscopic and molecular levels; however, success with 3-D crystals of membrane proteins has been limited. Here, we employ AFM to study the morphology of bacteriorhodopsin (bR) crystals grown from a lipidic cubic phase. We address the following issues, essential for the understanding of the mechanisms of crystal growth in cubo: How does the structure of the cubic phase influence the transport of proteins and small molecules? How are the transport conditions modified by the defects in the lipid bilayer network? Do the crystals grow by spreading of layers and, if so, what are the mechanisms of generation of these layers? Are the typical types of crystalline defects, point (vacancies and misplaced molecules), linear (dislocations), planar (stacking faults, block boundaries), and three-dimensional (voids and occlusions), present in lipid cubic phase grown crystals? How is defect formation influenced by the conditions of growth?

Results

Morphology and packing of bR crystals

Hexagonal bacteriorhodopsin crystals (Figure 1) were grown in the lipidic cubic phase (60–70% (w/w) monoolein) according to the published procedure. Crystals belong to space group P63, with unit cell dimensions \( a = b = 62 \text{ Å}, c = 108 \text{ Å}, \gamma = 120^\circ \). Molecules pack in a polar lamellar structure, with planar hexagonal arrays of the protein trimers stacked on top of each other, related by a 6-fold screw axis along the \( c \)-direction. The packing arrangement of the protein within each layer is identical to that found in native purple membranes, where bacteriorhodopsin trimers are arranged in a hexagonal lattice with \( a = 62(\pm 2) \text{ Å} \).

Typical images of the hexagonal face of bR crystals in 1.8 M Sørensen buffer (see Materials and Methods) are shown in Figure 2. The low-magnification image in Figure 2(a) reveals that the face consists of co-planar blocks of approximately 700 nm by 900 nm, separated by channels. The surface roughness, seen in Figure 2(a) and addressed further below, was limited to the mesoscopic lengthscale; on the molecular level, the surface was relatively smooth and the crystal lattice was clearly visible using a higher magnification (see Figure 2(b)). The lattice constants, determined from the Fourier transform (see the inset in Figure 2(b)), \( a = 86(\pm 4) \text{ Å}, b = 89(\pm 5) \text{ Å}, \gamma = 126^\circ \) are larger than those determined by X-ray crystallography, most likely due to distortions.
caused by the use of an O-ring during imaging,\textsuperscript{54,55} which was required to prevent evaporation of the buffer.

In agreement with the X-ray data, the crystals were found to consist of stacks of lamellae (see Figure 3). The thickness of the individual lamellae was determined from over 50 images and the statistical distribution of the data is shown in Figure 4. Thickness values of 11 nm, 21 nm, and 31 nm represented by the peaks of the histograms in Figure 4. These thicknesses correspond approximately to one, two, and three lattice parameters, respectively, in the \( c \)-direction, \( c = 108 \text{ Å} \).\textsuperscript{20} Lamella thickness of \( \sim 5-6 \text{ nm} \), which corresponds to 0.5\( c \), or the thickness of one purple membrane sheet,\textsuperscript{56} is indicated with a red arrowhead in Figure 4(c). Thus, the overall organization of the \( P_{63} \) bR crystals grown in the lipidic cubic phase as observed by AFM is consistent with that derived from X-ray crystallographic analysis.

The overall appearance of the large hexagonal (001) face of bR crystals is quite unusual. In contrast to the molecularly smooth terraces separated by unimolecular steps that are observed typically on surfaces of crystals of soluble proteins,\textsuperscript{32,35,46,48–50} the stacks of crystalline layers (e.g. see Figure 3), have various heights and sizes. As a result, the crystal face exhibits an uneven morphology, illustrated in Figure 2(a). The lamellae appear to have random shapes and are remarkably rough at the edges (Figure 3(b)), another stark contrast to what is observed commonly with soluble proteins. Between the stacks of lamellae, gaps that penetrate to depths \( \sim 1 \text{ μm} \) into the crystal face are seen. In comparison, stacks of straight, uniform edges of the lamellae were observed at the intersection of the hexagonal and prismatic faces (see Figure 3(e)).

### Etching of bR crystals

To further characterize the defect structure of bR crystals grown \textit{in cubo}, we monitored their dissolution \textit{in situ}. For this, bR crystals were exposed to a 3 mg/ml solution of \( \beta \)-octylglucoside (OG) in 1.8 M Sørensen buffer. A time-lapse series of images recorded at intervals of approximately two minutes shortly after the crystal was exposed to the solution of OG is shown in Figure 5. The lamella edges retreat at approximately similar rates. On some of the lamellae, it was possible to observe rows spaced \( \sim 5.4 \text{ nm} \) apart (Figure 5(h)). The spacing, determined from the Fourier transform in the inset in Figure 5(i), is comparable to the lattice constant \( a = 6.2 \text{ nm} \).\textsuperscript{20} On the basis of this observation, we conclude that these are molecular rows of bR trimers.

The characteristic roughness of the features in Figure 5(a)–(g) is similar to that in Figure 3 and, importantly, is preserved during the dissolution process.

Comparing the locations of the layers in Figure 5(a)–(g), we estimate the velocity of step retraction as \( 3(\pm 1) \text{ nm/s} \). These rates are comparable to the rates of step propagation during growth and dissolution of soluble proteins.\textsuperscript{32,57}

The etching experiments offer further support to the correspondence of the surface morphology of the mature crystal, as observed with the AFM, to the morphology of a growing crystal. Indeed, the
detergent treatment illustrated by Figure 5 leads to a rather orderly dissolution of the crystal that could not have produced any of the features observed prior to the treatment. This is not surprising: lipase, used to digest the lipid phase for the purpose of extracting the crystals from the lipidic matrix prior to AFM examination, does not hydrolyze the purple membrane lipids present within the crystals. Thus,

**Figure 3.** Structure of bR crystals revealed by AFM. (a), (b) and (c) Tapping mode AFM images of a bR crystal in 1.8 M Sørensen buffer (pH 5.6) showing stacks of lamellae. Frame sizes: (a) 10 μm, (b) 5 μm, and (c) 1.6 μm. (d) A tapping mode image (frame size 5.4 μm) of a different bR crystal in 1.8 M Sørensen buffer (pH 5.6) showing stacks of lamellae. Facets with characteristic ~120° angles are observed (indicated by arrowheads). (e) A contact mode image (frame size 5 μm) of the tapered region at the intersection of the hexagonal face with the prismatic face. The image was acquired in 25 mM Sørensen solution (pH 5.6). Z-scales: (a) 900 nm, (b) 350 nm, (c) 200 nm, (d) 300 nm, and (e) 1200 nm.
hydrolysis of the cubic phase matrix likely leaves the crystal largely intact.

**Nucleation of salt crystals on the surface of bR crystals**

Angular objects with a characteristic angle of 72° between the edges were observed on the hexagonal (001) face of bR crystals held at a high concentration (1.8 M) of Sørensen buffer (Figure 6). This angle is characteristic of potassium dihydrogen phosphate (KH$_2$PO$_4$). We assume that these triangular structures are small crystals of KH$_2$PO$_4$ that have nucleated on the surface of the bR crystal. To test this assumption, bR crystals with such structures were kept for two days in a solution containing a lower concentration (0.5 M) of Sørensen buffer, and then imaged. The triangular structures were no longer present. bR crystals are grown from saturated solutions of the phosphate salts and the nucleation and growth of the phosphate crystals is faster by orders of magnitude than those of bR. Since the solubility of the phosphates is strongly dependent on temperature, minor temperature instabilities can lead to supersaturated phosphate solutions and formation of the phosphate crystals. These phenomena could take place as bR crystals grow in the lipidic cubic phase in the presence of high concentrations of Sørensen salt, and can result in the incorporation of salt crystals in the bulk of the bR crystal. This would stress the bR material severely, and the strain may in turn be resolved by the formation of other two-dimensional and three-dimensional defects. Thus, the incorporation of KH$_2$PO$_4$ crystals would ultimately lead to poor diffraction quality of some of the bR crystals.

**Defects**

Besides the block boundaries, discussed above, the defects detected on the (001) face of the bR crystals include irregularly shaped holes (Figure 7(a)) and cracks (Figure 7(c)–(e)). Exposing crystals to various concentrations of salt revealed the elastic character of the lamellae: in response to the osmotic stress imposed by immersing the crystal grown in a solution of 1.8 M Sørensen salt into a solution of 25 mM Sørensen salt, the surface wrinkled, but did not fracture (Figure 7(b), (e) and (f)). We attribute the wrinkling to separation between adjacent lamellae in the regions where the interactions between them is weak, likely due to voids or other defects.

**Discussion**

The mechanisms of crystallization in cubo

A molecular mechanism for crystallization in cubo, based on polarization microscopy and low-angle X-ray diffraction studies, was put forth by Nollert et al. The basic premise of that model is that protein crystallization in cubo is initiated by dehydration of the protein-containing lipidic cubic phase, which triggers a decrease in unit cell size, an increase of membrane curvature, and a hydrophobic mismatch between the membrane protein and the curved lipid bilayers. Dehydration is induced by the added salt. It was speculated that crystallization is accompanied by a cubic-to-lamellar transformation in lipid structure, and that this transformation is facilitated by the protein, probably due to the increased stiffness of the protein–lipid layers relative to their protein-free counterparts.

The main elements of this mechanism triggered by the salt-induced curvature increase can be classified as follows: (i) fluctuation of concentration leads to a locally high concentration of bR at random locations in the lipidic cubic phase; (ii) the high concentration of protein triggers a local transformation of the cubic phase into a lamellar structure; (iii) crystal nucleation occurs in the lamellar region; (iv) the growth of the crystal is fed from the bulk cubic phase via a several micrometer-wide lamellar region, which is
preserved around the crystal as its dimensions increase.

An alternative to the sequence of steps (i)–(ii) would be a mechanism whereby random appearances of lamellar regions within the dehydrated lipidic phase (which can be viewed as fluctuations of the structure of the lipidic phase) lead to a local increase in protein concentration and crystal nucleation. While there is no experimental evidence to distinguish the (i)–(ii) and (ii)–(i) scenarios, this question may have significant practical consequences. For instance, the (ii)–(i) scenario implies that numerous cubic phase structure fluctuations occur and are not “filled” by a nucleation event, so efforts should be applied towards enhancing the nucleation of the crystals within existing lamellar regions. If the (i)–(ii) scenario operates, one should try to enhance the protein density fluctuations in the lipidic cubic phase, for instance by increasing the attraction between the protein molecules suspended in it. Since the primary fluctuations lead to either isotropic or anisotropic objects, the issue could be resolved by experiments with scattering of polarized radiation.

Prior to this work, element (iv) of the above mechanism has been supported by just one piece of

**Figure 5.** Etching of bR crystal observed by addition of octyl glucoside. (a)–(g) A time-lapse series of 403 × 403 nm² (Z-scale: 50 nm) contact mode images of the hexagonal face of a bR crystal acquired shortly after exposure to 1.8 M Sørensen buffer containing 3 mg/ml of OG. The images were recorded consecutively, each at an acquisition time of two minutes. Edges of lamellae, visible in these images, are changing shape and retracting. The distances between a defect (blue arrowhead) and an edge of a lamella (red arrowhead) was found to increase from 34 nm in (c) to 48 nm in (e). (h) A contact mode image (frame size 627 nm) revealing molecular rows indicated with the black rectangle. (i) A magnification (frame size 160 nm) of the area is indicated with the black rectangle in (h). The image reveals rows separated by ~5.4 nm determined by the Fourier transform shown in the inset. Z-scale: (h) 70 nm and (i) 30 nm.
evidence: the finding that bR crystals within the bulk cubic phase are surrounded by a halo of bi-refringent material.\textsuperscript{25} In further support, we note that the surface of bR crystals is exceedingly rough, comprised of stacks of unfinished crystalline layers that are non-contiguous, of arbitrary shapes and sizes, and characterized by excessive edge roughness. This likely is a consequence of transport towards individual layers via the lamellar phase so that transport of protein within each layer is essentially independent of that in other layers. Thus, the mesoscopic and macroscopic processes of nucleation and growth in each layer are independent of those occurring in other layers. The only coordination between layers occurs at the molecular lengthscale, within the crystal lattice, ensuring that every new layer is aligned with the underlying layers. This is similar to what is observed in soluble proteins, for which growth via layers in registry with underlying layers is one of the two major growth modes.\textsuperscript{29,33–36} However, with soluble proteins, coordination between successive layers occurs also on the macroscopic lengthscale as a result of the overlap of the supply fields of the individual steps.

According to this model, each growing embryo of a crystalline layer is fed by protein molecules only from within the lamellar layer to which this embryo belongs. If several co-planar nucleation events occur, the regions between them will become depleted of protein. The outward growth of these sources will be faster than the inward growth, and inner regions of the crystal face will contain areas that may never be filled with crystalline material. During our observations, the lipid phase filling the non-crystalline voids is digested by the lipase and appears as gaps in the crystal (Figure 3). In agreement with this explanation, in Figure 3 the edges of crystalline layers facing other crystalline layers within the same lamellar layers are rough. This suggests cessation of growth due to exhaustion of the material. Such growth cessation is the likely reason for the presence in Figure 3 and of ~5-6 nm thick layers, a high free-energy configuration, corresponding to half of the thickness of one purple membrane sheet.\textsuperscript{56} The edges of the crystalline layers that are open for supply of building blocks within the same lamellar layer as seen in Figure 3(d) are faceted, likely because their growth stopped in a slowly decreasing concentration environment as equilibrium between the crystal and the cubic phase was approached.

This mechanism suggests that edges of the crystalline layers at the crystal’s facets, which are well supplied with protein, will be oriented along crystallographic directions, as in the case of crystals of soluble proteins. Accordingly, the overall shape of the crystal in Figure 1 is faceted. Furthermore, at the intersection between the prismatic and the hexagonal faces of bR crystals, shown in Figure 3(e), the crystalline layers have straight edges, likely along a crystallographic direction.

Another consequence of the two-dimensional mode of transport is the possibility of new crystals nucleating in the immediate vicinity of a growing crystal. Such a process is unlikely with isotropic three-dimensional transport, where a growing crystal depletes the solution around it, but is possible if crystallization in one lamella does not compete for supply with growth in neighboring lamellae. An example is presented in Figure 8, where a small crystal incorporated into the larger crystal at an inclination of about <5° with respect to the crystal face is visible. Judging from the size of
the smaller crystal, it likely nucleated as close as 1 μm away from the surface of the large one.

Lipidic phase rearrangement and crystal nucleation

The morphological features of bR crystals observed with AFM offer a strong support to element (iii) of the above mechanism, that the rearrangement of the lipid phase into a lamellar structure precedes crystal nucleation. The observation of smaller crystal nucleated within the lamellar structure surrounding a larger crystal discussed above, shows the feasibility of crystal nucleation within lamellar lipid layers. Furthermore, none of the investigated bR crystals revealed any screw dislocations, despite an extensive search for them. In crystals of soluble proteins, screw dislocations have been observed if the crystal layer parallel to the growing face is thinner than 5.5–6 nm, and not found in crystals of any protein or virus with thicker layers.35 This has been explained with the quadratic dependence of the dislocation energy on its Burgers vector, equal to a whole number of layer thicknesses,62 so that the dislocation energy in crystals consisting of thick layers is high. If one assumes that the elastic properties of the bR crystals are similar to those of soluble proteins,23 one should expect screw dislocations in bR crystals whose layer thickness is ~5 nm (Figures 3 and 4). The lack of screw dislocations is likely due to the nucleation of the first several crystalline layers within the parallel lipid lamellar layers, so that the lamellar structure serves as a template for perfect interlayer alignment.

Attachment of molecules to growing crystals

The observations of the characteristic roughness in Figure 5 suggest that dissolution proceeds via the loss of single bR molecules, or of trimers from the edges of the crystalline layers to the solution. Also, dissolution is faster at the lattice defects, where the lattice strain increases the chemical potential of the molecules in the crystal and the driving force for dissolution.32 This leads to etching at the defects. In these respects, this dissolution process is similar to dissolution of crystals of soluble proteins and small molecules,63 which is not surprising, because addition of detergent used in the dissolution experiments disrupts the lipidic cubic phase structure. In the case of bR crystals, the lattice defects that are preferentially etched are the boundaries between the blocks shown in Figure 2. The dense network of the block boundaries, spaced at

Figure 7. Block boundaries, holes, and cracks observed on the surface of bR crystals. White arrowheads point to holes in the surface; green arrowheads point to cracks. (a) A tapping mode AFM image (frame size 5 μm, Z-scale 500 nm) and (b) a contact mode AFM image (frame size 8.81 μm, Z-scale: 400 nm) both in the presence of 25 mM Sørensen buffer. (c) A tapping mode AFM image (frame size 10 μm, Z-scale: 300 nm) and (d) a tapping mode AFM image (frame size 10 μm, Z-scale: 500 nm) both in 1.8 M Sørensen buffer. (e) and (f) Images revealing folding and swelling of the surface, seen along with cracks shown in (f). Image sizes: (a) 5 μm, (b) 8.81 μm, (c) 10 μm, (d) 10 μm, (e) 5.68 μm, and (f) 8.85 μm. Z-scale: (a) 500 nm, (b) 400 nm, (c) 300 nm, (d) 500 nm, (e) 1600 nm, and (f) 750 nm.
700–900 nm apart, leads to uniform dissolution of the hexagonal face.

Another conclusion that can be drawn from the preservation of the characteristic roughness is that defects on a scale smaller than those seen in Figure 2 (a), down to the molecular scale seen in Figure 5 (h), are not present; they would serve as additional sources of etching and increase the roughness. The perfection of the crystalline layers at the molecular level suggests that they grew by the attachment of single molecules, rather than clusters preformed in the lipidic matrix.

Despite some similarities, the dissolution of the bR crystals grown in cubic differs from that of soluble proteins: bR molecules released to solution are likely embedded in mixed detergent–protein micelles, and the micelle formation likely affects the dissolution kinetics. Furthermore, while in the case of soluble proteins in isotropic solution dissolution is symmetric to growth on the molecular level, with deviations from the symmetry at coarser-scale phenomena, with lipidic cubic phase-grown crystals, dissolution processes have no growth equivalent at any lengthscale. Thus, during growth of the bR crystals form the lipid phase, the molecules are supplied to the crystals via the lipid network, while during dissolution they are released into solution. These differences affect only the rate of dissolution and do not weaken the conclusions based on observations of the layer morphology during etching.

**Defect formation during in cubo growth**

The observation that the major defects in lipidic cubic phase-grown bR crystals are of a characteristic size of a few micrometers, i.e. have the lengthscale of the transport processes, suggests that the defects are due to imperfect supply of material to the growing crystal layers. Two features of protein transport through the network of lipid bilayers merit discussion. If the rate of modified protein diffusion is significantly faster or slower than the rate of incorporation into the crystal, the non-linear coupling of the transport and growth processes may lead to kinetic instability, similar to the step bunching instability often observed with soluble proteins. This instability results in a variable growth rate and non-uniform concentration of protein in the crystal vicinity. Such phenomena may underlie the nucleation of multiple co-planar crystalline layers, seen in Figure 3. Furthermore, the inevitable defects in the cubic and lamellar lipid phases modify the distribution of the protein in the crystal growth medium and may lead to blocked structures and other undesirable phenomena.

These considerations suggest that crystal quality may be improved by controlling the rate of transport, i.e. by selecting a lipid substance ensuring the optimal rate of membrane protein diffusion through its ordered phases. Another means of improvement may be by controlling the structure of the lipid phases so that the concentration of defects in them is minimized. For successful implementation of these pre-conditions, we need insights into the diffusion of membrane proteins through the lipid phases, as well as into the defect structure of these phases.

**Materials and Methods**

**Crystallization of bR from lipidic cubic phases**

Crystallization was conducted according to published procedure. Purple membranes from *Halobacterium salinarum* were solubilized with β-octylglucoside (OG). Bacteriorhodopsin (bR) was purified by gel-filtration chromatography as described. Purified bR was mixed with melted monoolein (MO) from NuCheck (Elysian, MN) in a glass microtube (1 mm/15 mm). The pre-crystallization mix consisted of approximately 60% (w/w) MO and 40% (w/w) bR solution. The microtubes were sealed with Parafilm and centrifuged three or four times at 10,000 g for five minutes, in a temperature-controlled microcentrifuge at 20 °C, until a purple transparent gel (lipidic cubic phase) was formed at the bottom of the microtube. A Sørensen salt mixture composed of 9.48 g of KH₂PO₄ and 0.52 g of NaH₂PO₄·H₂O was added to the lipidic cubic phase at a ratio of 3 : 7 (w/w) to initiate the crystallization of bR. Crystal growth was examined by optical microscopy as described.

**Harvesting of bR crystals**

The bR crystals were freed from the surrounding lipidic matrix by digesting the lipids with *Candida rugosa* lipase.
Mounting of bR crystals for AFM imaging

Metal disks used as supports in the AFM experiments were coated with Teflon adhesive tape (BYTAC, Sigma, St. Louis, MO). The Teflon surface was cleaned with 2% (w/v) SDS (Sigma, St. Louis, MO) and rinsed with copious amounts of de-ionized water. bR crystals, harvested as described above, were mounted on the Teflon-coated metal discs with a two-component Epoxy glue (Cole-Parmer, Vernon Hills, IL). The epoxy was allowed to set for ~15 minutes, after which the samples were mounted in the atomic force microscope. The crystals were kept in Sørensen buffer (pH 5.6) throughout the experiment. The crystals were imaged at various time intervals.

Dissolution of bR crystals

To monitor the dissolution of bR crystals, they were exposed to 3 mg/ml of OG (Sigma, St. Louis, MO) solution in Sørensen buffer of appropriate ionic strength over periods ranging from a few minutes to several days and imaged at various time intervals.

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