A novel microseeding method for the crystallization of membrane proteins in lipidic cubic phase

Stefan Andrew Kolek,^a Bastian Bräuning^b and Patrick Douglas Shaw Stewart^{a*}

^aDouglas Instruments Ltd, East Garston, Hungerford RG17 7HD, England, and ^bDepartment of Chemistry, Technische Universität München, Lichtenbergstrasse 4, 85748 Garching, Germany. *Correspondence e-mail: patrick@douglas.co.uk

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Random microseed matrix screening (rMMS), in which seed crystals are added to random crystallization screens, is an important breakthrough in soluble protein crystallization that increases the number of crystallization hits that are available for optimization. This greatly increases the number of soluble protein structures generated every year by typical structural biology laboratories. Inspired by this success, rMMS has been adapted to the crystallization of membrane proteins, making LCP seed stock by scaling up LCP crystallization conditions without changing the physical and chemical parameters that are critical for crystallization. Seed crystals are grown directly in LCP and, as with conventional rMMS, a seeding experiment is combined with an additive experiment. The new method was used with the bacterial integral membrane protein OmpF, and it was found that it increased the number of crystallization hits by almost an order of magnitude: without microseeding one new hit was found, whereas with LCP-rMMS eight new hits were found. It is anticipated that this new method will lead to better diffracting crystals of membrane proteins. A method of generating seed gradients, which allows the LCP seed stock to be diluted and the number of crystals in each LCP bolus to be reduced, if required for optimization, is also demonstrated.

1. Introduction

The random microseed matrix screening (rMMS) approach to protein crystallization involves adding seed stocks containing crushed crystals to random crystallization screens (Ireton & Stoddard, 2004; D'Arcy *et al.*, 2007, 2014). The method is used routinely for protein structure determination by both industrial companies (Obmolova *et al.*, 2010, 2014) and academic laboratories (Rumpf *et al.*, 2015; Tanaka *et al.*, 2015; Petkun *et al.*, 2015; Abuhammad *et al.*, 2013; Stieglitz *et al.*, 2012). The method has at least three important advantages: (i) it picks up additional crystallization conditions that would not be found by conventional screening, including conditions that are chemically unrelated to the conditions in which the seed crystals were obtained (D'Arcy *et al.*, 2007), (ii) it reduces the need for optimization, with diffracting crystals being more likely to appear spontaneously than in conventional screening (D'Arcy *et al.*, 2007), and (iii) by diluting the seed stock, the number of crystals per drop can usually be controlled (D'Arcy *et al.*, 2007; Shaw Stewart & Mueller-Dieckmann, 2014). These three advantages are the result of increasing the chance that crystals will grow in the metastable zone of the phase diagram (Shaw Stewart *et al.*, 2011). Since the method requires little preparation or planning, is simple and can be automated, it can dramatically increase the productivity of a typical structural biology laboratory. In one industrial laboratory, 38 of 70 structures produced over roughly a three-year period benefited from the method (Obmolova, 2011). The method has also been spectacularly effective when other methods had failed

(Obmolova *et al.*, 2010; Abuhammad *et al.*, 2013), and in one study allowed every member of a set of 16 Fab constructs to be crystallized (using widely varying chemical conditions) and their structures determined (Obmolova *et al.*, 2014).

Membrane proteins perform critical functions in all organisms, and represent about 60% of approved drug targets in humans (Yıldırım *et al.*, 2007). In spite of its widespread success in crystallizing soluble proteins, we know of no examples where the rMMS approach generated diffracting crystals of membrane proteins, although promising preliminary results have been seen (Moraes *et al.*, 2014). This lack of success may be partly owing to the difficulty in making stable membrane-protein seed stocks. In particular, detergent is expensive and is not normally added to the reservoirs of vapour-diffusion experiments. If, therefore, seed stocks are suspended in the reservoir solution without detergent, they may become unstable and may dissolve (Moraes *et al.*, 2014).

Separately, the high-resolution structures of many membrane proteins have been generated in recent years by crystallizing out of the lipidic cubic phase (LCP; Caffrey *et al.*, 2015). We therefore set out to apply the rMMS approach to the crystallization of membrane proteins in LCP to supplement the conventional vapour-diffusion method and bypass the problems with membrane-protein seed crystal stability. To demonstrate the method, we used rMMS with LCP (LCP-rMMS) to find new crystallization hits of outer membrane protein F (OmpF) from *Escherichia coli*.

2. Methodology for scaling up and using LCP seed stocks

There are at least two important problems that can prevent the production and use of crystal seed stocks for membrane proteins: (i) seed crystals that are added to samples that contain insufficient precipitant may dissolve and (ii) if condi-

tions that normally give crystals in nanolitre volumes are scaled up to microlitre volumes without preserving the most important physical dimensions, crystals may not grow.

In order to maximize the chance of obtaining useful seed crystals, we followed the general approach that Weierstall *et al.* (2014) used to grow crystals of membrane proteins for serial data collection using a free-electron laser. This allowed us to ensure that sufficient precipitant was always present in the LCP seed stock, while at the same time scaling up the crystallization conditions established in a sandwich-plate setup (Cherezov & Caffrey, 2003) and maintaining the physical dimensions that are critical for crystallization. We therefore injected LCP into a gas-tight Hamilton syringe containing the same aqueous crystallization solution that we used in the plate (Fig. 1), ensuring that the diameter of the 'worm' of LCP formed was similar to the diameter of the LCP bolus in the original plate. We also ensured that the diameter of the syringe was similar to the diameter of the drop of aqueous solution in the plate. The result was a crystallization setup that was equivalent to the sandwich-plate setup but with a volume that was approximately 200 times greater. In particular, the characteristic time of the diffusion of the precipitant was similar.

Note that once the seed stock has been made, any LCP crystallization setup can be used for LCP-rMMS; the exact dimensions of the physical setup are no longer important.

To ensure that plenty of uncrystallized protein remains in the LCP and is available to form novel crystals in the various cocktails of the crystallization screen, it is essential that the LCP seed stock should be harvested and used as soon as, or even before, the first small crystals become visible (Fig. 2b).

Note also that in the original paper describing the rMMS method for proteins in solution, D'Arcy *et al.* (2007) recommended the following volumes: 0.3 μl protein solution + 0.2 μl reservoir solution + 0.1 μl seed stock. Larger or smaller volumes can be used, but volumes in the extended ratio 3:2:1

Figure 1

Overview of the LCP-rMMS method. (a) The method starts with a condition in a sandwich plate that yields crystals of the target membrane protein in LCP. (b) The conditions are scaled up to make an LCP seed stock in a much larger volume by injecting protein-laden LCP into a gas-tight syringe containing the same aqueous solution as was used to grow the crystals in the sandwich plate. It is important that the physical dimensions that are critical for crystallization in the sandwich plate match the corresponding dimensions of the scaled-up experiment. The syringes should be held vertically and the LCP seed stock injected upwards (because LCP normally floats in aqueous solutions) with a steady dispense action at a rate of roughly $2.0 \mu\text{l s}^{-1}$. (c) As soon as crystals become visible the LCP seed stock should be harvested by slowly pushing down the plunger of the gas-tight syringe to eject the aqueous solution, leaving the LCP seed stock in the syringe. (d) The LCP containing protein and seed crystals is dispensed into random screens in sandwich plates or other plates.

are generally used (D'Arcy *et al.*, 2014). This means that an additive experiment is carried out at the same time as the seeding experiment, since roughly one third of the precipitant in the final drop is delivered *via* the seed stock. This increases the chances of obtaining crystals because a set of reagents (precipitants, salts, buffers *etc.*) that have previously crystallized the target protein is added to every drop (D'Arcy *et al.*, 2014; Shaw Stewart & Mueller-Dieckmann, 2014). The

procedure outlined above has a similar effect in the case of LCP-rMMS experiments because the LCP seed stock also contains a set of reagents that have previously crystallized the target protein. Note that these reagents will diffuse out of the LCP bolus and into the surrounding aqueous solution, but that around 10% of the original precipitant remains in the bolus after equilibration. Like the classical rMMS method for soluble proteins (D'Arcy *et al.*, 2007), this method therefore combines the benefits of seeding and additive experiments.

3. Materials and methods

3.1. Protein production

The integral membrane protein OmpF from *E. coli* was purified from native membranes following the protocol described by Efremov & Sazanov (2012). Briefly, crude outer membranes from *E. coli* strain BL21(DE3) were isolated following lysate clarification *via* centrifugation at 25 000g. The pellet was resuspended in 20 mM Tris pH 8.0, 1.0 mM EDTA and 1% Triton X-100 was added subsequently. After stirring for 20 min at RT, outer membrane fractions were pelleted by ultracentrifugation at 200 000g for 45 min. Following another round of pellet resuspension, extraction (this time with 2% Triton X-100) and ultracentrifugation, the pellet comprised highly enriched outer membranes. For OmpF extraction, this outer membrane pellet was subsequently solubilized in 20 mM Tris pH 8.0, 1.0 mM EDTA, 2% DDM at RT for 2 h. Un-solubilized material was then removed *via* ultracentrifugation. The supernatant was loaded onto a 5.0 ml HiTrap Q HP anion-exchange column (GE Healthcare) and eluted with a linear salt gradient from 0 to 1.0 M NaCl in 20 mM Tris pH 8.0, 1.0 mM EDTA, 0.05% DDM. Fractions corresponding to the major eluting peak were pooled and concentrated to

Figure 2

The appearance of protein-laden LCP in the syringe used to prepare LCP seed stock. (a) Protein-laden LCP has just been injected into the gas-tight syringe containing the aqueous crystallization solution. (b) After incubation for 3 d, small crystals are visible using crossed polarizers (red arrows). The LCP seed stock is ready to be harvested and used. (c) The seed stock has been left too long (7 d) and large crystals of OmpF have grown, making the material unsuitable for LCP-rMMS experiments. This sample was not used. The solid bars represents 1.0 mm.

Figure 3

The OmpF crystallization condition that we used to prepare the LCP seed stock. These crystals grew in 4 d. The aqueous solution contained 1.25 M potassium thiocyanate, 2.1 M lithium nitrate and 0.1 M sodium acetate pH 4.6. (a) Appearance with normal optics. (b) Appearance with crossed polarizers above and below the plate. The solid bar represents 200 μ m.

Table 1

Summary of new hits obtained in 120 conditions using LCP-rMMS.

Crystallization screen	Condition No.	Chemical composition	Without seeding		With seeding	
			Crystals?	Crystal size (μm)	Crystals?	Crystal size (μm)
JCSG+	17	40% MPD, 5% PEG 8K, 0.1 M sodium cacodylate pH 6.5	—		Yes	20–30
JCSG+	18	40% ethanol, 5% PEG 1K, 0.1 M phosphate–citrate pH 4.2	—		Yes	20–30
JCSG+	22	50% PEG 200, 0.2 M MgCl_2 , 0.1 M sodium cacodylate pH 6.5	—		Yes	5–10
JCSG+	30	40% PEG 300, 0.1 M phosphate–citrate pH 4.2	—		Yes	10–20
JCSG+	43	40% PEG 400, 0.2 M lithium sulfate, 0.1 M Tris–HCl pH 8.5	—		Yes	10–20
JCSG+	53	40% MPD, 0.1 M CAPS pH 10.5	—		Yes	20–30
JCSG+	64	20% Jeffamine M-600 pH 7.0, 0.1 M Na HEPES pH 7.5	Yes	10–20	—	
JCSG+	66	10% MPD, 0.1 M Na bicine pH 9.0	—		Yes	10–20
Membrane 1	5	48% PEG 400, 0.2 M CaCl_2 , 0.1 M HEPES pH 7.5	—		Yes	10–20

60 mg ml^{−1} using 100 kDa cutoff Amicon concentrators. Aliquoted samples were snap-frozen in liquid nitrogen and stored at −80°C.

3.2. Screens and crystallization reagents

The JCSG+ crystallization screen was purchased from Molecular Dimensions Ltd, UK, while JBScreen Membrane 1 was purchased from Jena Bioscience GmbH, Germany. Monoolein was purchased from Glycon GmbH, Germany.

3.3. Preparation of LCP seed stock

A batch of LCP containing protein was prepared with an LCP Mixer (Douglas Instruments Ltd, UK) using the standard method in which the protein solution and lipid are passed repeatedly through a 22-gauge steel needle (internal diameter 0.41 mm; Caffrey *et al.*, 2015).

Crystals of OmpF were grown in LCP using previously published conditions (Efremov & Sazanov, 2012). We used Laminex glass bases and covers with 0.2 mm spacers (Molecular Dimensions Limited, UK), referred to in this report as

‘sandwich plates’. Some optimization was necessary and the best crystals grew in 4 d using an aqueous solution containing 1.25 M potassium thiocyanate, 2.1 M lithium nitrate and 0.1 M sodium acetate pH 4.6 (Fig. 3). We recommend using 40 nl boluses surrounded by 300 nl aqueous drops to match the geometry of the scaled-up crystallization setup in the incubation syringe, as described below. In practice, we mistakenly optimized with 200 nl boluses surrounded by 1.0 μl aqueous solution, as shown in Fig. 3. These volumes are not recommended.

LCP seed stock was produced by incubating the protein-laden LCP in the same aqueous crystallization solution. 15 μl of OmpF LCP was injected through a 22-gauge needle into a syringe containing 50 μl of the crystallization solution using an LCP Mixer (Caffrey *et al.*, 2015). This resulted in a continuous ‘worm’ of LCP in the incubation syringe (Fig. 2a). The LCP worm was then incubated until small crystals were observed using crossed polarizers (Figs. 1b and 2b). After 3 d, the LCP seed stock was collected from the syringe by slowly advancing the plunger (Fig. 1c). As the plunger advances LCP seed stock collects on the plunger, while the aqueous solution is displaced and moves out of the syringe. Just over 12 μl of useable LCP seed stock was collected and was used immediately for experiments.

We combined the resulting LCP seed stock with the JCSG+ crystallization screen (96 conditions) and the Membrane 1 crystallization screen (24 conditions) using 60 nl LCP boluses and 1.0 μl aqueous screen solution (Fig. 1d). For comparison, the experiment was repeated with protein-laden LCP that did not contain seed crystals.

3.4. Preparation of an LCP seed gradient

The ability to control the number of crystals per drop by diluting the LCP seed stock is an important advantage of the rMMS method as applied to soluble proteins (D’Arcy *et al.*, 2007). We used the following approach to create a gradient of seeds in LCP. (i) At least 5.0 μl of LCP seed stock in a first syringe was prepared as described above. (ii) This syringe was coupled to a second syringe containing 10.0 μl of protein-laden LCP, and the protein-laden LCP was injected into the first syringe. The syringe at this stage (*i.e.* ready for dispensing) is shown schematically in Fig. 4. (iii) An LCP microseeding experiment was set up using the mixture, with a constant

Figure 4

A schematic view of a syringe, loaded with LCP seed stock and protein-laden LCP, which is ready for dispensing a seed gradient. Near the plunger there are many seed crystals and a higher concentration of precipitant, whereas near the needle the protein-laden LCP contains few seed crystals and little precipitant.

Figure 5

New hits found for the membrane protein OmpF using LCP-rMMS. The solid bar represents 200 μm . Column *A*, condition 17 of JCSG+, consisting of 40% MPD, 5% PEG 8K, 0.1 *M* sodium cacodylate pH 6.5. Column *B*, condition 18 of JCSG+, consisting of 40% ethanol, 5% PEG 1K, 0.1 *M* phosphate-citrate pH 4.2. Column *C*, condition 53 of JCSG+, consisting of 40% MPD, 0.1 *M* CAPS pH 10.5. Rows 1 and 2 show experiments without seeds (row 1 with normal optics, row 2 with crossed polarizers). Rows 3 and 4 show experiments with the LCP seed stock (row 3 with normal optics, row 4 with crossed polarizers).

aqueous solution that had previously given crystals with the LCP seed stock. This resulted in an observable gradient where the number of seeds increased as the seed stock mixture was dispensed.

4. Results

Table 1 summarizes the results obtained with LCP-rMMS. Out of the 120 conditions that were set up, only one hit was found using the regular LCP without seeding, which contained very small crystals. Using the LCP seed stock, eight hits were found, including three that contained crystals that were slightly larger than the crystals grown in the condition that was originally used to make seed crystals (Fig. 3). The crystals grown in these three conditions are shown in Fig. 5. Clearly, crystals grew in

several new hit conditions using the LCP-rMMS method that were not found without it. The main precipitant was in all cases organic (PEG, ethanol or MPD), whereas the seed crystals and the other crystallization conditions reported by Efremov and Sazanov were all high-salt conditions (Efremov & Sazanov, 2012).

Fig. 6 shows that a gradient of seeds can be dispensed to a plate. The boluses near the middle of the gradient contained only a few crystals, with crystals being confined to one part of the bolus.

5. Conclusions

We conclude that an LCP seed stock can easily be produced within gas-tight syringes by following a simple protocol. This

Figure 6

A seed gradient on a sandwich plate. See the main text for a detailed description of the method. Briefly, protein-laden LCP was added to a syringe that already contained an LCP seed stock. The resulting mixture was dispensed to a sandwich plate with 200 nl boluses covered by 1.5 µl aqueous drops. 48 boluses were dispensed, and the aqueous solution used was 40% ethanol, 5% PEG 1K, 0.1 M phosphate-citrate pH 4.2. Six representative boluses are shown in the order that they were dispensed. It can be seen that the first boluses dispensed contained no crystals (*a, b*), while the final ones contained many small crystals (*e, f*). Near the middle of the gradient, a few crystals were present that were confined to one region of the bolus (*c, d*). The solid bars represent 200 µm.

protocol allows LCP crystallization conditions to be scaled up very easily and reliably because the physical dimensions that are critical for crystallization are maintained. The crystallization conditions can therefore be tested and if necessary optimized in conventional sandwich plates before the LCP seed stock is made.

We tested the method with the membrane protein OmpF from *E. coli*. The method worked well, increasing the number of hits from one to nine and identifying several new precipitants that supported crystallization. We conclude that our method can increase the number of hits of target proteins that are available for optimization. This increases the chance of obtaining crystals that diffract well since each hit can be optimized independently.

As noted above, an important advantage of conventional rMMS with soluble macromolecules is that the number of crystals per drop can (in general) be controlled by diluting the seed stock. We therefore investigated dilution of the LCP seed stock using an approach in which both LCP seed stock and LCP without seeds were loaded together into a single syringe (Fig. 4). The mixture was dispensed into a sandwich plate where all wells contained an aqueous solution that had previously given crystals with the LCP seed stock (Fig. 6). This showed that the number of crystals per bolus can be reduced and controlled. Note, however, that the length of the gradient was comparatively short, resulting in wells with clusters of crystals within clear LCP (Figs. 6*c* and 6*d*). We would have preferred to see individual crystals that were well separated from other crystals to encourage the growth of large crystals. However, we envisage approaches that might give more gradual gradients of seed crystals. For example, the LCP seed stock and the LCP without seeds could be blended together by passing them several times through the coupling needle. Another approach would be to incubate the mixture for a few hours or days to allow diffusion of the precipitant from the LCP seed stock to the LCP without seeds. New seed crystals may then form in regions that previously contained no crystals, or existing crystals may dissolve as the precipitant concentration decreases. Practical trials of different variations of the

method need to be carried out to find the best protocol for this variation of the method.

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