Growth of Diffraction-Quality Protein Crystals Using a Harvestable Microfluidic Device

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ABSTRACT: Protein crystallization is the major bottleneck in the entire process of protein crystallography, and obtaining diffraction-quality crystals can be unpredictable and sometimes exceptionally difficult, requiring many rounds of high-throughput screening. Recently, a more time- and cost-saving strategy to use the commercially available microfluidic devices called Crystal Formers has emerged. Herein we show the application of such a device using a protein from Legionella pneumophila called LidL that is predicted to be involved in the ability to efficiently manipulate host cell trafficking events once internalized by the host cell. After setting up just one 96-channel Crystal Former tray, we were able to obtain a diffraction-quality crystal that diffracted to 2.76 Å. These results show that Crystal Formers can be used to screen and optimize crystals to directly produce crystals for structure determination.

Protein crystallization is one of the major bottlenecks in the entire process of protein crystallography, and obtaining diffraction-quality crystals can be very unpredictable and sometimes exceptionally difficult, requiring many rounds of high-throughput screening and optimizations with no guarantee of success. Even once a condition for protein crystallization is identified, not all crystals will be of high quality, meaning single and well-ordered crystals that diffract to suitable resolution with low mosaicity. Common methods to crystallize proteins include vapor diffusion, batch crystallization, and seeding. Before the development of high-throughput methods, capillary-based counter-diffusion methods were not uncommon, but these methods were not amenable to high-throughput screening because each experiment had to be set up one by one in single capillaries, in addition to the significant amount of protein sample required. Since then, vapor diffusion has become the most popular method of crystallization, including hanging drop and sitting drop methods. Use of traditional, 96-well plates for sitting drop vapor diffusion experiments is the common place to start for high-throughput screening. However, some proteins do not crystallize even after utilizing many different screening kits, or the crystals obtained do not diffract to sufficient resolution necessary for structure determination.

Recently, the development of microfluidic systems for crystallizing proteins through the diffusion method at the nanoliter scale has provided alternative methods. One such device is Crystal Formers (Microlytic North America, Burlington, MA), which utilize liquid–liquid diffusion methods through loading nanoliters of protein to one side of the channels and precipitant to the other side. The mixing of these two solutions within the channels creates thousands of different combinations of conditions, which is more advantageous than conditions in a more discrete method as with vapor diffusion. Furthermore, crystallization at the microscale allows slow and well-controlled dispersive mixing of the solutions, which can promote growth of high-quality crystals. This method of crystallization is becoming more common in the search for initial crystallization conditions, as well as crystal growth optimization.

Before proceeding with crystallization, it is essential to first have a pure, homogeneous sample of protein. The protein we used for this experiment was LidL, which is a 489-residue protein from Legionella pneumophila that has been implicated in the ability to efficiently manipulate host cell trafficking events once internalized into host cells. L. pneumophila is the causative agent of a potentially fatal disease called legionellosis. In order for bacteria like L. pneumophila to survive within phagocytic host cells, bacterial pathogens have evolved strategies to avoid fusion with lysosomes. The main determinant of the infectious cycle of L. pneumophila is the existence of the Dot/Icm type IV secretion system (T4SS). The T4SS is a macromolecular transport system that translocates bacterial proteins called “effectors” into the host cell, and is required for intracellular survival and replication of the bacteria. More than 100 effector proteins have been identified in L. pneumophila, with LidL being one of the prime examples. Many of these effectors were determined to have

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distinctive eukaryotic domains, such as kinase, ligase, lyase, and phospholipase domains, as well as domains involved in protein–protein interactions, such as ankyrin repeats, leucine-rich repeats, and coiled coils.\(^{16,17}\) LdL itself contains 12 Sel1-like repeats, which are named after the extracellular protein from *Caenorhabditis elegans* for which it was first described.\(^{11,18}\) Sel1 motifs are important mediators of protein–protein interactions and are mostly found in eukaryotic proteins such as elongation factor 2 kinase (EF2K).\(^{11,19}\) Therefore, LdL may be important for diverting certain host cell proteins from participating in their normal defense mechanisms. Although the existence of a wide variety of effectors is known, only a minority of them have been structurally or functionally characterized.\(^{16}\) As a result, further characterization of these effectors, including LdL, is necessary to understand their modes of action within the infection cycle of *L. pneumophila*.

A recombinant, truncated form of LdL, residues 27–192 encompassing 4 Sel1-like repeats, with a C-terminal 6xHis-tag was overexpressed using *Escherichia coli* BL21 (DE3) cells. One liter cultures in lysogeny broth and 0.1 mg/mL ampicillin were grown at 37 °C with shaking to an optical density at 600 nm (OD\(_{600}\)) between 0.6 and 1.0, at which point the cells were induced overnight with the addition of 0.1 mM isopropyl β-D-1-thiogalactopyranoside and lowering the temperature to 20 °C. Cells were then harvested by centrifugation and the cell pellet was lysed with 1.5 mg/mL lysozyme in the presence of 100 mM thiogalactopyranoside and lowering the temperature to 20 °C. The soluble fraction was kept for puriﬁcation via nickel-nitriloacetic acid beads using 50 mM Tris (pH 8), 0.3 M sodium chloride, and 10 mM imidazole. This lysis solution was incubated on ice with occasional shaking before sonication. After sonication, the lysed cells were centrifuged to separate the soluble and insoluble fractions. The soluble fraction was kept for size-exclusion chromatography using a HiLoad 16/60 Superdex 75 prep-grade column (GE Healthcare) gave pure protein (Figure 1). The protein was then concentrated to a range between 10 and 114 mg/mL using a centrifugal concentrator.

In protein crystallization, not only is it important to have the right combination of buffer, salt, and precipitant, but the method by which the crystallization condition is subjected to the protein sample can have a great impact on crystal growth and quality. Furthermore, usage of highly concentrated protein is ideal, but not a prerequisite with Crystal Formers as long as the protein sample can have a great impact on crystal growth and quality. Furthermore, usage of highly concentrated protein is ideal, but not a prerequisite with Crystal Formers as long as the sample is soluble. Diffusive mixing with a protein of higher concentration allows even more possibilities of conditions within each channel due to the progressive dilution of the protein toward the middle of the channel and the creation of a wider gradient. In our experiment specifically, we obtained our crystal in a condition which samples the PEG crystallization space in a fairly complete and complex way. The process of

![Figure 2](http://dx.doi.org/10.1021/cg500450b) Crystals of LdL 27–192 obtained from the Crystal Former in a condition consisting of 0.3 M calcium acetate, 0.1 M Bicine, 22.5% PurePEGs (PEGs 0.3–8 kDa), final pH 6.4.

![Figure 1](http://dx.doi.org/10.1021/cg500450b) SDS-PAGE of the purified LdL 27–192 protein following size-exclusion chromatography. Lane 1, molecular weight ladder; Lane 2, purified protein.

Through the use of high-throughput, 96-channel Crystal Formers, we were able to obtain a crystal for a protein that has failed to produce crystals through conventional vapor diffusion methods (Figure 2). Numerous vapor diffusion experiments were initially set up at room temperature using commercially available crystallization screening kits, as well as utilizing five different constructs of LdL (full length and various truncations) at concentrations typical of most vapor diffusion experiments (<20 mg/mL).\(^{20}\) Protein crystals were obtained, but were too small and not of sufficient quality for high resolution data collection. Experiments using higher concentrations of protein were also set up but not pursued due to large amounts of precipitation forming within the crystallization drops. Consequently, using 114 mg/mL of LdL 27–192 in 50 mM Hepes (pH 7), 20 mM sodium chloride, a Crystal Former tray was set up by first pipetting 0.5 μL of protein to one side of the channels, and then 0.5 μL of each of the conditions from the Smart Screen and the PurePEGs screen to the other side. The channel inlets were sealed with the sealing tape provided and the tray was left at room temperature. The protein crystal hit was found in a PurePEGs condition consisting of 0.3 M calcium acetate, 0.1 M Bicine, 22.5% PurePEGs (PEGs 0.3–8 kDa), final pH 6.4. The crystal was large enough to test using our home source X-ray machine, which was harvested from the Crystal Former through the bottom of the tray by cutting the peelable film surrounding the reaction chamber using a razor blade. Once the film was peeled off, cryoprotectant was immediately applied to both the open capillary and the cut film to prevent dehydration of the crystal. From here, the crystal was looped and mounted on the X-ray machine. The crystal diffraction to a maximum resolution of 2.76 Å (Figure 3, Table 1), and diffraction data were collected at 100 K for 340° total, with 1° oscillation, using the home source X-ray machine equipped with a Rigaku rotating copper anode and a MarResearch345 detector. Data were indexed, integrated, and scaled using XDS.\(^{21}\) Crystals belonged to the orthorhombic space group C222\(_1\) with unit cell dimensions \(a = 28.4\) Å, \(b = 152.3\) Å, and \(c = 77.6\) Å.

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equilibration in the channel creates overlapping waves of PEG molecules diffusing at different rates determined by their varying molecular weights. This is a very powerful screening approach that is achieved by the combination of the PEG cocktail with a capillary-based crystallization method.

The crystal grown and harvested from the Crystal Former allowed for collection of a full data set useful for potential structure determination. The use of Crystal Formers essentially screens and optimizes crystals in a single channel, which can be a time- and cost-saving strategy for protein crystallization. Our work demonstrates the effectiveness of using Crystal Formers as an autonomous crystallization screening tool for producing diffraction-quality crystals and can be the stepping stone for future structure determination.

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**Notes**  
The authors declare no competing financial interest.

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