MICROFLUIDIC PLATFORMS FOR MEMBRANE PROTEIN CRYSTALLIZATION
AND IN SITU CRYSTALLOGRAPHY

BY

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DISSERTATION

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ABSTRACT

Membrane proteins, biological macromolecules that reside in cellular membranes, play critical roles in many biological process, including signaling, transport, and intercellular communication. The malfunction of membrane proteins has been linked to the initiation or progression of many diseases (e.g. autism, diabetes), so the study of their precise structure is of critical interest in the field of drug discovery and structure-based drug design. Structure-based drug development relies on the knowledge of atomic resolution 3D protein structures, the relationship between protein function and structure, and how these proteins interact with potential drug molecules. X-ray crystallography, presently the most common and robust method for solving structures, relies on the growth of high quality protein crystals. The structural pipeline reaches a bottleneck during X-ray crystallography because conditions for protein crystallization cannot be determined a priori – extensive screening methods (i.e. trial and error) across a multi-parametric chemical space must be conducted to discover appropriate crystallization conditions using limited amounts of precious membrane protein sample. Analysis of eukaryotic genomes that 30% of all proteins are membrane proteins, however <2% of all known structures are membrane proteins. Given their significant role in disease and the slow pace of structure elucidation, new methods are needed to accelerate structure discovery for membrane proteins.

The membrane protein crystallization toolbox contains many powerful, yet difficult-to-use tools. For example, nucleation and growth are typically coupled during crystallization experiments, which limits the degree of control over the quality and size of crystals grown in solution. Seeding techniques, where crystals grow from existing nuclei, provide a straightforward route to large, diffraction quality crystals. While simple in principle, seeding is difficult in practice because the experimental procedure requires the crystallographer to disrupt the equilibrium of the crystallization droplet, and oftentimes ruining the crystallization
experiment. This difficulty often leaves seeding as a ‘last resort’ technique. Another technique, *in meso* crystallization, maintains membrane proteins in a native-like membrane throughout the process of crystallization and has yielded very high quality crystals and structures of previously intractable membrane proteins. Unfortunately, the *in meso* method requires handling highly viscous lipid phases with specialized mixing and dispensing tools, and is thus limited to dedicated crystallographers and labs with robotic formulation systems. Regardless of the crystallization technique used, membrane protein crystals are incredibly fragile, so the final step of harvesting and mounting crystals prior to X-ray diffraction also hampers progress in structural studies.

*This dissertation details the development and application of a suite of microfluidic crystallization platforms designed to overcome technical difficulties in membrane protein crystallization. Specifically, these platforms enable crystallization condition screening for either seeding techniques or *in meso* techniques and subsequent in situ X-ray crystallography.*

This work improves upon the construction of X-ray transparent devices previously designed in the Kenis group and applies them to membrane protein crystallography. In Chapter 2, devices for separating nucleation and growth via crystal seeding were developed and applied to a model soluble protein and a target membrane protein. In Chapter 3, a novel microfluidic method for formulating *in meso* crystallization trials was developed and used to crystallize and solve the structure of a membrane protein. In Chapter 4, *in meso* crystallization devices for high-throughput screening and optimization experiments were designed, and crystallization conditions for several membrane proteins of unknown structure were discovered. In the interest of directly studying protein-ligand or protein-drug interactions, a novel microfluidic method for growing and subsequently soaking crystals *in meso* was developed and applied to a model crystallization system in Chapter 5.
In summary, this work details the development of microfluidic platforms that automate membrane protein crystallization through a variety of techniques. These devices incorporate fine-control at the nanoliter scale and *in situ* analysis into high-throughput arrays to facilitate membrane protein structure determination. The development of platforms for *in meso* crystallization is particularly significant, as they represent the first X-ray transparent microfluidic platforms for *in meso* crystallization which also push the limits of scale and throughput when compared to state-of-the-art robotic *in meso* techniques. Further, when extended to studying protein-ligand and protein-drug systems via soaking, the *in meso* approach demonstrated here presents an attractive route to develop and study pharmaceuticals.
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CHAPTER 1

Introduction

Proteins are biological macromolecules that carry out a large diversity of functions in living organisms, from enzymes that regulate biochemical activity to structural proteins that maintain the shape and integrity of cells.¹ A pool of 20 unique amino acids and their specific arrangement in each protein confers on them this wide diversity of functions.² Specific linear sequences of amino acids for each protein are encoded in genes on DNA, and the human genome, for example, contains genes for 20,000 unique proteins.³ Even though the exact amino acid sequences of proteins can be determined from the DNA, only the 3-dimensional structures of proteins at atomic resolution can provide the key to understanding how and why proteins function.

A more specific class of proteins, membrane proteins, reside in biological membranes where

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Figure 1.1: Progress in membrane protein structure determination. (a) All deposited structures as of April 2017. Membrane proteins represent <2% of all protein structures in the Protein Data Bank, while 30% of all proteins are membrane proteins. (b) Accumulated number of protein structures in the Protein Data Bank since 1970 (inset: membrane proteins). The pace of soluble protein structure solution exceeds that of membrane proteins by 2 orders of magnitude. Figure created with data extracted from the Protein Data Bank (www.rcsb.org) and Membrane Proteins of Known Structure (http://blanco.biomol.uci.edu/mpstruc/)
they act as cellular gatekeepers and perform other pivotal functions in cellular energy production and regulation. While the discovery of protein structures has progressed and accelerated over the past 80 years, the number of published structures of membrane proteins lags behind those of water-soluble proteins – as of April 2017, over 120,000 protein structures have been solved and deposited in the Protein Data Bank, of which only 2,194 are membrane protein structures (Figure 1.1). While membrane proteins represent 20-30% of all proteins in prokaryotic or eukaryotic organisms, they only represent 1.8% of all atomic-resolution structures. While trending upwards from ~1.0% in 2010 thanks to dedicated structural biology initiatives, such as the National Institutes of Health (NIH) Protein Structure Initiative (PSI), significant bottlenecks in membrane protein expression, purification, and crystallization have hampered the rate of structure solution for membrane proteins. Herein lies a discussion of the high impact nature of membrane protein structures, the reasons for the lack of 3-D structures, and recent efforts to overcome bottlenecks.

1.1 Structural biology and membrane proteins

Protein structure is classified on four levels: (1) primary structure, the sequence of amino acids of the protein backbone, (2) secondary structure, the local folding and assembly into substructure elements such as α-helices and β-sheets, (3) tertiary structure, the full protein molecule fold in three-dimensional space, and (4) quaternary structure, the arrangement of protein molecules into a multi-subunit complex (Figure 1.2). Primary structure elucidation is easily and routinely accomplished by sequencing either the DNA that codes for the protein or the protein itself. Secondary structure elucidation is also straightforward to solve through predictive and spectroscopic methods (such as circular dichroism and infrared spectroscopy). Large macromolecular assemblies can be observed with a variety of analytical techniques to determine quaternary structures. Tertiary structures, however, are typically the most difficult as
they must be solved at atomic resolution using techniques such as nuclear magnetic resonance (NMR), cryogenic electron microscopy (cryo-EM), or, most commonly, X-ray crystallography.

Without their unique 3-D folds, membrane proteins would not be able to serve diverse, specific and efficient biological roles. Located in cellular membranes, membrane proteins’ roles range from transporters of ions/molecules to signal receptors to respiratory enzymes. Consequently, membrane protein misfolding or malfunction has been tied to the initiation and progression of many diseases, thereby making membrane proteins attractive drug targets (>60% of all drug targets). Conversely, drugs can be targeted to inhibit MPs in pathogenic bacteria to reduce the spread of certain diseases. Atomic resolution three-dimensional structures reveal key features of proteins, such as binding pockets for small molecules, docking locations on the protein surface for other molecules or proteins, and conformational changes. A thorough understanding of these structural features and interactions can accelerate structure-based drug design to discover new cures for membrane protein linked diseases.

Photosystems and reaction centers are amongst the most well-studied membrane proteins, representing >150 structures in the Protein Data Bank. Photosynthetic proteins can transform
light energy into metabolic energy – when triggered with light, electron transfer propagates across a series of protein-bound co-factors, ultimately converting photons into chemical bonds.\textsuperscript{16} In 1984, photosynthetic reaction center was the first membrane protein to have its 3-D X-ray crystal structure solved,\textsuperscript{17} and this crystallization has been replicated many times. Because the crystallization system is robust and repeatable, photosystems and reaction centers are often used to test new membrane protein crystallization techniques\textsuperscript{18–22} and crystallographic methods.\textsuperscript{23–27}

Several noteworthy structural studies into membrane proteins have come from the G protein-coupled receptor (GPCR) family. GPCRs are a large family of receptors that recognize extracellular stimuli, transmit signals over \~30 Å across the cell membrane, and trigger a cellular response with the aid of G proteins.\textsuperscript{28} An estimated 30-40\% of all drugs currently on the market target GPCRs.\textsuperscript{29} Until recently, biologists understood broadly the function of GPCRs but not specifically, structurally, how GPCRs convert a signaling stimulus into a cellular response. As a culmination of decades of research, 2011 marked a breakthrough for GPCR structures. The crystal structure of the \(\beta_2\) adrenergic receptor was captured while the protein was activated by a hormone agonist, providing the first high-resolution insight into the mechanism of signal transduction by a GPCR.\textsuperscript{30} When bound to an agonist, a transmembrane helix, TM5 extends by two rotations while TM6 shifts outward 14 Å, opening a docking site for a G protein which then initiates a downstream function (\textbf{Figure 1.3}). The impact of this work earned Brian Kobilka and Robert Lefkowitz the 2012 Nobel Prize in Chemistry.\textsuperscript{31} Further, several other researchers have designed new agonists and antagonists based on the structure of the active site – for example, through structure-based drug design, several high efficacy and high potency agonists were designed based on the 3-D structure of the active \(\beta_2\) adrenergic receptor, including boric analogues of the native molecule\textsuperscript{32} and newly designed molecules.\textsuperscript{33}
Heme-copper oxidases are another important superfamily of membrane proteins that couple oxygen reduction with transmembrane proton pumping during aerobic respiration.\(^{34-36}\) Recent advances have resulted in an X-ray crystal structure of a member from each of the four heme-copper oxidase families, although the total number of structures, number of structures in an active state, and in some cases, structural resolution are low.\(^{37-41}\) For example, the structure of cytochrome *bo*\(_3\) oxidase (PDB: 1FFT) is only partially complete, although it provided enough evidence to propose the location of a quinone binding site.\(^{40}\) Notably, a follow-up report showed that a secondary, low affinity quinone binding site exists in this same membrane protein, although the structural quality was too poor to distinguish the second site in the partial structure.\(^{42}\) In other studies, structures of cytochrome *c* oxidase do not directly show the capacity for the currently proposed mechanisms – further studies and higher resolution structures are required to further elucidate mechanisms in these cases.\(^{43}\)

Figure 1.3: 3.2 Å resolution structure of the β₂ adrenergic receptor from X-ray crystallography. (a,b) Side and cytoplasmic views of the active receptor (green) compared to inactive structure (blue). Significant structural changes are seen for the intracellular domains of TM5 and TM6. TM5 is extended by two helical turns whereas TM6 is moved outward by 14 Å as measured at the α-carbons of Glu 268 (yellow arrow) in the two structures. (c) The helix of a G-protein docks into a cavity formed on the intracellular side of the receptor by the opening of TM5 and TM6. As the helix exits the receptor it forms a network of polar interactions with TM5 and TM 3. Figures and caption adapted from Nature Publishing Group (Rasmussen et al., Nature 477, 549 (2011).)
1.2 The challenge of obtaining membrane protein structures

Several methods have been successfully employed to solve 3-D structures of membrane proteins, most notably NMR, cryo-EM, and X-ray crystallography. NMR is an attractive technique for obtaining structures under physiological conditions (i.e., conformational mobility, not constrained to a crystal lattice), but NMR studies of large, transmembrane proteins has been impaired by difficulties in preparing sufficient amounts of isotopically labeled proteins, limited thermal stability, and sample heterogeneity.\textsuperscript{44,45} While still in its infancy, structural biology with cryogenic electron microscopy (cryo-EM) has made many exciting advances over the past several years, from large to small membrane proteins.\textsuperscript{9} New detectors and computational methods have allowed the solution of “near atomic” resolution structures as low as 3.4 Å.\textsuperscript{46,47} While at modest resolutions compared to X-ray structures (<2.0 Å), cryo-EM directly images single molecules and thus does not require crystals nor experimental phasing. While the field of cryo-EM seems to have many exciting years ahead, there are still many technical hurdles to overcome before cryo-EM becomes the preferred technique.\textsuperscript{48} The focus of the work herein utilizes the current state-of-the-art, X-ray crystallography, which has the widest applicability, best resolution, and is responsible for ~90% of membrane protein structures to date. X-ray crystallography measures the diffraction of X-rays from the lattice of a protein crystal (Figure 1.4a). The theoretical limit in resolution is half the wavelength (\(\lambda\)) of light used for diffraction (for atomic resolution crystallography, \(\lambda = 1\) Å). Full molecular structures are built by solving electron density maps from diffraction data collected at many orientations of a crystal or multiple crystals, and then fitting the sequence of amino acids to complete the overall 3-D fold of the protein (Figure 1.4b).

In preparation for a crystallization experiment, proteins are expressed, purified and concentrated in a solution, and then mixed with precipitant (a mixture of salts, polymers, or small molecules that drive supersaturation). Water-soluble proteins are prepared in a buffered
solution, while membrane proteins are prepared in a buffered solution with detergent.

Crystallization experiments are typically prepared in standard well plates in one of four methods: (1) vapor diffusion, (2) batch, or microbatch, (3) free interface diffusion, or (4) dialysis. In principle, in each method, the crystallization experiment enters supersaturation differently, either by slow gradient mixing (free interface diffusion, dialysis, vapor diffusion), or rapid mixing (microbatch). Under the ‘right’ conditions, protein crystals will grow out of solution. Unfortunately, there is no a priori method for determining what precipitants will drive crystal formation, so a wide variety of precipitants are tested via a sparse matrix screening approach.49

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**Figure 1.4:** Schematic of monochromatic X-ray crystallography and structure solution. (a) For X-ray data collection from protein crystals, crystals are first looped onto a crystal mount and placed on an X-ray goniometer in the path of an X-ray beam. The incident X-rays diffract into many specific directions and the scattering pattern is measured using an X-ray detector. The crystal is then rotated into selected orientations and many more diffraction images are recorded. (b) For each image, the angles and intensities of the diffracted X-rays are identified and measured to determine the space group and lattice of the crystal. For each image, every reflection is recorded, scaled, and reduced into a tabular format that lists the Miller index (h,k,l) and intensity for each reflection. Next, computational software package is used to determine the phase of the reflections, build electron density maps, build the 3-D protein structure, and refine the structure.
After crystallization, the crystals are then harvested, flash frozen, and probed with X-rays to collect diffraction data.

Protein sample preparation and crystallization gain an additional layer of complexity for membrane proteins due to their inherent amphiphilicity – as membrane proteins are natively situated in biological membranes, a portion of every membrane protein’s surface is hydrophobic, which renders them insoluble in aqueous solution. Amphiphilicity presents new challenges for expression, purification, and crystallization. The amount of membrane protein expressed is limited to the volume of two-dimensional membranes, yielding up to milligram quantities of precious membrane protein sample. As a result, the majority of structures come from membrane proteins that naturally express at high concentrations, or for membrane proteins that have been overexpressed in a homologous system. Before crystallization, membrane proteins must be removed from their native membrane through the addition of detergents and purified. Many proteins rapidly denature and aggregate when solubilized in detergents, although a variety of solubility enhancing tags and affinity tags have been developed to stabilize membrane proteins throughout the purification process.

Even when a membrane protein sample has been expressed and purified successfully, amphiphilicity still disturbs the crystallization process. Crystallization can be performed using the same methods as water-soluble protein crystallization, although the process is termed “in surfo” crystallization (in surfactant). Crystal formation requires protein molecules to form close crystal contacts, but these contacts are inhibited by the presence of stabilizing detergents on the hydrophobic transmembrane portions of the proteins. Consequently, crystals contacts form almost exclusively at the hydrophilic head and tail sections of the protein, resulting in low crystallization success rates and fragile, weakly diffracting Type II crystals. Comparatively, for water-soluble proteins, the entire surface is available for crystal contacts, and strongly diffracting
Type I crystals typically form (Figure 1.5). Despite the inherent reduced quality of crystals, many crystal structures have been solved from crystals grown \textit{in surfo}.

Due to the fragility of membrane protein crystals, the process of harvesting and mounting crystals are a significant experimental step prior to X-ray crystallography. Harvesting is performed with a pin mount that has crystal loop or a plastic mesh at the tip. The crystallization well is de-sealed and then crystals are manually picked out of the mother liquor with the mount and then flash frozen in liquid nitrogen to mitigate radiation damage.\textsuperscript{56} Crystals often sustain damage during the harvesting and freezing process which negatively impacts structural

\textbf{Figure 1.5:} Schematic of two approaches for membrane protein (MP) crystallography. (Top) Detergent solubilized MP is mixed directly with precipitant in the \textit{in surfo} method. Vapor diffusion crystallization trials are set up as either a ‘hanging drop’ or a ‘sitting drop’ and sealed in a closed well with precipitant as an equilibration buffer. When crystals form in the drop, the detergent molecules at the MP surface typically inhibit close crystal contacts, forming Type II crystals where the hydrophilic surfaces primarily make crystal contacts. (Bottom) Detergent solubilized MP is mixed with a lipid, forming a cell membrane-like bicontinuous lipid phase. Crystallization trials are typically set up as sitting drops or in a microbatch well. When crystals form in \textit{meso}, they crystallize stacked bilayers which facilitates formation of close crystal contacts on both hydrophobic and hydrophilic surfaces, forming high quality Type I crystals.
Although not widely accessible, new techniques mitigate some of these issues such as serial crystallography to mitigate radiation damage, automated harvesting approaches, and liquid jets or fixed targets to avoid crystal handling.

1.3 Advanced techniques for membrane protein crystallization

While *in vitro* crystallization methods have yielded many structures, several crystallization methods that enable crystallization success rates and grow higher quality crystals (larger or Type I crystals) have been successfully applied to membrane proteins. Notably, the application of two methods, microseeding and *in meso* crystallization, have both resulted in increased rates of crystallization and structure solution of previously intractable proteins.

**Microseeding membrane protein crystallization.** Microseeding of protein crystallization, the process of supplementing a crystallization experiment with previously grown crystal nuclei, decouples nucleation and growth. A fixed number of crystal nuclei are supplied to a supersaturated protein solution, resulting in the growth of a limited number of large crystals for X-ray diffraction experiments.

The microseeding method has been extended to microseed matrix screening, where a homologous microseed (same protein) or heterologous microseed (e.g., other proteins, hairs, fibers) are introduced to high throughput sparse matrix screens for new crystallization conditions. With microseeds, crystals grow at both high and low levels of supersaturation which increases hit rates during sparse matrix screening. The best crystals are identified from the screen, and then the condition(s) are repeated and re-screened to obtain diffraction quality crystals as demonstrated in several reports.

The experimental methods for microseeding are difficult, and despite numerous successes with soluble proteins, just one membrane protein has been crystallized with microseeds. While simple in principle, the addition of microseeds is difficult in practice. Microseeds will often...
dissolve in unmixed precipitant or protein solutions, thus the process of adding microseeds is delicate and time sensitive. Further, manual skill is required to de-seal crystallization wells to add nanoliters of microseed solution, a process which also inadvertently shifts the equilibrium of the crystallization experiment through incidental exposure to air. Specialized well plates and robotic technologies have been developed to increase the throughput of in situ and microseeded crystallization by automating the formulation of vapor diffusion and microbatch droplets. A few robotic techniques have been designed specifically for microseeding, (e.g., robots from Douglas Instruments and TTP Labtech) but these robots cannot solve the time-sensitive dissolution inherent to microseed addition.

**Crystallization of membrane proteins in meso.** Bilayer methods for MP crystallization make use of extended bilayers composed of lipid, detergent, and proteins to stabilize and facilitate the process of crystallization. The most impactful of these methods, in situ crystallization, reconstitutes and stabilizes membrane proteins in a lipidic cubic phase, a bicontinuous lipid crystal mesophase that mimics a cell membrane throughout the crystallization process (Figure 1.6). Although determining appropriate crystallization conditions still requires extensive sparse

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**Figure 1.6:** Cartoon representation of the events proposed to take place during crystallization in meso. The process begins with the protein reconstituted into the curved bilayer of the ‘bicontinuous’ cubic phase (tan). Added ‘precipitants’ shift the equilibrium away from stability. This leads to phase separation where molecules (a) diffuse from the cubic phase into a sheet-like domain and (b) locally concentrate therein in a process that progresses to nucleation and crystal growth... An expanded view of the various components in the system is shown in (c). Figure reproduced from Li, Shah et al. (2013). Copyright 2013 American Chemical Society.
matrix screening, the primary advantages of crystallization in meso arise from the membrane protein remaining in a membrane throughout the crystallization process. Due to their confinement in a membrane, when MPs crystallize in lipidic mesophases they typically form closely packed and strongly diffracting crystals. The absence of detergent micelles enables both the hydrophilic and hydrophobic domains to make crystalline contacts in “Type I” crystals. Since the discovery of in meso crystallization in the 1990s, many noteworthy crystal structures of membrane proteins, including the aforementioned GPCRs and heme-copper oxidases, were crystallized in lipidic mesophases.

The success of the in meso method relies on screening a large number of crystallization conditions, which requires repetitive use of specialized tools (e.g., coupled syringes, ratcheting syringes) to forcibly mix and dispense lipid and protein and overcome the high viscosity and non-Newtonian behavior of the mesophase (Figure 1.7). Automated screening approaches, robots, and novel crystallization protocols alleviate some of these problems, but harvesting protein crystals from mesophases is still performed manually. The typically small size

![Figure 1.7: Setting up an in meso crystallization trial involves (a) placing membrane-protein solution and lipid into syringes connected by a coupler, passing the protein from one syringe to the other via the coupler to mix the mesophase and reconstitute the protein, (b) transferring the mesophase into one syringe, (c) replacing the empty syringe to a dispensing micro-syringe mounted in a repeat dispenser, and (d) dispensing mesophase followed by precipitant solution into the wells of a glass sandwich plate. Figure and caption reproduced from the International Union of Crystallography (Caffrey, Acta Cryst F 71, 3 (2015)).]
of *in meso* MP crystals, 2-70 µm,\(^{86}\) which are embedded in the toothpaste-like mesophase makes harvesting them even more problematic than for *in surfo* crystallization. Both microseeding and *in meso* crystallization would benefit from easy-to-use technologies or devices to automation crystallization and facilitate analysis.

### 1.4 Microfluidic devices for protein crystallization

Microfluidic devices are tools to precisely control flow phenomena at sub-milliliter volumes, and have been applied in many research fields and commercial applications, from the analysis of single cells and molecules to next generation gene sequencing technologies. Polydimethylsiloxane (PDMS) is commonly used for developing and prototyping microfluidic devices because it is biocompatible, non-toxic, and optically transparent. Microchannels can be patterned in PDMS via soft lithography, and PDMS also has adhesive and elastomeric properties that enable facile bonding and the incorporation of integrated microvalves, enabling PDMS microfluidic devices to manipulate and mix just nanoliters of fluid with precision (Figure 1.8). Microfluidic devices built out of thermoplastics (e.g., polymethylmethacrylate, cyclic olefin copolymer) have grown in popularity due to their compatibility with commercial manufacturing.

![Figure 1.8: Two common types of valves used in microfluidic chips. (a) A normally-open valve – when the control channel is pressurized with air, the fluid channel will flatten and stop the flow of a liquid. (b) A normally-open valve – when vacuum is applied to the control channel, adhesive forces between PDMS and the substrate are overcome and the valve stop raises to allow fluid flow. Panel a reproduced from Multidisciplinary Digital Publishing Institute (Au et al., Micromachines 2, 179 (2011).), panel b reproduced from Elsevier (Mohan et al., Sens. Actuators, B 160, 1216 (2011)).](image-url)
processes, excellent optical properties, and impermeability. Microfluidic devices provide a facile route to scale down and scale out – through etching or lithography, channels and compartments at the micron scale hold just nanoliters of fluid and can be reproduced in highly parallel arrays. Due to the need for high-throughput screening at nanoliter-to-microliter scales, microfluidic devices are an attractive option for membrane protein crystallization.

Compared to protein crystallization robots, microfluidic methods offer equivalent throughput (number of trials) and can use sample more efficiently (volume consumed per trial). Several efforts have been made to increase throughput and lower the usage of precious protein sample – in a notable report, Quake and co-workers developed a microfluidic crystallization device that

**Figure 1.9:** Three microfluidic chips for protein crystallization. (a) Microfluidic array chip for protein crystallization via free interface diffusion using as little as 10 nL of protein solution in arrays 144 wells. Normally-open microfluidic valves separate protein and precipitant during filling, and is released for mixing. (b) Microfluidic droplet mixer to formulate crystallization trials – droplets of protein and precipitant are separated by water-permeable oil across which water will diffuse to induce crystallization. (c) X-ray transparent microfluidic array chip for protein crystallization by free interface diffusion. Normally closed valves separate protein and precipitant during filling. After crystallization, the entire X-ray transparent chip is mounted in front of an X-ray beam for analysis. Panel a reproduced from Hansen et al., Proc. Natl. Acad. Sci. USA 99, 16531 (2002), Copyright 2002 National Academy of Sciences. Panel b reproduced from Wiley (Zheng et al., Angew. Chem. Int. Ed. 43, 2508 (2004)). Panel c reproduced from Elsevier (Guha et al., Sensor. Actuat. B-Chem 174, 1 (2012)).
formulated 144 parallel free interface diffusion crystallization trials where each used just 10 nL of protein sample (Figure 1.9a). The automation and throughput were achieved by 480 active valves, and at its time, this device outperformed comparable methods in identifying crystallization hits while reducing sample consumption by two orders of magnitude. In another report, Ismagilov and co-workers developed a microfluidic system that formulated microbatch crystallization trials in 7.5 nL aqueous droplets at a rate of “several per second” under computer control (Figure 1.9b). In a third approach, Fraden and co-workers developed a device that formulated 1000 crystallization conditions by dialysis, storing 1 nL crystallization. These, among several other methods, demonstrate the translation of a variety of bench-top crystallization methods onto microfluidic chips.

To avoid damaging crystals during the process of crystal harvesting (Section 1.2), many recent microfluidic devices are designed for X-ray transparency to allow in situ X-ray diffraction experiments. Crystals grown in microcapillaries have been used to successfully used for in situ crystallography. Thin thermomoplastic X-ray compatible well plates, such as the X-CHIP, have also been successfully used to solve crystal structures in situ. Sauter and co-workers performed a thorough study of chip materials and developed counter-diffusion chips for in situ X-ray analysis fabricated from PDMS, polymethylmethacrylate (PMMA), or cyclic olefin copolymer (COC) – the devices most compatible with X-ray crystallography were determined to be the ones made of COC and PMMA as determined by the quality of protein structure collected on each chip. Thick layers of PDMS were found to significantly scatters X-rays, increasing background noise when analyzing crystals in situ. A few other reports also focus on device development and advantages with COC and other impermeable, X-ray transparent materials. Kenis and co-workers developed X-ray transparent microfluidic devices for crystallization by free interface diffusion with an overall thickness of <200 µm of COC and PDMS (Figure 1.9c). A series of reports demonstrated that these devices are effective tools for serial crystallography,
de novo structure solution via anomalous diffraction,99 time-resolved crystallographic experiments with Laue pump-probe crystallography,100 and cryo-crystallography.101

While numerous microfluidic technologies have automated nanoliter-scale fluid handling for protein crystallization in aqueous solutions, few methods have addressed the difficulties of multi-step crystallization experiments such as microseeding or in meso crystallization. Microseeded crystallization trials are typically prepared manually or with a robot, with a few notable exceptions. Ismagilov and co-workers demonstrated a two-step seeding method for protein crystallization that modulated the concentrations of nanoliter sized droplets,102 and Kenis and co-workers demonstrated microfluidic microseed screening by free-interface diffusion to optimize crystallization for active pharmaceutical ingredients.103 Similarly, only two non-robotic methods have been reported for in meso crystallization: a nanoliter-scale droplet-based method for crystallization in pre-mixed or diffusively-mixed mesophases (Figure 1.10),19 and a high-viscosity microfluidic mixer (Figure 1.11).84 These microfluidic approaches successfully

![Figure 1.10: Plug-based microfluidic system for in meso crystallization. (a) Schematic of the system. Small mesophase plugs (~1 nL) were formed in a PDMS flow-focusing device using fluorinated carbon (FC) as a carrier fluid and then they were merged downstream with the streams of protein and precipitant to form LCP-containing aqueous plugs (~80 nL). The plugs of the crystallization trials were stored and incubated at 23°C in Teflon tubing to allow crystals to grow. (b) A micrograph showing that LCP plugs formed in the flow focusing device. (c) A micrograph showing that LCP plugs successfully merged with precipitant and protein solutions. (d) A micrograph showing that there was no cross contamination between plugs. (e) Crystallization of reaction center from Blastochloris viridis. Figure and caption adapted from Springer (Li et al., Microfluid Nanofluid 8, 789 (2010)).]
crystallized membrane proteins \textit{in meso}, although their application has not been expanded to high-throughput screening and \textit{in situ} crystallography.

In summary, significant efforts have produced a wide diversity of microfluidic devices for protein crystallization, and a few have reached commercialization. To eliminate the steps of crystal handling and mounting, X-ray transparency is a highly desirable feature for these devices. Despite the advantages of microfluidic devices for manually intensive techniques such as microseeding or \textit{in meso} membrane protein crystallization, there has been far less development in user friendly and high-throughput microfluidics. Further, due to the fragile nature of membrane protein crystals, \textit{in situ} analysis on X-ray transparent devices is a highly attractive route to 3D membrane protein structures. The melding of scalability, fluidic control, and X-ray transparency for advanced crystallization techniques presents a great potential for new devices that can advance the frontier of structural biology of membrane proteins.
1.5 Summary and key challenges

The identification of crystallization conditions is a significant bottleneck in the membrane protein structural biology pipeline because conditions cannot be determined a priori. High-throughput screening is a necessity, and microfluidic devices have facilitated screening for numerous soluble proteins. Advanced crystallization techniques, such as microseeding or in meso methods, can offer a higher probability for success of membrane protein crystallization, but there is a noteworthy lack of accessible enabling technology for crystallization trial formulation, condition screening, and in situ crystallography. The ideal microfluidic tools to accelerate structural biology with these advanced crystallization techniques would: (1) automate the multi-step mixing process for aqueous or lipidic cubic phase crystallization trials, (2) enable in situ crystallography via X-ray transparency, and (3) be simple and easy-to-use for the average crystallographer.

Microseeded crystallization offers a reliable route to large, diffraction quality crystals by decoupling nucleation and growth, although the process of adding microseeds to an equilibrium crystallization experiment is a challenge. Chapter 2 focuses on the development and application of X-ray transparent microfluidic devices that control the difficult process of adding microseeds to crystallization experiments for both soluble proteins and membrane proteins in surfo.

In meso crystallization requires the preparation of highly viscous mesophases, and then subsequent addition of precipitant to trigger crystallization. Previous approaches have faced significant challenges in formulation of mesophases (<10 nL) in a high-throughput manner for crystallization and in situ diffraction. Chapter 3 details the development and application of in meso crystallization devices to simplify and automate the process of mesophase formulation and solve a membrane protein structure from on-chip data. Chapter 4 details the extension of
this work into high-throughput arrays and applies the devices to the crystallization of several membrane proteins of unknown structure.

After crystal growth, soaking small molecules into crystals can provide structural evidence for binding or docking sites, or even mechanistically important structural changes. Crystal soaking, however, has been shown to be prohibitively difficult when \textit{in meso} crystals are grown traditionally in glass-sandwich plates and beyond the capabilities of automated \textit{in meso} crystallization robots.\textsuperscript{104} \textbf{Chapter 5} discusses the development and application of a straightforward method for soaking inhibitor molecules into membrane protein crystals grown with \textit{in meso} crystallization devices.
1.6 References

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CHAPTER 2

X-ray Transparent Microfluidic Platforms for Microseeding Membrane Protein Crystallization*

2.1 Introduction

Protein crystal formation can be conceptualized in terms of composition-composition phase diagrams in which concentrations of protein and precipitant map the solubility and aggregation properties of protein solutions. Such a crystallization phase diagram (Figure 2.1) is constructed by mixing a protein and a precipitant at specific, increasing concentrations until the cloud point is observed to identify the precipitation boundary, and the dissolution of crystals to identify the solubility boundary. Between these two extremes are two distinct zones of supersaturation: the

Figure 2.1: Simplified protein crystallization phase diagram. The phases, in increasing orders of supersaturation are: soluble, metastable, labile, and precipitation. (a) The formulation of two microbatch droplets, points C and F, is illustrated. Composition C is formulated by mixing a droplet of composition A with a droplet of composition B (1:1 mixing ratio), while composition F is formulated by mixing a droplet of composition D with a droplet of composition E (1:1 mixing ratio). (b) A typical trajectory during a microbatch crystallization experiment. Droplet F will form new nuclei and grow crystals, while droplet C will not form new nuclei and remain at a metastable concentration, growing no crystals. (c) The same crystallization experiments supplemented with microseeds – pre-existing nuclei grow into large, ordered crystals.
metastable region and the labile region. Crystallization trials formulated at concentrations in the labile region result in spontaneous nucleation and successful crystallization, while those formulated in the metastable region result in no new nucleation (Figure 2.1b). Conceptually, the formation of new nuclei is unfavorable in the metastable region, but favorable in the labile region. Oftentimes, a crystallization trial formulated in the labile region results in uncontrolled nucleation producing hundreds of tiny crystals that are unfit for single crystal X-ray diffraction. These problems can be overcome through careful inspection of the phase diagram and through techniques such as microseeding to separate the processes of nucleation and growth.2

During initial crystallization experiments, any resulting microcrystals, irregular crystals, or other semi-crystalline phases, while not useful for X-ray diffraction, can be collected and crushed to form a solution of submicroscopic crystal nuclei.2,3 Microseeding, the process of introducing such nuclei into a crystallization mixture, has been demonstrated to be a powerful tool for growing large, diffraction-ready protein crystals.4–9 The process of microseeding decouples two steps in crystal formation – nucleation and growth – by supplying a finite number of nuclei into a metastable protein-precipitant mixture. In principle, when microseeds are introduced into a metastable mixture, no self-nucleation events occur in the droplets and only the supplied nuclei will grow into crystals (Figure 2.1c).

The application of microseeds also extends to routine screening experiments in a method known as microseed matrix screening. When screening for crystallization of a new protein target with an unknown crystallization phase diagram, the addition of microseeds can promote ordered crystal growth and enhance screening success rates.10–13 Microcrystals from an initial screen are crushed to form microseeds, and then used during re-screening of the same or a different set of conditions. The best conditions from the resulting screen are harvested and the process is iterated to produce diffraction-quality crystals. With the aid of microseeds, the number of protein crystallization conditions discovered increases because both metastable and labile
compositions will produce crystals, effectively broadening the chemical space for successful crystallization screening. Microseed matrix screening has been used successfully in cases where the best conditions for crystal growth without seeding lead to precipitation,\(^7\) to accelerate crystal growth of proteins complexed with hydrolysable substrates where the absence of seeding resulted in a hydrolyzed substrate,\(^14\) to crystallize antibody-antigen complexes,\(^4\) and many more cases.\(^{15-18}\)

Many efforts to simplify and automate microseeding have been successfully implemented for various proteins.\(^{11,12,19,20}\) Microseeding robots pre-mix the protein, precipitant and microseed solution during crystallization set up.\(^{11}\) Femtosecond laser ablation has been used to eject crystal fragments that serve as seed in the same crystallization drop.\(^{21}\) Acoustic matrix microseeding utilizes acoustic waves to deliver nanoliter volumes of seed suspension into protein drops.\(^{22}\) Despite the promise of improved crystallization success with microseeding, it remains an under-utilized tool and is often chosen as the last resort when all other attempts to grow high quality crystals have failed. In all methods, the procedure for making microseed stock solutions is the easy part, while the introduction of microseeds to the crystallization droplet requires skill and experience when performed manually. Alternatively, microseeding robots can perform routine microseed matrix screening, but robots with this capability are not commonly available in structural biology labs. Further, no method provides a non-intrusive method for introducing microseeds after a droplet reaches metastable equilibrium.

In this work, we report two microfluidic methods to simplify the process of microseeding and the application of methods to photoactive yellow protein (PYP, soluble protein) and cytochrome \(b_{03}\) oxidase (cyt \(b_{03}\), membrane protein). Microfluidic array chips are easy to use for researchers of any skill level and enable non-invasive incorporation of microseeds during an in-progress crystallization experiment by free interface diffusion across normally closed microvalves. In the first method, metastable mixtures of PYP and cyt \(b_{03}\) were formulated off-
chip, introduced into a microfluidic chip, and mixed with several different microseed dilutions to observe the impact of microseeds and find the best conditions for crystallization. For both proteins, microseeds at greater dilutions resulted in larger, less clustered crystals. A separate chip was developed to perform microseed matrix screening – in this approach, protein and precipitant were mixed on-chip at various compositions, and then, after incubation, mixed with microseed solutions. Importantly, this chip gives full control over the timing of seed introduction, preventing incidental seed dissolution during mixing. Results with cyt $bo_3$ showed localized areas of the composition-composition crystallization phase diagram where crystals of different diffraction quality and morphology could be grown. While the crystals obtained in this work did not result in an atomic resolution crystal structure, the method presented here demonstrates the use of these microfluidic chips as enabling technologies to reliably screen, troubleshoot, and analyze the crystallization of finicky membrane proteins with microseeds. The activation barrier for using this technology is low, as the only peripherals necessary for operation are pipettes to place solutions at the inlets, a small vacuum pump to actuate valves for filling and mixing, and a stereo-zoom microscope to observe the chips for crystal growth.

2.2 Materials and methods

Protein preparation. Photoactive yellow protein from *Halorhodospira halophila* was cloned and expressed in *Escherichia coli* (strain BN9626) and purified as published previously.$^{23}$ Briefly, polyhistidine-tagged apoPYP heterologously over-expressed in *E. coli* was reconstituted *in vitro* with the anhydrous derivative of the chromophore p-coumaric acid and the polyhistidine-tag was cleaved by incubating it with enterokinase.

Cytochrome $bo_3$ oxidase (cyt $bo_3$) from *Escherichia coli* (strain C43(DE3)) was purified as published previously.$^{24}$ Briefly, polyhistidine-tagged cyt $bo_3$ was overexpressed by IPTG induction in *E. coli* and solubilized in dodecylmaltoside. Prior to crystallization, the sample was
treated with 1 mM potassium ferricyanide to fully oxidize the protein and exchanged into 20 mM Tris-HCl, pH 8 with 0.7% beta-octylglucoside.

**Fabrication of photoresist-on-silicon masters for replica molding.** Photoresist-on-silicon masters were created by photolithography with SU8-2050 photoresist\(^{25}\) (Microchem) for patterns with 50-100 µm-tall vertical features. All photoresist-on-silicon masters were treated with (tridecafluoro-1,1,2,2-tetrahydrooctyl) trichlorosilane (Gelest, Inc.) in a vacuum chamber for 4 h for easy release of soft lithographic replicas.\(^{26}\)

**Fabrication of thin PDMS/COC microfluidic devices.** The PDMS layers were fabricated using standard replica molding procedures\(^{26,27}\) by spin-coating the photoresist-on-silicon masters with PDMS to obtain a PDMS film height ~10 µm thicker than the corresponding photoresist feature height. For the fluid layer PDMS with the monomer:cross-linker ratio of 15:1 was cured at 90 °C for 7-9 min. For the control layer PDMS with the monomer:cross-linker ratio of 5:1 was cured at 90 °C for 3 min.

The chips were assembled as follows: (i) a flat COC sheet was irreversibly bonded to the PDMS control layer, and (ii) the resulting COC-PDMS assembly was irreversibly bonded to the PDMS fluid layer. Permanent COC-PDMS bonding in step (i) was achieved by activating the surfaces using atmospheric plasma treatment\(^{28}\) in a plasma cleaner (Harrick, Model PDC-001) for 1 min at 500–700 mTorr. Permanent PDMS-PDMS bonding in step (ii) was created via the standard multilayer soft lithography approach\(^{27}\) by placing layers of PDMS with different monomer:cross-linker ratios in conformal contact and heating them at 70°C for 2 h. Inlet holes for the control and the fluid layer were drilled in the COC-PDMS-PDMS assembly using a 300 or 750 µm drill bit (McMaster-Carr). The assembly was then placed on an unpatterned COC substrate and a reversible bond between the PDMS fluid layer and the COC substrate formed spontaneously.
Crystallization in well plates. Vapor diffusion in hanging drops was set up as published previously. Un-seeded crystallization in well plates yielded clusters of crystals after 7-10 days of incubation for PYP, and microcrystalline showers for cyt bo3. These crystals, while unsuitable for X-ray analysis because of difficulties in isolating individual crystals from the clusters or microcrystalline showers, were used to create microseed solutions. Crystals from 2-3 crystallization wells each for PYP and cyt bo3 were harvested from hanging drops and transferred into 500 µL of a concentrated precipitant solution (PYP: 3M ammonium sulfate, cyt bo3: 13% PEG 1500), and then transferred to a tissue grinder (Kontes Duall model K885460-0021, Thermo Fisher Scientific Inc) where they were crushed gently to make the stock microseed solution. Various concentrations of the microseed solutions were prepared by serially diluting the stock solution in concentrated precipitant solutions at ratios of 1:1, 1:2, 1:4, 1:10, 1:20, and 1:50. Repeating the same crystallization experiments at reduced concentrations in well plates with microseeds successfully yielded crystallization after 2 days. Microseed stocks and dilutions were either used immediately or flash frozen in liquid nitrogen and stored at -80°C until use.

Computational fluid dynamic simulations of mixing time. Diffusive mixing of protein in microfluidic compartments was simulated using a 2D finite element solver, COMSOL Multiphysics (COMSOL Ltd.). The lateral dimensions of six different sets of protein and precipitant compartments in this model are identical to those in the actual chip. A ‘No Flux’ boundary condition was applied to external walls and non-mixing interfaces. When mixing, the valves areas raise and leave an open liquid-liquid interface for free interface diffusion. The model simulated 60 minutes of mixing at 1 minute intervals. The mesh was “finer”, with a total of 1319 elements. The initial concentrations of cyt bo3 oxidase were 10 mg/mL – 50 mg/mL, and the diffusion coefficient was $4 \times 10^{-7}$ cm$^2$/s. The initial concentration of PEG 1500 was 0.07 mM, and the diffusion coefficient was $3 \times 10^{-6}$ cm$^2$/s. The solutions were assumed to dilute and
that the diffusion coefficients were independent of concentration. For each half-well, the average concentration was calculated using a surface integral and scaled to the actual height of the microfluidic well (50 µm).

**Synchrotron X-ray data collection and analysis.** X-ray diffraction data were collected at APS (Advanced Photon Source) synchrotron, Argonne National Laboratory at beamline 23-ID-B GM/CA (General Medical Sciences and Cancer Institutes Structural Biology Facility). Data were collected from all cyt bo$_3$ crystals that produced diffraction in 0.2–0.4° steps with 0.2–0.4 s exposures and a sample-to-detector distance between 500–600 mm with a Pilatus 6M detector. All data were collected at room temperature. During diffraction experiments, each crystal in each well was numbered and recorded to match diffraction data to its corresponding protein-precipitant composition.

**Image analysis of cytochrome bo$_3$ oxidase crystals.** Images of cyt bo$_3$ crystals were captured using an upright stereo microscope (Leica MDG33) equipped with a macro lens and a digital camera (Leica DFC295). Images were analyzed manually using ImageJ. The length and width of up to 30 crystals in each well were recorded and correlated to the protein-precipitant condition used for crystal growth.

**2.3 Microseeded crystallization of photoactive yellow protein and cytochrome bo$_3$ oxidase**

Multilayered microfluidic chips designed for high X-ray transmission were fabricated to facilitate the addition of microseeds at variable concentrations to crystallization experiments. In a 24-well array, sets of integrated microvalves controlled the metering and mixing of metastable crystallization mixtures and microseed solutions. Devices were comprised layers of PDMS, a flexible polymer that enables the functionality of valves, sandwiched between thin layers of cyclic olefin copolymer, a rigid polymer that acts as an impermeable barrier to air and water (Figure 2.2a). At a total thickness of ~200 µm, these chips have been demonstrated to be
effective all-in-one tools for crystal growth and in situ serial X-ray data collection at room

Figure 2.2: A simple approach for microseeding on a microfluidic chip. (a) Exploded view of a single crystallization well comprised of three thin layers: a PDMS fluid layer containing compartments and valves for liquid solutions, a COC layer containing vacuum lines for valve actuation and windows, and a COC substrate to seal the chip for experiments. This work substitutes a COC-PDMS control layer for the shown COC control layer. (b) Schematic of the design of a 24-well array chip used for microseeding. Fluid layer is shown in black, and the various valve lines, V1, V2 and V3, are colored based on their function (see legend). The window structures (yellow) are present to decrease the total material present in the path of the X-ray beam. For microseeding experiments, a gradient of microseed dilutions were loaded (inlets 2-7) and mixed with a pre-mixed protein-precipitant solution (inlet 1). (c,d) Results from screening several microseed dilutions – at greater seed dilutions (lower seed concentration), PYP crystals (c) grew into fewer, larger crystals and cyt $b_0$ oxidase crystals (d) grew into fewer, thicker crystals. Figure 2.2a and 2.2b adapted with permission from Elsevier (S. Guha, S.L. Perry, A.S. Pawate, and P.J.A. Kenis, Sens. Actuators, B 174, 1, 2012)
temperature (>75% X-ray transmittance at $\lambda = 1\text{Å}$).

The operation of a similar device has been described in previous work.\textsuperscript{33} Briefly, 3 µL of a pre-formulated protein-precipitant mixture was placed onto inlet 1 and introduced to the protein-precipitant half-wells by dead-end filling (Figure 2.2b), a vacuum-actuated process that displaces air in each compartment with the protein-precipitant mixture as air permeates through a thin PDMS layer separating the fluid layer from the control layer. 1 µL of a microseed solution was placed onto each microseed inlet (ports 2-7), and they were also introduced to microseed half-wells by dead-end filling. The normally-closed valve separating the two half-wells was then opened for 5 minutes to allow mixing of microseeds and the protein-precipitant mixture by free interface diffusion.

As validation, the effect of microseeds on crystallization was tested for two proteins: (1) photoactive yellow protein, a protein that grows into well-ordered crystals only in the presence of microseeds, and (2) cytochrome $b_{0,3}$ oxidase, a fragile membrane protein with a known crystallization condition that has previously produced some crystals and an incomplete, low resolution structure. Microseeding experiments on-chip were performed by first preparing metastable 1:1 solutions of protein and precipitant in an Eppendorf tube (20 mg/mL PYP with 2.6 M ammonium sulfate, 15 mg/mL cyt $b_{0,3}$ with 10% w/v PEG 1500, 100 mM HEPES pH 7.0, 100 mM NaCl, 100 mM MgCl$_2$, and 5% ethanol) and allowing the solution to equilibrate for 1 hour on ice. Each mixture was then introduced to a microfluidic chip. Serial dilutions of microseed solution were introduced through inlets 2 through 7 to fill the half-wells adjacent to the protein-precipitant mixtures (Figure 2.2b). The metastable protein-precipitant mixture and microseed wells mixed by diffusion for 5 minutes by opening the microvalve that separated the wells. Control experiments without microseeds resulted in no crystal growth, verifying that the mixtures were metastable rather than labile.
A range of microseed dilutions were tested, from 1:1 (high concentration) to 1:50 (low concentration). With microseeds, crystals first appeared after 1 day and reached full size after 4 days (PYP) or 7 days (cyt bo₃). For both proteins, high concentrations of microseed resulted in many small or needle-like crystals. As microseed concentration decreased, fewer, larger crystals grew (Figure 2.2c,d). These observations agree with expected trends for crystal growth with microseeds – at lower microseed concentrations, fewer crystals grow to larger sizes, while at higher microseed concentrations, more crystals grow to smaller sizes. A 1:20 microseed dilution yielded large, isolated crystals for PYP, while a 1:50 ratio yielded the thick, individual crystals of cyt bo₃. The disparity in these optimal dilution ratios has two origins: first, depending on the protein and its solubility, some number of microseeds may dissolve during the mixing of the metastable mixture with microseeds. Second, an imprecise number of crystals were used to generate microseed stock solutions, so the true microseed concentration varies from seed stock to seed stock. Regardless, once an ideal microseed dilution was determined, large crystals could be reliably grown by repeating crystallization with the same seed stock.

X-ray diffraction data were collected for PYP and cyt bo₃ crystals on-chip. The entire microfluidic chip was mounted on a magnetic cap mount with a set screw and placed onto the goniometer. Due to the construction from X-ray transparent polymers and low thickness, crystals were targeted and analyzed at room temperature using the on-axis microscope available at the beamline. PYP crystals diffracted to a maximum resolution of 1.19 Å, and a structure was solved at 1.32 Å. The diffraction data set had high quality diffraction spots and good signal-to-noise, characteristic of good data collected from this on-chip approach and comparable to other crystallographic studies of PYP. The crystals from the membrane protein, cyt bo₃, however, diffracted poorly, with sparse spots up to ~12 Å. While this microseeding method aided the growth of large cyt bo₃ crystals, the diffraction reveals that even with microseeds, the quality of the crystals was poor. This crystallization condition requires further
optimization – a search across the cyt bo₃ / PEG 1500 composition-composition phase diagram may lead to higher quality crystals.

2.4 Microseed-assisted re-screening of cytochrome bo₃ oxidase

Typically, microseed matrix screening is performed by: (1) pre-mixing microseeds into the protein solution of precipitant solution, which results in partial or complete dissolution of microseeds in undersaturated solutions (Figure 2.1), or (2) pre-mixing microseeds into the protein-precipitant mixture immediately after mixing, which faces similar potential problems of microseed dissolution, or (3) adding microseeds at some time after equilibration, which requires that the crystallographer opens the crystallization well leading to the unwanted effect of upsetting the equilibrium between the crystallization drop and the vapor phase around it. The automation and precision of microfluidic chips provide an alternative route to incorporate

Figure 2.3: (a) Schematic of the design of a 24-well three-component array chip used for microseeding, with fluid lines in black, and the various valve lines, V1, V2, and V3, colored based on their function (see legend). (b) Exploded view of a single well, showing device construction with a total material thickness of ~200 µm. (c) Top view of the aligned fluid layer and control layer, showing relative positions of protein, precipitant, and microseed compartments and the two separate mixing valves.
microseeds into a metastable mixture without significantly disturbing the crystallization droplet.

To evaluate the sensitivity of diffraction resolution to position on a composition-composition phase diagram for cyt bo₃, a 24-well microseed matrix screening array was employed using a new microfluidic chip with two separate free-interface diffusion mixing steps: (1) protein and precipitant mixing, waiting for equilibration, and then (2) protein, precipitant and microseed mixing (Figure 2.3). Compared to the earlier microfluidic approach where a single point on the composition-composition phase diagram was generated by pre-equilibrating protein and precipitant off-chip, this chip generates an array of protein-to precipitant ratios on-chip for optimizing a precipitant or a screening random matrix of precipitants with microseeds. The keys to this design are the arrangement of microfluidic wells to facilitate filling and mixing, and the separation of mixing valves to introduce the microseed at any time after protein-precipitant mixing (Figure 2.3c).

Three sets of normally closed microvalves control filling and mixing. First, protein, precipitant, and microseed are simultaneously introduced into separate microfluidic compartments by dead-end filling initiated by actuation of valve set 1 (Figure 2.4b-i). Next, protein and precipitant mix by free-interface diffusion by opening valve 3 for 30 minutes (Figure 2.4b-ii). Then the valve is closed and the protein and precipitant wells incubate for 1 hour to reach a metastable composition. Valve 2 is then opened for 5 minutes to introduce microseeds to the metastable protein-precipitant mixtures (Figure 2.4b-iii). This final mixing step for microseeds introduction is short to introduce just a few seeds to the metastable mixture and to prevent large composition shifts in the metastable droplets. The chips are then sealed and incubated at 4°C or 20°C, and monitored daily for crystallization.

The amount of time for crystallization and the final concentrations of protein and precipitant were determined through computational fluid dynamics simulations. Diffusion coefficient for cyt bo₃ in water was estimated to be similar to other large proteins (hemoglobin: 64.5 kDa, 6.9 x 10⁻
A reported value for the diffusion coefficient of the protein is $7\, \text{cm}^2/\text{s}$; catalase: $247\, \text{kDa}$, $4.1 \times 10^{-7}\, \text{cm}^2/\text{s}$).

Figure 2.4: Sequence for mixing and filling steps for microseeding in three-component array chip. (a) Schematic of array chip showing inset of a single crystallization well – three sets of mixing valves are indicated on the inset, (1), (2), and (3). (b) Schematics (1 – before filling or mixing; 2 – after filling or mixing) and optical micrographs (right) of step. Arrows indicate free interface diffusion interfaces (b-i) Protein, precipitant, and microseed are placed on their respective inlets, and actuation of valve line 1 initiates dead-end filling. (b-ii) Valve line 2 is actuated for 30 minutes to mix protein and precipitant. (b-iii) After 1 hour of incubation, metastable protein-precipitant mixtures have formed (light and dark purple). Valve line 2 is then actuated for 5 minutes to introduce microseeds to each metastable mixture.
precipitant, polyethylene glycol, in water was modeled \((3.2 \times 10^{-6} \text{ cm}^2/\text{s})\). The geometry of microfluidic wells at each protein-to-precipitant ratio were constructed and free-interface diffusion was simulated for 60 minutes at 1 minute intervals (Figure 2.5a). Diffusion of protein and precipitant were modeled, although precipitant rapidly mixes to completion on time-scales for protein mixing, so this discussion will focus on protein concentrations. Time-concentration plots were constructed by a surface integral of concentration in the protein well and precipitant well separately (Figure 2.5b). Diffusive mixing of protein nears completion after 60 minutes. Mixing for 30 minutes (as indicated, Figure 2.5b) results in an intermediate where two different metastable compositions are generated in each ‘half-well’ (Figure 2.5c). As a sample point, after 30 minutes with a 40 mg/mL protein solution, mixing results in 12 different protein

![Figure 2.5](image)

**Figure 2.5:** Computational fluid dynamics simulations using COMSOL to determine mixing times and final concentrations. (a) Snapshots of protein concentrations over a 60-minute free interface diffusion simulation. (b) Integral concentrations at each time point in the protein compartment (left) and the precipitant compartment (right) for each of 6 different compartment sizes. On-chip experiments ran for 30 minutes, indicated by the red dashed line. (c) Concentration surfaces of three intermediate-sized crystallization wells after 30 minutes.
concentrations ranging from 13 mg/mL to 32 mg/mL. At this timescale, the precipitant mixes to completion in all wells. This method was used to quantify concentrations for further discussions of composition-composition phase diagrams.

Cytochrome bo$_3$ oxidase microseeding experiments were set up as discussed with a range of stock protein concentrations between 10 mg/mL and 50 mg/mL. Stock precipitant solutions contained 9-12% w/v PEG 1500, while the other components (NaCl, MgCl$_2$, HEPES, ethanol) were held constant. The optimal microseed dilution from the previous study, 1:50, was also found to produce large, individual crystals in these chips and is used for all experiments discussed herein. Crystallization results were recorded after 7 days and pictures were taken of each crystallization well for image processing. X-ray diffraction experiments were conducted within 14 days of setting up crystallization trials. As a control experiment, a separate chip was set-up with 30 mg/mL cyt bo$_3$ and 9-12% w/v PEG 1500 and no microseeds. Crystals did not grow in any wells for this experiment, validating that either undersaturated or metastable compositions (no native nucleation) were formulated at these intermediate concentrations.

Diffraction data and crystal aspect ratios were mapped on protein composition / precipitant composition phase diagram to evaluate the influence of microseed screening on crystal habit and diffraction resolution (Figure 2.6). A solid line to indicate protein solubility was drawn on phase diagrams by inspection based on experiments that did not grow crystals with microseeds. The phase diagram with diffraction data shows that diffraction quality segregates into a few distinct regions – at the high and low ends of protein-to-precipitant ratio, crystals diffracted strongly up moderate resolutions (9 Å), while at intermediate protein-to-precipitant ratios, crystals diffracted to much poorer resolutions (>12 Å) (Figure 2.6a,c). This measure indicates that the best regions for crystallization lie close to either axis at high concentrations of either protein or precipitant. Further, a phase diagram that maps crystal aspect ratios (long axis to short axis) shows differences in crystal habit that depend on crystallization composition. At
low-to-moderate protein concentrations and high precipitant concentrations, crystals formed in shorter, rectangular or cubic shapes. At moderate-to-high protein concentrations and low-to-moderate precipitant concentrations, crystals formed in long, needle-like shapes (Figure 2.6b,d). Interestingly, the needle-like crystals typically diffracted poorly, while the
rectangular/cubic crystals diffracted better. This process shows that the quality and morphology of crystallization can be controlled by microseed screening over the crystallization phase diagram. The higher quality crystals, while not of sufficient for structure solution, were sufficient for data indexing due to strong, albeit low resolution diffraction. Further, the regions of the 'best' crystals indicate compositions that future experiments should sample. This set of experiments with cytochrome bo$_3$ oxidase reached the upper limits of protein concentration, so further crystallization experiments with this protein should search for new precipitants through random microseed matrix screening for the best chance of uncovering crystals that diffract to atomic resolution.

2.5 Conclusions

In summary, microseed-assisted crystallization of soluble and membrane proteins was demonstrated on two different microfluidic array chips. These approaches exploit the phenomena of nucleation and growth to prevent the growth of small, uncontrolled showers of crystals in favour of large crystals that can be used for X-ray diffraction experiments. First, 24-well array chips with wells comprised of two compartments, one for a metastable protein-precipitant mixture and the other for a microseed solution, were used to crystallize photoactive yellow protein (PYP) and cytochrome bo$_3$ oxidase. Optimal seed dilutions were determined to grow the best crystals for known crystallization conditions. Microseeded PYP crystals diffracted very strongly to 1.19 Å, however cytochrome bo$_3$ oxidase crystals diffracted poorly to ~12 Å despite their large size, indicating that despite microseeds, this composition of protein and precipitant does not support the growth of high-quality crystals.

To screen for better crystal growth with microseeds, a new 24-well microfluidic array chip was developed with three separate compartments for protein, precipitant, and microseed. In two separate mixing steps, the new array first mixes a gradient of metastable protein-precipitant solutions, and then introduces microseed through a separate mixing step. The results from
these experiments showed that diffraction quality and aspect ratio (shape) depend on the composition of protein and precipitant, where some composition ranges favor small aspect ratio crystals with decent diffraction, and other composition ranges favor large aspect ratio crystals with poor diffraction. While even the best crystals from this screen were not suitable for solving the structure of cytochrome bo$_3$ oxidase, this microfluidic method demonstrates that the oftentimes unpredictable crystallization behaviour of fragile membrane proteins can be controlled through a systematic search of a composition-composition phase diagram with microseeds.

Looking forward, this technique can be used widely as an effective, non-invasive method of incorporating microseeds into crystallization droplets. A membrane protein like cytochrome bo$_3$ oxidase could be re-screened with a new set of precipitants with routine addition of microseeds – such a method has been demonstrated to increase crystallization hit rates. Further, for processes that require tight control over crystal quality and morphology, these microfluidic chips are useful analytical tools for generating gradients of conditions to determine phase diagrams.
2.6 References


CHAPTER 3

X-ray Transparent Microfluidic Device for Mesophase-Based Crystallization*

3.1 Introduction

While the dual hydrophobic-hydrophilic characteristic of membrane proteins imparts them with unique characteristics and functions, it also hinders membrane protein expression, purification, and crystallization. To handle membrane proteins in solution, detergents are introduced and to stabilize the hydrophobic core of membrane proteins. Detergents have unfortunately have negative ramifications for crystallization experiments - in aqueous solutions, the presence of detergents has been shown to inhibit the formation of close crystal contacts, generally resulting in crystals with mediocre diffraction quality.1 While techniques like in surfo crystallization and more advanced microseeding methods have attempted to overcome the problem through exhaustive screening and manipulation of the crystallization phase diagram, many membrane proteins remain intractable to crystallization in aqueous solution.

Lipidic mesophases are bicontinuous lipid bilayers that spontaneously assemble when lipids of the monoacylglycerol family are mixed with aqueous solutions, forming either a lamellar, cubic, or hexagonal bilayer phase.2 In 1996, the first report on the use of lipidic cubic phases (LCP) for membrane protein crystallization was published.3 Over the next two decades, the LCP method, also known as the in meso method, emerged as a powerful alternative to membrane protein crystallization from detergent solutions.4 By first reincorporating membrane proteins into a cell membrane-mimetic bilayer, amphiphilic membrane proteins stabilize and concentrate in the membrane prior to the addition of precipitants to trigger crystallization. When the addition of

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salts and precipitants swell or contract the cubic phase, membrane protein molecules can nucleate and grow to form a high-quality crystals.\textsuperscript{5,6} The robustness and effectiveness of the LCP method has been highlighted through the structural determination of several previously intractable membrane proteins, most notably the $\beta_2$-adrenergic receptor-Gs protein complex.\textsuperscript{7}

Prior to an LCP crystallization experiment, mesophases are prepared by \textgreater 50 cycles of mixing in coupled-syringes. Subsequent manipulation of the mesophase is difficult due to the high viscosity and stickiness of lipidic mesophases, which requires specialized tools such as ratcheted syringe dispensers or dispensing robots.\textsuperscript{8,9} Even with these tools, fragile crystals must be manually harvested from mesophases prior to X-ray analysis, a challenging task even for many experienced crystallographers. Next, harvested crystals are flash-frozen in liquid nitrogen and maintained under cryogenic conditions to prevent radiation damage during data collection.\textsuperscript{9} The typically small size, 2-70 µm,\textsuperscript{10} of membrane protein crystals makes this procedure highly challenging. The damage caused to the delicate crystals during these steps may severely compromise the quality of resulting diffraction data.

Numerous examples of microfluidic technologies that automate fluid metering and drastically reduce sample consumption have been demonstrated for various applications, including protein crystallization from aqueous solutions.\textsuperscript{11–14} While a number of X-ray transparent microfluidic devices that eliminate manual crystal handling, none have been applied to X-ray analysis of crystals in LCP.\textsuperscript{15–23} Further, only 2 prior approaches have attempted to simplify and automate the formulation of and crystallization in LCPs,\textsuperscript{24,25} and neither overcomes the hurdle of crystal damage sustained through harvesting. Both platforms rely on complex operation strategies and have numerous limitations due to the difficulties of manipulating viscoelastic materials in microscale compartments.

Herein, we present the first microfluidic device that combines LCP crystallization capabilities with X-ray transparency in a simple design that requires only a small vacuum pump to introduce
reagents.\textsuperscript{26} To overcome the viscoelasticity of the lipidic mesophases, this approach mixes membrane protein and lipid \textit{passively} by free-interface diffusion,\textsuperscript{27,28} after which crystallization is triggered by the introduction of precipitants. As validation, we crystallized the photosynthetic reaction center from \textit{Rhodobacter sphaeroides} and solved its structure to a resolution 2.5 Å using data collected on-chip at room temperature. We also compared our room temperature structure (298 K) to cryogenic structures (100 K) and made observations about the effect of temperature on diffraction data quality and structural flexibility.

3.2 Materials and methods

\textbf{Protein preparation.} \textit{Rhodobacter sphaeroides} photosynthetic reaction center (RC) was expressed and purified as described previously.\textsuperscript{29} The RC solution at an initial concentration of 6 mg/mL in 10 mM Tris pH 7.8, 280 mM NaCl, 0.05% LDAO (N,N-dimethyldodecylamine N-oxide) was concentrated in a Microcon centrifugal filter device (Millipore Corp.) with a 10,000 Da cut-off by spinning in a microcentrifuge at 10,000 \textit{g} in a cold room maintained at 4°C. The volume of the concentrate was measured after every 5-min spin and the centrifugation was stopped when the final volume reached ¼ of the initial volume, yielding a solution with a calculated final concentration of 24 mg/mL. The filtrate was used to dilute this solution to obtain samples with RC concentration of 10–24 mg/mL. The solutions were either used immediately for crystallization trials or separated into 2–3 µL aliquots and kept in the freezer at -12°C before using.

\textbf{Precipitant preparation.} Precipitants for protein crystallization trials were formulated by first preparing an aqueous solution of 1 M HEPES (Sigma Aldrich) and 1.15 M (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} (Fisher) and adjusting the pH to 7.5. Jeffamine M-600 (Hampton Research) was then mixed with the aqueous solution to obtain mixtures containing 11, 12, 13, and 14% w/v of Jeffamine.

\textbf{Lipids.} Monoolein (Sigma Aldrich, 99%) was used as received. 5% v/w and 10% v/w solutions of β-octylglucoside (OG, Anatrace, Anagrade) were prepared by dilution from a 20% v/w OG
solution in 25 mM NaH_2PO_4, pH 5.5. NaH_2PO_4 was obtained from EMD Chemicals.

**Equipment.** Filling and mixing of microfluidic devices were monitored using an upright microscope (Leica MDG33) equipped with a macro lens and a digital camera (Leica DFC295). Valves were actuated using a vacuum pump (GAST, Model DOA-P704-AA) or house vacuum.

**Fabrication of photoresist-on-silicon masters for replica molding.** Photoresist-on-silicon masters were created with SU8-2050 photoresist (Microchem) for patterns with 40-65 µm-tall vertical features and with SU8-25 photoresist (Microchem) for patterns with 25 µm-tall vertical features. All photoresist-on-silicon masters were treated with (tridecafluoro-1,1,2,2-tetrahydrooctyl) trichlorosilane (Gelest, Inc.) in a vacuum chamber for 4 h for easy release of soft lithographic replicas.

**Fabrication of patterned cyclic olefin co-polymer (COC) substrates.** Patterned COC

![Figure 3.1: Photomasks for producing silicon-on-photoresist masters with SU-8 negative photoresist for 12-well mesophase-based microfluidic devices. (A) Fluid layer, for routing protein and precipitant solutions, (B) the control layer, for vacuum actuation, (C) the substrate, for the lipid chambers, and (D) an auxiliary chip for filling lipid into the substrate.](image)
substrates were fabricated by hot embossing using the procedure developed by Guha et al. Briefly, polydimethylsiloxane (RTV-615 PDMS, Momentive Performance Materials) with 10:1 monomer:crosslinker ratio was used to fabricate a thick (several mm) inverse master of the photoresist-on-silicon master. PDMS was held at 75 °C for 2 h for curing. An epoxy master (Conapoxy FR 1080) for use in hot-embossing was then molded from the PDMS master. The epoxy components (83:100 hardener:epoxy, by mass) were mixed using a planetary centrifugal mixer (Thinky USA) for 15 min at 2000 rpm with rotation and then de-foamed for 12 min at 2200 rpm. The epoxy mixture was poured into the inverse PDMS master and cured on a level hot plate at 120 °C for 4 h. To prevent evolution of gas bubbles from the PDMS master during epoxy curing, the mold was degassed either for ~10 min under vacuum at room temperature or heated on the hot-plate at 120°C for 20 min prior to filling it with epoxy. The PDMS master could be re-used several times for epoxy molding. Hot embossing was done in a laminating press (Carver, Model 3851) under the load of 150–200 kg. For high-fidelity pattern transfer, a stack of (i) 7–10 mm PDMS slab, (ii) an epoxy mold, (iii) a COC sheet, and (iv) a 7.5 × 5 cm microscope glass slide was placed between the platens of the press. The temperature of the assembly was brought to 177°C and then down to below 121°C before removing the patterned layer. Holding at 177°C for an extended period was not required for accurate pattern transfer. Non-patterned COC sheets were flattened between glass slides at 177°C and a load of 150–200 kg in the laminating press prior to use to obtain sheets with smooth surface.

**Fabrication of thin PDMS/COC devices.** Hybrid microfluidic devices consisted of a flat COC top layer, a thin PDMS control layer, a thin PDMS fluid layer, and a patterned COC bottom layer (Figure 3.1A, B, C). The fabrication method for these thin PDMS/COC microfluidic devices is discussed in detail in Chapter 2.2. For details in the usage of this device, see Section 3.3.1.

**Fabrication of auxiliary PDMS devices for lipid filling.** Thick PDMS devices were fabricated for lipid filling into the patterned COC substrate. The devices consisted of a several mm-thick
PDMS layer with patterned control and fluid channels (Figure 3.1D) and of a 20 µm-thick unpatterned PDMS film. The thick PDMS layers were prepared by pouring a mixture with the monomer:cross-linker ratio of 5:1 on the photoresist-on-silicon master followed by curing at 90°C for 5-10 min. The thin unpatterned layers were spin-coated onto an unpatterned silane-treated silicon wafer using PDMS with the monomer:cross-linker ratio of 15:1 and cured at 90°C for 7-9 m.

The devices were assembled as follows: (i) inlet ports were punched in the thick layers using a 20 AWG needle with a thin wire plunger, and (ii) the thick layers were irreversibly bonded to the thin layers by placing the two layers in conformal contact and heating at 70 °C for 2 hours following the standard soft lithography approach.\textsuperscript{32}

For filling the COC substrates with lipid the parts of thin membranes to be located over the lipid chambers were pulled out carefully with sharp tweezers. The part of the membrane enclosing the channels remained intact in this step because of the different lateral dimensions of the channels and the circular endpoints. The assembly was then placed a patterned COC substrate, and a reversible bond between the PDMS fluid layer and the COC substrate formed spontaneously. \textit{For details in the usage of this device, see Section 3.3.2.}

**Crystallization of photosynthetic reaction center in well plates.** Protein crystallization in 96-well flat bottom microplates (Corning Crystal\textit{EX} 3785) replicated the protocol and crystallization conditions reported by Wallace \textit{et al.}\textsuperscript{27} although dry monoolein was used instead of preparing the lipid mesophase with water. Monoolein was dispensed into the well plate (0.2 µL/well) using a ratchet dispenser (Hampton Research), covered with Crystal Clear Sealing Film (Hampton Research) and stored at -12°C for up to 3 weeks before crystallization experiments. Prior to use, the plates were brought to room temperature before removing the tape.

For crystallization, 0.4 µL of the RC protein solution was added on top of dry monoolein in the wells, sealed, and incubated for 4–12 h at 20°C. Afterward, 2 µL of the precipitant solution
was added to the crystallization well and 5-10 µL of the same solution was added to the reservoir well. The plates were sealed and incubated at 20°C. Protein crystals of 5-60 µm in size formed in 24-48 h.

**On-chip crystallization of photosynthetic reaction center.** Protein crystallization on-chip was carried out with the same materials and incubation times as used in the crystallization in well plates. The volumes of protein and precipitant used for crystallization on-chip were determined by the volume of the chambers of the chip (~60 nL for protein, 244 nL for precipitant). After the chips were filled, the inlets were carefully sealed with Crystal Clear tape to minimize dehydration.

Protein crystals of 5 – 80 µm in size appeared within 48 h and continued growing through 96 h. Crystal X-ray diffraction data were collected within 10 d.

**Synchrotron X-ray diffraction data collection.** Following our previously developed on-chip data collection strategy, small wedges of data from multiple crystals were collected and merged into a single data set for building electron density maps. The ease of growing and analyzing multiple (tens to hundreds) isomorphous crystals in a single chip enables data collection under ambient conditions with minimal radiation damage. In contrast, traditional crystallographic protocols rely on the harvesting and mounting of a single crystal at a time, followed by the analysis of that crystal under cryogenic conditions to minimize radiation damage. The challenges associated with this one-at-a-time manual protocol render analysis of multiple LCP-grown crystals at ambient conditions impractical.

Protein crystal X-ray diffraction data were collected at macromolecular crystallography beamline 21-ID-F of the Life Sciences Collaborative Access Team (LS-CAT), Advanced Photon Source, Argonne National Lab. The beamline is equipped with a microdiffractometer (MAATEL MD2) consisting of a goniometer, XYZ micropositioner and an on-axis video microscope. The beamline operates at a fixed wavelength (\(\lambda = 0.979 \text{ Å}, 12.7 \text{ keV}\)) and has a MarMosaic 225
detector (Rayonix). A beam-defining aperture of 50 µm in diameter was used to control the footprint of the beam.

Data were collected using an unattenuated X-ray beam with a 2–4 s exposure and 1° rotation per image with a sample-to-detector distance of 200 mm. Crystal quality typically declined after 5 exposures. Optimal exposure settings were determined by testing exposures on additional crystals grown on-chip. Data were collected from 56 crystals grown in 12 different wells in 3 different chips for these experiments, and 23 crystals were selected to form an optimal dataset for the final structure. Crystals had moderate variability in size (60–100 µm in the longest dimension) and produced diffraction data with comparable quality, resolution and lattice parameters.

A second dataset was collected off-chip from protein crystals grown in well plates, where crystals were harvested using microloops (Hampton Research), flash-frozen in liquid nitrogen without the addition of cryoprotectant, and kept under a cryostream during data collection.

**Analysis of X-ray diffraction data.** Analysis of X-ray diffraction data collected at the synchrotron was performed using HKL2000 software for indexing, refinement, integration, and scaling (HKL Research Inc.). The resolution range of the data was established based on the point at which the highest resolution shell’s I/σ fell below 3 provided that Rsym was also less than 0.7. Subsequent processing of crystallography datasets was done using the Phenix suite of programs. Molecular replacement was done in Phaser using PDB structure 2UWW as a model. Model refinement was performed using phenix.refine. Electron density maps were displayed using Coot and PyMOL. Ligands bound to the structure were identified in the electron density and built into the final model. The loosely bound ubiquinone (Qb) was not fully resolved in the structure, but it is modeled into its approximate position.

**3.3 Microfluidic device for crystallization in lipidic mesophases**

In the 12-well chip presented here (Figure 3.2A), each well relies on diffusion to mix 60 nL
of protein solution and 10.5 nL dry lipid for mesophase formulation, and 244 nL of precipitant solution to induce crystallization. *The protein solution is layered on top of the lipid, significantly reducing the diffusional path and, consequently, the mixing time compared to the traditional side-by-side placement of microfluidic compartments.*\(^{33,41–43}\) The chip screens two crystallization conditions in parallel and can be easily modified for more extensive screening.

For X-ray transparency, the chip is assembled (as discussed in Section 3.2.2) from four polymeric layers with a combined thickness of only ~200 µm. Fluid flow and precise dispensing in the chip relies on channels, compartments and normally-closed valves patterned in two layers of elastomeric polydimethylsiloxane (PDMS) (**Figure 3.2B, C**). Top and bottom cyclic olefin co-polymer (COC) layers (**Figure 3.2C**) impart rigidity as well as a barrier against water evaporation. Fluid flow and compartment filling in the PDMS fluid layer is achieved by applying negative pressure (vacuum) to microfluidic control lines in the PDMS control layer (**Figure 3.2B, C**).

After fabrication, the chip is comprised of two separable pieces: (1) a three-layer assembly

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**Figure 3.2:** (A) A photograph of the 2 x 6-well array chip. Lilac dotted lines (1-5) indicate the different control lines connected to control valves. (B) Magnified top view schematic of a single crystallization well comprised of three patterned layers and a top COC layer: first layer (PDMS, lilac) contains control lines and valves, second (PDMS, black) and third (COC, red outlines) layers contain sample compartments. (C) Cross section of a crystallization well showing the layered assembly of the chip.
(TLA, with a COC impermeable cover layer, the PDMS control layer, and PDMS fluid layer), and (2) the COC bottom substrate containing solid lipid. For crystallization experiments, the TLA is reversibly sealed to the COC substrate, exploiting the adhesive properties of PDMS. All COC films used in this work had a thickness of 50 µm (2 mil) and were of grade 6013. The chamber height in PDMS fluid layers was 40–65 µm, and the chamber height in PDMS control layers and embossed COC sheets was 25 µm.

Figure 3.3: Device architecture and the sequence of operations in the lipid filling step. (A) Superimposed patterns in the hot-embossed COC substrate (red) and in the auxiliary thick PDMS layer (green) of the 2 x 6-well microfluidic platform for LCP crystallization. (B) A magnified top view of a single well and (C) the cross-section of the PDMS assembly aligned with the patterned COC layer. Features in the top PDMS layer correspond to lines 1 and 2 as indicated in (A-C). (D) The sequence of steps in the lipid-filling protocol. (D1) The part of the thin PDMS membrane corresponding to the circular end of line 1 in (A) is removed with sharp tweezers. (D2) The PDMS assembly is placed on the COC substrate. Application of negative pressure (vacuum) to line 2 results in air withdrawal from line 1 due to air permeability of PDMS and draws molten lipid into the chamber through line 1. (D3) The lipid fills the entire chamber in 5-10 min. After filling, the lipid is frozen and remains solid at room temperature. (D4) The auxiliary PDMS layer is removed, the crystallization layer is aligned over the filled substrate and the chip is ready for crystallization.
Deposition of lipid into COC substrates. Prior to combining the TLA and COC substrate, an auxiliary PDMS device was used to deposit monoolein into the respective 25 µm-deep chambers of the COC substrate (Figure 3.3). Monoolein was melted (T_melt = 37 °C), filled into each 25 µm-deep chamber, and then frozen by applying dry ice for 10 minutes. The auxiliary device was then removed. Handling lipid in the molten state enabled accurate metering of nanoliter (microgram) quantities. Because monoolein is solid at room temperature, the lipid-filled substrates could be easily manipulated in subsequent steps once monoolein was solidified.

The auxiliary PDMS device contained two sets of channels patterned in the thick PDMS layers and sealed by bonding a thin PDMS layer to the thick layer (Figure 3.3C). One set of channels was used as a fluidic line and consisted of branched channels that terminated above the patterned chambers in the COC substrate (Figure 3.3D). The thin PDMS membranes were carefully removed at the ends of the channels to form a continuous fluidic path between the inlet port and the chambers patterned in COC (Figure 3.3D). The other set of channels, used as a control line, passed over the patterned chambers and was connected to the vacuum source.

To fill monoolein, negative pressure (vacuum) was applied to the control line. Although the line was separated from the chambers with the thin PDMS membrane, pressure gradient between the chambers and the inlet port of the fluidic line formed due to the air permeability of PDMS, and monoolein was drawn into the chambers through the fluidic line (Figure 3.3-D2), eventually filling all chambers in the substrate (Figure 3.3-D3). Device architecture ensured that monoolein only remained in the COC chambers once the PDMS layer was removed (Figure 3.3-D4) as the fluidic lines themselves were sealed from the contact with the substrate.

Formulation of mesophase-based crystallization trials on-chip. After the lipid is filled into the COC substrate, the TLA is aligned over the bottom COC substrate (Figure 3.4A). The on-chip crystallization protocol presented here mimics a LCP crystallization variant that was performed in well plates.27 Through a single inlet (Figure 3.2A), the protein solution is
introduced into the 12 crystallization wells and is brought into direct contact with the lipid (Figure 3.4B, G). In the crystallization compartment, only the area above the lipid, the mesophase chamber (Figure 3.2B), is filled. Filling of the rest of the crystallization well with protein solution is prevented by a capillary valve geometry44 (obtained by appropriate choice of channel dimensions and wall angles) located between the mesophase and the precipitant chambers (Figure 3.2B). Incubation of the protein solution with the lipid results in spontaneous formation of the protein-enriched mesophase27,28 (Figure 3.4). After a 4–12 h incubation, precipitant solution is introduced. The mesophase stays in place due to its high viscosity45 (Figure 3.4D). Under favorable conditions, incubation of the precipitant with the protein-

**Figure 3.4:** (A-E) Sequence of steps in the LCP protein crystallization protocol on-chip; (F-H) corresponding optical micrographs of the mesophase chamber. (A,F) The hybrid COC/PDMS/PDMS assembly is placed on the COC substrate pre-filled with lipid. COC, yellow; PDMS, blue; lipid, gray. (B,G) Protein solution is combined with the lipid through the corresponding fluid line by applying negative pressure (vacuum) to control lines 1 and 2. (C) Protein-enriched mesophase forms spontaneously upon incubation and (D) precipitant is introduced by applying negative pressure to control lines 3, 4, and 5. Line 2 in (B) and lines 3 and 5 in (D) serve to increase the rate of air withdrawal from respective sample chambers. (E,H) Protein crystals form in the mesophase after incubation. (I) Representative example of RC crystals grown on-chip.
enriched mesophase results in crystal formation in the mesophase (Figure 3.4E, l).

Like the strategy presented for lipid filling, this protocol also fills solutions into their respective chambers through actuation of negative pressure (vacuum) to control lines (Figure 3.4). The formation of a pressure gradient in the fluid layer due to the air permeability of PDMS displaces air pockets and enables complete filling of each device compartment.

3.4 On-chip crystallization and X-ray analysis of *R. sphaeroides* reaction center

*Note: Although RC crystallization conditions likely produce a “sponge phase” rather than a true LCP, the term “LCP crystallization of proteins” is commonly used regardless of the exact mesophase type formed under specific crystallization conditions, and is utilized throughout this discussion of RC crystallization.*

Figure 3.5: (A) Optical micrograph of an X-ray transparent chip for LCP crystallization mounted on beamline 21-ID-F-at LS-CAT, ANL. (B) Section of a crystallization well with crystals as seen in the on-axis video microscope during X-ray data collection. The red circle represents the location and the footprint of the 50 μm X-ray beam. (C) Example of X-ray diffraction data from an RC crystal on-chip at RT. (D) RMSD visualization along the periplasmic side of our RT structure and a cryogenic structure (PDB ID: 2GNU). Residues in grey were not included in calculation. Image was generated using the ColorByRMSD script in PyMOL.

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Room temperature X-ray diffraction data collection for reaction center. We validated our approach by crystallizing photosynthetic reaction center (RC), a membrane protein from *Rhodobacter sphaeroides*, using previously reported crystallization conditions to obtain crystals of up to 80 µm in size (Figure 3.4I). We collected X-ray diffraction data at room temperature (RT) from crystals in the chips (“on-chip”) and solved the crystal structure of RC to a resolution of 2.5 Å (Table 1). The chips were mounted directly, without modification, on the goniometer at beamline 21-ID-F at the Advanced Photon Source (APS), Argonne National Lab (ANL) (Figure 3.5A). Diffraction data from the crystals were easily resolved (Figure 3.5C) despite background scattering from chip materials (Figure 3.6). The excellent optical properties of the chips facilitated crystal targeting during data collection (Figure 3.5A, B). In contrast, crystals grown in parallel using the classical LCP method in microplates were difficult to locate in standard loop mounts because of the opacity of the mesophase (Figure 3.7).10,47

![Figure 3.6](image-url)

**Figure 3.6:** A. A scattering pattern obtained with an empty chip shows two diffuse scattering rings. B. Scattering profile for 50- and 100-µm-thick COC films and for a hybrid PDMS/COC microfluidic chip (cumulative PDMS thickness 20 µm, cumulative COC thickness 75 µm); q is the scattering vector related to d-spacing as \( q = \frac{2\pi}{d} \). COC films show background scattering centered at the value of q of 1.2 Å\(^{-1}\) (d-spacing of 5.2 Å). Standalone thin PDMS films were not probed due to their lack of rigidity. However, comparison of background scattering from the COC films with that from the chip indicates that the scattering from PDMS is centered at the value of q of 0.835 Å\(^{-1}\), corresponding to d-spacing of 7.5 Å.
Comparison of on-chip room temperature and cryo-RC structures. The structure determined from on-chip data (Table 3.1) agreed well with previously published structures of LCP-crystallized RC obtained using the traditional crystallization and data collection approach (PDB ID: 2GNU, PDB ID: 1OGV). The merged dataset for our structure was complete (Table 3.1), indicating that on-chip crystals were oriented randomly. Our RC structure was isomorphous with the structures reported previously and had comparable structural statistics, refinement parameters, and final structural resolution. Values for $R_{sym}$ and $I/\sigma$ for the on-chip structure were typical of good diffraction data.

We also observed several important differences between cryogenic structures of RC and our RT structure. First, the lattice parameters of our RC structure were up to 1.8% larger than those reported previously, indicating unit cell contraction upon flash cooling. Second, the mosaicity (long-range order) of crystals analyzed on-chip was nearly an order of magnitude lower (better order) compared to other high-resolution RC structures crystallized in LCP. Higher mosaicity (poorer order) is typically related to contraction of the unit cell caused by flash-cooling to cryogenic temperatures. Availability of low-mosaicity crystals and non-cryogenic...
Root mean square deviation (RMSD) comparisons revealed noticeable non-uniformly distributed deviations in the positions of backbone alpha-carbons in our RC structure compared to both available cryo-structures\textsuperscript{48,49} (Figure 3.5D, Table 2). The greatest deviations were located along the hydrophilic chains at the periplasmic and cytosolic sides of the protein (RMSD = 0.45 Å, Table 3.2) and were as large as 2 Å at residues 268-271 of the L-subunit (Figures 3.5D, 3.8). The hydrophobic chains embedded within the lipid bilayer showed a significantly smaller deviation (RMSD = 0.28 Å, Table 2). Conversely, the RMSD values for superimposed cryogenic RC structures did not exceed 0.23 Å anywhere (Table 3.2). The overall RMSD of 0.36-0.37 Å between the RT and the cryogenic structures was in the range reported for independent structure determinations of an identical protein\textsuperscript{53} (Table 3.1).
For soluble proteins, cryo-cooling has been shown to affect mechanistically relevant side-chain conformations and, in extreme cases, backbone conformations. Similar analyses for LCP-crystallized membrane proteins are largely unavailable because of the difficulties of

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<th>Table 3.1: Crystallographic data(^a) and refinement statistics</th>
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<tr>
<td><strong>4TTQ (On-chip)</strong></td>
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<td>Unit cell dimensions</td>
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<td>Resolution (Å)</td>
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<td>No. unique reflections</td>
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<td>Completeness(^b)</td>
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<td>(R_{\text{sym}})(^b)</td>
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<td><strong>Refinement</strong></td>
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<td><strong>Overall</strong></td>
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\(^a\) Merging of small data sets from multiple crystals analyzed on-chip at room temperature

\(^b\) Values in parentheses indicate values for the highest resolution shells

| Table 3.2: Root-mean square deviations (RMSD) for structure comparison |
|------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                        | **RMSD**        | **RMSD**        | **RMSD**        | **RMSD**        | **RMSD**        | **RMSD**        | **RMSD**        | **RMSD**        |
| **Structures**          | **Target 1**    | **Target 2**    | **All (833 Ca)**| **H-Chain (281 Ca)**| **M-Chain (302 Ca)**| **Hydrophobic (438 Ca)**| **Hydrophilic (395 Ca)**| **L 264-275 (12 Ca)**|
| 4TQQ\(^b\)             | 0.36            | 0.39            | 0.32            | 0.29            | 0.27            | 0.44            | 0.62            |
| 1OGV\(^c\)             | 0.38            | 0.39            | 0.35            | 0.29            | 0.28            | 0.45            | 0.69            |
| 4TQQ\(^b\)             | 0.19            | 0.22            | 0.18            | 0.18            | 0.17            | 0.23            | 0.24            |

\(^a\)Measured in angstrom (Å). RMSD calculated between alpha-carbons of target 1 and target 2 in PyMOL using the super function.

\(^b\)Protein Data Bank identification tag for on-chip (this work) structure

\(^c\)Protein Data Bank identification tags for cryogenic RC structures
obtaining RT crystal structures that require screening of a larger number of crystals, as highlighted in the impressive recent study of a human membrane protein under non-cryogenic conditions.\textsuperscript{54,55} While the transmembrane chains of the proteins are likely to be constrained by the membrane-like LCP environment, hydrophilic segments may undergo significant conformational changes upon flash-cooling, as also observed by Liu \textit{et al.}\textsuperscript{54} These changes may be of importance for mechanistic studies and for protein docking, and the analysis of LCP-grown crystals under near-physiological temperatures, as enabled by our on-chip approach, may provide new insights into these phenomena.

### 3.5 Conclusions

\textit{In summary, we demonstrated the first X-ray transparent microfluidic chip for LCP crystallization of membrane proteins and subsequent on-chip X-ray diffraction data collection of multiple crystals on a single chip at room temperature for protein structure determination.} We validated our approach by crystallizing a membrane protein, photosynthetic reaction center, and solving its structure to a resolution of 2.5 Å. The chip automates metering and sample formulation, eliminates manual mesophase handling, and reduces the amount of sample per trial \textasciitilde7-fold compared to similar macroscale protocols,\textsuperscript{27,28} and \textasciitilde3-fold compared to standard protocols with pre-mixed mesophase.\textsuperscript{8,56} \textit{In situ} X-ray data collection on multiple crystals obviates cumbersome manual harvesting of fragile protein crystals. These features make our chips a valuable tool for the analysis of membrane proteins by providing a facile route to crystal structures and potentially to time-resolved studies of LCP-embedded proteins. For example, our on-chip analysis of RC revealed conformational flexibility in its hydrophilic chains at RT.
3.6 References


CHAPTER 4

High-throughput *In Meso* Crystallization Screening and Optimization of Membrane Proteins

### 4.1 Introduction

To trigger crystallization in lipidic cubic phases (LCP, or *in meso* crystallization), a multicomponent precipitant mixture of salts and other additives must be introduced into a protein-laden mesophase.\(^1\) Unfortunately, protein crystallization conditions cannot be determined *a priori*, thus requiring extensive screening of hundreds to thousands of potential precipitant mixtures to find one that yields diffracting crystals.\(^2,3\) Further, to obtain crystals that diffract to atomic resolution, crystallization conditions must also be optimized through fine-gradient re-screening of crystallization conditions\(^4,5\) and precipitants\(^6\). Unfortunately, the *in meso* crystallization method is tedious: the preparation of high viscosity protein-laden lipidic mesophases requires specialized tools and manual expertise for precise handling and dispensing, which complicates the set-up and repeatability of high-throughput screens and optimization at nanoliter volumes. Further difficulties arise after crystallization when crystals are usually physically transferred by the operator to a synchrotron-compatible holder and then flash frozen for X-ray analysis, a process which may damage the crystal and decrease the quality of X-ray diffraction data.\(^7,8\)

For traditional *in meso* crystallization experiments, several microliters of protein and lipid are mixed in coupled-syringes to form a highly viscous protein-laden mesophase. A protein-to-lipid ratio of 2:3 is typically selected from the temperature-composition diagram of lipid in water to form a stable cubic mesophase at room temperature for crystallization. In multicomponent

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crystallization mixtures of lipid, protein, salts, detergents, and other additives, the mesophase behavior can change and ratios other than 2:3 may be ideal for stable cubic phases and crystallization, and in extreme cases unexpected phases will form, such as sponge phases.9–11 In a few reports, other mixing ratios, formulations, and variant in meso methods have demonstrated successful crystallization: a reaction center was crystallized by overlaying protein over lipid for passive mixing by diffusion at a 2:1 protein-to-lipid ratio;12 a separate effort demonstrated passive mixing followed by controlled dehydration of the mesophase for crystallization.13 These methods showed that (1) crystallization-compatible mesophases can be formed reliably by passive mixing rather than coupled-syringe mixing, and (2) crystallization success can be affected by mesophase formulation and mesophase/protein mixing ratio, probably by influencing the amounts of protein and detergent incorporated in the same volume of mesophase.

State-of-the-art methods for high-throughput screening and optimization of in meso crystallization often rely on robots to dispense mesophase boluses.14,15 The most common type of well-plates for crystallization with robots are hermetically-sealed glass-sandwich crystallization plates. Glass-sandwich plates are incompatible with in situ X-ray analysis, and the high viscosity of the mesophase plus the need to cut through the glass-sandwich plate to gain access to each crystallization well requires manual expertise to harvest the crystals, and is time-consuming.14,16 Recent reports demonstrate in meso in situ X-ray crystallography by replacing glass plates with thin layers of either cyclic olefin copolymer (COC)17 or Mylar18, both materials with low background scattering and high transmission for X-rays. While a significant step forward for high-throughput in meso screening, the use of these X-ray transparent plates still relies in many cases on dispensing robots which perform crystallization in mesophases as small as 30–40 nl.
As an alternative approach, several microfluidic devices have successfully addressed formulation of in meso crystallization trials. The first microfluidic devices for in meso crystallization formulated mesophases by passive mixing in droplets\textsuperscript{19} or chaotic mixing in microchambers.\textsuperscript{20} Both microfluidic approaches successfully yielded diffraction quality crystals, but the chips were an additional barrier to solving crystal structures as neither approach facilitated the process of crystal harvesting and X-ray analysis. To overcome this hurdle, a set of design principles were developed to build thin X-ray transparent microfluidic devices for crystal growth and in situ X-ray analysis.\textsuperscript{21,22} For X-ray diffraction experiments, the entire chip was mounted in front of an X-ray source, and the resulting crystal structures had very good resolution and mosaicity, two parameters that often suffer as a result of crystal harvesting. An X-ray transparent device for in meso crystallization for passive mixing of protein and lipid was developed and validated by crystallizing and solving a high-resolution structure of the membrane protein, photosynthetic reaction center (\textbf{Section 3.4}).\textsuperscript{23} Formulation was automated by loading protein directly on-top of lipid to create a large, open-interface for mixing by diffusion; the subsequent addition of a precipitant into a connected adjacent well triggered crystallization. The application of these in meso devices as all-in-one mesophase formulation, crystallization, and X-ray diffraction tools for routine experiments, such as screening and optimization, is limited by scale (50–70 nL mesophases in each well, 12 well arrays).

Building on these prior efforts, this project overcomes the scaling limitations of X-ray transparent in meso crystallization devices and demonstrates their applicability in routine crystallization experiments, specifically high-throughput condition screening and optimization. First, the limitations of scale-down were addressed to efficiently utilize precious membrane protein solution. To achieve this, a capillary-valve strategy was developed to deliver as little as 8 nL of membrane protein (to form 13 nL of protein-laden mesophases) into open-interface crystallization wells. Second, the limitations of scale-out were addressed by designing densely-
packed microfluidic arrays to screen up to 192 potential crystallization conditions and combinatorial microfluidic arrays to generate 16 fine-gradient condition variants to optimize crystallization conditions. To validate the effectiveness of these microfluidic devices for routine crystallization experiments, they were used: (1) as high-throughput screening tools to identify new crystallization hits of three membrane proteins with limited or no structural data: quinol-dependent nitric oxide reductase (qNOR), cytochrome bo₃ oxidase, and the LM-dimer of photosynthetic reaction center, and (2) as crystallization optimization tools to increase the size and diffraction quality of crystals of a reaction center mutant, L223SW, from which the structure was solved on-chip to a resolution of 3.5 Å.

4.2 Materials and methods

Chemicals. For the experiments described here, we used screening kits (Cubic Screen 96-well screening kit from Emerald Biosystems, now distributed as Wizard Cubic Screen from Molecular Dimensions, Altamonte Springs, FL), monoolein (1-Oleoyl-rac-glycerol) and cholesterol (Sigma–Aldrich, St. Louis, MO), polydimethylsiloxane (PDMS) (RTV-615 from Momentive Performance Adhesives, Waterford, NY), negative photoresists (SU-8 25 and SU-8 2050 from MicroChem Corporation, Newton, MA), cyclic olefin copolymer films (COC) (Grade 6013, 2 mil from TOPAS Advanced Polymers, Florence, KY), silanizing agent (tridecafluoro-1,1,2,2-tetrahydrooctyl)trichlorosilane from Gelest, Inc., Morrisville, PA), and epoxy (Conapoxy FR 1080 from Cytec Industries, Woodland Park, NJ). All other salts, precipitants and solvents were purchased from Sigma–Aldrich and Hampton Research and used without further purification.

Preparation of protein samples. Photosynthetic reaction center (L223SW) and LM dimer were produced through site-directed mutagenesis in the pUCLHRC plasmid as described previously.²⁴ pUCLHRC is based on the commonly used cloning vector pUC19 and has the photosynthetic puh and puf operons integrated as a 5kb BamHI/EcoRI fragment. After verifying the mutation with DNA sequencing, the mutated fragment was cleaved and incorporated to
vector pATP19P\textsuperscript{25}, which, unlike pUC19 based plasmids, can be maintained in *Rhodobacter sphaeroides*. This mutated plasmid was then transferred to the *R. sphaeroides* strain ΔLHRC (knockout of reaction center and both light harvesting complexes) via conjugation. Transformed *R. sphaeroides* was cultured in Sistrom medium supplemented with 0.5% yeast extract. Expression of the photosynthetic reaction center was induced automatically at high cell density under low oxygen tension. The purification of mutant photosynthetic reaction center follows the same protocol for the wild type\textsuperscript{26}. Starting with the purified bacterial photosynthetic reaction center which contains three subunits, the LM dimer core complex was prepared by precipitating the H subunit in the presence of lithium perchlorate and ethanol.\textsuperscript{27} Extensive dialysis against 10 mM Tris, 0.03% LDAO (N,N-dimethyldodecylamine N-oxide) pH 8 was then carried out to remove these chaotropic agents.

Cytochrome bo\textsubscript{3} ubiquinol:oxygen oxidoreductase (cytochrome bo\textsubscript{3} oxidase) was overexpressed by IPTG (isopropyl β-D-1-thiogalactopyranoside) induction in *E. coli* strain C43(DE3) transformed with plasmid pETcyo. Bacteria were cultured in M63 minimal medium at 37 °C as previously reported.\textsuperscript{28} When the cell density reached an OD\textsubscript{600} of ~0.6, 0.5 mM IPTG was added to the culture. After 4–6 h of induction to allow protein expression, cells were harvested by centrifugation at 5,000 g for 20 min at 4°C. Cells were then disrupted with a French press. After 10 min of low speed spin at 5,000 g to remove cell debris, the cytoplasmic membrane was pelleted at 180,000 g for 4 h at 4°C. The membrane was then resuspended in 50 mM potassium phosphate pH 8 and solubilized with 1% DDM (n-dodecyl β-D-maltoside), followed by 1 h centrifugation at 180,000 g at 4°C to remove insoluble membrane fractions. The supernatant containing bo\textsubscript{3} oxidase in DDM micelles was loaded onto a Ni-NTA affinity column. After washing with 5 column volumes of 10 mM imidazole, cytochrome bo\textsubscript{3} oxidase was eluted with 50 mM imidazole. Extensive dialysis in either 50 mM potassium phosphate, 0.05% DDM or 20 mM Tris-HCl, 0.7% n-octyl-β-D-glucoside (OG) was performed to remove imidazole.
Quinol nitric oxide reductase (qNOR) was prepared using the 2,200 base pair qNOR gene from *Persephonella marina* which was amplified from genomic DNA using PCR, cloned into the plasmid pET-22b (Novagen), and overexpressed by IPTG induction in *E. coli* strain C43(DE3). Bacteria were cultured in LB media at 37°C while shaking at 200 rpm. Once the cultures reached an OD$_{600}$ of 0.7, 1 mM IPTG was added to the culture. Cells were harvested after 4 h of induction using centrifugation at 5,000 g for 10 min at 4°C, and broken by passing the cell suspension through a Microfluidizer three to four times at a pressure of 80,000 psi. The membrane fraction was collected by centrifugation of the disrupted cell membranes at 180,000 g for 4 h at 4°C. Isolated membranes were resuspended in buffer containing 100 mM Tris-HCl pH 8, 100 mM NaCl and solubilized with 1% DDM. Insolubilized membrane was pelleted with a 30 min centrifugation at 180,000 g. The supernatant containing qNOR was applied to a Ni-NTA affinity column. The resin was washed in the buffer containing 50 mM imidazole and 0.05% DDM, then the protein was eluted in the buffer containing 100 mM imidazole and 0.05% DDM. The protein was concentrated using 100 kDa molecular weight cutoff centrifugal filter units (Millipore) and dialyzed against 100 mM Tris-HCl pH 8, 100 mM NaCl, 0.05% DDM, and 10% glycerol.

For all proteins, small aliquots (<10 µL) of the protein sample were flash-frozen in liquid nitrogen and stored at –80°C until use.

**Flash-induced optical spectroscopy for reaction centers.** Flash-induced optical spectroscopy was carried out with an in-house designed apparatus. The saturating excitation light pulse, which initiates electron transfer within the photosynthetic reaction center, comes from a Xenon flash lamp controlled by a LabJack U3-LV via TTL signal. The continuous measuring beam from a 12 V tungsten lamp first goes through a Bausch & Lomb monochromator, then passes through the sample cuvette. The transmitted fraction was measured with a photomultiplier as voltage. This signal was recorded by a PicoScope (Model
4424) as a function of delay after the excitation pulse and converted into absorbance using value prior to the excitation pulse as a reference.

**Synchrotron X-ray diffraction data collection:** X-ray diffraction data were collected at APS (Advanced Photon Source) synchrotron, Argonne National Laboratory, specifically beamlines 21-ID-F, LS-CAT (Life Sciences Collaborative Access Team) and 23-ID-B GM/CA (General Medical Sciences and Cancer Institutes Structural Biology Facility). In-line microscopes aided in focusing on crystallization wells and centering on crystals. At 21-ID-F, a 20 x 20-µm microbeam with a wavelength of 0.9795 Å was used, and data were collected in 0.5–1° steps with 1–2 s exposures and sample-to-detector distance between 200–500 mm with a MARmOsAic 300 detector. At 23-ID-B, data were collected in 0.2–0.4° steps with 0.2–0.4 s exposures and a sample-to-detector distance between 400–500 mm with a Pilatus 6M detector. All data were collected at room temperature, and an on-chip serial crystallography approach was utilized to mitigate the effect of radiation damage as demonstrated in prior work.23

**Data reduction and structure determination for L223SW:** From the 50 crystals of L223SW mutant, X-ray data from 19 crystals that diffracted to a maximum of 3.2 Å were used for structure determination. HKL2000 software was used for indexing, refinement, integration, and scaling multiple data sets together (HKL Research Inc.).29 Molecular replacement, structure building, and refinement were done using the Phenix suite of programs.30 Molecular replacement was performed with Phaser using PDB structure 4TQQ as a model.23 Ligands bound to the structure were identified in the electron density and built into the final model. Electron density maps were displayed and compared using PyMOL.

**Fabrication of high-throughput X-ray transparent microfluidic chips.** Microfluidic chips were fabricated using a strategy adapted from previous work.23 For a full description, see Chapter 3. Briefly, patterned COC substrate layers were fabricated by hot embossing 50 µm thick sheets of COC with a high temperature epoxy master mold in a laminating press (Carver).
Photoresist-on-silicon master molds were fabricated by photolithography using transparency photomasks (Figure 4.1). Polydimethylsiloxane (PDMS), an elastomeric thermoset, was spin-coated on photoresist-on-silicon master molds to fabricate thin, patterned PDMS fluid and control layers by soft lithography. The chip layers were assembled through a combination of irreversible and reversible bonds. A blank COC layer was irreversibly bonded to the PDMS control layer by activating the surfaces with an atmospheric plasma treatment (Harrick) and then bringing them into direct contact. This COC–PDMS control layer was then irreversibly bonded to the PDMS fluid layer by a thermal bonding to form a COC–PDMS–PDMS three-layer assembly (TLA). Prior to crystallization experiments, the compartments in the patterned COC substrate layer were filled with lipid. The crystallization wells in the TLA were aligned to the lipid compartments and reversibly bonded by surface adhesion to complete the construction of the...
chip. Vacuum interconnects were fabricated from PDMS blocks attached to PTFE tubing to facilitate vacuum actuation.

4.3 Design and operation of \textit{in meso} crystallization wells and arrays

Design of \textit{in meso} crystallization well. The design goal was to automate the sequential mixing of three components—protein, lipid, and precipitant—in a compact and modular well for scale-out into microfluidic arrays (Figure 4.2b). Individual wells for formulation of \textit{in meso} crystallization trials rely on passive mixing, as demonstrated in prior work (Chapter 3). In a microfluidic well, protein solution was layered on top of dry lipid in the chip’s substrate layer (Figure 4.2a, c) for passive formulation of a protein-laden mesophase by mixing across a large

![Architecture of a single microfluidic in meso crystallization well.](image)

\textbf{Figure 4.2: Architecture of a single microfluidic in meso crystallization well.} (a) Optical micrograph of a single crystallization well during a crystallization experiment. Protein and precipitant meet at a mixing interface in an open-ended capillary valve for passive mixing by free interface diffusion. (b) Photograph of a microfluidic array chip for \textit{in meso} crystallization, showing a chip filled with protein (red solution) and precipitants (blue, green, yellow, and clear solutions). (c) Exploded schematic of a single crystallization well. X-ray transparency is achieved by construction with <200 \( \mu \text{m} \) of low-scattering materials. (d) Inset of the open-ended capillary valve, highlighting key optimization parameters indicated with capped red lines or red angles. Experimental studies of width (\( w \)), expansion angle (\( \theta \)), and length (\( L \)) resulted in an optimal valve geometry for high-throughput chips.
l lipid-protein interface. After protein-lipid mixing, the protein-laden mesophase becomes very viscous and remains immobile. During mixing, a concentration gradient may form into the 25 µm depth of the lipid layer, although this effect is difficult to visually observe with light microscopy. Next, a precipitant solution was filled in the other half of the well. The two half-wells (protein/lipid and precipitant) meet in a 75 µm-wide ‘S’-shaped channel where mixing of the protein-depleted solution and precipitant occurs by diffusion across a liquid–liquid interface. As the precipitant mixes with the protein-depleted solution, it begins to diffuse into the protein-laden mesophase to trigger crystallization. While mixing of precipitant into the mesophase is expected to be complete within a few minutes, the mesophase and precipitant reservoir stay in contact indefinitely across the normally-open capillary valve to ensure mixing to completion. A multilayer hybrid chip architecture of two polymers is required for chip functionality: (1) elastomeric polydimethylsiloxane (PDMS) for the fluid layer and control layer, which enables the use of normally-closed, vacuum-actuated valves for filling and fluid routing, and (2) rigid, impermeable cyclic olefin copolymer (COC), which enables long-term incubations (>1 month) for crystal growth. Further, the chip construction of thin PDMS layers (X-ray path length: ~20 µm) sandwiched between two COC layers (X-ray path length: ~75 µm) impart the chips with X-ray transparency for in situ crystal diffraction experiments (Figure 4.2c).\textsuperscript{21,23}

In contrast to prior work, this approach addresses the challenges of scale-down (volume of reagents) and scale-out (number of tests) in the design of the chip. To automate high-throughput screening, a large number of wells are needed on a single chip. To accomplish this, the footprint of each microfluidic well was scaled-down and re-designed to fit modularly in dense arrays. Features in the vacuum control layer were reconfigured for facile operation: in these scaled-out chips, as few as 2 control lines automate filling and mixing in every well. Additionally, the filling strategy was modified to use an open-ended capillary valve to optimize handling volumes as small as 8 nL. Capillary valves are routinely employed in the field of centrifugal
microfluidics, but in this report, the strategy is adapted to vacuum-actuated microfluidics. The open-ended capillary valve balanced the vacuum-induced force that drives dead-end filling with resistance to flow in a narrow microfluidic channel. Test arrays that varied the width, length, and expansion angle of the open-ended capillary valve were used to determine an optimal valve geometry, and after testing, a long ‘S’-shaped channel resulted in the best performance (Figure 4.2d, 4.3).

Microfluidic arrays were built using these wells to address two challenges in membrane protein crystallization: high-throughput screening for crystallization hits (Section 4.4), and optimization of crystallization hits (Section 4.5).

**Deposition of lipid into the COC substrate.** Lipid was filled as described in Chapter 3 with two key differences to address the scale and throughput for screening and optimization. First, up to 192 lipid wells were filled in parallel using a single lipid-filling chip. Second, substrates held ~5 nL of lipid due to the reduction in preparative scale. After filling and solidifying the lipid, the lipid-filling chip was removed from the substrate, and each crystallization well of the TLA was

![Figure 4.3: Microfluidic test arrays to determine optimal characteristics for an open-ended capillary valve. (a) Schematic of a filled protein compartment (bottom, green) separated from the precipitant compartment (top, empty) by an ‘S’-shaped capillary valve. The width (w), length(L), and expansion angle (θ) of the capillary valve are noted. (b) Optical micrographs from testing of each valve length, width, and expansion angle. Greater expansion angles and longer channel lengths had the most significant improvements on creating resistance to flow and thus fluid retention. The final valve geometry combined these elements into a compact design with a long, ‘S’-shaped channel and an expansion angle greater than 90°.](Image)
aligned over each lipid-filled chamber in the substrate to complete the assembly of a full crystallization device.

**Setting up crystallization screening and optimization trials on-chip.** A vacuum source, a vacuum interconnect, and a pipette were required to operate microfluidic screening and optimization chips. First, 1–3 µL of solution were pipetted onto each protein inlet. A vacuum interconnect (a small PDMS block and tubing attached to a vacuum pump that reversibly sealed to the chip’s surface) was then placed on a protein-fill vacuum port, initiating liquid filling by simultaneously opening normally-closed microvalves and displacing air with protein solution. Vacuum actuation of the control layer creates a pressure gradient in the fluid layer due to the permeability of air through PDMS. Protein and precipitant solutions completely filled into a dead-end as air was withdrawn. Protein filling typically completed within 2 minutes, and the protein solution only filled the protein chamber up to the open-ended capillary valve (Figures 4.2, 4.3). Lipid and protein mixed passively by diffusion during a 4-8 h incubation. Next, 1 µL of each precipitant was pipetted onto each precipitant inlet and filled by vacuum actuation. Precipitant filling was typically completed within 5 minutes. The protein and precipitant inlets were then sealed with crystal-clear tape, and the entire chip was placed in a sealed Petri dish with a microcentrifuge tube containing 500 µL of water. Chips were stored in a 20°C incubator for up to 6–8 weeks. Crystallization was monitored with a microscope (Leica MDG33, DFC295) 1, 2, 4, and 7 d after filling, and then every three days afterward.

### 4.4 Screening of novel membrane proteins: cytochrome bo₃ oxidase, qNOR, and LM-dimer

To facilitate the discovery of suitable conditions for growing diffraction-quality crystals, dense microfluidic arrays of up to 192 wells for *in meso* crystallization were designed (Figure 4.4a). These high-throughput devices mix a single membrane protein sample with up to 48 different precipitant solutions. Membrane protein solution fills through two inlets into 192 protein
compartments of varying sizes. Precipitants fill through 48 inlets into sets of 4 precipitant compartments. Prior to filling with membrane protein and precipitants, ~5 nL of monoolein was

Figure 4.4: High-throughput screening chips for membrane protein crystallization. (a) Designs for 192-well (left) and 48-well (right) screening chips. Fluid introduced through protein and precipitant ports mix to generate 192 unique conditions. (b) Variable protein:lipid ratios generated in high-throughput screens. As indicated in the table, the amount of lipid for mesophase formulation remains constant while the variable size of protein compartments generates a linear gradient of protein concentrations. Each precipitant mixes with four different mesophases for extensive screening. (c) Tabulated screening results for qNOR, screened at 20 mg/mL with Cubic Screen. 96 conditions (A1—H12) were screened in high-throughput chips. Scores are indicated with colors and numbers: red (0–1) and grey (2–3) for a negative result, yellow (4–6) for optimization candidates (low quality crystals, crystallites), and green (7–9) for diffraction ready crystals (not observed in shown qNOR screen). (d) Representative crystallization screening results and scores for qNOR, cytochrome bo₃ oxidase, and the LM-dimer (3 conditions each) as visualized on-chip with light or cross-polarized microscopy. Scale bar: 100 µm
deposited into compartments in the patterned COC substrate layer,\textsuperscript{23} after which the TLA was aligned and reversibly sealed to complete the assembly of high-throughput chips (Figure 4.2c).

To begin a high-throughput screening experiment, 2.5 µL of membrane protein solution was pipetted onto each protein inlet. Next, vacuum actuation of control lines 3 and 4 (Figure 4.4a) initiated filling of 8–17 nL of protein solution into each protein compartment directly on top of the monoolein. After a 4 h incubation to allow protein–lipid mixing by passive diffusion, 1 µL each of 48 different precipitant solutions were pipetted onto the precipitant inlets. Vacuum actuation of control lines 1 and 2 initiated filling of precipitants into each precipitant compartment. Chips were incubated for up to 8 weeks and regularly monitored for crystallization with polarized light microscopy. As previously discussed, the protein-to-lipid ratio (2:3 in coupled syringes, 2:1 for passive mixing) used traditionally in screening is derived from a binary monoolein–water phase diagram, the behavior of which may significantly change upon addition of salts, detergents, and precipitants. On microfluidic chips, mesophase mixing occurs independently in each well. To enhance and widen the extent of screening, chips are designed to vary the protein-to-lipid ratio from one well to the next based on the size of the protein compartment. In contrast to off-chip work where mesophases are usually prepared in bulk with coupled-syringes and to prior on-chip work where protein-to-lipid ratio was maintained at 2:1, high-throughput chips screen protein-to-lipid ratios from 1.5:1 to 3.3:1 for each precipitant (Figure 4.4b). To screen the same conditions via passive mixing \textit{in meso} crystallization in a well plate (with pre-deposited lipid), 284 individual pipetting actions would be required, while the microfluidic approach presented here both conserves protein sample and reduces the burden on the experimenter by requiring 50 pipetting actions.

To validate the screening capabilities of these high-throughput microfluidic chips, three membrane proteins that were not previously crystallized \textit{in meso} were screened: (1) quinol nitric oxide reductase (qNOR) from \textit{Persephonella marina}, (2) cytochrome \textit{bo}_3 oxidase from
Escherichia coli, and (3) the LM-dimer of reaction center from Rhodobacter sphaeroides. Protein concentrations ranged from 5–20 mg/mL for each of the proteins. Precipitants from an in meso compatible screening kit, Cubic Screen (Emerald Bioscience), were used. Chips were observed with optical and cross-polarized microscopy at scheduled intervals after the chips were set-up. Each well was evaluated and assigned a score using an in meso crystallization scoring system: scores from 0–1 indicated incompatibility with the mesophase, 2–3 indicated no notable crystal hit, 4–6 indicated a hit that may lead to crystals upon optimization, and 7–9 indicated crystal growth. For each protein, on-chip screening was conducted at variable protein concentrations and variable precipitant concentrations. Extended experimental details are included in the Supporting Information.

During screening with 192 well chips, each condition was sampled in duplicate. Using just two chips, 96 Cubic Screen conditions could be screened with one protein solution condition, and 4 chips were used to complete an entire screen in duplicate. After the first pass for screening, Cubic Screen conditions were diluted with MilliQ water in a 1:1 ratio and repeated. Crystallization hits (Table 4.1) were tested for diffraction, although no indexable diffraction patterns were identified. Several crystallization hit conditions for each protein were selected for optimization. First, the Cubic Screen hit condition was diluted with MilliQ water in ratios of 1:1, 1:2, 1:4, and 1:6. Further, each component of the crystallization hit conditions (pH and concentrations of buffer, salt, and precipitant) were systematically varied. Despite extensive screening and optimization attempts on crystallization hits, crystals did not yield high-quality diffraction, indicating that an expanded set of chemical compositions must be surveyed to grow crystals for structural studies.

Each crystallization condition was evaluated using a scoring system developed by Caffrey et al. 14 Each crystallization well was evaluated as a whole and assigned a score. Briefly, a score of 0–3 indicated a negative result, ranging from an incompatible lipidic phase (0) to a protein
precipitate (3). Scores from 4–6 indicated a result for further optimization, ranging from a birefringent precipitate (4) to microcrystals (6). Higher scores, 7–9, indicated positive crystallization results, either 1-D crystals (7), 2-D crystals (8), or well-defined 3-D crystals (9).

For some protein–detergent combinations, high concentration samples prepared in centrifugal concentrators resulted in a persistent bulk birefringent phase when observed through crossed polarizers. Birefringence indicates that a stable cubic phase has not formed, and the likelihood of crystallization is low. In previous literature, high detergent concentrations have been shown to destabilize cubic phases.34 This is particularly prevalent with some detergents commonly used in crystallization, including DDM and OG, which were used in this study to stabilize qNOR and cytochrome bo3 oxidase, as they either form large protein–detergent complexes that not pass through a centrifugal concentrator or as they have a high critical micelle concentration.35 Passive mixing approaches facilitate screening with lower protein

<table>
<thead>
<tr>
<th>Membrane protein sample</th>
<th>Crystallization condition hits</th>
</tr>
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<tbody>
<tr>
<td><strong>qNOR, 5–10 mg/mL</strong></td>
<td>20% (w/v) PEG 1000, 100 mM HEPES/NaOH pH 7.5, 200 mM Magnesium chloride</td>
</tr>
<tr>
<td>100 mM Tris-HCl pH 8.0, 100 mM NaCl, 0.05% DDM</td>
<td></td>
</tr>
<tr>
<td><strong>Cytochrome bo3 oxidase, 5–10 mg/mL</strong></td>
<td>30% (w/v) PEG 8000, 100 mM Sodium cacodylate/NaOH pH 6.5, 200 mM Calcium acetate</td>
</tr>
<tr>
<td>50 mM Potassium phosphate pH 8, 0.05% DDM</td>
<td></td>
</tr>
<tr>
<td><strong>Cytochrome bo3 oxidase, 5–10 mg/mL</strong></td>
<td>2.5 M NaCl, 100 mM Sodium citrate/citric acid pH 5.5, 200 mM Magnesium chloride</td>
</tr>
<tr>
<td>20 mM Tris-HCl pH 8, 0.7% OG</td>
<td></td>
</tr>
<tr>
<td><strong>LM-dimer, 5–20 mg/mL</strong></td>
<td>2.5 M NaCl, 100 mM MES/NaOH pH 6.0, 200 mM Lithium sulfate</td>
</tr>
<tr>
<td>10 mM Tris pH 7.8, 280 mM NaCl, 0.03% LDAO</td>
<td></td>
</tr>
<tr>
<td>1 M HEPES pH 7.5, 1.15 M (NH4)2SO4, 12–15% Jeffamine M-600</td>
<td></td>
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</table>

All 96 conditions from Cubic Screen were tested, and those producing crystals are listed here.
concentrations due to a concentrating effect during the mixing process.\textsuperscript{12} Compared to initial screens, reduced concentrations (qNOR: <10 mg/mL, cytochrome bo\textsubscript{3} oxidase: <15 mg/mL) yielded stable cubic phases and successful crystallization screening. Several crystallization hits were successfully identified for the target proteins within 14 d, and representative results and screening data are shown (Table 4.1, Figure 4.4c, d).

Next, diffraction quality of the hits discovered for each membrane protein was evaluated. Screening chips were directly mounted without modifying the goniometer at beamlines 21-ID-D or 23-ID-B for room-temperature X-ray diffraction experiments. To accomplish this, a magnetic cap mount was modified with a tube with a thin slit and a set-screw to hold the microfluidic chips. Crystal hits were identified and targeted via an on-axis video microscope and sequentially probed with X-rays: (1) qNOR crystal hits yielded either no diffraction or weak scattering rings, (2) cytochrome bo\textsubscript{3} oxidase crystal hits yielded weak, low resolution diffraction (<12 Å), and (3) LM-dimer crystal hits yielded no identifiable diffraction. Unfortunately, optimization of these hits did not yield crystals with better diffraction, indicating that either (a) a wider range of chemical compositions must be evaluated to uncover ‘good’ crystallization hits, or (b) these proteins or particular protein preparations (choice of buffer, detergents, and additives) are intractable and resistant to forming high-quality crystals. A more complex crystallization device could broaden the variety of crystallization conditions screened in parallel by either sampling more precipitant-to-mesophase ratios, or by introducing on-chip serial dilutions\textsuperscript{36} to screen a fine-gradient of concentrations for each precipitant. Regardless, through successfully screening crystallization conditions, identifying new hits, and evaluating their diffraction with X-rays, this work demonstrates an automated high-throughput \textit{in situ} approach for \textit{in meso} crystallization screening with minimal sample consumption.
4.5 Fine-gradient optimization of crystallization conditions: L223SW and microbial rhodopsin

Microfluidic optimization chips were developed to facilitate the optimization of poorly diffracting crystallization screening hits. These experiments sample a fine-gradient of protein and precipitant mixtures, based on the condition obtained in the initial trial, in fully combinatorial 48-well arrays. On a single chip, 4 concentration or composition variants of a protein solution are mixed with 4 variants of a precipitant solution in all possible combinations. Four inlets for protein solution (P1–P4) fill the protein compartments in each of 4 quadrants of the chip, while 7

Figure 4.5: Optimization chips for membrane protein crystallization. (a) Design of a 48-well chip (left) and fluid filling scheme (right) for a crystallization optimization experiment. The mixing array formulates fully combinatorial in meso crystallization experiments for 4 different levels of both protein and precipitant, aiding the search for diffraction-quality crystals once proper precipitant components are discovered via screening. (b) (left) Photograph of on-chip optimization experiment for L223SW observed 10 d after set-up. (right) Representative optimization results from each condition formulated on a single chip. No crystallization was observed at low protein concentration, and many small crystals appeared at high protein concentration and low precipitant concentration. The best crystals were observed at higher protein concentrations (15, 20 mg/mL) and high precipitant concentrations (14—15%). Scale bars: 100 μm
inlets for precipitant solutions fill the precipitant compartments (4 unique solutions, Ppt1–Ppt4) and formulate 16 unique combinations of in meso crystallization conditions in triplicate (Figure 4.5a).

The parameters to be optimized (e.g., protein concentration, pH, precipitant concentration, additive concentration) are chosen and prepared off-chip. The operation of the chip is similar to the operation described above (Section 4.3) with a few small differences. To begin an optimization experiment, first 1 µL of each protein solution was pipetted onto each of the protein inlets. Next, actuation of control line 2 initiated filling of protein into the protein compartment directly on top of the lipid. After a 4-8 h incubation to allow protein–lipid mixing by passive diffusion, 1 µL of each of 4 precipitant variants were pipetted onto their respective inlets (Figure 4.5a). Actuation of control line 1 initiated the filling of precipitant into each precipitant compartment. To perform the same optimization experiment for passive mixing in meso crystallization in a well plate (with pre-deposited lipid), 96 individual pipetting actions would be required, while the microfluidic approach presented here requires just 11 pipetting actions.

Two proteins were used for testing and validating these optimization chips: a microbial rhodopsin from Haloquadratum walsbyi and a photosynthetic reaction center mutant from Rhodobacter sphaeroides, denoted L223SW. Initial testing of crystallization and fluid flow for the optimization device were conducted with microbial rhodopsin, a photochemically active seven-transmembrane α-helical protein with a covalently bound retinal chromophore. Upon mixing of protein and precipitant with known crystallization conditions (7.5 mg/mL in 50 mM MES pH 6.5, 500 mM NaCl, 1% OG mixed with 7–10% Tacsimate pH 7, 20% PEG 3350), a birefringent mesophase formed. After 4 weeks of incubation with precipitant, the phase was no longer birefringent and microbial rhodopsin crystals ranging from 20–50 µm were observed on-chip. A coarse optimization was conducted with two protein concentrations (7.5 and 15 mg/mL) and 4 variations of precipitant (7–10% Tacsimate), although significant changes in crystal size and
quality were not observed (Figure S4.6a, b). X-ray diffraction data were collected at room temperature, but the crystals were highly susceptible to radiation damage and rapidly discolored from purple to pale blue, indicating damage or displacement of the chromophore (Figure 4.6c). Prior to radiation damage, crystals diffracted to ~2.5 Å (Figure 4.6d). Due to the lengthy crystallization time and fragility of microbial rhodopsin crystals, validation of optimization chips was instead carried out with the L223SW mutant.

The L223SW mutant was designed for fundamental structure–function experiments to study the mechanism of electron transport in photosynthetic reaction centers. The crystal structure and crystallization conditions are known for the wild-type protein, and we previously grew crystals on-chip and solved the structure for the wild-type reaction center (PDB: 4TQQ). As a

![Image](Figure 4.6: On-chip crystallization experiments of microbial rhodopsin. (a) Optical micrograph of crystals grown at 15 mg/mL microbial rhodopsin and precipitant containing 8% Tacsimate. (b) Optical micrograph of crystals grown at 7.5 mg/mL microbial rhodopsin and precipitant containing 8% Tacsimate. (c) A 50 µm hexagonal crystal (i) before X-ray exposure, observed with a light microscope, and (ii) after X-ray exposure, observed using the in-line microscope at beamline 23-D-D. Green circles indicate beam size at two exposure locations. (d) Sample diffraction pattern up to 2.5 Å at room temperature of microbial rhodopsin collected on-chip.)
starting point, crystallization was attempted with the condition used in prior work (15 mg/mL reaction center in 10 mM Tris pH 7.8, 280 mM NaCl, 0.05% LDAO mixed with 1 M HEPES, 1.15 M (NH₄)₂SO₄, 13% w/v Jeffamine M-600 adjusted to pH 7.5). However, this condition produced small crystals that diffracted only to 6 Å, while the wild-type crystals had diffracted to 2.5 Å. To obtain structure-quality crystallization conditions for L223SW, changes in protein concentration (5–20 mg/mL), pH (7.4–8.0), and precipitant concentration (Jeffamine M-600, 12–15% w/v) were analyzed with optimization chips (Figure 4.5b). Changes in pH either had a negative or no effect on crystallization. High concentrations of protein and low concentrations of precipitant resulted in small (~20 µm), poorly diffracting (~6 Å) crystals, while high concentrations of both protein and precipitant solutions resulted in the largest crystals (~60 µm) with good diffraction (~3.2 Å) (Figure 4.7c). The best diffracting crystals were grown with 20 mg/mL protein at pH 7.8

Figure 4.7: (a) Work station at beamline 23-ID-D, Advanced Photon Source. Targeting cameras for crystal centering are shown. On the left, a top-down low resolution camera for bringing the plane of the chip into focus. On the right, an on-axis high resolution camera for crystal targeting. The L223SW crystal pictured is ~50 µm in size. (b) An optimization chip mounted on the X-ray goniometer, enabling quick crystal identification and targeting for serial crystallography. (c) An X-ray diffraction pattern from L223SW, diffraction extends out past the 4 Å ring. Background scattering from the chip materials is present between 6 and 10 Å.
using high concentrations of Jeffamine M-600 (14–15% w/v).

Following a previously developed on-chip data collection strategy for room-temperature crystals (Figure 4.7),23 in situ data was collected from multiple crystals in small wedges and then merged together to minimize the effect of radiation damage on the structure. In total, 19 crystals from a small range of conditions (15–20 mg/mL for protein, 14–15 % w/v Jeffamine M-600) were selected to form an optimal dataset. Despite the small variations in chemical composition, the crystals had near identical lattice parameters (<0.5% variation) and a small variation in mosaicity. Interestingly, crystallization at 20 mg/mL L223SW and 15% w/v Jeffamine M-600 yielded the lowest mosaicity crystals and the highest quality of diffraction (3.2 Å). The crystal structure of L223SW was solved to a resolution of 3.5 Å (Table 4.2, PDB: 5V33). In the wild-type protein, a serine located in the L-subunit at position 223 facilitates the binding of a quinone (QB) molecule, which serves as the terminal electron acceptor for the reaction center. Both residue Ser223 and QB are resolved in the wild-type crystal structure (Figure 4.8a). In the mutant protein, the small polar residue Ser223, is replaced with tryptophan, a relatively large hydrophobic residue (Trp223, also denoted S223W). In the solved structure, Trp223 protrudes
into the binding pocket, which prevents Q$_B$ from binding (Figure 4.8b). This structural observation matches observations from flash-induced spectroscopy: with a Q$_B$ present in the wild-type protein, electron transfer has a lifetime of about 1 s, whereas, when Q$_B$ is absent in the mutant, electron transfer has a lifetime of about 100 ms (Figure 4.8c). Previous studies that used a molecular additive to inhibit charge transfer from Q$_A$ to Q$_B$ observed a 10-fold reduction in lifetime of the charge-separated state, agreeing with this structural and spectroscopic observation.\(^{38}\) Through successfully optimizing the diffraction quality of crystals, this work demonstrates the application of microfluidic optimization chips as effective tools for streamlining crystallography and for supporting structure–function studies that require atomic-resolution crystal structures.

<table>
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<tr>
<th>Table 4.2: Crystallographic data$^a$ and refinement statistics</th>
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<tr>
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<td>Space group</td>
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<td>Resolution (Å)</td>
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<tr>
<td>No. unique reflections</td>
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<tr>
<td>Completeness$^b$</td>
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<td>Redundancy$^b$</td>
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<td>Mosaicity (deg.)</td>
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<td>R$_{merge}$$^b$</td>
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$^a$ Merging of small data sets from 19 crystals analyzed on-chip at room temperature
$^b$ Values in parentheses indicate values for the highest resolution shells
4.6 Conclusions

In summary, X-ray transparent microfluidic devices for in meso crystallization screening and optimization were developed that automated the formulation of mesophases for crystallization experiments in a high-throughput manner while consuming just a few nanoliters of membrane protein solution per condition. The high-throughput screening devices were validated by discovering crystallization hits for qNOR, cytochrome bo3 oxidase, and the LM-dimer of reaction center. The ability to survey 192 unique crystallization conditions in parallel with an X-ray transparent platform expedites extensive screening, crystal hit identification, and diffraction testing, all without opening or altering the device. In addition, the optimization devices were validated using the crystallization of the L223SW mutant of reaction center. Starting from a condition that yielded low quality crystals, fine-gradient re-screening of L223SW led to large, diffraction quality crystals. Data from these crystals were used to resolve a room-temperature structure with a 3.5 Å resolution. Compared to the wild-type reaction center structure, the L223SW structure verified that the S223W mutation inhibits the binding of a secondary ubiquinone to reaction center. For optimum performance of both devices, simple vacuum actuation and diffusive mixing are key to facilitating device-wide filling and incubation of all wells. The open-ended capillary valve enabled these devices to precisely measure as little as 8 nL of protein solution to form a 13 nL crystallization-ready mesophase, a ~60% decrease in sample usage when compared to the most efficient in meso robots.\(^{39}\) While this technique is currently exclusive to laboratories with microfluidics expertise and their collaborators, the development of this technology into a commercially available product as an alternative to traditional in meso crystallization plates would provide immense advantages in user-friendliness and sample usage.

The precise dispensing, control over fluid routing, and X-ray transparency in this type of microfluidic device may facilitate their use for other delicate, complicated, and labor-intensive in
meso crystallization experiments. As the field of crystallography pushes toward brighter X-ray beams and smaller crystals for serial crystallography, high-throughput devices that can grow hundreds of crystals on the same device (as reported here) provide an excellent fixed-target platform that has key advantages over continuous liquid-injection jets. Further, a more advanced device could facilitate crystal soaking, i.e., the on-chip introduction of heavy atoms or anomalous scatterers into the crystal post-crystallization for in situ, de novo phasing experiments.
4.7 References


CHAPTER 5

Microfluidic Platforms for Crystallographic Studies of Protein-Ligand Interactions

5.1 Introduction

An area of significant interest in structural biology is the study of protein-ligand complexes, such as small drugs or biomolecules that bind or dock to the protein.\textsuperscript{1–3} Other important ligands include heavy atoms (metals) or other molecules to solve the crystallographic ‘phase problem’ for \textit{de novo} structure solution through anomalous scattering or isomorphous replacement.\textsuperscript{4–8} When planning a crystallization experiment, a key challenge is determining when and how to add ligand to the protein. Broadly, there are 4 methods of adding ligands to a crystallization experiment: (1) co-expression of protein with the ligand, (2) addition of ligands during purification, (3) co-crystallization of the protein with the ligand, or (4) soaking ligand into a grown crystal.\textsuperscript{9} Co-expression of protein with a ligand can increase levels of expression as well as increasing solubility. Ligand addition during purification is typically used when the expression system has been optimized, but the protein is still poorly behaved in solution. The remaining two methods, co-crystallization and soaking, can be tested during crystallization experiments. During co-crystallization experiments, ligand is added to the purified protein sample prior to crystallization, and is often used for ligands that are insoluble or aggregate easily. Soaking, the addition of ligand to grown crystals, is the most commonly used method because it does not require re-optimizing the expression, purification, or crystallization system and is typically easy to implement.

Soaking experiments are typically prepared during the process of crystal harvesting, just prior to flash-freezing crystals for X-ray crystallography. Either the ligand solution is directly added to the mother liquor, or the crystal is removed from its mother liquor and equilibrated in a
separate ligand solution. The crystal then remains in the soaking solution for several hours to several days, depending on the protein-ligand system. Upon completion of the soak, the crystals are picked up with a crystallography loop and immediately plunged into liquid nitrogen as the final preparation step for X-ray crystallography. While straightforward for proteins crystallized in aqueous solution, extending this method to membrane proteins crystallized *in meso* adds several layers of complication. In *meso* crystallization is performed in hermetically sealed glass-sandwich plates, which are comprised of two thin layers of glass separated by an adhesive spacer. For soaking crystals *in meso*, the glass-sandwich plates must be carefully opened with a diamond-tipped scribe and forceps. Next, the soak solution is pipetted directly into the well, after which the glass is replaced and re-sealed with tape. After the soak (hours to days), the well is re-opened, the crystal harvested and then flash-frozen for X-ray crystallography. In a 2015 report from Caffrey and co-workers, the success rate for adding soak solutions was 5-10%, meaning that in 90-95% of all crystallization wells chosen for soaking, the crystal or crystallization well was compromised during the experiment (e.g., the glass-sandwich plate cracked, glass shards entered the well, crystals were lost, etc.). With the current state-of-the-art *in meso* technology, ranging from specialized manual tools to robots, crystal soaking is prohibitively difficult.

In this work, we report a new microfluidic method for manipulation of crystals *in meso* after crystallization is complete and its application to the crystallization and soaking of a model protein-ligand system. In a 6-well X-ray transparent microfluidic array chip, crystallization and soaking were performed sequentially in a two-step process. First, crystals were first grown in a lipidic mesophases using the passive-mixing microfluidic method established in prior work (Chapter 2). Next, the mother liquor (remaining precipitant and depleted protein solution) was exchanged for a ligand-rich solution containing the inhibitor molecule using a new flow-through method. Protein crystals, which were embedded in the highly-viscous mesophase, remained
immobile while the mother liquor was displaced by the soak solution. The result was a facile and non-invasive method for solution exchange and ligand incorporation via soaking, where on-chip automation limited the possibilities for failure. The method was applied to the protein lysozyme and its inhibitor, triacetylchitotriose (tri-NAG), and validated through on-chip X-ray crystallography. The observed occupancy of tri-NAG in the crystal structures varied as expected with soaking time and tri-NAG concentration in the soak solution.

5.2 Materials and methods

For experiments described here, we used the following materials and chemicals: monoolein (1-Oleoyl-rac-glycerol) and cholesterol (Sigma–Aldrich, St. Louis, MO), polydimethylsiloxane (PDMS) (RTV-615 from Momentive Performance Adhesives, Waterford, NY), negative photoresists (SU-8 25 and SU-8 2050 from MicroChem Corporation, Newton, MA), cyclic olefin copolymer films (COC) (Grade 6013, 2 mil from TOPAS Advanced Polymers, Florence, KY), silanizing agent (tridecafluoro-1,1,2,2-tetrahydrooctyl)trichlorosilane from Gelest, Inc., Morrisville, PA), and epoxy (Conapoxy FR 1080 from Cytec Industries, Woodland Park, NJ).

Sample preparation. To prepare the protein solution, powdered hen egg white lysozyme (Hampton Research, HR7-110) was used as received and dissolved in E-pure water (18.2 MΩ) at concentrations of either 50 mg/mL or 100 mg/mL. The precipitant solution was prepared with 9% (w/v) sodium chloride in 0.1 M sodium acetate, pH 4.5 in E-pure water. The soaking stock solution was prepared at 16 μM tri-NAG (Toronto Research Chemicals, T735000) in E-pure water. Ligand-rich solutions for soaking experiments were prepared in a buffer with a similar composition to the mother liquor, 9% sodium chloride, 0.1 M sodium acetate, pH 4.5.

Absorbance difference spectroscopy. Protein-ligand binding interactions in solution were verified by measuring the absorption spectra of lysozyme, and an equimolar mixture of lysozyme and tri-NAG. Measurements were performed with an Agilent 8453 UV-vis
Spectrophotometer. Spectra for each sample were measured from 280nm – 320nm, and the resulting difference spectrum was analyzed.\textsuperscript{17}

**Fabrication of soaking chips.** Microfluidic chips were fabricated using a strategy adapted from previous work.\textsuperscript{18} For a full description, see Section 3.2. Briefly, patterned COC substrate layers were fabricated by hot embossing 50 µm thick sheets of COC with a high temperature epoxy master mold in a laminating press (Carver). Photoresist-on-silicon master molds were fabricated by photolithography using transparency photomasks (Figure 5.1). Compared to prior work, these photomasks have an extra feature which is key for flow-through soaking: an additional inlet hole located across from the protein inlet. Polydimethylsiloxane (PDMS), an elastomeric thermoset, was spin-coated on photoresist-on-silicon master molds to fabricate thin, patterned PDMS fluid and control layers by soft lithography. The chip layers were assembled through a
combination of irreversible and reversible bonds. A blank COC layer was irreversibly bonded to the PDMS control layer by activating the surfaces with an atmospheric plasma treatment (Harrick) and then bringing them into direct contact. This COC–PDMS control layer was then irreversibly bonded to the PDMS fluid layer by a thermal bonding\(^{19}\) to form a COC–PDMS–PDMS three-layer assembly (TLA). Prior to crystallization experiments, the compartments in the patterned COC substrate layer were filled with lipid (see Section 3.3.2). The crystallization wells in the TLA were aligned to the lipid compartments and reversibly bonded by surface adhesion to complete the construction of the chip. Vacuum interconnects were fabricated from PDMS blocks attached to PTFE tubing to facilitate vacuum actuation.

**Synchrotron X-ray data collection and analysis.** X-ray diffraction data were collected at APS (Advanced Photon Source) synchrotron, Argonne National Laboratory at beamline 21-ID-F, LS-CAT (Life Sciences Collaborative Access Team). In-line microscopes were used to center and focus on crystals with a 60 µm microbeam. The beamline operated at a fixed wavelength (\(\lambda = 0.9795\ \text{Å}\)). Data were collected in 1° steps with 1 s exposure and a sample-to-detector distance of 250 mm with a Rayonix MX-300 detector. Data from 10-15 crystals were collected for each of the 6 soaking conditions tested (Soaking time: 24 h, 48 h; Ligand-to-protein ratio: 1:1, 5:1, 10:1). All data were collected at room temperature, and an on-chip serial crystallography approach was utilized to mitigate the effect of radiation damage as demonstrated in prior work.\(^{18}\)

HKL2000 software was used for indexing, refinement, integration, and scaling multiple data sets together (HKL Research Inc.).\(^{20}\) Molecular replacement was performed with Phaser using PDB structure 5D5C as a model.\(^{18}\) Molecular replacement, structure building, and refinement were done using the Phenix suite of programs.\(^{21}\) Ligands bound to the structure were identified in the electron density and built into the final model. Electron density maps were displayed and compared using PyMOL. For each soaking condition, an optimal data subset was constructed
with diffraction from a minimum of 7 crystals for each of the 6 structures built. The maximum resolution of the structures ranged from 1.5 Å to 1.9 Å.

5.3 Crystallization and soaking of a lysozyme-trisaccharide complex on-chip

The goal of this work was to develop an on-chip, two-step process for (1) crystallization of proteins in a lipidic mesophase, and (2) subsequently soaking the newly grown crystals in a ligand-rich solution (Figure 5.2a). Similar to previous reports (Chapter 2), the soaking chip automates mesophase-based crystallization by passive mixing of protein, lipid, and precipitant. The second step, soaking of ligands, is enabled by a new fluidic path for flow-through filling through which the mother liquor can be displaced by the ligand-rich solution.

For a crystallization-soaking experiment, first pre-deposited dry lipid was mixed with protein solution by diffusion across a large interface for several hours, which resulted in the formation of a stationary, protein-laden lipidic cubic phase. Crystallization was then triggered by the introduction of a precipitant solution – the precipitant compartment and protein-laden mesophase compartment meet in an open-interface ‘capillary valve’, which enabled complete mixing of precipitant into the mesophase. Crystals typically grow in the mesophase and reach their full size after a few days of incubation at 20°C. The mother liquor is then replaced with the ligand-rich solution. Ligands then diffuse through the mesophase and into crystal over several minutes to several days. Because the soaking chip is X-ray transparent, there is no need to harvest crystals for mounting – the entire microfluidic chip was mounted in an X-ray beam path for diffraction experiments to study protein-ligand interactions on a structural level.

The soaking chip employs two different methods for filling solutions in sequence: first, dead-end filling to formulate the crystallization trial, and then through-filling to replace the mother liquor with a ligand-rich solution (Figure 5.2b). To dead-end fill, vacuum is actuated adjacent to a microfluidic compartment to withdraw air, but not liquid, through a thin membrane. A solution
fills from a single inlet hole into the compartment in response to the displaced air, and the compartment will completely fill with liquid. Dead-end filling is the preferred method for accurately metering nanoliter volumes into a microfluidic chip because the size of the microfluidic compartment determines the amount of fluid filled. To through-fill, there must be a

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**Figure 5.2:** Schematics for *in meso* crystal soaking platform. (a) (left) An array of 6 parallel crystallization wells for *in meso* crystallization and soaking. The fluid layer (black) contains channels and compartments for all liquid solutions (inlets F1-F4). The control layer (pink) contains channels for vacuum actuation (inlets C1-C3) – these features overlay fluid compartments and valves to automate filling and mixing. (right) Inset of the top-down view for a single crystallization well showing the relative positions of the control layer over the fluid layer. The protein and lipid chamber meets with the precipitant chamber across a normally-open ‘S’-shaped capillary valve. Cross section X-X’ shows the layer-by-layer architecture of the chip – from top to bottom, an impermeable COC (50 µm), the control layer (50µm), the fluid layer (50 µm), and an embossed substrate to hold lipid and additional precipitant (50 µm). (b) The platform operates in two distinct modes: crystallization mode and soaking mode. (left) To operate in crystallization mode, vacuum actuation at inlet C1 initiates protein filling through inlet F1. After a four-hour incubation, vacuum actuation of inlet C2 fills precipitant solution from inlets F3 and F4. Inlets C3 and F2 are not used in this mode. (right) To operate in soaking mode, vacuum actuation at inlet C1 and C3 opens the fluidic path between F1 and F2. Withdrawal at inlet F1 will induce flow of a ligand-rich solution through the crystallization chambers, from F2 to F1.
direct fluidic path between two inlet holes. For displacement of the mother liquor with a ligand-rich solution, the ligand-rich solution is pipetted onto one inlet hole, and withdrawn through the other inlet hole (Figure 5.2c). Through-filling utilizes samples less efficiently, although it is the preferred method for rapid filling, or for when entire volumes need to be displaced.

The use of dead-end filling is well-characterized for fluid filling and metering, and has been extensively tested for on-chip in meso crystallization. Three different methods of through-filling were evaluated for their compatibility with the mesophase and crystals in this study: (1) vacuum suction, (2) syringe pump withdrawal, and (3) wicking with an absorbent material. After the ligand-rich solution was deposited on the soaking inlet, the syringe pump withdrawal method was applied to the soaking outlet, resulting in flow from the inlet, through each of the 6 parallel crystallization chambers, and exiting through the outlet. Each method was tested experimentally with lysozyme crystals grown in lipidic mesophases with a dye solution in place of the ligand-rich solution to visualize the exchange of solutions (Table 5.1, Figure 5.3).

With vacuum suction, the ligand-rich solution exchanged very rapidly (1 – 2 s) and crystals sustained significant damage (e.g., cracking, partial dissolution) during the exchange. Further, exchange by vacuum suction occurs very fast, meaning that a short extension of actuation time will fully evacuate the chip and ruin the crystallization experiment. Alternatively, syringe pump withdrawal resulted in slow (2 – 10 min), controlled solution exchange. These crystals sustained either no damage, or minor surface cracks which, later, did not impact X-ray diffraction experiments. Wicking with an absorbent material resulted in slow solution exchange (<15 min). Of the three, wicking is the most attractive method because no ancillary equipment is required.

Table 5.1: Summary of solution exchange methods

<table>
<thead>
<tr>
<th>Method</th>
<th>Flow rate</th>
<th>Time for exchange</th>
<th>Crystal Behavior</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vacuum suction</td>
<td>&gt;5 µL/s</td>
<td>&lt;2 s</td>
<td>Cracks, dissolution</td>
</tr>
<tr>
<td>Syringe pump</td>
<td>1 µL/min – 20 µL/min</td>
<td>2 – 10 min</td>
<td>Minor cracks</td>
</tr>
<tr>
<td>Wicking</td>
<td>~ 1 µL/min</td>
<td>&lt;15 min</td>
<td>No change</td>
</tr>
</tbody>
</table>
for solution withdrawal, with primary drawbacks being that it is slow relative to the other methods and the flow rate is not controllable.

The lysozyme/tri-NAG complex is a well understood protein-inhibitor system and an ideal model crystallization system due to the robustness of lysozyme crystals. Further, although lysozyme is a soluble protein, it crystallizes in the aqueous channels of lipidic cubic phases and is thus a suitable target for the initial application of soaking chips. Lysozyme natively

Figure 5.3: Schematics (left) and optical micrographs (right) for operation of microfluidic platform in soaking mode at various time points. (a) Schematic cross section of a microfluidic device after crystallization – protein crystals are embedded in the mesophase. (b,e) 1 minute after ligand-rich solution is placed at inlet F2, vacuum actuation is applied to inlets C1 and C3, and suction is applied with a syringe pump in withdrawal mode at F1. Ligand-rich solution (red) begins to displace the mother liquor (clear) in all 6 wells. (c,f) 5 minutes after initiation of flow, showing the soak solution further displacing the mother liquor. (d,f) After ten minutes, flow-through reaches completion and the mother liquor is fully replaced with ligand-rich solution. During an incubation step (several hours to days), the ligand diffuses into the crystal.
functions as an antimicrobial protein by hydrolyzing 1,4-beta-linkages between certain saccharides in cell walls. Lysozyme’s active site has six locations where each saccharide can bond, and previous work has shown that tri-NAG: (a) is unhydrolyzable by lysozyme, and (b) binds preferentially in locations 2, 3 and 4 in the active site, and therefore acts as an inhibitor. Lysozyme/tri-NAG binding in solution was validated by absorbance difference spectroscopy. When mixed at a 1:1 molar ratio, the difference spectrograph showed the distinct ‘fingerprint’ of lysozyme/tri-NAG binding as demonstrated in literature.\textsuperscript{17}

Lysozyme crystallization was performed using the previously established on-chip \textit{in meso} crystallization protocol. First, \textasciitilde50 nL of 50 mg/mL lysozyme solution was filled in each mesophase chamber to passively mix with \textasciitilde10 nL of solid monoolein. After a 24 hour pre-incubation to allow for mesophase formation,\textsuperscript{27} \textasciitilde240 nL of 9\% (w/v) sodium chloride, 0.1 M sodium acetate, pH 4.5 was introduced to each crystallization well. Crystals were first observed after 12 hours and grew to their full size within 1-2 days. To evaluate the reliability of on-chip soaking of ligands into crystals in mesophases, two parameters were evaluated: (1) duration of soaking and (2) ligand-to-protein molar ratio. 6 different soaks were tested on-chip (in duplicate)

Figure 5.4. 1.53 Å structure of the lysozyme/tri-NAG complex. (a) Ribbon diagram of 3-D structure, with secondary structure elements highlighted in red (\(\alpha\)-helices) and yellow (\(\beta\)-strands). The tri-NAG molecule is shown as a stick representation at the binding site. (b) Surface rendering of the lysozyme/tri-NAG complex. (c) Electron density map of tri-NAG (yellow) in the binding site (blue), showing how tri-NAG aligns with residues on the surface of lysozyme. 2Fo - Fc density map is contoured to \(\pm1\sigma\).
with 24 or 48 hour soaks, and molar ratios of 1:1, 5:1, and 10:1. For all conditions, crystals did not sustain observable cracking or damage, and crystals were stable on-chip for at least 2 weeks. Synchrotron X-ray diffraction experiments were performed after the conclusion of the soaks. For each condition, >10 crystals were analyzed using an established on-chip, room temperature method for serial crystallography. All crystals diffracted well (<2 Å), and the optimal data sets for each soaking condition were selected for structure solution (Figure 5.4).

A structure was built for each of the 6 soaking conditions, and tri-NAG was bound to lysozyme at the lysozyme binding site in all structures. The structures had good overall quality with high resolution, high completeness, high I/σ, and excellent R-factors (Table 5.2). There was minimal variation between the quality of crystal structures for bound and unbound lysozyme. While the tri-NAG was observed in all six soaking conditions, there were notable differences that depended on soaking time and ligand-to-protein ratio (Figure 5.5). At long soak

![Figure 5.5: Stick representations of tri-NAG from lysozyme structures at six different soaking conditions: (a) 24 hour soak and (b) 48 hour soaks at ligand-to-protein ratios of (i) 1:1, (ii) 5:1, and (iii) 10:1. The ligand is resolved in the structure best at high ligand-to-protein ratios, while lower occupancy or conformational flexibility is seen at lower ratios and soaking times.](image-url)
durations and high ligand:protein ratios, the ligand electron density was easily recovered and the three rings of the tri-NAG were resolved in the crystal structure. At short soak durations and low ligand:protein ratios, only two of the three rings of tri-NAG were resolved in the crystal structure. This result was expected, and can be explained by two phenomena: first, the process of soaking is diffusion limited at low concentrations of tri-NAG as the ligand must diffuse into the crystal from the solution, resulting in a heterogeneous crystal with many proteins bound to tri-NAG, and many proteins unbound. Thus, the final structure represents an average of both populations, resulting in a lowered tri-NAG occupancy. Second, the third ring of tri-NAG does not bind strongly to lysozyme and exists in many conformations when observed in the crystal structure, which results in structural disorder and reduced occupancy.

Table 5.2: Crystallographic data\(^a\) and refinement statistics for lysozyme/tri-NAG complex

<table>
<thead>
<tr>
<th>Soak conditions:</th>
<th>1:1, 24h</th>
<th>5:1, 24h</th>
<th>10:1, 24h</th>
<th>1:1, 48h</th>
<th>5:1, 48h</th>
<th>10:1, 48h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unit cell (Å)</td>
<td>a = b = 78.8</td>
<td>a = b = 78.9</td>
<td>a = b = 79.0</td>
<td>a = b = 78.8</td>
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<td>a = b = 78.8</td>
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<tr>
<td>c = 38.3</td>
<td>c = 38.2</td>
<td>c = 38.2</td>
<td>c = 38.3</td>
<td>c = 38.2</td>
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<tr>
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<td>α = β = γ = 90</td>
<td>α = β = γ = 90</td>
<td>α = β = γ = 90</td>
<td>α = β = γ = 90</td>
<td>α = β = γ = 90</td>
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<tr>
<td>Space group</td>
<td>P 4(_2)2(_1)2</td>
<td>P 4(_2)2(_1)2</td>
<td>P 4(_2)2(_1)2</td>
<td>P 4(_2)2(_1)2</td>
<td>P 4(_2)2(_1)2</td>
<td>P 4(_2)2(_1)2</td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>25.9 – 1.64</td>
<td>25.9 – 1.90</td>
<td>25.0 – 1.60</td>
<td>25.9 – 1.70</td>
<td>31.5 – 1.53</td>
<td>25.9 – 1.53</td>
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<tr>
<td>No. unique reflections</td>
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<td>9338</td>
<td>15391</td>
<td>13763</td>
<td>18534</td>
<td>17549</td>
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<tr>
<td>Completeness (%)(^b)</td>
<td>98.8 (91.7)</td>
<td>94.22 (94.5)</td>
<td>93.4 (85.2)</td>
<td>99.3 (95.9)</td>
<td>98.8 (92.4)</td>
<td>93.4 (89.0)</td>
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<td>Redundancy(^b)</td>
<td>13.3 (13.8)</td>
<td>7.6 (7.7)</td>
<td>8.7 (7.8)</td>
<td>14.3 (12.9)</td>
<td>6.8 (6.6)</td>
<td>8.0 (7.4)</td>
</tr>
<tr>
<td>I/σ(^b)</td>
<td>28.5 (5.6)</td>
<td>23.7 (9.7)</td>
<td>20.5 (4.4)</td>
<td>31.1 (7.5)</td>
<td>20 (2.0)</td>
<td>26.5 (6.5)</td>
</tr>
<tr>
<td>R-merge(^b)</td>
<td>0.111 (0.927)</td>
<td>0.160 (0.637)</td>
<td>0.156 (0.978)</td>
<td>0.209 (0.217)</td>
<td>0.159</td>
<td>0.090 (0.453)</td>
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<td>Mosaicity (°)</td>
<td>0.05 – 0.73</td>
<td>0.04 – 0.87</td>
<td>0.07 – 0.41</td>
<td>0.11 (0.05)</td>
<td>0.03 – 0.39</td>
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<td>Number of atoms</td>
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<td>1142</td>
<td>1176</td>
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<td>1151</td>
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<tr>
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<td>98</td>
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<td>89</td>
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<td><strong>Refinement</strong></td>
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<tr>
<td>R-work(^b)</td>
<td>0.185 (0.199)</td>
<td>0.164 (0.178)</td>
<td>0.175 (0.190)</td>
<td>0.185 (0.175)</td>
<td>0.174 (0.210)</td>
<td>0.171 (0.201)</td>
</tr>
<tr>
<td>R-free(^b)</td>
<td>0.215 (0.232)</td>
<td>0.218 (0.232)</td>
<td>0.206 (0.263)</td>
<td>0.223 (0.235)</td>
<td>0.194 (0.250)</td>
<td>0.203 (0.253)</td>
</tr>
<tr>
<td>Ramachandran stats</td>
<td></td>
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<td></td>
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<td></td>
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</tr>
<tr>
<td>Most favored (%)</td>
<td>99</td>
<td>98</td>
<td>98</td>
<td>99</td>
<td>98</td>
<td>98</td>
</tr>
<tr>
<td>Allowed (%)</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Disallowed (%)</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Average B-factors</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein (Å(^2))</td>
<td>14.7</td>
<td>18.2</td>
<td>16.0</td>
<td>13.7</td>
<td>17.6</td>
<td>15.5</td>
</tr>
<tr>
<td>Hetero (Å(^2))</td>
<td>30.6</td>
<td>34.0</td>
<td>27.9</td>
<td>36.1</td>
<td>27.7</td>
<td>26.4</td>
</tr>
</tbody>
</table>

\(^a\) Merging of small data sets from multiple crystals for each structure, all analyzed on-chip at room temperature

\(^b\) Values in parentheses indicate values for the highest resolution shell
5.4 Conclusions

In summary, an X-ray transparent device for in meso crystallization and ligand soaking was developed and applied to a protein and its molecular inhibitor. The device was designed to operate in two complementary modes: (1) crystallization via dead-end filling and (2) soaking via through-flow displacement. After crystals were grown on-chip as previously discussed (Chapter 3), a ligand-rich solution was drawn from one end of the crystallization chambers to the other with the aid of suction to effectively displace the mother liquor with a new solution for the crystals to soak in. Three methods of suction were evaluated, and using a syringe pump in withdrawal mode and wicking with an absorbent material were the most effective for exchanging solutions without cracking crystals. The effectiveness of the soaking method and its sensitivity to ligand concentration and soaking time were evaluated through X-ray diffraction and structure building of a soaked lysozyme/tri-NAG complex. Notably, tri-NAG was observed in the structure at all soaking conditions, and increased occupancy was observed at higher ligand:protein ratios and soaking durations. Altogether, the microfluidic platform presented here provides a facile route to crystal growth and the incorporation of ligands in meso – compared to literature where many crystals are lost or damaged during the soaking process,11 crystals are never physically manipulated throughout the on-chip soak procedure. This feature preserves the quality of tens to hundreds of crystals as they are soaked simultaneously and then analyzed via in situ serial X-ray crystallography.

The on-chip crystal soaking procedure can be extended to study many proteins that crystallize in lipidic cubic phases and for other crystallographic techniques beyond ligand incorporation. For instance, to solve a membrane protein crystal structure de novo, crystallographic phasing data must be collected to solve the ‘phase problem’ through the soaking of heavy atoms or other anomalous scatterers into existing crystals. Alternatively, the growth of large crystals facilitates X-ray crystallography experiments, but membrane protein
crystals typically grow to small sizes. Pre-grown crystals can potentially be used as macroseeds, and the replenishment of membrane protein concentration in the mother liquor may result in the growth of larger crystals.
5.5 References

22. Schieferstein, J. M. et al. X-ray transparent microfluidic chips for high-throughput screening and


CHAPTER 6

Summary and Future Directions

6.1 Introduction

Three-dimensional structures of membrane proteins reveal key insights into molecular function, although crystallization is a major bottleneck to structures. Microfluidic platforms can address these bottlenecks and provide many advantages in scalability and automation for membrane protein crystallization. Through my PhD work, I have expanded on these capabilities by developing X-ray transparent microfluidic platforms that facilitate powerful, yet cumbersome crystallization techniques. The work presented in previous chapters represents methods to facilitate multistep protein crystallization formulation for two primary techniques: (1) microseeded and (2) in meso crystallization. The thin construction (<200 µm) of these devices facilitates serial in situ X-ray crystallography, a key technique that prevents both physical damage and radiation damage from accumulating in crystals. Further, novel features such as microwell layouts and the development of an ‘always-open’ capillary microvalve enable multi-step mixing and simplified handling of non-Newtonian lipidic phases.

6.2 Microfluidic Platforms for Microseeding Protein Crystallization

For most protein crystallographers, microseeding is typically used as a last resort optimization step due to the difficulty of implementation. The development of array chips is a noteworthy step toward routine usage for microseeded membrane protein crystallization. Using microfluidic array chips, microseeding was first demonstrated by pre-formulating a metastable protein-precipitant mixture for a model protein and its precipitant off-chip, and then filled into a microfluidic chip and mixed with dilutions of microscopic crystal nuclei solutions (microseed). This microfluidic technique provides a significant advantage for growing crystals for X-ray
diffraction because large crystals diffract better during X-ray crystallography. A second microfluidic device enabled microseed matrix screening (MMS) for screening many different compositions of crystallization solutions with microseeds. This devices separated mixing into a two-step process for on-chip formulation of metastable mixtures followed by addition of microseeds. In collaboration with the Gennis group, microseeding devices were applied to a membrane protein with a crystallization condition known to produce mediocre crystals, cytochrome $b_{o3}$ oxidase. While not resulting in a high-resolution crystal structure after optimization, a thorough search of the protein-precipitant phase diagram with microseeds helped identify distinct composition regions where large crystals could be reliably grown.

The studies here demonstrate the proof-of-principle for microseeding and MMS in microfluidic compartments applied to both soluble and membrane proteins. These devices can be further applied for (1) screening for and optimizing crystallization conditions for proteins of unknown structure, (2) surveying crystallization phase diagrams and solubility boundaries, and (3) implementing heterogeneous microseeds (non-protein, e.g. pulverized horse hair, cat whiskers, or seaweed) for proteins that do not produce crystalline results for microseed stocks.

6.3 Microfluidic Platforms for In Meso Crystallization

In meso crystallization remains the preferred technique for membrane protein crystallization because it typically produces high quality crystals. Despite the development and implementation of special tools and robots over the past 20 years, in meso crystallization remains a difficult technique to use for many crystallographers. Building from the efforts in the development of a microfluidic lipidic mixer by Perry et al.,¹ the work detailed in Chapter 3-5 represent the development and applications of the first microfluidic X-ray transparent devices for in meso crystallization. By relying on passive mixing,²,³ where membrane protein solution mixes into the lipid by diffusion, rather than active mixing,¹ on-chip formulation became a much simpler and repeatable process. After the development and application of the chip for proof-of-principle
studies, the work was expanded into routine crystallization tasks of (1) high-through screening and optimization, and (2) in situ crystal soaking. Notably, the work here meets or exceeds the sample usage (<10 nL of protein per well) and level of automation (high-throughput formulation, soaking) in state-of-the-art in meso crystallization techniques. Through these applications, three protein structures were solved: room temperature structures of a reaction center, a mutant of a reaction center, and a lysozyme/inhibitor complex. Several crystallization hits were identified for proteins of unknown structure, although none yielded a crystal structure.

There are a few future directions for in meso microfluidic devices. The development of these in meso microfluidic devices is mature enough where they can be implemented for routine use in research labs on a small-scale. The Kenis group has shared devices and documentation with a few research labs in the crystallographic community for crystallization screening and optimization for membrane proteins of unknown structure, as well as for soaking of membrane protein crystals, and these works are ongoing. There are opportunities to extend the capabilities of in meso soaking devices to new applications, such as de novo phasing and in meso seeding. De novo phasing techniques, such as isomorphous replacement or anomalous dispersion, are also difficult tasks for in meso crystallization because they require post-crystallization incorporation of heavy atoms or other scatterers. A method for in meso seeding is also appealing because membrane protein crystals are often small or too small for crystallization. Recently, a benchtop approach has been demonstrated where small crystals are pre-grown in a mesophase, which is then used for a crystallization trial. With the soaking chip, a two-step seeding process can be developed where small crystals are first grown on-chip, then those crystals act as seeds for larger crystals when the protein concentration is replenished by solution replacement.
6.4 Concluding Remarks

Despite the noted advantages to using microfluidics for microseeding and in meso crystallization, most laboratories do not have the capabilities of producing devices for their own experiments. A commercial route to crystallization chip production would be ideal, although most companies in the microfluidic sector refrain from using PDMS due to issues with scale-up and manufacturability. The industry standard materials, such as polymethylmethacrylate or polycarbonate, do not exhibit the same elastomeric behavior as PDMS which is necessary for the implementation of normally open or normally closed valves. Either new manufacturing methods need to be explored or developed for producing multilayer PDMS elastomer devices, or thermoplastic elastomers (e.g., SEBS) that are more amenable for processing must be substituted for PDMS.

In summary, the work presented here represents a significant step forward in the implementation of microseeded and in meso crystallization coupled with in situ crystallography. These microfluidic approaches address key experimental requirements of low sample consumption, high-throughput automation, and in situ analysis. There are several future directions for these projects, and the solution of a membrane protein target of unknown structure would demonstrate the full potential for microfluidic crystallization devices. A wide-scale implementation of the devices detailed in this dissertation, which would get these technologies into the hands of expert protein crystallographers, could accelerate the membrane protein structural pipeline and help alleviate the crystallographic bottleneck.
6.5 References


