CRYSTALLIZATION OF ACTIVE PHARMACEUTICAL INGREDIENTS USING MICROFLUIDIC PLATFORMS AND MENISCUS-GUIDED COATING

BY

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DISSERTATION

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Chemical Engineering in the Graduate College of the University of Illinois at Urbana-Champaign, 2017

Urbana, Illinois

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Abstract

Long lead times and large development cost has led the pharmaceutical industry to evaluate and improve pharmaceutical development and manufacturing procedures. Emphasis has been placed on the connection between drug development and discovery, especially on characterization of candidate drugs earlier in drug development. High throughput screens yield multiple solid forms of candidate drugs that are later characterized and prioritized for further development. Another focus within the pharmaceutical industry has been to improved manufacturing practices with continuous manufacturing and online monitoring for quality control. Additive manufacturing, where drugs are assembled layer-by-layer, is a technique used to improve process development.

Microfluidics platforms have been validated for high throughput crystallization of pharmaceutical solid form screening of polymorphs, salts, and cocrystals, however the solid forms obtained are often poor quality crystals that can only be characterized by vibrational spectroscopy (e.g., infrared or Raman). In this work X-ray compatible microfluidic platforms were develop for on-chip characterization. Crystal growth was controlled by on-chip crystal seeding. This allowed for controlled growth of only one polymorph and growth of large diffraction quality crystals. Diffraction data was collected from crystals residing within the chip and the resulting crystal structure had similar resolution to the previously reported structures. This work validates the use of X-ray characterization on-chip.

Solid form screens of two model systems, piroxicam cocrystals and clofazimine salts, were conducted on and off-chip. The goal of this work was to determine (1) if microfluidic platforms would lead to good quality crystals suitable for X-ray diffraction and (2) if microfluidic platforms could facilitate solid form discovery. Concentration and solvent were varied to elucidated different crystal structures. Four solid forms of piroxicam cocrystals were identified via the on-chip screen. Three of the solid forms were good enough crystals for X-ray diffraction, and two of those solid forms had not been identified in off-chip experiments. Four solid forms of clofazimine salts were observed, three were grown in traditional glass vials and one was grown in a microfluidic platform. Three of the solid forms had not been previously reported including the solid form grown in the microfluidic platform. Both works yielded the discovery and crystal structure of solid forms from crystals grown within a microfluidic platform. This work emphasizes the need for multiple crystallization techniques used for high throughput screening of pharmaceuticals, possibly including the use of microfluidic platforms.
Meniscus-guided coating was explored as an additive manufacturing technique to make pharmaceutical thin films. Crystalline films were grown on a biocompatible polymer substrate and characterized by polarized optical microscopy to determine the film morphology and X-ray diffraction (powder and grazing incidence) to determine the molecular packing. Process parameters such as concentration of drug in solution and shearing speed were varied resulting in changes in the film morphology, thickness, and polymorph. Aspirin thin films displayed spherulitic and oriented morphology, however the molecular packing was of both morphologies was consistent with the reported crystal structure for aspirin. Films of ellipticine, a model drug, were found to also display multiple morphologies, and again the molecular packing of the different morphologies was the same. However, the ellipticine films did not match the reported crystal structure, the films pack in a second polymorph of ellipticine. Through this work meniscus-guided coating was a useful technique for growing thin pharmaceutical films and controlling the molecular arrangement of the model drugs. Meniscus-guided coating can expand the manufacturing approaches for pharmaceutical additive manufacturing.
To my family.
Acknowledgements

This work would not have been possible without the support, guidance, and encouragement of many people. First, I would like to thank my advisor, Dr. Paul J.A. Kenis, for his help navigating the ups and down of my graduate school journey. I thank him for being a constant supporter of my professional and personal development though graduate school. He has challenged me to become an independent thinker and strive toward excellence in anything I undertake. I would also like to acknowledge the support of Dr. Ying Diao, who provided me with an enormous amount of support and has treated me like one of her own students. I appreciate the great example she sets as an advisor and mentor.

I would like to thank Dr. Sachit Goyal who helped train me during my first year of graduate research. My undergraduate researcher assistants Lucas Gonzalez, Eric Kim, and Yifu Zhang were instrumental in the completion of my experimental research and success of my projects. Experimental help and support from Dr. Fengjiao Zhang, Tim Chung, Ge Qu, and Erfan Mohammadi was greatly appreciated.

Support from the Support for Under-Represented Groups in Engineering Fellowship Program and the National Science Foundation Graduate Research Fellowship Program helped fund my graduate studies and provided freedom to pursue interesting research directions. I would also like to acknowledge AbbVie Inc. who sponsored my first year of research. Working with Dr. Yuchuan Gong and Dr. Geoff G.Z. Zhang provided insight into real issues in pharmaceutical crystallization. Both contributed greatly to my development as an independent researcher. A recent collaboration with Dr. Rahul Keswani and Dr. Gus Rosania at the University of Michigan has led to some interesting work on biocrystallization, I thank them for the support and input on our work together. I would like to thank Dr. Danielle Grey, Dr. Jeffery Bertke, and Dr. Toby Woods from the School of Chemical Sciences X-ray Lab. All have been exceedingly helpful with crystal structure determination and ideas for growing better crystals. I greatly appreciate their patience with me and my unconventional crystallization platforms.

Lastly, I would like to thank all of the friends I have made throughout my graduate career; they have contributed to so many happy memories. Through the Graduate Committee in the Society of Women Engineers I have met inspirational women who have become great friends. Hector, my fiancée, is a constant source of support who was always there with a helping hand and a loving word. My parents and brother have supported and continue to motivate me throughout my career.
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Chapter 1

Introduction

The estimated cost of drug development has increased from $138 million to $1.3 billion from 1975 to 2005.\textsuperscript{1, 2} Increasing development costs and long turnaround times have led to an increased focus during drug discovery to ensure viability of the drug candidate prior to bringing the drug to market.\textsuperscript{3} In the early stages of drug discovery thousands of candidate drugs are screened for desirable physiochemical, biopharmaceutical, toxicity, and efficacy. Through this process thousands of candidate drugs are eliminated in from the development pipeline (Figure 1.1\textsuperscript{4}, image adapted).\textsuperscript{5} During the pre-clinical stage the candidate drugs are screened for scalability, intellectual property, and economic impact. Only few candidate drugs make it past the clinical studies and are federally approved. While more than 50\% of the candidate drugs entering clinical trials are lost due to efficacy and safety concerns, nearly 40\% are lost to patent issues and poor biopharmaceutical and physiochemical properties.\textsuperscript{6} The biological efficacy of an active pharmaceutical ingredient (API) is determined by the nature of the interaction with the physiological target (e.g., receptors and enzymes). However, the ability to synthesize, store, and administer the drug to the patient in a safe, stable, and cost effective manner depends largely on the material properties of the API.\textsuperscript{6-8}

Oral administration of pharmaceuticals is the preferred route when developing a conventional dosage for a new drug. In 2015, 53\% of novel drugs approved by the United States Federal Drug Administration (U.S. FDA) were orally administered, with injectable making up 40\%, and other making up 7\%. Although oral dosages can be formulated in several ways, about 60\% of orally administered new drugs are solids (tablets, capsule, and powder).\textsuperscript{9-12} The percentage of solid dosage forms for novel drugs approved by the U.S. FDA has been between 54-70\% from 2012-2015. Solid formulation is advantageous because it is often more stable than liquid counterparts, APIs are often manufactured, transported, and sold as solids, and manufacturing capacity to form tablets, capsules, and powders is in place.
1.1 Solid Form Screening

To strengthen the discovery and development interface, a ‘developability’ screen has been implemented.\textsuperscript{3, 13} The screen allows for sufficient pre-clinical studies to determine the physiochemical properties of candidate drugs. The knowledge gained during the discovery phase screen is used to develop drug formulations, storage, and delivery strategy.\textsuperscript{3} Ultimately the screen allows researchers to prioritize the selection of candidate drugs based on their probability of success and their potential for returns. Several physiochemical properties assessed during the developability screen are: solubility, ionization constant, surface activity, and stability. The aforementioned properties are dictated by the molecular interactions within the solid form. Candidate drugs can crystallize into multiple solid forms including polymorphs (same molecules with different molecular packing), cocrystals (multi-component solid with non-covalent interaction between candidate drug and small molecule), salt (multi-component solid with a proton transfer between the candidate drug and small molecule), solvate/hydrate (non-ionic multi-component solid with candidate drug and solvent or water), Figure 1.2\textsuperscript{14}, image adapted. To complicate the crystallization even further, salts, cocrystals, and solvates/hydrates can also exist as different polymorphs. It is advantageous to elucidate many solid forms to allow for better selection of the candidate drug, and to remove the possibility of forming a more thermodynamically stable solid form later in drug development. Additionally, if a candidate drug does not crystallize it can be formulated as an amorphous material (no long range order) and the physiochemical properties of the material will be different than the crystalline solid forms.

<table>
<thead>
<tr>
<th>Discovery</th>
<th>Pre-clinical</th>
<th>Clinical Trials</th>
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<td>10,000 compounds</td>
<td>250 compounds</td>
<td>5 compounds</td>
<td>1 approved drug</td>
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\textbf{Figure 1.1.} Pharmaceutical drug development pipeline showing the attrition of candidate compounds throughout the different stages of development.
Often, the solid forms of a candidate drug are screened using solution-based crystallization. Crystallization occurs when a solution is supersaturated (the concentration of the solute in solution is higher than the solubility limit). Techniques to supersaturate a solution include anti-solvent addition, solvent evaporation, and heating/cooling of the solution. In each of these routes, the rate of supersaturation is a critical factor that can lead to different crystallization outcomes. The candidate drug will be crystallized with different solvents, at different concentrations, and in the presence of different small molecules. The physiochemical properties of the resulting solid forms will be characterized. The solid forms are typically characterized with optical microscopy to note the crystallinity of the sample, crystal habit, crystal color, and average crystal size. Vibrational spectroscopy (IR or Raman) is commonly used to identify solid forms, and when the spectrum does not match any reported spectra a new solid form is identified.

**Figure 1.2.** Diagram illustrating the wide variety of solid forms that can be used to formulate pharmaceuticals.
Solid form screening is limited by the quantity of material present at early stages in drug discovery (~ several milligrams) and time consumption for large number of screening conditions. An ideal screen would require small amounts of material while maintaining a high level of accuracy in testing. Manual screening is often performed; however, it is time consuming and subject to error in measurements. Large robotic fluid handling systems designed for high throughput screens have shown to perform more screening conditions and require smaller amounts of material.\textsuperscript{7, 15, 16} However, the large robotic systems are expensive and do not provide the flexibility sometimes needed to induce crystallization for some drug candidates. An ideal screening technology would require minimal amounts of material, allow for high throughput screens, allow for easy characterization of solid forms, be easy to use and implement, and be adaptable to multiple crystallization routes. An enhanced screening technology could be instrumental in early stage drug discovery.

1.2 Microfluidic Platforms for Solid Form Screening

Microfluidic platforms that utilize less material per condition screened and are compatible with analytical techniques (e.g., optical microscopy, Raman spectroscopy, and X-ray diffraction) may be able to facilitate early solid form screening. Progress in microfluidics has resulted in the development of microfluidic platforms for pharmaceutical crystallization of different solid forms, such as polymorphs, salts, and cocrystals.\textsuperscript{17-24} Utilization of microfluidic platforms allows for solid form screening at earlier stages of drug development when much smaller quantities of APIs are available. For example, platforms based on droplets,\textsuperscript{21, 23} patterned surfaces,\textsuperscript{24} free interface diffusion,\textsuperscript{18, 20} antisolvent addition,\textsuperscript{17} temperature control,\textsuperscript{22} and evaporation\textsuperscript{19} have been employed for pharmaceutical crystallization. Some of the aforementioned platforms allow for crystal harvesting and subsequent analysis via traditional approaches\textsuperscript{25-27} and some allow for on-chip characterization which eliminates the need for manual crystal harvesting.\textsuperscript{18, 19, 28-30} In prior work, the Kenis group developed microfluidic platforms for pharmaceutical solid form screening employing FID, antisolvent addition, and solvent evaporation that allow for solid form identification via on-chip Raman spectroscopy.\textsuperscript{17,20} Part of the work described in this thesis focused on comparing on-chip and off-chip crystallization outcomes to ensure that similar solid forms can be generated in both approaches.\textsuperscript{31, 32} On-chip the crystallization occurred more frequently and the crystals that were obtained were higher quality than the crystals obtained during the off-chip experiments. The rate of supersaturation on-chip is slower compared to the off-chip experiments resulting in better crystals.
In the previous work, Raman spectroscopy was the analytic technique used to differentiate solid forms on-chip, but this technique cannot provide structural information. Microfluidic platforms comprised of X-ray transparent materials have been used for crystallization screening and X-ray data collection for protein crystals on-chip.\textsuperscript{28-30, 33} To move toward discovery and characterization of novel solid forms, microfluidic platforms need to be compatible with X-ray diffraction. It is imperative that the composition and structure of the solid form is known before moving to the next stage of development. Single crystal X-ray diffraction is the best way to determine the composition and structure of a solid form, but this technique requires a pristine crystal larger than 30 µm not typically obtained in a high throughput screen.\textsuperscript{34} A microfluidic approach for pharmaceutical solid form screening that (1) enables crystallization optimization by crystal seeding and (2) allows for on-chip X-ray data collection from crystals formed on-chip is described. The microfluidic seeding approach was validated using a model drug, carbamazepine, and a model cocrystal, carbamazepine / 4-hydroxy benzoic acid.

Previous work validated that microfluidic platform can be used for crystallization screens. In this work, I will use microfluidic platforms for solid form discovery. In this work, a cocrystal screen was performed using a simple microfluidic platform. This single well platform simulates the experimental conditions of a 96 well plate (typically used for high-throughput screening), while utilizing less mass of sample per condition screened and providing more control over the rate of solvent evaporation. A model cocrystal, piroxicam / 2,5-dihydroxybenzoic acid was chosen since 3 solid forms were identified, but no crystal structures were reported.\textsuperscript{35} In this work a solvent and concentration screen led to the discovery and of new cocrystals.

1.3 Additive Manufacturing of Pharmaceuticals

The US FDA has encouraged pharmaceutical companies to improve product quality by focusing on process development and manufacturing.\textsuperscript{36} These efforts have stimulated innovation in more efficient pharmaceutical manufacturing processes, one of which being the implementation of additive manufacturing techniques. Additive manufacturing is a general term used to describe the assembly of 3-dimensional objects by joining multiple 2-dimensional objects, or layer-by-layer assembly. This technique allows for the rapid manipulation of design since there is no need for retooling of machines. Process parameters can quickly be changed providing more diverse products. Instead of a pharmaceutical company making drug formulations with two or three concentrations of API, a specific formulation can be made for each patient. Customize dosage and design of drug
release profiles for a specific purpose and person are two of the claims achievable through additive manufacturing.  

Most of the current additive manufacturing techniques for pharmaceuticals is based on a drop-on-demand production,\textsuperscript{38, 39} where material is added dropwise onto a substrate until a final formulation is achieved. These techniques provide highly controllable API deposition onto an edible substrate, and can be made compatible with solvent-based and melt-based systems. These systems often incorporate some type of on-line monitoring for quality-by-control purposes. An integral step of additive manufacturing will be the use of real-time monitoring for product control and quality.\textsuperscript{40} The US FDA is encouraging consideration of several factor during development including, materials, design, printing, and post printing validation, printing characteristics, printing parameters, and physical and mechanical properties.\textsuperscript{41} Different techniques for additive manufacturing are desired to increase the applicability and scope of additive manufacturing. An ideal technique would allow for large scale production, control over the crystal form and morphology, and compatibility with a wide range of drugs.

1.4 Meniscus-guided Coating for Additive Manufacturing

Meniscus-guided coating is a technique previously used to tune molecular packing and crystalline thin film morphology of small molecules and polymers. In this technique a thin layer of solution is deposited on a substrate and crystallization occurs as the solution is deposited. Commonly used for small molecule organic semiconductors, meniscus-guided coating may also be a good technique for pharmaceutical small molecule deposition. Growth of thin films have been documented to trap or stabilize metastable polymorphs,\textsuperscript{42} grow with different crystal morphology\textsuperscript{43} likely impacting the physiochemical properties of the material.

In this work meniscus-guided coating was investigated as a possible technique for additive manufacturing of APIs. Pullulan, a biocompatible polymer, will be used as the substrate and crystalline films of aspirin and ellipticine, two model drugs, will be investigated. The focus of this work will be to understand how process parameters such as solution concentration and shear rate affect crystallization outcomes. Control of film morphology, thickness, and polymorph will be discussed.
1.5 References

Chapter 2

X-ray Compatible Microfluidic Platforms

2.1 Introduction

To determine the crystal structure of API’s with X-ray diffraction, high quality crystals need to be obtained. This can be challenging because a wide range of supersaturation levels need to be screened to obtain detailed phase behavior information, which can then guide crystallization of diffraction quality crystals. Diffraction quality crystals can be grown by supersaturating a solution until it reaches the labile zone (nucleation zone) where nuclei form, then reduction of the supersaturation level of the solution to the metastable zone (crystal growth zone) will ensure growth of the nuclei into crystals, without additional nuclei being formed. In principle this sequence of steps can lead to monodisperse, diffraction quality crystals; however, precise control of supersaturation is not trivial. Furthermore, the stochastic nature of the nucleation process often results in a broad crystal size distribution as well as poor control over the solid forms obtained.

Some of the aforementioned issues can be overcome by implementing advanced protocols to decouple crystal nucleation and growth leading to more narrow crystal size distribution and better control over the specific solid form obtained. For example, the seeding approach, in which previously formed microcrystals are used to initiate crystal growth (thus eliminating the stochastic nucleation process), can be used to grow large and mono-disperse crystals. During initial screening, pharmaceuticals and proteins tend to crystallize as microcrystals, spherulites, fine needles, or poorly formed crystals. These crystalline solids can be micronized and then used as seeds in subsequent crystallization trials to enhance crystal quality. This process is reiterated until diffraction quality crystals are formed. The process of consistently introducing microseeds in a crystallization trial is challenging, although robots to automate this process have been used with some success. Still, obtaining single and isolated diffraction quality crystals remains a challenge. An inexpensive, high throughput technology that can simplify the seeding protocol and enable good control over seed

transfer would be instrumental in the crystallization and structure determination of solid forms.

Progress in microfluidics has resulted in the development of microfluidic platforms for pharmaceutical crystallization of different solid forms, such as polymorphs, salts, and cocrystals.\textsuperscript{9,16} Utilization of microfluidic platforms allows for solid form screening at earlier stages of drug development when only very small quantities of APIs are available. For example, platforms based on droplets,\textsuperscript{13,15} patterned surfaces,\textsuperscript{16} free interface diffusion,\textsuperscript{10,12} antisolvent addition,\textsuperscript{9} temperature control,\textsuperscript{14} and evaporation\textsuperscript{11} have been employed for pharmaceutical crystallization. Some of the aforementioned platforms allow for crystal harvesting and subsequent analysis via traditional approaches\textsuperscript{17-19} and some allow for on-chip characterization which eliminates the need for manual crystal harvesting.\textsuperscript{10,11,20-22} In prior work, the Kenis group developed microfluidic platforms for pharmaceutical solid form screening employing free interface diffusion, antisolvent addition, and solvent evaporation that allow for solid form identification via on-chip Raman spectroscopy.\textsuperscript{9,12} Similarly, microfluidic platforms comprised of X-ray transparent materials have been used for crystallization screening and X-ray data collection for protein crystals on-chip.\textsuperscript{20-23}

Here, a microfluidic approach for pharmaceutical solid form screening that (1) enables crystallization optimization by crystal seeding, and (2) allows for on-chip X-ray data collection from crystals formed. The approach was validated using a model API, carbamazepine (CBZ) as well as a cocrystal carbamazepine-4-hydroxybenzoic acid (CBZ-4HBA). The chemical structure for these

\textbf{Figure 2.1.} Chemical structures of carbamazepine and 4-hydroxybenzoic acid.
compounds can be found in Figure 2.1.

2.2 Experimental

2.2.1 Platform Design

The 72-well microfluidic platform used here (Figure 2.2) is an adaptation of a previously reported design. Briefly, each well was comprised of adjacent chambers for an API and microseed solution (Figure 2.2a). The dimensions of the adjacent chambers were varied to screen different volumetric ratios of the seed solutions and API solutions. Each well has a height of 50 µm, a width of 1 mm and a total length of 2 mm. The total volume of the wells was approximately 100-nL. The ‘length’ of the API and microseed chambers were varied to achieve dilution on-chip in ratios of 2:1, 1:1 and 1:2, as schematically shown in a 3 × 3 array in Figure 2.2b. Varying the relative size of the chambers changes the concentration of seeds transferred between the chambers. In this design, the microchannels utilize a ‘zig-zag’ to connect adjacent wells reducing issues with dead volume and bubbles and ensuring faster and complete filling of the chambers (Figure 2.2c). This platform design was ideal for performing crystallization screening experiments because numerous conditions could be screened within one experiment without consuming large quantity of sample. Further description of platform fabrication and utilization can be found in Appendix A.1.

2.2.2 Crystallization

Saturated Solution. A saturated solution of the CBZ in acetonitrile (0.16M) was prepared for crystallization experiments. For cocrystal experiments, a 4HBA (cocrystal former) solution in acetonitrile (0.16 M) was prepared.

Off-chip Crystallization. Seeds for on-chip seeding trails were obtained from crystallization of the CBZ and CBZ / HBA off-chip. A saturated solution of CBZ was allowed to evaporate in a glass vial covered with parafilm and a small hole poked in the top. The measured solvent evaporation rate was 6-24 µL/hr. The solvent was allowed to fully evaporate resulting in a deposition of CBZ crystals on the walls and bottom of the glass vial. The crystals were removed and powder X-ray diffraction (PXRD) data was collected from the off-chip sample and compared to published data to confirm the identity of the off-chip solids. To prepare seeds of the CBZ-4HBA cocrystal, an equal volume of CBZ and 4HBA in acetonitrile was dispensed into a glass vial and allowed to evaporate through the same procedure as described above.
Figure 2.2. (a) Cross sectional view of three microfluidic crystallization wells showing the layered assembly of the platform. Diffusional mixing occurs between adjacent chambers when the mixing valve (2) is actuated. (b) Top view of a 3x3 array of microfluidic crystallization wells showing adjacent chambers and two sets of valves for introducing solutions and mixing solutions. (c1-6) Schematic demonstration of how to operate the platform. (c1) Empty platform; (c2) valve set 1 is actuated to start filling of the wells; (c3) microseed solution or API solution is loaded into the crystallization wells; (c4) crystallization wells are completely filled, after which valve set 1 is closed; (c5) valve set 2 is opened and diffusional mixing between the adjacent chambers begins; (c6) valve set 2 is closed and diffusional mixing within the microseed and API chambers.

Seed Solution. Crystals were harvested from the off-chip crystallization experiments. First, approximately 1.5 mg of the API or cocystal crystals grown off-chip were harvested from the glass vial and carefully placed in the glass tube of a tissue homogenizer, and 600 μL of saturated API solution was added to the glass vial, yielding a solution with an approximate seed concentration of 2.5 mg/mL. The crystals were then pulverized using the homogenizer until no crystal agglomerates could be seen. The resulting seed stock solution was serially diluted with saturated drug solution with volumetric ratios of 1:2 (1.25 mg/mL), 1:5 (0.5 mg/mL), and 1:10 (0.25 mg/mL). Each
successive dilution contains fewer and fewer seeds. Seed stock solution preparation and off-chip dilutions are shown schematically in Figure 2.3.

Figure 2.3. (a) The identity of crystals grown off-chip were verified via powder X-ray diffraction. (b) The solid form crystals were crushed in a saturated CBZ solution to prepare a seed solution. (c) The seeding solutions are introduced on-chip along with CBZ solution (or premixed CBZ/4HBA solutions). Subsequently, the mixing valve is opened for 3 minutes to introduce seeds from the seed solution chamber to the adjacent chamber.

2.2.3 Characterization

Optical Microscopy. Optical micrographs of crystals were taken with a Leica M205 C stereo microscope. The number of crystals, crystal quality (clarity, defects, single), and crystal number were noted to determine the effect of seed concentration on crystallization outcome.

Powder X-ray Diffraction. Powder X-ray diffraction data was collected on a Rigaku Miniflex 600 in the Bragg-Brentano geometry. The data was collected from 3° – 80° 2θ with 0.02° steps and a 1.00 s detection time.

On-chip X-ray Diffraction. X-ray data was collected on crystals while they still resided within the microfluidic platform. Typically, crystals are mounted on a loop, cryo-cooled, and rotated 180-360° in the X-ray beam to collect a full data set. The typical data collection strategy would not work for our samples due to geometric constraints of the microfluidic platform. A strategy previously used for protein crystal data collection within a microfluidic platform was adapted to accommodate small molecule x-ray data collection on a microfluidic platform.²⁰,²⁴ Data collection was performed at the synchrotron source at Argonne National Laboratory. The modified data collection strategy is as follows. First, the size of the microfluidic platform was reduced by cutting out a few wells. The
section of the platform was mounted on a standard magnetic goniometer with a metal tube and set screw to secure the platform. Data was collected at room temperature using 1° steps with a 1s exposure at an X-ray energy of 18 keV ($\lambda = 0.689$ Å, LS-CAT 21-ID-D). The geometry of the microfluidic platform made complete rotation of the chip ($-180^\circ$ to $180^\circ$) for complete dataset collection challenging. Instead, small wedges of data (10-40° per crystal) were collected from a number of crystals. The frames were later merged together to form a complete dataset. This approach allowed for the collection of a complete dataset provided that the crystal orientation was random (not templated by the microfluidic platform). The employment of thin layers of PDMS and X-ray transparent COC maximizes transmission of X-rays through the chip materials, thereby enabling collection of high quality X-ray data from crystals formed on-chip. The characteristic scattering pattern of PDMS and COC observed in diffraction data collected on-chip occurs at relatively low angles of q-spacing or areas of low resolution diffraction (rings at 5.2Å for COC and at 7.5Å for PDMS) and does not affect the data collected at higher resolution.

X-ray diffraction data was indexed, refined, integrated, and scaled using HKL2000 software. The resolution range of the data was established based on the resolution shell at which $I/\sigma$ fell above 2.0. Subsequent processing of crystallography datasets was done using several programs including WinGx, Shelx, Shelxe, Shelxl, and Shelxs to identify the space group, generate the electron density maps, and generate the final crystal structure images.

Figure 2.4. (a) The microfluidic chip mounted at the Argonne beam line (LS CAT 21-ID-D). (b) Example of diffraction data for CBZ-4HBA. The background from the chip material can be observed in the form of dark rings at low resolutions. (c1) Crystals of different compounds in individual wells: (c1) CBZ-4HBA, and (c2) CBZ.
2.3 Results

2.3.1 On-chip Seeding

CBZ-4HBA. The co-crystal of CBZ-4HBA is known to have 3 polymorphic forms, designated as forms A-C. Two of the polymorphs are made up of a 1:1 ratio of CBZ : 4HBA (form A and form C), and the third polymorph is composed of a different ratio (1:X) of CBZ : 4HBA (form B).\textsuperscript{25} Form A is the most stable polymorphic form, with form C being metastable relative to form A at room temperature. In on-chip seeding experiments for CBZ-4HBA, Figure 2.5, fewer and larger crystals were observed upon increasing the dilution ratio from 1:2 to 1:10. This was expected since crystallization is primarily controlled by crystal growth of the co-crystals seeds. Similarly, when fewer co-crystal seeds were present (at higher dilution ratio) we saw a higher number of high quality crystals.

**Figure 2.5.** Results of the on-chip seeding experiment for CBZ-4HBA. The variation in the number and size of crystals can be observed as a function of seed dilution ratio (blue box) and microseed to crystallization solution chamber ratio (red box).
crystals, Figure 2.5. Polymorph form A was predominantly observed when microseeds of the same polymorph were used in the experiments. The seeds were confirmed to be form A by comparing experimental PXRD with simulated powder patterns from the known crystal structures. These results imply that seeding not only aids in growing single, isolated crystals but it also aids crystallization of one particular polymorph or form of the cocrystal. Seeding drastically increased the number of crystals compared to the control, Figure 2.5, and variation in the well geometry helped to control the number and size of crystals generated. When the length of the seed chamber to the adjacent chamber was varied from 1:2 to 2:1 the size of the resulting crystals decreased while the number of crystals increased for all dilution ratios, Figure 2.5.

**CBZ.** Four polymorphic forms of CBZ, designated as form I – form IV, have been reported.\(^{25}\) The most stable polymorph is form III, however the difference in energy from the most stable to the least stable polymorph is less than 0.7 kcal/mol.\(^{25}\) Crystallization control experiments on-chip resulted in a mixture of form II (needles) and form III (prisms) crystals. Employment of the seeding method directed the crystallization towards predominant formation of form III crystals. The seeds used in our experiment were polymorph form III confirmed with PXRD. In the control experiment, unit cell analysis of diffraction quality crystals was used to distinguish between crystal forms. Due to the presence of multiple solid forms, no on-chip diffraction data was collected from the control wells.

### 2.3.2 Structure Determination

The data in this study was collected at room temperature using the method described Section 2.2.3 under On-chip X-ray Diffraction. The crystal structure of CBZ-4HBA and CBZ were solved to a resolution of about \(\sim0.7\) Å. The on-chip crystals were randomly oriented and data collected from 10-15 crystals resulted in a completeness of 99.7% for CBZ-4HBA and 89.4%. CBZ-4HBA was found to have two CBZ and

**Figure 2.6.** Asymmetric units of CBZ-4HBA and CBZ solved from diffraction data collected on-chip.
2 4HBA molecules in the asymmetric unit, and packs in a monoclinic unit cell. The solved crystal structure closely resembles the reported crystal structure. The crystal structure for CBZ was found to have two CBZ molecules in the asymmetric unit and pack in a monoclinic unit cell. The solved crystal structure closely resembles the form III crystal structure previously reported. Relevant crystallographic data can be found in Table 2.1. The crystal structure were compared to previously reported structures deposited in the Cambridge Crystal Structure Database. The reference codes for the reported structures are MOXVIF and CBMZPN02, for comparison with CBZ-4HBA and CBZ respectively.

### 2.3.3 Advantage of Microfluidic Platform

The microfluidic platform developed for crystallization optimization provides a simplified crystal seeding method for growth of diffraction quality crystals. Use of pneumatic valves allows precise metering of small solution volumes with ease. The design of the platform with varying volumetric ratios of microseed solution and adjacent solution allows a wide range of crystallization conditions to be screened without extensive manual experimental setup (solution preparation and pipetting). In addition to simple experimental set-up, the microfluidic platform provides greater control over solution mixing and solvent evaporation. Diffusional mixing between seed solution and crystallization solution allows the concentrations within each chamber to gradually change, enhancing the chances of the supersaturation level maintaining the crystal growth zone instead of traversing into the crystal nucleation zone, Figure 2.7. By incubating the platforms in a solvent vapor atmosphere, the rate of solvent evaporation is further reduced leading to better crystal growth.

A key advantage of this microfluidic platform is the compatibility with solid form characterization methods (optical microscopy, Raman, and X-ray diffraction) eliminating the time consuming task of crystal harvesting followed by off-chip analysis of each crystal individually. In an on-chip experiment, identification of diffraction quality crystals with optical microscopy is relatively straightforward. With off-chip experiments, identification of single diffraction quality crystals among many poorly formed crystals can be challenging. In addition, thin crystals, often too fragile to be harvested, can be analysed on-chip.
Table 2.1. Crystallographic information for CBZ-4HBA and CBZ.

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<th>CBZ-4HBA</th>
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<td>298</td>
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<td>P 21/n</td>
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<td>density\textsubscript{calc} (mg/m³)</td>
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<td>goodness-of-fit of F²</td>
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<td>final T indices [I &gt; 2σ(I)]</td>
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<td>R₁ = 0.0395</td>
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<tr>
<td></td>
<td>wR₂ = 0.1512</td>
<td>wR₂ = 0.1119</td>
</tr>
</tbody>
</table>

Comparing the results of off-chip seeding experiments (in 1mL glass vials) for CBZ and CBZ / 4HBA with those of on-chip seeding, revealed that the outcomes of on-chip experiments typically are better in terms of the quality of the crystals obtained, the ease by which they can be found within a well, and the number of experiments leading to crystals. These outcomes of on-chip experiments are the result of better control over solvent evaporation and the addition of seeds (often too many seeds are added in an off-chip experiments, leading to showers of crystals), as well as better control over mixing of the seed solution with the crystallization solution.
2.4 Conclusion

In this chapter, a microfluidic approach to optimize crystallization and subsequently determined structures of pharmaceutical solid forms via on-chip X-ray analysis was reported. Specifically, a 72-well (8 x 9) array-based microfluidic platform was used to screen unique seeding conditions of CBZ and CBZ-4HBA by varying the volumetric ratio of the microseed solution to crystallization solution. Screening microseed dilutions along rows helped determine optimized conditions for crystallization of numerous crystals that are large enough for X-ray diffraction. In the case of carbamazepine, the seeding approach also aided in producing crystals of a specific solid form (form II) over a mixture of forms II and III. The platform was engineered to enable X-ray data collection on-chip by minimizing materials thickness which maximizes the transmission of X-rays. Data was collected on different crystals and later merged together to form a complete dataset and resulted in the determination of the structure of pharmaceutical solid forms. Key attractive features of these platforms are easy handling and simplified seeding while using small quantities of CBZ (about 5 microgram/well), and compatibility with X-ray diffraction. Ease of operation, requiring only a vacuum source, enables immediate application of these chips in laboratories for solid form crystallization.

Going forward, better control over crystallization outcomes would be expected if temperature control was integrated with the microfluidic platforms. A wider range of
supersaturation conditions could be screened and experiments could be conducted in the metastable zone (growth zone) to maximize the advantages of seeding. One can also foresee using this platform to study the effects of additives (polymers, surfactants, and antisolvents) on crystal morphology, polymorphism, and amorphous form stabilization.
2.5 References

Chapter 3
Microfluidic Platforms for Solid Form Discover

3.1 Introduction

In the pharmaceutical industry BCS Class II drugs are reported to make up about 60-90% of drugs in the pipeline. These drugs are characterized by low solubility across physiological pH range. Crystallization of these parent compounds with cocrystal formers (CCFs) provides a way to enhance the physiochemical properties of the solid form and improve their performance. Thousands of food ingredients and additives have been recognized by the U.S. Food and Drug Administration as being safe for human consumption and a majority of this list may be acceptable for crystal engineering of pharmaceutical solids. Since predictive cocrystal formation has not been fully developed, high throughput cocrystal screens with a large number of CCFs are often used to discover novel solid forms of a parent compound. Many techniques have been developed for cocrystal screening including solution based (e.g. evaporation, solvent diffusion, anti-solvent addition) and mechanochemical based (e.g., grinding, twin screw extrusion) approaches.

Although high throughput cocrystal screens can be effective at generating novel cocrystals, the success of finding a cocrystal often depends on the screening method chosen. Deeper understanding of cocrystal formation is needed to aid the more rational design of cocrystal screening experiments. For example, MacGillivray et al. has examined cocrystals of theophylline and caffeine to characterize intermolecular and intramolecular synthons. This work also provided a systematic study to determine synthon hierarchies in molecules with multiple potential hydrogen bonding sites. Additionally, researchers at times analyze the several hundred thousand crystal structures of organic compounds available in the Cambridge Crystal Structure Database (CCSD), to help predict possible new cocrystals between CCFs and target parent compounds, based on hydrogen bond lengths and angles observed in previously reported structures. Despite these efforts, current design of cocrystal screens typically incorporates minimal rational input, resulting in low success rates in most current

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cocrystal screening efforts.

Developing a deeper understanding of cocrystallization also relies on the ability to obtain the structure of the solid forms obtained. Many cocrystal screens, however, do not result in the formation of diffraction quality crystals. In those cases, bulk characterization methods such as powder X-ray diffraction (PXRD) or Raman spectroscopy are used to differentiate among starting materials, cocrystals, and cocrystal polymorphs. Knowing the crystal structure of the cocrystal is the only way to unequivocally identify what solid form has been crystallized. In the case of AMG 517, the API surprisingly cocrystallized with sorbic acid (present in only trace quantities) in the final formulation. The cocrystal of AMG 517 and sorbic acid drastically changed the physiochemical properties including increased exposures following an oral dosing to rats. This incident highlights the challenges in predicting solid formation and underscores the importance of solid form screening during drug development.

Several microfluidic approaches have been investigated as a way to study formation of pharmaceutical solid forms, enabling the screening of more conditions with small amounts of material. For example, in prior work we have demonstrated the use of microfluidic platforms to screen for cocrystals by diffusional mixing of an API solution and a CCF solution in adjacent compartments, at times in combination with solvent evaporation.

Here we investigate cocrystals of piroxicam (PRX) and 2,5-dihydroxybenzoic acid (HBA), Figure 3.1. PRX is a non-steroidal anti-inflammatory BCS Class II drug. Three cocrystals of PRX and HBA have been previously reported. All three solid forms were obtained by solution mediated phase transformation and characterized via Raman spectroscopy and PXRD. In this work, a simple

![Figure 3.1. Chemical structures of piroxicam and 2,5-dihydroxybenzoic acid.](image)
microfluidic platform consisting of arrays of single wells was used to screen for solid forms of PRX and HBA and identify crystallization conditions to grow diffraction quality crystals. This platform simulates the experimental conditions of a 96 well platform, while utilizing less volume of sample per condition screened and providing more control over the rate of solvent evaporation. We discovered several solid forms of PRX and HBA, two being cocrystals that had not been reported and another being a cocrystal that incorporates an impurity into its structure forming a solvated cocrystal. The aforementioned solid forms were characterized further via thermogravimetric analysis (TGA), powder X-ray diffraction (PXRD), and differential scanning calorimetry (DSC).

3.2 Experimental

3.2.1 Platform Design

The crystallization platform used in this study is composed of a 6 x 6 micro-well array and one set of valves to allow fluidic routing, Figure 3.2. Each micro well is about 1250 µm x 1500 µm x 60 µm and houses approximately 110 nL of solution. The platform fabrication and operation is detailed in the Appendix A1.\textsuperscript{19,28} Briefly, the platform is comprised of a thin poly-dimethylsiloxane (PDMS) control layer to control fluid routing and a fluid layer to provide fluid storage. The PDMS layers are sandwiched between a cyclic olefin copolymer (COC) support layer and a substrate layer. The COC layers provide physical rigidity and reduced solvent loss. The support, control, and fluid layers were irreversibly bound together, then this assembly was reversibly bound to the substrate.

![Figure 3.2](image)

**Figure 3.2.** (a) A 3x3 array of the microfluidic platform showing that the platform can be peeled apart to harvest crystals grown on-chip. (b) Magnified view of a single well as well as a cross sectional view of the chip to highlight the position of the valves, and the ‘zig-zag’ design used to decrease trapping of bubbles when filling the chip.
layer to allow for easy access to the solids crystallized on-chip by peeling apart the chip, Figure 3.2.

### 3.2.2 Crystallization Trials

*Single Crystal Growth.* Single crystals of PRX-HBA were grown on-chip from an acetonitrile solution. PRX was added to acetonitrile until saturation (~0.034M), and then an equimolar ratio of HBA was added to form a crystallization solution. The PRX and HBA crystallization solution was introduced on-chip. After filling, the chip was placed inside a Petri dish and sealed with Parafilm. After complete solvent evaporation (1 day), PRX-HBA crystals were observed on-chip via optical microscopy. Initially, the crystals were characterized on-chip using Raman spectroscopy; then, crystals were harvested from the chip for structure determination using X-ray diffraction.

Single crystals of PRX-HBA-ACT 2PRX-HBA were grown on-chip from an acetonitrile solution. PRX was added to acetonitrile until saturation (~0.034M), then a 1.5 molar ratio of HBA was added to form a crystallization solution. The PRX and HBA crystallization solution was introduced on-chip. After filling, the chip was placed inside a Petri dish and sealed with Parafilm. After complete solvent evaporation (1 day), the PRX-HBA-ACT crystals were observed on-chip. Only several 2PRX-HBA crystals were observed. The PRX-HBA-ACT and 2PRX-HBA crystals have different crystal habits and are easily distinguished with optical microscopy. The solid forms were characterized with on-chip Raman spectroscopy. The Raman spectra of PRX-HBA-ACT matched previously reported spectra.\(^\text{14}\) Crystals of both forms were harvested from the chip for structure determination.

*Bulk Crystallization.* Equimolar ratios of PRX and HBA were slurried in acetonitrile for 12 hours, to obtain gram scale quantities of PRX-HBA. The slurry was filtered to isolate the solids and the solids were allowed to dry. Bulk PRX-HBA-ACT cocrystal was crystallized by slurrying equimolar ratios of PRX and HBA in acetonitrile/acetone mixture (v/v = 2/1) for 12 hours. Excess acetone was added to the slurry to ensure complete conversion of the PRX-HBA-ACT cocrystal. The slurry was then filtered to isolate the solids and the solids were allowed to dry.

### 3.2.3 Characterization

*Optical Microscopy.* Optical micrographs of crystals were taken with a Leica M205 C stereo microscope to observe crystal habit and crystal color.
**Raman Spectroscopy.** On-chip Raman spectra was collected using a Renishaw microPL/Raman microscope equipped with a 785 nm excitation source and a Renishaw NIR 100 mW diode laser. Data was collected in the range of 600−1800 cm\(^{-1}\) at a spectral resolution of \(\sim 0.5\) cm\(^{-1}\) at 1800 gratings/mm. Spectra were obtained over a 10 s exposure and averaged over 2 accumulations.

**X-ray Diffraction.** Single crystal X-ray diffraction data was collected on a Bruker D8 Venture equipped with a four-circle kappa diffractometer and Photon 100 detector. An I\(\mu\)s microfocus Cu source supplied the multi-mirror monochromated incident beam. A combination of Phi and Omega scans were used to collect the necessary data. A single crystal was picked and mounted on a 0.3mm loop using paratone oil. The samples were cooled to 100 K or 222K (PRX-HBA or PRX-HBA-ACT, respectively) in a nitrogen supplied Oxford 700 Cryostream. Data was integrated using SAINT and absorption corrected using SAINT/SADABS v2012/1 or SAINT/SADABS v2014/2. The final structure was solved using SHELX-2013-4 or SHELX-2014-3.

**Powder X-ray Diffraction.** Powder X-ray diffraction data was collected on a Rigaku Miniflex 600 in the Bragg-Brentano geometry. The data was collected from 3° – 80° 20 with 0.02° steps and a 1.00 s detection time.

**Thermogravimetric Analysis.** Mass loss data was measured with a TA Q50. Approximately 10 mg of material was measured and placed in an alumina cup and loaded on a platinum pan. Samples were heated from 25°C to 250°C at 5 °C /min. Dry nitrogen was used as a purge gas with a balance purge flow rate of 40 mL/min and sample purge flow rate of 60 mL/min.

**Differential Scanning Calorimetry.** Cocryystals thermograms were obtained using a TA Q2000. Approximately 5 mg of material was measured and crimped into a small aluminium pan. Samples were heated from 25 °C to 225 °C at 5 °C /min. An empty sealed aluminium pan was used as a reference. Dry nitrogen was used as a purge gas at a flow rate of 20 mL/min.

### 3.3 Results

#### 3.3.1 Cocrystal Screen

Crystallization of PRX-HBA cocrystal was first carried out on-chip at 1:1 molar ratio in six solvents (mixtures), i.e. methanol, trifluoroethanol, acetonitrile, methanol/trifluoroethanol (v/v = 1/1), methanol/acetonitrile (v/v = 1/1), and trifluoroethanol/acetonitrile (v/v = 1/1). Thin needle-like crystals were observed in all solvents (mixtures) except in acetonitrile. The crystals collected in
the three solvent mixtures were too small to give acceptable Raman scattering. Raman spectra of the needle-like crystals grown in methanol and trifluoroethanol were collected and found to matched with that of Form 18B1 previously reported by Childs et al.\textsuperscript{14} Crystallization of PRX and HBA (PRX-HBA) in acetonitrile led to large prismatic crystals (Figure 3.3a) with an unique Raman spectra that had not been reported (Figure 3.3).

On-chip crystallization of PRX and HBA was then repeated in acetonitrile with a molar ratio of 1:1.5 and 1:2, respectively, to determine the impact of HBA concentration on the solid form. At both conditions, crystals collected on-chip presented two different morphologies, i.e. prism and plate, with more plate-like crystals at higher HBA concentrations. The Raman spectra of both crystals were collected on-chip. The plate-like crystals had the same Raman spectra as that of Form 18C as previously reported by Childs \textit{et al.} (Figure 3c).\textsuperscript{14} Several of the plate-like crystals had a different morphology and an unique Raman spectra that had not been previously reported. The prismatic crystals gave the same Raman spectra as the crystal identified in acetonitrile with PRX/HBA ratio of 1:1.

\subsection*{3.3.2 Structure Determination}

Single crystal X-ray diffraction was used to determine the crystal structure for the three solid forms identified in the crystallization screen. The three solid forms were found to be a 1:1 molar ratio of PRX:HBA, a 1:1:1 molar ratio of PRX:HBA:ACT, and a 2:1 molar ratio of PRX:HBA. Crystallographic data is summarized in Table 3.1.

The data collection for the PRX-HBA-ACT crystal proved to be challenging since the unit cell of the cocrystal changed with temperature. The first data collection was carried out at -200 °C, and although the diffraction data was not high enough quality to

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{example.png}
\caption{Figure 3.3. Images of (a) PRX-HBA , (b) PRX-HBA (2:1) and (c) PRX-HBA-ACT grown in acetonitrile and their Raman spectra.}
\end{figure}
resolve the crystal structure, the unit cell was determined to be triclinic. To facilitate single crystal data collection, crystallization was repeated off-chip with molar ratio of PXR/HBA = 1:1.5. A larger crystal with the same morphology (plate-like) was collected and tested using single crystal x-ray diffraction at -200 °C. The data suggested the same unit cell (triclinic) but again, did not give a reliable structure. Data collection was repeated after bringing the same crystal back to room temperature. Optical inspection of the crystal showed signs of crystal degradation. However, a fresh

### Table 3.1. Crystallographic information for all solid forms.

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crystal with the plate-like morphology collected from the same crystallization batch was found to have a monoclinic unit cell at room temperature. Following this observation, x-ray diffraction data of the monoclinic crystal was collected again at -50 °C to reduce the thermal motion. A full data set was collected and the structure of the crystal was successfully solved.

Acetone incorporation in the crystal structure was a surprise since acetone was not used in crystallization. The acetone is believed to be introduced as an impurity with HBA. This may be an excellent example on the impact of trace amount of impurity on the result of crystallization. To confirm the source of acetone further experiments were performed. Repeating the previously mentioned experiments using a different batch of acetonitrile yielded the same outcome, namely that the PRX-HBA-Act cocrystal was observed in the experiments with a molar ratio of PRX/HBA = 1:1.5 and 1:2. Since the acetone was believed to be present in the HBA, the HBA was heat-treated at 100 °C for 3 hours, presumably removing any remaining acetone. When crystallization experiments were repeated using this heat-treated HBA, only PRX-HBA cocrystals were obtained, strongly suggesting that the acetone indeed was an impurity in the HBA.

3.3.3 Crystal Structure Analysis

PRX-HBA. This cocrystal grows in the monoclinic space group P2 1/c with one PRX and one HBA molecule in the asymmetric unit. Hydrogen bonding occurs between the pyridine and amide nitrogen atoms of PRX and the carboxylic acid of HBA. The hydroxy substituent in the 5-

![Figure 3.4](image-url)
position on the HBA in the PRX:HBA cocrystal is hydrogen bonded to the hydroxyl substituent in the 2-position of a neighboring HBA. All hydrogen bonding occurs in one plane creating a 1-D wavy structure (Figure 3.4b).

**PRX-HBA-ACT.** This solvated cocrystal also crystallizes in the monoclinic space group P2₁/c with one PRX, HBA, and ACT molecule in the asymmetric unit. Hydrogen bonding occurs
between the pyridine and amide nitrogen atoms of PRX and the carboxylic acid of HBA. The hydroxyl on the 5 position on the HBA is hydrogen bonded to the carbonyl oxygen in ACT. The ACT molecules are arranged along the a-axis, which is evident when looking at a stack of several unit cells of this crystal structure along this axis (Figure 3.4d). This arrangement possibly allows the ACT molecules to escape from the crystal structure when heated (experiment described below) without interrupting the hydrogen bonding between PRX and HBA.

**PRX-2HBA.** The cocrystal contains a 2:1 ratio of PRX to HBA. Hydrogen bonding between the PRX and HBA forms layers in approximately the ab plane. The asymmetric unit of this cocrystal consists of two PRX molecules and one HBA molecule, with all atoms residing on general positions. The PRX molecules exhibit two different conformations in the crystal structure. In one conformation intramolecular hydrogen bonding exists between the amide oxygen atom and the hydroxide proton. Free rotation about the C—C bond allows a second zwitterionic conformation in which intramolecular hydrogen bonds exist between the amine proton and the ketone oxygen atom. An additional intramolecular hydrogen bond exists between the pyridinium proton and the amide oxygen atom. The HBA molecule is disordered over two positions rotated by approximately 180° in the plane of the aromatic ring with site occupancies of 0.809:0.191. The major orientation participates in intramolecular hydrogen bonding between the hydroxide substituent of the benzene ring and the oxygen atom of the carboxylic acid. The major orientation also participates in intermolecular hydrogen bonding. The minor orientation of the HBA molecule also displays intramolecular hydrogen bonding between the hydroxide substituent and the oxygen atom of the carboxylic acid but does not participate in intermolecular hydrogen bonding. Intermolecular hydrogen bonds between the PRX and HBA form hexameric units that propagate along the a-axis. These units pack into layers in the ab plane; the layers stack along the c-axis.

### 3.3.4 Thermal Characterization

A slurry method (see Experimental section) was used to obtain gram-scale quantities of PRX-HBA and PRX-HBA-ACT for thermal characterization. Raman spectroscopy was used to confirm the identity the slurried products.
**Thermogravimetric Analysis (TGA).** Thermogravimetric analysis was used to determine the temperature at which significant weight loss is observed, Figure 3.7. Heated at 5 °C/min, solids of the starting materials PRX and HBA, and of the PRX-HBA cocrystal remained thermally stable until 200 °C, at which temperature the solids degraded. No mass loss was detectable due to acetone desolvation from the HBA, possibly due to detection limits of the instrument. The solid of PRX-HBA-ACT lost ~10.4% of its weight at 80 °C, presumably due to the loss of acetone from its lattice. This mass loss is in close agreement with the theoretical value of 10.7%. PRX-HAB-ACT degraded at 200 °C when heated further.

**Powder X-ray Diffraction (PXRD).** PRX-HBA-ACT solids were held at 100 °C for 1 hour and at room temperature for 1 month to evaluate the physical stability of the solid form. Complete desolvation of acetone is expected in the heat treated sample based on the TGA data. The heat treated sample was cooled to room temperature and PXRD data was collected for all the samples, Figure 3.8. The heat treated sample was compared to the PRX-HBA and PRX-HBA-ACT starting materials. The spectra for the heat treated cocrystal solvate correspond exactly to the peak positions observed for PRX-HBA. The heated samples show slightly broader peaks when compared to PRX-HBA indicating that the heated samples have a smaller crystal size. Additionally, peak intensities of the heated samples do not directly correspond to the PRX-HBA, possibly due to preferred orientation of the heated samples. Comparison of these patterns with those of the non-solvated cocrystal PRX-HBA, suggests that the solid of PRX-HBA-ACT converts to the non-solvated cocrystal at elevated temperature. The PRX-HBA-ACT sample held at room temperature for 1 month showed similar spectra to the starting material showing that the acetone within the structure will not leave the material under standard conditions.
Differential Scanning Calorimetry (DSC). The thermal events of the cocrystals and each of their components were recorded while heated to 250 °C, Figure 3.9. Solids of PRX-HBA exhibited a sharp melting endotherm at 194 °C followed by a weaker endothermic peak at 202 °C. These peaks are tentatively attributed to sublimation of HBA, which shows peaks at 202 °C and 206 °C, or melting of PRX, 201 °C, that partially crystallized after melting of the cocrystal. Solids of PRX-HBA-ACT showed a broad endothermic peak at beginning at 65 °C and continuing to 104 °C likely due to desolvation of the cocrystal. The desolvated cocrystal melts at 194 °C followed by a weaker endothermic peak at 202 °C. The two cocrystals had the same melting temperatures and profiles suggesting that the solvate may have partially converted to the non-solvated cocrystal upon heating.

3.4 Conclusion

In this work, a cocrystal screen of PRX and HBA was used as a model system to investigate cocrystallization of BCS Class II compounds. Class II compounds make up the majority of the drugs currently in the pharmaceutical pipeline and increasing the solubility of the API, for example via
cocrystallization, could ultimately determine the fate of the API. A simple microfluidic platform was developed to perform a cocrystal screen by solution mediated phase transformation through solvent evaporation. The platform is comprised of a 6 x 6 array of single micro-wells that are isolated with normally closed valves to replicate the experimental conditions of a 96 well plate. The shallow wells (~60 μm) and transparent chip materials allow for easy detection of solid forms on-chip as well as subsequent characterization of these solid forms with Raman spectroscopy. The platform is easy to manufacture and operate and only requires a vacuum source and pipette for filling.

The crystallization screen yielded high quality crystals of three solid forms: a PRX-HBA cocrystal, a PRX-HBA-ACT solvated cocrystal and a 2PRX-HBA cocrystal. Acetone incorporation into one of the solid forms was a surprising find, and was attributed to an impurity in the HBA starting material. Bulk quantities of PRX-HBA and PRX-HBA-ACT were synthesized for physical characterization via TGA, PXRD, and DSC. With TGA the only observed noticeable mass loss from PRX-HBA-ACT, 10.4 wt%, attributed to desolvation of acetone. Investigation of the stability of PRX-HBA-ACT revealed that this solid form is stable at 25 °C. Upon increasing the temperature to 100 °C the PRX-HBA-ACT desolvated resulting in a PXRD pattern that is similar to the spectra obtained for PRX-HBA. In this work we demonstrated the utility of microfluidics in pharmaceutical drug discovery, in particular with respect to solid form screening and subsequent solid form characterization. The results presented here also underscore the importance of crystal structure determination to confirm solid form.
3.5 References

Chapter 4

Biomimetic Crystallization and Solid Form Screen

4.1 Introduction

Abnormal morphology of crystals has been a subject of significant study over the last century. Particularly, crystalline growth and distribution within living organisms such as cells, tissues or even clinically in humans has allowed us to understand how solid-state crystal chemistry can modulate their biological and biophysical environments. Specifically, the interaction of crystalline matter with cells such as macrophages within clinical microenvironments has garnered much attention. Macrophages (MΦs) are critical self-nonself recognizing immune cells capable of maintaining mammalian homeostasis and resolving inflammatory conditions responsible for many diseases. Concurrently, MΦs are cellular “vacuum cleaners” eliminating foreign matter, typically referred to as xenobiotics. Such elimination may also be preceded by massive bioaccumulation, self-assembly and intracellular crystallization of sequestered foreign molecular agents such as clofazimine (CFZ, Figure 4.1), a red-pigmented, anti-inflammatory, FDA-approved drug molecule. Specifically, CFZ accumulates within MΦs upon prolonged oral dosage in humans and rodent models (protocol in Appendix A.2) to form biocrystals containing clofazimine hydrochloride (CFZ-HCl) crystalline domains.

Mechanical properties of crystals (moduli, plasticity, elasticity) are known to be dependent on the atoms, ions or molecules forming the crystals and the interactions between these particles (molecular packing and intramolecular bonding). Further, recent work has explored the relationship between elasticity in organic crystals and their molecular and structural properties. Ghosh and Reddy observed an elastic organic crystal of caffeine-4-chloro-

Figure 4.1. Chemical structures of clofazimine.  

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3-nitrobenzoic acid cocystal with weak and dispersive C-H···π interactions in three nearly perpendicular directions, suggesting that elastic deformation is due to isotropic molecular packing.\textsuperscript{28, 30} Subsequently, Ghosh \textit{et al.} studied n-benzylidene anilines and describes design rules for elastic organic crystals.\textsuperscript{27-29} According to this study, elastic crystals should have (1) multi-directional weak dispersive bonds that can be easily broken to dissipate energy as the crystal is bending and that can be easily formed when no force is applied and (2) corrugated packing along a crystallographic face to prevent long range dislocation within the crystal.\textsuperscript{27-29} In their studies, when crystals did not align with these design rules the crystals did not demonstrate elastic behavior. As such, we explored if elasticity and curvature of biocrystals could be explained via these design parameters.

Additionally, in the prior work by Ghosh \textit{et al.}, they noted that polymorphs of the elastic organic crystals were not observed despite repeated trial.\textsuperscript{27} An additional aim of this work was to grow polymorphs of the biomimetic CFZ-HCl crystal with the hypothesis that a non-biomimetic CFZ-HCl crystal would be characterized by the reduced presence or absence of the weak, non-covalent interactions setup by the salt chloride ions.

4.2 Experimental

Information on biocrystal preparation can be found in Appendix A.2.

4.2.1 Crystallization

\textit{Solution Preparation}. Crystallization solutions were made by dissolving CFZ in methanol or acetone to a concentration of 2 mM (or 8 mM). When the CFZ was dissolved a small amount of 12 M HCl was added to the solution to create a 0.1 M HCl concentration. After the HCl is added the solution will change color, typically from a bright red to a dark red or purple depending on the solvent. This crystallization solution was allowed to evaporate in a glass vial and in a microfluidic device. Glass vial crystallization: Approximately 500 μL of the crystallization solution was dispensed into a 20 mL glass vial and allowed to evaporate very slowly. Once crystals were observed, the lid of the vial was tightened to prevent further solvent evaporation. Crystals are harvested with a metal spatula and mounted for XRD. Microfluidic crystallization: Approximately 30 μL of crystallization solution was used to fill a 6 x 6 array of single micro-wells (volume ~100nL). Once the device was filled, the device was placed in a petri dish and the petri dish was sealed with parafilm to reduce the rate of solvent evaporation. The solvent was allowed to completely evaporate (typically takes 1 day). Crystals were harvested by cutting the microfluidic device and peeling the device apart. Crystals are
mounted on a traditional magnetic loop for XRD. Detailed descriptions of microfluidic device fabrication and utilization have been previously reported in Chapter 3: Experimental Section and Appendix A.1.

**Crystallization of Bulk CFZ-HCl polymorph I.** Bulk CFZ-HCl polymorph I crystals were grown by adding HCl to a 2mM CFZ in methanol solution until the HCl concentration was 0.1M. After the solution sat for 5 minutes, water is added to the solution to double the solution volume. Within minutes, thin dark red crystals were observed. To grow diffraction quality crystals of CFZ-HCl, 2mM CFZ was dissolved in benzene and 0.1M HCl was added to the solution. The solution was allowed to slowly evaporate and red, rectangular plate-like crystals were observed.

### 4.2.2 Characterization

**Microscopy of Synthetic Crystals.** Optical micrographs of crystals were taken with a Leica M205 C stereo microscope.

**Powder X-ray Diffraction.** Powder diffraction data of isolated biocrystals was carried out as published26 with Bruker D8 Advance: Cu Kα radiation (λ = 1.5406 Å), tube voltage = 40 kV, and tube current = 40 mA. Data were collected at 2θ = 4° to 40° at a continuous scan rate of 2.5°/min. For CFZ-HCl crystals - data was collected on a Rigaku Miniflex 600 in the Bragg-Brentano geometry. The data was collected from 5° – 40°, 2θ with 0.02° steps and a 1.00 s detection time.

**Single Crystal X-ray Diffraction.** Diffraction data for CFZ-HCl (both polymorphs and CFZ-2HCl was collected on a Bruker D8 Venture equipped with a four-circle kappa diffractometer and Photon 100 detector. An Iμs microfocus Cu source supplied the multi-mirror monochromated incident beam. Diffraction data for CFZ-HCl-2MeOH was collected on a Bruker Kappa/ApexII CCD with Molybdenum source. A combination of Phi and Omega scans were used to collect the necessary data. A single crystal was picked and mounted on a 0.3mm loop using paratone oil. The samples was cooled to 100 K in a nitrogen supplied Oxford 700 Cryostream. Data was integrated using SAINT and absorption corrected using SAINT/SADABS v2014/4 or SAINT/SADABS v2014/2. The final structure was solved using SHELX-2014-4 or SHELX-2014-6.

**Curvature of Biocrystals.** The diattenuation images obtained using Polarization microscopy were used to quantify for curvature of biocrystals using a ThreePointROI plugin for ImageJ. Briefly, three points were marked along the maximum Feret’s length of the high diattenuation signal of the crystal.
which was used by the plugin to draw a circle through the three points and accordingly provide a radius of the circle (r). Curvature for this circle is then defined as $\kappa = 1/r$. Co-linear points that define an impossible circle resulted in a circle with $r = \pm 1$ or $2147483647$ pixels and were marked as $\kappa = 0$. A line was then drawn through the three points used to generate the circle to generate a chord of length (x) for that circle. Using the length of the chord and the radius, the angle formed by the biocrystal arc through that circle was then computed using the formula $\theta = 2\sin^{-1}(x/2r)$. The arc length of the biocrystal was then computed as $L = \theta . r$ where $\theta$ is in radians. Linear curvature density was computed as $\kappa/L$.

**Qualitative Bending of Synthesized Crystals.** Crystals that were about 2 mm long and ~20 µm thick were isolated from the crystallization solution and manipulated to demonstrate the crystal’s elasticity. Crystals were placed on a microscope slide in a small amount of water to prevent the crystal from moving off the slide. A pair of tweezers and a crystallization probe were used to manipulate the crystals. Video of this procedure was taken with a Leica M205 C stereo microscope.

### 4.3 Results

#### 4.3.1 Flexibility of Biocrystals

The physical morphology of biocrystals were analyzed from by harvesting peritoneal and alveolar MΦs from an 8-week CFZ-fed mouse, Appendix A.2. Through brightfield microscopy, several cell-associated dark red crystals were identified, some of which were surprisingly curved (Figure 4.2a). Moreover, after 10 minutes, the crystals turned over likely due to mechanical forces exerted by its parent MΦ (Figure 4.2a). Upon closer inspection, we observed that another biocrystal in close proximity to the curved biocrystal also had a minor curvature that straightened out when the curved biocrystal flipped (Figure 4.2a). In light of this empirical evidence, we further characterized the biocrystals for their curvature ($\kappa$) within or isolated from MΦs (Figure 4.2b). The curvature per unit length ($\kappa/L$) of biocrystals was significantly higher when present in peritoneal MΦs (4.8-fold change in mean) or alveolar MΦs (3.6-fold change in mean) compared to when isolated from MΦs. A greater number of biocrystals were found to be inherently straight or having zero curvature when isolated (26) than when present in peritoneal MΦs (10) or alveolar MΦs (18) (Figure 4.2b). Such a reduction in curvature of isolated biocrystals is indicative of forces acting on the crystals by MΦs.
The primary component of the biocrystals is CFZ-HCl, thus it was hypothesized that the inherent crystal structure of CFZ-HCl may play a role in the flexibility (being able to adopt non-linear morphologies) and elasticity (being able to return to the straight conformation) of the biocrystals. A synthetic crystallization route was developed to grow CFZ-HCl crystals with a similar crystal structure to the biocrystals. The synthetic CFZ-HCl crystals form dark red rectangular plates (Figure 4.3a) that closely resemble the crystal habit and color of the biocrystals (Figure 4.3b) as observed with brightfield microscopy. The CFZ-HCl crystals can grow to be much larger than the biocrystals allowing for optimal structural characterization. The p-XRD (Figure 4.3c) suggests a strong agreement in the observed peaks of CFZ-HCl with those from the biocrystals previously reported. Both samples show strong preferential orientation of the crystals along the (001) face. The most intense peak on the p-XRD spectra is the (002) peak due to a systematic absence at (00l) when l=2n for Pbcn space groups. Thus, the synthesized CFZ-HCl crystals are a suitable model for characterizing the mechanical behavior of the biocrystals.

**Figure 4.2.** (a) Peritoneal macrophages from a CFZ-fed mouse with the curvature of the curved biocrystal marked with dotted lines (white arrows show cell membrane, black arrows mark the crystal in physical contact with the first curved crystal). Scale bar – 10 μm. (b) Curvature per unit arc length (κ/L) of biocrystals in peritoneal Mφs or alveolar Mφs or when isolated from splenic Mφs. (n=100 biocrystals, * - p<0.005. The wider and thick horizontal line indicates the Mean of the distribution whereas the other smaller two lines indicate the Mean ± SE. Number in brackets indicates the number of biocrystals measured to have zero curvature.

### 4.3.2 CFZ-HCl polymorph I – Biomimetic Crystals

The primary component of the biocrystals is CFZ-HCl, thus it was hypothesized that the inherent crystal structure of CFZ-HCl may play a role in the flexibility (being able to adopt non-linear morphologies) and elasticity (being able to return to the straight conformation) of the biocrystals. A synthetic crystallization route was developed to grow CFZ-HCl crystals with a similar crystal structure to the biocrystals. The synthetic CFZ-HCl crystals form dark red rectangular plates (Figure 4.3a) that closely resemble the crystal habit and color of the biocrystals (Figure 4.3b) as observed with brightfield microscopy. The CFZ-HCl crystals can grow to be much larger than the biocrystals allowing for optimal structural characterization. The p-XRD (Figure 4.3c) suggests a strong agreement in the observed peaks of CFZ-HCl with those from the biocrystals previously reported. Both samples show strong preferential orientation of the crystals along the (001) face. The most intense peak on the p-XRD spectra is the (002) peak due to a systematic absence at (00l) when l=2n for Pbcn space groups. Thus, the synthesized CFZ-HCl crystals are a suitable model for characterizing the mechanical behavior of the biocrystals.
Single crystal XRD was also performed to determine the crystal structure of CFZ-HCl. The CFZ-HCl crystals grow in an orthorhombic Pbca space group with unit cell parameters; a=10.266 Å, b=19.828 Å, and c=24.156 Å, α=β=γ=90° and Z=8 (CCDC number: 1497722). One CFZ and one HCl molecule make up the asymmetric unit (Figure 4.4a). Cl3 hydrogen bonds with the CFZ through two N-H···Cl interactions. The data set was solved to 99.8% completeness. Some water was incorporated into the crystal structure that caused disorder in the isopropyl group, and the water had an occupancy of 0.13. A summary of the unit cell parameters and diffraction data can be found in Table 4.1.

Figure 4.3. (a) Optical micrograph of synthesized CFZ-HCl crystals and (b) harvested biocrystals from a CFZ-fed mouse spleen. (c) Powder X-ray diffraction data from both biocrystals and CFZ-HCl crystals. Both samples display preferential orientation favoring the (001) face. The diffraction data from 2θ=8-40° is zoomed in for convenient comparisons.

A key design feature of the CFZ-HCl crystal structure is the corrugated packing along the (001) face (Figure 4.4b,c). One CFZ-HCl molecule makes up one step of the zig-zag and together the steps make up one corrugated sheet. The steps of the zig-zag are held together with C-H···π and C-H···Cl interactions highlighted by light blue dotted lines and orange solid lines respectively (Figure 4.4b). Cl3 is situated in the crease of the zig-zags and serves an anchor by coordinating with 3 additional molecules strengthening interactions within the crease and coordinating with adjacent sheets. These interactions are in 3 nearly perpendicular directions. Weak dispersive bonding between
the sheets of the zig-zags from π····π interactions between the aromatic rings is also present (grey dotted lines, Figure 4.4b). Cl1 forms a C-H···Cl interaction that creates a ribbon of CFZ molecules when you look down the b-axis. When viewed along the c-axis this interaction is located in the crease and it stabilizes interactions between the sheets of the zig-zag. The weak multi-directional interactions are located at critical parts of the zig-zag and support the previously proposed design rules for crystal elasticity.27 The angle between the corrugated steps is ~82°. Face indexing of the crystal during XRD showed that the (001) face is the largest surface and which corresponds to the previous p-XRD data. The projection of the (001) face is shown in Figure 4.4c to better show the corrugated packing.

### Impact of Bending Crystals

Interestingly, while harvesting CFZ-HCl crystals for single crystals XRD, the crystals would noticeably bend when force was applied to pick up the crystals. As soon as the force was removed the crystals would promptly return to their original linear morphologies. Subsequently, video microscopy was performed of bending crystals with tweezers to further demonstrate their observed elastic response. Crystallization experiments yielded a very wide size distribution of crystals out of which crystals between 500 μm and 2 mm long and ~20 μm thick were chosen. The CFZ-HCl crystals typically lie with the (001) face normal to the substrate (Figure 4.5a). When a slight force was
applied, the crystal rotated 90° about the a-axis (Figure 4.5b). When a force was applied to the (001) face, the crystal bent (Figure 4.5c-d) and assumed its original shape upon removal of the force (Figure 4e). The crystal arched again when a second force was applied, Figure 4.5f. As such, the crystals can be bent many times without noticeable deformation to the crystal. In one experiment a crystal was bent 8 times without noticeable deformation. However, upon applying a larger force, the crystal snapped and the crystalline fragments adopted the original straight configuration.

To confirm the elastic deformation of CFZ-HCl, polarization microscopy was performed as a way of measuring the photoelastic phenomena that is commonly observed with application of stress and development of strain. In this particular study, linear dichroism could be measured using hardware configured on an epifluorescence-brightfield microscope (Appendix A.2). Crystals were imaged under polarized light following which the anisotropy in transmittance and importantly, the azimuth – defined as the polarization orientation that results in maximum transmittance were computed (Figure 4.6). Crystals were first identified as regions with low transmittance (Figure 4.6ac). Given the structural packing of CFZ-HCl lattice and absence of polycrystalline elements in the biocrystals, the azimuth distribution was expectedly isotropic across the crystal plane when unstressed and straight (Figure 4.6b). In contrast, the azimuth was anisotropic in the elastically deformed region of the crystal wherein two approximately orthogonal azimuthal orientations were measured (Figure 4.6d). In the inward region, the azimuth was perpendicular to

Figure 4.5. (a-d) A CFZ-HCl crystal was bent by applying a force with a crystallization probe while the crystal was held stationary against a pair of tweezers. While the crystal is bent it is thought that the angle between the corrugated packing on the (001) face decreases on the concave side and increases on the convex side of the crystal. (e) The force was removed and the crystal quickly regained the original position before (f) another force was applied to the crystal. Scale bar is 100 µm.
the long axis of the crystal whereas in the outer region, the azimuth was retained along the long-axis of the crystal as observed in the straight crystals. Such anisotropy is clearly indicative of a stress network resulting in the development of an elastic strain through the crystal.\textsuperscript{36} As such, given the extent of cross-interactions within the crystal lattice as described before (Figure 4.4), the development of an elastic stress-strain network through an expectedly isotropic crystal structure was easily accomplished and verified through photoelastic azimuth measurements.

Further, no differences in vibrational spectra (Raman) were evident before and after bending at the point of maximum curvature in these crystals (Figure A2.1). Naturally curved crystals also showed no differences in molecular vibrations compared to the reference CFZ-HCl crystals. Finally, the melting point for CFZ-HCl was measured to be 275°C (Figure A2.2), which is 53 °C degrees higher than CFZ while other salts of CFZ also have melting points <246 °C.\textsuperscript{37} As indicated by their melting points, the salts of CFZ are more stable than CFZ. The stabilization of CFZ as CFZ-HCl within mammalian cells therefore favors the formation of a highly stable crystalline polymorph and as reported here has appropriate structural packing features that allow it to adapt to intracellular mechanical stressors.

4.3.4 Salt Screen

Clofazimine is a weakly basic drug, and salt formation is initiated by a proton transfer to the imine nitrogen in CFZ (Figure 4.7). In a previous study where 6 salts of CFZ were grown and

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{image.png}
\caption{(a) Computed transmittance Image of a (a-b) straight vs. (c-d) bent crystal. Calibration bar in (a,c) shows the range of transmittance. Dark regions (with low transmittance) follow the crystal with the dotted tangential line from one edge of the crystal confirming the curvature in the crystal. Azimuth – polarization orientation of maximum transmittance overlayed on the transmittance image indicating unidirectional axes in the (b) straight crystal while in the (d) bent crystal, there is a bidirectional axes depending on the direction of compressional (inward bending edge leads to an orthogonal axes) or tensile stress (outward bending edge leading to a longitudinal axis).}
\end{figure}
analyzed, all the salts exhibited similar hydrogen bonding between the CFZ and the counter ion. The scheme in Figure 4.7 shows the expected proton transfer and hydrogen bonding to the Cl\(^{-}\) ion at high and low pH. The crystallization landscape of CFZ and HCl salts was explored as solvent, concentration and crystallization environment were varied. Crystallization was induced through solvent evaporation conducted in 20 mL glass vials and single well microfluidic platforms. Prior work indicates that microfluidic platforms facilitate the growth and identification of solid forms not observed in an initial off-chip experiments, possibly by changing the rate of supersaturation or decreasing the nucleation rate in a small volume. In glass vial experiments, solvent evaporated through a small hole poked in parafilm that covered the vial while in the microfluidic chip the solvent evaporated slowly through the PDMS. The microfluidic platform used in this work has been previously described. Crystallization experiments were conducted in methanol and acetonitrile, where the solubility of CFZ is sparingly soluble.

**Crystallization from Methanol.** Slow evaporation of a 2 mM CFZ and 0.1 M HCl methanol solution in a glass vial yielded long, thin, red rectangular plates near the edge of the solution. After a few days, very dark red rectangular plates were observed at the bottom of the glass vial, Figure 4.8. The crystals were extracted and XRD was used to determine the identity of the crystals. The long thin needles had a orthorhombic unit cell that matched previously reported data for CFZ-HCl; however the crystals did not diffract well enough to collect a full data set to publishable standards. The dark red crystals harvested from the bottom of the vial were found to be a polymorph of the biomimetic CFZ-HCl crystal structure which we designate as CFZ-HCl polymorph II. When the same crystallization solution was allowed to evaporate in the microfluidic device, only a few crystals were observed and the crystals had the same crystal habit as CFZ-HCl polymorph I. A different
concentration of CFZ in methanol was probed to determine the solid form dependence on concentration. Immediately after an 8mM CFZ and 0.4M HCl solution was prepared in methanol block-like red crystals were observed on the side of the glass vial (Figure 4.8). The crystals were a CFZ-HCl salt that incorporated 2 methanol molecules (CFZ-HCl-2MeOH) within the crystal structure. The solvent incorporation in the crystal structure was attributed to the rapid crystallization at high concentrations, so further experimentation at higher species concentrations was not pursued. When this solution was crystallized in a microfluidic platform no crystals were observed.

**Crystallization from Acetonitrile.** Slow evaporation of a 2 mM CFZ and 0.1 M HCl acetonitrile solution in a glass vial resulted in small dark red crystals suspended in the crystallization solution. The crystals had the same unit cell as CFZ-HCl polymorph II, but were much smaller. Crystallization of this solution in a microfluidic platform resulted in dark purple prismatic crystals, Figure 4.8. The crystals were cut from the microfluidic platform with a razor blade and harvested for XRD. The crystals were a higher order salt; a CFZ salt with two exogenous chlorine atoms (CFZ-2HCl). Crystallographic data for all crystal structures can be found in Table 4.1.

### 4.3.5 Structure Determination and Analysis

**CFZ-HCl polymorph II.** The asymmetric unit of CFZ-HCl polymorph II is made up of one CFZ molecule with N1 protonated and one Cl atom. There are two hydrogen bonds between N1-H···Cl3 and N2-H···Cl3 with bond distances of (D, d, θ: 3.153Å, 2.32Å, 167° and 3.197Å, 2.41Å,
There are two asymmetric units per unit cell and the ion pair crystallizes in the triclinic \( \text{PT} \) space group. Although there are no other hydrogen bonding interactions, there may be weak dispersive interactions (C-H⋯Cl) between Cl1 and the propyl group on a neighboring CFZ and Cl3 and neighboring CFZ. The crystal has long \( \pi \cdots \pi \) interactions along the (111) face, Figure 4.9. The distance between the ring centroids is 3.769(1)\(\text{Å} \), which is exceedingly far for \( \pi \cdots \pi \) stacking interactions.\(^{43, 44}\) The phenyl rings provide some steric hindrance which does not allow the CFZ molecules to come into closer contact.

CFZ-HCl polymorphs I and II seem to have different supramolecular features, even though the hydrogen bonding is very similar. Both crystals are largely held together by weak electrostatic interactions (C-H⋯Cl) with Cl3 and Cl1 interacting with neighboring CFZ molecules. One unique feature of the polymorph I crystal structure is that the CFZ molecules \( \pi \cdots \pi \) stack into a corrugated structure that allows the crystal to elastically

\[ \text{Figure 4.9. Asymmetric unit of CFZ-HCl solid forms plotted as } 50\% \text{ ellipsoids. The hydrogen bonding interactions are shown with orange dotted lines. The packing of several molecules for each crystal structure are shown. In all cases, the CFZs seem to create a layered structure with packing hindered by the phenyl rings of CFZ. In the CFZ-HCl polymorph structure the packing seems to be along the (111) face, while the other two structures show alignment up the } b \text{-axis.} \]
deform when a force is applied and return to the original structure when the force is removed.\textsuperscript{45} Elasticity within CFZ-HCl polymorph I is a unique property governed by the crystal packing and intramolecular interactions.\textsuperscript{27} Polymorph II does not have the necessary crystal packing features to allow for elastic crystals.

**CFZ-HCl-2MeOH.** The asymmetric unit of CFZ-HCl-2MeOH is made up of one CFZ molecule with N1 protonated and hydrogen bonded to a Cl molecule and two methanol molecules. Cl3 is hydrogen bonded to N1 and N2 in a similar fashion to the non-solvated solid forms with N1-H····Cl and N2-H····Cl bonds (3.168 Å, 2.33 Å, 174° and 3.231 Å, 2.36 Å, 168°, respectively), Figure 3. One of the methanol molecules is disordered over two positions and Cl3 is hydrogen bonded to the methanol molecules through a O-H····Cl bond (3.030 Å, 2.18 Å, 172° and 3.093 Å, 2.28 Å, 160°, for positions A and B respectively), Figure 4.9. The second methanol was highly disordered and atomic positions could not be resolved. The electron density from the disordered methanol was removed from the structure using the SQUEEZE function. The crystal grows as an orthorhombic Pbcn space group with 8 asymmetric units per unit cell.

**CFZ-2HCl.** CFZ-2HCl is comprised of one CFZ molecule with N1 and N4 protonated and 2 Cl molecules that are held together with hydrogen bonds. One of the hydrogen bonds is similar to the CFZ-HCl (1:1) salts where Cl3 is hydrogen bonded to N1 and N2 through N-H····Cl (3.147 Å, 2.27 Å, 176° and 3.139 Å, 2.30 Å, 173°, respectively), Figure 4.9. The second hydrogen bond is between N4 and Cl4 with a N-H····Cl (2.949 Å, 2.13 Å, 170°), Figure 4.9. Cl4 has occupancy of 0.76. The crystal grows in a P2\textsubscript{1}/c monoclinic space group with 4 asymmetric units in the unit cell. There are no other hydrogen bonding interactions within the crystal structure. The crystal is likely held together with weak electrostatic interactions with all the Cls participating. As in polymorph II, steric hindrance from the phenyl rings prevents the CFZ molecules from packing close enough to π····π stack along the (010) face.

The protonation of the second nitrogen was not necessarily expected because the computed pK\textsubscript{a} of that nitrogen is 2.31.\textsuperscript{42} The formation of this solid form within the microfluidic device could be due to nucleation suppression within the small volume.\textsuperscript{38-40} If enough solvent evaporates, the remaining solution will become increasingly acidic until the second nitrogen is protonated. Growth of CFZ-2HCl was repeatable within a microfluidic platform, but was not observed in our glass vial experiments.
### Table 4.1. Crystallographic information for all solid forms.

<table>
<thead>
<tr>
<th></th>
<th>CFZ-HCl (I)</th>
<th>CFZ-HCl (II)</th>
<th>CFZ-2HCl</th>
<th>CFZ-HCl-2MeOH</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>empirical formula</strong></td>
<td>C_{27}H_{23.26}Cl_{3}N_{4}O_{0.13}</td>
<td>C_{27}H_{23}Cl_{3}N_{4}</td>
<td>C_{29}H_{31}Cl_{3}N_{4}O_{2}</td>
<td></td>
</tr>
<tr>
<td><strong>formula weight</strong></td>
<td>512.18</td>
<td>509.84</td>
<td>537.46</td>
<td>573.93</td>
</tr>
<tr>
<td><strong>temperature (K)</strong></td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td><strong>wavelength (Å)</strong></td>
<td>1.54178</td>
<td>1.54178</td>
<td>1.54178</td>
<td>0.71073</td>
</tr>
<tr>
<td>Crystal system</td>
<td>Orthorhombic</td>
<td>Triclinic</td>
<td>Monoclinic</td>
<td>Orthorhombic</td>
</tr>
</tbody>
</table>
| **space group**      | Pbc
a (Å) | 10.266            | 8.754             | 12.073            | 14.151           |
| b (Å)                | 19.828             | 12.178            | 15.270            | 13.796           |
| c (Å)                | 24.156             | 12.589            | 14.612            | 28.103           |
| α (°)                | 90                 | 63.841            | 90                | 90               |
| β (°)                | 90                 | 80.740            | 111.04            | 90               |
| γ (°)                | 90                 | 83.940            | 90                | 90               |
| **volume (Å³)**      | 4917.10            | 1188.00           | 2514.20           | 5486.20          |
| **Z**                | 8                  | 2                 | 4                 | 8                |
| **density calc (mg/m³)** | 1.384            | 1.425             | 1.420             | 1.390            |
| **absorption coefficient (mm⁻¹)** | 3.562            | 3.679             | 4.229             | 0.369            |
| **θ range for data collection (°)** | 3.659 to 68.333 | 3.944 to 68.391 | 3.923 to 68.286 | 2.062 to 25.348 |
| **data/restraints/parameters** | 4500 / 134 / 365 | 4332 / 0 / 315  | 4613/0/329        | 5025 / 33 / 362  |
| **completeness to theta (%)** | 99.8             | 99.4              | 99.8              | 99.9             |
| **goodness-of-fit of F²** | 1.069            | 1.074             | 1.052             | 1.040            |
| **final T indices [I > 2σ(I)]** | R₁ = 0.0392 | R₁ = 0.0357 | R₁ = 0.0463 | R₁ = 0.0319 |
|                      | wR₂ = 0.0982      | wR₂ = 0.0800      | wR₂ = 0.0816      | wR₂ = 0.0823     |
| CCDC                 | 1497722           | 1507038           | 2507039           | 1507040          |

### 4.4 Conclusion

The inherent structural packing features of CFZ-HCl within biocrystals contribute to their elasticity and naturally adjustable curvature. As such, formation of biocrystals mediated by Mφs leads
to the development of elastic and curved crystalline elements with structural packing features that showcase the classical design rules designated for organic crystal flexibility\textsuperscript{27–30}. The presence of CFZ biocrystals in the Mϕ endows them with potent anti-inflammatory characteristics\textsuperscript{46, 47} and specific fluorescent\textsuperscript{48} and photoacoustic\textsuperscript{49, 50} signatures for cellular optical tracking. Such dual therapeutic and diagnostic (theranostic) applications make biocrystals a potent cellular device that could be harnessed for biomedical applications. Mϕ-mediated crystallization is an important example of how these cells self-assemble a crystal with features that allow for easier adaptability to the mechanical environment of the cell, thereby allowing a massive drug loading within the cell. As such, mechanical flexibility of exogenous elements within cells could be a critical design parameter toward engineering organic intracellular constructs to endow cells with unnatural yet stable and beneficial features for therapeutic applications. Finally, viscoelasticity and related mechano-transductive elements are innately connected to Mϕ inflammatory phenotype\textsuperscript{51, 52}. As such, the characterization of intracellular mechanical properties could be important for cell-based mechano-biological applications leading to a new class of pharmaceutics: mechano-pharmaceutics.

Additionally, the crystallization screen was successful at identifying other solid forms of biomimetic clofazimine hydrochloride salts. Solvent, concentration, and crystallization condition (glass vial or microfluidic platform) all played a role in solid form elucidation. Analysis of the crystal packing showed similarities between the hydrogen bonding and electrostatic interactions with all solid forms compared to the bio-accumulated CFZ-HCl crystal. However none of the solid forms discussed had the unique corrugated crystal packing within the crystals. Despite the multitude of interactions driven by the salt chloride ions within the crystal, the solid forms are structurally different from the original biomimetic CFZ-HCl crystal as well as the biocrystal itself.
4.5 References

Chapter 5
Additive Manufacturing of Pharmaceuticals by Meniscus-guided Coating

5.1 Introduction

The United States Federal Drug Administration (U.S.F.D.A.) has encouraged pharmaceutical companies to improve their manufacturing procedures through process analytical technology and quality-by-design procedures. An outcome of this effort has been the development of a new manufacturing strategy in the pharmaceutical industry called additive manufacturing. Through additive manufacturing a 3-dimensional object is made by the addition of multiple 2-dimensional objects, or layer-by-layer assembly. Additive manufacturing has been implemented in industries such as aerospace and medical device. One of the most attractive features of additive manufacturing is continuous processing and the ability to quickly change manufacturing conditions without the need to create new tooling. Coupled with online monitoring of product quality, it is possible to monitor product and quickly adapt process parameters to meet specifications. Additive manufacturing can lead to a decrease in waste and could solve some of the scale-up issues seem when moving from bench to pilot or full-scale production. A potential benefit of additive manufacturing is the potential of personalized medicine. Currently pharmaceutical companies determine the average recommended dosage based on clinical studies, however ideally the dosage and time-release profile would be tailored to the patient’s needs. Batch technologies do now allow for much flexibility in processing conditions without large changes to product quality.

Additive manufacturing has been previously demonstrated for pharmaceuticals. Many of the techniques are based on droplet-on-demand technology and utilize similar technology as an ink jet printer. Additional additive manufacturing techniques could help broaden the scope of and impact of use in the pharmaceutical industry.

Meniscus-guided coating is a solution deposition technique commonly used for alignment of small molecule organic semiconductors and conductive polymers. Through meniscus-guided coating thin crystalline layers of material are deposited on a substrate. Controlling process parameters, such as concentration of the solution and shearing speed the crystallinity and morphology of the film can be manipulated. In prior work, growth of thin films have been documented to trap or stabilize
metastable polymorphs\textsuperscript{6} and grow with different crystal morphology\textsuperscript{7} likely impacting the physiochemical properties of the material. This work explores meniscus-guided coating of pharmaceuticals as an additive manufacturing technique. Aspirin and ellipticine were used as model compounds to determine process parameters and printability for pharmaceutical thin films.

5.2 Experimental

5.2.1 Meniscus-guided coating

\textit{Solution Preparation.} Solutions of aspirin in ethanol were prepared by measuring out amounts of aspirin and adding the appropriate amount of ethanol to achieve the needed concentration in small vials. Aspirin would first be weighed out in a small scintillation vial, and then that value was divided by the desired concentration to obtain the volume of ethanol needed. The ethanol was then pipetted into the scintillation vial. The vial was lightly agitated to promote dissolution. It was important to be cautious to prevent solution from making contact with the lid of the vial in order to prevent contamination.

For ellipticine, the same method was applied. An appropriate amount of ellipticine was weighed out in a small scintillation vial, and then that value was divided by the desired concentration to obtain the volume of DMSO that needed to be added. The DMSO was then pipetted into the scintillation vial and the vial was lightly agitated to promote dissolution. This solution was then used so that dilutions could be made in order to obtain more samples of varying concentrations.

\textit{Substrate Preparation.} A 1x1 cm SiO\textsubscript{2} wafer was cleaned with toluene, acetone, and isopropanol, and was placed exposed to an oxygen plasma. Once the machine was started and the gas stabilization phase was turned on, the pressure was checked to ensure that it is around 200 mmHg. The plasma cleaned substrate was then spin coated with a solution of 5 wt\% pullulan in water at 1200 rpm for 30 seconds.

\textit{Printing on Wafer.} The computer programs EP901 and StreamPix were opened. In EP901, the settings were changed to Remote Mode and the motor must be turned on. The motor apparatus, the camera overhead light, the background light, blade and stage vacuums, and the heating system were also turned on. The heating system was set to 25 °C. On StreamPix, PixelLink and AVTMako cameras should both be turned on. “Set new sequence in RAM” was also selected. The SiO2 wafer was placed on the stage, on top of the vacuum, and aligned using the AVTMako camera. The
cleaned Si blade was placed on the blade vacuum, with the reflective side facing down, towards the stage. The blade was aligned with the wafer by looking at the AVTMako and adjusting the orientation of the blade until it was parallel to the wafer. The blade was then lowered to the wafer until the two almost touch, then it was raised by 10 µm. Through EP901, the blade was aligned to around 1 mm of the wafer front and the printing speed was then changed to 0.1 mm/s, or any of the speeds that were being screened. A pipette was used to pull 5 µm of aspirin solution and was dropped in a line on the wafer. As the printing speed decreased, more solution was needed to be deposited onto the substrate. On EP901, the printer was then set to drag 25 mm to the right. On SteamPix, the recording was then started. This procedure was repeated for all concentrations of aspirin. After each print of aspirin, the recording was stopped and the file was saved into .sequence and .jpg formats. Before starting to record a new print, “Set new sequence in RAM” had to be selected. For ellipticine, the same steps were repeated, however the temperature of the printing stage was set to 110 °C.

_Taking and Making Videos._ The .jpg files from a print were imported into a Windows Movie Maker new file. The duration was set to 1.00 and the file was saved as a Windows Movie Maker file. The images were then deleted from the Windows Movie Maker program and the new movie file was imported. The speed was then adjusted so that the overall movie duration was approximately 10 to 15 seconds long. This file was then saved again in a Windows Movie Maker file.

### 5.2.2 Characterization

**Polarized Optical Microscopy.** The light source was turned on and the dial was adjusted accordingly to the amount of light needed. The substrate was placed on the stage with a 10x magnification and then NIS Elements Viewer was opened on the computer. The live viewer was then enabled. The cross polarization was adjusted until the display of the microscope was as dark as possible. Likewise, the stage was rotated until the crystals displayed on the screen were also as dim as possible. The fine tune dial was turned until the image displayed on the screen was as clear as possible, and then the image was saved. The same was repeated for the 50x magnification.

**Powder X-ray Diffraction.** Data was collected on a Rigaku Miniflex 600 in the Bragg-Brentano geometry. The data was collected from 5° – 40°, 2θ with 0.02° steps and a 8.00 s detection time. The entire SiO₂ wafer/polymer/crystalline film was placed in a standard powder diffraction sample holder and the crystalline film was flush with the surface of the sample holder.
Single Crystal X-ray Diffraction. Diffraction data for ellipticine polymorph II was collected on a Bruker D8 Venture equipped with a four-circle kappa diffractometer and Photon 100 detector. An Iμs microfocus Cu source supplied the multi-mirror monochromated incident beam. Diffraction data for CFZ-HCl-2MeOH was collected on a Bruker Kappa/ApexII CCD with Molybdenum source. A combination of Phi and Omega scans were used to collect the necessary data. A single crystal was picked and mounted on a 0.3mm loop using paratone oil. The samples was cooled to 100 K in a nitrogen supplied Oxford 700 Cryostream. Data was integrated using SAINT and absorption corrected using SAINT/SADABS v2014/4 or SAINT/SADABS v2014/2. The final structure was solved using SHELX-2014-4 or SHELX-2014-6.

5.3 Results

5.3.1 Meniscus-guided coating Setup

The meniscus-guided coating setup is composed of a SiO₂ blade coated with a crystalline self-assembled monolayer (octadecyltrichlorosilane) to prevent wetting of the ink solution. The blade was moved with a linear stage motor digitally programed. The substrate is a SiO₂ wafer coated with a water soluble and biocompatible polymer, pullulan. Pullulan is a polysaccharide polymer often used in orally dissolving films for pharmaceuticals or novelty items such as mints. The solution that

Figure 5.1. Meniscus-guided coating diagram and chemical structures of aspirin, ellipticine, and pullulan. Depicting a crystalline film of aspirin or ellipticine on a pullulan substrate. As the coating blade is moved, the drug is solution crystallized to form a thin film of the model drug.
is sheared over the substrate was aspirin dissolved in ethanol or ellipticine dissolved in dimethyl sulfoxide (Figure 5.1).

5.3.2 Aspirin Thin Films

Processing conditions can play a large role in the packing and morphology of crystalline thin films. Concentration of the ink solution and the rate of shearing were varied to determine the effect of these processing conditions on film morphology. A morphology diagram was created to visualize the effect of these parameters on film morphology. Non-dimensional units were used for the diagram. Concentration of the drug in solution was reported as a weight percent which equals the weight of the drug divided by the total weight of the solution and the blade shearing speed was reported as a capillary number (Ca) which equals the shearing velocity multiplied by the viscosity and divided by the surface tension. Standard values for ethanol the viscosity and surface tension of ethanol are used in this calculation. Optical micrographs along with video taken during the printing are used to determine the morphology regime (Figure 5.2). For aspirin thin films there are two primary regimes, spherulitic growth and oriented growth. Between these regimes there is a transition region where both morphologies are observed. Spherulitic growth occurs by a two-step mechanism where a thin film of the drug in solution is deposited across the substrate. After a few seconds, spherulites begin to form and crystal growth is isotropic form the nucleation point. On the other hand, in the oriented growth regime crystal growth tracks the meniscus during printing. In this regime crystals growth is mainly in the direction of the blade shearing. Optical polarized microscope images of films in each regime can be found in Figure 5.2. At lower concentrations and faster shearing speeds (higher Ca number), spherulitic growth is expected. At higher concentrations and slower shearing speeds (lower Ca number) oriented growth is expected. From the morphology diagram, shearing speed seems play a larger factor in the film morphology.

Manipulating process parameters can change the thickness of the crystalline films. Shearing speed and concentration were varied and film thickness was measured with profilometry (Figure 5.2). Shearing speed was manipulated over almost four orders of magnitude from 3,000 µm/s to 5 µm/s while the concentration of the solution was held constant at 5 mg/ml. At slower speeds the film thickness increases and the standard error in the measurement is much larger due to uneven morphology of the oriented films. At faster speeds the film thickness decreases until it reaches a critical value of about 50 nm. After this point, even as shearing speed increases the film thickness is
not affected. This could be due to dewetting of the solution from the polymer substrate. Varying the shearing speed resulted in aspirin thin films with thicknesses from 50 nm to over 1.5 µm. Concentration of aspirin solutions varied from 2 mg/ml to 100 mg/ml with shearing speed held constant at 0.1 mm/s. Higher concentration solutions yielded higher concentration films with most films having oriented morphology. Concentration and film thickness have a linear relationship. In this study, films were printed on pullulan and plasma treated SiO$_2$. Wetting properties for the ethanol on both of these substitutes is slightly different resulting in a different slope of the

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**Figure 5.2.** Morphology diagram of aspirin thin films as a function of weight percent and capillary number. Optical polarized microscopy images are used to represent each film morphology. Film thickness was measured as a function of shearing speed and concentration of drug in solution. In the thickness versus speed graph the concentration of aspirin was 5 mg/ml and in the constant velocity graph the shearing speed was 100 µm/s.
concentration thickness graph (Figure 5.2).

Grazing incidence X-ray diffraction (GIXD) and powder X-ray diffraction (PXRD) data was collected for the spherulitic and oriented aspirin films, Figure 5.3. The collected spectrum were compared to the simulated power pattern for aspiring (CCDC ASCALA01). Data for both morphologies correspond well the simulated pattern, indicating they have the same crystal packing. The films are preferentially oriented with the (100) face growing out of plane.

**5.3.3 Ellipticine Thin Films**

Ellipticine is an anti-cancer drug with only one reported polymorph. A morphology diagram was created to visualize the effect of concentration and shearing speed on film morphology, Figure 5.4. In a similar fashion to aspirin distinct morphologies are observed. Dots are observed at high shearing speed and low concentration. The formation of dots is likely due to spherulites breaking into smaller pieces and dewetting from the substrate. Spherulites are not independently observed, but spherulites coupled with needles are observed. When both needles and spherulites are observed at low concentration the needles are randomly oriented and do not preferentially grow in the
As the concentration increases and speed decreases, the needles become more prevalent and the amount of spherulites decreases. The needles will also preferentially grow in the direction of meniscus-guided coating. With continued increase in concentration and reduction in speed, the needles grow into plates. At high concentration and the slowest printing speed, multi-phase crystals are observed where yellow needles grow on top of the plate morphology, Figure 5.4.

GIXD from samples of plates with yellow needles, plates, and needles with spherulites was collected to determine if the crystal morphology has the same molecular packing. The data was plotted with a simulated powder pattern for the reported crystal structure of ellipticine (CCDC ELLIPT). Data from the thin films did not match the reported crystal structure. A single crystal of a new polymorph of ellipticine was found and characterized (Appendix Table A.1). The simulated powder pattern of the new polymorphs was compared with the thin films spectra, Figure 5.5. The
spectra for plate and needles with spherulites morphology correspond well to each other and compare well with the peaks of the new polymorph. The plates with yellow needles have diffraction peaks that mostly align with polymorph II but a few that align well with polymorph I. From this information, the yellow needles that grow on top of the plates are thought to be polymorph I, while the underlying film is polymorph II.

5.4 Conclusion

Through this work, meniscus-guided coating was demonstrated as an additive manufacturing technique for pharmaceuticals. Meniscus-guided coating allowed for the control of film morphology, film thickness, and molecular stacking. Aspirin was used as a model compound to demonstrate the effect on film morphology and thickness as concentration and shearing speed were varied. Film thickness ranged from 50 nm to 1.5 \( \mu \)m. Ellipticine was used as a model compound to demonstrate control over molecular packing. Thin films of ellipticine polymorph II were primarily obtained, however at high concentration and low shearing speed multi-phase films of both polymorph I and II were observed. Both model compounds displayed different film morphologies as process parameters were varied, although the molecular arrangements for these morphologies were the same. Although much more work should be done to determine the viability of meniscus-guided coating for industrial

Figure 5.5. GIXD and simulated powder diffraction data for ellipticine films. The simulated polymorph I data corresponds to ELLIPT from the CCDC. The numbers at the top of the graph indicate the (hkl) values of the diffraction peak. The red (hkl) values correspond to peaks from polymorph II while black (hkl) values correspond to polymorph I. Ellipticine films with needle, or needle and spherulites seem to be polymorph II while the plates are composed of mainly polymorph II with a small amount of polymorph I. Along with the morphology diagram, this data can be used to grow a specific phase and morphology of ellipticine.
pharmaceutical application, meniscus-guided coating has proven to be a promising technique for controlling small molecule molecular arrangement for pharmaceutical thin films.
5.5 References

Chapter 6

Conclusions and Future Directions

6.1 Expanding Microfluidic On-chip Characterization

The crystallization platforms used in the Kenis group have evolved from traditional thick polydimethylsiloxane (PDMS) based devices to multi-layer platforms made from cyclic olefin copolymer and thin PDMS layers. These engineered platforms allowed for on-chip characterization with polarized optical microscopy and Raman spectroscopy.\textsuperscript{1, 2} The platforms have normally closed valves that allow for specific and precise fluid routing. Polar organic solvents such as alcohols and acetonitrile are compatible with the platforms, and in recent work platforms made from a perfluoropolyether instead of PDMS provide a much wider range of solvent compatibility to include benzene and hexane.\textsuperscript{3} Part of this work was to determine if the microfluidic platforms are compatible with X-ray diffraction.

The first challenge of doing X-ray diffraction on-chip is the need for large, single, unblemished crystals. Typically, the crystallization screens result in a mixture of small poorly formed crystals and single crystals. To improve crystallization outcomes, a crystal seeding approach was taken to decouple crystal nucleation and growth. Crystal seeding resulted in larger higher quality crystals as compared to the control experiments with no seeds. Crystal seeding also allowed for growth of only one polymorph of the carbamazepine – 4-hydroxybenzoic acid cocrystal compared to the control experiment where two polymorphs were observed. Diffraction data was collected from multiple crystals and merged to create a complete electron density map.\textsuperscript{4} The resulting crystal structure was comparable quality to the previously reported data.\textsuperscript{5} This demonstration underlies the powerful capability of on-chip characterization. This technique would be largely useful for small crystals that are not amenable with traditional X-ray crystallography, since data can be taken from multiple crystals and merged to create a whole dataset.

6.2 Microfluidics for Solid Form Screening

In previous work microfluidic platforms have been used to screen for polymorphs, salts, and cocrystals of APIs.\textsuperscript{1-3} This previous work was done to validate that microfluidic
systems could achieve the same crystallization outcomes as off-chip screens. However, often the crystals grown in the microfluidic platform were larger and better quality than the crystals off-chip. Part of my work was to determine if microfluidic platforms could be used for solid form discovery, instead of simply verification. A model system where 3 cocrystals of piroxicam – 2,5-dihydroxybenzoic acid were reported with Raman spectra but no crystal structure. Although Raman spectroscopy is good for differentiating solid forms, it is not able to provide structural information about the composition or the packing of the solid. Single crystal X-ray diffraction is an ideal technique to gain structural information about the solid. The goal of this work was to determine if a crystallization screen on a microfluidic platform would yield crystals of high enough quality for X-ray diffraction. A solvent screen and concentration screen yielded four different solid forms, two of them previously reported. Three of the crystals were high quality and characterized by X-ray diffraction. Interestingly, one of the previously reported crystals was a solvated cocrystal that incorporated an impurity, acetone, into the crystal structure even though acetone was not the solvent used during the crystallization. Through this work, microfluidic platforms are validated for improving crystal quality, when compared to the previous crystal screen. Additionally, the microfluidic screen identified two new solid forms of the cocrystal system.

### 6.3 Solid Form Screening for Biomimetic Crystals

After realizing that bioaccumulated crystals of clofazimine hydrochloride (CFZ-HCl) are bent by their environment, a collaborative project between the Kenis group and the Rosania group at the University of Michigan began. The goal of the work was to determine the underlying mechanism behind the flexibility of the bioaccumulated crystals. The biocrystals as well as CFZ-HCl crystals grown with the typical protocol were too small for single crystal X-ray diffraction. A solvent and concentration screen was performed on and off-chip. Four solid forms of clofazimine hydrochloride were observed: biomimetic CFZ-HCl polymorph I, CFZ-HCl polymorph II, CFZ-2HCl, and CFZ-HCl-2MeOH. Three of the diffraction quality crystals were grown on-chip and one of the solid forms was only observed on-chip. The inherent elasticity of the crystal was determined to be caused by two unique features of the crystal packing (1) weak multi-directional bonds and (2) corrugated packing. The additional solid forms form the screen may open up new therapeutic avenues
and again the use of microfluidic platforms in the screen helped elucidated additional solid forms.

6.4 Microfluidic Implementation in the Pharmaceutical Industry

Although there are many reasons to use microfluidic technology in the pharmaceutical industry, the industry has been slow adopt this technology. Lack of dedicated vendors selling microfluidic platforms, protocols outlining how the product should be used, and training on platform use could be the largest hurdle. Many microfluidic platforms claim to be easy to use or require few ancillaries, however an experienced used is often still required to consistently set-up the experiment. Additionally, each candidate drug often requires a slightly different crystallization protocol based on solubility or ease of crystallization and a standard microfluidic platform may not be able to accommodate these diverse needs. Even though there are existing challenges preventing microfluidic platforms from entering the pipeline in the pharmaceutical industry, microfluidics seem to be a beneficial supplementary technique for solid form screening or growth of single crystals.

6.5 Meniscus-guided Coating for Additive Manufacturing

Additive manufacturing of pharmaceuticals currently is dominated by drop-on-demand technology where active pharmaceutical ingredients (APIs) are dissolved in a solvent or polymer mixture and sequentially dropped onto an edible substrate. Manufacturing advantages to this technique are continuous processing, allowing for online monitoring and quality-by-control, and easy manipulation of process parameters. This would allow pharmaceutical companies to create personalized dosages and specific time release profiles, compared to the traditional one-size fit all approach. Meniscus-guided coating to create pharmaceutical thin films was explored as a technique to expand the additive manufacturing space. Aspirin and ellipticine were explored as model compounds to determine process parameters and printability for creating thin films. Printing conditions were varied to change the film thickness of aspirin from 50 nm to over 1.5 μm. Concentration and shearing velocity were varied to create a morphology diagram for both drugs. In both cases, distinct regions of film morphology were observed ranging from thick plates (high concentration, low speed) to anisotropic growth of spherulites or multi-directional needles (low concentration, high speed), and transition zones between them. For aspirin and ellipticine
both morphologies (oriented and spherulites) have the same crystal packing, although interestingly the crystal packing for the ellipticine film corresponds to an unreported second polymorph. Through this work meniscus-guided coating was demonstrated to be a useful technique for growing thin pharmaceutical films, and expanding the manufacturing approaches for pharmaceutical additive manufacturing.

6.6 Future Directions of Meniscus-guided Coating for Pharmaceuticals

There are many possible directions for meniscus-guided coating of pharmaceuticals. (1) Determine increase in solubility. Nanosizing is a technique currently employed in the pharmaceutical industry to increase the solubility of poorly water soluble drugs, however currently the methods to create nanosized particles are high energy and lead to a wide dispersion of particle size. Creating nanoscale thin films via meniscus-guided coating may be able to address the current challenges. (2) Creating multi-layered films. One challenge when formulating multicomponent API tablets or capsules is finding processing conditions that are appropriate for both APIs, another challenge is preventing the APIs from cocrystallizing. Creating multi-layered films separated by thin polymer sheets could avoid these challenges. (3) Roll-to-roll printing. Continuous manufacturing is of interest in the pharmaceutical community, to deduce the time and effort associated with batch processing. Roll-to-roll printing is a common technique used from the manufacturing of newspapers to organic semiconductors. This technique could be implemented for scaling the production of pharmaceutical thin films. (4) Online monitoring of product quality. Implementing analytical techniques into the production line could reduce product waste and improve manufacturing procedures.
6.7 References

Appendix A

A.1 Platform Fabrication and Assembly

Negative photoresist masters were fabricated using SU-8 2050. Two unblemished wafers (which will serve as the control layer and fluid layer masters) were rinsed with acetone and isopropyl alcohol, then dried with nitrogen gas and placed on a 95°C hotplate for at least 2 minutes so that they completely dry. Once the wafers were dried, SU-8-2050 was poured on the center of the wafer, then placed on the spin-coater. The control layer master was programmed to spin for 35 seconds at 2500 rpm. The fluid layer master was programmed to spin for 35 seconds at 2800 rpm. Once the wafers were coated, they are set aside for 5 minutes. The wafers were soft baked uncovered for 1.5 minutes at 65°C then 6.5 minutes at 95°C then allowed to cool. The appropriate mask and wafer were paired and a solid quartz block was placed on top of the mask. The assembly was exposed to the UV lamp for 23 seconds. Care was taken to not place the mask backwards and to not damage the quartz crystal. The masters were then hard bake for 1.5 minutes at 65°C then 6.5 minutes at 95°C, then allowed to cool. The masters were developed in a PGMEA bath for roughly 5 minutes with occasional stirring. Once all of the photoresist was dissolved, the wafers were washed with IPA and dried with nitrogen. Finally, the wafers were silanize to reduce adhesion of the PDMS used in the next stage (Figure A.1).

The control layer was made my mixing PDMS part A and B in a 5:1 ratio, and the fluid layer was made by mixing PDMS part A and B in a 15:1 ratio. The PDMS was vigorously stirred to ensure good mixing of the monomer and cross-linker, and then placed in the vacuum chamber to eliminate air in the PDMS. The masters were cleaned using tape to remove any particulate matter or fibers that may have fallen on them during storage. PDMS was then poured over the fluid and control layers and any bubbles or particulate matter was removed with a pipette. The fluid layer was spin coated at 1550rpm for 35 seconds and the control layers at 1150rpm for 35 seconds. The wafers were baked on a hot plate at 80°C, for 12 minutes and 5 minutes respectively. A sheet of COC and the control layer were activated with plasma. The COC was quickly aligned on top of the control layer and any bubbles were smoothed out. The assembly was baked at 80°C for 3 minutes, making sure to smooth out any additional bubbles that formed. The COC-control layer combination
was peeled off of the master, making sure not to let the device rip. Holes were drilled into the COC-control layer assembly to provide access to the vacuum lines and allow for operation of the valves. The COC-control layer sheet was aligned with the fluid layer under a microscope, heated at 80°C for 5 minutes, and placed in an oven at 60°C overnight. The COC-control layer-fluid layer assembly was peeled off the master and holes were drilled to provide fluid inlet ports. The assembly was then placed on a COC substrate layer and was ready to use.

A.2 Biocrystals

A.2.1 Biocrystal Harvesting

Mouse Model for Generating Biocrystals. Mice (4 week old, male C57Bl/6) were purchased from the Jackson Laboratory and acclimatized for 1 week in a specific-pathogen-free animal facility. Animal care was provided by the University of Michigan’s Unit for Laboratory Animal Medicine (ULAM), and the experimental protocol was approved by the Committee on Use and Care of Animals. Clofazimine (CFZ) was dissolved in sesame oil to achieve a concentration of 3 mg/ml, which was mixed with Powdered Lab Diet 5001 to produce a 0.03% drug to powdered chow feed.

Peritoneal Lavage to obtain Peritoneal Macrophages. Peritoneal lavage was done 8 weeks after the initiation of CFZ treatment. Mice were euthanized by exsanguination while deeply anesthetized by an
intraperitoneal injection of ketamine (100 mg/kg)/xylazine (10 mg/kg) followed by sterilization of the outer skin with 70% of ethanol. A small incision was made along the midline of the abdomen followed by abdominal skin retraction up to the thoracic boundary and the animal extremities to expose the intact peritoneal wall. A smaller incision was then made on the peritoneal wall to expose the cavity. The entire peritoneal cavity was washed with ice-cold sterile Phosphate Buffered Saline (PBS) + 5% of Fetal Bovine Serum (FBS) (5–10 ml) and collected as peritoneal exudate. The exudate was then centrifuged (100 x g for 5 min, 4 °C) and suspended in 1.5 ml of PBS + 5% of FBS. Cells were counted using a hemocytometer for viable cells using Trypan Blue and for biocrystal-containing cells. For preparation of microscopy slides, a 20 μl drop of cell suspension was placed on a glass slide and allowed to dry overnight in the dark. The following day, a single drop of Prolong® Gold was added to the dry slide and a cover slip was applied prior to imaging.

Alveolar Lavage to obtain Alveolar Macrophages. Mice were euthanized as described above and the trachea was surgically exposed and cannulated with an 18G luer stub and the lungs were lavaged to obtain alveolar exudate by instilling PBS containing 0.5 mM of EDTA in 1 ml aliquots for a total of 6 ml. The alveolar exudate fluid was centrifuged (400 x g, 10 min, 4 °C) and resuspended in RPMI 1640 media. Viable (using Trypan Blue staining method) and biocrystal containing cells were counted using a hemocytometer followed by preparation of microscope slides as mentioned above.

Isolation of Biocrystals. At 8 weeks post-drug feeding, mice were euthanized as described above and spleens were harvested and cut open to prepare tissue homogenate in phosphate-buffered saline (PBS). The tissue homogenate was sonicated for 30 min and centrifuged (100 x g for 1 min) to remove large cell debris. A solution of 10% sucrose in PBS was added to the acquired supernatant and the mixture was centrifuged (100 x g). The resulting supernatant was centrifuged (3200 x g for 20 min) to pellet drug inclusions which were then suspended in 2 ml of 10% sucrose in water (w/v). Biocrystals were further purified using a three-layer discontinuous gradient (50, 30, and 10% sucrose (w/v) in PBS) centrifugation method (3200 x g for 30 min, no brakes).

Microscopy of Biocrystals. Optical micrographs of crystals were taken with a Leica M205 C stereo microscope. The combination brightfield and diattenuation LC-Pol-Scope microscope set-up is a custom built microscopic imaging system similar to the birefringence LC-PolScope designed by Oldenbourg et al, but without the polarization analyzer. Our LC-PolScope is built on the Nikon Eclipse Ti inverted microscope, with the computer-controlled universal compensator placed between the interference filter (546±22 nm) and condenser lens. Illuminating light is narrowed to
546 nm by the interference filter, and the light is linearly polarized by passing it through a universal compensator, allowing for the diattenuation of the sample to be measured. The LC in the universal compensator is controlled by Image J “Micro-manager” software (Vale Laboratory, UCSF) and is automatically rotated to produce polarized light at 0°, 45°, 90° and 135° angles, respective to the horizontal, during image acquisition. The image maps of diattenuation, mean transmittance, and angle of high transmittance are generated by image analysis algorithms followed by calibration. Brightfield and fluorescence images were captured using the Nikon DS-U3 camera and Photometrics CoolSnap MYO camera system, respectively, under the control of Nikon NIS-Elements AR software. Microscopy slides of samples were prepared as mentioned before.

**A.2.2 Characterization of Biomimetic CFZ-HCl Crystals**

*Differential Scanning Calorimetry (DSC).* Samples were analyzed using a TA Instruments 2910 MDSC system equipped with a refrigerated cooling unit. All experiments were performed by heating the pre-weighed samples at a rate of 10 °C/min under a dry nitrogen atmosphere. Temperature and enthalpy of the instrument were calibrated using high purity indium standard.

*Raman Spectroscopy.* Crystal samples were synthesized and physically manipulated before being transferred onto a pure silicon wafer. Confocal Raman microscopy was performed using a WITec

![Graph showing Differential Scanning Calorimetry of CFZ-HCl. Melting Point indicated with curved arrow – 275.80 °C.](image)
alpha300 R equipped with a near-IR 785nm to minimize clofazimine’s fluorescence signal. Samples were positioned on the stage for spectral data acquisition and were observed using the reflectance illumination mode of the microscope. Once positioned, the 2 µm diameter 785 nm laser illumination spot was directed to the sample, and the Raman spectrum was acquired. Raw data were background subtracted from the signal obtained from pure silicon wafers and further baseline-corrected using Origin®.

Figure A.3. (a) Raman Reflectance Brightfield images of (top) short CFZ-HCl crystals and (bottom) bent portion of a long CFZ-HCl elastic crystal. Blue arrows indicate moving away from the confocal plane used to obtain the point spectra of CFZ-HCl. (b) Raman Spectra of CFZ-HCl reference crystals with crystals that were subjected to mechanical bending (before and after) and crystals that had an inherent curvature.
A.3 Ellipticine Polymorph II

Table A.1 Crystallographic information for ellipticine polymorph II.

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