X-RAY COMPATIBLE MICROFLUIDIC PLATFORMS FOR SCREENING, CRYSTALLIZATION AND *DE NOVO* STRUCTURE DETERMINATION OF PROTEINS

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ABSTRACT

We present here an X-ray compatible, array based microfluidic chip that functions as a complete crystallization platform. It is capable of screening multiple crystallization conditions using minimal amount of protein solution per trial and facilitates *de novo* structure determination by collecting and merging X-ray data from multiple crystals grown on-chip. Our approach bypasses issues associated with the need to grow large, high quality crystals, crystal damage during handling, cryocooling, and radiation exposure and enables *in situ* data collection at biologically relevant temperatures. To validate the method, we determined the structure of PhnA to 2.30Å resolution solely using data collected on-chip.

KEYWORDS: microfluidics, protein crystallization, *in situ* structure determination, SAD

INTRODUCTION

Protein crystallography is an important aspect of structural biology that provides information on the 3D structure of proteins and insight into their function. This intensive, multi-step process includes expression, purification, and crystallization of protein material, followed by harvesting of the crystals for X-ray analysis. Some of these steps have been improved recently, but major bottlenecks still exist in identifying crystallization conditions with limited material available and in avoiding crystal damage while manually harvesting the oftentimes small, delicate crystals for X-ray analysis [1].

Current methods in crystallography rely on a single protein crystal to give the entire structural data at cryogenic temperatures to avoid radiation damage [2]. This requires physically harvesting individual crystals from crystallization droplets. The manual handling, exposure to the environment during harvesting and cryo-cooling has the potential to lead to degradation of the crystal thereby affecting the quality of data collected. As a result of these issues, researchers try to find alternative crystallization conditions yielding more robust crystals of the same protein or the protein is abandoned in favor of crystallization of other, hopefully more robust proteins. Structural data from a protein crystal needs to be coupled with phase information which can be obtained experimentally through methods like single wavelength anomalous diffraction (SAD) or multi-wavelength anomalous diffraction (MAD), or through computational methods like molecular replacement [3]. For *de novo* structure determination, experimental methods are more useful since homologous structures of novel targets, as needed for molecular replacement, may not be readily available.

Microfluidics offers more precise control over composition and kinetics of a crystallization trial than what is possible using conventional methods such as vapor diffusion. Most of the microfluidic platforms for protein crystallization reported in literature to date require crystals to be harvested from the chip before they can be analyzed. Thus, the aforementioned issues handling, mounting and cryo-cooling encountered with traditional structure determination approaches still remain. These microfluidic platforms are usually made out of PDMS and/or glass, materials which both attenuate X-rays significantly. Alternative microfluidic chips fabricated out of more X-ray transparent polymers like cyclic olefin copolymer (COC), polyimide, poly(methyl methacrylate) have been largely simple in design and have not been able to capitalize on the integrated fluid handling capabilities that multi-layer microfluidics offers [4-7]. The microfluidic platform presented here retains the integrated fluid handling capabilities of traditional multi-layer devices necessary for high throughput crystallization, while achieving X-ray transparency, thus enabling *in situ* analysis.

EXPERIMENTAL DESIGN

Our hybrid chip consists of three layers of materials: COC for the control layer, a thin PDMS fluid layer for valve actuation, and a bottom substrate of COC or Duralar (Figure 1a). The control layer is made by hot pressing flat sheets of COC (4 mil, Topaz Chemicals) using an epoxy mold [8]. The feature height in this layer is 25 µm. The fluid layer is fabricated using standard soft lithographic techniques with a feature height of 50/100 µm and a total thickness of 70/120 µm. The control layer is aligned with the fluid layer and bonded using a silane based chemical surface treatment [9] and are placed on a COC/Duralar substrate. A dense network of normally closed valves (actuated with a vacuum pump) is used to fill in the chip with protein and precipitant solutions, as well as to mix the two solutions by actuating a valve between adjacent chambers, thereby inducing free interface diffusion (Figure 1b). The orange window structures (Figure 1) in the control layer serve to reduce the path length of X-rays through non- crystalline material.

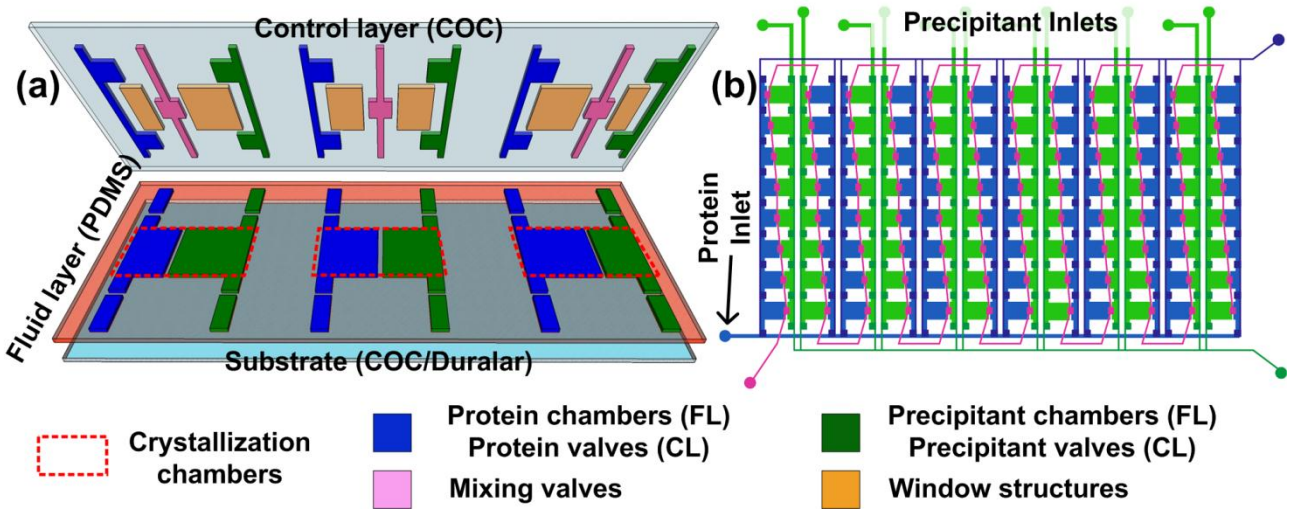

Figure 1: (a) *A 3D schematic view of 3 crystallization chambers of a microfluidic array chip for protein crystallization. The blue chambers in the fluid layer are filled with protein solution and the green chambers with precipitant solution. The ratio of protein-to-precipitant is varied in different chambers on the chip to allow for screening of different conditions. The three different sets of normally closed valves in the control layer are used to fill in the protein and precipitant, and to bring adjacent solutions into contact for mixing.* ***(b)*** *Schematic of a 96-well array chip with the ratio of protein-to-precipitant volume varying from 1:4 to 4:1 moving vertically down each column. (Window structures omitted for clarity)*

Attenuation of the X-rays when passing through the chip material, X-ray scattering due to the chip material and relative path length of the X-rays through protein crystal vis-à-vis chip material are the main issues, which have been addressed in our chip design. The attenuation of X-rays by the assembled layers (125 µm COC and a 20 µm PDMS membrane) is minimal and results in the transmission of 75-78% of the incident X-rays at an energy of 12.4 keV. The chip materials also produce a scatter ring (Figure 2c) contributing to background noise but this scatter is at a relatively low diffraction resolution and does not affect structure determination from high resolution data.

The 24- to 96-well array chips can be utilized for both screening and structure determination, using either varying or identical well ratios. The varied well design (Figure 1b) allows for covering a larger sample space for possible crystallization conditions using minimal protein solution. Once identified, the appropriate crystallization condition is repeated in a chip with identical wells to obtain a large number of isomorphic crystals. Slices of data are collected from these crystals at room temperature and merged to obtain the final structure of the protein, whereas traditionally a full dataset is collected from a single, large cryocooled crystal.

RESULTS AND DISCUSSION

We use 3x2-cm^2 array chips of 24 to 96 individually addressable wells, ~50 nL in volume each, to screen multiple crystallization conditions (Figure 1b). Each screen uses less than 5µL of protein solution. To validate our approach we set up the 50 condition Crystal screen kit (Hampton Research) using lysozyme in both our array chips and traditional microbatch well plates. After a week, crystals were observed in 32 out of the 50 conditions in our chip as compared to 26 on the well plate. More unique hits were observed in the chips, validating the performance of these platforms for *de novo* crystallization trials. The differences between the microfluidic and traditional results can be explained in terms of the difference in mixing of protein and precipitant solution, generation of a controlled and slow concentration gradient in the chip and stochastic nature of the crystallization trials. Because the chips are X-ray transparent, easy differentiation between protein and salt crystals as wells as an initial estimate of the crystal quality can be made very easily.

The size of the chip and spatial limitations at the beamlines make cryo-cooling the entire chip infeasible. We use an alternate strategy wherein, a large number of isomorphic crystals are grown in an array chip and data is collected at room temperature. The whole chip is mounted in the X-ray beam (Figure 2a) and 10 shots of data (± 5° from normal, 1s) are collected from each crystal. The low number of X-ray shots per crystal ensures that radiation damage is kept to a minimum. These wedges of data are merged together using standard macromolecular crystallography software to give the final structure.

For novel protein targets, obtaining experimental phase information is necessary for structure determination. Phasing methods such as SAD require a large amount of high quality data to be collected that is free from radiation damage and crystal-to-crystal variation so that accurate measurement of the anomalous diffraction signal measurements can be made. Here we applied SAD phasing for the first time in a microfluidic platform.

We fully validated this methodology by determining the structure of a novel bacterial lyase PhnA to 2.30 Å resolution using SAD phasing methods that make use of data collected entirely on-chip at room temperature. Initial screening using the

96-well varied array chip. Subsequently we used chips with identical wells to grow crystals at the optimized crystallization condition to collect data for phasing and structure determination. A large number of high quality isomorphic crystals (Figure 1b) were grown and analyzed at LS-CAT, Argonne National Lab, yielding high quality diffraction data (Figure 2c). The structure of PhnA was solved to 2.30Å resolution (Figure 2d-e) from data collected on-chip.

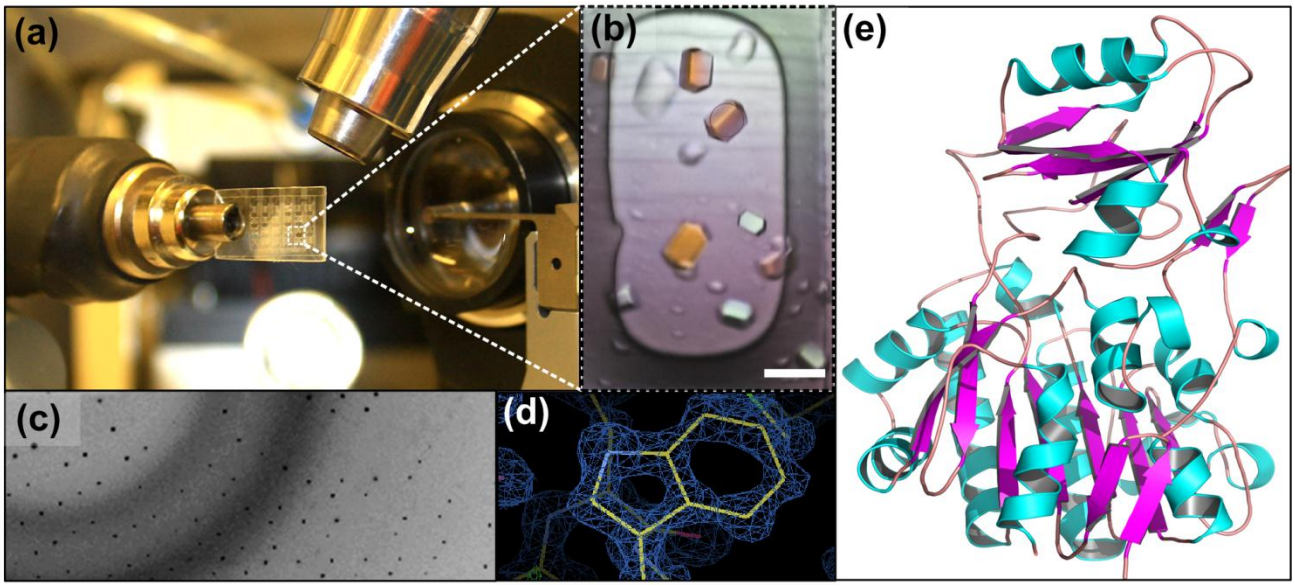

Figure 2: ***(a)*** *A 24-well hybrid array chip mounted at LS-CAT, APS using a modified goniometer mount.* ***(b)*** *Optical micrograph of PhnA crystals grown on-chip for in situ data collection (scale bar corresponds to 100µm).* ***(c)*** *Part of an in situ diffraction pattern from a PhnA crystal. Diffraction spots are clearly visible, even on the scatter ring from the chip material.* ***(d)*** *Electron density map (σ = 2.0) of a Tryptophan residue.* ***(e)*** *Cartoon of the structure of PhnA at 2.30Å.*

CONCLUSION

Here we have presented a microfluidic platform that allows for screening of crystallization conditions, together with on-chip X-ray analysis of the crystals formed. Not only do these chips eliminate crystal handling, they also allow for room temperature data collection from multiple small crystals, as opposed to collecting data from single large crystals, which requires cryocooling to avoid radiation damage. Efforts on extending the capabilities of this platform to perform a variety of other studies on different novel protein targets are currently underway.

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REFERENCES

[1] Z. E. R. Newby, J. D. O'Connell, F. Gruswitz, F. A. Hays, W. E. C. Harries, I. M. Harwood, J. D. Ho, J. K. Lee, D. F. Savage, L. J. W. Miercke and R. M. Stroud, Nature, **4**, 619-637 (2009).
[2] D. H. Juers and B. W. Matthews, Quarterly Reviews of Biophysics, **37**, 105-119 (2004).
[3] G. L. Taylor, Acta Crystallographica Section D-Biological Crystallography, **66**, 325-338 (2010).
[4] L. Li and R. F. Ismagilov, Annual Review of Biophysics, **39**, (2010).
[5] C. L. Hansen, S. Classen, J. M. Berger and S. R. Quake, Journal of the American Chemical Society, **128**, 3142-3143 (2006).
[6] C. J. Gerdts, M. Elliott, S. Lovell, M. B. Mixon, A. J. Napuli, B. L. Staker, P. Nollert and L. Stewart, Acta Crystallographica Section D, **64**, 1116-1122 (2008).
[7] K. Dhouib, C. K. Malek, W. Pfleging, B. Gauthier-Manuel, R. Duffait, G. Thuillier, R. Ferrigno, L. Jacquamet, J. Ohana, J.-L. Ferrer, A. Theobald-Dietrich, R. Giege, B. Lorber and C. Sauter, Lab on a Chip, **9**, 1412-1421 (2009).
[8] G. Mehta, J. Lee, W. Cha, Y. C. Tung, J. J. Linderman and S. Takayama, Analytical Chemistry, **81**, 3714-3722 (2009).
[9] L. T. a. N. Y. Lee, Lab on a Chip, **10**, 1274 - 1280 (2010).

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