to the wild-type C05 IgG mAb, which bound to and neutralized 8 out of 24 tested strains with at least 100 nM binding affinity. The two cyclic peptide designs showed 100 nM binding to at least 11 and 10 strains, respectively. However, when the biological activity of these peptides was tested in influenza HN1 hemagglutinin neutralization assay, these designed peptides showed no activity. Among several factors, the authors attribute the lack of avidity in neutralizing assays to the monomeric nature of the cyclic peptide designs, which can be addressed in the future by using a scaffold protein.

Although the newly designed reagents in this study would require additional modifications before biological activity is achieved, this work was successful in delivering cyclic peptides that assume biologically active conformations. Further, the authors confirmed the hypothesis that by focusing on the more conserved part of the paratope, a molecule can be created that binds to a wide range of influenza strains. This work showcases the benefit and viability of rationally designing new biologics for desired therapeutics purposes.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health (NIH) grants GM118709, GM136357R01, and AI141816.

REFERENCES

Sevy, A.M., Gilchuk, I.M., Brown, B.P., Bozhanova, N.G., Nargi, R., Jensen, M., Meiler, J., and Crowe, J.E., Jr. (2020). Computationally Designed Cyclic Peptides Derived from an Antibody Loop Increase Breadth of Binding for Influenza Variants. Structure *28*, this issue, 1114–1123.

Usmani, S.S., Bedi, G., Samuel, J.S., Singh, S., Kalra, S., Kumar, P., Ahuja, A.A., Sharma, M., Gautam, A., and Raghava, G.P.S. (2017). THPdb: Database of FDA-approved peptide and protein therapeutics. PLoS One *12*, e0181748.

Structures from the Mesophase: MicroED Targets Crystals Extracted from LCP

Marcus Gallagher-Jones[1] and Jose A. Rodriguez[1,*]
[1]Department of Chemistry and Biochemistry, UCLA-DOE Institute for Genomics and Proteomics, STROBE, NSF Science and Technology Center, University of California, Los Angeles, Los Angeles, CA 90095, USA
*Correspondence: jrodriguez@mbi.ucla.edu
https://doi.org/10.1016/j.str.2020.09.005

The determination of protein structures from nanocrystals grown in lipidic cubic phase (LCP) is a promising crystallographic approach. In this issue of *Structure*, Zhu et al. (2020) extract crystals from the dense matrix of monoolein LCP for interrogation by micro electron diffraction (MicroED) and yield a 2 Å structure of Proteinase K.

Over the past 20 years, lipidic cubic phases (LCPs) have changed a facet of membrane protein crystallography. The observation in 1996 that 3D crystals of bacteriorhodopsin grow in an LCP matrix (Landau and Rosenbusch, 1996) presented new opportunities for the crystallization of challenging membrane proteins. LCP-based crystallization has since been successful in generating over 200 structures of membrane proteins by a variety of crystallography techniques. In this issue of *Structure*, Zhu and coworkers demonstrate that protein crystals can be extracted from LCP in a manner suitable for high-resolution structure determination by micro electron diffraction (MicroED).

At its core, the LCP method involves the reconstitution of solubilized membrane proteins into the curved lipid bilayers of a monoacylglycerol in the cubic phase (Figure 1A). Membrane proteins may find a more hospitable environment in this phase than in the detergents used for their solubilization. The addition of precipitants that locally destabilize the cubic phase promote the formation of a lamellar phase enriched in protein that acts as a nucleus for crystal formation. Wider adoption of LCP as a crystallization medium has coincided with the development of automated sample handling and serial X-ray crystallography, alleviating some of the challenges caused by the highly viscous nature of LCP (Cherezov et al., 2004). While crystals grown in LCP can be small and challenging for conventional X-ray diffraction data collection and require extensive optimization, they are suitable for serial crystallography techniques at both synchrotrons and X-ray free electron lasers. The lower rates at which LCP can be extruded by sample injectors, compared to aqueous solutions, allows for greater hit rates between X-ray pulses and crystals, drastically reducing sample consumption (Weierstall et al., 2014). This is particularly useful for membrane proteins that may be produced or isolated in smaller quantities than soluble proteins. In an important milestone for serial crystallography, LCP has enabled its determination of G protein-coupled receptor (GPCR) structures (Liu et al., 2013).

To adapt LCP crystallography to MicroED, Zhu et al. (2020) tackle hurdles in sample preparation that have traditionally rendered LCP-grown crystals incompatible with this technique. As a cryo-EM method, MicroED inherited sample preparation techniques that were developed to interrogate single molecules or

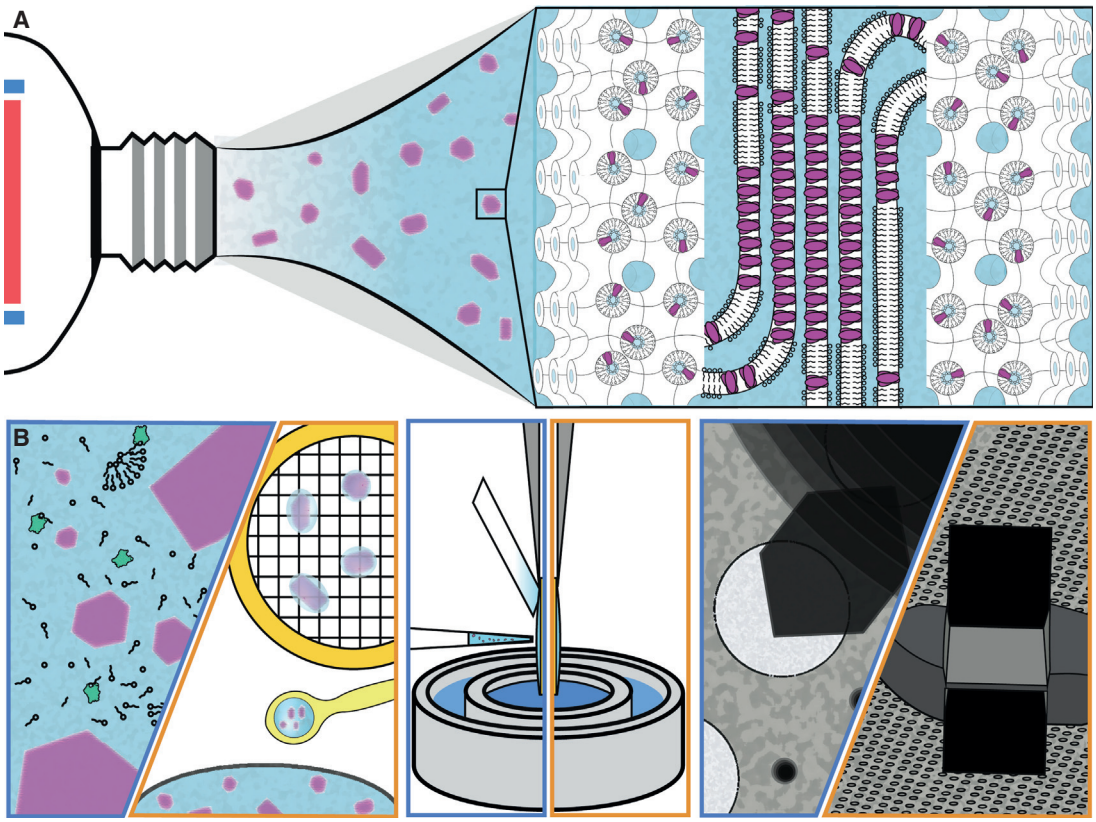

Figure 1. Extraction of Crystals from LCP for MicroED Experiments

(A) Illustration of crystal growth in the mesophase. Protein crystals are shown growing in highly viscous LCP. A magnified view illustrates proteins assembling into a single crystal within lipid bilayers in the LCP, which is locally destabilized to promote formation of a lamellar phase. Proteins are shown diffusing freely within bilayers and appending to a growing lattice.

(B) Side-by-side comparison of two potential methods for extracting LCP grown crystals for use in MicroED experiments. Blue panels illustrate instances of the workflow presented by Zhu et al. (2020) where the LCP is first degraded by treatment with lipase (green globules) reducing the viscosity of the solution (left). EM grids can then be prepared by conventional plunge freezing (middle) and small, thin crystals are identified for diffraction by TEM (right). Orange panels illustrate instances of the workflow presented by Polovinkin et al. (2020). Crystals embedded in LCP are transferred directly onto EM grids using a mitogen loop (left) and flash frozen without blotting (middle). A lamella of suitable thickness for MicroED is obtained from a microns-thick crystal after focused ion beam milling (right).

molecular crystals in a thin layer of vitreous ice on a transmission electron microscopy (TEM) grid (Shi et al., 2013). If protein crystals tolerate being crushed or collected, crystal suspensions can be deposited onto a grid for MicroED analysis. They are subjected to wicking or blotting to remove as much of the mother liquor as possible, without compromising their integrity; blotting conditions must be optimized against mother liquor composition during freezing. Once a specimen is sufficiently thin, diffracted beams can be collected by the lens system of a TEM and recorded on a camera as the illuminated crystal is rotated unidirectionally within the beam (Nannenga et al., 2014).

Removal of nearly all excess material enveloping a crystal by blotting is poorly effective with especially dense, viscous, or quasi-solid media. Perhaps no medium presents a greater challenge to blotting than quasi-solid LCP. While the extrusion of semi-solid crystal suspensions in LCP is beneficial to serial crystallography, the viscoelastic properties and metastable nature of LCP challenges wicking, dilution, centrifugation, and other methods of crystal harvesting. When these methods are used to extract crystals from a dense LCP matrix, the integrity of the crystals may be compromised by the process, ultimately affecting the quality of their diffraction. Zhu et al. overcome these challenges by carrying out a systematic search for appropriate methods to extract protein crystals from monoolein LCP in a manner compatible with cryo-EM methods (Zhu et al., 2020).

To achieve their goal, Zhu et al. (2020) screen both chemical and enzymatic routes for crystal extraction from LCP, attempting to achieve the thinnest possible layer of material surrounding crystals of interest (Figure 1B). Zhu and colleagues first attempt to reduce the viscosity of the LCP matrix by phase conversion by mixing with a panel of additives. They find that addition of 2-Methyl-2,4-pentanediol (MPD) facilitates efficient blotting of LCP and enables extraction of intact Proteinase K crystals from the mesophase. In a second strategy, Zhu et al. (2020) use lipase to hydrolyze lipids in the LCP and facilitate its conversion into a two-phase liquid. This second strategy is also successful in rendering Proteinase K crystals free of their LCP environment. As proof of principle, Zhu et al. (2020) then determine the structure of Proteinase K from LCP-extracted crystals after MPD or lipase treatment. Zhu et al.

(2020) further demonstrated the generality of MPD addition to extract crystals from LCP on crystals of cholesterol grown in the mesophase. With atomic resolution diffraction from the LCP-extracted cholesterol crystals Zhu et al. (2020) enable a potentially broad scope of targets for their technique.

Zhu and coworkers offer a glimpse of the applicability of their approach to membrane protein crystals with diffraction from LCP-extracted microcrystals of the human adenosine A_{2A} receptor. Although their diffraction is presently limited to 4.5 Å and only a narrow wedge of data could be collected, this effort represents a step toward a first GPCR structure by MicroED. Structures of GPCRs remain notably absent from the MicroED catalog, while structures of other membrane proteins determined from crystals grown in more conventional mother liquors have already been determined by the technique. Structures of both the Ca^{2+} ATPase and the non-selective sodium-potassium (NaK) channel determined by MicroED both align well to analogous structures determined by X-ray crystallography from larger crystals (Nguyen and Gonen, 2020). Recently, in a companion experiment presented prepublication, Polovinkin and coworkers use focused ion beams (FIBs) to machine thick vitrified crystals of bacteriorhodopsin (BR) grown in LCP and demonstrate ~2.5 Å diffraction (Polovinkin et al., 2020). FIB milling of frozen-hydrated biospecimens, an approach initially developed for the tomographic exploration of cellular substructure, has recently been repurposed to carve out lamella from microns-thick crystals (Martynowycz et al., 2019). The process turns impenetrable blocks of hydrated material into thin slabs suitable for high-resolution data collection. For samples whose size or mother liquor composition cause challenges during preparation, few alternatives exist. A combined approach of LCP extraction and FIB milling might therefore provide a more accessible route to structures of molecules whose crystals are sensitive to LCP extraction or where complete removal of the LCP cannot be achieved. In that hypothetical procedure, any LCP remaining on a grid would be further ablated by FIB milling (Figure 1B).

As we await the first MicroED structure of a membrane protein from crystals grown in LCP, the need for high-resolution structures of membrane proteins continues to inspire innovative advances. Thanks to Zhu and coworkers, MicroED structures from these and other crystalline residents of the mesophase may now be on the horizon.

ACKNOWLEDGMENTS

J.A.R. is supported as a Pew Scholar, a Beckman Young Investigator, and a Packard Fellow and by STROBE, an NSF Science and Technology Center Grant (DMR-1548924), DOE Grant DE-FC02-02ER63421, and NIH-NIGMS Grant R35 GM128867.

REFERENCES

Cherezov, V., Peddi, A., Muthusubramaniam, L., Zheng, Y.F., and Caffrey, M. (2004). A robotic system for crystallizing membrane and soluble proteins in lipidic mesophases. Acta Crystallogr. D Biol. Crystallogr. *60*, 1795–1807.

Landau, E.M., and Rosenbusch, J.P. (1996). Lipidic cubic phases: a novel concept for the crystallization of membrane proteins. Proc. Natl. Acad. Sci. USA *93*, 14532–14535.

Liu, W., Wacker, D., Gati, C., Han, G.W., James, D., Wang, D., Nelson, G., Weierstall, U., Katritch, V., Barty, A., et al. (2013). Serial femtosecond crystallography of G protein-coupled receptors. Science *342*, 1521–1524.

Martynowycz, M.W., Zhao, W., Hattne, J., Jensen, G.J., and Gonen, T. (2019). Qualitative Analyses of Polishing and Precoating FIB Milled Crystals for MicroED. Structure *27*, 1594–1600.e2.

Nannenga, B.L., Shi, D., Hattne, J., Reyes, F.E., and Gonen, T. (2014). Structure of catalase determined by MicroED. eLife *3*, e03600.

Nguyen, C., and Gonen, T. (2020). Beyond protein structure determination with MicroED. Curr. Opin. Struct. Biol. *64*, 51–58.

Polovinkin, V., Khakurel, K., Babiak, M., Angelov, B., Schneider, B., Dohnale, J., Andreasson, J., and Hajdu, J. (2020). Demonstration of electron diffraction from membrane protein crystals grown in a lepidic mesophase after lamella preparation by focused ion beam milling at cryogenic temperatures. bioRxiv. https://doi.org/10.1101/2020.07.03.186049.

Shi, D., Nannenga, B.L., Iadanza, M.G., and Gonen, T. (2013). Three-dimensional electron crystallography of protein microcrystals. eLife *2*, e01345.

Weierstall, U., James, D., Wang, C., White, T.A., Wang, D., Liu, W., Spence, J.C.H., Bruce Doak, R., Nelson, G., Fromme, P., et al. (2014). Lipidic cubic phase injector facilitates membrane protein serial femtosecond crystallography. Nat. Commun. *5*, 3309.

Zhu, L., Bu, G., Jing, L., Shi, D., Lee, M.-Y., Gonen, T., Liu, W., and Nannenga, B.L. (2020). Structure Determination from Lipidic Cubic Phase Embedded Microcrystals by MicroED. Structure *28*, this issue, 1149–1159.