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Microfluidics: From crystallization to serial time-resolved crystallography

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Capturing protein structural dynamics in real-time has tremendous potential in elucidating biological functions and providing information for structure-based drug design. While time-resolved structure determination has long been considered inaccessible for a vast majority of protein targets, serial methods for crystallography have remarkable potential in facilitating such analyses. Here, we review the impact of microfluidic technologies on protein crystal growth and X-ray diffraction analysis. In particular, we focus on applications of microfluidics for use in serial crystallography experiments for the time-resolved determination of protein structural dynamics. © 2017 Author(s). All article content, except where otherwise noted, is licensed under a Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).

I. INTRODUCTION AND BACKGROUND

The development of increasingly bright and micro-focused synchrotron X-ray beams, as well as the advent of X-ray free-electron lasers (XFELs), has enabled structure determination using ever-smaller crystals and more challenging targets.1,2 However, the direct observation of functional motions within proteins and other biomolecules remains an ultimate goal. The challenge of obtaining dynamic structural information stems from (i) the large X-ray dose required for the collection of data at multiple time points during a reaction, (ii) sample handling strategies for multi-crystal experiments, and (iii) difficulties associated with synchronizing structural dynamics within protein crystals and matching X-ray exposure times to the timescale of the relevant structural dynamics.

Many of these challenges have been addressed through serial data collection strategies,3,4 which extend the concept of combining data from multiple crystals5–13 with the limit of a single frame of data per crystal. This type of single-frame-per-crystal data collection has been critical for structural biology efforts at X-ray free-electron laser (XFEL) sources where radiation damage may require a "diffraction before destruction" approach3,14 and has subsequently been extended for use at synchrotron sources.15–18 Furthermore, these approaches have enabled time-resolved structural determination for a much broader range of targets than had previously been accessible.19–30

However, these large-scale serial methods suffer from the need to manipulate crystals and/or from inefficient sample utilization3,31–36 The material-intensive nature of these experiments extends the long-standing challenge of growing a well-diffracting crystal to reproducibly generating a large number of high quality, isomorphous crystals. These issues are then further compounded by the need to deliver those samples efficiently to the X-ray beam.5,7–11,18,37–42 Microfluidic and microscale technologies have played a critical role in facilitating both protein crystallization and structure determination, with the breadth and variety of reported solutions demonstrating the challenging nature of the field.
In this review, we discuss the utility of microfluidic technologies for addressing the challenges of crystal growth and sample manipulation for applications in serial crystallography and time-resolved structure determination. We begin by considering the benefits of working at the microscale and how these advantages facilitated the development of a wide range of microfluidic platforms for protein crystallization. We then examine the evolution of these crystallization-centric technologies for in situ X-ray analysis and contrast such devices with a range of platforms developed to facilitate the efficient, high-throughput delivery of crystals for both static and time-resolved structure determination. Finally, we discuss the potential for these various microfluidic strategies to address future challenges related to the study of protein structures and dynamics. As this review is focused on the technologies surrounding crystal growth and delivery, and not on the larger aspects of serial or time-resolved X-ray methods, we refer the reader to other excellent reviews on these topics.

II. MICROFLUIDICS FOR PROTEIN CRYSTALLIZATION, AN HISTORICAL PERSPECTIVE

A. The benefits of working at the microscale

Undoubtedly, the most obvious benefit of transitioning from the laboratory-scale to microscale experiments is the potential for dramatically decreasing the quantity of a sample needed for a given experiment. Decreasing the physical size (i.e., the length, $L$) of a sample results in a decrease in the volume that scales as $L^3$ (Table I). Thus, for a cubic geometry, decreasing the size of the cube from 1 mm to 0.1 mm results in a 1000-fold volume change, from 1 ml to 1 nl. However, the benefits of working at the microscale extend far beyond sample conservation. Microfluidic technologies are able to take advantage of small geometries by enabling large surface-area to volume ratios ($SA/V$, Table I), eliminating convective effects to create a purely diffusive environment, generating extremely sharp and controllable gradients in concentration and temperature and harnessing interfacial tension effects.

B. Capillary-based counter-diffusion methods

Much of the motivation and inspiration for early microfluidic efforts in protein crystallization were connected with experiments performed in microgravity. The excitement regarding these experiments was fueled by the production of higher quality diffraction for nearly 35% of the targets investigated in space, as compared to ground-based methods. The advantage conferred by microgravity was a tremendous reduction in buoyancy-driven convection and sedimentation effects and the subsequent dominance of diffusive mixing. These microgravity experiments were typically performed in a counter-diffusion style geometry, where protein and precipitant solutions are placed into contact in a capillary and allowed to slowly intermix (Figure 1(a)). The resulting diffusional profile continuously samples a wide range of supersaturation conditions. While high levels of supersaturation close to the initial point of contact will

<table>
<thead>
<tr>
<th>Length ($L$)</th>
<th>Volume ($V$)</th>
<th>$SA/V^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>mm</td>
<td>$\mu$m</td>
<td>$nm^3$</td>
</tr>
<tr>
<td>10</td>
<td>100000</td>
<td>$10^3$</td>
</tr>
<tr>
<td>1</td>
<td>1000</td>
<td>1</td>
</tr>
<tr>
<td>0.1</td>
<td>100</td>
<td>$10^{-3}$</td>
</tr>
<tr>
<td>0.01</td>
<td>10</td>
<td>$10^{-6}$</td>
</tr>
<tr>
<td>0.001 (1 $\mu$m)</td>
<td>1</td>
<td>$10^{-9}$</td>
</tr>
<tr>
<td>0.0001</td>
<td>0.1</td>
<td>$10^{-12}$</td>
</tr>
</tbody>
</table>

$^a$SA/V is the surface area to volume ratio specified based on the geometry of a cube.
tend to result in amorphous precipitates, lower levels of supersaturation allow for continuous sampling and the identification of optimal rates of nucleation and growth.52

In translating counter-diffusion style experiments for use in earth-bound laboratories, it is necessary to utilize a strategy to minimize the effects of convection. While smaller capillaries and microfluidic channels can achieve this condition directly as a consequence of their geometry,49,55–63 larger capillaries typically utilize a gel matrix to eliminate convection.64–69 These techniques have been applied to a range of proteins and can be compatible with in situ X-ray analyses, depending on the thickness of the surrounding material.55,56,58,60–62,65–68,70

C. Integrated microfluidic devices

While simple channel and capillary-based approaches are effective strategies for protein crystallization, more integrated microfluidic devices have been used to enable higher-throughput screening of a broad range of conditions and have the potential to enable the reproducible growth of a large number of isomorphous crystals for serial crystallography efforts. Advances in the microfabrication techniques known as soft lithography and replica molding71–74 facilitated the straightforward manufacture of microscale features from poly(dimethylsiloxane) (PDMS), an optically transparent elastomer. Furthermore, the elastomeric nature of PDMS...
enabled the development of a range of multi-layer microfluidic device structures such as integrated valves and pumps based on the deflection of a thin PDMS membrane. 75–78

Probably, the most well-known microfluidic device for protein crystallization was developed by Quake et al., where pairs of fluidic chambers were used to screen for crystallization conditions via free-interface diffusion (FID). 54,79–85 For a single crystallization experiment, three different volumetric ratios of protein and precipitant were tested, as defined by the size of the molded chamber. Control of fluid flow and isolation of the chambers were achieved with integrated pneumatic valves (Figure 1(b)). By scaling out, the components of an individual crystallization experiment were replicated across a larger area to create an integrated array chip (Figure 1(c)). This device architecture was commercialized by Fluidigm Corp., along with the necessary ancillary equipment for valve control as the Topaz System, one of the first commercially available microfluidic platforms for protein crystallization. 86 Recognizing the challenge of harvesting crystals from such a device, a thinner X-ray compatible version was also fabricated. 84

One of the limitations of many first-generation integrated microfluidic devices, including the initial FID chip, was the use of valves that required the application of pressure in order to remain sealed. The subsequent development of vacuum activate-to-open valves enabled the design of array-style counter-diffusion chips that could be set up and then allowed to incubate without the need for ancillary pressure sources (Figures 1(d) and 1(e)). 18,19,39,40 Furthermore, this new generation of devices was designed specifically with in situ X-ray analysis in mind. These chips retained only the thin layer of PDMS necessary for valve actuation, replacing the remaining device layers with thin films of cyclic olefin copolymers (COCs) to alleviate the potential for sample dehydration during incubation and/or data collection (Figure 1(d)). 18,19,39,40 Similar device architectures have also been applied to microfluidic platforms to facilitate the lipidic cubic phase (LCP) method for crystallizing membrane proteins. 87–89 A wide range of other integrated microfluidic devices have also been developed to help survey crystallization phase space and protein solubility behavior. 54,79,89

D. SlipChip devices

However, it should not be supposed that multi-layer architectures are the limit of integrated microfluidics. Ismagilov et al. developed an elegant platform for sample formulation termed SlipChip. 57,90–92 SlipChip-based devices consist of channel and well features fabricated into the top and bottom layers of a device. A lubricating layer of a fluorocarbon oil is used to facilitate slipping of the two plates relative to one another, allowing for the alignment of fluidic channels with microfluidic wells for filling and subsequent “slipping” of the two plates to align features containing, for instance, protein solution and precipitant solution (Figure 2). A variety of device designs have been reported, including geometries for both microbatch and FID. 57 While X-ray compatible SlipChips have not been reported, such devices could serve as interesting platforms to create dense sample arrays for serial crystallography experiments.

E. Capillary valves and centrifugal devices

The small length scales of microfluidic geometries also allow for the use of surface tension-based or capillary pressure-based valving. In these types of devices, the ability of a liquid to flow past either a hydrophobic surface patch or a constriction in the channel is determined by the applied pressure. 93 While this pressure can be applied by any source, it is convenient to utilize a CD-based form factor and use centrifugal force to drive fluid flow (Figure 3). Thus, the force needed to “open” such a capillary valve is determined by the spin rate of the device and the radial location of the valve. Lower-force valves can be used to facilitate initial metering applications while higher-force valves can be used to control subsequent mixing events. These types of centrifugal devices have been reported in both microbatch and vapor diffusion configurations and can be made X-ray compatible. 94,95
F. Droplet microfluidics

The last major class of microfluidics is based on the formation of droplets in two-phase flow (Figure 4). A tremendous advantage of droplet-based microfluidics over the types of prefabricated chips described previously is their ability to change experimental parameters such as the protein-to-precipitant ratio or the total sample volume by simply adjusting the flowrates of

![Diagram](image1)

**FIG. 2.** A SlipChip (well-based) system. Schematics showing (i) loading of protein into a SlipChip that has already been preloaded with precipitants and (ii) slipping to combine protein and precipitants to form trials. (iii) Optical micrograph showing the loading of a green food dye (mimicking the protein) into a SlipChip that has already been preloaded with colored dyes (mimicking precipitants). (iv) Optical micrograph of the SlipChip after slipping to combine the solutions. (v) Crystals of the photosynthetic reaction center from *Blastochloris viridis* obtained using this device. Adapted with permission from Du et al., Lab Chip 9(16), 2286–2292 (2009). Copyright 2009 Royal Society of Chemistry.

**FIG. 3.** Schematic depiction of a vapor diffusion-style centrifugal microfluidic device, showing (a) the top view and (b) the vapor diffusion chamber. Reprinted with permission from Wang et al., Sens. Actuators, B 219, 105–111 (2015). Copyright 2015 Elsevier.
the various component streams during device operation. Furthermore, such trials can be formulated either as microbatch or pseudo-vapor diffusion trials, based on the permeability of the oil phase and/or the container used to house the final droplets. Additionally, the two-phase nature of this method ensures that the formation of precipitants and/or crystals will not result in clogging of the device. This flexibility has been extended to the formulation of LCP trials for membrane proteins. Beyond formulation, storage of the resulting droplets can be performed either in two-dimensional arrays in a chip-like structure or in flexible tubing. Thus, it is straightforward to formulate crystallization trials into X-ray compatible capillaries, tubing, or planar arrays for subsequent in situ analysis.

**III. DESIGN CONSIDERATIONS FOR X-RAY COMPATIBILITY**

The design and optimization of any X-ray experiment relies on finding a balance between the observed signal and contributions to background noise. For protein crystallography...
experiments, this translates to balancing the strength of the diffraction from the crystal with the attenuation of both the incident and diffracted beam and contributions to background noise. These background effects can come from the direct beam itself, if the beam size is not matched to the sample size, or can be the result of X-ray scattering due to the presence of air, liquid around or within the crystal, and/or the materials used to mount the crystal. Thus, the design of an X-ray compatible microfluidic device for the delivery of crystals to the X-ray beam can have a tremendous impact on the quality of the resulting data.

While many of the early microfluidic devices for protein crystallization were focused solely on crystallization screening and optimization, the need for X-ray compatible microfluidics was quickly recognized because of the challenge in translating crystallization results obtained on-chip to more traditional, larger-scale platforms. However, at the time, most traditional microfluidic chips were manufactured from millimeter-thick layers of PDMS and/or glass that were incompatible with the requirements for X-ray diffraction experiments, requiring a reexamination of the materials and fabrication strategies for such devices.

A. Design considerations

From a design perspective, X-ray compatibility must address the attenuation of both the incident and the diffracted X-ray beam and scattering resulting from the material of the device itself. Attenuation results from the absorption of photons into the material, thereby decreasing the intensity of both the incident X-ray beam and the resultant signal. Scattering is an elastic redirection of photons based on the internal structure of the material that scales with the thickness of the material and can increase the observed background. The strength of the diffraction signal from a crystal is related to not only the degree of order within the crystal but also the packing density and the size of the crystal.1,2,39,46,123

Attenuation can be calculated for a particular energy based on the exponential decay in intensity of a narrow beam of monochromatic photons from an incident intensity $I_0$ as it passes through a material of thickness $x$ and density $\rho$ with a mass attenuation coefficient of the material $\mu$,124

$$I = I_0 \exp \left(\frac{-\mu}{\rho}x\right).$$

(1)

Thus, decreasing the thickness of any material interacting with the X-rays can mitigate both the attenuation and scattering effects. Eq. (1) also demonstrates the reason why X-ray experiments are frequently done in a vacuum, enabling a decrease in the density of air present between the sample and the detector.

Attenuation coefficients have been well studied and documented for elemental materials.124 The mass attenuation coefficient for a compound or a mixture can be calculated based on the sum of the contribution to attenuation from each of the individual elements $i$, weighted based on their mass fraction $w_i$,

$$\mu = \sum \mu_i w_i.$$  

(2)

It should be noted that the attenuation coefficient varies significantly as a function of photon energy, with stronger attenuation observed for soft X-rays compared to hard X-rays.

Thus, from Eqs. (1) and (2), the transmission factor $I/I_0$ can be calculated as a function of thickness for any material. Analysis of a plot of transmission vs. thickness (Figure 5) highlights the importance of both the material composition and the density in signal attenuation. The low density of gases such as air and helium facilitates a much higher level of transmission than solid materials; however, the lower atomic number of helium dramatically decreases attenuation effects. Similarly, thin films of organic polymers such as cyclic olefin copolymer (COC) and poly(methyl methacrylate) (PMMA) show significantly lower attenuation compared to silicon-based elastomers like poly(dimethylsiloxane) (PDMS) or hard materials such as quartz (SiO$_2$), silicon, or silicon nitride (Si$_3$N$_4$), while atomically thin films such as graphene mitigate...
It is interesting to note that water, the presence of which is practically inescapable in protein crystallography, attenuates the X-ray signal to an equal or greater degree than most organic polymers. Table II summarizes the results shown in Figure 5, listing both the density and the absorption coefficient for each material and quantifying the thickness associated with varying levels of X-ray transmission.

Thus, the solution to creating X-ray compatible microfluidics typically involves decreasing the thickness of the various device layers and possibly substituting dense or high atomic number materials (such as silicon-containing PDMS) for lighter organic polymers. Indeed, Quake et al. were able to transform their approximately 1 cm thick FID chip to a device where a ~250 μm thick section of the device could be easily removed and mounted for X-ray analysis. Similar strategies were adopted by Kenis et al., Perry et al., and later Fraden et al., keeping a minimal amount of elastomeric PDMS in the device to facilitate microfluidic valving and capping the device on top and bottom with a more X-ray compatible film of optically transparent COC to minimize the potential for sample dehydration due to water loss through the thin PDMS layers. The quality of diffraction observed from these chips has been sufficient to solve the structures of novel proteins, including via single-wavelength anomalous diffraction methods and time-resolved Laue diffraction studies.

B. Device materials for microcrystallography

Thin polymeric materials have proven to be a successful solution for the challenge of in situ crystallography. In particular, COC has seen widespread adoption as the polymer film of choice for X-ray compatible devices, including simple channel structures for counter-diffusion, droplet-based devices, and larger-scale X-ray compatible well-plates. However, further decreasing the device thickness to achieve the signal-to-noise levels required for microcrystallography is a significant materials’ challenge. Typical reports of X-ray compatible microfluidics describe results where the path length of the device materials is nearly twice that of the crystal of interest. Thus, the analysis of micron-scale or smaller crystals would suggest the need to shrink device materials to a total thickness of 1–10 μm. Unfortunately, although ultra-thin polymeric films are available, such materials tend to suffer from poor mechanical stability and more critically show poor barrier performance against sample dehydration.

While the popularity of polymeric materials has stemmed from their low cost and ease of use, micromachining of hard materials has been demonstrated as a viable solution for the creation of stable, X-ray compatible devices for the in situ analysis of microcrystals. Various deposition, photolithography, and etching strategies have been utilized to fabricate a range
<table>
<thead>
<tr>
<th>Transmission factor</th>
<th>Helium (He)</th>
<th>Air N\textsubscript{2}O\textsubscript{2}</th>
<th>CO\textsubscript{2}H\textsubscript{4}</th>
<th>PMMA C\textsubscript{2}H\textsubscript{4}O</th>
<th>Water (H\textsubscript{2}O)</th>
<th>Graphene (C)</th>
<th>PDMS Si\textsubscript{61}O\textsubscript{60}C\textsubscript{124}H\textsubscript{388}</th>
<th>Quartz (SiO\textsubscript{2})</th>
<th>Silicon nitride (Si\textsubscript{3}N\textsubscript{4})</th>
<th>Silicon (Si)</th>
</tr>
</thead>
<tbody>
<tr>
<td>99.9%</td>
<td>26701</td>
<td>345</td>
<td>0.88</td>
<td>0.68</td>
<td>0.62</td>
<td>0.15</td>
<td>0.136</td>
<td>0.107</td>
<td>0.093</td>
<td>0.060</td>
</tr>
<tr>
<td>99%</td>
<td>268223</td>
<td>3468</td>
<td>8.9</td>
<td>6.8</td>
<td>6.3</td>
<td>1.5</td>
<td>1.37</td>
<td>1.08</td>
<td>0.93</td>
<td>0.60</td>
</tr>
<tr>
<td>90%</td>
<td>2811861</td>
<td>36356</td>
<td>93</td>
<td>72</td>
<td>66</td>
<td>16</td>
<td>14.4</td>
<td>11.3</td>
<td>9.8</td>
<td>6.3</td>
</tr>
<tr>
<td>Density ρ (g/cm\textsuperscript{3})</td>
<td>1.66 × 10\textsuperscript{-4}</td>
<td>1.23 × 10\textsuperscript{-3}</td>
<td>1.02</td>
<td>0.94</td>
<td>1.00</td>
<td>1.8</td>
<td>0.92</td>
<td>2.65</td>
<td>3.2</td>
<td>2.3</td>
</tr>
<tr>
<td>μ/ρ at 1 Å (cm\textsuperscript{-1})</td>
<td>3.747 × 10\textsuperscript{-5}</td>
<td>2.898 × 10\textsuperscript{-3}</td>
<td>1.13</td>
<td>1.47</td>
<td>1.607</td>
<td>6.51</td>
<td>7.33</td>
<td>9.33</td>
<td>10.8</td>
<td>16.6</td>
</tr>
</tbody>
</table>
of microstructures out of silicon or other hard materials. The resulting devices can then be sealed with \( \sim 50 \) to 500 nm-thick silicon nitride windows.\(^{34,46,141-144}\) This approach allows for the creation of extremely small features, such as wells to capture individual microcrystals. However, the devices are relatively time and labor intensive and expensive to produce.

Recently, Perry et al. reported the use of large-area graphene films as both ultra-thin X-ray transparent windows and vapor diffusion barriers.\(^ {123}\) This proof-of-concept work, inspired by earlier reports on the stability of graphene-wrapped crystals,\(^{145,146}\) utilized windows of single-layer graphene backed by a 500 nm-thick layer of PMMA for structural stability, surrounding a microfluidic channel cut into 100 \( \mu \)m-thick COC. The resulting microfluidic chambers were shown to be stable against dehydration for at least two weeks and were robust enough to survive overnight shipping to the synchrotron. Using lysozyme as a model system, albeit with relatively large crystals, the authors then analyzed the resulting levels of background scattering and the observed signal-to-noise in their diffraction data, comparing diffraction through graphene/PMMA-only windows with the observed signal obtained through graphene/PMMA and a 100 \( \mu \)m COC layer (Figure 6). Both the two-dimensional diffraction images and the corresponding one-dimensional integrated intensity plots showed a tremendous enhancement in the observed signal-to-noise, particularly at high resolution and in the regions where a significant scattering signal from COC was observed. This initial work shows tremendous promise for the use of graphene-based windows in other areas of X-ray compatible microfluidics.\(^ {43}\)

IV. PLATFORMS FOR SERIAL CRYSTALLOGRAPHY

Thus far, this review has discussed the general use of microfluidics in the context of protein crystallization and the potential for adapting such technologies to facilitate the growth of a large number of isomorphous crystals. However, for serial crystallography, much of the technological focus has been less on crystal growth and more on the efficient delivery of crystals into the X-ray beam. This discontinuity in the use of microfluidic technology for structural biology represents an opportunity to couple exquisitely controlled microfluidic methods for crystallization with high-throughput strategies for sample analysis. While most of the microfluidic crystallization platforms were originally designed for more traditional-scale experiments on the order of 1–100 crystals, strategies for “scaling out” could be used to facilitate the controlled, large-scale preparation of crystals at the scale necessary for serial or even time-resolved serial crystallography experiments.

Looking beyond crystal growth, the priorities of a serial crystallography experiment and/or a sample delivery technology include (i) maximization of diffraction data quality, (ii) maximization of data collected per quantity of the sample, and (iii) maximization of experimental capacity by enabling efficient data collection and minimization of down time.\(^ {141}\) Although these priorities might appear somewhat obvious and represent a common sense approach to experimental design, the challenge lies in actualizing such goals in the context of the high repetition rates of current and planned X-ray sources. For instance, while fast readout detectors enable data collection on the order of 10 Hz at synchrotron sources, X-ray pulses are currently delivered at a rate of 120 Hz for the LCLS and are anticipated in the MHz regime at the European XFEL and the LCLS II.

Successful sample delivery methods for serial crystallography at both XFEL and synchrotron sources have utilized both fixed-target and injector technologies, nearly all of which qualify as microfluidic or microscale in nature. As with the X-ray compatible microfluidic devices discussed earlier, the thickness of any material surrounding the crystals and the stability of the crystals in the resulting environment must be carefully addressed, particularly for weakly diffracting microcrystals. Here, we will briefly summarize the various technologies that have been used to date. We also refer the reader to other recent review articles on sample delivery techniques.\(^ {141,147}\)
A. Injectors

1. Gas dynamic virtual nozzle (GDVN)

The most common delivery device for serial crystallography experiments at XFELs has been the gas dynamic virtual nozzle (GDVN, Figure 7(a)). The original GDVN design consisted of two relatively large-diameter concentric capillaries. The slurry of crystals flows through the inner capillary, while a sheath gas such as helium or nitrogen flows through the outer one. More recently, soft lithographic and 3D printing methods for the robust manufacture of nozzles have been reported. For all these various GDVN designs, the pressure of the flowing sheath gas is used to focus the liquid stream into a narrower jet that could be achieved by the features of the nozzle alone. This gas-driven focusing also allows for the use of larger capillaries or injector features, relative to the size of the crystals, to decrease the

FIG. 6. (a) One-dimensional integrated X-ray intensity profiles showing the relative strength of the observed (Laue) diffraction signal from a lysozyme crystal compared to the noise resulting from background scattering due to the presence of device materials as a function of resolution. The corresponding two-dimensional diffraction images for a crystal (b) located between two 500 nm-thick PMMA/graphene windows and a 100 μm-thick COC layer (orange) and (c) a crystal located between two 500 nm-thick PMMA/graphene windows (magenta). Adapted with permission from Sui et al., Lab Chip 16, 3082–3096 (2016). Copyright 2016 Royal Society of Chemistry.
potential for clogging and allow for the modulation of the jet size, relative to the crystal size. Furthermore, the presence of the sheath helps to delay ice formation during data collection performed in a vacuum. Typical jet sizes are 1–5 μm in diameter, facilitating minimization of signal attenuation and background noise from the carrier fluid itself. The tunability and low background of GDVN-based systems have even enabled the analysis of sub-micron crystals at XFEL sources. Despite these benefits, the most significant disadvantage of GDVN injectors is poor efficiency in terms of sample utilization. The current data acquisition rate at the LCLS is typically 120 Hz, resulting in estimated crystal hit rates on the order of 0.01%–0.1%. Thus, sample utilization at synchrotron sources would be prohibitive. However, for the higher MHz-level data acquisition rates anticipated at the European XFEL and the LCLS II, more optimal sample utilization should be achievable.

2. Electrokinetic injectors

To increase the efficiency of data collection at currently available sources, a major goal has thus been to decrease the rate of sample delivery. One potential solution to this challenge has been the use of electrospinning or electrokinetic injection of the crystal slurry (Figure 7(b)). In this method, an applied voltage is used to extend the liquid emerging from a capillary into a fine Taylor cone. The addition of viscosity modifiers such as glycerol or poly(ethylene glycol) (PEG) can facilitate the formation of a liquid jet, rather than a spray of droplets. While electrospinning can be performed directly on the crystal slurry, data collection performed in a vacuum can result in freezing of the jet. Similar to the GDVN, coaxial flow where an outer “sister liquor” can be used to mitigate freezing

FIG. 7. An overview of injector technology for serial crystallography. (a) GDVN liquid injector schematic for use at the LCLS, including images of the observed diffraction data. (b) Electrospinning jet schematic depicting the injection of a crystal suspension and the resulting powder diffraction pattern. (c) Acoustic injector for on-demand drop injection. The setup can be operated either vertically (purple) or inverted (red). (d1) Schematic depiction of the LCP injector. Cross-polarized micrographs of the extruded LCP with (d2a) helium as the co-flowing gas, resulting in the dessication and formation of the birefringent crystalline lipid, and (d2b) nitrogen as the co-flowing gas, showing no visible birefringence. Scale bar: 100 μm. The corresponding diffraction images showing (d3a) diffraction spots from A2A adenosine receptor microcrystals and (d3b) diffraction from serotonin receptor 5-HT2B with no visible powder diffraction. (a) Adapted with permission from Chapman et al., Nature 469, 73–77 (2011). Copyright 2011 Royal Society of Chemistry. (b) Adapted with permission from Sierra et al., Acta Crystallogr., Sect. D: Biol. Crystallogr. 68, 1584–1587 (2012). Copyright 2012 International Union of Crystallography. (c) Adapted with permission from Roessler et al., Structure 24, 631–640 (2006). Copyright 2016 Elsevier. (d) Adapted with permission from Weierstall et al., Nat. Commun. 5, 3309 (2014). Copyright 2016 Royal Society of Chemistry.
effects and to facilitate focusing of the jet.\textsuperscript{164} This electrokinetic method of sample injection allows for a 10-fold decrease in the overall flowrate, helping to improve sample utilization to around 5\%.\textsuperscript{164}

3. On-demand droplet delivery

With both the GDVN and electrokinetic injection methods, a slurry of crystals is delivered to the X-ray beam as a continuous jet. The potential for inefficient sample utilization is strongly coupled to the continuous nature of such a delivery method. An alternative delivery strategy is the on-demand delivery of discrete liquid droplets (Figure 7(c)). This approach has been demonstrated using both acoustic\textsuperscript{32,167,169} and piezoelectric\textsuperscript{36} droplet injectors, resulting in a hit rate of close to 90\%. However, the diameter of the delivered droplets has typically been at least an order of magnitude larger than for the GDVN or electrokinetic methods, representing a significant increase in the background. Thus, on-demand droplet delivery could be used for serial analysis of larger-scale crystals but would be extremely challenging for the analysis of weakly diffracting or submicron crystals.\textsuperscript{32}

4. Aerosol injection

Sample nebulization has also been proposed as a sample delivery strategy for serial crystallography. The advantage of working with an aerosolized sample is the absolute minimization of background scattering and signal attenuation effects from the carrier solvent.\textsuperscript{170} This type of approach could be used to enable analysis of nanocrystals; however, the speed of sample delivery would most likely be the fastest of all the methods discussed heretofore and would result in the lowest hit rates. Additionally, the effect of nebulization on protein crystal quality and hydration has not yet been fully explored\textsuperscript{161} although the injector shows promise for single particle diffractive imaging experiments.\textsuperscript{170}

5. Viscous media extrusion

Stepping aside from advanced injector technologies, one of the simplest ways to improve sample efficiency is to slow down the overall flowrate by increasing the viscosity of the stream. Using positive displacement extrusion, a variety of high viscosity materials including the lipidic cubic phase (LCP) matrix used in membrane protein crystallization (Figure 7(d)),\textsuperscript{27,141,147,161,163,171–173} a grease matrix,\textsuperscript{175–177} agarose,\textsuperscript{178} and hyaluronic acid have been used as carrier materials for serial crystallography.\textsuperscript{179} In these methods, the aqueous slurry of crystals is mixed with the higher viscosity component. One reason why a variety of high viscosity matrices have been explored is the challenge of finding a carrier material that is compatible with each particular protein system and crystallization condition. Transfer of crystals into a viscous medium has the potential to cause osmotic stress and potentially alter protein solubility.\textsuperscript{179} Additionally, the LCP method in particular can be sensitive to crystallization conditions. For instance, the LCP is not compatible with ammonium sulfate,\textsuperscript{171,176,180,181} while agarose requires gentle heating of the sample.\textsuperscript{178}

With respect to signal-to-noise, as with any method, the diameter of the extruded material will directly affect the level of background scattering. One drawback of this approach is the difficulty in extruding small columns of viscous materials. For instance, pressures of several thousand psi are required to extrude the LCP through a capillary of diameter 10–50 \(\mu\)m.\textsuperscript{147,163} Thus, while viscous jets have the potential to enable structure determination of small and weakly diffracting crystals, a significant loss in signal-to-noise is expected because of the larger diameter of the carrier material. The level of background noise resulting from the carrier material is further exacerbated by the fact that nearly all the materials used to date have characteristic molecular length scales that result in an observable background scattering signal. For instance, the LCP shows characteristic diffuse scattering signals at approximately 4.5 \(\AA\),\textsuperscript{172,178} grease at around 5 \(\AA\), agarose at 3.2 \(\AA\),\textsuperscript{178} and hyaluronic acid at 3.3 \(\AA\).\textsuperscript{179} Depending on the expected resolution of the protein crystals, this scattering ring could dramatically affect the quality of the observed diffraction. Preliminary comparison work has suggested that the scattering intensity of
agarose is lower than that of the LCP\textsuperscript{178} and that hyaluronic acid showed weaker scattering than grease-based matrices.\textsuperscript{179}

B. Fixed-target approaches

While a degree of uncertainty regarding the crystal location exists with the majority of injector technologies, the most sample-efficient delivery strategy would involve preparing an ordered array of crystals onto a fixed-target type of mount and then rapidly rastering through the various sample locations (Figure 8(a)).\textsuperscript{161} However, three potential challenges must be considered. Firstly, such an approach requires the development of a method for creating such an array of crystals. Secondly, the batch nature of this kind of fixed-target device could necessitate frequent exchange, thereby increasing the dead time during an experiment. Thirdly, while these types of mounts are typically robust in the context of synchrotron radiation, the intensity of the unattenuated X-ray pulses generated at XFEL sources is likely to destroy any sort of window material, which has the potential to compromise the entire array of samples.

![Fixed-target approaches](image)

**FIG. 8.** An overview of fixed-target mounting strategies for serial crystallography. (a) Schematic depiction of a crystal slurry embedded in a micro-manufactured silicon mesh with silicon nitride windows being rapidly translated relative to the beam path for data collection at the LCLS. (b) A droplet-microfluidics based X-ray compatible array chip mounted on the goniometer inside the Cornell CHESS F1 beamline. The inset shows an optical micrograph of glucose isomerase crystals inside the microfluidic device. (c) X-CHIP with 24 crystallization drops mounted on a goniometer. (d) Optical micrograph of a plastic grid fabricated from SU-8. (e) A sample-mounting grid affixed to a standard magnetic base. For comparison, a Hampton Research-style copper magnetic sample pin is also shown. Samples fit into a Uni-Pack enclosure. The optical microscopy image shows lysozyme crystals growing in sitting-drops mounted on a grid. (a) Adapted with permission from Hunter et al., Sci. Rep. 4, 6026 (2014). Copyright 2014 Royal Society of Chemistry.\textsuperscript{34} (b) Adapted with permission from Heymann et al., IUCrJ 1, 349–360 (2014). Copyright 2014 International Union of Crystallography.\textsuperscript{15} (c) Adapted with permission from Kisselman et al., Acta Crystallogr., Sect. D: Biol. Crystallogr. 67, 533–539 (2011). Copyright 2011 International Union of Crystallography.\textsuperscript{135} (d) Adapted with permission from Feld et al., J. Appl. Crystallogr. 48, 1072–1079 (2015). Copyright 2015 International Union of Crystallography.\textsuperscript{243} (e) Adapted with permission from Baxter et al., Acta Crystallogr., Sect. D: Biol. Crystallogr. 72, 2–11 (2016). Copyright 2016 International Union of Crystallography.\textsuperscript{195}
Interestingly, nearly all the fixed target solutions for serial crystallography have been developed independent of previous X-ray transparent microfluidic array chips for protein crystallization. This is most likely a consequence of the emphasis on sample preparation for use at XFELs, where large-scale methods have been used to grow the millions of crystals needed for such high-throughput experiments. However, a consequence of this type of \textit{ex situ} crystal growth is the need to transfer crystals into an injector or sample array, which can cause damage and/or sample loss.\ citation{18,19,40,123,133,171,176} Thus, depending on the injection or mounting strategy, it may also be necessary to pre-prepare and/or sort crystals by size for subsequent use.\ citation{182,183} Finally, as in any protein crystallography experiment, samples must be protected against dehydration or degradation during data collection, all while addressing experimental priorities related to data quality and signal-to-noise.\ citation{159}

1. Goniometer-based approaches

One of the simplest approaches to mounting a large number of crystals is the use of traditional loops,\ citation{17,184} micromounts,\ citation{185} or capillaries\ citation{16} to mount a slurry of crystals.\ citation{169} It is also possible to mount crystals grown directly on the mount, as in the case of the X-CHIP, where pinned droplets are protected against dehydration by a thin coating of oil (Figure 8(c))\ citation{186,187} or the aforementioned graphene-based microfluidic devices.\ citation{123} Such samples can be cryocooled, protected by a capillary sheath or other materials, or analyzed directly using a traditional goniometer setup for sample manipulation. Again, the presence of extra material and/or solvent will increase background noise and decrease the resultant data quality. Thus far, these types of smaller-scale mounting approaches have been favored more at synchrotron-based sources because of the lower rates of data acquisition and the higher availability of beam time. Serial approaches have also been reported for larger loop-mounted crystals, where a fresh crystal volume is sampled each time.\ citation{188–190}

2. Sample arrays

To increase the number of crystals available for analysis, a variety of mesh and array-based approaches have been developed.\ citation{25,31,33,34,37,143,185,191–195} The simplest mounting strategy involves the deposition of a crystal slurry between two thin silicon nitride wafers.\ citation{143} However, most approaches have utilized microfabricated mesh-type structures. One advantage of a mesh-type structure is the potential to localize individual crystals within each small micro-well. This process, along with the removal of excess mother liquor to reduce the background, can be facilitated by the application of vacuum to the mesh structure.\ citation{37} Mesh structures have been fabricated out of silicon\ citation{25,31,37,192,193} and polymers such as SU-8 (Figure 8(d))\ citation{191} and polycarbonate (Figure 8(e)).\ citation{185,195} The material of the mesh itself should not affect the quality of the observed diffraction, provided that the crystal is well located within the center of the well. However, care must be taken when crystalline materials such as silicon or metal are used, as intense diffraction from the mesh structure can potentially damage the detector.\ citation{37,191,193} One particular advantage of this type of mesh, or well-based approach, is its ability to insulate crystals in one region of the device from the destructive aspects of the intense XFEL beam when applied to a neighboring well. Such isolation strategies would be critical in adapting any of the aforementioned protein crystallization devices for use as fixed-target XFEL delivery platforms.

Once mounted, a variety of approaches have been utilized to stabilize the slurry of crystals during analysis. From a signal-to-noise perspective, the use of either cryocooling\ citation{37} or a humidified stream of air\ citation{193} circumvents the need to seal the device, helping to minimize the background. However, it is more common to provide some layer of protection, particularly, as data collection at XFEL sources is often performed under vacuum. Examples have been reported for crystals embedded in oil;\ citation{34} however, most reports have sealed the device using thin films of plastic, such as polyimide,\ citation{31} mylar,\ citation{25} PMMA,\ citation{192} or polycarbonate.\ citation{195} These films have the advantage of being relatively cheap and easy to use. However, the physical strength and barrier properties of most polymer films decrease significantly as a function of film thickness, suggesting the possibility of a lower limit on the crystal size for mounting and stabilization strategies.
that rely on the use of polymer films. Alternatively, micromachining can be used to fabricate ultra-thin (e.g., 20–50 nm-thick) silicon nitride windows, which provide rigidity, sample stability, and excellent X-ray transmission. These hard materials have the potential to facilitate the analysis of nanocrystals but are more expensive and labor-intensive to manufacture than polymer films. Ultimately, it should be possible to use atomically thin barrier materials such as graphene for this purpose. While proof-of-concept results have been reported for the in situ analysis of crystals grown on a microfluidic chip at a synchrotron source, graphene-based materials have not yet been extended for use in high-throughput serial crystallography experiments at XFELs.

Generally, mesh structures represent a passive strategy for the creation of an ordered array of samples. Disadvantages of this approach are the potential for either clustering and overlapping of crystals at high concentrations or empty wells and less efficient data collection at lower concentrations. Active strategies for the arrangement of single crystals have also been reported. Brunger et al. reported a microfluidic trap array which utilized hydrodynamic forces to arrange microcrystals grown off-chip into an ordered array (Figure 9). In contrast, Fraden et al. reported that kinetic optimization of droplet-microfluidics was used to ensure the growth of only a single in each droplet. The droplets were then arranged into a microfluidic array for subsequent analysis (Figure 8(b)). Both of these approaches have tremendous promise as fixed-target delivery strategies. However, a key advantage of the droplet-based approach described by Fraden et al. is its ability to both grow and then analyze protein crystals in place, without the need for sample manipulation. Further studies would be needed to explore the limits of the crystal size and signal-to-noise levels achievable using this droplet-based approach, as well as an examination of whether such arrays of droplets would be stable in the destructive context of ultra-brilliant XFEL pulses.

Ultimately, a combination of factors must be considered in terms of experimental design and the development of a sample delivery strategy. The physical robustness, size, and available quantity of a particular protein target will dictate, in part, an optimal sample delivery strategy.

FIG. 9. A microfluidic trap array for protein microcrystals. (a) Schematic representation of the overall crystal-capturing device design. (b) Close-up of the general scheme for trap-and-bypass hydrodynamic crystal capture. (c) Schematic of a single hydrodynamic trap (labeled in (b); Wt is the width of the trap channel and Lt is the length of the trap channel). (d) Optical micrograph of a representative section of a fabricated crystal-capture chip. (e) Optical micrograph series showing single and multiple lysozyme microcrystals immobilized in hydrodynamic traps. Adapted with permission from Lyubimov et al., Acta Crystallogr., Sect. D: Biol. Crystallogr. 71, 928–940 (2015). Copyright 2015 International Union of Crystallography.33
for serial analysis. However, for hard-to-handle samples, there is tremendous opportunity to integrate microfluidic platforms for protein crystallization using in situ, high-throughput serial crystallography at both synchrotrons and XFELs. Furthermore, there is also tremendous potential to harness the complex fluid handling capabilities of microfluidic devices for more challenging time-resolved crystallography experiments.

V. TIME-RESOLVED STRUCTURE DETERMINATION

In addition to the aforementioned experimental design challenges for serial crystallography, experiments geared towards the elucidation of protein structural dynamics must also address the need for reaction initiation for time-resolved measurements. The detection of transient structural intermediates in time-resolved crystallography requires a rapid triggering event or pump (e.g., a laser pulse, temperature jump, or substrate addition) to synchronize reaction events within a crystal. This process must then be repeated to facilitate data collection at the various time points of interest, as well as at different crystal orientations. Prior to the development of serial methods for crystallography, these types of experiments were only feasible on very large, stable crystals where the structural change or enzymatic reaction was reversible and would naturally reset to the initial state, allowing for a large number of repetitions of this pump-probe cycle.\textsuperscript{19,196–203} The advent of serial crystallography has opened up the potential for time-resolved experiments to smaller crystals and targets that are highly sensitive to radiation damage, as well as reaction pathways that are irreversible within the limitations of the crystal lattice.\textsuperscript{177,201,204–206} Furthermore, the development of next-generation X-ray sources has expanded the range of available timescales from femtoseconds to seconds or longer.

A. Laser triggering

The challenge associated with reaction triggering is the necessity for the triggering event to occur at a faster rate than the kinetics of the structural changes in question. Because of this experimental requirement, a vast majority of time-resolved crystallography experiments have been performed on targets where a fast laser pulse can be used to trigger the reaction. Examples include photolysis-induced dissociation of CO from myoglobin\textsuperscript{207,208} and the photocycles of photoactive yellow protein (PYP),\textsuperscript{19,199,200,209–215} photosystem II,\textsuperscript{22,165–167} and bacteriorhodopsin.\textsuperscript{174} It is also possible to use laser triggering to initiate a reaction through the photorelease of a caged compound.\textsuperscript{202,216} However, many caged compounds suffer from poor solubility, and the resulting rate of initiation is limited by the rate of diffusion.

Serial approaches to time-resolved crystallography have recently been demonstrated using laser photoinitiation at XFELs (Figure 10). Initial studies on photosystem II,\textsuperscript{20,22} myoglobin,\textsuperscript{151} and photoactive yellow protein (PYP),\textsuperscript{23,24} took advantage of a GDVN injector. Photoactivation was achieved by laser pumping of the sample jet at a distance upstream of the X-ray pulse corresponding to the delay time of interest. The fast flowrates of GDVN injectors are such that time delays longer than $\sim$100 $\mu$s would require sample illumination within the jet itself\textsuperscript{22,141} or the use of a different sample delivery method. For example, the LCP injector has been used to facilitate the time-resolved analysis of the light sensitive membrane protein bacteriorhodopsin,\textsuperscript{27,174} and a fixed-target approach utilizing a silicon mesh with mylar windows was used to study CO dissociation from myoglobin.\textsuperscript{25} More recently, a combination of electrokinetic injection\textsuperscript{164} and an on-demand droplet injector with a conveyor-belt system\textsuperscript{169} was used with multiple laser pulses to drive the activation of varying states of photosystem II at delay times ranging from 0.5 s to 1 s.\textsuperscript{156,167}

B. Chemical triggering

While light is the fastest and simplest method for initiating a reaction, the vast majority of protein targets require a chemical trigger, such as the addition of a substrate, a pH jump, or a change in ionic strength. Experimentally, it is much more difficult to affect such a change, requiring mounting of crystals within a flow-cell to facilitate the fast delivery of the triggering
molecules. However, even under ideal conditions, the accessible experimental timescales are much slower for chemical triggering than for light-initiated reactions.

The rate at which reaction initiation can be carried out limits the accessible range of structural information. For chemical reaction initiation, the limiting rate is the timescale for the triggering molecules to penetrate the crystal. Thus, only structural changes that are slower than the diffusion time will be observable. If typical enzyme cycle times are on the order of microseconds to milliseconds, one can then calculate the timescale for the diffusion of the triggering molecule and determine the size of protein crystals, which will avoid diffusional limitations. Calculations by Schmidt, based on the diffusivity of glucose and assuming no significant barriers to diffusion within the crystal, suggest that the size of microcrystals is critical for the success of chemically triggered time-resolved experiments (Table III). Furthermore, the use of high concentrations of triggering molecules is important to facilitate diffusion and achieve effective reaction initiation. With these experimental requirements in mind, many of the injection strategies for serial crystallography are uniquely posed to usher in a new era of dynamic protein structural studies.

Preliminary reports have described the use of GDVN injectors to facilitate chemical triggering. By including an additional capillary into the GDVN architecture, diffusive mixing between the crystal slurry and an outer sheath of triggering solution can be achieved. The time for mixing can then be altered by adjusting the location of the mixing point within the larger GDVN structure. This approach has been used to study the reaction of β-lactamase microcrystals from M. tuberculosis with an antibiotic solution of ceftriaxone and the binding of ligands to the adenine riboswitch adaptor domain. For both these reports, crystals on the order of 1–10 μm in size were analyzed at time-delays on the order of 2–10 s. The use of the GDVN facilitated matching of the jet diameter to that of the crystals. While the mixing injector
used in these studies is expected to enable the study of kinetics as fast as 500 μs, significantly smaller crystals and a commensurate decrease in the jet diameter would be needed to maintain signal-to-noise quality.

A similar approach could be applied to the other injector architectures. However, for the high viscosity jets, diffusion through the highly viscous matrix material is expected to limit the range of accessible timescales to only very slow reactions. Similarly, the relatively large droplet size for on-demand droplet injectors may represent significant limitations in terms of the smallest possible crystals that can be analyzed and the timescales for chemical triggering. However, the potential exists to probe faster enzyme kinetics if fast mixing strategies can be coupled with electrokinetic or aerosol injectors that are compatible with nanocrystals.

To date, there have been no reports of fixed-target strategies for chemical triggering. The use of individually addressable sample locations within an integrated microfluidic platform has the potential to enable extremely precise and potentially automated control. Unfortunately, there are currently no reports of microfluidic integration within the types of ultra-thin devices that would be necessary for the study of typical enzyme kinetics. Thus, it is difficult to estimate the potential rate of data collection using such a platform.

VI. SUMMARY AND OUTLOOK

We have discussed the impact and potential of microfluidic technologies related to protein structure determination. The small scale of these devices, coupled with exquisite control over flow patterns and local concentrations, has been instrumental in the development of technologies for both protein crystallization and serial crystallography. Looking forward, the push to enable structure determination on even more challenging targets will require additional innovation regarding the design and fabrication of such devices. In particular, new strategies are needed to enable the use of chemical reaction initiation for time-resolved experiments in fixed-target devices, while ultra-thin materials such as silicon nitride membranes and large-area graphene sheets are critical to enable the analysis of ever smaller and more weakly diffracting targets. Strategies that couple in situ crystallization and analysis may also prove to be critical for targets where either physical handling and/or environmental exposure are a challenge. It is also exciting to consider the potential application of these materials in related experiments coupled to protein structural dynamics, including X-ray scattering and continuous diffusion/diffuse scattering, where the signal-to-noise and low background are critical.

To date, the majority of serial crystallography efforts have been directed towards XFEL sources because of the requirements of such “diffraction before destruction” experiments. However, there are growing efforts to extend these types of data collection strategies to synchrotron sources. Beyond static structure determination, the even more intense, microfocused third generation synchrotron sources also have the potential to enable time-resolved experiments using both monochromatic and polychromatic Laue diffraction strategies over timescales.

<table>
<thead>
<tr>
<th>Crystal size</th>
<th>$\tau_D$</th>
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<tr>
<td>$300 \times 400 \times 500 \mu m^3$</td>
<td>9.5 s</td>
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<td>$10 \times 20 \times 30 \mu m^3$</td>
<td>15 ms</td>
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<td>$3 \times 4 \times 5 \mu m^3$</td>
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<tr>
<td>$0.5 \times 0.5 \times 0.5 \mu m^3$</td>
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<tr>
<td>$0.1 \times 0.2 \times 0.3 \mu m^3$</td>
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*aBased on the diffusivity of a typical substrate, such as glucose ($5 \times 10^{-6} \text{ cm}^2/\text{s}$) assuming no barriers to diffusion within the crystal lattice. Tortuosity and/or hindered diffusion may result in increased diffusion times. Variations in diffusivity scale inversely with the effective radius and/or molecular weight of the molecule.

*bWith much smaller crystals, mixing times might be slower than diffusion times.
from the sub-nanosecond-scale and longer. Technological and software developments in the field are rapidly advancing to the point where the automated collection of dynamic structural information has the potential to become as accessible as cryocrystallographic structure determination is today.


