Grease matrix method for serial femtosecond crystallography using XFELs

Using femtosecond X-ray pulses from X-ray free-electron lasers (XFELs), serial femtosecond crystallography (SFX) offers a route to overcome radiation damage to small protein crystals via the "diffraction-before-destruction" approach. A single-pulse X-ray exposure will completely destroy small individual crystals; therefore, fresh specimens must be supplied for subsequent X-ray pulses to continue data acquisition (Fig. 1). Diffraction signals up to a few angstroms in resolution can be obtained from even submicrometer-size crystals, thereby greatly reducing the difficulty of producing large crystals. SFX has expanded the window for obtaining room temperature structures of proteins. More recently, it has also been applied in time-resolved studies of light-driven structural changes and chemical reaction dynamics.

SFX is contingent on a reliable and tractable supply of protein crystals to complete the data collection. Tens of thousands of diffraction patterns from specimens in random orientations are required to obtain a structure. At SACLA (SPring-8 Angstrom Compact Free Electron Laser), SFX has been carried out using a diverse application platform for hard X-ray diffraction in SACLA (DAPHNIS) system, which consists of a sample chamber, injectors and an MPCCD detector [1]. Liquid jet injection of small protein crystals is often exploited for serial sample loading. A continuous flow of the liquid-jet injectors at a relatively high speed (~10 m/s) consumes 10–100 mg of the protein sample; consequently, the applicability of SFX to proteins with low expression or poor crystallization is limited. Although a rather large amount of sample is needed to form a continuous jet stream, less than 0.01% of the crystals are typically exposed to X-ray pulses. On the other hand, the microextrusion of specimens using viscous media such as monoolein, which is used for the crystallization of membrane proteins in the lipidic cubic phase (LCP), can maintain a stable stream at a low flow rate of 0.02 – 0.5 μl/min, which helps to reduce sample consumption (~0.3 mg) [2]. However, this approach is probably limited to proteins crystallized in the LCP. A more universal method that is applicable to a wide variety of proteins is essential to firmly establish SFX.

In this study, we introduce an oil-based grease matrix as a generic carrier of protein microcrystals for SFX using XFELs [3]. In protein X-ray crystallography, a mineral oil is used as a versatile cryoprotectant for a wide variety of proteins without serious damage to crystals. A grease matrix provides maximum adaptability for most classes of proteins with a straightforward sample-loading procedure, protection against the cracking and dissolution of protein crystals due to various physical or chemical events such as osmotic shock arising from the properties of other viscous media (e.g., hydrogels), and preservation of the aqueous environment of the native protein molecules. We successfully applied a grease-matrix carrier to various proteins including lysozyme, glucose isomerase, thaumatin and fatty acid–binding protein type 3 (FABP3) in SFX experiments and obtained electron density maps beyond 2 Å resolution using less than 1 mg of micrometer-size protein crystals.

We performed the SFX experiments using femtosecond X-ray pulses from SACLA. Each X-ray pulse delivered ~7×10^{10} photons within a 10 fs duration (FWHM) to the samples with a grease matrix. The experiments were carried out using a DAPHNIS at **BL3**. We suspended protein microcrystals in the grease medium. An aliquot of the sample was loaded into a syringe. We used storage solutions of microcrystals of the proteins lysozyme (size 7–10 μm, Fig. 2(a)), glucose isomerase (10–30 μm), thaumatin (10–30 μm) and FABP3 (10–20 μm). For SFX data

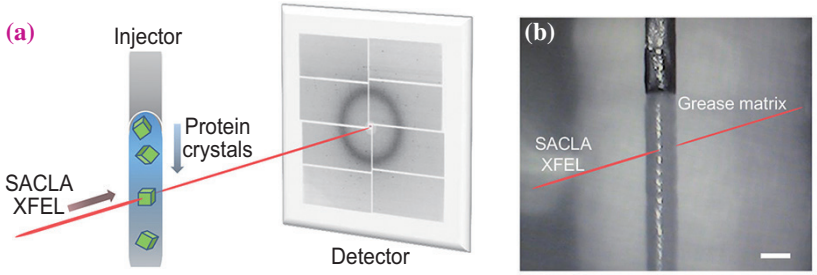

Fig. 1. Serial femtosecond crystallography. **(a)** Fresh nano/microcrystals are supplied for subsequent X-ray pulses to continue data acquisition. **(b)** Sample extrusion of the grease matrix through a syringe needle. Grease matrix was extruded as a continuous column to intersect with the XFEL beam. Scale bar represents 240 μm.

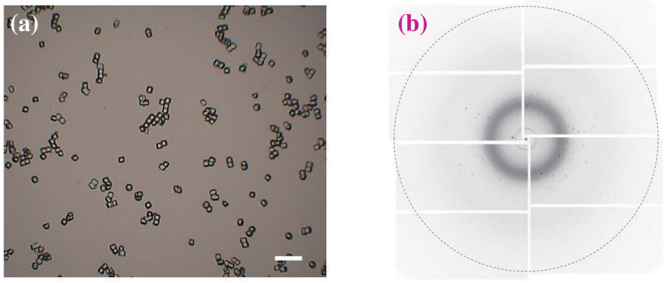

Fig. 2. **(a)** Lysozyme microcrystals used for SFX measurements. Scale bar represents 40 μm. **(b)** Typical XFEL single diffraction pattern from grease matrix. The resolution at the edges corresponds to ~1.6 Å (dashed circle).

collection, we used a sample volume of ~30 μl (10^7 crystals/ml). The grease matrix containing randomly oriented protein microcrystals was continuously delivered to the 1.5-μm (FWHM)-diameter focal spot of the XFEL radiation using a syringe injector with a 110-μm-i.d. needle installed in a diffraction chamber with a helium atmosphere. The sample flow rate was 0.5 μl·min^{-1}. The grease formed a stable flow for all protein samples (Fig. 1(b)). Diffraction patterns were collected using a custom-built multiport CCD.

We collected single-pulse X-ray diffraction patterns of microcrystals from the four different proteins (Fig. 2(b)). With the SACLA running at a 30 Hz repetition rate, ~100,000 diffraction patterns were collected within 1 h using a sample volume of less than 30 μl. We successfully indexed and integrated 8,000–30,000 patterns for each of the proteins. Lysozyme, glucose isomerase, thaumatin and FABP3 crystals yielded data sets beyond 2.0 Å resolution. We determined the room-temperature crystal structures of lysozyme (PDB ID: 3WUL), glucose isomerase (4W4Q), thaumatin (3WXS) and FABP3 (3WXQ) at 2.0, 2.0, 2.0 and 1.6 Å, respectively. We were able to observe clear electron density maps; an example is shown here for lysozyme (Fig. 3). An increase in the crystal hit rate (indexed patterns/collected diffraction patterns) can be achieved with increased crystal number densities, which would, however, cause multiple sample hits in one shot and complicate the indexing procedures. We found that a crystal number density of ~10^7 was suitable for SFX data collection under our experimental conditions using the 110-μm-i.d. needle and a 0.5 μl·min^{-1} flow rate.

In summary, using a grease matrix as a general carrier of protein microcrystals for serial sample loading in SFX, we successfully obtained room-temperature structures beyond 2 Å resolution from four different proteins in 7 – 30 μm microcrystals using less than 1 mg of a sample. More recently, using the grease matrix technique, Yamashita and coworkers have demonstrated a single isomorphous replacement with anomalous scattering (SIRAS) phasing for a Hg-derivatized luciferin-regenerating enzyme [4]. In addition, we have successfully determined the structure of native lysozyme with single-wavelength anomalous diffraction (SAD) by utilizing the weak anomalous signals of sulfur and chlorine [5]. One of the major challenges of phasing in SFX is to improve the signal-to-noise ratio. This technique using the matrix with low background scattering noise will contribute significantly to measuring weak anomalous signals for *de novo* phasing from SFX data. Our grease matrix-based approach should be applicable to a wide range of proteins as well as inorganic nanocrystals to facilitate investigations to determine structures with atomic resolution using single-pulse exposure.

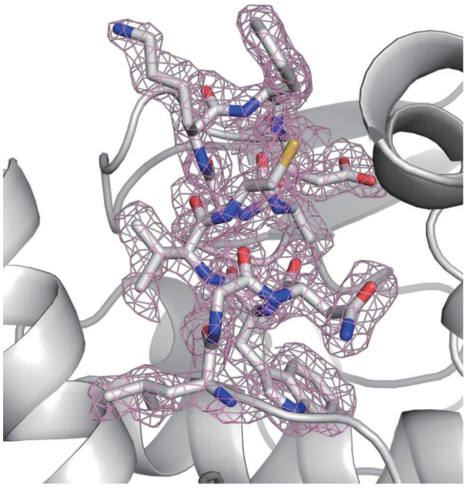

Fig. 3. Close-up view of the lysozyme structure with a $2F_o$ -F_c electron density map contoured at the 1.0σ level.

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