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Transcription with a Laser: Radiation-Damage-Free Diffraction of RNA Polymerase II Crystals

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Keywords

Free electron laser; Nano-crystals; radiation-damage-free data collection; RNA polymerase II

Introduction

DNA-directed RNA Polymerase II (Pol II) is a highly-conserved protein among eukaryotic organisms and plays a fundamental role in cellular life, specifically the transcription of genes into messenger RNA (mRNA). Structural studies of Pol II have been very successful and have allowed observation of Pol II in its apo form [1, 2], in the process of initiation and elongation [3–5], and during backtracking [6] or paused by DNA lesions [7–11]. Moreover, the remarkable single particle cryo-electron microscopy structures of Pol II in complex with the general transcription factors and mediator (preinitiation complex) [12–14]; and with the elongation factors [15–17] have provided snapshots of the complexities of the initiation and elongation steps. A molecular picture of the role of the individual factors during transcription initiation and elongation is beginning to emerge and has generated tremendous progress towards our understanding of gene processing and regulation. Similarly, structural studies of Pol II bound to a matched nucleotide have revealed the architecture (at 4.0 Å resolution) of Pol II during synthesis of the nascent transcript [18]. This structure illustrated for the first time the trigger loop in the “on” or closed conformation, and the matched nucleotide in the substrate binding pocket; however, these studies were hampered by the low resolution of the data. Moreover, electron density for the two Mg²⁺ ions in the active site was not clearly discernable, which could be possible attributed to the effect of radiation damage during data collection. Thus, in order to study the molecular mechanism of nucleotide addition in greater detail, i.e., at near atomic resolution, Pol II crystals are suitable candidates for 1) visualization and optimization of crystal lattice quality using TEM [19–21]; and 2) FEL experiments at the LCLS to improve resolution (since diffraction of Pol II crystals is dose dependent), and to obtain radiation-damage-free structures [22, 23] that could allow observation of metals and solvent molecules in Pol II’s active site.

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#Equal contribution

1. TEM guided optimization of Pol II crystals

Crystallization of protein targets remains the most significant challenge in the process of structure determination by macromolecular crystallography. Recent efforts towards improving sample “crystallizability” using techniques such as alanine scanning mutagenesis [24–26] or the use of chimeric proteins to promote/improve crystal packing [27–31] has led to structures of important targets. However, less effort has been applied towards the discovery, evaluation and optimization of crystals and nano-crystals (NCs). Transmission electron microscopy (TEM) is one of the best techniques to do so [19–21, 32]. Here, we will describe reproducible protocols, using TEM to visualize lattices from fragmented crystals and to study details of the crystallization process. Our experiments have shown that for all protein crystals tested, TEM images reveal details of the crystal lattices that prove to be accurate qualitative indicators of the potential diffraction of the crystal. In general, the detection by negative stain TEM methods of well-ordered lattices with third order Bragg spots was a good predictor of X-ray diffraction, while samples with disorganized lattices yielded no diffraction [19]. Moreover, TEM has been shown to have an important role to identify crystal pathologies that contribute to poor X-ray diffraction data [32]. These include: 1) crystal lattice defects; 2) anisotropic diffraction; and 3) crystal “polluting” by heavy protein aggregates and micro-crystal nuclei. Detection of lattice defects in some crystals could point to the presence of samples containing protein contaminants, aggregates or partially proteolyzed protein as well as discrepancies in the stoichiometry of the sample. Similarly, identifying crystals that possess anisotropic diffraction may indicate that steps to improve crystal contacts, such as altering or adding reagents to the crystallization conditions or modification of the protein itself, may be advisable. Importantly, this information cannot be observed with other techniques. The use of TEM has also enabled qualitative estimation of crystal solvent content and allowed the study of lattice dehydration on crystal diffraction [32]. This application was particularly noteworthy since 1) crystal dehydration protocols have proven very useful in the improvement of X-ray diffraction, and 2) negative staining with uranyl acetate provides very high contrast between solvent channels and biological macromolecules. Overall Information obtained by TEM experiments could provide critical advice to the experimenter about crystallization conditions to be pursued and would also allow monitoring of crystal optimization protocols.

2. FEL data collection of Pol II crystals

X-ray crystal structures are generally limited by the ability to obtain large, well diffracting crystals. The emergence of FEL based serial femtosecond crystallography (SFX) opens the possibility of solving the three-dimensional structures of samples that can only crystallize as nano-crystals (NCs). FEL sources provide X-rays ten times more brilliant than modern day synchrotrons [33]. SFX takes advantage of these ultra-short pulses to collect single diffraction images from crystals 200–10000 nm in size crystals [22, 34]. SFX relies on gas dynamic virtual nozzle (GDVD) liquid jet which injects a NC slurry into the path of a synchronized ~50 femtoseconds long X-ray pulse under vacuum [35, 36]. With each pulse a single diffraction image is collected before the intensity of the beam destroys the NC. Successful implementation of these technologies has yielded high profile structures with several groups involve in its implementation [37].

However, in spite of the success of the GDVD setup, large amounts of protein are required to produce several milliliters of a 20–30% crystal slurry solution for SFX experiments. For some proteins this is a viable option, however for challenging proteins that require special conditions for expression and purification, such as Pol II, this is technically difficult to achieve. Another hardship of this method includes the potential damage that mechanically sensitive crystals could sustain during pressurization and “flight” [32] affecting crystal diffraction and resolution.

In addition to the liquid injector setup, the X-ray pump-probe (X-PP) features FEL data collection at cryogenic temperatures using a 50 fs beam pulse of approximately 12–30 micrometers in diameters [38]. Crystals are mounted on cryo-loops and diffracted individually. However, since the intensity of the beam creates a 20–30 micron damage area [23] around the target spot, the number of useful diffraction images per crystal is limited, and therefore, a large number of cryo-loops holding multiple crystals are required to collect a full dataset.

Here, we describe a new methodology to optimize Pol II crystals quality using TEM, and introduce a method to improve FEL data collection. Our results show that TEM guided crystal growth allowed consistent diffraction of Pol II crystals to up to 3.3 Å resolution for conventional synchrotron radiation and to 3.0 Å resolution for FEL radiation.

Results

1. Visualization and evaluation of Pol II nanocrystals using TEM.

1.1 Visualization of crystal drops to detect UV-positive granular aggregates.

- a. Hanging drop crystallization experiments using 24 well-plates are performed by adding 1 µl of Pol II (10 mg/ml) or Pol II complex (at the desired concentration) to 1 µl of crystallization screen solution on siliconized cover-slips. Trays are inspected daily using brightfield microscopy to screen for conditions with crystals or granular aggregates.
- b. Crystal drops containing granular aggregates under brightfield microscopy (Fig. 1, A and B) are selected for further evaluation using ultra violet (UV) microscopy (Jansi, Molecular Dimensions). UV positive granular aggregates can be easily discerned with the use of a 10 X objective (Fig. 1, C and D). Note 1: for very fine granular aggregates use of a 40 X objective might be required. Note 2: False UV-positive granular aggregates can be observed in crystallization conditions that include calcium salts [21].

1.2 Negative stain TEM studies of crystal drops with UV-positive granular aggregates.

- a. Four hundred square mesh copper grids with continuous carbon film (CF400-CU, Electron Microscopy Sciences) are negatively glow discharged for 1 min at 25 mV (EmiTech KX 100) not more than 20 min before applying the samples.

- b. The coverslip of a well of interest (bearing UV-positive granular aggregates) is opened, and 4–6 μ l of mother liquor are added to the crystal drop and mixed carefully by gently pipetting the drop up and down several times.
- c. Approximately 2 μ l of the granular mix are added to the carbon film electron microscopy grid and incubated for 1 min before the excess liquid is blotted away with P2 filter paper (Fisherbrand) from the side. Then the grid is stained twice by placing it (carbon side down) on top of 200 μ l of a 2 % (w/v) uranyl acetate solution for 40 seconds. After incubation, the grid is blotted from the back to remove the staining solution and dried on air for 20 minutes.
- d. The nature of the granular aggregates can be inspected by collecting TEM images on a FEI TECNAI T12 electron microscope operating at 120 kV using a single-tilt specimen holder (Fig. 1E). Images are acquired with a $2k \times 2k$ Gatan UltraScan 1000 CCD camera, typically at magnifications between 11000 and 52000 x. FFT calculations of the lattices are used to determine the crystal quality [21].
- e. Observation of crystal fragments on EM requires preparation of several grids before mastering the technique, we urge the experimenter to spend a considerable amount of time searching the grid for crystals.

1.3 Negative stain TEM studies of fragmented crystals.

- a. Four hundred square mesh copper grids with continuous carbon film (CF400-CU, Electron Microscopy Sciences) are negatively glow discharged for 1 min at 25 mV (EmiTech KX 100). Glow discharged grids should be used promptly, we suggest to proceed within 20 min.
- b. Fragmentation of crystals is achieved by adding approximately 10 μ l of glass beads (Research Products International) to a 1.5 ml Eppendorf tube and washed with 500 μ l of 20% (v/v) ethanol, followed by dH₂O washing and equilibrated with 20 μ l of stabilizing buffer (well buffer). The size of glass beads (0.5 mm or 1.0 mm diameter, Fig. 2A) is selected depending on the stability of the protein crystal; smaller beads result in harsher crystal fragmentation [32]. For fragile crystals (needles and plates) we recommend using 1.0 mm beads; for sturdier and “chunkier” crystals, 0.5 mm beads are generally needed.
- c. The coverslips of wells of interest are opened and 6–8 μ l of mother liquor are added to each crystal drop in order to allow crystal transfer to the Eppendorf tube containing the glass beads (which should be pre-equilibrated with the corresponding mother liquor or stabilizing solution). The crystals are fragmented by vortexing (about 2 seconds to 2 min) until UV microscopy images reveal a homogeneous slurry of high density crystal fragments with edge lengths of around 1–5 μ m (Fig 2B–D). Alternatively, in the absence of an UV-source, slurries should always be inspected using brightfield microscopy to ensure that proper lysis has occurred. For scarce material containing fewer granular aggregates, it is recommended to use stainless steel beads that can be removed from the tube using a strong magnet. Concentration of the slurry can be achieved

by removing the magnetic beads, followed by low speed centrifugation (1500 rpm) to pellet down crystal fragments followed by removal of excess stabilizing solution. Note 3: crystallization conditions that have highly viscous solutions (for example 25% polyethylene glycol 8000 (peg8K)) are difficult to handle or visualize on EM grids. For such conditions it is convenient to find a stabilizing solution with low viscosity where aggregates do not dissolve. A solution containing 25% peg8K could be exchanged with a stabilizing solution containing 30–40% methyl pentanediol (MPD).

- d. Approximately 2 μ l of the fragmented crystal slurry are added to the carbon film electron microscopy grid and incubated for 1 min before the excess liquid is blotted away with P2 filter paper (Fisherbrand) from the side. Then the grid is stained twice by placing it (carbon side down) on top of 200 μ l of a 2 % (w/v) uranyl acetate solution for 40 seconds. After incubation the grid is blotted from the back to remove the staining solution and dried on air for 20 minutes.
- e. The nature of the crystal fragments can be inspected by collecting TEM images on a FEI TECNAI T12 electron microscope operating at 120 kV using a single-tilt specimen holder. Images are acquired with a $2k \times 2k$ Gatan UltraScan 1000 CCD camera, typically at magnifications between 11000 and 52000 x. FFT calculations of the lattices are used to determine the crystal quality [21].
- f. Evaluation of crystal lattices using TEM (Fig. 2E) can reveal valuable information regarding crystal properties including potential diffraction quality, lattice anomalies such as anisotropy, solvent content and multinucleation [32]. Moreover, the effects of serial seeding or crystal dehydration on Pol II crystals can also be monitored through TEM studies [32]. Crystal slurries from TEM inspected samples with high quality lattices were used for quantitative seeding experiments [32] to generate Pol II crystals for FEL diffraction experiments. In our experience multiple rounds of seeding or “serial seeding” produce the best crystals yielding data sets diffracting consistently to 3.3 Å at conventional X-ray sources.

2. Data Collection strategies of Pol II crystals.

2.1 Design and use of customized multi-crystal holders (MCHs) to optimize data collection at LCLS.

- a. Given the scarcity of beamtime at free electron laser facilities, it is highly desirable to optimize data collection in order to use the largest number of crystals available for diffraction experiments. Thus, in order to maximize the number of crystals mounted per loop, we designed diamond-shaped multi-crystal holders (MCHs) measuring 1.5 mm $\times$ 1.5 mm in length and 50 μ m thickness (Fig. 3A).
- b. Crystal mounting using MCHs. Crystals of interest are set on hanging drop crystallization trays using 1 μ l protein and 1 μ l mother liquor. Once crystals achieve the desired size, approximately 6–8 μ l of stabilizing solution (containing a previously screened cryoprotectant) are added to crystal drops. Multiple crystals are picked in one single scoop. Using a thin piece of Whatman filter

paper, excess fluid is removed carefully from the MCHs to avoid drawing crystals out of the loop during this procedure.

- c. Immediately after excess fluid removal, loops can be flash-frozen (Fig 3B). Alternatively, crystals can be UV-imaged to determine their loop positions for automated data collection (Barnes et al, under review) (Fig. 3C).
- d. Crystals mounted on MHCs can be easily detected at the MFX station (LCLS) (Fig. 3B), thus allowing selection of multiple targets for efficient data collection. For small crystals difficult to visualize, UV images of MCHs can be used to determine the positions of the crystals on the holder to allowed automated data collection (Barnes et al, under review). Use of FEL diffraction images of high quality Pol II crystals showed spots beyond 3.0 Å at the LCLS (Fig. 3D). Note 4: We have successfully used MHCs for conventional X-ray data collection at the APS and SSRL.
- e. Data collection of Pol II crystals at LCLS was carried out using 9.5 keV X-ray pulses (40 fs duration) and an 8 μm beam. Diffraction images were recorded on a Rayonix MX325 detector and processed using the cctbx.xfel software package [39]. The combination of crystal optimization using high quality crystal fragments and data collection at FEL allowed data reduction and scaling to 3.0 Å (Fig. 3E).

Discussion

High quality crystals are critical to obtain structural information from Pol II complexes. Crystal optimization is traditionally performed using a “trial and error” approach where the initial conditions that produced visible crystals (under brightfield microscopy) are modified with the goal of obtaining well-diffracting crystals. Here we present a rational approach where 1) the crystallization space is expanded with the inclusion of screening of UV-positive granular aggregates which upon negative stain TEM studies can yield NCs with well-ordered lattices. These NCs can be used for seeding experiments or optimized for X-ray or electron diffraction experiments [32, 40–44]. 2) Optimization of crystallization conditions can be monitored and guided through the use of TEM, i.e., TEM-guided crystal optimization. This strategy allowed consistent diffraction of Pol II crystals to 3.3 Å.

The study of crystal fragments using TEM is another important tool in the crystallographer’s arsenal since it allows observation of the crystal lattices and therefore provides a unique opportunity to evaluate its diffraction potential. Well-ordered crystal lattices with high order Bragg spots are usually associated with well-diffracting crystals [20, 32]. Thus, poorly diffracting crystals with well-ordered lattices can improve diffraction by adjusting cryo-conditions.

Given that diffraction of WT Pol II crystals is dose dependent, optimization of data collection at FEL sources allowed diffraction of Pol II crystals to 3.0 Å.

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Highlights

1. Use of negative stain transmission electron microscopy (TEM) allows visualization of Pol II nano-crystals (NCs) and evaluation of crystal lattices for wild type (WT) Pol II crystals optimization
2. Use of novel data collection protocols at LCLS to obtain radiation-damage-free structure of Pol II.
3. Data collection of radiation free damage data to 3.0 Å, the highest resolution for WT Pol II crystals collected to date.

Fig 1.
Discovering Pol II nano crystals from crystallization screens. A and B, conditions bearing granular aggregates showing positive UV images (C and D, respectively) illustrated as white granular patterns. E, Negative stain TEM of Pol II NC showing a well order lattice.

Fig 2.

Fragmentation of Pol II crystals. A Glass beads used for crystal fragmentation measuring 0.5 and 1 mm (right to left). A. Teflon ball measuring 5 mm (white) is illustrated for comparison. B. Pol II crystals before fragmentation. C. Pol II crystals after fragmentation, D UV image of C to illustrate size homogeneity of crystal fragments. Nano crystals observed after fragmentation illustrating the presence of a well-ordered lattice. Individual Pol II particles can be observed in the micrograph, arrows

Fig 3.

FEL data collection on multi-crystal holders (MCH). MCHs (left) used for crystal mounting, a mitegen loop (0.3 mm) is illustrated for comparison. B. Brightfield image of crystals (of approximately 0.2 μm) mounted on MCHs. C. UV image of B, Crystals adopt random orientations on the loops. D. FEL diffraction of Pol II crystals mounted on MCHs. E. Sigma-A weighted electron density map ($2F_0 - F_c$) counteracted at 1.2σ from FEL data illustrating the presence of solvent molecules and density for Pol II residues. Water molecules interact with neighboring residues to form hydrogen bonds (black broken lines).