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High-Resolution Electron Diffraction of Protein Crystals in Their Liquid Environment at Room Temperature Using a Direct Electron Detection Camera

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The study of proteins and their structural conformation is an important step towards understanding the different mechanisms that organisms use in order to keep life going. Besides X-ray crystallography and nuclear magnetic resonance, transmission electron microscopy (TEM) has been extensively and successfully used and has become an essential tool over the past decade. In this respect, the pioneering work of Richard Henderson, Jacques Dubochet and Joachim Frank in the development of cryo-electron microscopy (cryo-EM) pushed the broad adoption of this technique for the visualization and reconstruction of soft matter [1, 2, 3]. However, the use of vitrified samples kept at liquid nitrogen temperature limits the imaging of dynamics, and also raises the question if crystalline samples would have the same crystal structure as in their natural environment. For this reason, a new field called liquid phase EM is expanding to answer these questions by means of specifically-designed liquid holders [4], but also through the encapsulation of biological specimens with graphene sheets [5]. Although plenty of works have been published on the imaging of liquid samples, very few have been focused on the potential to obtain crystallographic information via electron diffraction (ED). Keskin et al. [6] showed that a number of reflections from catalase crystals could be acquired if wrapped with two graphene layers, yet the number of ED reflections appear quite limited for possible structure analysis. Here, we demonstrate how improved ED quality on lysozyme crystals can be obtained using a direct electron detection camera and a special specimen preparation using one-layer or three-layer graphene sheets supported by lacey C on Cu grids, as well as ultrathin continuous C films (3-4 nm in thickness) supported on Cu grids.

Lysozyme was crystallized as urchin-type needle-like crystals (orthorhombic crystal form) as a test sample. Less than 0.5 µL of crystals in crystallization buffer (Na citrate 100 mM pH 5.5, NaCl 1M) was placed on one of the coated Cu grids and then another one was quickly positioned at top (with the film side facing the liquid) to seal the sample, i.e., the crystals in their buffer. After 5 minutes, the resulting "sandwich" liquid cell (LC) was placed on a standard single-tilt holder for TEM measurements on a FEI Tecnai F20 FEG operated at 200 kV. Notably, the grids were previously treated with a mild glow discharge (25mA) for 30 s to increase the hydrophilicity, leading to a better dispersion of the sample. Figures 1A and 1B show the typical view of these LCs with one layer graphene grids and ultrathin continuous C grids, respectively. In some cases, the needle-like crystals are surrounded by salt crystals, which suggests that the protein crystals have been dried out and denatured (indicated with red arrows in figures), while others appear to be encapsulated in liquid.

In this work, a FEI 4k CETA-M CMOS-based camera was first used to acquire an image of about 30 µm x 30 µm with a very low illumination to check the availability of thin crystals. Later, a selected area aperture that covered a circular area of around 1 µm in diameter was positioned to the most promising crystal, and diffraction patterns were acquired with a Medipix CheeTah M3 (512x512 pixels) direct electron detector (ASI). The integration mode at 5 ms exposure time for each frame and a very low illumination (< 0.01 e-/A^2s) were used for their collection. The sample was not exposed to the beam during the intermediate steps between image and pattern acquisitions, minimizing any beam damage. Figures 1C to 1F show the obtained ED patterns with the different kind of grids that reveal the Bragg reflections from lysozyme crystals. Up to 100 reflections can be found per pattern at a maximum resolution of ~ 3 Å. Although the ED intensities are weak, they are clearly visible, and we believe that their detection can be further enhanced by the optimization of sample preparation and data acquisition procedures. The use of this specific "sandwich" LCs and direct detection cameras open the possibility of room temperature electron crystallography with protein crystals kept in their mother liquor [8].

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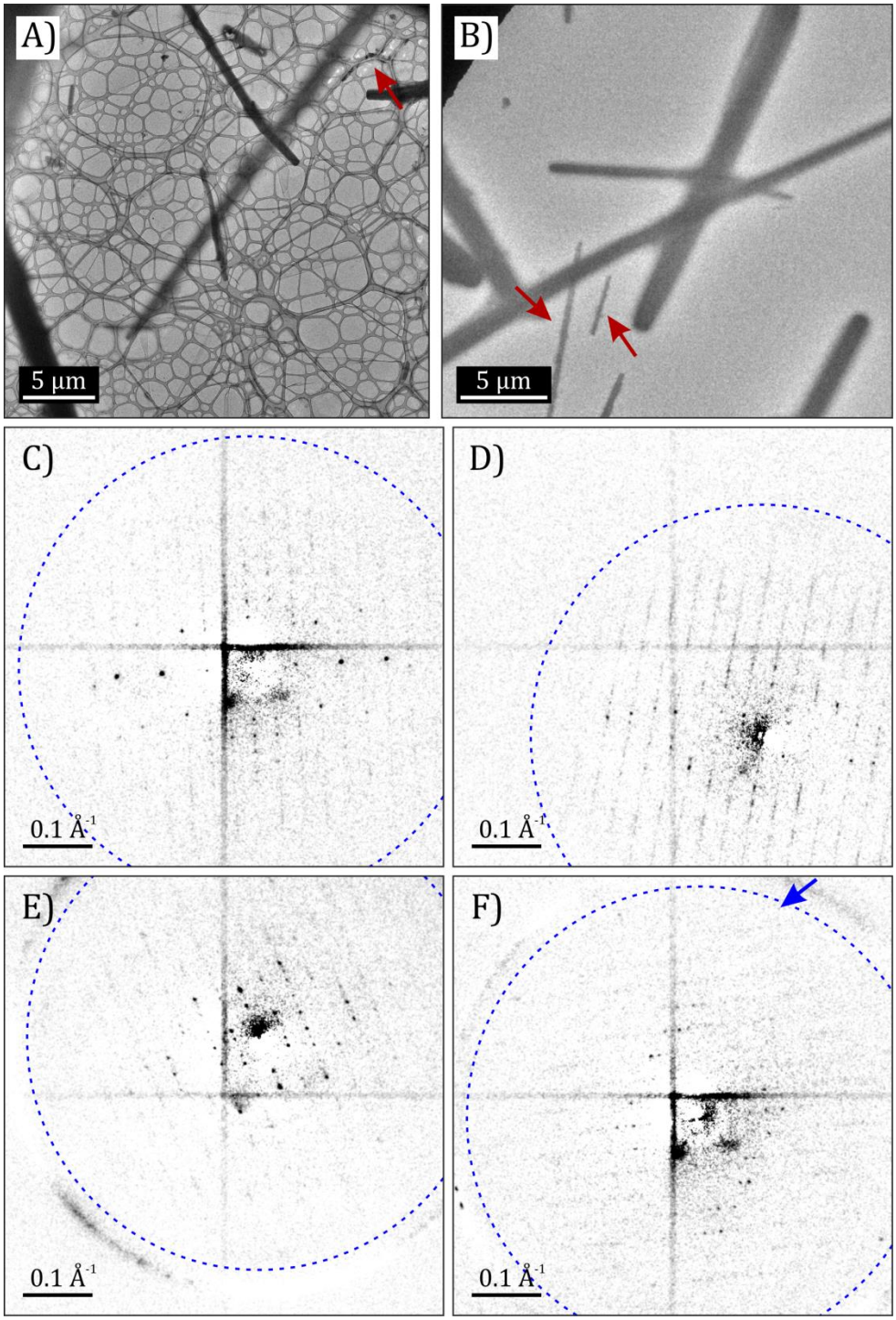

Figure 1. TEM images of lysozyme crystals encapsulated by the "sandwich" approach with A) one layer graphene Cu grids and B) ultrathin continuous C films (3-4 nm) on Cu-grids. Diffraction patterns from encapsulated crystals with C)-D) continuous C, E) one layer graphene and F) three layers graphene grids. Red arrows indicate salt crystals caused by the drying of the buffer liquid. Blue dashed circles mark the 3 Å resolution limit, and the blue arrow points to the reflection observed at the highest resolution. The pattern backgrounds were removed with the radial-background subtraction tool from the *diffractem* software package [7].

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