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Solving Protein Nanocrystals by Cryo-EM: Multiple Scattering artifacts.

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Glaeser et.al.[1] have evaluated the importance of multiple elastic scattering on transmission electron diffraction (TED) patterns from 2D protein crystals. At 200KeV, for monolayers (< 10nm thick) they find only small perturbations. But at larger thicknesses, large perturbations generate Bragg intensities that bear no relationship to the kinematic or single-scattering values needed for structure analysis [2,3]. Recently however, Shi et.al.[4] have reported 3D electron density maps from 0.5um thick lysozyme crystals at 3Å resolution using TED at 200KeV. Since multiply scattered TED intensities are known to agree with high accuracy with experimental intensities in CBED (see [2,5] for reviews), we use ZMult (multislice program in [3]) to simulate diffraction intensities as a function of thickness for a 200 kV beam oriented along (001) in lysozyme. As is common practice in the cryo-EM community, we assume the Bragg beam intensities are proportional to the X-ray stucture factor moduli (however, see [3]). The x-ray crystallographic program PHENIX was used to study the accuracy of these structure factors and their effect on charge density maps for various thicknesses of protein.

First, we study the variation of diffracted beam intensity with thickness for lysozyme crystals. Using this, we identify the kinematic limit at various resolutions. For example, in fig (1a), we see the characteristic pendellosung oscillations for high (23 13) and low (4 4) resolution diffracted beams (3Å and 14Å respectively). Allowing 10% error in the intensities, we calculate the kinematic (single-scattering) limit and tabulate the results as in fig (1b). At 3Å resolution (23 13), this is 22.74nm. The limit increases with incident beam energy (2), as shown in fig (1b). The reduction in ionization damage, but rapid increase in "knock-on" ballistic damage, with increasing beam energy has been thoroughly studied and reported in the literature for beam energies up to 3 MeV (see [2,6] for reviews).

In protein crystallography, it is widely accepted that about 2/3 of the information in a charge density map comes from structure factor phases (commonly obtained using the molecular replacement method, with a similar protein as model) and 1/3 from the intensities. To evaluate this, we introduce random errors to the x-ray intensities from lysozyme (4ET8.pdb) and maintain accurate phases. We then plot an omit map (omitting residues 50-60), as in fig 1(c), where the charge densities (wireframes) are laid over the ideal structure (rods) from the model. For a perfect fit these should agree. At the region of interest (r.o.i) marked with a star, with 30% error in structure factor amplitudes (|F|) or greater, the wire frames substantially fail to overlap with the rods. We can also identify the threshold error beyond which, similar (but not identical) amino acid sequences from hen and turkey lysozymes cannot be differentiated. For a model based on turkey-lysozyme and experiments based on hen-lysozyme, as in fig (2a), the F_o–F_c difference map produces positive charge densities (wire frame) falling outside the model (error in |F| up to 34%). The absence of such positive density (at 41% error) indicates an inability to distinguish the hen lysozyme (egg white) from the turkey lysozyme.

Finally, we use multiple diffracted beams (90 in total) to study the variation in R_{factor} (based on |F|) with thickness at 200KeV. Fig (2b) shows that the maximum thickness of lysozyme crystal that can be studied

for $R_{factor} \leq 0.3$ (large, but often accepted) is 90nm. For thicknesses ≥ 230nm, $R_{factor} \geq 0.59$. This, for a non-centrosymmetric crystal such as lysozyme, corresponds to completely random values of |F|.

In summary, (i) the TED single-scattering limit at 3Å resolution for lysozyme at 200KeV is about 23nm at a zone-axis orientation. (i) At higher incident beam energy, a larger thickness can be used at the risk of a high probability for knock-on damage. (iii) By introducing random errors to |F| for lysozyme, while keeping the phases unaltered, we find that (a) at around 30% error in |F|, the charge densities obtained from the erroneous intensities start to substantially deviate from the ideal model. (b) the ability to distinguish hen and turkey lysozyme is lost at about 40% error in |F|. (iv) $R_{factors}$ vs. thickness calculations show that the maximum crystal thickness that can be used to maintain a reasonable R_{factor} value ($R_{factor} < 0.3$) is about 0.1 μm. Because of the similarities in atomic density and composition, these limitations of TED for solving lysozyme should apply to all proteins, which do not contain heavy atoms.

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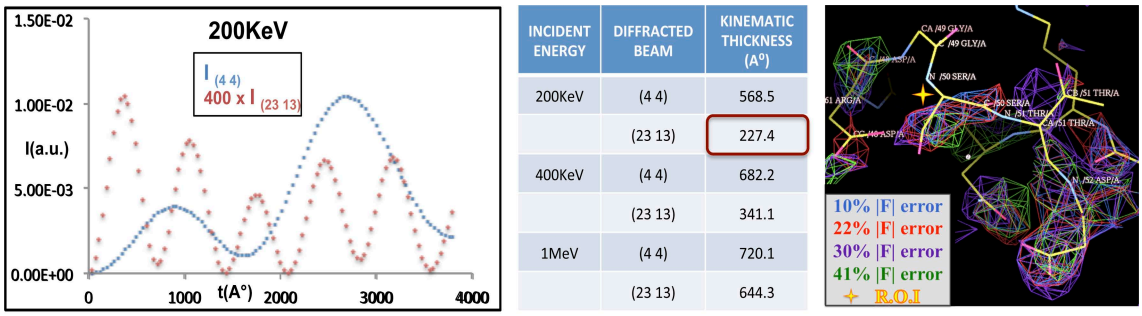
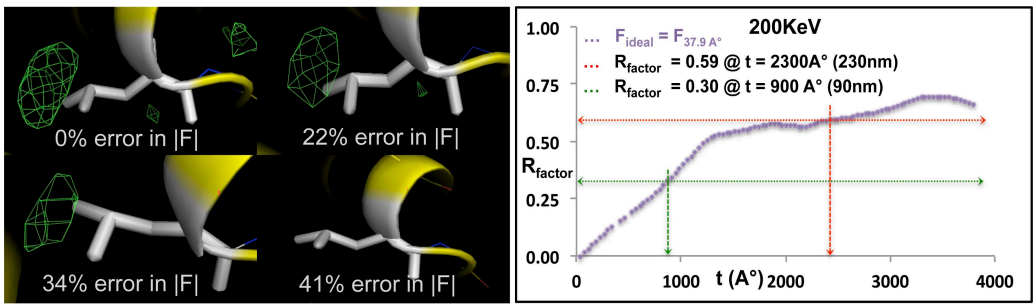


INCIDENT ENERGY	DIFFRACTED BEAM	KINEMATIC THICKNESS (A°)
200KeV	(4 4)	568.5
	(23 13)	227.4
400KeV	(4 4)	682.2
	(23 13)	341.1
1MeV	(4 4)	720.1
	(23 13)	644.3

(a) **(b)** **(c)**

Figure 1. (a) Diffracted beam intensity vs thickness of lysozyme crystal for low and high resolution. **(b)** Variation of kinematic limit with incident beam energy of lysozyme. **(c)** Omit map for lysozyme with random errors introduced in the intensities. The star shows the region of interest.



(a) **(b)**

Figure 2. (a) Fo-Fc difference maps for Turkey (model) - Hen (experiment) lysozyme at 3.0σ. Density outside the model (wire frame) indicates distinguishability, absent with 41% error in |F|. **(b)** R_{factor} vs thickness for lysozyme @ 200keV.

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