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Protein Three Dimensional Nano-Crystallography by Imaging and Diffraction

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Chapter 1

Introduction¹

1.1 Outline

The biological activity of most proteins is determined by their 3D structure. Determining the 3D structure of proteins can therefore be very useful to show how they function: protein structures can be used to interpret molecular processes of life. This can, not only give us a better understanding of life itself, but also can facilitate discovering new drugs or improving existing ones. For instance, a substantial number of molecular diseases are caused by protein structural alterations, which are genetically encoded. Drugs operate by binding to proteins, inducing changes to their functional structure and thereby affecting their biological activity. Hence the design and improvement of drugs is greatly facilitated by knowledge of the 3D structures of their macromolecular targets. In the light of these considerations, it is clear that elucidation of the 3D structure of proteins is of prime importance for understanding the underlying mechanisms of molecular diseases.

The most widely used techniques for the structure determination of proteins are X-ray crystallography, NMR and single particle cryo-EM. X-

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ray crystallography is the most powerful in terms of resolution but requires well diffracting protein crystals. NMR yields structures of proteins in solution but is limited by the size of proteins that can be analysed. Larger protein structures and structures of protein complexes can be solved using cryo-EM but the resolution is not as high compared to the other methods.

Using crystals for structure determination has major advantages. As all the molecules are in the same orientation, their signal adds up and is easier to distinguish from the noise. Hence crystallographic methods have so far yielded the most detailed information of the structures of the molecules of life. I will first discuss how we can generate protein crystals, including pitfalls and bottlenecks of current procedures. Then, after a brief discussion of how crystals are currently used for structure determination, I will address new methods discussed in this thesis that aim at allowing structure determination even if crystals are sub-microscopic.

1.2 Protein 3D Micro- and Nano-Crystallogenesis

1.2.1 Relevance

It was initially believed that any soluble protein that could be purified would be relatively easy to crystallize. However, the results have indicated that solubility and purity of proteins, although being important factors, do not secure a yield of useful crystals. The crystallization behaviour of proteins turns out to be very complex.

In an effort to identify the naturally occurring protein folds, large structural genomics consortia were set up. The somewhat disappointing outcome of these efforts is that only about 3% of all proteins that were targeted by these consortia yielded a crystal structure, despite massive investments in high-throughput, automated protein production, purification and crystallization. It is clear that in order to improve the current situation, better strategies for protein crystallization are required, combined with techniques that allow the use of smaller nano-crystalline material.

1.2.2 Background and Bottlenecks

Crystallization of proteins involves the three classical steps of nucleation, growth and cessation of growth, even though the protein crystals contain on average 50% of disordered solvent (1). However, crystal growth of biological molecules differs substantially from small molecule crystallogenesis. The reason is the much larger number of parameters involved in protein crystallization, as well as the specific physico-chemical properties of the biological compounds. The main difference with small molecule crystal growth is the conformational flexibility and chemical versatility of macromolecules and their greater sensitivity to external factors. An overview of different parameters affecting the crystallization of bio-macromolecules is presented in table 1 (2).

Intrinsic physicochemical properties • Supersaturation • Temperature, pH • Ionic strength and purity of chemicals • Pressure, electric and magnetic fields • Vibration and sound	Biochemical and biophysical parameters • Sensitivity of conformation to physical parameters • Binding of ligands • Specific additives • Aging of samples
Biological parameters • Rarity of biological macromolecules • Bacterial contaminants • Biological sources of organisms and cells	Purity of macromolecules • Macromolecular contaminants • Sequence (micro) heterogeneity • Conformational (micro) heterogeneity • Batch effects

Table 1.1 *Parameters affecting crystal growth.*

Another important prerequisite for successful crystallization is the quality of the macromolecular samples. Bio-macromolecules are extracted from living cells or synthesized *in vitro* and they are frequently difficult to prepare at a high degree of purity and homogeneity. Besides traces of impurities, the different treatments proteins are subjected to may decrease their stability and activity through different kinds of alterations. As a general rule, purity and homogeneity are of prime importance. Accordingly, purification, stabilization, storage and handling of macromolecules are essential steps prior to crystallization.

Purity

The concept of purity has a special meaning in biological crystallogenesi. Molecular samples need to be not only chemically pure, but they must also be conformationally uniform (3). This concept is based on the fact that the best crystals are grown from solutions containing well-defined entities with identical physico-chemical properties. For X-ray crystallographic studies, the aim is to grow 'single crystals' diffracting to high resolution with a low mosaicity and prolonged stability in the X-ray beam. However, contaminants may compete for sites on the growing crystals and generate lattice errors leading to internal disorder, dislocations, poor diffraction or early cessation of growth (4). Because of the high molecular weight of molecules in a single crystal (up to millions of Daltons), and hence low molarity of their solutions, even relatively small amounts of contaminant may induce formation of non-specific aggregates, alter macromolecular solubility, or interfere with nucleation and crystal growth (5,6).

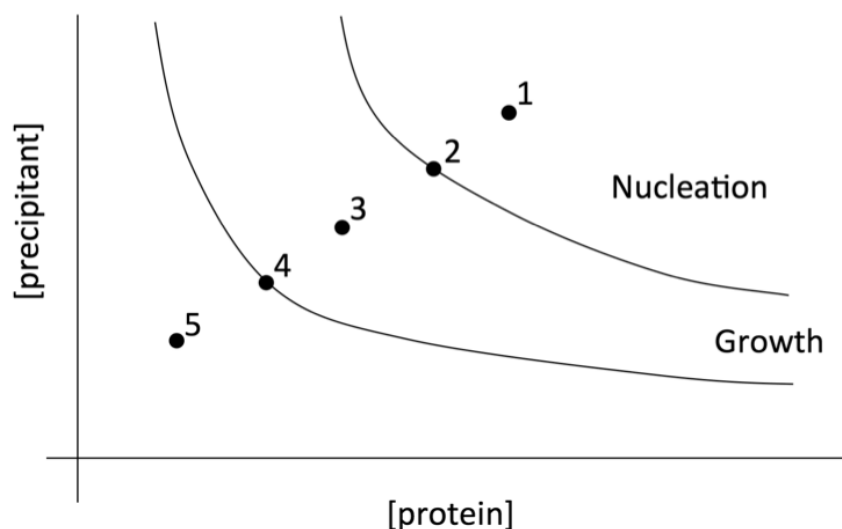


Figure 1.1 Solubility curve of a protein, where the phase state of the protein is plotted against the concentration of both protein and precipitant. At point [1], the protein may precipitate so fast that an amorphous precipitate or at best shower of micro-crystals is formed. At [2] the conditions are just right for the protein to form a stable crystal nucleus, which will start to grow – passing [3] – into a stable protein at equilibrium with the mother liquor [4]. At [5], the concentration of protein and precipitant are too low for crystal nucleation or growth, and the solution will remain clear. Note that the true solubility curve of any protein is highly multidimensional, with every parameter affecting protein solubility (cf. Table 1) representing a different independent axis.

Solubility, Supersaturation and Phase Transition

Biological macromolecules follow the same thermodynamic rules as inorganic or organic small molecules concerning supersaturation, nucleation and crystal growth. However, protein macromolecules are organized in tertiary and quaternary structures. The intra-molecular interactions responsible for their tertiary structure, the intermolecular interactions involved in the crystal contacts, and the interactions necessary to solubilise them in a solvent are similar.

To crystallize a biological macromolecule, its solution must have reached supersaturation, which is the driving force for crystal growth. The under- and supersaturated states are defined by the solubility of the macromolecules. When the concentrations of the crystallization agent and the macromolecules correspond to the solubility condition, the saturated macromolecule solution is in equilibrium with the crystallized macromolecules. Below the solubility curve (Figure 1.) the solution is under-saturated and the system is thermodynamically stable. In this case, phase transition (crystallization) will not occur. Above the solubility curve, the concentration of the biological macromolecules is higher than the concentration at equilibrium. A supersaturated macromolecular solution contains an excess of macromolecules that will fall out of solution as a solid phase, reducing the concentration of the dissolved species until the macromolecular concentration reaches the solubility value. The higher the supersaturation, the faster this solid phase appears. However, at very high supersaturation levels, precipitation, rather than crystallization occurs, as insoluble macromolecules rapidly separate from the solution in an amorphous state.

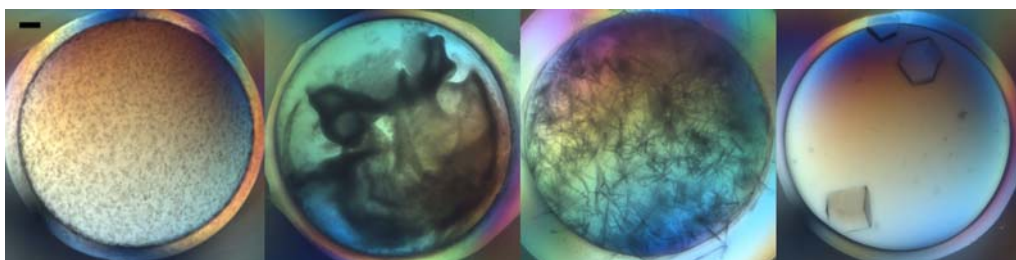


Figure 1.2 *Glucose Isomerase crystallization condition yielding phase separation (far left) amorphous precipitation (near left) micro-crystals (near right) and macro-crystals (far right) bar on the top left represents 200 micrometer.*

1.2.3 Growing and Handling Protein Micro- and Nano-Crystals

Crystallization of biological macromolecules is still one of the major bottlenecks in X-ray crystallography. There are many proteins of which the structures cannot be solved using current techniques due to their inability to grow well diffracting crystals. Protein crystals that are smaller than 1 micrometer are beyond the scope of standard X-ray techniques. Membrane proteins and large protein complexes often have stacking faults decreasing the size and diffraction potential of their crystals. Membrane proteins are located in the lipid layer that separates the cell from its environment and are essential in many signalling pathways, making them important drug targets. Structures of large (dynamic) protein-nucleic acid complexes can provide more information on how proteins and DNA interact with each other.

Many projects are abandoned when proteins yield crystals that cannot be optimized to diffraction grade, and thus structural information on these important drug targets is therefore severely lacking. Current trends in X-ray crystallography focus on decreasing the size limit of protein crystals, proving it is worthwhile to focus on this big supply of

poorly diffracting protein crystals. Micro-focused X-ray beams and improved quantum area detectors, like the Pilatus, have decreased the size limits of protein crystals (7,8). Also serial femtosecond crystallography using X-ray free-electron lasers will help in expanding the methods towards smaller crystals (9,10).

1.2.3.1 Nucleation

Nucleation is the rate-limiting step in protein crystallization (11,12). When a supersaturated protein solution is obtained, as described in 1.2.2, the growth of crystals is dependent on precipitant concentration, pH and temperature. However for crystal growth to be induced there needs to be a starting point, a crystal nucleus. These nuclei are formed in random events when a number of protein molecules come together in the same region in the crystallization drop and by chance coalesce into an ordered or semi-ordered cluster. If these nuclei are stable, more protein molecules can stick to them in the same orientation as the proteins of the nucleus and form a protein crystal. Increased nucleation usually increases the chances of nano- and micro-crystal growth at the expense of the growth of large single crystals, since multiple nucleation sites are competing for the same protein.

Heterogeneous nucleation, which is induced by introducing a properly chosen nucleant into a crystallization condition, may promote nucleation. The first report of a nucleant inducing crystallisation of macromolecules was the epitaxial growth of protein crystals on minerals (13). Other nucleants followed, like zeolites, silicates, charged surfaces, and other porous materials, that all have been tested for

multiple proteins (14,15). Previous results showed that horsehair and dried seaweed showed increased hits when added to sparse-matrix crystallization trials. The increase in crystallization was 35% when horsehair was added to 10 test proteins (16). The underlying mechanism is explained with epitaxial nucleation in the case of minerals, electrostatic interactions if the nucleants contain charged surfaces, nucleation through specific favourable protein-protein interactions or physical entrapment in the caves of porous materials. Introducing heterogeneous nucleants can increase nucleation sites decreasing the size of the crystals obtained.

1.2.3.2 Automation

In recent years, setting up protein crystallisation trials and analysis of the results have become largely automated. Lab automation includes the use of dispensing robots, imaging robots, *in situ* crystal analysis as well as automated diffraction analysis (17,18).

In general, as a beginning, one or more standard screens are used with pre-defined crystallization conditions. These screens are designed on the basis of statistical analysis of results obtained at structural genomics initiatives. When initial hits are found with screens like these, optimisation screens need to be performed to produce diffracting crystals. In this stage, use of a Lab Information Management System (LIMS), where experimental design can be coupled to experiment preparation and analysis, greatly enhances the potential throughput in a lab and thereby the success rate (19).

1.2.3.3 Detecting Crystals in Crystallization Drops

The increase in throughput obtained by using dispensing robots makes it impossible to routinely scan the results manually/interactively using a standard microscope. The dynamic nature of these experiments can cause the crystallographer to miss events, even crystals, especially micro- or nano-crystals that are often difficult to see by eye. Imaging robots vary from semi-automated microscopes with a camera and a moving stage, to fully automated incubators that are capable of following all lab experiments from start to finish without human intervention.

In recent years, three new techniques have been developed for detecting crystals and assess their quality while they are still in the crystallization plates. These techniques are *in situ* diffraction analysis, UV detection and second harmonic microscopy. Several years ago a device for X-ray diffraction analysis of crystals in the plate where they were grown was developed (20,21). Crystals are centred using visual alignments and X-ray diffraction patterns are obtained. When suitably diffracting crystals are found they still need to be harvested and frozen for complete diffraction analysis. UV detection is becoming more popular for detecting crystals. This technique makes use of the fluorescence in UV by proteins, mostly caused by tryptophan (22). Having visible light and UV cameras integrated in a single imaging device greatly enhances its usefulness for distinguishing protein from salt crystals. Second harmonic generation (SHG), more often referred to as “frequency doubling” (23) is an even more recent development. When an intense laser pulse travels through a crystal with a non centrosymmetric space group, light emerges with exactly half of the

wavelength of the incident beam. The explanation is that two photons of the incident beam merge, creating a single photon with twice the energy. If the incident beam is near-infrared, the emerging beam will be in the visible range. The technique is very sensitive, making it suitable for detecting nano- and micro-crystals that cannot be seen using conventional UV and light microscopy.

1.2.3.4 Harvesting and Mounting

X-ray diffraction data is collected at liquid nitrogen temperature (-196 °C) to reduce radiation damage to the crystal and to reduce noise caused by thermal motion. Crystals are transferred to a cryo-protectant solution before flash-freezing to prevent them from cracking. The cryo-protectant also minimizes ice-crystals from forming which interfere with data collection (24).

Protein crystals are traditionally harvested using a cryo-loop. A cryo-loop is a small pin with a nylon loop at the end to “scoop” a single protein crystal. This step is done by hand and can be quite tedious, especially for very small crystals. When a single crystal is captured using a cryo-loop, it can be transferred into an X-ray beam for data collection. Micro-crystals too small to be transferred with a cryo-loop can be transferred to a micro-mesh by transferring the whole or part of the droplet they grew in. They can then be examined using UV or SHG methods and data can be collected using a micro-focus X-ray beam. If data is collected on an X-ray Free Electron Laser source using femtosecond exposures, nano-crystals in suspension are “sprayed” through the beam using a liquid jet (25).

For examining nano-crystals and 2D crystals by cryo-electron microscopy, a drop of mother liquor containing the nano-crystals is pipetted onto an EM-grid. However since electron microscopes operate using vacuum, the drop needs to be vitrified. This is done by blotting away excess liquid and plunge-freezing the sample in liquid ethane cooled by liquid nitrogen. The thin aqueous layer is cooled so abruptly, there is no time for ice crystals to form and the water turns vitreous. The sample is now encased in a 10 – 100 nm layer of amorphous ice thin enough to let electrons through.

1.3 X-ray Micro- and Nano-Diffraction

1.3.1 Background

X-ray diffraction is the most widely used technique for solving crystal structures. If a well-ordered protein crystal is obtained that is large enough, it can diffract incoming X-rays resulting in a diffraction pattern. From a set of these diffraction patterns an electron density map can be calculated into which the protein structure can be modelled.

Bragg Model of Diffraction

When a protein crystal diffracts an incoming X-ray beam, it is diffracted into well-defined angles. The further away from the centre the X-rays are scattered (or: the higher the scattering angle), the better the resolution of the data set. The maximum angle to which the crystal diffracts the incoming X-rays determines how fine the details are that can be distinguished in the protein structure. High resolution is thus

desirable. Knowing the wavelength λ of the X-rays and the diffraction angle $2q$ of a reflection, its resolution d can be calculated using the following equation:

$$d = \frac{1}{2} \left(\frac{\lambda}{\sin \theta} \right)$$

This is just a reformulation of the famous Bragg's Law, formulated almost exactly 100 years ago (26):

$$\lambda = 2d \sin \theta$$

In this Bragg equation the variable d is the distance between atomic layers in a crystal. In a diffraction experiment we measure the intensities of diffraction spots and the position of the reflections. From the position of a reflection we can determine its index (h,k,l) , which is associated with the direction of the parallel atomic planes from which the diffraction spot is reflected. By integrating the signals of each of the reflections, we can assign the appropriate intensities to them. This intensity is proportional to the square of the structure (factor) amplitude, $F(hkl)$, and is a scalar value, a simple number. One dataset usually contains a few ten thousand of reflections collected for each crystal.

The structure factor $F(hkl)$ for a reflection h,k,l itself is a *complex* number and is derived as follows:

$$F_{(h,k,l)} = \sum_{j=1}^{atoms} f_{(j)} \exp \left[2\pi \cdot i \left(hx_{(j)} + ky_{(j)} + lz_{(j)} \right) \right]$$

This summation extends over all atoms j of the repeating unit of the crystal (x, y , and z are the fractional coordinates of the atoms). The parameter $f(j)$ is the scattering factor of atom j and depends on the kind of atom and the diffraction angle of the corresponding reflection (h,k,l) . For X-ray crystallography, at $h,k,l = 0$, f equals the atom's number of electrons times the unit scattering of a single electron ($2.82 \cdot 10^{-15}$ m). The i in the equation is the unit complex number: the square root of (-1) . This equation shows that if the structure is known the structure factors can easily be calculated.

However since diffraction is measured in Fourier space and not in real space the Fourier summation is used:

$$\rho(x,y,z) = \frac{1}{V} \sum_{HKL} |F(h,k,l)| \exp \left[-2\pi \cdot i(hx + ky + lz) + ia(h,k,l) \right]$$

In which ρ is the electron density, $|F(h,k,l)|$ is the structure factor amplitude of reflection (h,k,l) , which includes a temperature factor correction; $\alpha(h,k,l)$ is the phase angle; as in the previous equation, x, y, z are the fractional coordinates of the atoms in the unit cell. From the diffraction pattern $I(h,k,l)$ can be obtained after applying several correction for experiment-specific parameters. Because $I(h,k,l) = |F(h,k,l)|^2$ the amplitudes $|F(h,k,l)|$ can be calculated. Unfortunately, no

information is available on the phase angles $\alpha(h,k,l)$. Without this information the structure can't be solved. Four techniques are used for solving this so-called the 'phase problem'. They are molecular replacement (MR), multiple wavelength anomalous diffraction method (MAD), multiple / single isomorphous replacement method (MIR, SIR) and finally direct methods, which require resolutions that are mostly not reached by protein crystals.

Molecular Replacement

This technique is used if a structure of a highly homologous protein is available, the phases calculated from the model can be combined with the experimental amplitudes, allowing the calculation of an initial electron density map. Through model building and refinement, this map is improved in an iterative way, in several rounds until the process converges and leads to a final model or structure.

Single or Multiple Isomorphous Replacement (SIR or MIR)

If heavy, electron dense atoms (lead, mercury, etc.) are soaked into the protein crystal without changing the crystal lattice, the positions of the heavy atoms can be calculated directly from difference Patterson maps. From the differences between the native and derivative data sets, the phases of the native data set can be inferred. These heavy atoms also cause anomalous dispersion of X-rays, causing differences between the Friedel pairs of reflections. This additional information is also used to estimate starting phases.

Multiple Anomalous Dispersion (MAD)

For MAD-phasing, a variant of the protein is produced that contains a seleno-methionine (methionine in which the sulphur atom is replaced by a selenium atom) instead of methionine. If X-Rays are used at a specific wavelength, these selenium atoms will produce a large enough anomalous signal to obtain phases. A “native” dataset collected at a different wavelength is also needed. The advantage of MAD over MIR (or SIR) is that the data sets can be recorded from the same crystal, thereby ensuring that the datasets are isomorphous (apart from radiation damage).

Model Building and Refinement

If an electron density map is obtained the protein model can be automatically built using ARP/wARP, buccaneer or other programs (27,28). If an initial model is obtained this can be refined with the original data using refinement software like Refmac or Phenix (29,30). This is done to better fit the model to the measured diffraction data. The model can be manually checked using coot (31), a graphical model building software tool. Coot can also be used for rebuilding and refining selected parts of the protein model.

1.3.2 Micro-Diffraction

Obtaining diffraction grade crystals is a real bottleneck in macromolecular crystallography, thus it has become a trend to improve current techniques to make structure solution of suboptimal crystals possible. In the last ten years micro-focus beams have become routinely used for data collection of micro-crystals, which are too small for

structure solution using traditional synchrotron X-ray beams. Micro-focus beams were developed at the ESRF (32) and use apertures and mirrors to focus beams, thus decreasing their size and increasing their flux. Improvements not only increase maximum resolution, but also isotropy of diffraction data. Since diffraction data can be obtained from only a small part of the crystal, also twinned or mosaic crystals can now yield useful data. Radiation damage is reduced since less of the crystal is submerged to X-rays. Many synchrotrons now have micro-focused beams incorporated at their macromolecular beamlines (33).

1.3.3 Nano-Diffraction

A novel technique called serial femtosecond crystallography used X-ray Free Electron Lasers (XFELs) to collect diffraction data of protein crystals ranging from 200 nm to 2 μm . Protein crystals in suspension are sprayed through the XFEL beam and millions of randomly oriented diffraction patterns are collected (9). Exposures of 10 to 200 fs are used at power densities of $10^{16} \text{ W Cm}^{-2}$, which vaporizes the protein crystals. Although this method is still in the development stage, it shows high potential for the future.

1.3.4 Detecting X-rays

In an X-ray experiment a crystal diffracts an incoming beam, which is scattered into reflections or spots. In the early days these spot patterns of diffracted X-rays were collected on photographic film. These have since been replaced by image plate scanners and CCD detectors. The latest developments have been pixel detector like the Pilatus (34),

which significantly increase the signal to noise ratio, thus decreasing exposure times and beam damage.

1.4. Electron Diffraction and Imaging of Nano-Crystals

1.4.1 Relevance

Since the development of the electron microscope by Max Knoll and Ernst Ruska in the early 1930's, the applications of transmission electron microscopy (TEM) have been extensive and impressive. Originally, material scientists used it for examining metals, alloys, ceramics, glasses, polymers, semiconductors and mixtures of these materials. With the development of cryo-electron microscopy in the 1970's (35-37) the applications have also shifted to biological samples. The advantages of using TEM for the structure determination of proteins and protein complexes over X-ray diffraction is that when employing single particle analysis, no crystals are needed. In electron crystallography thinner crystals can be used in the form of 2D crystals or 3D nano-crystals. Electrons are less damaging to proteins than X-rays by several orders of magnitude per diffracted quantum (Table 2. (38)), minimizing beam damage by decreasing energy deposited in the sample.

	Electrons (200 keV)	X-rays (1.5 Å)
Ratio (inelastic/elastic) scattering events	~3	~10
Energy deposited per inelastic event	~20 eV	8 keV
Energy deposited relative to electrons per elastic event	1	~10 ³

Table 1.2 *Electrons are less damaging to proteins than X-rays (38).*

1.4.2 Principles of Electron Diffraction and Imaging

Since the wavelength of electrons is significantly smaller than that of X-rays (or visible light), a much higher resolution can be obtained in theory. The higher the accelerating voltage of the electrons, the smaller their wavelength, which increases the maximum resolution that can be obtained. Unlike X-rays, electrons are charged, and therefore they can be focused by electromagnetic lenses. This means an electron microscope can be build in similar fashion to a light microscope, see figure 3.

1.4.2.1 Basic Principles of a Transmission Electron Microscope

We distinguish three basic systems in an electron microscope: the illumination system, the image formation system and the image detection system (for the latter two, see figure 3).

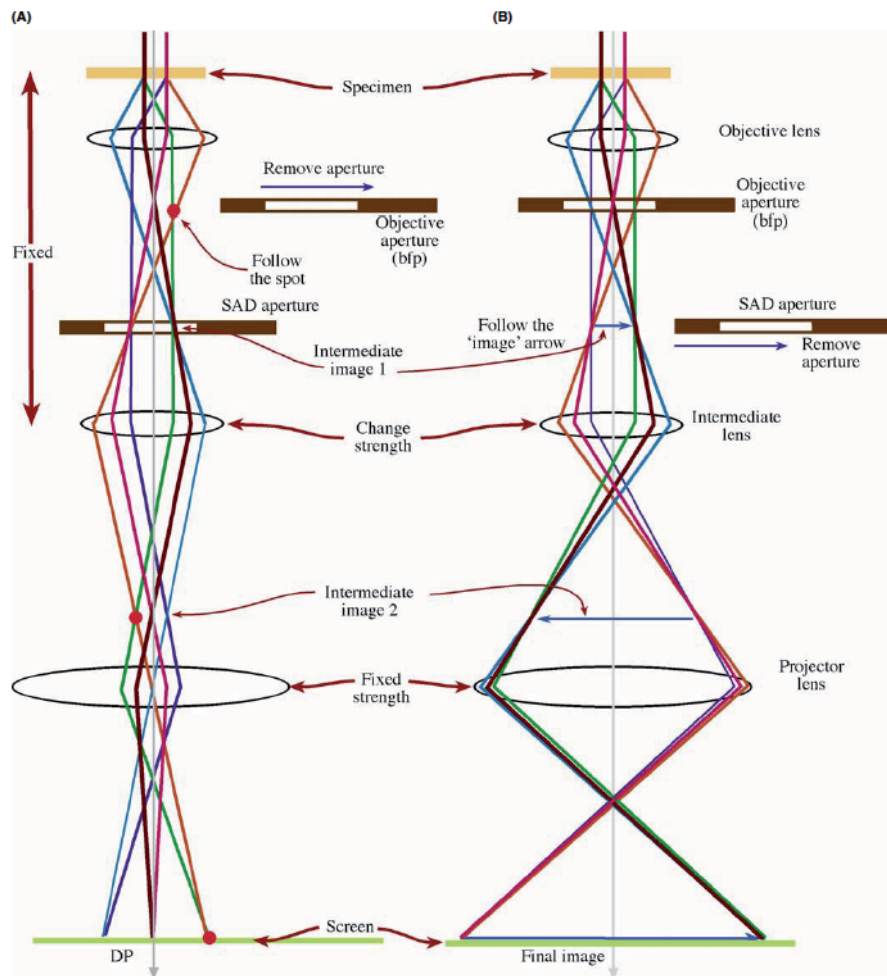


Figure 1.3 Formation of diffraction pattern (left) and image (right) in a TEM. (From Williams and Carter 2nd edition 2009(58)).

The illumination system consists of an electron source and a condenser system. The electron source is either an emission source (either tungsten or LaB₆ crystal) or field emission gun. The condenser consists of one or more condenser lenses and apertures and delivers a beam of electrons to the specimen. Depending on the optical settings, this beam

can be parallel, di- or convergent, can be precessing over the sample or scan the sample.

After the electrons have passed through the specimen they travel through the image formation system, which contains an objective lens an objective aperture, a selected area aperture, intermediate lenses and a projector lens.

The image is then formed on a fluorescent screen or a detector. In the past photographic film was used for detection but it has been replaced by image plates and CCD cameras. Recently direct electron detectors like the Falcon or the K2 were developed (39,40). For measuring diffraction data, a quantum area detector like the Medipix is ideal, which significantly increases the signal to noise ratio, thereby decreasing exposure times and hence beam damage.

Since electrons are charged, they interact much stronger with matter compared to light or X-rays. This property means that electrons can be used to study much smaller objects. There are different ways electrons can interact with matter: they can scatter elastically, inelastically and dynamically.

Elastic scattering occurs when the electron conserves its kinetic energy but changes direction due to interaction with the electrons and nuclei of the specimen. If an electron loses kinetic energy, inelastic scattering has occurred. Inelastic scattering is the main scattering process of electrons in high voltage electron microscopy (see table 2) but for high-resolution imaging, the inelastic scattering events are mostly ignored. The reason is that elastic scattering, but not inelastic scattering, can be analysed

using Bragg's law, in a similar manner as was described for X-rays in 3.1.

Elastically scattering electrons can interact multiple times with atoms in the specimen, giving rise to dynamic scattering. These multiple scattered electrons can only be analysed using complex calculations and there is no straightforward relation between the structure of the specimen and the location where they end up in a diffraction spot or image.

Spherical aberration (Cs) is the limiting factor for high resolution in modern day electron microscopy. However Electron microscopes can be equipped with a Cs-corrector system to correct for spherical aberration. A Cs-corrector consists of two hexapole and four additional lenses, which make it possible for high-resolution images to be obtained.

1.4.2.2 High Resolution Imaging of Biological Samples

Single particle analysis is the most widely used imaging technique for determining the structure of large protein complexes, viruses and other biological samples that are too large to be analysed by X-ray diffraction. Samples are negatively stained or vitrified and transferred into the electron microscope. Thousands of electron-images are taken at low dose to minimize beam damage. To increase the signal, multiple images are averaged and analysed using various image analysis techniques.

The first step in image processing is to group images of different orientation. This is done by classifying the images using multivariate statistical analysis. The images in each class are aligned and through averaging increase the signal to noise ratio. To reduce the influence of

high or low spatial frequency information a band pass filter is usually applied to images. These filters make use of the Fourier transform algorithms to weight or improve the results of alignment. To enhance the contrast in single particle analysis, EM images are taken 'under-focus'. This means images are blurred by a point spread function. This can be corrected for using the contrast transfer function, which is a function in reciprocal space and can allow higher resolution reconstructions. Since the images contain particles at random projections, their orientation angles need to be inferred in order to make a three dimensional reconstruction. To assign these 'Euler angles' common projection lines can be used. In the case of a sample containing symmetry like viruses or crystals this can enhance reconstructions by assigning angles in reciprocal space.

1.4.2.3 Electron Crystallography of Protein 2D Crystals

Electron crystallography can be used to determine the structure of two-dimensional (membrane) protein crystals. Resolutions better than 3 Ångstrom can be achieved (41,42). The method has similarities to single particle analysis but because of the internal order of the sample, the resolution can be enhanced through crystal unbending, internal symmetry and measuring diffraction patterns. First electron images are collected. These contain phase and amplitude information. Then electron diffraction patterns are obtained to further improve the resolution of the data. Since the crystals consist of one flat layer that is horizontally aligned they are often tilted during data collection to obtain three-dimensional datasets. 2D crystals of membrane proteins generally

need lipids and detergents to grow and are generally vitrified, negatively stained or embedded in sugar.

1.4.2.4 Electron Crystallography of 3D Crystals (Materials vs. Proteins)

In material sciences, electron diffraction is a well-developed tool for structure determination of inorganic crystals. Selected area electron diffraction (SAED) can be used to determine three-dimensional unit cells from different zone axes. To obtain more information about the symmetry of the crystal convergent beam electron diffraction (CBED) (43) can be used. The development of precession electron diffraction (PED) (44,45), automated diffraction tomography (ADT) (46,47) and rotation electron diffraction (RED) (48) have contributed to the toolbox of electron crystallographers, for inorganic or small organic molecules. However 3D nano-crystals of large macromolecules have so far resisted structure determination. The main reasons are the beam sensitivity, the large unit cell and the thickness of the crystals. The latter factor contributes to increased multiple scattering and non-linear effects in electron diffraction and imaging. However multi-slice least square methods (49) have tackled such problems in electron crystallography of small molecules. To increase resolution and refine structural data, electron diffraction data can be combined with electron microscopy images, which contain phase information (50-53).

4.3 Trends

Current trends in Cryo-EM are going towards sorting images to find more than one heterogeneous group of molecules (54). This means

dynamic processes of biological systems can be studied. Another development is the use of phases from electron micrograms to solve structures with X-ray diffraction where phases are missing (55,56).

In this thesis I describe my work on the analysis of 3D protein nano-crystals using cryo-EM imaging and electron diffraction. I discuss the use of heterogeneous nucleants to increase the success of crystallization in automated trials. The structural studies of the enzyme Small Laccase and the roll of Tyr108 in its enzyme mechanism are also discussed.

References

- (1) Matthews, B. W. (1968). *Journal of Molecular Biology* **33**, 491-497.
- (2) Bergfors, T. M. (2009). *Protein Crystallization: Second Edition*. International Univ Line.
- (3) Giegé, R., Dock, A. C., Kern, D., Lorber, B., Thierry, J. C. & Moras, D. (1986). *Journal of Crystal Growth* **76**, 554-561.
- (4) Vekilov, P. G. & Rosenberger, F. (1996). *Journal of Crystal Growth* **158**, 540-551
- (5) Skouri, M., Lorber, B., Giegé, R., Munch, J. P. & Candau, J. S. (1995). *Journal of Crystal Growth* **152**, 209-220.
- (6) Kuznetsov, Y. G., Malkin, A. J., Glantz, W. & McPherson, A. (1996). *Journal of Crystal Growth* **168**, 63-73.
- (7) Cusack, S., Belrhali, H., Bram, A., Burghammer, M., Perrakis, A. & Riek, C. *Nat Struct Mol Biol*.
- (8) Eikenberry, E. F., Bronnimann, C., Hulsén, G., Toyokawa, H., Horisberger, R., Schmitt, B., Schulze-Bries, C. & Tomizaki, T. (2003). *Nuclear Instruments and Methods in Physics Research Section A: Accelerators, Spectrometers, Detectors and Associated Equipment* **501**, 260-266.
- (9) Chapman, H. N., Fromme, P., Barty, A., White, T. A., Kirian, R. A., Aquila, A., Hunter, M. S., Schulz, J., DePonte, D. P., Weierstall, U., Doak, R. B., Maia, F. R. N. C., Martin, A. V., Schlichting, I., Lomb, L., Coppola, N., Shoeman, R. L., Epp, S. W., Hartmann, R., Rolles, D., Rudenko, A., Foucar, L., Kimmel, N., Weidenspointner, G., Holl, P., Liang, M., Barthelmess, M., Caleman, C., Boutet, S., Bogan, M. J., Krzywinski, J., Bostedt, C., Bajt, S., Gumprecht, L., Rudek, B., Erk, B., Schmidt, C., Homke, A., Reich, C., Pietschner, D., Strüder, L., Hauser, G., Gorke, H., Ullrich, J., Herrmann, S., Schaller, G., Schopper, F., Soltau, H., Kühnel, K.-U., Messerschmidt, M., Bozek, J. D., Hau-Riege, S. P., Frank, M., Hampton, C. Y., Sierra, R. G., Starodub, D., Williams, G. J., Hajdu, J., Timneanu, N., Seibert, M. M., Andreasson, J., Röcker, A., Jonsson, O., Svenda, M., Stern, S., Nass, K., Andrich, R., Schrotter, C.-D.,

- Krasniqi, F., Bott, M., Schmidt, K. E., Wang, X., Grotjohann, I., Holton, J. M., Barends, T. R. M., Neutze, R., Marchesini, S., Fromme, R., Schorb, S., Rupp, D., Adolph, M., Gorkhover, T., Andersson, I., Hirsemann, H., Potdevin, G., Graafsma, H., Nilsson, B. & Spence, J. C. H. (2011). *Nature* **470**, 73-77.
- (10) Boutet, S. b., Lomb, L., Williams, G. J., Barends, T. R. M., Aquila, A., Doak, R. B., Weierstall, U., DePonte, D. P., Steinbrener, J., Shoeman, R. L., Messerschmidt, M., Barty, A., White, T. A., Kassemeyer, S., Kirian, R. A., Seibert, M. M., Montanez, P. A., Kenney, C., Herbst, R., Hart, P., Pines, J., Haller, G., Gruner, S. M., Philipp, H. T., Tate, M. W., Hromalik, M., Koerner, L. J., van Bakel, N., Morse, J., Ghonsalves, W., Arnlund, D., Bogan, M. J., Caleman, C., Fromme, R., Hampton, C. Y., Hunter, M. S., Johansson, L. C., Katona, G., Kupitz, C., Liang, M., Martin, A. V., Nass, K., Redecke, L., Stellato, F., Timneanu, N., Wang, D., Zatsepin, N. A., Schafer, D., Defever, J., Neutze, R., Fromme, P., Spence, J. C. H., Chapman, H. N. & Schlichting, I. (2012). *Science* **337**, 362-364.
- (11) Georgieva, D. G., Kuil, M. E., Oosterkamp, T. H., Zandbergen, H. W. & Abrahams, J. P. (2007). *Acta Crystallographica Section D* **63**, 564-570.
- (12) Saridakis, E. & Chayen, N. E. (2009). *Trends in Biotechnology* **27**, 99-106.
- (13) McPherson, A. & Shlichta, P. J. (1987). *J. Cryst. Growth*, **85**, 206-214.
- (14) Sugahara, M., Asada, Y., Morikawa, Y., Kageyama, Y. & Kunishima, N. (2008). *Acta Crystallographica Section D* **64**, 686-695.
- (15) Takehara, M., Ino, K., Takakusagi, Y., Oshikane, H., Nureki, O., Ebina, T., Mizukami, F. & Sakaguchi, K. (2008). *Analytical Biochemistry* **373**, 322-329.
- (16) Thakur, A., Newman, J., Martin, J. & Kobe, B. (2008). *Methods in Molecular Biology*, Vol. 426, *Structural Proteomics*, edited by B. Kobe, M. Guss & T. Huber, pp. 403-409. Humana Press.
- (17) Stevens, R. C. (2000). *Current Opinion in Structural Biology* **10**, 558-563.
- (18) Berry, I. M., Dym, O., Esnouf, R. M., Harlos, K., Meged, R., Perrakis, A., Sussman, J. L., Walter, T. S., Wilson, J. & Messerschmidt, A. (2006). *Acta Crystallographica Section D* **62**, 1137-1149.
- (19) Bard, J., Ercolani, K., Svenson, K., Olland, A. & Somers, W. (2004). *Methods* **34**, 329-347.
- (20) Skarzynski, T. (2009). *Acta Crystallographica Section A* **65**, s159
- (21) le Maire, A., Gelin, M., Pochet, S., Hoh, F., Pirocchi, M., Guichou, J.-F., Ferrer, J.-L. & Labesse, G. (2011). *Acta Crystallographica Section D* **67**, 747-755.
- (22) Judge, R. A., Swift, K. & Gonzalez, C. (2005). *Acta Crystallographica Section D* **61**, 60-66.
- (23) Wampler, R. D., Kissick, D. J., Dehen, C. J., Gualtieri, E. J., Grey, J. L., Wang, H.-F., Thompson, D. H., Cheng, J.-X. & Simpson, G. J. (2008). *Journal of the American Chemical Society* **130**, 14076-14077.
- (24) Helliwell, J. (2005). *Acta Crystallographica Section D* **61**, 793-798.
- (25) DePonte, D. P., Weierstall, U., Schmidt, K., Warner, J., Starodub, D., Spence, J. C. H. & Doak, R. B. (2008). *Journal of Physics D: Applied Physics* **41**, 195505.
- (26) Thomas, J. M. (2012). *Nature* **491**, 186-187.
- (27) Langer, G., Cohen, S. X., Lamzin, V. S. & Perrakis, A. (2008). *Nat. Protocols* **3**, 1171-1179.
- (28) Cowtan, K. (2006). *Acta Crystallographica Section D* **62**, 1002-1011.

- (29) Murshudov, G. N., Skubak, P., Lebedev, A. A., Pannu, N. S., Steiner, R. A., Nicholls, R. A., Winn, M. D., Long, F. & Vagin, A. A. (2011). *Acta Crystallographica Section D* **67**, 355-367.
- (30) Adams, P. D., Mustyakimov, M., Afonine, P. V. & Langan, P. (2009). *Acta Crystallographica Section D* **65**, 567-573.
- (31) Emsley, P. & Cowtan, K. (2004). *Acta Crystallographica Section D* **60**, 2126-2132.
- (32) Evans, G., Axford, D., Waterman, D. & Owen, R. L. (2011). *Crystallography Reviews* **17**, 105-142.
- (33) Sanishvili, R., Yoder, D. W., Pothineni, S. B., Rosenbaum, G., Xu, S., Vogt, S., Stepanov, S., Makarov, O. A., Corcoran, S., Benn, R., Nagarajan, V., Smith, J. L. & Fischetti, R. F. (2011). *Proceedings of the National Academy of Sciences* **108**, 6127-6132.
- (34) Kraft, P., Bergamaschi, A., Broennimann, C., Dinapoli, R., Eikenberry, E. F., Henrich, B., Johnson, I., Mozzanica, A., Schlepütz, C. M., Willmott, P. R. & Schmitt, B. (2009). *Journal of Synchrotron Radiation* **16**, 368-375.
- (35) Taylor, K. A. & Glaeser, R. M. (1974). *Science* **186**, 1036-1037.
- (36) Klug, A. (1979) "Image analysis and reconstruction in the electron microscopy of biological macromolecules." *Chemica Scripta* 14:245-256.
- (37) Knapke, E. & Dubochet, J. (1980). *J. Mol. Biol.* **141**, 147-161.
- (38) Henderson, R. (1995). *Q Rev Biophys* **28**, 171-193.
- (39) FEI Falcon press release, <http://investor.fei.com/releasedetail.cfm?ReleaseID=399045>
- (40) <http://www.gatan.com/company/news/news06231001.php>
- (41) Henderson, R., Unwin P. N. (1975). *Nature* **257**, 28.
- (42) Gonen, T., Cheng, Y., Sliz, P., Hiroaki, Y., Fujiyoshi, Y., Harrison, S. C. & Walz, T. (2005). *Nature* **438**, 633-638.
- (43) Spence, J. C. H. & Zou, J. M. (1992). *Electron microdiffraction*. New York: Plenum.
- (44) Vincent, R. & Midgley, P. A. (1994). *Ultramicroscopy* **53**, 271-282.
- (45) Oleynikov, P., Hovmöller, S. & Zou, X. D. (2007). *Ultramicroscopy* **107**, 523-533.
- (46) Kolb, U., Gorelik, T., Kübel, C., Otten, M. T. & Hubert, D. (2007). *Ultramicroscopy* **107**, 507-513.
- (47) Mugnaioli, E., Gorelik, T. & Kolb, U. (2009). *Ultramicroscopy* **109**, 758-765.
- (48) Zhang, D., Oleynikov, P., Hovmöller, S. & Zou, X. (2010). *Zeitschrift für Kristallographie* **225**, 94-102.
- (49) Jansen, J., Tang, D., Zandbergen, H. W. & Schenk, H. (1998). *Acta Crystallographica Section A* **54**, 91-101.
- (50) Henderson, R., Unwin P. N. (1975). *Nature* **257**, 28.
- (51) Weirich, T. E., Zou, X., Ramlau, R., Simon, A., Cascarano, G. L., Giacovazzo, C. & Hovmöller, S. (2000). *Acta Crystallographica Section A* **56**, 29-35.
- (52) Tsuda, K. & Tanaka, M. (1995). *Acta Crystallographica Section A* **51**, 7-19.
- (53) Zuo, J. M. & Spence, J. C. H. (1991). *Ultramicroscopy* **35**, 185-196.

(54) Van Heel, M.; Portugal, R. Rohou, A., Linnemayr, C., Bebeacua, C., Schmidt, R., Grant, T., and Schatz, M. (2010). "Four-Dimensional Cryo Electron Microscopy at Quasi Atomic Resolution: "IMAGIC 4D"". *International Tables for Crystallography, Vol. F*.

(55) Dodson, E. (2001). *Acta Crystallographica Section D* **57**, 1405-1409.

(56) Navaza, J. (2008). *Acta Crystallographica Section D* **64**, 70-75.

Textbooks:

(57) Rhodes, G. (2006). *Crystallography made crystal clear: a guide for users of macromolecular models*. Academic press.

(58) Williams, D. B. & Carter, C. B. (2009). *Transmission electron microscopy: a textbook for materials science*. Springer.

(59) Zou, X., Hovmöller, S. & Oleynikov, P. (2011). *Electron Crystallography: Electron Microscopy and Electron Diffraction*. OUP Oxford.

Chapter 2

A Straightforward and Robust Method for Introducing Human Hair as a Nucleant into High Throughput Crystallization Trials²

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Abstract

Growing X-ray grade crystals of a specific protein is a process of trial and error. Usually, hundreds or even thousands of conditions are screened in order to identify useful crystallization conditions. Heterogeneous nucleants have been shown to increase the success rate of crystallization trials, and (human) hair has previously been identified as a promising nucleant. Here, we describe and evaluate a method for preparing crystallization plates that are locally coated with fragments of human hair, allowing automated, high-throughput crystallization trials in a fashion that is entirely compatible with standard hanging or sitting drop crystallization techniques. We assessed the effect of these nucleants on the crystallization of 11 different proteins in more than 4000 crystallization trials. We found additional crystallization

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conditions for 10 out of 11 proteins when using the standard JCSG+ screen (96 different conditions). In total, 34 additional crystallization conditions could be identified (13.1% of the total number of successful crystallizations). The increase in crystallization conditions ranged between 33.3% (2 additional conditions were identified for myoglobin on top of 4 homogeneous crystallizations) to 1.2% (we identified a single additional condition for insulin, which crystallized in 85 out of 96 conditions) – the median increase in crystallization hits was 14%. On the basis of these numbers, we conclude that the inclusion of human hair fragments in high throughput crystallization screens may be beneficial. The method is inexpensive, straightforward with standard equipment and uses materials available in any crystallization lab. Furthermore, initial experiments with the crystallization of membrane proteins on hair show the technique may also be beneficial for growing membrane proteins.

2.1 Introduction

Crystallization of biological macromolecules is still one of the major bottlenecks in X-ray crystallography (1,2,3,4). The process of obtaining diffraction-grade crystals comprises a screening and an optimization step (2). First nucleation of a microscopic crystallite must occur. Such a nucleus often exists of only a few hundred molecules and is not visible with an optical microscope. The screening step aims at identifying suitable nucleation conditions. It should provide directions for further crystal optimizing experiments, required for producing well diffracting single crystals (5).

A number of studies have shown that often the initial nucleation phase of macromolecular crystals is the limiting step in the formation of X-ray grade crystals. The reason is that, whilst the protein should be in the meta-stable phase for crystal growth, much higher levels of saturation are needed for nucleation (6). Spontaneous formation of a crystal nucleus by the ordered aggregation of supersaturated proteins (homogeneous nucleation) is an unlikely event (7), but it is well known that surfaces can help in the formation of crystal nuclei (8).

The first report of a nucleant inducing nucleation of macromolecules on heterogeneous material was the epitaxial growth of protein crystals on minerals, published more than 20 years ago (9). Other candidate nucleants followed (e.g. zeolites, silicates and dried seaweed) and have been tested for multiple proteins (10,11,12,13.). Previous results have proven that hydroxy-apatite, cellulose, horsehair and dried seaweed, showed increased hits when added to Sparse Matrix crystallization screening. The observed increase in crystallization was 35% when horsehair was added to ten test proteins before screening with standard methods (10,11). Specially designed bio-glass (amorphous-mesoporous bioactive gel-glass) has recently been found to be a nucleation-inducing material with greater universality than other studied materials before (14,15,16,17). Porous materials as nucleants for protein crystallization have been intensively studied for years. In short, results and calculations indicate that the cavities of nucleants might entrap the protein molecules and thereby support nucleation and crystal growth (18).

For many years, natural hairs (cat's whiskers, horse hair, human hair) have been used successfully in macromolecular crystallization for transferring crystal seeds (i.e. critical sized nuclei) into solutions (4). The microstructure of hair contains overlapping cuticles and thus is ideal for trapping the micro fragments of a crystal (19). Horsehair was shown to be effective as a nucleant for three model proteins as well as for a difficult to crystallize Fab-D protein (6). Recently, the nucleation properties of human hair have been investigated, showing that various surface properties of human hair (cavities within the cuticles, an ordered keratin surface, preferred entrapment of protein at specific sites resulting in concentration gradients of protein) all contribute to effective protein crystal nucleation (19).

Ideally, heterogeneous nucleants in initial screening should be combined with automated, miniaturized crystallization (20,21). In this study a method is described that introduces fragments of human hair in miniaturized high throughput crystallization experimentation. The method is straightforward and can be preformed in any crystallization lab with materials readily available. To keep the nucleating properties of the hair surface and surface patterning intact, thin slices of hair were produced with a microtome. As an alternative, we also screened using hair fragments that were too small to distinguish by optical microscopy and were obtained by crushing hair in liquid nitrogen, using a mortar and pestle.

Our method was tested by screening 11 proteins with 96 crystallization conditions (JCSG+ screen 22,23) in wells that either contained hair particles (of either type: crushed or sliced), and comparing them to

experiments that did not contain heterogeneous nucleants. The results show an increase in hits in initial screening of 12 % when drops containing either sliced or crushed hair are compared to drops without purposefully added nucleants. This percentage of increase is lower than previous results (10,11), however the total number of additional hits is significantly higher. These differences are probably due to the different screen that was used as well as different proteins. Since heterogeneous nucleation often resulted in small, needle-shaped crystals, the described high-throughput method promoted growth of crystals suitable for electron diffraction (24).

Since the crystallization of membrane proteins is known to be problematic (26), we also performed initial crystallization experiments using hair as a nucleant with the membrane protein Diacylglycerol Kinase (DgkA). The fact that DgkA crystallized on the hair surface suggests the method to also be beneficial for membrane proteins, but in the case of membrane proteins data on additional proteins needs to be gathered.

2.2 Materials and Methods

2.2.1 Preparation of Hair Particles

Three different hair samples were collected, prepared and cut. A small tuft with a length of 2 cm was washed extensively with petroleum ether to remove any remaining proteins and lipids. The hair was then cut using a microtome (Leica) into pieces with a diameter ranging between 25-45 μm , and a length of 25 μm . The hair slices were extensively

washed with Milli-Q water to remove any polymers and re-suspended in ethylene glycol. Of the three different types of hair used, the hair with a diameter of 25-30 μm showed the best properties for dispensing with our dispensing robot.

2.2.2 Preparation of Crystallization Plates Dusted with Hair Particles

A suspension containing around 12 hair particles per μl was used for dispensing. The hair fragments stay in suspension for a few minutes in ethylene glycol before they aggregate and then precipitate. The hair slices were added to designated wells on Pzero 3550 plates (Corning) using an ORYX 6 robot and XStep software (both Douglas Instruments). In each well 0,5 μl of hair suspension (around 6 particles) was deposited and 0,1 μl of water using the second micro-tip was added in order to speed up ethylene glycol evaporation at 37 °C. After 3 days both ethylene glycol and water had evaporated and the plates were ready for experiments. In order to have reproducible amount of particles, all wells were imaged and occasionally wells with less than 3 hair particles were reprocessed as described above.

2.2.3 Preparation of Crushed Hair with Liquid Nitrogen

For crushed hair, thicker hair was used with a diameter of around 100 μm . Hair was crushed using a mortar under liquid nitrogen until a powder was formed. Around 25 ml of ethanol was added to form a suspension of the crushed hair powder. The suspension was mixed and transferred to tubes (Eppendorf) with 500 μl of this solution. After evaporation of ethanol, for each experiment the protein solution was

added to these tubes in order to mix with crushed hair before dispensing with an ORYX crystallization robot into clean crystallization plates.

Table 2.1 *Proteins used for screening experiments.*

Protein	Concentration (mg/ml)	Buffer (mM)	pH	Ions (mM)	Source
Lysozyme	10	Acetate (20)	4.6		Fluka
Glucose Isomerase	22	Tris (6)	7	(NH ₄) ₂ SO ₄ (90)	Hampton Research
Trypsin	50	Tris (20)	8	CaCl ₂ (3)	Sigma
Ccp-Cc	38	H ₂ O			Qamar et al. (25)
Insulin	30	Ches (25)	8.4		Sigma
Myoglobin	25	Tris (10)	7.4		Sigma
(Ov)albumin	20	Tris (10)	7.4		Sigma
α -Lactalbumin	25	Tris (10)	7.4		Sigma
α -Chemotrypsin	50	Tris (10)	7.4		Sigma
Alcohol dehydrogenase	20	Tris (20)	7.4	MgCl ₂ (1)	Sigma
Pepsin	25	Tris (10)	7.4		Sigma

2.2.4 Scanning Electron Microscopy

Scanning electron microscopy (SEM) was performed on the sliced hair particles as well as the crushed hair to determine the surface structure of hair particles. Experiments were performed on a Nova Nanosem 200 (FEI). After applying a 20 nm layer of evaporated carbon on the particles, images were obtained at different magnifications. The goal was to investigate whether the properties of hair that are known to promote nucleation (19) remained intact after preparing the hair for high throughput experiments.

2.2.5 Crystallization Screening

Crystallization experiments were carried out using the sitting drop vapor diffusion technique in Pzero 3550 plates (Corning). Rockmaker software (Formulatrix) was used for designing the experiments. We used a Genesis (Tecan) for dispensing the screening solutions in the reservoirs. The ORYX 6 (Douglas Instruments) crystallization robot was used for transferring 200 nl reservoir solution and 200 nl protein solution in sitting drop wells. Plates were stored at 20 °C and imaged using the automated imaging system Rock Imager (Formulatrix). The JCSG+ screen (Qiagen) was used for random screening experiments and chemicals used for grid experiments were purchased from Sigma.

A list of the proteins used in the screening experiments and their source is shown in table 1. In all cases, the proteins were not further treated nor were any inhibitors used. By direct dissolution of dry material most of the proteins were made up as 10 mg/ml stock solutions unless a concentration more suitable for crystallization was described in the literature. For most proteins the buffer used was 10-20 mM TRIS buffer at pH 7,4 and salt ions were kept to a minimum to prevent false positives in the form of salt crystals.

2.2.6 Crystallization Scoring and Validation

Crystallization plates were imaged using an automated imaging system but scored manually. To prevent false positive hits several precautions were taken. The control conditions were set up in duplicate, if in one of the two (or in both) controls a crystal was observed, the condition was labeled as a hit. All images were recorded using a polarization filter to check birefringence. If it was still unclear if a hit was a protein crystal or a false positive, UV images were collected using an Olympus BX51 microscope. UV imaging distinguishes between protein and salt crystals since the tryptophane residues cause the crystals to fluoresce under UV-light.

2.2.7 *Escherichia coli* Diacylglycerol Kinase (DgkA)

Crystallization on Human Hair

His-tagged DgkA was expressed in BL21 strains. Growth and expression methods were implemented as previously described (27). The DgkA protein was purified and solubilised with DM (n-Decyl-beta-D-Maltopyranoside) according to literature (28). Crystallization was performed above a 75 μ l reservoir buffer containing 33% (v/v) PEG 300, 0.05 M glycine pH 9.65, 0.1 M NaCl. Crystallization drops consisted of 2 μ l of reservoir solution and 2 μ l of 16 mg/ml DgkA. Human hair was prepared as described above (without slicing) and added to crystallization experiments in NeXtal evolution μ plate from Qiagen.

2.3 Results and Discussion

2.3.1 Scanning Electron Microscopy

The scanning electron microscopy images (figure 1) prove that when hair is sliced with a microtome it retains cuticles on the edges. These cuticles have been found to be beneficial for nucleation (19). Additional positive effects of cutting hair in small slices could be caused by the increased ratio between the cortex surface (the cutting planes of the sliced hair) and the cuticle surface. This cortex surface, including the medulla, is porous and shows cavities of many different sizes. These cavities have been known to induce nucleation (6). With the method described here for including hair particles in micro-crystallization drops, it is now also possible to control the amount of sliced hair particles in the drop.

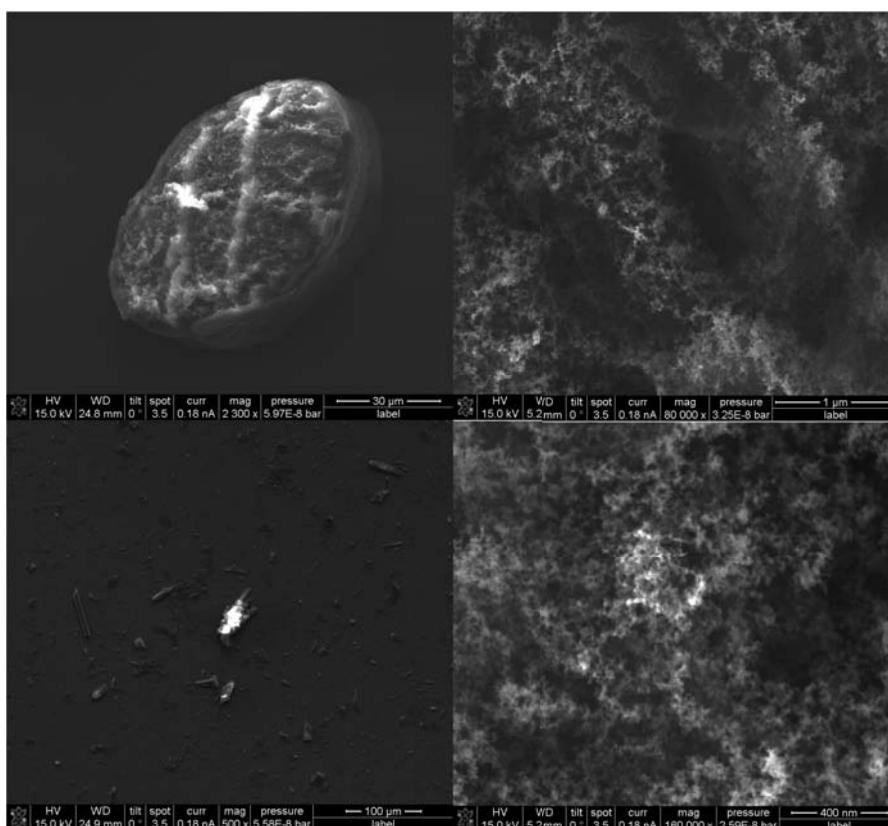


Figure 2.1 SEM images of hair. Top Left; Hair particle sliced using microtome. Top Right; High magnification image of hair slice showing spongy center that promotes nucleation. Bottom Left; Image of scattered crushed hair particles. Bottom Right; High magnification image of crushed hair shows spongy properties are maintained.

The SEM images of crushed hair (figure 1) show that when hair is crushed, particles are obtained of 20 μm and smaller. When using this method there will be hundreds of these particles scattered around the drop. The spongy structure of the keratin fibers in the centre of the hair, containing the cavities is maintained. A major advantage of using crushed hair is that due to the small size of the particles they could easily be added to the protein prior to setting up experiments. A disadvantage is that sometimes the particles are too small to see with

the optical microscope, making it is impossible to check how much particles are present in the drop.

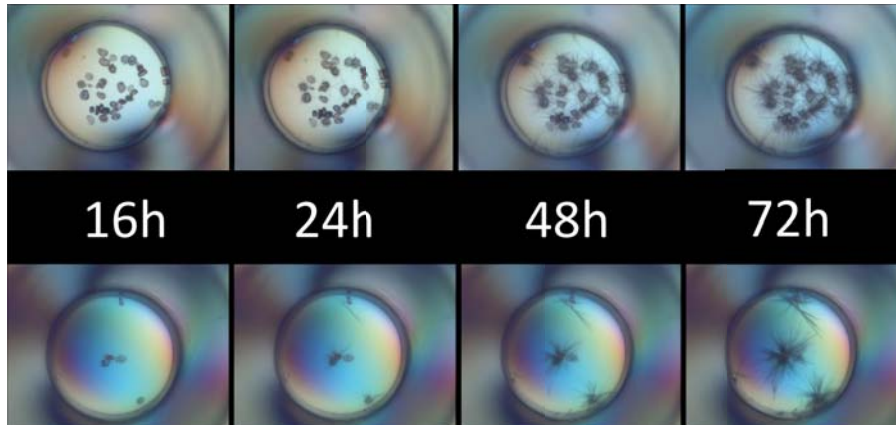


Figure 2.1 *Lysozyme needles growing in a condition with 5 hair particles (below) as well as in a condition with approximately 30 particles (above).*

2.3.2 Hair Slices Induce Nucleation

The nucleation ability of protein crystals on sliced hair was tested using a single buffer condition with different amounts of hair fragments. In a total of 288 drops with increasing amounts from zero to up to 45 hair particles, lysozyme was crystallized. It was expected that higher amounts of hair particles might disrupt nucleation and crystal formation by capturing a substantial proportion of the protein molecules on the hair surface, thereby reducing the protein concentration. However, also in all drops with more than 40 particles crystal formation was observed. Time-lapse images show that needle-shaped crystals start growing from the hair particles (figure 2). The accuracy of dispensing these particles is shown in figure 3.

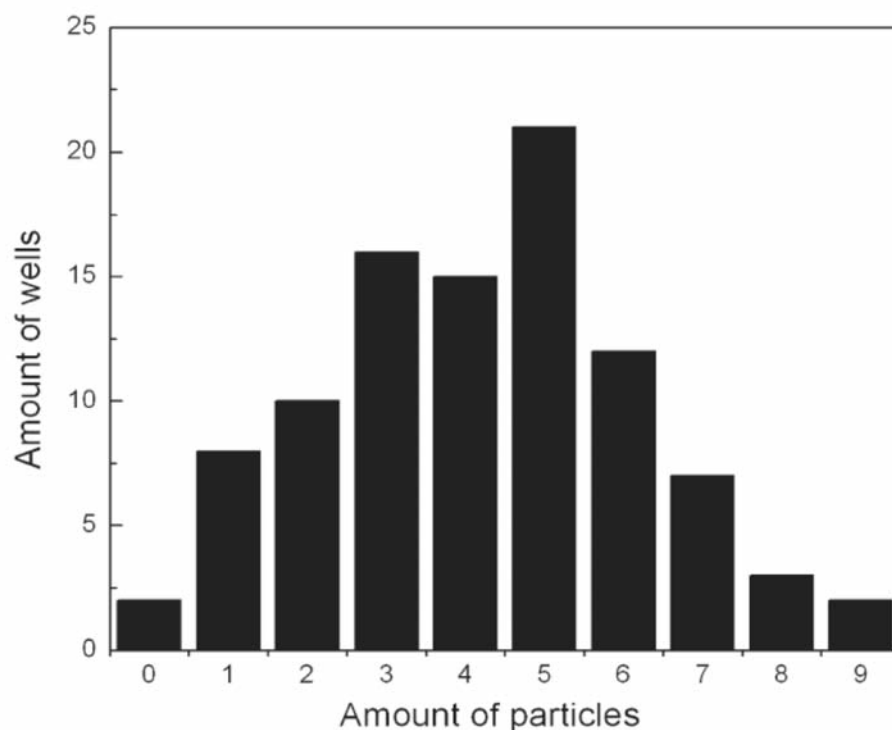


Figure 2.3 Amount of sliced hair particles dispensed in each well of a 96 well plate.

The needle-shaped crystals seem to prefer growing on the cuticle surface, confirming previous observations on long strands of hair (19). However, needle-shaped crystals were also observed to be growing from the spongy surface at the cutting planes of the hair particles. Hair slices of 25 microns in length and an average a radius of 25 microns (with an approximately equal ratio between hair cuticle surface and edge surface) were used. Problems were encountered when using our robotics to dispense hair with a larger radius. For subsequent experiments, only a few hair particles per trial were introduced in the crystallization experiments, in order to prevent disruption of the crystallization process by excess absorption of protein.

2.3.3 Heterogeneous Crystallization with JCSG+ Screen Using Hair as a Nucleant

The influence of the crushed and sliced hair on 11 different proteins was tested using the broad JCSG+ Screen. In total, including controls, we scored in excess of 6000 crystallization trials. Table 2 shows the results of screening with either sliced hair or crushed hair. The control conditions are in duplicate, if in one of the two (or in both) controls a crystal was observed, the condition was labeled as a hit. Furthermore, in certain crystallization solutions both crushed hair and sliced hair induced nucleation and crystal formation for the same protein. Subtracting these two events if they were found to overlap yields the number of unique extra crystallization hits, which are also included in table 2.

Proteins	Conditions crystallized in control	Additional conditions Crystallized with crushed hair	Additional conditions Crystallized with Sliced hair	Additional Unique Conditions Crystallized
Insulin	85	1	1	1
Lysozyme	17	7	3	7
Trypsin	34	2	0	2
CCp-CC complex	43	4	3	5
Myoglobin	4	2	1	2
(Ov)Albumin	11	0	3	3
Glucose Isomerase	31	2	6	8
α -Lactalbumin	12	2	0	2
Alcohol dehydrogenase	9	1	0	1
α -Chemotrypsin	11	1	2	3
Pepsin	2	0	0	0
Total	259	22	19	34

Table 2.2 Additional hits in JCSG+ screening for 11 different proteins after adding hair particles. Additional hits were counted if a protein crystallized in a condition where it did not crystallize without the addition of the hair particles. A condition was considered unique if the extra condition with crushed hair and with sliced hair were different. Ten out of eleven proteins produced a wider range of crystallisation hits after hair particles were introduced in crystallization trials.

We found additional crystallization conditions for 10 out of 11 proteins when using the standard JCSG+ screen (96 different conditions). Pepsin crystallized only in 2 conditions, and the inclusion of nucleants did not affect this outcome, but of the remaining proteins, 34 additional crystallization conditions could be identified (13.1% of the total number of successful crystallizations). The increase in crystallization conditions ranged between 33.3% (2 additional conditions were identified for

myoglobin on top of 4 homogeneous crystallizations) down to 1.2% (we identified a single additional condition for insulin, which crystallized in 85 out of 96 conditions) – the median increase was 14%.

UV imaging proved a useful tool to determine whether crystals were protein crystals or false positives in the form of salt crystals. Figure 4 shows five crystals of which doubt emerged whether they were protein crystals or salt. UV illumination of the proteins removed this doubt, as tryptophan fluorescence clearly lights up the crystals. If the crystals had been salt, they would not have shown UV fluorescence.

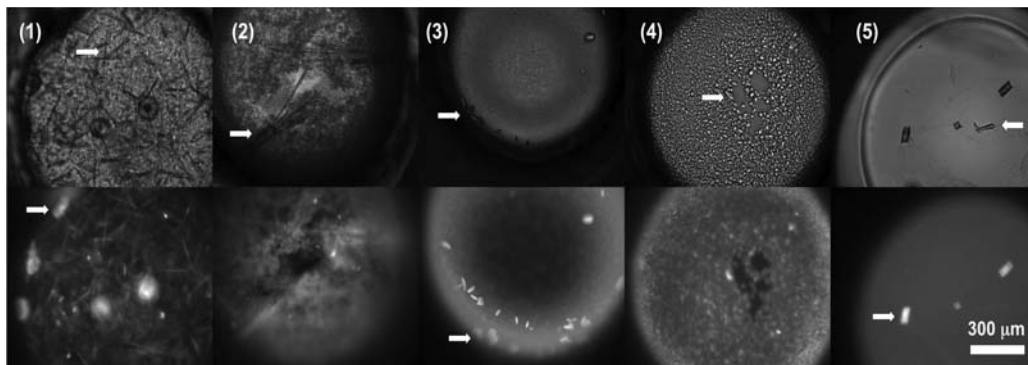


Figure 2.4 Bright field (above) and UV (below) Images of protein crystals suspected of being false positives. In the bright field images the arrows point to the suspected crystals in the UV these crystals fluoresce. In the UV images the arrows point to fluorescent hair slices. 1; The small needles are Glucose Isomerase crystals. 2; Crystals of alpha lactoglobulin at right and bottom left, under a cloud of precipitation. 3; Small crystalline discs of alcohol dehydrogenase around bottom edge of plate where hair (bigger discs) are also present. 4; Small crystals of insulin, the biggest crystals fluoresce the most in UV light. 5; Salt crystals these crystals do not fluoresce.

2.3.4 Experiments at Suboptimal Conditions

Experiments with sliced and crushed hair were set up at sub-optimal conditions using lysozyme. It was clear that at sub-optimal conditions crystal growth could be promoted by adding heterogeneous nucleants.

Figure 5 shows an example: no crystals grow in a suboptimal crystallization condition without hair. The exact same condition containing hair slices yields a single crystal after 36 hours and a total of 2 crystals after 60 hours. In the well with crushed hair there are a lot more nucleation sites scattered over the well. In this well a lot more crystals can be observed at a much shorter time starting around 12 hours and the crystals keep growing until 60 hours. This indicates that increased heterogeneous nucleation increases the growth of crystals in conditions that under normal circumstances do not yield crystals. When too much heterogeneous nucleation sites are included smaller crystals and irregular crystal growth are observed.

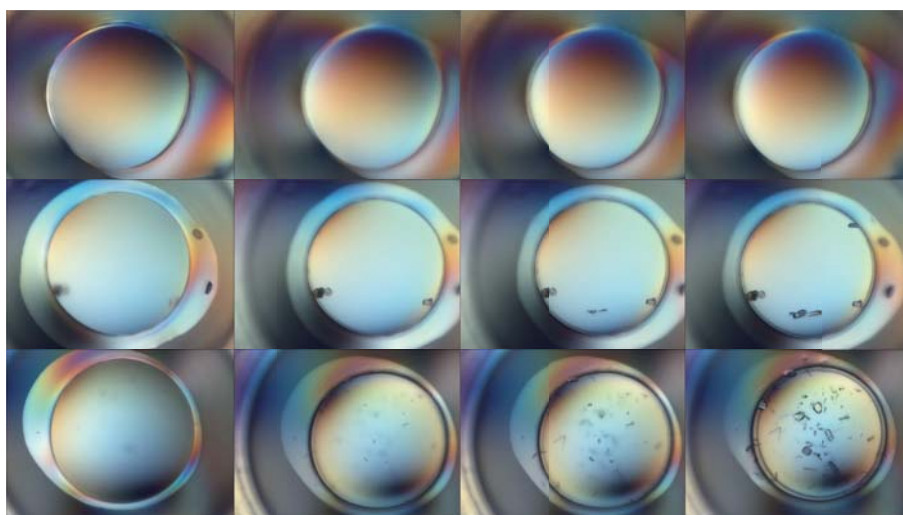


Figure 2.5 Lysozyme crystals growing at a suboptimal condition (0.1 M Na-Acetate buffer pH 4.2, 1 M NaCl) at a concentration of 8 mg/ml. Images of a well containing crushed hair (bottom row), a well with hair slices (middle row) and of a control with no added heterogeneous nucleants. Images were taken at 0, 24, 36, and 60 hours.

2.3.5 Heterogeneous Crystallization Using a Membrane Protein

DGKA crystals grew overnight at 20° C, using conditions described above. An image of DgkA crystals grown on human hair is shown in figure 6. From the image it is clear that nucleation is taking place on the hair as most crystals grow on the surface. Heterogeneous crystallization may also be beneficial for membrane proteins. Experiments performed with hair slices also showed crystallization localized around the chips as is visible in figure 6 UV imaging proved the crystals to be protein.

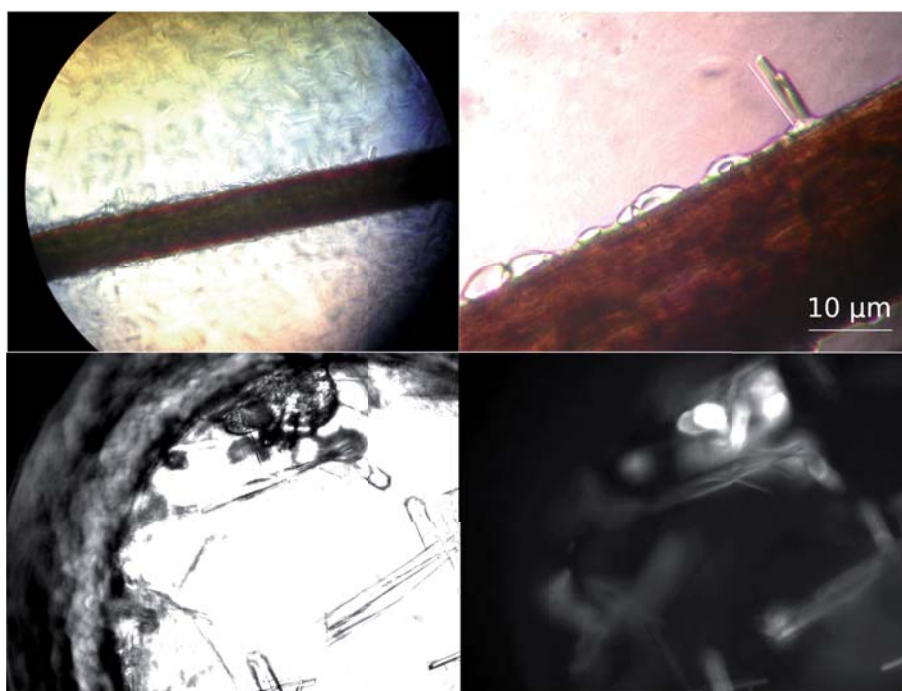


Figure 2.6 Image of DgkA crystals growing on human hair as a nucleant. Top Left: small crystal grew on human hair and rather big crystal grew in the drop solution. Top Right: Nucleation of DgkA on human hair and small crystal from nucleation. Bottom Left: DGKA crystals growing around hair chips. Bottom Right: UV-illuminated Image of DGKA around hair chips proves crystals are protein.

2.4 Discussion/ Conclusion

Overall a versatile high-throughput method is described for introducing hair as heterogeneous nucleants in micro-crystallization. The method is straightforward and can be done in almost any crystallization lab with available materials. The nucleants showed an increase in number of crystallization hits in standard screening experiments, compared to controls without the specific addition of heterogeneous nucleants. These additional crystallization conditions would have been missed without addition of nucleants, even upon repeated testing with the same screening solutions. Initial experiments show that the method may also be beneficial for membrane protein crystallization, but further experiments need to be done in order to validate this observation with additional proteins and extensive screening. Scanning electron microscopy revealed that both crushed hair and sliced hair show a surface containing small cavities suitable for crystal nucleation. The advantage of using sliced hair particles over crushed hair is that the hair slices can easily be seen by light microscopy, the crushed hair cannot. This makes it easier to check and control the amount of nucleants per well. However making the plates containing hair slices is more work and a microtome is needed. If a microtome is not available the crushed hair method is the only option. The increase in hits of both methods is similar, though can vary per protein, thus ideally a plate should be set up with both methods.

Human keratin is one of the major contaminants in mass spectroscopy (29) and its most likely source is dander (shed fragments of skin) and hair fragments of the experimenters. We did not perform our

crystallization experiments in a clean-room facility hence we cannot exclude the possibility that a proportion of the hits we observed in the control experiments (to which we did not deliberately added hair fragments), may in fact have been induced by ambient, keratinous dust. In that case, the increase in nucleation due to human hair fragments that we report here may even be an underestimation. In any case, our experiments indicate it may not be a good idea to work in a clean-room environment for protein crystallization experiments, or to be very rigorous about filtering all crystallization buffers. Our results also lend some credence to aspects of folklore in protein crystallography (for instance: “Good crystallographers have beards”) that indicate that the experimenter may influence the outcome of protein crystallization trials in unexpected ways.

References

- (1) Bolanos-Garcia, V. M.; Chayen, N. E., New directions in conventional methods of protein crystallization. *Progress in Biophysics and Molecular Biology* **2009**, 101 (1-3), 3-12.
- (2) McPherson, A., Introduction to protein crystallization. *Methods* **2004**, 34 (3), 254-265.
- (3) Eisenstein, M., The shape of things. *Nat Meth* **2007**, 4 (1), 95-102.
- (4) Bergfors, T., Seeds to crystals. *Journal of Structural Biology* **2003**, 142 (1), 66-76.
- (5) Chernov, A. A., Protein crystals and their growth. *Journal of Structural Biology* **2003**, 142 (1), 3-21.
- (6) D'Arcy, A.; Mac Sweeney, A.; Haber, A., Using natural seeding material to generate nucleation in protein crystallization experiments. *Acta Crystallographica Section D* **2003**, 59 (7), 1343-1346.
- (7) Cacciuto, A.; Auer, S.; Frenkel, D., Onset of heterogeneous crystal nucleation in colloidal suspensions. *Nature* **2004**, 428 (6981), 404-406.
- (8) Ducruix, A.; Giegé, R. In *Crystallization of Nucleic Acids and Proteins: A Practical Approach*; Ducruix, A., Giegé, R., Eds.; Oxford University Press: New York, **1992**
- (9) McPherson, A.; Shlichta, P., Heterogeneous and Epitaxial Nucleation of Protein Crystals on Mineral Surfaces. *Science* **1988**, 239 (4838), 385-387.

- (10) Thakur, A. S.; Robin, G.; Guncar, G.; Saunders, N. F. W.; Newman, J.; Martin, J. L.; Kobe, B., Improved Success of Sparse Matrix Protein Crystallization Screening with Heterogeneous Nucleating Agents. *PLoS ONE* **2007**, 2 (10), e1091.
- (11) Thakur, A. S.; Newman, J.; Martin, J. L.; Kobe, B., Increasing Protein Crystallization Screening Success with Heterogeneous Nucleating Agents. In *Structural Proteomics*, Kobe, B.; Guss, M.; Huber, T., Eds. Humana Press: **2008**; Vol. 426, pp 403-409.
- (12) Sugahara, M.; Asada, Y.; Morikawa, Y.; Kageyama, Y.; Kunishima, N., Nucleant-mediated protein crystallization with the application of microporous synthetic zeolites. *Acta Crystallographica Section D* **2008**, 64 (6), 686-695.
- (13) Takehara, M.; Ino, K.; Takakusagi, Y.; Oshikane, H.; Nureki, O.; Ebina, T.; Mizukami, F.; Sakaguchi, K., Use of layer silicate for protein crystallization: Effects of Micromica and chlorite powders in hanging drops. *Analytical Biochemistry* **2008**, 373 (2), 322-329.
- (14) Rong, L.; Komatsu, H.; Yoshizaki, I.; Kadowaki, A.; Yoda, S., Protein crystallization by using porous glass substrate. *Journal of Synchrotron Radiation* **2004**, 11 (1), 27-29.
- (15) Saridakis, E.; Chayen, N. E., Towards a 'universal' nucleant for protein crystallization. *Trends in Biotechnology* **2009**, 27 (2), 99-106.
- (16) Chayen, N. E.; Saridakis, E.; Sear, R. P., Experiment and theory for heterogeneous nucleation of protein crystals in a porous medium. *Proceedings of the National Academy of Sciences of the United States of America* **2006**, 103 (3), 597-601.
- (17) Chayen, N. E.; Saridakis, E.; El-Bahar, R.; Nemirovsky, Y., Porous silicon: an effective nucleation-inducing material for protein crystallization. *Journal of Molecular Biology* **2001**, 312 (4), 591-595.
- (18) Page, A. J.; Sear, R. P., Heterogeneous Nucleation in and out of Pores. *Physical Review Letters* **2006**, 97 (6), 065701.
- (19) Georgieva, D. G.; Kuil, M. E.; Oosterkamp, T. H.; Zandbergen, H. W.; Abrahams, J. P., Heterogeneous nucleation of three-dimensional protein nanocrystals. *Acta Crystallographica Section D* **2007**, 63 (5), 564-570.
- (20) Chayen, N. E.; Saridakis, E., Protein crystallization for genomics: towards high-throughput optimization techniques. *Acta Crystallographica Section D* **2002**, 58 (6 Part 2), 921-927.
- (21) Bodestaff, E. R.; Hoedemaeker, F. J.; Kuil, M. E.; de Vrind, H. P. M.; Abrahams, J. P., The prospects of protein nanocrystallography. *Acta Crystallographica Section D* **2002**, 58 (11), 1901-1906.
- (22) Page, R.; Stevens, R. C., Crystallization data mining in structural genomics: using positive and negative results to optimize protein crystallization screens. *Methods* **2004**, 34 (3), 373-389.
- (23) Newman, J.; Egan, D.; Walter, T. S.; Meged, R.; Berry, I.; Ben Jelloul, M.; Sussman, J. L.; Stuart, D. I.; Perrakis, A., Towards rationalization of crystallization screening for small- to medium-sized academic laboratories: the PACT/JCSG+ strategy. *Acta Crystallographica Section D* **2005**, 61 (10), 1426-1431.

- (24) Jiang, L.; Georgieva, D.; Zandbergen, H. W.; Abrahams, J. P., Unit-cell determination from randomly oriented electron-diffraction patterns. *Acta Crystallographica Section D* 2009, 65 (7), 625-632.
- (25) Bashir, Q.; Volkov, A. N.; Ullmann, G. M.; Ubbink, M., Visualization of the Encounter Ensemble of the Transient Electron Transfer Complex of Cytochrome c and Cytochrome c Peroxidase. *Journal of the American Chemical Society* 2009, 132 (1), 241-247.
- (26) Caffrey, M., Membrane protein crystallization. *Journal of Structural Biology* 2003, 142 (1), 108-132.
- (27) Gorzelle BM, Nagy JK, Oxenoid K, Lonzer WL, Cafiso DS, Sanders CR. Reconstitutive refolding of diacylglycerol kinase, an integral membrane protein. *Biochemistry*. 1999 Dec 7;38(49):16373-82
- (28) Lau FW, Bowie JU. A Method for Assessing the Stability of a Membrane Protein. *Biochemistry*. 1997 May 13;36(19):5884-92.
- (29) Ross, A. R. S.; Lee, P. J.; Smith, D. L.; Langridge, J. I.; Whetton, A. D.; Gaskell, S. J., Identification of proteins from two-dimensional polyacrylamide gels using a novel acid-labile surfactant. *PROTEOMICS* **2002**, 2 (7), 928-936.

Chapter 3

A Medipix2 Quantum Area Detector Allows Rotation Electron Diffraction Data Collection of Sub-Micron 3D Protein Crystals

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Abstract

When protein crystals are sub-micron sized, X-ray radiation damage precludes conventional diffraction data collection. For crystals that are in the order of 100 nm in size, at best only single-shot diffraction patterns can be collected and rotation data collection has not been possible, irrespective of the diffraction technique used. Here we show that at a very low electron dose (at most $0.1 \text{ e}^-/\text{\AA}^2$), a Medipix2 quantum area detector is sufficiently sensitive to allow collecting a 30 frame rotation series of 200 keV electron diffraction data from a single $\sim 100 \text{ nm}$ thick protein crystal. A highly parallel 200 keV electron beam ($\lambda=0.025 \text{ \AA}$) allowed observation of the curvature of the Ewald sphere at low resolution, indicating a combined mosaic spread/beam divergence of at most 0.4 Degrees. This result shows that volumes of crystal with low mosaicity can be pinpointed in electron diffraction. We also show that strategies and data analysis software (MOSFLM and Scala) from X-ray protein crystallography can be used in principle for analysing electron diffraction data of 3D nano-crystals of proteins.

3.1 Introduction

Protein crystallography is a major justification for large-scale X-ray facilities like synchrotrons and free electron lasers. However, 3D protein crystals that are smaller than about 0.5 μm are too small for standard X-ray crystallography, although XFEL sources are expanding the method towards smaller crystals (1). This is a serious bottleneck, as about 30% of proteins that crystallize, do not grow crystals of a sufficient size or quality for X-ray structure determination (2). Especially membrane proteins and large (dynamic) protein-nucleic acid complexes fail to grow into crystals of sufficient size. Structural information on these important drug targets is therefore severely lacking.

Electrons are less damaging to proteins than X-rays by several orders of magnitude per elastically diffracted quantum (3). This property of electrons explains the successes of 2D electron crystallography. For instance, 45 $\text{\AA}$ thick, sub-micron patches of bacteriorhodopsin 2D crystals yielded images with a resolution of 2.8 $\text{\AA}$ (4). 3D crystals with an equivalent volume would measure approximately $150 \times 150 \times 150 \text{ nm}^3$. Recent data demonstrates that useful, high-resolution electron diffraction data (up to 2.5 $\text{\AA}$) can be obtained from nano-sized 3D protein crystals, where synchrotron X-rays fail (5).

However, only single diffraction shots could be collected. Collection of rotation data of protein nano-crystals was not possible because the signal-to-noise ratio and dynamic range of CCD detectors and image plates was insufficient. Very recently, direct electron detectors became available, which have a better signal-to-noise ratio and which may be better suited. These

new detectors are very expensive and are probably not sufficiently radiation-hard to be routinely exposed to the direct electron beam.

Electrons can also be detected by quantum area detectors like Medipix2 (6,7). The Medipix2 detector is more radiation hard than other direct electron detectors, like the Falcon, because its read-out electronic circuitry (which is sensitive to radiation damage and interference) is shielded by a semiconductor sensor layer, to which it is bump-bonded (6). The detector has a very high signal-to-noise ratio because each pixel has its own readout electronics that measures the hole-charges that are produced by an incident electron hitting the sensor layer within 10^{-5} s. If the integrated energy after amplification is above a set threshold corresponding to the energy of a 200 keV electron, the incident quantum is counted as a “hit”. Thus the Medipix2 chip only counts 200 keV electrons, and, unlike many other detectors, is blind to soft X-rays of lower energy that are also produced in great abundance inside any EM. In this fashion its noise is almost exclusively determined by the counting statistics of the electrons. This gives a significant improvement over conventional CCD cameras in electron microscopes. (8,9).

We recently showed that a Medipix2 detector with a 500 μm sensor layer can detect 200 keV electrons with a signal-to-noise ratio that is at least an order of magnitude better than that of image plate (9). The Medipix detector is highly sensitive at low count rates, allowing accurate measurement of the high-resolution terms. In addition, with a 500 μm sensor layer it is sufficiently radiation hard to routinely be exposed to a direct beam of 200 keV electrons. It also has a high dynamic range allowing accurate measurement of the intense, highly peaked dose it receives in the low-

resolution diffraction spots. For 200 keV, the point spread of the detector is increased, but if the point spread is not much higher than the spread of the Bragg spots this would not matter for collecting diffraction data.

In view of these desirable characteristics of the Medipix2 detector, we investigated whether it would be possible to reduce the electron dose per diffraction frame by an order of magnitude and still measure high-resolution diffraction data, as this would, for the first time, allow collecting rotation diffraction data of single protein crystals.

3.2 Materials and Methods

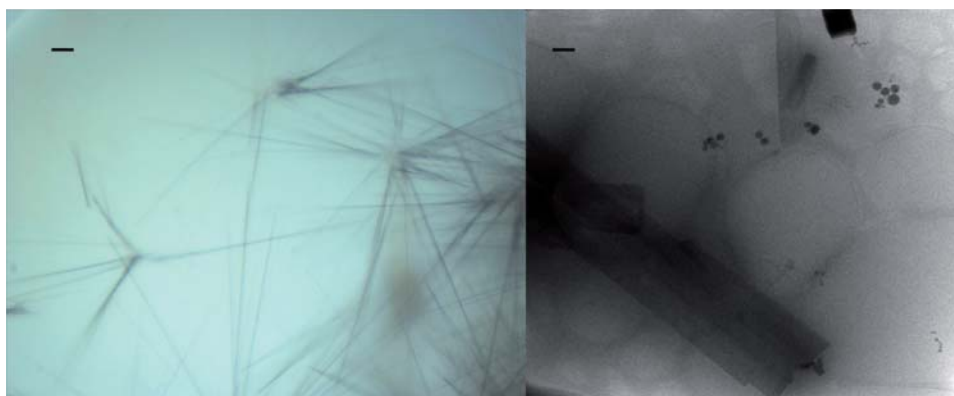


Figure 1. *Lysozyme nano-crystals in a crystallization drop (left) and an electron micrograph of vitrified crystals (right). Scale bars correspond to 10 μm (left) and 500 nm (right).*

3.2.1 Crystallization

Crystallization experiments were carried out using the sitting drop vapour diffusion technique in Innovadyne SD-2 plates. Rockmaker software (Formulatrix) was used for designing the experiments. A Genesis (Tecan) robot was used for dispensing the screening solutions in the reservoirs of MRC2 plates (Swissci). The ORYX 6 (Douglas Instruments) crystallization

robot was used for transferring 500 nl reservoir solution and 500 nl protein solution in sitting drop wells. Plates were stored at 18 °C and imaged using the automated imaging system Rock Imager (Formulatrix). Lysozyme (8 mg/ml) formed needle-shaped crystals after 48h when mixed 1:1 with well solution containing 0.1 M Sodium Acetate pH 3.8 and 1.0 M Potassium Nitrate.

3.2.2 Vitrification

Protein crystals were vitrified using a Vitrobot (FEI). 3 µl of well solution was mixed with the drop containing nano-crystals and transferred to a 3mm glow-discharged holey carbon grid (AGAR). Excess liquid was blotted away (blot time 3 sec blot force 5) and the sample was plunge frozen in liquid ethane cooled by liquid nitrogen.

3.2.3 Rotation Electron Diffraction

Diffraction data were collected at 200 keV on a CM200FEG (Philips) transmission electron microscope at the National Center for High Resolution Electron Microscopy in Delft. Samples were cooled to -180 Celsius in liquid nitrogen in a home-modified cryo-transfer holder (Gatan). Diffraction patterns were collected using a Medipix2 detector. We created a narrow, highly parallel electron beam with limited intensity by using a small (10 µm) condenser aperture and spotsize 11 (which controls the first condensor lens). The beam was hardly convergent, as indicated by the data analysis described below (< 0.4 Degrees). An automated compu-stage allowed crystal alignment and rotation.

3.2.4 Measuring Electrons Using a Medipix2 detector

Diffraction patterns were collected with a CMOS Medipix2 detector mounted on a Nikhef carrier board and the data were transferred to a Windows PC using USB 1.1 read out electronics (10). Four abutting Medipix2 ASICs, each with 256x256 pixels and a pixel size of 55 μm make a Medipix2 Quad. The Quad was covered by a single custom-made 500 μm semiconductor sensor chip. The distance between two neighbouring single Medipix2 ASICs is approximately 250 μm , so the edge pixels are about 125 μm wide. Therefore they will capture more signal than the other pixels resulting in a bright cross that quarters the raw images. This cross can be corrected for as discussed below.

The detector has a big dynamic range where each pixel consists of a separate 14 bit pseudo-random counter. The test circuitry and four bit trimming system are able to compensate for most fabrication variations and therefore the overall global threshold has a variation of 95 eV. The electronics noise can account for another 110 eV. These two values combined result to a dynamic range of no lower then 900 eV between channels. In silicon it requires ~ 3.6 eV to produce one electron-hole pair, therefore a 200keV electron can produce $\sim 55,000$ pairs. This means that the dynamic range of the electronic noise accounts for less than 1.6% of the total deposited energy per electron incident and even less when there is more than one electron incident per clock cycle (11,12).

The thickness of the sensor layer (500 μm) is larger than for stock Medipix2 chips (which is 300 μm). We selected this larger thickness to prevent the 200 keV electrons from penetrating through the sensor layer and damaging

the underlying electronics. Monte Carlo simulations (300 μm Si sensor layer; 120 keV, 200 keV and 300 keV) predicting such events have been previously described (7,13,14). Most importantly it also describes the events where these higher energy electrons scatter in the Silicon layer to neighbouring pixels. As long as this scattering stays close to the boundaries of the Bragg spots, this would give no major point spread problems.

There is a need to set the threshold settings such that the gain of the detector is close to or slightly lower than one. Setting the threshold too low will result in multiple pixels recording a single high energy electron hit; setting the threshold too high will not count electrons, or (at sub-optimally high thresholds) only count electrons that deposit all their energy into a single pixel. It is important that the threshold is chosen in such a way that when the electron partly scatters to a neighbouring pixel it still is counted. The chance of a single high-energy electron scattering in the sensor layer to neighbouring pixels increases with higher energies. (8). To get a higher electron counting gain, a compromise threshold level was chosen where also lower energy particles would be counted in each pixel. This, however is not much of influence for electron diffraction studies with the Medipix, since most other particles (X-ray) will stay well below an equivalent 100 keV threshold setting. This would be an undesirable setting in any case, because that would enable double counting in neighbouring pixels for a single electron hit. To minimise the radiation damage the experiment was conducted under such low dose conditions that the chance that a two electrons hit within the same pixel within a clock cycle of the camera is almost non-existent (e.g. less than 0.1%). We validated our gain estimate with MOSFLM. The spot profile statistics predicted a gain of 0.816 counts/electron. But since the measurement was performed at very low dose

conditions (the average count per pixel per frame is around five) it is no longer possible to estimate the gain by fitting a Gaussian (the MOSFLM method) instead of a Poisson distribution. Therefore the true gain must be higher than 0.816 but not higher than 1.00.

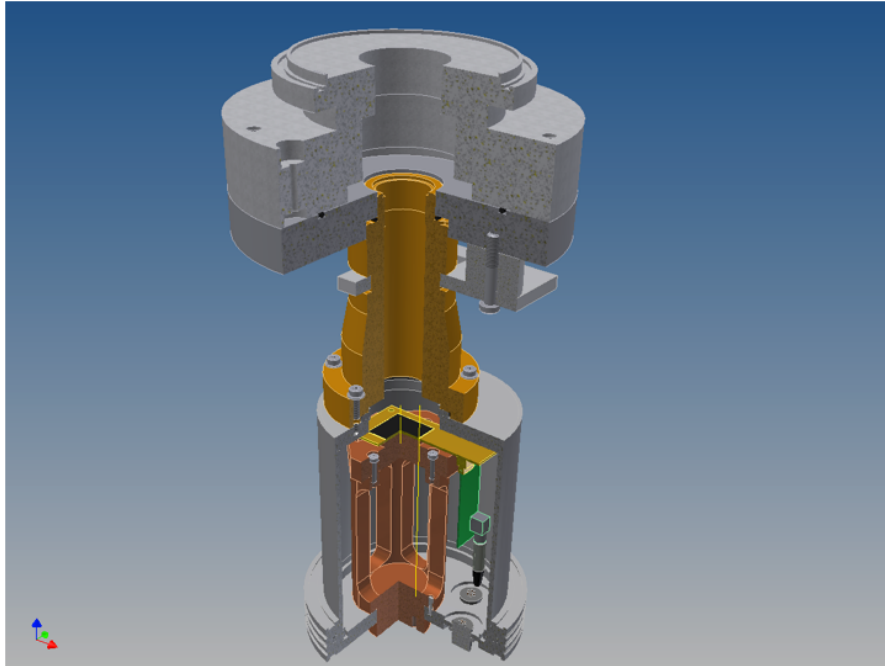


Figure 2. Cut-out view of the Medipix2 camera assembly. Electrons enter the tube from the top and hit the detector (black square) on the NIKHEF assembly board (15), which is connected through the USB read-out electronics and a vacuum connector to the computer.

The vacuum pod in which the Medipix2 was mounted on the on-axis port of the CM-200 FEG electron microscope is shown in figure 3. It houses both the carrier and USB1.1 readout board. Damage to the electronics of these boards by the direct electron beam was prevented by the small window of the pod, which only allowed illumination of about 80% of the total area of the Medipix2. Out-gassing of the electronics at 1.10^{-6} Pa vacuum did not prove to be a problem for both the electronics and the TEM. The bias voltage on the Medipix2 was set to 100V to be able to count the holes after

the electron incidents (9). The 4 chips are set to non-parallel read-out by the Pixelman v2.0 software (14) on a PC with windows 7 in Java mode. The Medipix2 Quad was calibrated using the standard tools that are included in Pixelman:

- DACs were scanned to determine the noise edge of the different ASICs.
- Threshold equalization was performed to remove small standard discrepancies between individual pixels (3 bits). This procedure adjusts each pixel so that its threshold is as close as possible to the average and was performed with the standard settings supplied with the Pixelman package.
- Each lower threshold (THL) was set to a value close to the noise edge in order to acquire as much signal as possible from the incident electrons.
- Flat field images obtained by illumination with a uniform beam allowed equalizing the four chips.

3.2.5 Preparing Diffraction Patterns for Data Processing

The Medipix2 images need some pre-processing before they can be read into MOSFLM. Three problems needed to be addressed: (i) the cross needs to be removed, (ii) dead pixels needed to be corrected and (iii) the image needed to be centred, as there was a substantial drift of the direct beam. To this aim, we wrote a program that solved these issues as described below.

Removing the Cross

A bright cross appears in the raw Medipix2 Quad images, as it is a tiled assembly of four single Medipix2 ASICs. The edge pixels of each single Medipix2 ASIC that abuts another in the Quad assembly are larger than

the other pixels (they are 125 mm wide, rather than 55 mm). Therefore, the pixels at the interface between single Medipix2 ASICs capture more electrons. This results in a spatial distortion and a non-uniformity of the measured signal. In order to correct for the spatial distortion, our program shifts pixels with an x-value smaller than 256 by one pixel to the left and it shifts pixels with a value of 256 or higher by one pixel to the right. It applies vertical shifts in a similar fashion. This procedure duplicates the pixels adjacent to the horizontal and vertical bisecting lines (and quadruples the four centre pixels). Their value was divided by 2.3 (or 5.3 for the quadrupled centre pixels) to correct the increased measured intensities of these pixels.

Correcting Bad Pixels

Despite tuning the pixels of the Medipix2 detector, it can still have dead pixels (or bright pixels). Since positions of dead pixels are fixed and their values do not follow a Poisson distribution over time, it is possible to identify these dead pixels. For each pixel position, we calculated its variance over a range of images, and compare this variance to the variances at the other positions. 'Good' pixels will have a much higher variance than 'bad' pixels since the values of the latter tend to be relatively constant and largely independent of the signal. If the variance value was below a set threshold the pixel was be labelled as 'bad'. After having identified the bad pixels, every image was corrected. For every bad pixel in every image, its value will be replaced by the mean value of the good pixels that surrounded it. If a bad pixel had no good neighbours, the procedure was iterated after reclassification of the pixels.

Centring the Images

The position of the beam centre shifted as a function of the tilt of the sample holder. The shift was not huge (15 pixels maximally), but sufficiently large to interfere with data processing. Images were centred by calculating their cross-correlation functions with a 2D Gaussian function peaking at pixel (256, 256) and applying the calculated shift. After centring the images, they were written to disk in the CCP4 ‘.pck’ format (16) as 1200 by 1200 pixel images, so that they could be processed as small MAR images by MOSFLM (17).

3.3 Results

3.3.1 Collecting Diffraction Data

Prior to installing the Medipix2 detector in the EM, we collected diffraction data on image plate, film and CCD, but with these detectors we could never collect multiple diffraction patterns of single protein crystals to high resolution because of beam damage (5). The Medipix2 detector has an efficiency at low count rates that is at least an order of magnitude higher than that of image plate (8,9). This meant that we could for the first time collect rotation data over multiple images without severe beam damage.

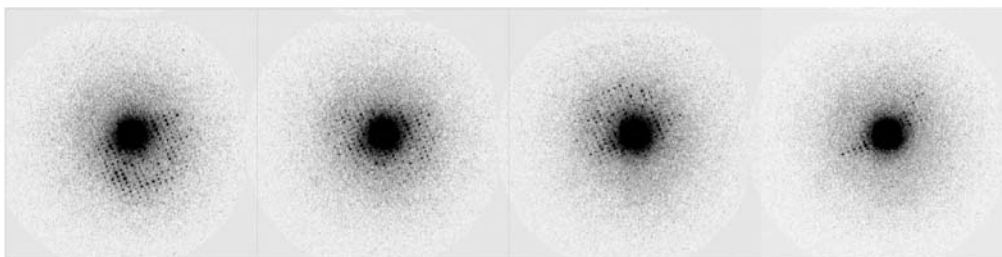


Figure 3. Four diffraction patterns from a series of 23 subsequent frames (0.05 Degrees per frame, 0.083 Degrees between adjacent images, see text for details) collected at 200 keV with a Medipix2 Quad detector (512 by 512 pixels). The crystal was orthorhombic lysozyme, maximum resolution $\pm 1.8 \text{ \AA}$. The frame on the utmost right is after 30 seconds of data collection and shows the degradation in signal due to beam damage. Per second each frame was exposed to between 0.05 and 0.1 $\text{e}^-/\text{\AA}^2$.

Unfortunately, the hardware prevented us from interfacing the detector with the electron illumination and the rotation of the sample holder. This meant in practice that we had to start rotating the crystal slowly, switch on the beam and start collecting images until the crystal had sustained too much damage. Since transferring a single Medipix2 frame into the computer takes 0.7 s (we were using a USB1 interface), this meant that we could only collect rotation data with angular gaps between adjacent images. By using fine phi slicing, we reduced the systematic errors that were introduced by this unfortunate, but in the circumstances inevitable experimental flaw.

We collected rotation data of many crystals, improving data collection strategies and sample handling iteratively. Here we describe representative results using two rotation data sets from the same single crystal, but collected at different positions, using a fresh part of the crystal. We rotated in the positive direction for the first wedge of data and in the negative direction for the second wedge. Both wedges started at the same goniometer setting, but processing revealed their orientations to be about 1.5 Degrees apart. The first wedge was measured with a rotation speed of 0.083

degrees/image (0.05 Degrees/s), reading out an image every second. This resulted in a data set in which each image had recorded about 0.050 degrees of rotation data, with a gap of 0.033 degrees until the next image. For the second data set, the crystal was rotated at a speed of 0.26 degrees/s, reading out an image every second. This resulted in a data set with 0.15 degrees per image with gaps between images of 0.11 degrees. Both data sets had diffraction spots to a resolution of 1.8 Ångstrom in the early images (see figure 4).

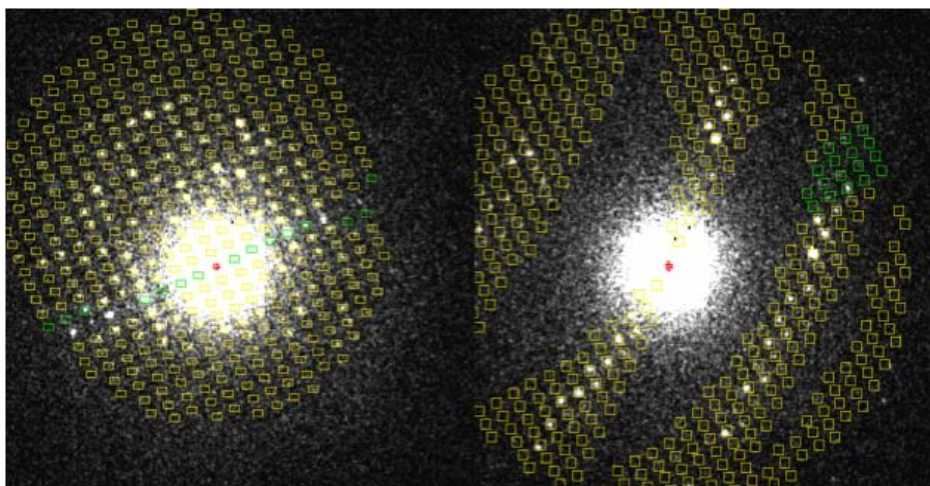


Figure 4. *Overlay of predicted spot positions (resolution threshold 2 Ångstrom) with observed diffraction patterns of lysozyme nano-crystals, indicating that the 'a'-axis could be close to 125 Ångstrom, while the 'b' and 'c' axes are about 34.2 and 25.5 Ångstrom. For the Images screen shots of MOSFLM runs are used.*

The diffraction spots were analysed and profiles of two single spots are shown in figure 5. Low intensity spots (15 to 20 electrons in the peak) that are far from the central beam clearly show up above the noise (Figure 5a). These spots still show a significant level over the surrounding background pixels, which on average count 2 to 3 electrons. To compare, a bright spot with a maximum intensity of 100 to 120 electrons per pixel is shown in Figure 5b.

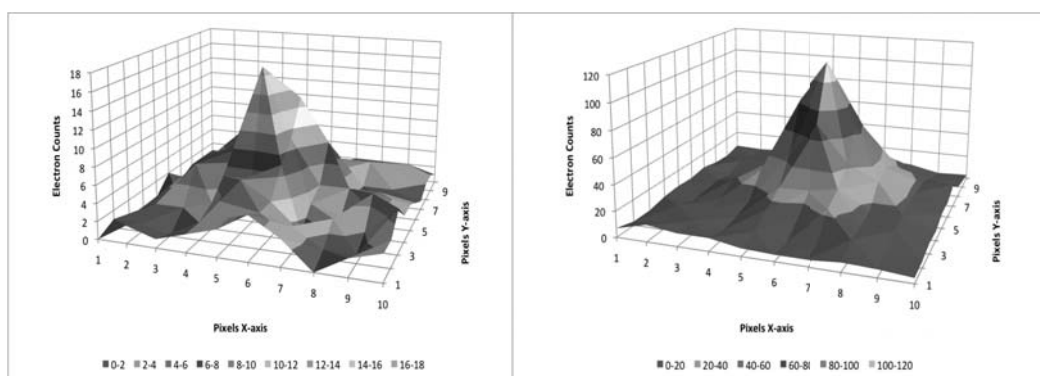


Figure 5. Two typical spot profiles in a 10 by 10 pixel frame. (a) A low intensity spot profile at 2.0 Å resolution with a maximum of 15-20 electrons. (b) A high intensity spot profile with a maximum of 100-120 electrons close to the central beam.

3.3.2 Processing the diffraction data with MOSFLM

Several essential parameters had to be extracted from the diffraction patterns: the angle of the rotation axis relative to the detector coordinate frame, the beam centre, the beam divergence, the unit cell parameters and the orientation of the crystal. The determination of each of these parameters will be discussed in more detail below.

Beam centre

We observed the beam centre to shift considerably (about 15 pixels) between the starting and ending rotation angles. Since we did not use a

backstop, this shift could readily be corrected for after data collection by shifting back the images accordingly (as described in the Methods section). At the time of data collection, it was unclear what the cause of the beam shift was, but later it transpired that a screw of the EM stage close to the crystals had become slightly magnetised.

Angle of the rotation axis

As the electrons spiral their way through the magnetic lenses of the microscope, the rotation axis that is observed on the detector is not necessarily an orthogonal projection of the physical rotation axis. It needs to be calibrated for every camera distance. Careful analysis of the diffraction patterns using the human eye, a ruler and a protractor indicated that at a virtual camera length of 700 mm and an electron energy of 200 keV, the angle between the rotation axis and the x-direction of the detector was around 115 Degrees. We could refine this initial estimate to 116.5 Degrees by overlaying predicted diffraction patterns on observed diffraction patterns once we had good estimates of the other parameters. When rotating in the negative direction, the rotation axis was redefined to be -63.5° .

3.3.3 Unit cell parameters

Using our knowledge of the virtual crystal to film distance and the wavelength (which is $0.0251 \text{ \AA}$ for electrons at 200 keV energy), we interactively estimated the two spacings in the plane of the detector using the ‘measure’ facility in MOSFLM. These parameters (34.2 and $25.5 \text{ \AA}$ with an angle of 90 degrees) were consistently found in both rotation ranges. No low index spacings of the third axis were present in the images, and its magnitude could only be estimated from the location of the lunes in the second data set. A reasonable, but by no means perfect overlay between

observed and predicted spot positions could be obtained for a unit cell of $125 \times 34.2 \times 25.5 \text{ \AA}^3$, with all cell angles 90 Degrees (figure 4). In the past we found orthorhombic nano-crystals of lysozyme to have $P2_12_12_1$ symmetry, with a unit cell of around $31.5 \times 52.5 \times 89 \text{ \AA}^3$ (5). However, we could not index the rotation data with the latter cell parameters.

3.3.4 Determining the orientation of the crystal

Due to radiation damage, we could only measure small wedges of data. Auto-indexing routines could therefore not cope with the data. We indexed interactively and let MOSFLM refine the orientation using post-refinement, which, despite the angular gaps between images, managed to improve the fit between observed and predicted patterns. The wedges contained sufficient data for orientation refinement, but did not allow for the refinement of cell parameters.

3.3.5 Beam divergence

Once we had satisfactory predictions of the diffraction patterns, we adjusted the beam divergence (or mosaic spread: the two phenomena cannot be disentangled easily) in MOSFLM so that all spots were covered by the predicted pattern. The combined beam divergence/mosaic spread was about 0.3 to 0.4 Degrees.

Typical profiles of the spots are shown in figure 5. There were a number of bad spots, mainly because of a high gradient in the background, but by setting the maximum gradient to 5 (instead of the default value 3), all spots were accepted. One likely cause of the high background gradient for some of the spots was the very intense zero order reflection: the direct beam. Removing this reflection with a backstop was not possible without also

removing all the reflections with a resolution lower than about 5 Ångstrom. The reason for this is the low scattering angle and the position of the backstop in the CM-200 microscope. For protein crystallographic data collection the position of the backstop should have been much closer to the detector, but hardware restrictions prevented us from moving the backstop.

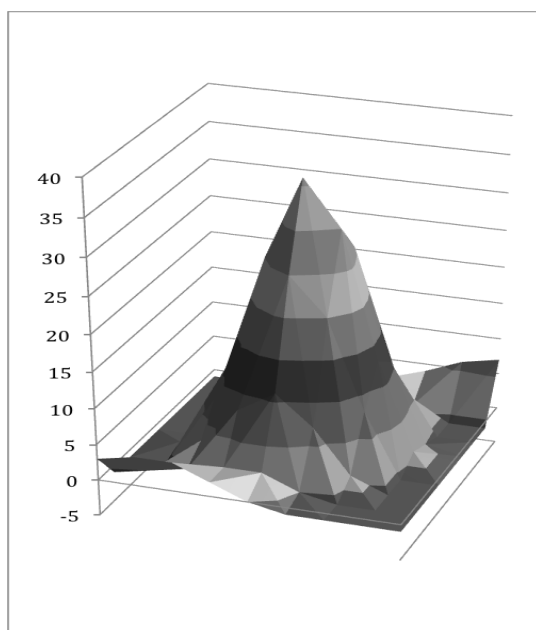


Figure 6. Spot profile from MOSFLM for the first data set. The second data set had a similar spot profile (not shown).

Scaling the data was problematic in view of the high partiality, the small wedge size the scarcity of data and the angular gaps between frames. For this purpose, we used the CCP4 program Scala (18). The statistics were extremely poor because we had to scale the partials using their calculated partiality and because of the large missing gaps of data between adjacent images. Figure 6 shows the merged and scaled ‘h=0’ zones of both data sets. Note that we processed all the data as P1 with anomalous signal. We did so in order to identify potential dynamic scattering, which results in a breakdown of point group symmetry in the diffraction patterns (19). The

symmetries shown in figure 6 are therefore intrinsic to the diffraction experiment and not imposed by symmetry averaging of corresponding reflections.

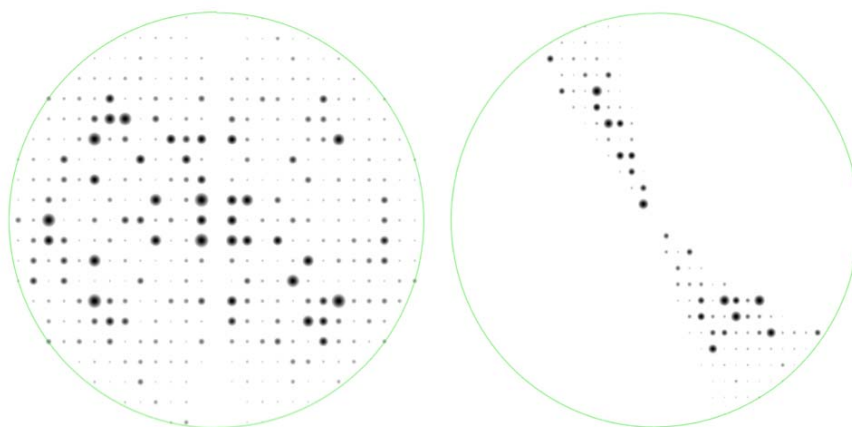


Figure 6. *Laue zones at $h=0$ of the scaled data, as displayed by 'viewhkl'. Note that the second data set (right pattern) does not have a complete ' $h=0$ ' Laue zone.*

3.3 Discussion and Conclusion

The 3D nano-crystals of lysozyme have successfully been grown, vitrified and transferred to an electron microscope at liquid nitrogen temperature. Rotation electron diffraction data was obtained at multiple places of a single sub-micron crystal to resolutions better than 2 Å. For the first time, we could collect multiple frames of protein nano-crystals. This was made possible by the much higher sensitivity and signal-to-noise ratio of the Medipix2 detector, combined with its radiation hardness at 200 keV.

By using a parallel electron beam, we could for the first time observe the curvature of the Ewald sphere in our electron diffraction patterns of protein crystals. At 200 keV, electrons have a wavelength of only about

0.025 Angstrom, causing the Ewald sphere to be extremely flat at low resolution. Yet the data show that the curvature is still significant.

When the curvature of the Ewald sphere cannot be ignored, both members of a Friedel pair cannot simultaneously be in full diffraction. Geometric considerations imply that the rotation angle between the two reflections of a Friedel pair is always at least their diffraction angle. Consider for instance a reflection at 5 Å resolution and assume it is optimally aligned. At ($\lambda=0.025$ Angstrom) the crystal would need to be rotated by almost ($\sin^{-1}(0.025 / 10) = \sim 0.3$) Degrees in order to move its Friedel mate into full diffraction. The fact that we can clearly see deviations in intensity between Friedel mates even at 5 Å resolution (figure 4), confirms the low beam divergence and mosaic spread suggested by the data processing. Clearly, the rocking curve of the crystal (with a maximal width of less than 0.4 Degrees) is sufficiently peaked to allow observing these fine angular differences in orientation. This implicates it should be possible to pinpoint areas of low mosaicity in the crystals. This may have an advantage if the crystalline order varies within a single nano-crystal, in which case its best parts can be selected.

These results are encouraging enough to consider fixing the technical problems that prevent optimal data collection. The lack of a scheduling interface between detector and the goniometer that controls the crystal orientation, combined with the relatively slow readout of the Medipix2 quad (0.7 seconds), is an evident cause of many of these problems. As the rotation of the sample could not be interrupted during the readout time, there were angular gaps between subsequent frames. We anticipate fixing this problem by employing a novel Medipix2 detector on a RELAXd read-out board (20)

with improved readout time (up to 200 frames per second) that can also read data while collecting the next frame. This would significantly decrease illumination time and subsequent beam damage. We plan to mount the detector on a Titan Krios machine, so data collection will be improved due to an autoloader, a programmable goniometer and a programmable beam blanker. For small molecule crystals, it has been reported that collecting data in STEM-diffraction mode further reduces beam damage (21), so we plan incorporating the Medipix2 detector in a microscope that has this option.

In view of the suboptimal data collection, we were happy being able to integrate the data with one of the standard programmes for processing X-ray data: MOSFLM. However, we cannot exclude the possibility that the results we report here do not reflect the true crystal structure. We noticed unexpected correlations between parameters. For instance, rotation of the crystal around ϕ was accompanied by an apparent rotation (as suggested by post refinement) around Ψ , which is a rotation of the crystal around the beam. Perhaps this is caused by eucentric height variation of the crystal as it is being rotated. Since the electrons are focused spirally, rotation of the image or diffraction pattern with height is expected. These and other correlations, which were independent of the unit cell or crystal orientation parameters, may indicate that the indexing solution we present here is wrong. However, we think that at least in part, these correlations are caused by distortions specific for electron diffraction. These exist and we unequivocally identified at least one. For instance, the correlation of the beam shift with rotation of the crystal, which we observed (and corrected for), would never be encountered in X-ray diffraction.

Together with beam damage, the angular gaps between adjacent images may explain the observed breakdown in point symmetry observed in the 'h=0' Laue zones (figure 7). An alternative explanation may be found in the effects of dynamic scattering, which also causes such a breakdown in symmetry (19). With improved hardware we anticipate to be able to distinguish between these effects.

We have demonstrated that it is possible, at least in principle, to collect rotation electron diffraction data of protein nano-crystals. This prompts the question concerning the usefulness of such data sets for solving protein crystal structures. Problems with dynamic scattering have been anticipated, yet these did not prevent the structure solution to 3.3 Å of the aquareovirus using single particle analysis (22), which also has a size of approximately 100 nm, just like the crystals analyzed here. If at higher resolution the effects of dynamical scattering cannot be ignored anymore, they can be modeled by the multi-slice approach (23). This is common practice in electron crystallography of small molecules. Furthermore, there are theoretical considerations that imply that dynamical scattering may yield phase information (19), and a limited amount of such dynamical scatter may therefore even be beneficial.

Electron area detectors – like Medipix – that allow distinguishing the signal of high-energy electrons from that of other types of radiation are likely to have a major impact on structural biology. Our results imply that also protein crystallography stands to benefit from these technical advances.

References

- (1) Chapman, H. N., Fromme, P., Barty, A., White, T. A., Kirian, R. A., Aquila, A., Hunter, M. S., Schulz, J., DePonte, D. P., Weierstall, U., Doak, R. B., Maia, F. R. N. C., Martin, A. V., Schlichting, I., Lomb, L., Coppola, N., Shoeman, R. L., Epp, S. W., Hartmann, R., Rolles, D., Rudenko, A., Foucar, L., Kimmel, N., Weidenspointner, G., Holl, P., Liang, M., Barthelmess, M., Caleman, C., Boutet, S., Bogan, M. J., Krzywinski, J., Bostedt, C., Bajt, S., Gumprecht, L., Rudek, B., Erk, B., Schmidt, C., Homke, A., Reich, C., Pietschner, D., Struder, L., Hauser, G., Gorke, H., Ullrich, J., Herrmann, S., Schaller, G., Schopper, F., Soltau, H., Kuhnel, K.-U., Messerschmidt, M., Bozek, J. D., Hau-Riege, S. P., Frank, M., Hampton, C. Y., Sierra, R. G., Starodub, D., Williams, G. J., Hajdu, J., Timneanu, N., Seibert, M. M., Andreasson, J., Rocker, A., Jonsson, O., Svenda, M., Stern, S., Nass, K., Andritschke, R., Schroter, C.-D., Krasniqi, F., Bott, M., Schmidt, K. E., Wang, X., Grotjohann, I., Holton, J. M., Barends, T. R. M., Neutze, R., Marchesini, S., Fromme, R., Schorb, S., Rupp, D., Adolph, M., Gorkhover, T., Andersson, I., Hirsemann, H., Potdevin, G., Graafsma, H., Nilsson, B. & Spence, J. C. H. (2011). *Nature* **470**, 73-77.
- (2) Rupp B (2004) "A Guide to Automation and Data Handling in Protein Crystallisation", in: "Protein crystallisation Strategies for Structural Genomics" (Chayen N, ed.), ISBN 0-9720774-3-X, p 9-56
- (3) Henderson, R. (1995). *Q Rev Biophys* **28**, 171-193.
- (4) Baldwin, J. M., Henderson, R., Beckman, E. & Zemlin, F. (1988). *Journal of Molecular Biology* **202**, 585-591.
- (5) Jiang, L., Georgieva, D., Zandbergen, H. W. & Abrahams, J. P. (2009). *Acta Crystallographica Section D* **65**, 625-632.
- (6) Llopart Cudié, X., Campbell, M., Dinapoli, R., San Segundo, D. & Pernigotti, E. (2002). *IEEE Transactions on Nuclear Science*(ISSN 0018-9499)(EISSN 1558-1578) **49**, 2279-2283.
- (7) Faruqi, A. R. & Henderson, R. (2007). *Current Opinion in Structural Biology* **17**, 549-555.
- (8) A. R. Faruqi and G. McMullan (2011). Electronic detectors for electron microscopy. *Quarterly Reviews of Biophysics*, **44**, pp 357-390.
- (9) Georgieva, D., Jansen, J., Sikharulidze, I., Jiang, L., Zandbergen, H. W. & Abrahams, J. P. (2011). *Journal of Instrumentation* **6**, C01033.
- (10) Vykydal, Z., Jakubek, J. & Pospisil, S. (2006). *Nuclear Instruments and Methods in Physics Research Section A: Accelerators, Spectrometers, Detectors and Associated Equipment* **563**, 112-115.
- (11) McMullan, G., Cattermole, D. M., Chen, S., Henderson, R., Llopart, X., Summerfield, C., Thustos, L. & Faruqi, A. R. (2007). *Ultramicroscopy* **107**, 401-413.
- (12) Plackett, R., Ballabriga, R., Campbell, M., Llopart, X., Tick, T., Thustos, L., Turecek, D., Vahanen, S. & Wong, W. (2010) *Proceedings of the 19th International Workshop on Vertex Detectors*, 30

- (13) McMullan, G., Chen, S., Henderson, R. & Faruqi, A. R. (2009). *Ultramicroscopy* **109**, 1126-1143.
- (14) Turecek, D., Holy, T., Jakubek, J., Pospisil, S. & Vykydal, Z. (2011). *Journal of Instrumentation* **6**, C01046.
- (15) San Segundo Bello, D., van Beuzekom, M., Jansweijer, P., Verkooijen, H. & Visschers, J. (2003). Nuclear Instruments and Methods in Physics Research Section A: Accelerators, Spectrometers, Detectors and Associated Equipment **509**, 164-170.
- (16) Abrahams, J. P. (1993). *Joint CCP4 and ESF-EACBM Newsletter on protein crystallography*, **28**, 3-4.
- (17) Leslie, A.G.W., Powell, H.R. (2007), Evolving Methods for Macromolecular Crystallography, **245**, 41-51.
- (18) Evans, P. (2006). *Acta Crystallographica Section D* **62**, 72-82.
- (19) Abrahams, J. P. (2010). *Zeitschrift für Kristallographie* **225**, 67-76.
- (20) Visser, J., van der Heijden, B., Weijers, S. J. A., de Vries, R. & Visschers, J. L. (2011). Nuclear Instruments and Methods in Physics Research Section A: Accelerators, Spectrometers, Detectors and Associated Equipment **633**, Supplement 1, S22-S25.
- (21) Kolb, U., Mugnaioli, E. and Gorelik, T. E. (2011). *Cryst. Res. Technol.*, **46**: 542-554.
- (22) Zhang, X., Jin, L., Fang, Q., Hui, W. H. & Zhou, Z. H. (2010). *Cell* **141**, 472-482.
- (23) Jansen, J., Tang, D., Zandbergen, H. W. & Schenk, H. (1998). *Acta Crystallographica Section A* **54**, 91-101.

Chapter 4

Imaging Protein 3D Nano-crystals with Cryo-EM³

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Abstract

Flash-cooled, 3D crystals with a thickness in the order of 100 nm of the small protein lysozyme were imaged by 300 kV cryo-EM on a Falcon direct electron detector. The images were taken close to focus and to the eye appeared devoid of contrast. Fourier transforms of the images revealed the reciprocal lattice up to 3 Å in favourable cases and up to 4 Å for about half the crystals. The reciprocal lattice spots showed structure, indicating that the ordering of the crystals was not uniform. Data processing revealed details higher than 2 Å resolution and indicated, the presence of multiple mosaic blocks within the crystal which could separately be processed. We discuss the prospects of full 3D structure determination by electron imaging of protein 3D nano-crystals.

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4.1 Introduction

Three dimensional protein crystals that are smaller than about 1 μm are beyond the scope of the usual diffraction methods in Structural Biology. Since about 30% of proteins that crystallize, do not grow crystals of a sufficient size or quality for X-ray structure determination (1,2), this is a serious bottleneck. Structural information of important drug targets, like membrane proteins and large complexes, is often lacking due to their inability to grow sufficiently sized and ordered crystals. Current trends in X-ray crystallography focus on data collection of ever-smaller crystals. For example micro-focused X-ray beams and improved quantum area detectors, like the Pilatus, have decreased these limits (3,4). Especially free electron lasers may help in expanding the methods towards smaller crystals (5). We believe electron microscopy could have a big impact on the field of protein nano-crystallography since electrons are less damaging to protein crystals per diffracted quantum than X-rays by several orders of magnitude (6).

Since the development of cryo-electron microscopy and macromolecular reconstructions in the 1970's (7-9) there have been constant improvements in the maximum resolution that can be achieved with this method. Recent advances in 'single particle analysis' make the solution at atomic resolution of large molecular complexes imminent (10,11). Important recent improvements in single particle analysis have been automated sample handling (12,13), automated image processing (14-16) and direct electron detection (17,18). Automated data collection makes it possible to collect millions of images

and software can automatically average the signal and do angular reconstructions of the protein models. Beam damage can be minimized by using efficient direct electron detectors which significantly improve the signal to noise ratio at low electron dose conditions.

In material sciences, electron diffraction is a well-developed tool for structure determination of inorganic crystals where selected area electron diffraction from different zone axes can be used to determine three-dimensional unit cells. Convergent beam electron diffraction (19) can be used to obtain more information about the symmetry of the crystal. The development of precession electron diffraction (20,21), automated diffraction tomography (22,23) and rotation electron diffraction (24) have contributed to the toolbox of electron crystallographers. Applications for electron crystallography of small molecules are becoming robust, making 3D structure determination by electron diffraction of 3D nano-crystals a very attractive method.

Structure determination using electron diffraction of two-dimensional protein crystals has been used since the seminal work on bacteriorhodopsin in the 1970s (25). In 2005 the structure of 2D Aquaporin crystals was solved to a resolution of 1.9 Angstrom (26). However 3D nano-crystals of proteins have so far resisted structure determination. Main reasons are the beam sensitivity, the large unit cell and the thickness of the crystals. The latter factor contributes to increased multiple scattering and non-linear effects in electron diffraction and imaging. However multi-slice least square method (27) have tackled such problems in electron crystallography of small molecules. To increase resolution and refine structural data, electron

diffraction data can be combined with electron microscopy images, which contain phase information (25,28-30). Phases from these electron micrograms can also be used to solve structures with X-ray diffraction where phases are missing (31,32).

Here we report the electron imaging of three dimensional protein crystals. We have previously collected rotation electron diffraction data of similar lysozyme nano-crystals to a resolution of 1.8 Angstrom (33). We discuss preliminary image processing results and the possibility of integrating diffraction data with imaging data.

4.2 Materials & Methods

4.2.1 Crystallization

Crystallization experiments were carried out using the sitting drop vapour diffusion technique in Innovadyne SD-2 plates. Rockmaker software (Formulatrix) was used for designing the experiments. We used a Genesis (Tecan) for dispensing the screening solutions in the reservoirs. The ORYX 6 (Douglas Instruments) crystallization robot was used for transferring 500 nl reservoir solution and 500 nl protein solution in sitting drop wells. Plates were stored at 20 °C and imaged using the automated imaging system Rock Imager (Formulatrix). Lysozyme (8 mg/ml) formed needle crystals after 48h when mixed 1:1 with well solution containing 0.1 M Sodium Acetate pH 3.8 and 1.0 M Potassium Nitrate.

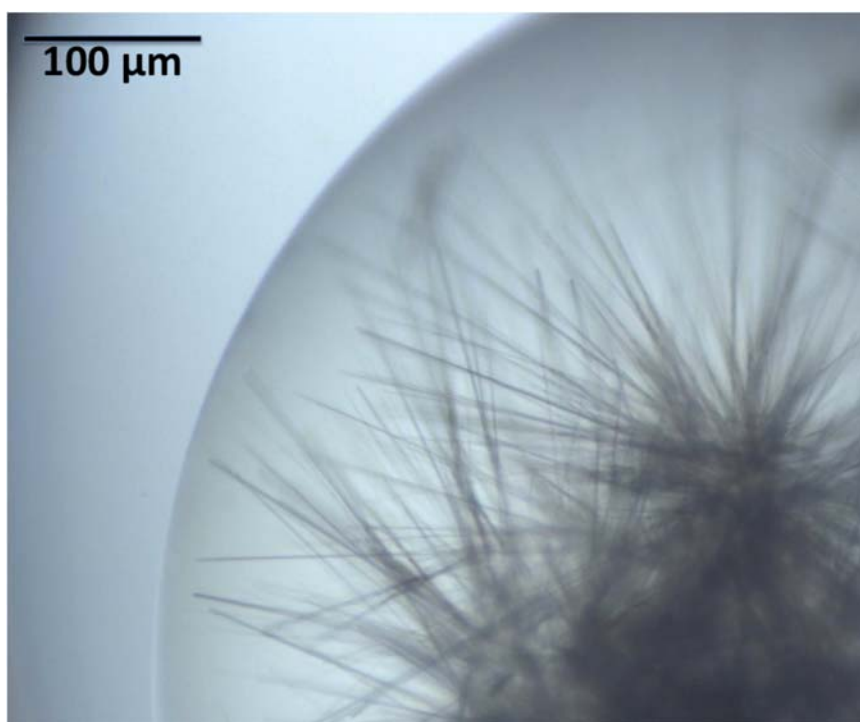


Figure 4.1 Lysozyme nano-crystals in crystallization drop imaged with a light microscope. The needle-shaped crystals that are visible under a light microscope are too thick for EM analysis because of absorption. Yet the crystallization drops also contain (much) smaller crystals with a thickness in the order of 100 nm, which could be imaged at high resolution by cryo-EM.

4.2.2 Vitrification

Protein crystals were vitrified using a Vitrobot (34,35). 3 μ l of well solution was mixed with the drop containing nano-crystals and transferred to a 3mm holey carbon grid (AGAR). Excess liquid was blotted away (blot time 3 sec blot force 5) and the sample was plunge frozen in liquid ethane. Samples transferred to the microscope immediately and loaded and kept at -180 degrees Celsius using an automated cryo-loader/ cryo-stage.

4.2.3 Electron Microscopy Data Collection

Electron images were taken on a Titan Krios (FEI) transmission electron microscope at NeCEN. The voltage was set to 300 keV and a Falcon (FEI) 4Kx4K direct electron detector was used at 0.822 and 1.055 Angstrom per pixel. The electron dose used was between 5 and 10 e⁻/Å.

4.3 Results

Image data of about 200 lysozyme 3D nano-crystals in random orientations and at tilt angles varying between -45 and +45 degrees, were collected. All images were collected close to Scherzer focus (which at 300 kV is ~70 nm) and were devoid of amplitude contrast (figure 2). However, Fourier transformation of the images indicated crystalline order (figure 3), with discernable Bragg spots going out to about 4 Ångstrom or better in about half of the crystals and in favourable cases even further (figure 4). Crystals that on average yielded the best resolution were about 100 nm thick, as determined by tomography (figure 5). We did not orient the crystals prior to high resolution imaging, so most of their Fourier transforms showed multiple Laue zones (figure 3). These multiple Laue zones appeared as 2D lattices of Bragg spots with three primitive spacings, the third spacing corresponding to the distance between lunes that are observed in diffraction patterns. This is different from 2D electron crystallography, as diffraction patterns of 2D protein crystals only have two primitive spacings. The presence of three primitive spacings predictably leads to moiré patterns in the real space image. The repeat of this complex pattern of fringes that is caused by the multiple Laue zones, is

determined by the smallest common denominator between the primitive spacings.

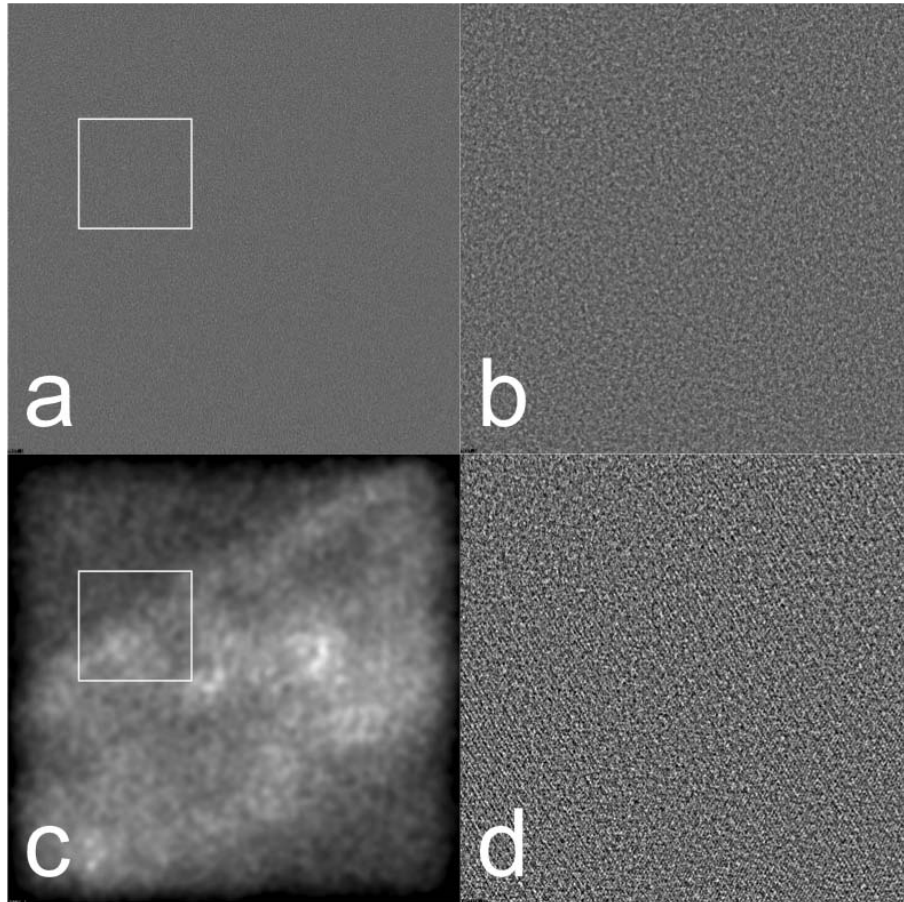


Figure 4.2 (a) An electron micrograph collected using a Titan cryo-EM on a Falcon camera (4096x4096 pixels, 0.9 Angstrom/pixel) of a lysozyme 3D nanocrystal (location of the zoomed-in figure 2b indicated) (b) Zoom in (1024x1024 pixels) of a. Although appearing just noise, the image shown here in fact does contain very significant high-resolution detail, as is revealed in its Fourier transform (figure 3). Calculating the local variance within a circular area with a diameter of 30 pixels reveals the shape of the crystal (c). A Wiener filter can be used to enhance the lattice contrast as is shown in panel 2d, which is equivalent to 2b after filtering.

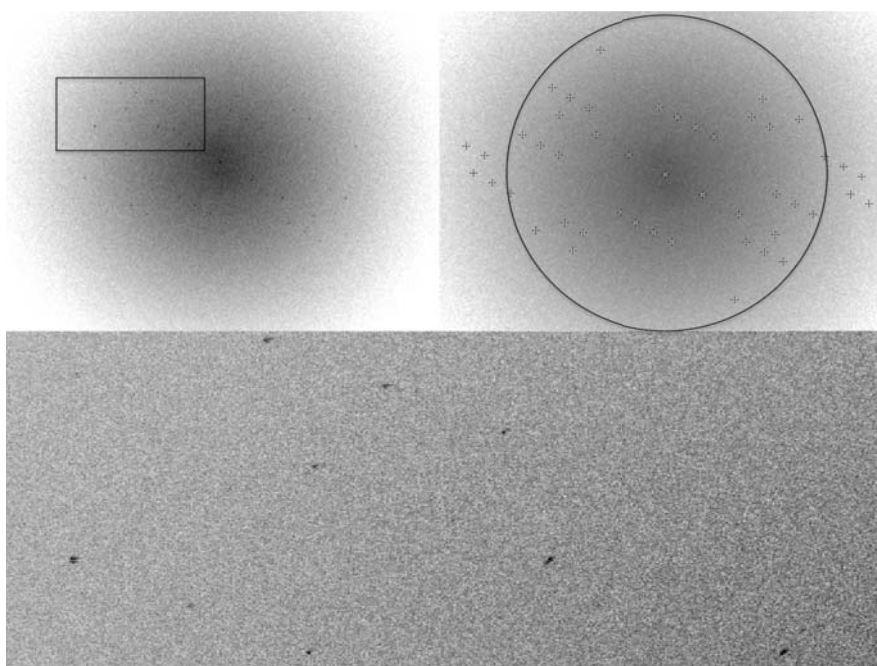


Figure 4.3 Top left: slightly enlarged Fourier transform of figure 2a. Top right: Fourier transform of figure 2, with peak positions indicated, showing a projection of a regular 3D lattice (ring represents 4.5 Å). Bottom: detail of top left, showing the structure of the Bragg spots.

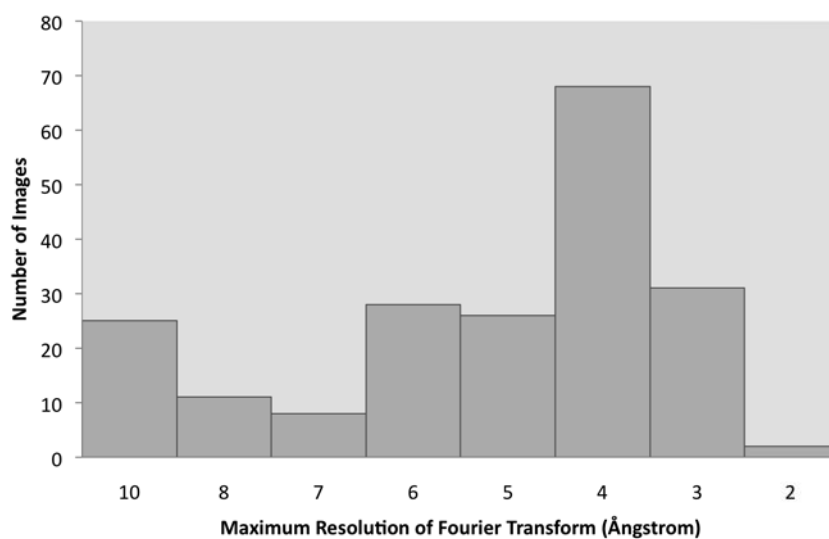


Figure 4.4 Histogram of the maximum resolution observed in electron images of 200 different lysozyme 3D nano-crystals.

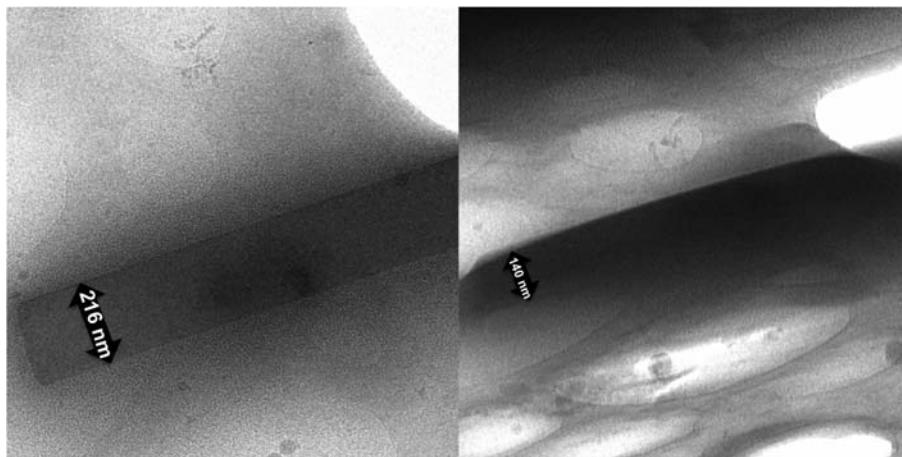


Figure 4.5 Two defocused ($-8.00\ \mu\text{m}$) frames from raw data of a tomographic series of one of the crystals that high-resolution data was collected on. Frames were taken at 0 Degrees (left) and at 44 Degrees (right), and shows the crystal was less than 150 nm thick.

4.3.1 Structure of the Bragg Spots

Detailed analysis revealed the Bragg peaks to be structured (figure 3). In diffraction studies, the shape (but not the intensity) of a Bragg peak is the product of the shape of the source and variation of the crystal spacing corresponding to the index of the Bragg peak within the diffracting crystal. As we did not measure the diffraction pattern, but instead calculated the Fourier transform of an EM image, the situation was slightly different. The shape of the source turned out to be irrelevant. The observed structure of the Bragg peaks therefore must, at least in part, be caused by the shape and internal ordering of the crystal. In some cases splitting of the diffraction spots was observed (e.g. the leftmost spot in figure 3 (bottom)), but splitting was by no means the norm. Like the other irregularities in the Bragg spots, splitting could be caused by non-uniform ordering of the crystal, but it

cannot be ruled out that Ewald sphere curvature was a contributing factor, as explained in the discussion and conclusion.

By Fourier transforming the images, we could not only calculate the intensity distribution within the Bragg spots, but also the phases of each reciprocal pixel. With ImageJ (36) both the intensity and the phase of each reciprocal pixel can be inspected in a number-field. Inspection showed that the phase correlation between adjacent reciprocal pixels was low within a single Bragg spot. This suggests that the observed spreading of the Bragg spots was probably caused by local differences in size and projected potential of the unit cells (resulting in subtle origin shifts of the unit cells with respect to the average lattice), rather than by in-plane rotations of unit cells relative to one another (which would have preserved the phase).

4.3.2 Centrosymmetry of the Fourier Transform

A detector placed in the diffraction plane of the microscope measures a diffraction pattern. Its intensities are those of the Fourier $F(\vec{h})$ of the plane electron wave function $\psi_e(\vec{x})$ that has passed through the sample:

$$F(\vec{h}) = \mathbf{F}^{-1}(\psi_e(\vec{x})) \quad [1]$$

This Fourier transform of the exit wave function $\psi_e(\vec{x})$ peaks at the Bragg positions determined by the periodicity of the crystal. In imaging mode, the lenses of the microscope recombine these structure factors through a Fourier transform into a real space defocused electron plane wave function $\psi_{\Delta f}(\vec{x})$. Distortions of this recombination are described by a centrosymmetric transfer function $\tau(\vec{h})$, which is determined by the wavelength of the electrons, aberrations of the electron lenses and

the defocus (the coherence of the illumination system, and the position of the detector relative to the focal imaging plane of the microscope):

$$\psi_{\Delta f}(\vec{x}) = \mathbf{F}\left(F(\vec{h})\tau(\vec{h})\right) \quad [2]$$

Note that $\psi_{\Delta f}(\vec{x})$ is a complex wave function. When $\psi_{\Delta f}(\vec{x})$ impinges on the detector, the wave function collapses and its intensities (which are calculated by multiplying $\psi_{\Delta f}(\vec{x})$ with its complex conjugate $\overline{\psi_{\Delta f}(\vec{x})}$) are measured as the electron image $M(\vec{x})$:

$$M(\vec{x}) = \psi_{\Delta f}(\vec{x})\overline{\psi_{\Delta f}(\vec{x})} \quad [3]$$

Note that $M(\vec{x})$ is a real function, so its Fourier transform $\mathbf{F}^{-1}(M(\vec{x}))$ must obey Hermitian symmetry: $\mathbf{F}^{-1}(M(\vec{x})) = \overline{\mathbf{F}^{-1}(M(-\vec{x}))}$. However, in the general case, $F(\vec{h})$ is not centrosymmetric. Hence, the Fourier transform of the measured image cannot be equivalent to $F(\vec{h})\tau(\vec{h})$. An example of a typical electron diffraction pattern of a non-oriented lysozyme 3D nanocrystal is shown in figure 6. The curvature of the Ewald sphere, in combination with the parallel electron beam, causes the Bragg spots to be limited to so-called lunes that are such a usual and prominent feature in X-ray diffraction patterns of non-oriented protein crystals. These lunes clearly break centrosymmetry.

Nevertheless, even though the crystallographic phases corrected by $\tau(\vec{h})$ are not necessarily always equivalent to the phases of $\mathbf{F}^{-1}(M(\vec{x}))$, it is possible (through iterative procedures) to retrieve crystallographic phase information from the inverse Fourier transform of $M(\vec{x})$. This is relatively straightforward if the weak phase object approximation holds. First, we have to realise that the Fourier transform of $M(\vec{x})$ is

equivalent to the autocorrelation of the Fourier transform of $\psi_{\Delta f}(\vec{x})$.

This follows from the convolution theorem:

$$\mathbf{F}^{-1}(M(\vec{x})) = \int \overline{F_{\Delta f}(\vec{h}_t)} F_{\Delta f}(\vec{h} + \vec{h}_t) \partial \vec{h}_t \quad [4]$$

The weak phase object approximation implies that most of the power of $F_{\Delta f}(\vec{h})$ is located at $\vec{h} = \vec{0}$. This implies that we can use the following approximation for weak phase objects:

$$\overline{F_{\Delta f}(\vec{h}_t)} F_{\Delta f}(\vec{h} + \vec{h}_t) \neq 0 \Rightarrow \vec{h}_t = \vec{0} \vee \vec{h} + \vec{h}_t = \vec{0} \quad [5]$$

So the product $F_{\Delta f}(\vec{h}_t) F_{\Delta f}(\vec{h} + \vec{h}_t)$ is only non-zero if either $\vec{h}_t = \vec{0}$ and/or if $\vec{h} + \vec{h}_t = \vec{0}$. With this restriction, the integral in lemma [4] can be simplified, as $\vec{h}_t$ can be substituted by $\vec{0}$ or $-\vec{h}$:

$$\mathbf{F}^{-1}(M(\vec{x})) = \int \overline{F_{\Delta f}(\vec{0})} F_{\Delta f}(\vec{h}) + \overline{F_{\Delta f}(-\vec{h})} F_{\Delta f}(\vec{0}) \partial \vec{h}_t \quad [6]$$

This reduces to:

$$\frac{\mathbf{F}^{-1}(M(\vec{x}))}{F_{\Delta f}(\vec{0})} = F_{\Delta f}(\vec{h}) + \overline{F_{\Delta f}(-\vec{h})} \quad [7]$$

In other words, provided that the weak phase object approximation holds, the reverse Fourier transform of the measured image is the same as the defocused set of structure factors of the crystal (in any orientation), added to the same set after a point inversion through the origin (which is equivalent to a rotation by π) followed by taking the complex complement.

The implications of this conjecture are:

- The reverse Fourier transform of the measured image has Friedel symmetry (which is another way of saying that the measured image is real).
- If the diffraction pattern would have recorded two Friedel mates simultaneously, the amplitude of these reflections in the inverse transform of the measured image is changed by the transfer function - it can become negative - but the phase is not affected.
- If the diffraction pattern would have recorded two Friedel mates simultaneously, but only partially (due to the presence of mosaic blocks, for instance), splitting can occur if the two reflections would not have been recorded on spot-on centrosymmetric locations.
- If the diffraction pattern would have recorded two reflections that are *not* Friedel mates, but that occur by chance on each others centrosymmetric location, the reverse transform of the image mixes the two by adding the one to the complex complement of the other, thus imposing Hermitian symmetry on the reverse transform.
- Because the imposition of centrosymmetry on the reverse transform, information on the handedness is lost.

Similar considerations, albeit in different formalisms, have been discussed in papers on the implications of the curvature of the Ewald sphere on single particle reconstructions (37) and in 2D crystallography (38). There are also parallels with single sideband approach in electron microscopy (39). The images of the crystals are essentially made up of

interference fringes between the unscattered electron beam and the diffracted beam that is in general single not accompanied by a Friedel mate. In other words, the images are all single sideband images. There is therefore no zero in the contrast transfer function (CTF) and the out-of-focus phase shift simply rotates the structure factor so that the correction to the phase due to defocus is a linear one proportional to defocus. As a result, it is perfectly reasonable to aim to record exactly in-focus images.

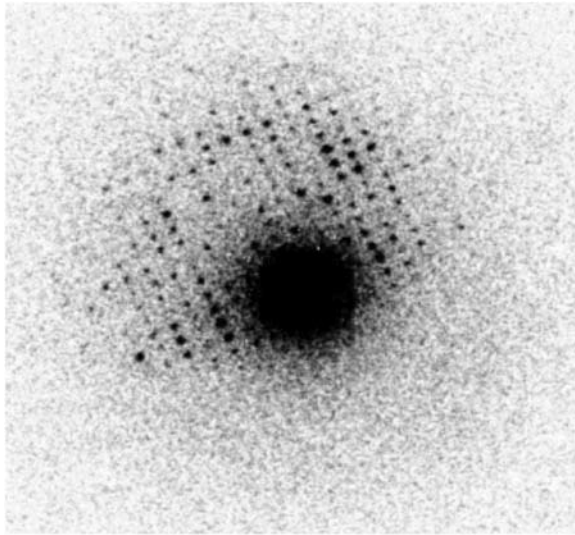


Figure 4.6 *Diffraction pattern showing lunes.*

4.3.3 Visualizing the lattice

The translational symmetry of an EM image of a (3D) nano-crystal can be enhanced by first Fourier transforming the image, then zeroing all reciprocal pixels that do not belong to the lattice and finally reversing the Fourier transform. This procedure has been applied with great success in 2D protein crystallography (e.g. 25). We can enhance this procedure by assuming that the signal of the crystal lattice is not correlated with the noise. This implies a phase difference of $\pi/2$

between the expected structure factors of the lattice on the one hand, and noise on the other. This implies⁴:

$$|F_m(\vec{h})|^2 = |\langle F_l(\vec{h}) \rangle|^2 + |\langle F_n(\vec{h}) \rangle|^2 \quad [8]$$

The phase of $F_m(\vec{h})$ is the best (and only) estimate of the phase of $F_l(\vec{h})$.

We therefore have to project $\langle F_l(\vec{h}) \rangle$ onto $F_m(\vec{h})$ to get the best estimate of structure factor corresponding to the lattice, denoted here by $F_{W,l}(\vec{h})$.

The right-angled geometry that imposed lemma [8] also implies:

$$F_{W,l}(\vec{h}) = F_m(\vec{h}) \frac{|\langle F_l(\vec{h}) \rangle|^2}{|F_m(\vec{h})|^2} \quad [9]$$

Substitution with [8] results in:

$$F_{W,l}(\vec{h}) = F_m(\vec{h}) \left(1 - \frac{|\langle F_n(\vec{h}) \rangle|^2}{|F_m(\vec{h})|^2} \right) \quad [10]$$

This is equivalent to an optimal (Wiener) filter, which requires calculating the ratio between an estimate of the power spectrum of the noise $|\langle F_n(\vec{h}) \rangle|^2$ and the smoothed power spectrum of the measured image $|F_m(\vec{h})|^2$ (40). We generated this optimal lattice filter by first smoothing the power spectrum of the image using a soft-edged circular window with a radius of 256 reciprocal pixels. We estimated the power spectrum of the noise to be the rotational average of this smoothed power spectrum. In order to prevent $F_m(\vec{h})$ to be multiplied by negative numbers in [10], we applied a threshold, limiting the range to of its scale factor to $[0;1]$. Application of [10] clearly revealed the lattice (figure

⁴ For each reciprocal pixel $\vec{h}$, we define $F_m(\vec{h})$ to be the measured structure factor and $|F_m(\vec{h})|$ is its amplitude; $|\langle F_l(\vec{h}) \rangle|$ is the amplitude of the expected structure factor of the lattice and $|\langle F_n(\vec{h}) \rangle|$ is the amplitude of the expected structure factor of the noise.

2c). The outline of the crystal now becomes apparent by calculating the local variance of the lattice-enhanced image (figure 2d).

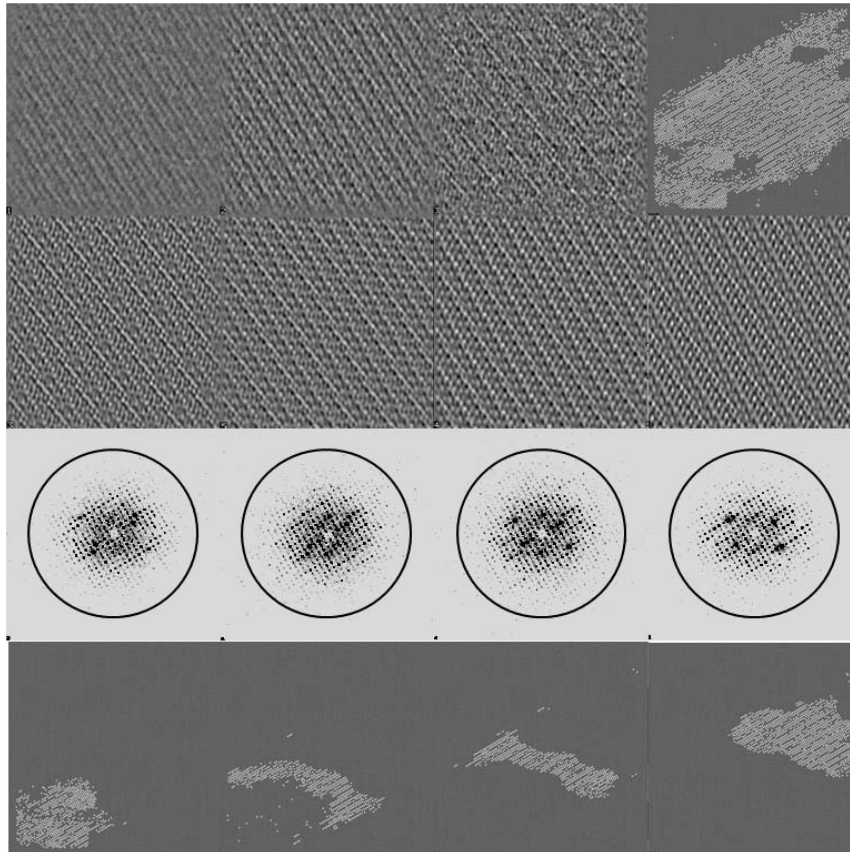


Figure 4.7 The top row of images shows the reconstructed projection classes of the electron image of the 3D nanocrystal of lysozyme in figure 2, using just 101 non-overlapping sub-images (patches) of 256x256 pixels, randomly selected from the Wiener-filtered image shown in fig. 2. Using these three initial projection classes, we selected high-correlation patches, their locations are shown top-right. These patches were classified into 5 classes, four of which are shown in the second panel from the top. The patches that contributed to each of these classes are located at positions plotted in the bottom row. Patches were cut out of the original, non-filtered image (fig. 2a) at exactly the same locations, and were assigned their corresponding shift – and class parameters. The second panel from the bottom shows the amplitudes of the Fourier transforms of their class averages at low contour level and suggest a resolution beyond 2 Å (the resolution of the circle is 2.1 Å and there are lattice spots well beyond this circle).

Subsequent image processing with IMAGIC (41) enhanced the resolution. We randomly picked 101 patches of 256x256 pixels from the lattice enhanced image, aligned them and classified them into three classes using eigenvector analysis (figure 7, top panel). We used these classes to identify 4500 equivalent patches in the lattice-enhanced image by correlation search. The locations with high correlations were in agreement with the contour of the nano-crystal (compare figure 2d with figure 7, top panel right).

Using IMAGIC, we classified the selected patches into five different classes of on average 900 patches. Four of the class averages are shown in figure 8, second panel from the top. A fifth class that had less pronounced contrast and was located at the edges of the crystals is not shown.

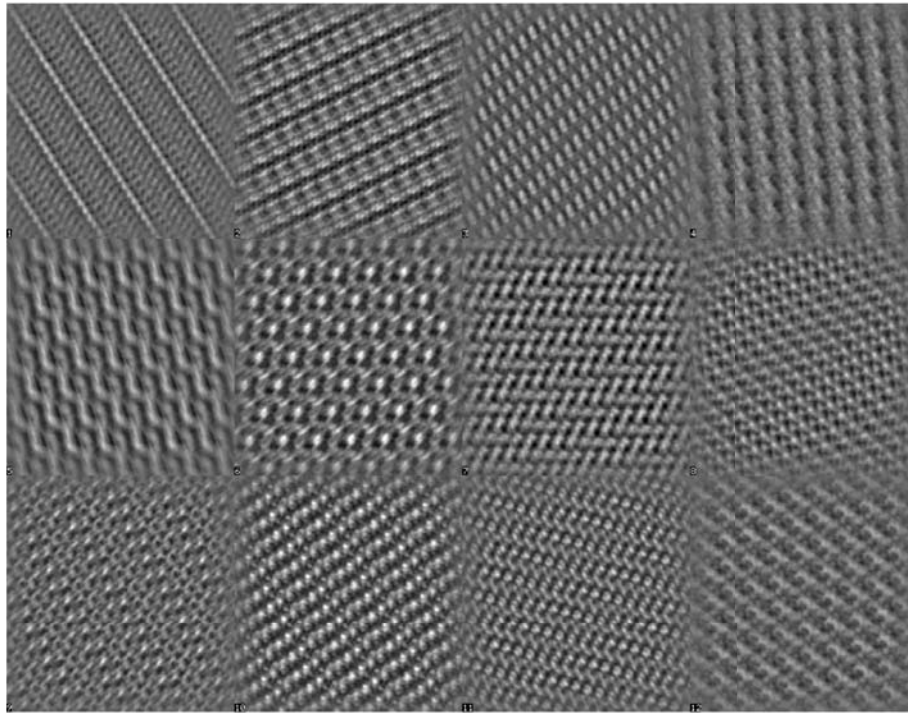


Figure 4.8 A dozen of the more than 200 high-resolution projection images (resolution better than 2.5 Å judging from their Fourier transforms) of different lysozyme 3D nano-crystals, all in different orientations. The data were collected as in figure 2, and processed as described in the text.

The resolution of the patch averages is illustrated using their Fourier transforms (figure 7, second panel from the bottom). At lower contour levels, spots can be seen extending almost to the Nyquist frequency (1.64 Å). The classes differ both in real space and in reciprocal space. In reciprocal space, Bragg spots prominent in one amplitude spectrum are hardly visible in other amplitude spectrum and *vice versa*. These differences in reciprocal space are not resolution dependent. Notice furthermore that the differences between classes 1 and 2 and between classes 3 and 4 are subtle, but that the differences between classes 1 and 2 and 3 and 4 are more pronounced.

We analysed all crystals of which we collected images that showed Bragg spots beyond 4 Å. All resulted in high resolution, high contrast classes, some of which are shown in figure 8.

4.4 Discussion & Conclusion

We have shown that 3D protein nanocrystals that are too small for conventional (synchrotron) X-ray analysis can robustly be imaged at high resolution with electrons. Fourier transforms of these images reveal the presence of moiré lattices, caused by the interference between the lattices of the zero-order Laue zone with higher order Laue zones, indicating that the crystals are not aligned with principal axes parallel to the electron beam. Furthermore, the Fourier transforms have Hermitian symmetry, which has some repercussions for extracting crystallographic phase information. As long as the weak phase object approximation is valid (which is the case when most electrons scatter not more than once within the sample), extracting phase information from the Fourier transform of the measured images is relatively straightforward.

The lattice can be enhanced by Wiener filtering. Knowledge of the lattice parameters is not required for this procedure. Hence the outcome is not biased by any imposed lattice parameters, which is an advantage.

With software designed for single particle cryo-EM data analysis, the contrast and resolution of the projection images can be dramatically improved by identifying and averaging within coherent domains of the crystals. To a similar aim, images of 2D crystals have been analysed in the past with multivariate statistical analysis (MSA) in order to analyse

differences between adjacent unit cells in the 2D lattice (42-44). However, here we analyse 3D nanocrystals, which have some distinguishing differences compared to 2D crystals. Since the third cell axis of the crystals is usually not aligned with the electron beam, the projection images usually show moiré patterns (see figure 2), which in Fourier transform manifest themselves as multiple reciprocal 2D lattices (figures 3 and 7). Please note that the moiré unit repeat in the electron projection is larger than a 3D single unit cell.

When crystals are also ordered in the third dimension they are less likely to be affected by warping and bending, or by in-plane rotations of the unit cell, because of increased crystal contacts. This will increase the probability of achieving high-resolution. The increased thickness of the crystals will cause more pronounced dynamical scattering and a different defocus at top and bottom of the crystal.

Detailed analysis revealed that within a crystal, we could observe multiple views of projected density, correlating with the location within the image (cf. figure 7). Potential causes are:

- the presence of mosaic blocks within the crystal, each with a slightly different orientation of the unit cells resulting different Bragg spots to be sliced by the Ewald sphere;
- the crystal was not normal to the electron beam, resulting in a different focus for different parts of the crystal;
- the crystal is not uniformly thick, resulting in a higher degree of dynamical scattering in the thicker parts of the crystal, compared to the thinner parts.

Differential defocus is a less likely explanation of the observed differences, as this reduces the intensity of Bragg spots in the reverse Fourier transforms uniformly within a resolution shell (provided the microscope is stigmatically aligned). Figure 7 does not suggest this to be the case. Differences in thickness of the crystal would result in a (subtle) density gradient of the image due to increased electron absorption by thicker parts of the crystal. We did not observe such gradients. Furthermore, increased dynamical scattering can only cause the intensities of spots to become more alike, and we observe some spots that are present in one reverse transform to be absent in the other, and *vice versa*, which cannot be explained by dynamical scattering effects. Hence, for the moment, we consider the presence of mosaic blocks the most likely explanation of the observed differences. Macromolecular X-ray crystallography explicitly assumes and models the presence of such mosaic blocks within crystals; here we could be seeing them directly for the first time.

Essential steps towards real space 3D nano-crystallography have been taken. Next, we aim to automate data analysis, so that projection views of many different orientations can straightforwardly be determined. We have made progress in this respect, as is shown in figure 7. We also aim to apply 3D reconstruction techniques that are well known in the single particle cryo-EM community, to the reconstruction of the 3D crystal structure, initially at low resolution. However, at the resolution that we can now achieve (figure 7), we will also have to take aspects into account that single particle analysis so far could ignore: dynamical scattering and focal differences between top and bottom of the sample (equivalent to considering Ewald sphere curvature).

In subsequent refinement procedures, aiming to achieve high (atomic) resolution, these aspects will also have to be modelled. We assume that the knowledge that we obtain in this venture will assist the field of single particle analysis for analysing structures of non-crystalline materials to resolutions where these aspects can no longer be ignored.

References

- (1) Rupp B. (2004) "A Guide to Automation and Data Handling in Protein Crystallisation", in: "Protein crystallisation Strategies for Structural Genomics" (Chayen, N. ed.), ISBN 0-9720774-3-X, p 9-56.
- (2) Quevillon-Cheruel, S., Liger, D., Leulliot, N., Graille, M., Poupon, A., De la Sierra-Gallay, I. s. L., Zhou, C.-Z., Collinet, B., Janin, J. I. & Van Tilbeurgh, H. (2004). *Biochimie* **86**, 617-623.
- (3) Cusack, S., Belrhali, H., Bram, A., Burghammer, M., Perrakis, A. & Riek, C. (1998) *Nat Struct Mol Biol.* **5**, 634 – 637.
- (4) Eikenberry, E. F., Brönnimann, C., Hülsen, G., Toyokawa, H., Horisberger, R., Schmitt, B., Schulze-Bries, C. & Tomizaki, T. (2003). Nuclear Instruments and Methods in Physics Research Section A: Accelerators, Spectrometers, Detectors and Associated Equipment **501**, 260-266.
- (5) Chapman, H. N., Fromme, P., Barty, A., White, T. A., Kirian, R. A., Aquila, A., Hunter, M. S., Schulz, J., DePonte, D. P., Weierstall, U., Doak, R. B., Maia, F. R. N. C., Martin, A. V., Schlichting, I., Lomb, L., Coppola, N., Shoeman, R. L., Epp, S. W., Hartmann, R., Rolles, D., Rudenko, A., Foucar, L., Kimmel, N., Weidenspointner, G., Holl, P., Liang, M., Barthelmess, M., Caleman, C., Boutet, S., Bogan, M. J., Krzywinski, J., Bostedt, C., Bajt, S., Gumprecht, L., Rudek, B., Erk, B., Schmidt, C., Homke, A., Reich, C., Pietschner, D., Struder, L., Hauser, G., Gorke, H., Ullrich, J., Herrmann, S., Schaller, G., Schopper, F., Soltau, H., Kuhn, K.-U., Messerschmidt, M., Bozek, J. D., Hau-Riege, S. P., Frank, M., Hampton, C. Y., Sierra, R. G., Starodub, D., Williams, G. J., Hajdu, J., Timneanu, N., Seibert, M. M., Andreasson, J., Rocker, A., Jonsson, O., Svenda, M., Stern, S., Nass, K., Andritschke, R., Schrotter, C.-D., Krasniqi, F., Bott, M., Schmidt, K. E., Wang, X., Grotjohann, I., Holton, J. M., Barends, T. R. M., Neutze, R., Marchesini, S., Fromme, R., Schorb, S., Rupp, D., Adolph, M., Gorkhovev, T., Andersson, I., Hirsemann, H., Potdevin, G., Graafsma, H., Nilsson, B. & Spence, J. C. H. (2011). *Nature* **470**, 73-77.
- (6) Henderson, R. (1995). *Q Rev Biophys* **28**, 171-193.
- (7) Taylor, K. A. & Glaeser, R. M. (1974). *Science* **186**, 1036-1037.
- (8) Klug, A. (1979) "Image analysis and reconstruction in the electron microscopy of biological macromolecules." *Chemica Scripta* **14**:245-256.
- (9) Knapik, E. & Dubochet, J. (1980). *J. Mol. Biol.* **141**, 147-161.

- (10) van Heel, M., Gowen, B., Matadeen, R., Orlova, E. V., Finn, R., Pape, T., Cohen, D., Stark, H., Schmidt, R., Schatz, M. & Patwardhan, A. (2000). *Q Rev Biophys* **33**, 307 - 369.
- (11) Zhou, Z. H. (2008). *Current Opinion in Structural Biology* **18**, 218-228.
- (12) FEI EPU press release, <http://investor.fei.com/releasedetail.cfm?ReleaseID=495245>
- (13) Carragher, B., Kisseberth, N., Kriegman, D., Milligan, R. A., Potter, C. S., Pulokas, J. & Reilein, A. (2000). *Journal of Structural Biology* **132**, 33-45.
- (14) van Heel, M., Harauz, G., Orlova, E. V., Schmidt, R. & Schatz, M. (1996). *Journal of Structural Biology* **116**, 17-24.
- (15) Van Heel, M.; Portugal, R. Rohou, A., Linnemayr, C., Bebeacua, C., Schmidt, R., Grant, T., and Schatz, M. (2010). "Four-Dimensional Cryo Electron Microscopy at Quasi Atomic Resolution: "IMAGIC 4D"". *International Tables for Crystallography, Vol. F*.
- (16) Tang, G., Peng, L., Baldwin, P. R., Mann, D. S., Jiang, W., Rees, I. & Ludtke, S. J. (2007). *Journal of Structural Biology* **157**, 38-46.
- (17) FEI Falcon press release, <http://investor.fei.com/releasedetail.cfm?ReleaseID=399045>
- (18) Llopart Cudié, X., Campbell, M., Dinapoli, R., San Segundo, D. & Pernigotti, E. (2002). *IEEE Transactions on Nuclear Science (ISSN 0018-9499)(EISSN 1558-1578)* **49**, 2279-2283.
- (19) Spence, J. C. H. & Zou, J. M. (1992). *Electron microdiffraction*. New York: Plenum.
- (20) Vincent, R. & Midgley, P. A. (1994). *Ultramicroscopy* **53**, 271-282.
- (21) Oleynikov, P., Hovmöller, S. & Zou, X. D. (2007). *Ultramicroscopy* **107**, 523-533.
- (22) Kolb, U., Gorelik, T., Kübel, C., Otten, M. T. & Hubert, D. (2007). *Ultramicroscopy* **107**, 507-513.
- (23) Mugnaioli, E., Gorelik, T. & Kolb, U. (2009). *Ultramicroscopy* **109**, 758-765.
- (24) Zhang, D., Oleynikov, P., Hovmöller, S. & Zou, X. (2010). *Zeitschrift für Kristallographie* **225**, 94-102.
- (25) Henderson, R., Unwin P. N. (1975). *Nature* **257**, 28.
- (26) Gonen, T., Cheng, Y., Sliz, P., Hiroaki, Y., Fujiyoshi, Y., Harrison, S. C. & Walz, T. (2005). *Nature* **438**, 633-638.
- (27) Jansen, J., Tang, D., Zandbergen, H. W. & Schenk, H. (1998). *Acta Crystallographica Section A* **54**, 91-101.
- (28) Weirich, T. E., Zou, X., Ramlau, R., Simon, A., Cascarano, G. L., Giacomazzo, C. & Hovmöller, S. (2000). *Acta Crystallographica Section A* **56**, 29-35.
- (29) Tsuda, K. & Tanaka, M. (1995). *Acta Crystallographica Section A* **51**, 7-19.
- (30) Zuo, J. M. & Spence, J. C. H. (1991). *Ultramicroscopy* **35**, 185-196.
- (31) Dodson, E. (2001). *Acta Crystallographica Section D* **57**, 1405-1409.
- (32) Navaza, J. (2008). *Acta Crystallographica Section D* **64**, 70-75.
- (33) Nederlof, I., Van Genderen, E., Li, Y. W., Abrahams, J.P. (2012) (submitted)
- (34) FEI Vitrobot press release, <http://investor.fei.com/releasedetail.cfm?ReleaseID=255249>

- (35) Frederik, P. M. & Hubert, D. H. W. (2005). Vol. Volume 391, *Methods in Enzymology*, edited by D. Nejat, pp. 431-448. Academic Press.
- (36) Abramoff, M. D., Magelhaes, P. J. & Ram, S. J. (2004). *Biophotonics Int* **11**, 36-42.
- (37) Wolf, M., DeRosier, D. J. & Grigorieff, N. (2006). *Ultramicroscopy* 106, 376-382.
- (38) Philippsen, A., Engel, H.-A. & Engel, A. (2007). *Ultramicroscopy* 107, 202-212.
- (39) Hohenstein, M. (1992). *Applied Physics A: Materials Science & Processing* 54, 485-492.
- (40) Press, W. H., Teukolsky, S. A., Vetterling, W. T. & Flannery, B. P. (2007). *Numerical Recipes 3rd Edition: The Art of Scientific Computing*. Cambridge University Press.
- (41) van Heel, Marin, Rodrigo Portugal, and Michael Schatz. "Multivariate statistical analysis in single particle (cryo) electron microscopy." *Handbook on DVD 3D-EM in Life Sciences* (2009).
- (42) Sherman, M. B., Soejima, T., Chiu, W. & van Heel, M. (1998). *Ultramicroscopy* **74**, 179-199.
- (43) Koeck, P. J., Purhonen, P., Alvang, R., Grundberg, B. & Hebert, H. (2007). *J. Struct. Biol.* **160**, 344-352.
- (44) Zeng, X., Stahlberg, H. & Grigorieff, N. (2007). *J. Struct. Biol.* **160**, 362-374.

Chapter 5

The structural effect of mutating Tyr108 on the Active Site conformation of the Small Laccase from *Streptomyces coelicolor*⁵

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Abstract

Close to the active site Cu's of the Small Laccase protein, a Tyr is found, which is likely involved in the enzyme mechanism. The Tyr was mutated (to Phe and to Ala) and the effect of these mutations on the conformation of the active site was shown by X-ray crystallography to be negligible. Comparison of the enzymatic activities of these mutants suggests that, contrary to the more common three-domain MCOs, the tyrosine participated in the enzyme mechanism by providing an electron during oxygen reduction, giving rise to the temporary appearance of a tyrosyl radical. The relatively low $k_{\text{cat}}/K_{\text{M}}$ of SLAC and the involvement of Y108 in the enzyme mechanism may reflect an adaptation to a milieu in which there is an imbalance between the available reducing and oxidizing co-substrates. The purported

⁵ In this chapter I present my contribution to the following paper:

Involvement of Tyr108 in the Enzyme Mechanism of the Small Laccase from *Streptomyces coelicolor*

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evolutionary relationship between the two-domain MCO's and human ceruloplasmin appears to extend not only to the 3D structure and the mode of binding of the Cu's in the trinuclear center, as noted before, but also to the enzyme mechanism. The enzyme mechanism of the multicopper oxidase (MCO) SLAC from *Streptomyces coelicolor* was investigated by structural (XRD) and compared to spectroscopic (optical,EPR), and kinetics (stopped-flow) experiments on variants in which residue Tyr108 had been replaced by Phe or Ala through site-directed mutagenesis.

5.1 Introduction

Multicopper oxidases (MCOs) catalyze the oxidation of a wide variety of substrates while reducing molecular oxygen to water. To achieve this, they utilize four copper atoms: a type 1 (T1) Cu which accepts reducing equivalents from the substrates and a trinuclear Cu cluster (TNC) where oxygen binds and gets converted to water. The TNC consists of a normal or type 2 (T2) Cu and a binuclear type 3 (T3) Cu pair. The mechanism of O₂ reduction at the TNC has been extensively studied by a variety of techniques (1). It has been proposed that O₂ first binds at the TNC and then gets reduced in two 2e⁻ steps (2). The conserved residues between T1 Cu and TNC form a covalent link and promote rapid electron transfer from T1 Cu to the TNC (3). However, views about O₂ binding and the mechanism of reduction have changed over time and still remain a topic of debate (4,5). Analysis of the genome of *Streptomyces coelicolor* revealed the presence of a gene possibly encoding an MCO (6), "small laccase" (SLAC), which owes its name to its smaller molecular weight as compared to the other well-known MCOs such as ascorbate oxidase,

laccase, Fet3p, and CueO. SLAC was found to be active as a homotrimer, unlike most other MCOs described until now, which are monomeric proteins in solution (7–10). It has been suggested that the three-domain ascorbate oxidase, the three-domain laccases, and the six-domain ceruloplasmins have evolved via formation of a trimer of two-domain cupredoxins (11). The recent crystal structure of SLAC clearly shows that the enzyme has such a trimeric form with a canonical TNC (12,13). This structure, together with structures of other two-domain MCOs, has been used in an attempt to fill in the gaps in the proposed evolution of MCOs (12,14,15). Thus, it was of interest to study SLAC not only from a fundamental point of view to understand the structure–function relationship of this new enzyme but also to seek footprints of the proposed ancestor that may have been carried over or discarded by the generations that followed after. Apart from that, SLAC holds potential for its applications in industry and its use as a cathode in biofuel cells to cater for the demands of green energy (16–17). SLAC was found to be excreted in the growth media of *S. coelicolor* cultures and, thus, was identified as an extracellular enzyme. The physiological roles of most extracellular enzymes, including SLAC, are unclear, and researchers have mixed views (18), but it is well known that members of the *Streptomyces* genus produce dozens of antibiotics as secondary metabolites using such secreted enzymes (19). For ease of expression and purification in higher yields, the gene encoding SLAC was isolated and recombinantly expressed in *E. coli* (6). Following the preliminary characterization, it was recently reported that reduced, type 1 depleted (T1D) SLAC, upon reaction with oxygen, forms an unusual biradical intermediate which has not been reported for the more common laccases (20). Spectroscopic signatures, when compared with those of

other enzymes and model systems, led to the hypothesis that a ferromagnetically coupled triplet state arises in SLAC due to exchange coupling of two unpaired spins, one residing on T2 Cu and the other on a tyrosyl radical ~ 5 Å away, possibly Tyr108. Similar spectroscopic features were also observed during turnover of the native enzyme, and a role of the radical in catalytic turnover was implicated (see also ref 21).

In order to examine the role of Tyr 108 by site directed mutagenesis, we had to confirm that mutating this residue did not affect the active site other than removing a potential location for the radical that forms during the reaction. For this purpose, we crystallized the SLAC mutants Y108F and Y108A, and determined their structure.

5.2 Materials and methods

5.2.1 Site-directed Mutagenesis

Site-directed mutagenesis was carried out to prepare SLAC variants in which the tyrosine is replaced by phenylalanine (Y108F) or alanine (Y108A), and was carried out using the Quick Change site-directed mutagenesis kit (Stratagene). The primers used for respective mutations are given below where mutations are in bold and underlined.

Y108F

Forward primer: 5'—G CAC GGC CTG GAC **TTC** GAG ATC TCC AGC
G—3'

Reverse primer: 5'—C GCT GGA GAT CTC **GAA** GTC CAG GCC GTG
C—3'

Y108A

Forward primer: 5'—G CAC GGC CTG GAC **GCC** GAG ATC TCC AGC
G—3'

Reverse primer: 5'—C GCT GGA GAT CTC **GGC** GTC CAG GCC GTG
C—3'

Desired mutations were confirmed by DNA sequencing (BaseClear).

The purification of the wildtype (wt) SLAC and the mutants was carried out as reported previously (6). The proteins were aliquoted and stored at -80 °C till further use. The absorption spectra of the wt SLAC, Y108A and Y108F mutants are shown in figure 1.

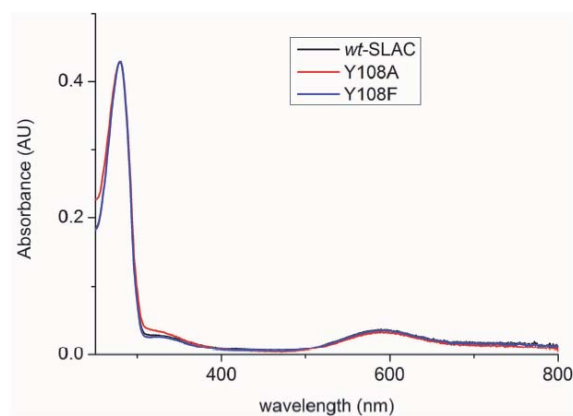


Figure 5.1. Absorption spectrum of the wt-SLAC overlaid with that of Y108A and Y108F. The spectra have been normalized at the 280nm absorption.

5.2.2 Crystallization and Data Collection

Crystallization experiments were carried out using the sitting drop vapor diffusion technique in SD-2 plates (Innovadyne). Rockmaker software (Formulatrix) was used for designing the experiments and tracking crystal growth. A Genesis (Tecan) was used for dispensing the screening solutions in the reservoirs. 200 nl of reservoir solution and 200 nl protein solution in sitting drop wells were dispensed by an ORYX 6 (Douglas Instruments) crystallization robot. Crystallization experiments with SLAC were setup around conditions previously published (12). Plates were stored at 20 °C and imaged using the automated imaging system Rock Imager (Formulatrix). Chemicals used for experiments were purchased from Sigma. Crystals of the SLAC mutants grew after 3 days in 35-45 % polyethylene glycol-550-monomethyl ether, 0.15-0.5 M NaCl and 0.1 M TRIS pH 8.5. Crystals were transferred to cryo-protectant containing 80 % mother liquor and 20 % glycerol, and frozen in liquid nitrogen. Single-crystal diffraction data were collected at the ESRF on beam line ID 14-4 (Grenoble) using an ADSC Q315 X-ray detector at a temperature of 100 K.

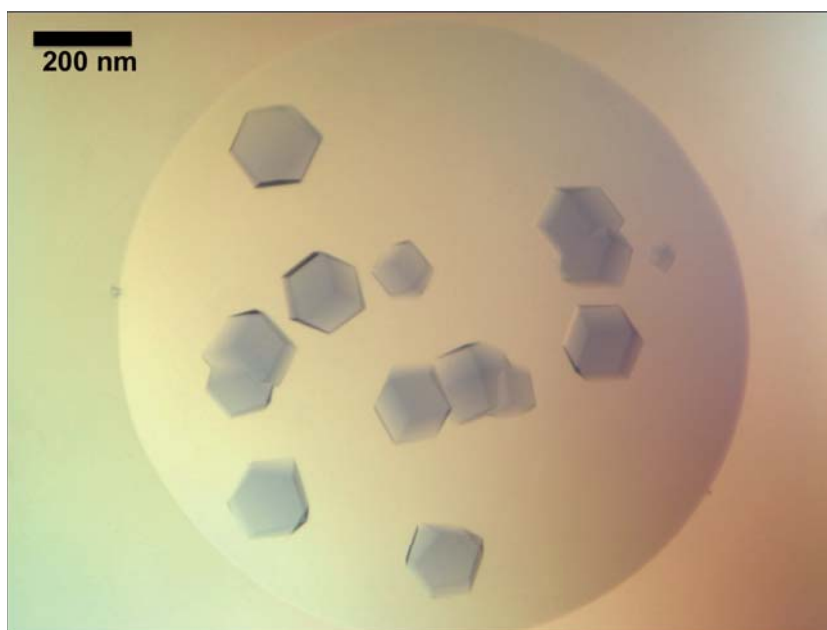


Figure 5.2 Crystals of SLAC mutant Y108A.

	SLAC Y108F	SLAC Y108A
Space group	$P4_32_12$	$P4_32_12$
Unit cell parameters $\text{\AA}$	$a = b = 176.66$ $c = 176.85$	$a = b = 178.44$ $c = 177.77$
Resolution range $\text{\AA}$	53.27 – 2.73 (2.88 – 2.73)	79.80 – 2.67 (2.81 – 2.67)
No. of observations	370849 (46537)	1047174 (131331)
No. of unique reflections	59361 (8756)	82018 (11803)
Data completeness	80.5 (82.3)	100.0 (100.0)
Redundancy	6.2 (5.3)	12.8 (11.1)
Mosaicity ($^\circ$)	0.45	0.65
Mean $I/\sigma(I)$	12.9 (3.5)	5.7 (0.9)
R_{pim}	0.043 (0.202)	0.099 (0.768)

Table 5.1 Data collection and processing statistics parameters for the SLAC variants studied (values in parenthesis correspond from highest resolution shell).

5.2.3 Data Analysis

Diffraction data were indexed and integrated using MOSFLM (22) and imported into the CCP4 software package (23). SCALA (24) was used for scaling the data. Crystals of SLAC variants belonged to the $P4_32_12$ space group and the cell dimensions are mentioned in table 1. Molrep (25) was run for molecular replacement using 3CG8 (12) as a homologous structure. 10 rounds of REFMAC (26) were run to refine the structure. Terminal residues, mutations and water molecules were built using Coot (27) and another 10 refinement runs were done using REFMAC. The final structure parameters are summarized in Table 2.

	SLAC Y108F	SLAC Y108A
R_{work}	0.18204	0.21452
R_{free}	0.19858	0.22710
R_{all}	0.18238	0.21515
Mean B -factor ($\text{\AA}^2$)	36.507	49.31
RMSD bond lengths from ideal ($\text{\AA}$)	0.010	0.020
RMSD bond angles from ideal ($^\circ$)	1.590	1.925
No. of non hydrogen atoms	6888	6694
No. of monomers in asymmetric units	3	3
No. of water molecules	280	232
Other localized molecules	12 x Cu, 3 O, 8 PEGs	12 x Cu, 3 O, 8 PEGs
Solvent content (%)	84	84
Matthews coefficient ($\text{\AA}^3\text{DA}^{-1}$)	7.52	7.78

Table 5.2 *SLAC variants structure parameters.*

5.2 Results

Site-directed mutagenesis was carried out to prepare SLAC variants in which the tyrosine is replaced by phenylalanine (Y108F) or alanine (Y108A) in both the wt and the T1D (C288S) sequences. All variants containing mutations in the wt sequence at position 108 were crystallized and analyzed by X-ray diffraction to a resolution of 2.7–2.8 Å. The diffraction data confirm single amino acid replacements at the desired position as well as intact active sites. No significant changes in the overall fold of the enzyme or near the active sites were observed (figure 3). This facilitates a direct comparison of the enzyme kinetics and spectroscopic features of the mutants to those of the wt SLAC.

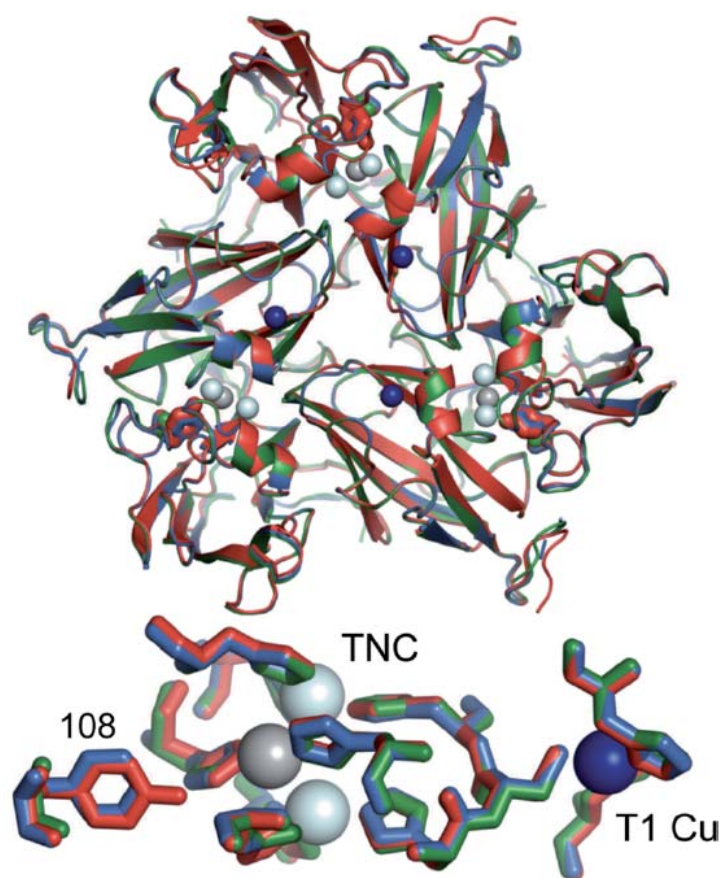


Figure 5.3 Ribbon representations of wt SLAC (red, PDB: 3CG8) overlaid with those of the mutants Y108F (blue, PDB: 4GXF) and Y108A (green, PDB: 4GY4). The bottom shows an expanded view of residues near the T1 Cu and TNC. The T3 Cu's are shown in light blue and the T2 Cu in gray. Clearly the overall fold and active sites are intact in the mutants.

Discussion and Conclusion

Mutation of Tyr 108 in SLAC to either Phe or Ala does not affect the other residues of the active site. This allows interpreting activity assays and spectroscopic analysis of these mutants in mechanistic terms. Below the implications of these additional experiments (described in

Gupta *et al*, 2012 (42)) are summarized.

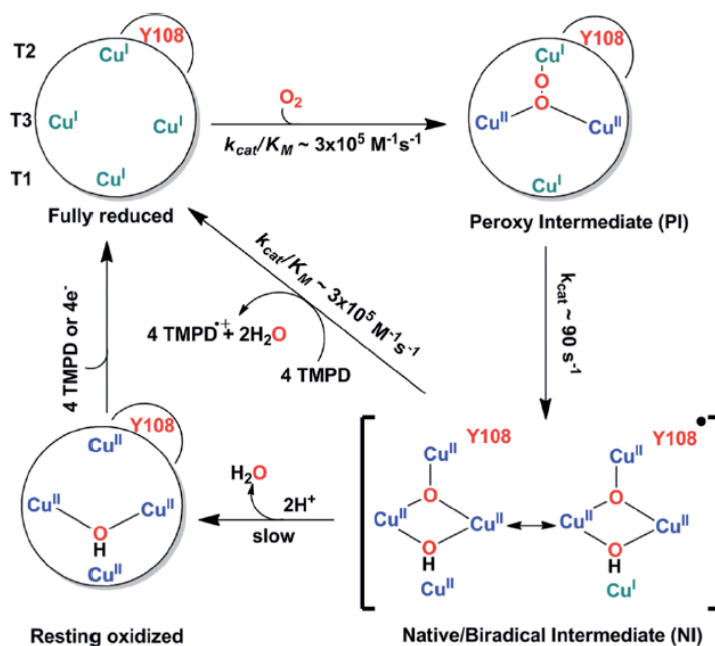
Kinetics

	$^{app}k_{cat}$ (s^{-1})		$^{app}k_{cat}/K_M$ ($\times 10^5 M^{-1} s^{-1}$)	
	O_2^b	TMPD ^c	O_2^b	TMPD ^c
wt SLAC	90 ± 3	315 ± 9	2.8 ± 0.2	3.2 ± 0.4
Y108A	34 ± 1	128 ± 3	2.7 ± 0.2	3.8 ± 0.4
Y108F	35 ± 1	132 ± 4	2.6 ± 0.2	2.4 ± 0.3

Table 5.3 Turnover Number (k_{cat}) and Second-Order Rate Constant (k_{cat}/K_M) of Wild Type and Mutant SLAC at 295 K in Phosphate Buffer at pH6.

It is evident from the data in Table 3 that the mutation affects only the turnover number (k_{cat}) of the enzyme and not the second-order rate constants (k_{cat}/K_M). While one would expect such a result for TMPD, as the mutations are far away from the T1 Cu reaction site for the TMPD (figure 3), it is not immediately obvious why the second-order rate constant for O_2 remains unaffected. After all, the mutations are close to the O_2 reaction site (i.e., the TNC). For any given enzyme, k_{cat}/K_M encompasses the steps from substrate binding up to and including the first irreversible step, whereas k_{cat} signifies the steps related to turnover of the ES complex and/or product release (28). From single-turnover experiments on SLAC, it is found that O_2 binding to the TNC is practically irreversible. This is in agreement with the enzymatic mechanism proposed for laccases, where the binding of O_2 followed by its reduction to the peroxide intermediate (PI) at the TNC was found to be irreversible (29). We conclude that the rate-limiting step(s) must occur after the binding of O_2 and reduction to PI and may involve the decay of PI to the native intermediate (NI) (30). TMPD or another co-substrate may then reduce the NI, thereby completing the reaction cycle and regenerating fully reduced SLAC ready to bind and reduce oxygen

(Scheme 1). Since k_{cat} is affected by the mutations at position 108, the rate-limiting step(s) must involve Y108 in the case of wt SLAC. Structural studies proved that no other alterations were present to the proteins active site or overall 3 tertiary structure.



Scheme 5.1 Proposed Pathway in the Reaction Mechanism of SLAC with a Role for Y108. Reduced copper sites are depicted in light green and oxidized ones in blue. Tyrosine is shown in red. The rate constants are those obtained for SLAC in this study. Work is going on to characterize the native/ biradical intermediate (NI) in more detail.

Spectroscopic Experiments

The results from transient absorption spectroscopy and EPR spectroscopy support the original hypothesis (20) about the localization of the unpaired spins on Y108 and T2 Cu in the T1D SLAC. Experiments analogous to those performed earlier with the T1D SLAC (20) were now carried out with the double mutants, T1D-Y108F and T1D-Y108A, i.e., where both C288S and Y108A or Y108F mutations are present. Pre-

steady-state kinetics experiments reveal that the absorption feature around 410 nm, which was earlier attributed to the formation of a tyrosyl radical, is not observed in these variants. Instead, an intermediate resembling the PI (31) (absorption maxima around 340, 470, and 710 nm) is observed (Figure 4b). In conjunction with the results above, no biradical signal is observed in the EPR spectrum of T1D-Y108A mutant (Figure 4b). It is interesting to note that a new radical signal is observed in the T1D-Y108F mutant, which is distinct from the signal reported for T1D itself (not shown). However, the intensity of the new radical signal compared to that of the total spin is very low (<10%). While similar turnover numbers are observed for Y108A and Y108F variants, we conclude that this signal cannot be catalytically relevant and is formed only in the absence of Y108. High-field magnetic resonance spectroscopy studies are underway to determine if this signal may correspond to a phenylalanine or possibly a tryptophan radical.

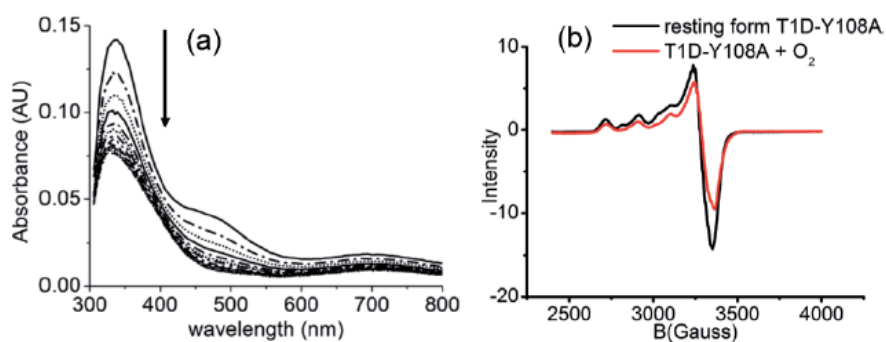


Figure 5.4 (a) Decay of the peroxide intermediate (PI) monitored by absorption spectroscopy following rapid mixing of reduced T1DY108A mutant with air-saturated phosphate buffer (pH 6.8) at 295 K. The PI is formed within 200 ms of mixing and then decays slowly with a half-life of 2.5 s. (b) X-band EPR spectrum of the resting form of T1D-Y108A mutant (black) overlaid with the spectrum of the same enzyme after it had been reduced, reoxidized, and frozen immediately (red). The spectra were recorded at 40 K.

It is important to note that Y108 is conserved among the homologous two-domain MCOs and also in human ceruloplasmin (hCp), for which a crystal structure has been published (32). The appearance of a 410 nm intermediate (presumably oxidized tyrosine) during oxidation of fully reduced hCp was observed several years ago (33,34) but was not investigated further owing to the challenging mutagenesis of the recombinant protein and its purification in soluble form (35). By analogy with SLAC, we now can assign, tentatively, this intermediate to a tyrosine radical. It is noteworthy that, among all known MCOs, only the six-domain ceruloplasmins and SLAC bind to the Cu sites with eight 2N ϵ -His coordination, whereas the three-domain laccases and ascorbate oxidase contain seven 2N ϵ -His and one 1N δ -His as a ligand, leading to a distinct asymmetry between the T3 α (two 2N ϵ -His and one N δ 1-His) and T3 β (three 2N ϵ -His) Cu's (36). It has been suggested that this structural difference between the T3 α and T3 β Cu's has important mechanistic consequences for O₂ binding and reduction in the three-domain laccases (36). It will be of interest to see if the more symmetric coordination of the T3 site in SLAC (as in hCp) leads to different reaction kinetics than in the three-domain laccases. Thus, SLAC and hCp share not only the conserved Y108 (Y107 in hCp) and the above same features of unique copper binding motifs but possibly also a similar enzyme mechanism. We conclude that, not only from a structural viewpoint but also from a mechanistic point of view, our experiments appear to support the earlier postulated evolution of copper proteins, where the two-domain MCOs are proposed to be ancestors to the sixdomain hCp (11,37,38).

While the physiological significance of SLAC in the morphological

development or metabolic system of *S. coelicolor* is not clear, along with the question of its natural substrates, it is evident that residue Y108 does form an integral part of the active site and is involved in the oxidase activity of this enzyme. If the natural substrate of SLAC has much higher turnover number for the wt SLAC, as known for the ferroxidase in yeast (Fet3p) and hCp (39), the effect of mutation may be much more pronounced across the mutants. While we have noticed that SLAC catalyzes oxidative coupling of o-phenylenediamines and o-aminophenols to phenazines and phenoxazines, respectively, which are speculated to be extracellular secondary metabolites for signaling or self-defense of *Pseudomonas aeruginosa* and *S. antibioticus* (40,41), their turnover numbers are too low to identify them as the natural substrates. The ability of SLAC to catalyze such reactions, along with a recombinant expression system, indeed holds new promises for the use of SLAC in industry for large-scale production of antibiotics for further studies or for human welfare. We are currently investigating other possible substrates and also attempting to identify the substrate-binding pocket using crystallography, which may shed more light on the enzyme function and its similarities and dissimilarities relative to the other members of the family of MCOs.

References

- (60) Solomon, E. I.; Sundaram, U. M.; Machonkin, T. E. *Chem. Rev. (Washington, DC, U. S.)* **1996**, 96, 2563.
- (61) Solomon, E. I.; Augustine, A. J.; Yoon, J. *Dalton Trans.* **2008**, 3921
- (62) Augustine, A. J.; Quintanar, L.; Stoj, C. S.; Kosman, D. J.; Solomon, E. I. *J. Am. Chem. Soc.* **2007**, 129, 13118.
- (63) Solomon, E. I.; Ginsbach, J. W.; Heppner, D. E.; Kieber-Emmons, M. T.; Kjaergaard, C. H.; Smeets, P. J.; Tian, L.; Woertink, J. S. *Faraday Discuss.* **2011**, 148, 11.
- (64) Kosman, D. J. *J. Biol. Inorg. Chem.* **2010**, 15, 15.
- (65) Machczynski, M. C.; Vijgenboom, E.; Samyn, B.; Canters, G. W. *Protein Sci.* **2004**, 13, 2388.

- (66) Messerschmidt, A.; Ladenstein, R.; Huber, R.; Bolognesi, M.; Avigliano, L.; Petruzzelli, R.; Rossi, A.; Finazzi-Agro, A. *J. Mol. Biol.* **1992**, *224*, 179.
- (67) Taylor, A. B.; Stoj, C. S.; Ziegler, L.; Kosman, D. J.; Hart, P. J. *Proc. Natl. Acad. Sci. U. S. A.* **2005**, *102*, 15459.
- (68) Roberts, S. A.; Weichsel, A.; Grass, G.; Thakali, K.; Hazzard, J. T.; Tollin, G.; Rensing, C.; Montfort, W. R. *Proc. Natl. Acad. Sci. U. S. A.* **2002**, *99*, 2766.
- (69) Hakulinen, N.; Kiiskinen, L. L.; Kruus, K.; Saloheimo, M.; Paananen, A.; Koivula, A.; Rouvinen, J. *Nat. Struct. Biol.* **2002**, *9*, 601.
- (70) Nakamura, K.; Go, N. *Cell. Mol. Life Sci.* **2005**, *62*, 2050.
- (71) Skalova, T.; Dohnalek, J.; Ostergaard, L. H.; Ostergaard, P. R.; Kolenko, P.; Duskova, J.; Stepankova, A.; Hasek, J. *J. Mol. Biol.* **2009**, *385*, 1165.
- (72) Skalova, T.; Duskova, J.; Hasek, J.; Stepankova, A.; Koval, T.; Ostergaard, L. H.; Dohnalek, J. *Acta Crystallogr., Sect. F: Struct. Biol. Cryst. Commun.* **2011**, *67*, 27.
- (73) Komori, H.; Miyazaki, K.; Higuchi, Y. *FEBS Lett.* **2009**, *583*, 1189.
- (74) Lawton, T. J.; Sayavedra-Soto, L. A.; Arp, D. J.; Rosenzweig, A. C. *J. Biol. Chem.* **2009**, *284*, 10174.
- (75) Rodriguez Couto, S.; Toca Herrera, J. L. *Biotechnol. Adv.* **2006**, *24*, 500.
- (76) Wheeldon, I. R.; Galloway, J. W.; Barton, S. C.; Banta, S. *Proc. Natl. Acad. Sci. U. S. A.* **2008**, *105*, 15275.
- (77) Bibb, M. J. *Curr. Opin. Microbiol.* **2005**, *8*, 208.
- (78) Horinouchi, S. *Biosci. Biotechnol. Biochem.* **2007**, *71*, 283.
- (79) Tepper, A. W. J. W.; Milikisyants, S.; Sottini, S.; Vijgenboom, E.; Groenen, E. J. J.; Canters, G. W. J. *Am. Chem. Soc.* **2009**, *131*, 11680.
- (80) Stubbe, J. A.; van der Donk, W. A. *Chem. Rev. (Washington, DC, U. S.)* **1998**, *98*, 2661.
- (81) Leslie, A. G. W. Joint CCP4 + ESF-EAMCB Newsletter on Protein Crystallography, **1992**, 26.
- (82) Collaborative Computational Project, Number 4. The CCP4 Suite: programs for protein crystallography, *Acta crystallogr. Sect. D* **1994**, *50*, 760.
- (83) Evans, P. R. "Data reduction", Proceedings of CCP4 Study Weekend on Data Collection & Processing, **1993**, 114.
- (84) Vagin, A.; Teplyakov, A. *Acta crystallogr. Sect. D* **2010**, *66*, 22.
- (85) Murshudov, G. N.; Skubak, P.; Lebedev, A. A.; Pannu, N. S.; Steiner, R. A.; Nicholls, R. A.; Winn, M. D.; Long, F.; Vagin, A. A. *Acta crystallogr. Sect. D* **2011**, *67*, 355.
- (86) Emsley, P.; Cowtan, K. *Acta crystallogr. Sect. D* **2004**, *60*, 2126.
- (87) Northrop, D. B. *J. Chem. Educ.* **1998**, *75*, 1153.
- (88) Cole, J. L.; Ballou, D. P.; Solomon, E. I. *J. Am. Chem. Soc.* **1991**, *113*, 8544.
- (89) Lee, S. K.; George, S. D.; Antholine, W. E.; Hedman, B.; Hodgson, K. O.; Solomon, E. I. *J. Am. Chem. Soc.* **2002**, *124*, 6180.
- (90) Shin, W.; Sundaram, U. M.; Cole, J. L.; Zhang, H. H.; Hedman, B.; Hodgson, K. O.; Solomon, E. I. *J. Am. Chem. Soc.* **1996**, *118*, 3202.
- (91) Bento, I.; Peixoto, C.; Zaitsev, V. N.; Lindley, P. F. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **2007**, *63*, 240.
- (92) Manabe, T.; Manabe, N.; Hiromi, K.; Hatano, H. *FEBS Lett.* **1972**, *23*, 268.
- (93) Manabe, T.; Hatano, H.; Hiromi, K. *J. Biochem.* **1973**, *73*, 1169.
- (94) Bielli, P.; Bellenchi, G. C.; Calabrese, L. *J. Biol. Chem.* **2001**, *276*, 2678.
- (95) Augustine, A. J.; Kjaergaard, C.; Qayyum, M.; Ziegler, L.; Kosman, D. J.; Hodgson, K. O.; Hedman, B.; Solomon, E. I. *J. Am. Chem. Soc.* **2010**, *132*, 6057.
- (96) Murphy, M. E. P.; Lindley, P. F.; Adman, E. T. *Protein Sci.* **1997**, *6*, 761.
- (97) Dwulet, F. E.; Putnam, F. W. *Proc. Natl. Acad. Sci. U. S. A.* **1981**, *78*, 2805.
- (98) Quintanar, L.; Stoj, C.; Taylor, A. B.; Hart, P. J.; Kosman, D. J.; Solomon, E. I. *Acc. Chem. Res.* **2007**, *40*, 445.
- (99) Le Roes-Hill, M.; Goodwin, C.; Burton, S. *Trends Biotechnol.* **2009**, *27*, 248.
- (100) Dietrich, L. E.; Teal, T. K.; Price-Whelan, A.; Newman, D. K. *Science (Washington, DC, U. S.)* **2008**, *321*, 1203.
- (42) Gupta, A.; Nederlof, I.; Sottini, S.; Tepper, A. W. J. W.; Groenen, E. J. J.; Thomassen, E. A. J.; Canters, G. W., Involvement of Tyr108 in the Enzyme Mechanism of the Small Laccase from *Streptomyces coelicolor*. *Journal of the American Chemical Society* **2012**, *134* (44), 18213-18216.

Chapter 6

Summary, Conclusion and Future Prospects

Macromolecular X-ray crystallography is a powerful tool for understanding the structures and functions of proteins. However, diffraction grade crystals need to be obtained for X-ray diffraction data collection. Crystals of membrane proteins and large protein complexes are often difficult to grow. If diffraction grade crystals suitable for X-ray diffraction experiments do not grow, projects are often discontinued. Compared to X-rays, electrons give information with a much higher signal-to-noise ratio. Therefore, they have a strong potential for determining the structure of 3D nano-crystals of biological macromolecules that are too small for X-ray experiments. Furthermore nano-crystals of proteins may be easier to grow, since all big crystals start from small crystals. If crystal growth is tracked, it can be stopped when the crystals reach a desired size, for example by freezing the sample.

This thesis focuses on new methods in (nano-) crystal growth, data collection and analysis using electron diffraction and imaging techniques. It also discusses an experiment where X-ray diffraction techniques among others were used to determine the role of Tyr108 in the enzyme mechanism of Small Laccase from *Streptomyces coelicolor*.

Nucleants induce (nano)- crystal growth

In protein crystallography one of the most serious bottlenecks is

obtaining initial hits in protein crystallization screens. Often hundreds and sometimes even thousands of crystallization trials are set up before such a hit is found. Human hair has previously been identified as a promising heterogeneous nucleant for initializing crystal growth, increasing the success rate of crystallization trials. Scanning electron microscopy images proved that hair fragments show small cavities that can trigger crystal nucleation. However, hair samples are difficult to introduce deliberately in every drop of a crystallization screen. Since crystallization experiments are nowadays mainly set up using automated dispensing robots, a new method was developed to incorporate human hair fragments in automated crystallization experiments. The method is straightforward and can be applied in almost any automated crystallization lab. It was evaluated by setting up standard crystallization experiments with and without hair fragments with 11 different proteins. Additional crystallization conditions were found for 10 of these 11 proteins using the standard JCSG+ screen containing 96 conditions. 34 additional conditions were identified meaning a total increase of new crystallization conditions of 13.1 %. These conditions would have been missed if screening had been performed without the addition of hair fragments. Since dust particles, which consist for a large part of microscopic hair fragments, are naturally present in crystallization labs, the effect of hair particles on crystallization experiments may be far greater than suggested by these tests.

Rotation electron diffraction of 3D protein nano-crystals

Protein crystals smaller than one micron are beyond the scope of X-ray diffraction techniques due to excessive radiation damage. Electron

diffraction data collection of 2D nano-crystals has shown the potential of electron diffraction methods as an alternative. In this thesis I describe the use of a highly sensitive medipix2 detector to collect rotation electron diffraction of 3D protein crystals, which have similar volumes to the 2D crystals for which the methods have already been developed. The data obtained could be analysed with MOSFLM and Scala, two data analysis programs used in X-ray crystallography. The data showed resolution better than 2 Ångstrom. Using a parallel electron beam the curvature of the Ewald's sphere could be observed, indicating low mosaicity. Data could be collected on multiple places of a single nano-crystal. Even though important steps were taken to develop electron diffraction of 3D protein crystals there is still work to be done. The installation of a Medipix2 camera, with an enhanced readout chip, on a Titan Krios microscope at NeCen will optimise data collection and increase reproducibility. This will allow more insight into what steps need to be taken to optimize software for data analysis.

Imaging protein 3D nano-crystals with cryo-EM

In this thesis I report that 3D protein nano-crystals that are too small for conventional (synchrotron) X-ray analysis can robustly be imaged to resolution higher than 3 Ångstrom with electrons. Data processing and averaging revealed details in the lattice beyond 2 Ångstrom. Analysis with Cryo-EM software showed that within one crystal multiple views of projected density appeared in different parts of the crystal. Potential causes can be the presence of mosaic blocks within the crystal, the crystal may not be completely parallel to the beam or the fact that the crystal is not uniformly thick, in which case the differences could be

caused by a higher degree of dynamic scattering in the thicker parts. Mosaic blocks are usually assumed in X-ray crystallography and I predict them to be the main cause here. The next steps would be to optimize and automate data collection and analysis. Then 3D reconstruction techniques from single particle cryo-EM could be applied to obtain an electron density map that can be used to build the macromolecular model.

In 2D crystallography the resolution from imaging techniques can be increased by applying diffraction techniques. The diffraction and imaging techniques described in this thesis, show high potential for similar techniques for 3D protein crystals.

Tyr108 involvement in enzyme mechanism of the Small Laccase from *Streptomyces coelicolor*.

Multicopper oxidases (MCO) are enzymes that oxidise their substrates while reducing molecular oxygen to water. This thesis describes the role of tyrosine 108 in the mechanism of the MCO, Small Laccase (SLAC) in *Streptomyces coelicolor*. Two mutants were constructed of SLAC where the tyr108 was substituted into a phenylalanine and an alanine. Structural studies using x-ray crystallography to a resolution of 2.7-2.8 Å confirmed the mutations and also that the active site had remained intact. However kinetic experiments showed a decreased oxygen turnover number for the Y108A and Y108F mutants. Spectroscopic experiments show that if the Type 1 copper site *and* the Y108 are both depleted a peroxy intermediate appears. An electron reduces this intermediate from provided that the tyrosine is present. It would be interesting to investigate the role of other substrates in the kinetics as well as their binding pockets with subsequent crystallographic studies.

Nederlandse Samenvatting

Röntgenkristallografie is de meest gebruikte techniek voor het bepalen van de drie-dimensionale structuur van eiwitten. Echter om diffractie data op te nemen is het noodzakelijk dat het te bestuderen eiwit kristalliseert. Het kristalliseren van bijvoorbeeld membraaneiwitten en grote eiwitcomplexen is een lastige taak. Dit terwijl vooral deze eiwitten veel informatie kunnen geven over de werking van ons lichaam en bijvoorbeeld interacties met medicijnen. Vaak groeien eiwitkristallen niet groot genoeg om met Röntgen kristallografie bestudeerd te worden, in deze gevallen kunnen elektronen wellicht uitkomst bieden.

In dit proefschrift worden nieuwe methoden beschreven voor het groeien van eiwit (nano-)kristallen en het verzamelen van experimentele data van dit soort kristallen met elektronen diffractie en imaging technieken. Ook wordt een experiment besproken waar o.a. Röntgen kristallografie gebruikt wordt om de rol van het aminozuur Tyr108 te bepalen in het enzym mechanisme van Small laccase, een koperbevattend oxidoreductase.

Nucleanten induceren de groei van (nano)-kristallen

Een van de grootste knelpunten in de Röntgenkristallografie van eiwitten is het verkrijgen van kristallen. Om kristallen te groeien moeten honderden en soms wel duizenden experimenten worden opgezet. Menselijk haar is al eerder geïdentificeerd als een nucleant dat eiwitgroei kan initiëren. Analyse met scanning elektronen microscopie bewijst dat haarfragmentjes kleine holten bevatten waar eiwit kan

kristalliseren. Kristallisatie experimenten zijn tegenwoordig geautomatiseerd en worden opgezet met gebruik van robots. Om haarfragmentjes als nucleant op te nemen in deze geautomatiseerde omgeving is een nieuwe methode ontwikkeld, die makkelijk toepasbaar is in elk kristallisatie lab. De methode is getest door standaard kristallisatie-experimenten op te zetten met 11 verschillende eiwitten. Extra condities zijn gevonden voor 10 van deze 11 eiwitten met de standaard JCSG+ screen die 96 condities bevat. In totaal werden er 34 extra condities gevonden, een toename van 13.1 %, als gevolg van het toevoegen van haarfragmentjes. Deze condities zouden anders gemist zijn. De invloed van haarfragmentjes is wellicht nog groter als er in acht wordt genomen dat stofdeeltjes, aanwezig in het lab, voornamelijk bestaan uit microscopische haardeeltjes.

Rotatie diffractie van 3D eiwit kristallen

Eiwit kristallen kleiner dan een micron zijn buiten het bereik van huidige Röntgendiffractie technieken, wegens excessieve stralingsschade. Elektronen diffractie kan hier een uitkomst bieden aangezien deze ook al succesvol wordt toegepast op 2D eiwit kristallen met vergelijkbare volumina. Dit proefschrift beschrijft het gebruik van een ultragevoelige Medipix2 detector voor het opnemen van rotatie elektron diffractie data van 3D eiwitten. De verkregen data kon geanalyseerd worden met MOSFLM en Scala, twee programma's voor data analyse die gebruikt worden in de Röntgen kristallografie. Met een parallelle elektronen bundel konden de kromming van de Ewald's bol worden waargenomen, een indicatie van een zeer lage mozaïeke structuur. De data kon op meerdere plaatsen van een kristal worden

opgenomen en vertoonde resolutie beter dan 2 Ångstrom. De installatie van een Medipix2 camera op een Titan Krios elektronen microscoop in NeCen zal er voor zorgen dat het verzamelen van data geoptimaliseerd kan worden en meer reproduceerbaar zal zijn. Dit zal duidelijk maken welke stappen er gezet moeten worden om software voor data analyse te optimaliseren.

Imaging van 3D eiwitten met cryo-elektronen microscopie

In dit proefschrift wordt gerapporteerd dat 3D eiwit kristallen, die te klein zijn voor conventionele Röntgenkristallografie, in een elektronenmicroscoop kunnen worden bestudeerd tot een resolutie van 3 Ångstrom. Met data analyse en middeling kunnen details in het kristalraster worden gezien tot een resolutie van 2 Ångstrom. Analyse met cryo-EM software gaf aan dat er meerdere projecties van elektronen dichtheid binnen een enkel kristal aanwezig waren. Redenen hiervoor kunnen zijn dat er binnen het kristal meerdere mozaïeke blokken aanwezig waren. Het kan ook zijn dat het kristal niet geheel parallel aan de elektronen bundel lag of dat het geen gelijkmatige dikte had. Dit laatste zou resulteren in verhoogd dynamische verstrooiing. Macromoleculaire blokken worden waargenomen in Röntgen kristallografie en ik verwacht dat deze ook hier de grootste oorzaak zijn. Volgende stappen zullen zich richten op het optimaliseren en automatiseren van de data verzameling en analyse technieken. Daarna kunnen 3D reconstructies technieken uit de “single particle cryo-EM” gebruikt worden om een macromoleculair model te bouwen.

In 2D kristallografie kan de resolutie van imaging technieken vergroot worden door diffractie data toe te voegen. De diffractie en imaging

technieken beschreven in dit proefschrift tonen aan dat er mogelijkheden zijn om vergelijkbare technieken toe te passen voor 3D eiwit kristallen.

Betrokkenheid van Tyr108 in het enzym mechanisme van Small Laccase van *Streptomyces coelicolor*

Koper bevattende oxidoreductasen (MCO) zijn enzymen die hun substraten oxideren terwijl ze zuurstof reduceren naar water. Dit proefschrift beschrijft de betrokkenheid van het aminozuur tyrosine 108 in het mechanisme van Small Laccase (SLAC). Twee mutanten van SLAC werden geconstrueerd waarbij tyr108 gesubstitueerd werd in een fenylalanine en een alanine. Met Röntgenkristallografie werden de mutaties bevestigd, en ook dat de rest van het actieve centrum in intact was gebleven. Echter een activiteit test van de Y108A en Y108F mutanten gaf aan dat omzet van zuurstof sterk verlaagd was. Spectroscopie experimenten lieten zien dat als de type 1 koper site en tyr108 afwezig zijn, dat er dan een peroxy intermediair aanwezig is. Dit tussenproduct zou door het elektron van Y108 gereduceerd worden (mits aanwezig). Het zou interessant zijn om te weten wat het effect van andere substraten zijn op de kinetiek en hoe deze binden in het actieve centrum.

Curriculum Vitae

Igor Nederlof was born in Stanford, California on the 29th of March 1979. In 1997 he graduated from het Baarnsch Lyceum and went to study Analytical Chemistry at the Institute for Life Science and Chemistry Utrecht. In 2003 he graduated obtaining a bachelor's degree in analytical chemistry. During this time he did a 10-month research internship in the Sacchettini Lab at Texas A&M University over-expressing, purifying and crystallising β -amyloid and α 2-Macroglobulin from Alzheimer's disease. He obtained a master's degree in Biological Chemistry in 2006 from Leiden University, after doing a 6-month internship in the Page Lab at Brown University. At Brown he studied the plasticity of neurons by doing structural studies of Spine Associated RapGAP. During his master's he worked part time for Key Drug Prototyping, a small "start-up" biotech company that focused on structure aided drug design through X-ray protein crystallography. After his graduation he worked full time at Key drug prototyping from April 2006 through December 2008. In January 2009 he started his PhD in the Biophysical Structural Chemistry group of Jan Pieter Abrahams at Leiden University. His research project focuses on electron microscopy and diffraction studies of 3D protein crystals of which the results are presented in this thesis. This project is still in full development as he has accepted a post-doc position in the Biophysical Structural Chemistry group continuing his work.

List of Publications

2013

Nederlof, I., Li, J.W., van Heel, M., Abrahams, J.P. (2013). *A Medipix Quantum Area Detector Allows Rotation Electron Diffraction Data Collection of Sub-Micron 3D Protein Crystals* (under review)

Nederlof, I., Van Genderen, E., Li, J.W., Abrahams, J.P. (2013). *Imaging Protein 3D Nanocrystals with Cryo-EM* (accepted for publication in Acta Cryst. Sec. D)

2012

Gupta, A., **Nederlof, I.**, Sottini, S., Tepper, A. W. J. W., Groenen, E. J. J., Thomassen, E. A. J. & Canters, G. W. (2012). *Journal of the American Chemical Society* **134**, 18213-18216.

Abrahams, J., Georgieva, D., Jiang, L. & **Nederlof, I.** (2012). *NATO Science for Peace and Security Series B: Physics and Biophysics, Uniting Electron Crystallography and Powder Diffraction*, edited by U. Kolb, K. Shankland, L. Meshi, A. Avilov & W. I. F. David, pp. 389-398. Springer Netherlands.

Nederlof, I., Van Genderen, E., Hoedemaeker, F., Abrahams, J.P., Georgieva, D., *Protein Crystal Growth, Modern Aspects of Bulk Crystal and Thin Film Preparation*, edited by N. Kolesnikov & E. Borisenko, pp. 463-476. InTech

2011

Jiang, L.H., Georgieva, D., **Nederlof, I.**, Liu, Z.F., Abrahams, J.P., *Image Processing and Lattice Determination for Three-Dimensional Nanocrystals, Microscopy and Microanalysis*, vol. 17, no. 6, pp. 879-885.

Nederlof, I., Hosseini, R., Georgieva, D., Luo, J.H., Li, D.F., Abrahams, J.P., *A Straightforward and Robust Method for Introducing Human Hair as a Nucleant into High Throughput Crystallization Trials, Crystal Growth & Design*, vol. 11, no. 4, pp. 1170-1176.

2010

Liu, Z.F., Jiang, L.H., Galli, F., **Nederlof, I.**, Olsthoorn, R.C.L., Lamers, G.E.M., Oosterkamp, T.H., Abrahams, J.P., *A Graphene Oxide center Streptavidin Complex for Biorecognition - Towards Affinity Purification, Advanced Functional Materials*, vol. 20, no. 17, pp. 2857-2865