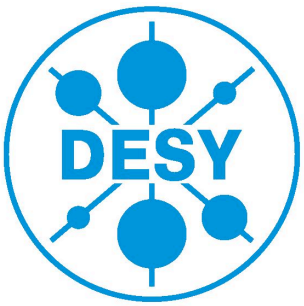

Creation and Characterisation of Nanoprotein Crystals for use in Serial Femtosecond Crystallography

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Abstract

This project was undertaken as part of the DESY Summer Student Programme 2013. It entailed making and characterising crystals for two categories of Serial Femtosecond Crystallography. The first type of crystallography required 2μm crystals. These were produced for the P11 beamtime and were based on tests carried out in this project. The second category required very small crystals that could be jetted in air. Research carried out by Falkner [3] was reproduced and adapted by changing crystallisation conditions to create smaller crystals. Crystals were characterised using Optical Microscopy, Dynamic Light Scattering (DLS) Spectroscopy and Scanning Electron Microscopy (SEM). The capabilities of two DLS devices were compared and the Libro plate instrument proved to be more reliable and easier to use than the cuvette instrument. Crystals were successfully cross-linked in order to improve visibility when using SEM.

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1. Introduction

This project entailed making and characterising nanoprotein crystals for the Coherent Imaging Division, CFEL for use in their serial femtosecond crystallography experiments.

The Coherent Imaging Division develop methods for imaging using X-ray Free Electron Laser (XFEL) and synchrotron sources, with an emphasis on bio-particles and macro-molecules. These methods are used to determine the structure of such molecules. However, these molecules are easily damaged through radiation exposure so the ultrafast X-ray pulses are being used to overcome this issue. The methods being developed offer new opportunities to examine the structure of biological particles whose structure is still unknown, providing insight into pharmaceutical design.

Crystallography methods currently used require mesoscopic crystals that may take many years of research to obtain. The Coherent Imaging Division is developing a new concept for structure determination. Single shot diffraction patterns are collected from a stream of nanocrystals using femtosecond pulses from an X-ray Free Electron Laser (XFEL). Researchers realised almost half a decade ago that very high dose rates delivered by their short and intense pulses might be suitable for reducing the amount of damage suffered by a specimen during its irradiation, allowing smaller crystals to be examined. The pulse is higher than what a specimen can normally tolerate before atomic rearrangements occur, the pulse is over and the structural information already measured, before being destroyed.[1]

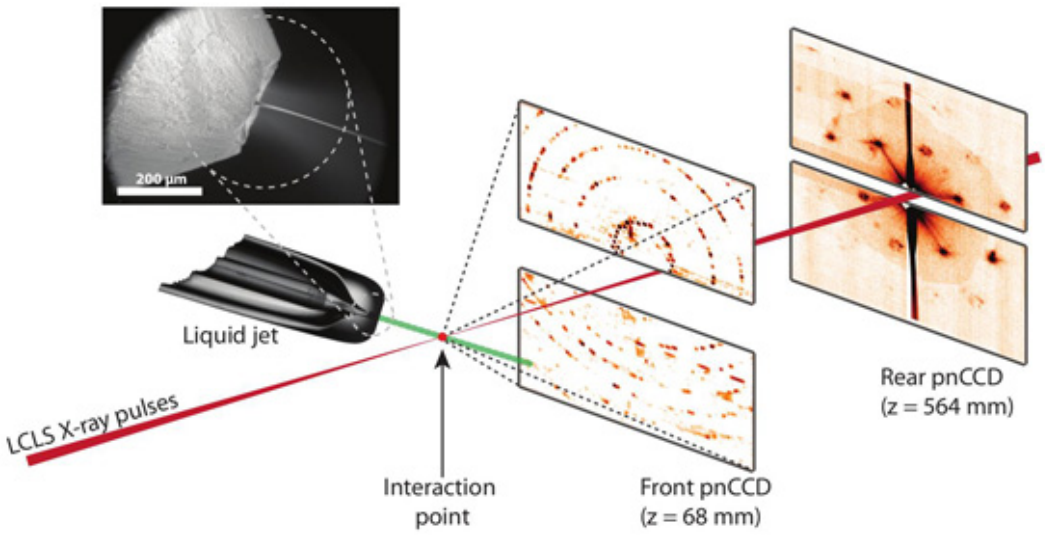

Figure 1: Set-up for a serial femtosecond crystallography experiment. [2]

The objective of this project was to make nanoprotein crystals suitable for serial femtosecond crystallography experiments and characterise them using the following methods: Optical Microscopy, Dynamic Light Scattering (DLS) Spectroscopy and Scanning Electron Microscopy (SEM).

2. Theory

2.1. Serial Femtosecond Crystallography

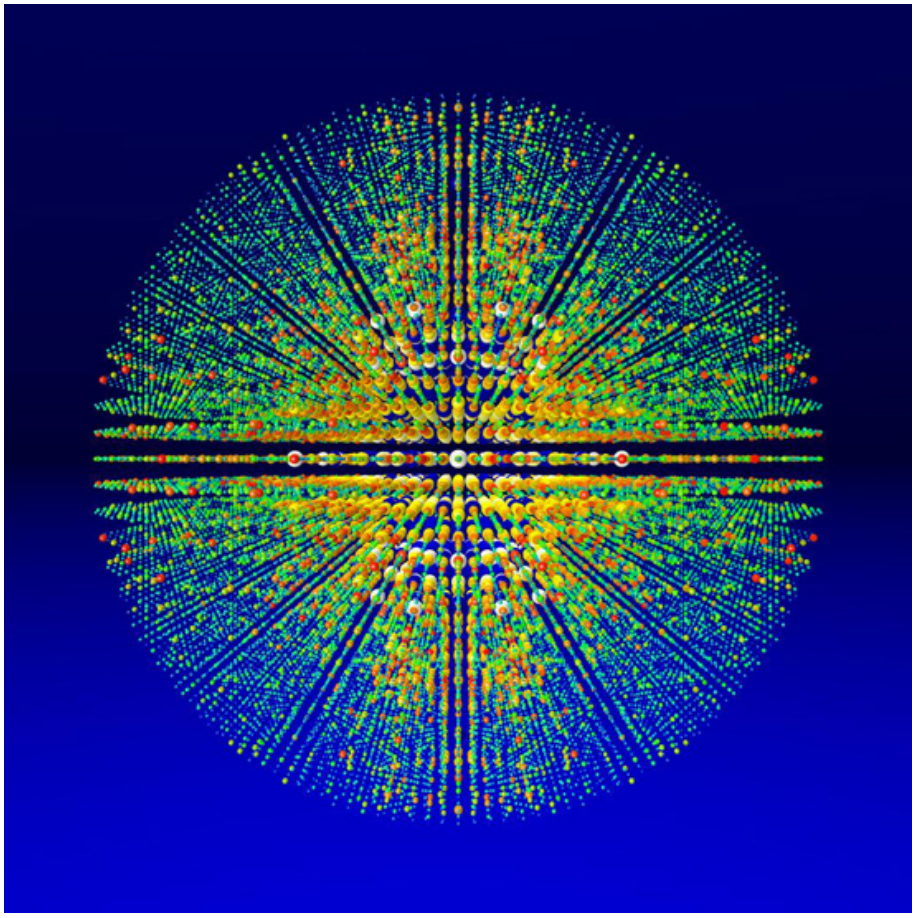

Figure 2: 3D diffraction pattern created by CrystFEL software suite from over 15,000 single-shot patterns acquired using LCLS. [1].

When a crystal is hit with an X-ray pulse, a diffraction pattern is created, before the crystal is vaporised. These diffraction patterns are read by a set of detectors like the two sets shown in Figure 1. The thousands of diffraction patterns obtained from each experiment are analysed by CrystFEL software suite, specialised software developed by CID. It combines the intensities from all of the patterns using Monte Carlo merging,

resulting in a 3D dataset like that in Figure 2. These are then further processed using standard macromolecular crystallography software to produce electron density maps an example of which is shown in Figure 3. From these maps, key features of protein complexes can be resolved and the structure determined.

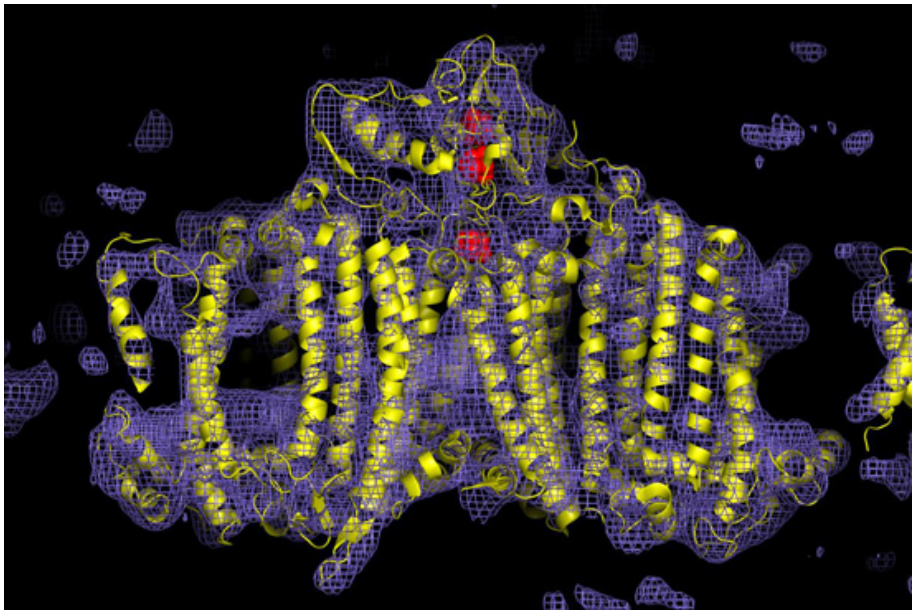

Figure 3: Electron density map of the photosystem I protein complex obtained from the LCLS diffraction data. First published in Nature 470, 73 78 (2011). Nanocrystals were grown by Petra Fromme of Arizona State University. [?]

This project entails making and characterising crystals for two sorts experiment. The first required crystal are very small crystals - on the nanoscale - to be used for experiments where the X-ray pulses hit crystals being jetted in the air from a nozzle. The experimental set up is shown in Figure 1. The second category of crystal is around 2μm and will be used in experiments where a capillary is used to deliver the crystals to the interaction point.

2.2. Protein Crystallisation

Lysozyme was the protein crystallised for the majority of this project. The form used was hen egg white lysozyme (HEWL) which is inexpensive when compared to other proteins. It was the first protein studied by X-diffraction and is still the most widely used for crystal growth studies [6].

Granulovirus was characterised using the Dynamic Light Scattering (DLS) tray instrument as part of the comparison with the DLS cuvette instrument.

The final part of the project was spent beginning tests crystallising other proteins. Glucose Isomerase was chosen as it is also commonly used for crystal growth research and has also been used in diffraction studies.

Research undertaken by Falkner et al in his paper 'Generation of Size-Controlled, Submicrometer Protein Crystals' was used as an outline for conditions that could be used to vary protein crystal size. It showed that crystal size decreases with:

- decreasing pH with the optimum at pH3.
- increasing NaCl concentration.
- increasing lysozyme concentration between 30-100mg/ml.
- decreasing temperature.[3]

'Effect of Additives on the Crystallization of Lysozyme and Chymotrypsinogen A' written by Lu et al. states that various additives in the precipitant cocktail have various effects on the crystallisation of Lysozyme and Chymotrypsinogen A. Those include changing the interactions between protein molecules, change in protein solubility, shift of thermodynamic equilibrium, altering surface energy of the crystal and affecting the nucleation in protein crystallisation [5]; all of which would affect the size and shape of crystals and hence the diffraction properties.

Reducing the salt concentration of the precipitant solutions is one of the objectives of this project, as this will lead to crystals that are better to jet. Alcohols have long been use as additives in crystallisation and there is evidence that certain alcohols could be used to replace the salt in the precipitant solutions [7, 8].

2.3. Characterisation using Dynamic Light Scattering

Dynamic Light Scattering (DLS) is a technique use to determine the size distribution of small particles or polymers suspended in a known solution. It measures the average intensity of light scattered by these particles, of a defined concentration, in excess to the background.

Small particles undergo Brownian motion when suspended in a fluid. So when the solution with particle suspension is hit by monochromatic and coherent from a laser, there is a time dependence of the fluctuation in the scattering intensity. The time variation in this random physical process can be described mathematically using the correlation function given by equation (1).

$$C = B[1 + a(C'(\tau))^2] \tag{1}$$

where B is the background, a is a constant which depends on the spatial coherence of detected scattered light, τ is the delay time and $C'(\tau)$ is called the normalised scattered electric field correlation function. For a monodisperse protein species, an exponential decay can be fitted to the normalised scattered electric field correlation function, as shown in equation (2).

$$C'(\tau) = e^{-\Gamma\tau} \tag{2}$$

where Γ is the decay constant. This is directly proportional to the diffusion coefficient, as given by equation (3).

$$\Gamma = Dq^2 \tag{3}$$

where q is the scattering vector. The hydrodynamic radius can be found once the diffusion constant has been determined, using the Stokes-Einstein equation.

$$R_H = \frac{kT}{6\pi\eta D} \tag{4}$$

where k is the Boltzmann constant, T is the absolute temperature and η is the viscosity of the solution. The hydrodynamic radius is the apparent radius of a suspended particle which includes the hydration and shape effects. Hence, analysing $C(\tau)$ provides an attractive method to estimate the radius of a protein solute [4].

2.4. Characterisation using Scanning Electron Microscopy

Scanning Electron Microscopy used a focused beam of electrons to image a sample. The sample must be electrically conductive and electrically grounded to prevent an electrostatic charge from accumulating on the surface. Cross-linking a sample, purifies the crystals and removes the salt, allowing them to be viewed clearly under the SEM.

3. Method

3.1. Protein Crystallisation

- A precipitant cocktail was prepared. This is a mixture of buffer, Polyethylene Glycol (PEG), salt and other additives which helps the protein crystallise. It was important that all composite solutions of the precipitant cocktail as well as the protein were filtered as to not interfere with results when it came to characterising the crystals.
- An eppendorf tube was filled with a quarter of the volume of the final solution with protein.
- The precipitant solution was added to make up the other three quarters of the final volume.
- This mixture was vortexed and allowed to rest so that protein had time to crystallise.

3.2. Optical Microscopy

After observing possible crystals with the naked eye in the form of the milky appearance of a crystal and supernatant solution, the crystals were preliminarily characterised using optical microscopy. A ZEISS 1022071042 1/1 optical microscope with Axiocam Mrm was used to view 5ml drops of samples on slides.

3.3. Dynamic Light Scattering

Two DLS instruments were used during this project. Firstly, tests were carried out to compare the capabilities of both pieces of equipment and then they were used to characterise nanoprotein crystal samples.

The first DLS device used a glass/quartz cuvette that was filled with at least 20μl of sample or diluted sample and inserted into the instrument. A cuvette is a small tube, specially designed to hold samples for spectroscopic experiments.

The second DLS device made use of a Linbro plate which was filled with oil and 4μ sample droplets were inserted into each oil well. It had an automated system that could do many measurements across the plate over a long period of time, meaning it could be left overnight or even over the weekend to collect data on numerous samples.

3.4. Cross-Linking

- A cross-linking solution was made by mixing one part 10% Glutaldehyde with three parts of the respective precipitant solution and vortexed.
- The crystals that required cross-linking were then centrifuged for two minutes at 2000 revs so that the crystals separated from the supernatant.
- The supernatant was removed and replaced with the same amount of relevant cross-linking solution.
- This new crystal mixture was then vortexed and left for 36 hours at room temperature.

3.5. Washing cross-linked crystals

- The crystals were vortexed and then centrifuged for two minutes at 2000 revs.
- The supernatant was removed and replaced with the same amount of buffer solution that the precipitant cocktail was made with. In this case 10mM NaOAc pH3.
- This process was repeated at least twice.

3.6. Scanning Electron Microscopy

0.5μl drops of sample were placed on a silicon wafer and allowed to dry under a fume hood. This wafer was then taken to the scanning electron microscope and carefully mounted in the device. Each sample droplet was viewed in turn and photographs taken for record.

4. Results

4.1. Dynamic Light Scattering Device Comparison

The cuvette DLS device was tested first. A series of measurements were carried out using Duke Standards Polymer Microspheres with known diameters. Figure 4 shows an example of results obtained using this device.

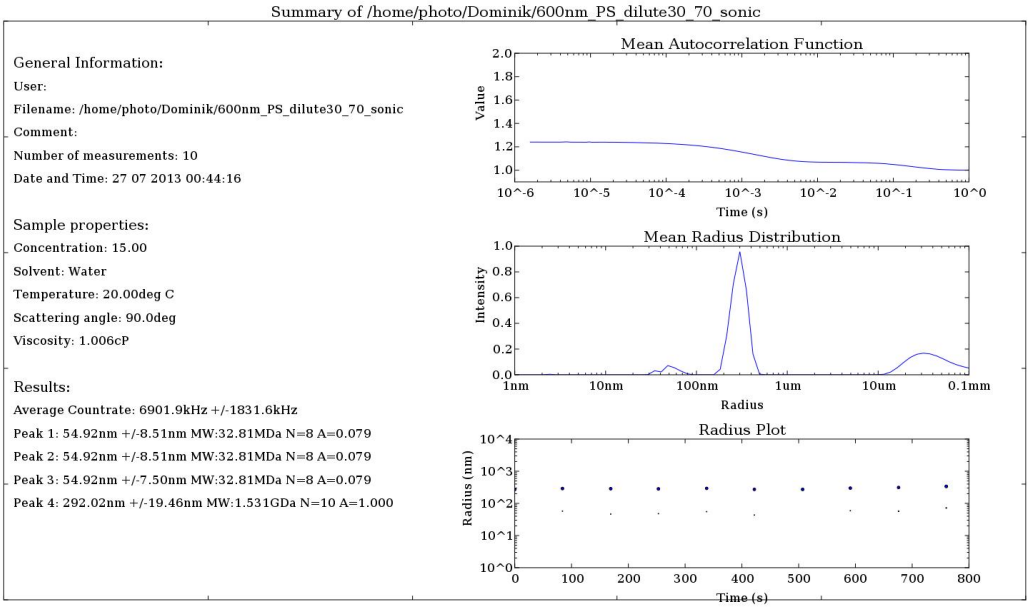

Figure 4: DLS cuvette results for 600nm Polymer microsphere nanoparticles.

When measuring Duke Standards Polymer Microspheres with a diameter of 600nm, results were expected to be a bit greater than 300nm, allowing for the hydronic shell. However, it can be seen from Peak 4 on Figure 4 that the observed radius is lower than the expected value.

It was found that diluting the sample with its respective precipitant solution produced the best results. It was observed that the amount a solution was diluted varied the results dramatically with the optimum being a 1/100th dilution. It was also found that the cuvette had to be thoroughly cleaned in order to provide a good result. The following cleaning regime was found too work: Wash with millipore water five or six times, remove all water with a pipette, wash once with isopropanol, remove all isopropanol with a pipette and finally wash with acetone, remove all the acetone with a pipette and the dry with canned air. Before putting the cuvette into the instrument, it must be cleaned and dried with a Kimtech tissue, wet with millipore water, to remove and

dust and marks. This whole cleaning regime made using the DLS cuvette instrument laborious to use.

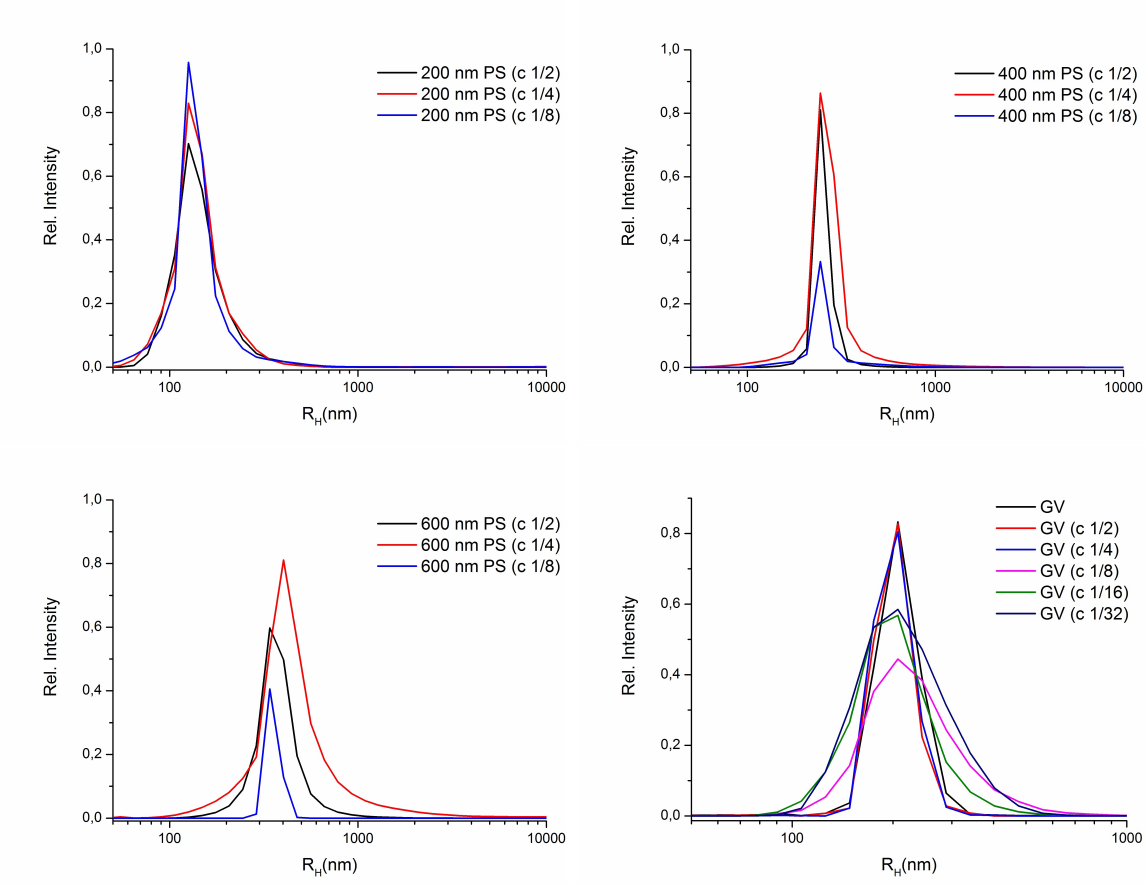

Figure 5: From left to right, top to bottom: DLS plate results for 200nm, 400nm, 600nm polymer microspheres and Granulovirus.

The Libro plate was then tested using Duke Standards Polymer Microspheres, nanocrystals and Granulovirus. Figure 5 shows some of the results obtained. Different dilutions were also tested to see what produced best results. The amount a solution was diluted by is denoted by its c value in the legend of the plots.

The results shown in Figure 5 are shown and compared with the results in Figure 4 in Table 1. It could be seen by comparing the results obtained from testing both pieces of equipment that results obtained by the DLS Libro plate instrument were closer to the expected values and with the advantage of being easier to use, it far outweighs the cuvette instrument as the preferred DLS device.

Unfortunately, even though the DLS plate instrument was found to be better, it could

Table 1: Table to show the results obtained when measuring Polymer Microspheres and Granoluvirus with the DLS cuvette and Libro plate instruments

Device	Particle	Expected radius (nm)	Average radius (nm)
Cuvette	600nm Polymer Microsphere	300	292
Plate	200nm Polymer Microsphere	100	135
Plate	400nm Polymer Microsphere	200	250
Plate	600nm Polymer Microsphere	300	349
Plate	Granoluvirus		200

not be used for further studies during this project as it got sent to England. Using this to measure protein nanocrystals could be investigated further. The DLS cuvette instrument was used in the DLS tray instrument's absence but results obtained can only be taken as an estimate.

4.2. Cross-linking

Cross-linking was carried out on crystals that were to be characterised with Scanning Electron Microscopy. The aim was to be able to reproduce the same quality of image as in the Falkner paper. This was achieved as shown by figure 9. It was found that crystals could be diluted by a tenth using the buffer that was used to make their respective precipitant solution to produce clearer images.

A crystal solution that was made before the project began was tested with the DLS cuvette instrument before and after being cross-linked. The results are shown in Figures 6 and 7 respectively and an SEM image of the crystals shown in Figure 8.

By comparing the results shown in Figures 6 and 7 with the SEM picture in Figure 8, it can be seen that the uncross-linked result of 619.30nm is much too high compared to the 126.50nm in the SEM picture. The cross-linked result of 253.72nm is much closer to the result observed in the SEM picture. Cross-linked crystals could be diluted with 0.1μm filtered millipore water for better DLS results.

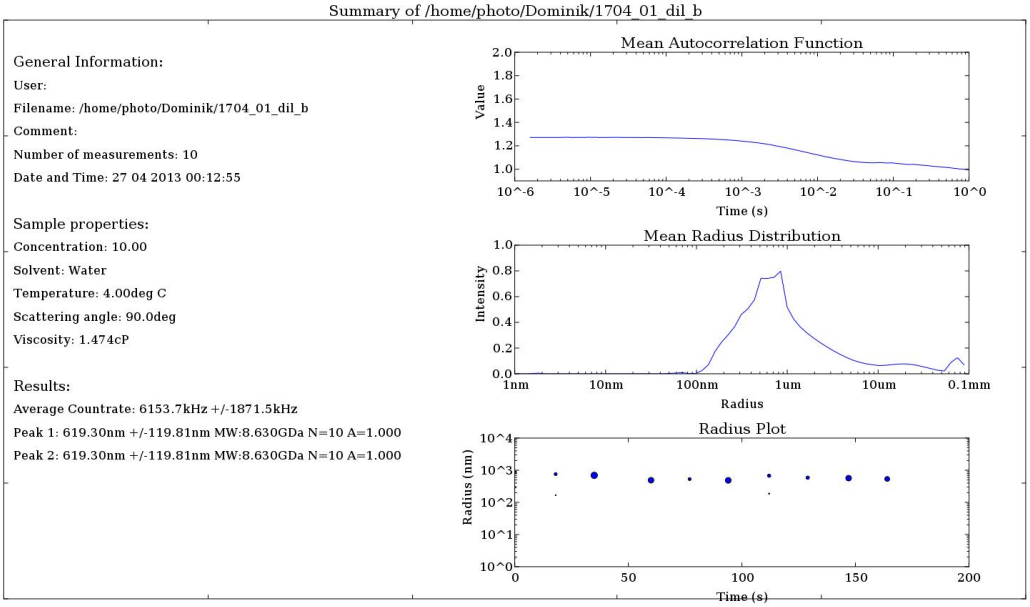

Figure 6: DLS cuvette results for uncross-linked crystals.

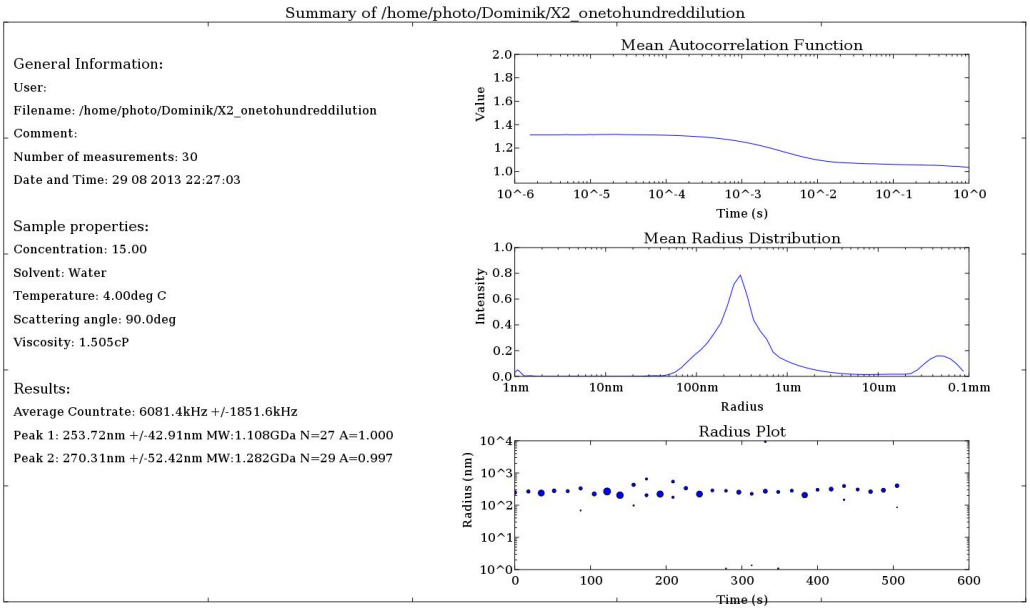

Figure 7: DLS cuvette results for cross-linked crystals.

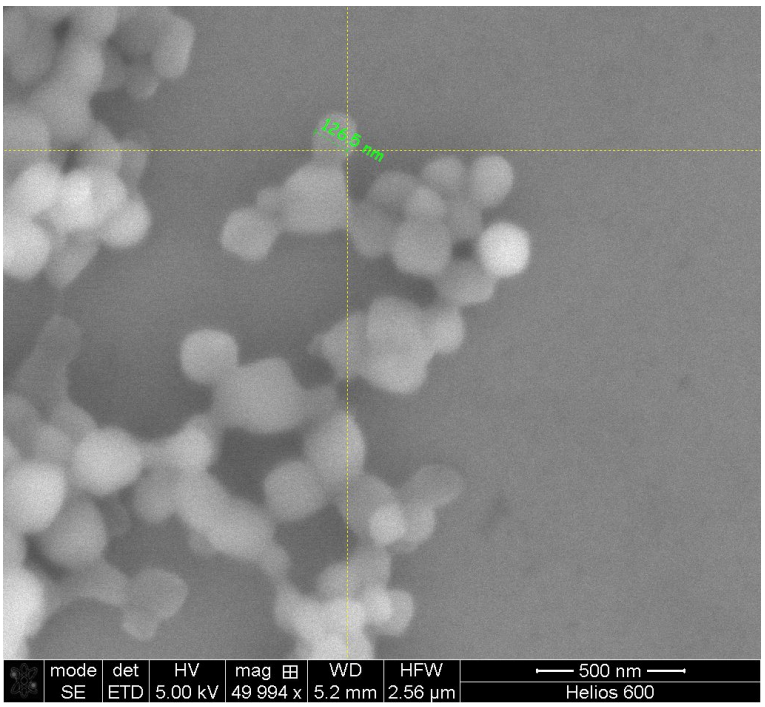

Figure 8: SEM image for cross-linked crystals.

Table 2: Table to show examples of favourable precipitant solutions.

Name of precipitant	Ethanol (100%) (ml)	PEG 8000 (30%) (ml)	NaCl (4.5M pH3) (ml)	Tacsimate (100% pH4) (ml)	Isopropanol (ml)
200813_5B	1.40	0.40	0.10	0.10	0.00
200813_5F	1.00	0.40	0.10	0.00	0.50
N5_270813	1.05	0.30	0.05	0.10	0.50
N6_270813	1.00	0.30	0.05	0.10	0.55

4.3. Lysozyme Nanocrystallisation - Recreating Falkner's Results

Tests were undertaken to recreate Falkner's results on using salt to control crystal size and PEG to control crystal quality. This was done successfully with solution series A-E which ran in order of decreasing salt and increasing PEG concentrations. Crystals B, C and E are depicted in Figure 9 in order from the top right hand corner, moving clockwise.

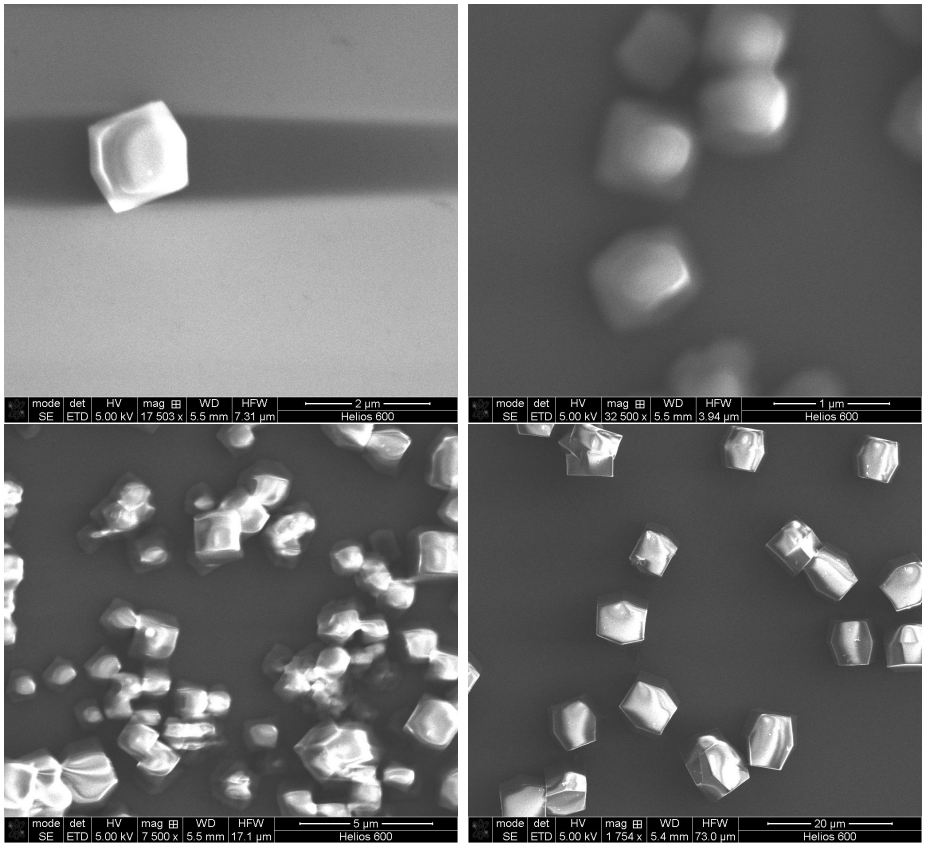

Figure 9: SEM images for lysozyme crystals made with precipitant solutions containing (top right, clockwise): 15% 4.5M NaCl & 3.5% PEG 8000; 7% 4.5M NaCl & 9% PEG 8000; 12% 4.5M NaCl & 5% PEG 8000; and 18% 4.5M NaCl, 4% PEG 8000 & Glutathione.

4.4. Lysozyme Nanocrystallisation - Additives and crystallisation conditions

Different additives and crystallisation conditions were screened to reduce the salt concentration in the precipitant cocktail as to make the crystals easier to jet in air.

The 5 solution series, based on previous screens, tests absolute ethanol, PEG 8000 50%, tacsimate 100% pH4 and poly-(propylenlykol) as additives whilst reducing the salt concentration to 5%. With preliminary analysis, 5B and 5F deemed to be the most successful precipitant solutions. Their compositions are outlined in Table prec.

A few of the 5 series precipitant solutions were used to crystallise lysozyme of concentration 100mg/ml. A larger volume of crystals was produced when using a higher concentration of lysozyme but these crystals need to be characterised in order to determine the effect on crystal size.

Based upon the two most favourable precipitant solutions, 5B and 5F, the 6 and N series were done with the aim of fine-tuning the concentrations of the successful additives to give the optimum crystallisation conditions. Isopropanol was also tested as an additive in this series and proved successful. The N series also reduces the salt concentration to 2.5%.

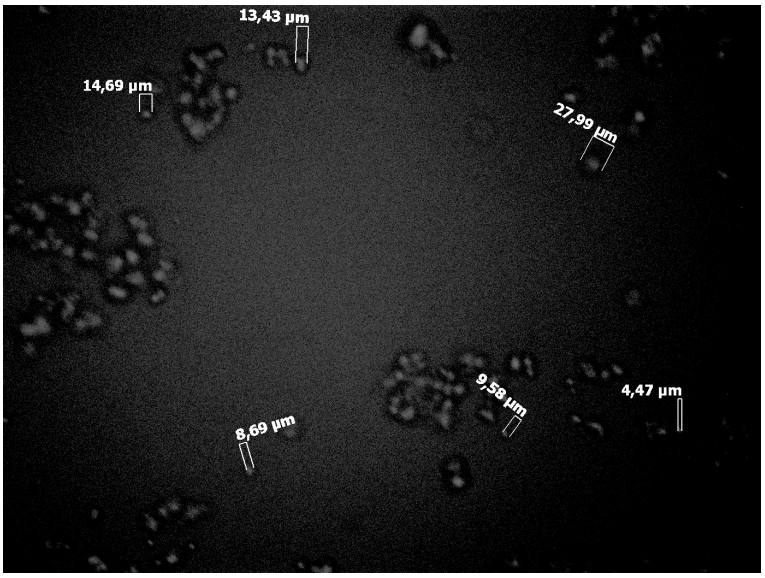

Figure 10: SEM image of protein nanocrystals made with alcohol additives at 4^oC.

Falkner found that smaller crystals were produced at lower temperatures so this theory was tested in the C series with alcohol additives at 4^oC that had already been tested

in the 5 and 6 series. At this lower crystallisation temperature, crystals were found to be larger than they previously had been when crystallisation occurred at room temperature. An example of this can be seen in Figure 10.

Lysozyme was also tested with a 1M TRIS buffer instead of the NaOAc buffer otherwise used throughout this project. It was tested with different PEG weights and 2-Methyl-2,4-pentanediol (MPD) at different concentrations. Only T1 out of the first four precipitant solutions in the T series successfully crystallised.

4.5. Microcrystals for P11 beamtime

2μm crystals were required for the P11 beamtime which took place between the 21st and the 26th August. Part of this project was to help make these crystals. Tests were carried out and results of these were used to choose crystallisation conditions for the crystals that were chosen for the beamtime. Figure 11 shows examples of beamtime crystals made including which were used HiPEG+ crystals that were used for the majority of the beamtime. Figure 12 shows one of the diffraction patterns obtained during this beamtime.

4.6. Test-protein Nanocrystals

The final part of the project was to carry out initial tests on other proteins for crystallisation additives. Glucose Isomerase was tested with precipitant cocktails adapted from Hampton Research data. This initial screening deemed successful, with numerous additives being tested.

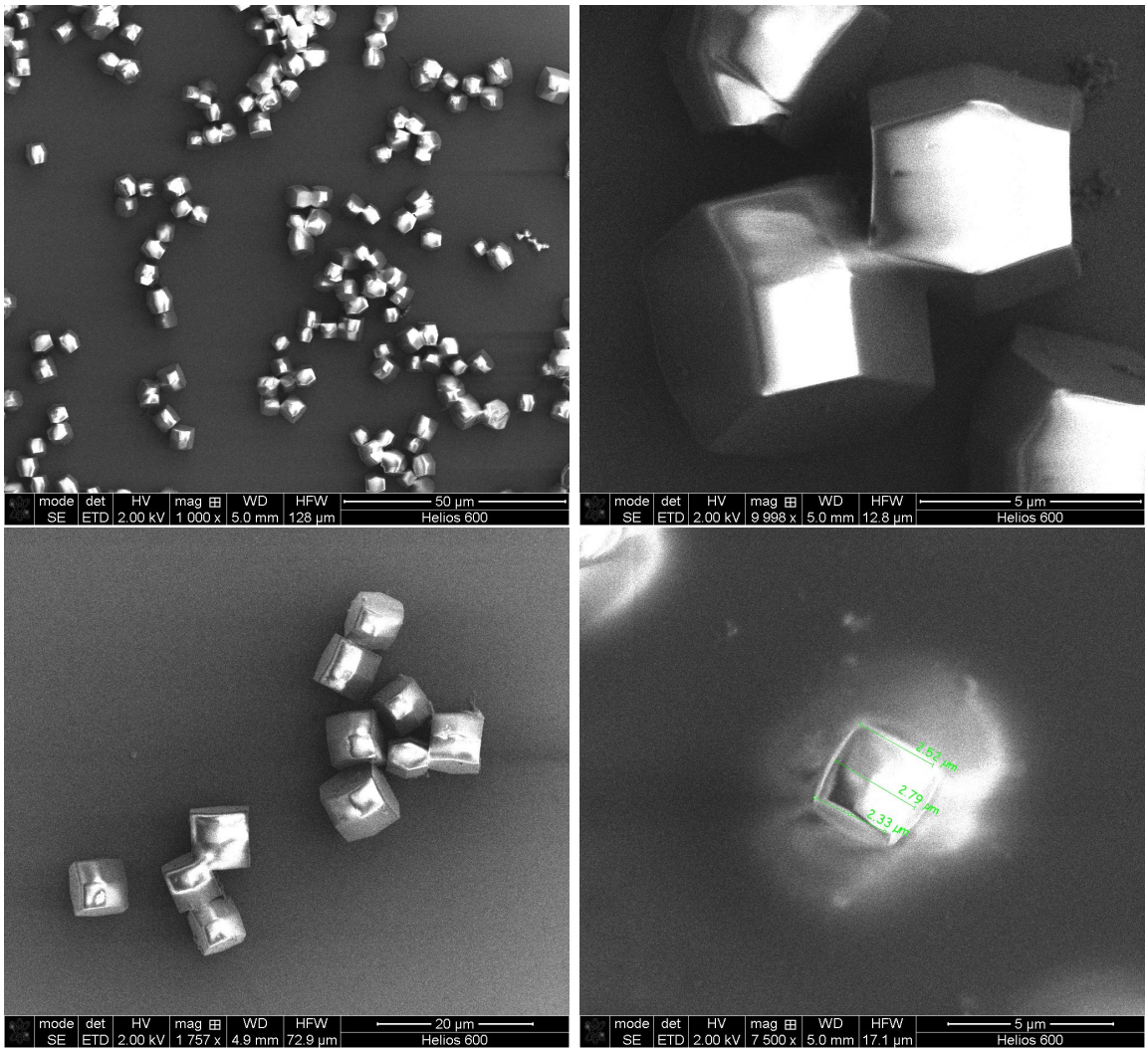

Figure 11: SEM images of beamtime crystals made with top: a Zinc additive; Bottom: HiPEG+

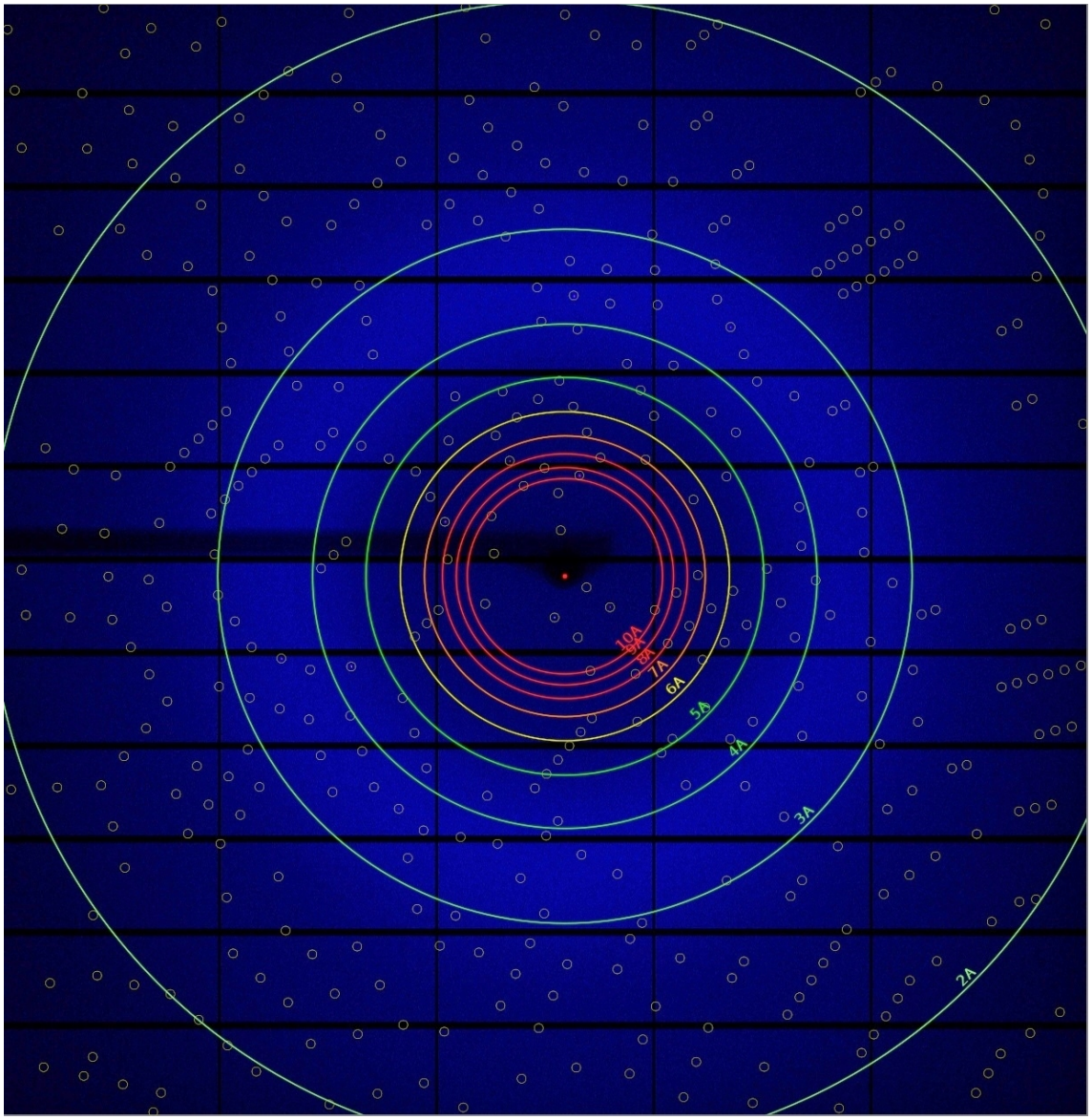

Figure 12: An example of the diffraction patterns obtained during August's P11 beamtime.

5. Conclusions

After eight weeks work on this project, the following conclusions can be drawn. The Libro plate DLS instrument is easier to use and produces better results than the cuvette DLS instrument. Cross-linking enabled good pictures of samples to be taken using the SEM and also gave a more accurate DLS measurement. Falkner's research on how salt and PEG concentrations affected crystal size and quality was reproduced. Ethanol, isopropanol and tacsimate were found to be the most promising when screening for additives to reduce the salt in precipitant solutions. When alcohols was used as an additive, crystal size appeared to increase with decreasing crystallisation temperature, contrary to Falkner's research. Initial tests were carried out using a TRIS buffer produced a positive result. A different concentration of lysozyme was also used for crystallisation, producing a greater volume of crystals for a greater concentration of lysozyme. Initial screening of Glucose isomerase was carried out, producing positive results. Tests were undertaken to help produce the crystals used in August's P11 beamtime.

Further work that could be carried out includes:

- Preliminary observations were noted during this project but further analysis should be done on series 5,6, C, N, G, T and GI before further tests are carried out.
- Characterisation of the samples made with 100mg/ml lysozyme and the 60mg/ml lysozyme samples made with the same precipitant solution. This will allow the effect of lysozyme concentration on crystal size to be determined. Then continue tests with 60, 100 and 120mg/ml lysozyme.
- Continue to screen Glucose Isomerase for favourable crystallisation conditions.
- Sputtering samples with gold before viewing under the SEM to produce higher quality images.
- Using a stirrer to mix the solutions during crystallisation for a less broad distribution of crystal sizes.
- Test how crystal size depends on crystallisation temperature with different additives.

6. Acknowledgements

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