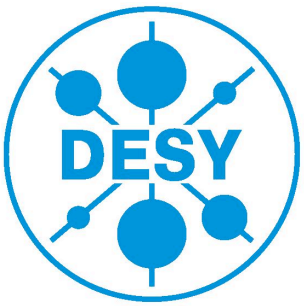

Creation and Characterization of Nanocrystals with in situ Dynamic Light Scattering

João Jacinto, Universidade Nova de Lisboa - Faculdade de Ciências e Tecnologias, Portugal

September 11, 2014

Abstract

This project was done as part of DESY Summer Student Programme 2014. The objective of the project was to perform in situ Dynamic Light Scattering,DLS measurements of different biomolecules. A flow system was used to characterized polymer nanospheres and lyzosyme crystals obtained ex situ and in situ. To perform in situ crystallization a precipitant solution of sodium chloride was added. Crystals obtained were characterised using Optical Microscopy, Dynamic Light Scattering and X-ray diffraction revealing a hydrodynamic radius of 165nm. This method has proven to be suitable for detection of in situ crystallization. Vitamin B12 crystallization trials were also performed without success in achieving relevant measurements.

Contents

1 Introduction

This work was conducted in Coherent Imaging group in CFEL. The main goal of the group is to realize Serial Femtosecond Crystalography. A new concept of structure determination is being developed, single shot diffraction patterns are collected from a stream of nanocrystals using Femtosecond pulses from an X-Ray Free Electron Laser (XFEL).

With this approach each crystal is hit by a single X-Ray pulse that forms a single diffraction pattern before being eliminated. The patterns are read by a set of detectors, one that is close, records with a high resolution since it detects the large scattered angles. The detector which is far away from the sample helps to reconstruct the shapes of the crystals using normal algorithms of diffractive imaging[1]. This setup is illustrated in Figure 1.

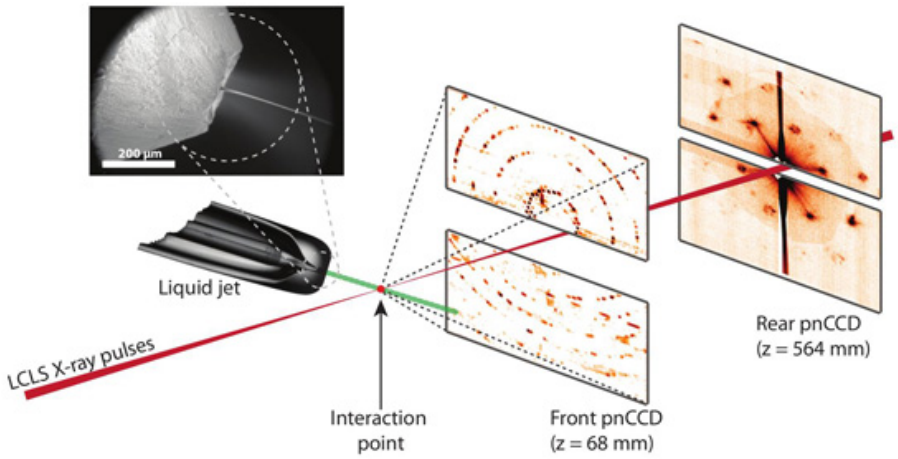

Figure 1: Experimental set-up for serial femtosecond crystallography.

The structure of biomolecules is of most importance. This knowledge is used by for instance the pharmaceutical industry, in order to design more efficient drugs.

The objective of the project was to adjust in the best way possible the crystallization of biomolecules that can in future be used as samples in the method above described. In situ DLS flow measurements of ongoing crystallization process were used to access the feasibility of the method.

2 Theory

2.1 Protein Crystallization

Producing high quality crystals has always been a bottleneck to structure determination. The task of producing suitable crystals may be tackled using two approaches. Empirical techniques, which are based in trial and error, or by gaining understanding of the fundamental principles that govern crystallization. The knowledge may be applied to design experimental methodology for producing high quality crystals of medical and industrial interest.

X-ray crystallography is one of the most powerful techniques that can be used to obtain the structure of a protein; however this method requires highly ordered crystals.

The crystallization process can be illustrated by a phase diagram, which indicates which state is stable under a variety of crystallization parameters. Such as concentration of protein, precipitants or additives.

Figure 2 shows the different degrees of supersaturation. High supersaturation where precipitation of protein will occur, moderate supersaturation, where spontaneous nucleation takes place, the metastable zone where crystals are stable and may grow but no further nucleation occurs(this zone is the best one for crystals formation), and undersatured region where no nucleation occurs.

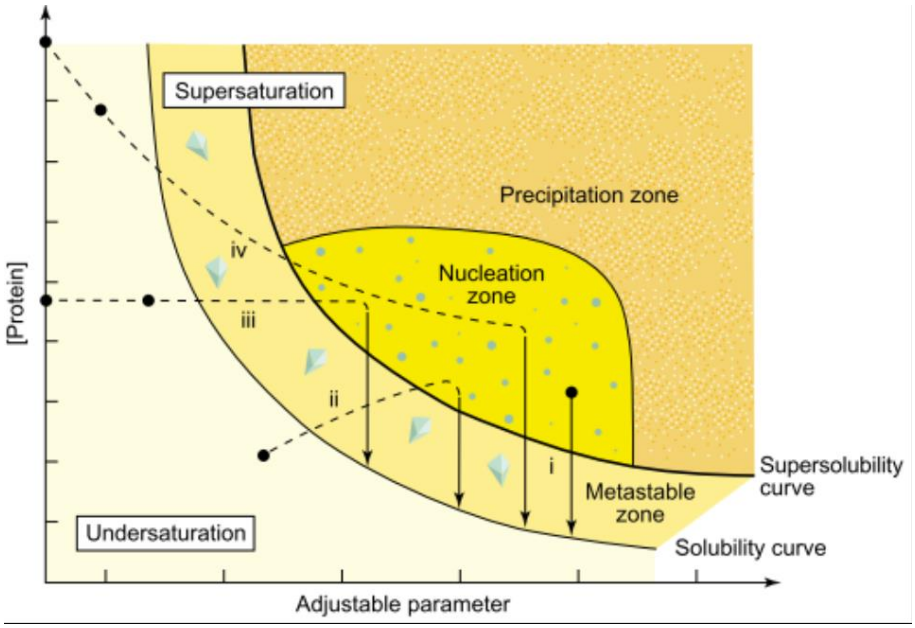

Figure 2: Scheme of crystallization diagram

The key to crystallization is nucleation. This can be of two types, homogeneous (in the bulk of the solution) or heterogeneous which can be caused by solid material in the medium, charged surfaces or the walls of the crystallization vessel. Even though it is hard, it is possible to control this various factors.

Every protein crystallizes differently, some with ease and other simply do not form suitbale crystals.[2]

2.2 Dynamic Light Scattering

When light impinges on matter, the electrical field of light will induce an oscillating polarization of the electrons in the molecules. These will act as a secondary source of light that will scatter it. The shifts in frequency, angular distribution, polarization and intensity of light are determined by size, shape and molecular interactions in the scattering material. Given this and with the aid of electrodynamics and theory of time-dependent statistical mechanics it is possible to obtain information regarding the structure and molecular dynamics of the scattering medium[3]. DLS is a powerful tool for characterizing small particles and a cheap useful method that can be incorporated into a wide range of instrumentation.

Particles that are small when suspended in a fluid undergo Brownian motion, therefore when hit by a laser there is a time dependence of the fluctuation in the scattering intensity. It is possible to describe this mathematicaly using the correlation function given by equation 1 .

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

Where B is the background, α is a constant which depends on the spatial coherence of detected scattered light, τ is the delay time and $C'\left(\tau\right)$ is the normalized scattered electric field correlation function.
It is possible to fit an exponential decay to $C'\left(\tau\right)$, for a monodisperse protein species, equation (2)

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

Where Γ, is the decay constant and directly proportional to the diffusion coefficient (3)

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

With q, being the scattering vector.
Using the Stokes-Einstein relation, equation (4), is possible to measure the hydrodynamic radius of particles, according to this equation k_b is Boltzmann's constant and η is the solvent viscosity.[4]

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

Given this the correlation function is a suitable method to estimate the radius of particles in solution.

3 Method

3.1 Instrumentation

SpectroSize 300 by Xtal Concepts was used to characterize the crystallization process. This instrument makes use of different glass/quartz cuvettes which have to be filled with 20 μl of the sample. In the work conducted the flow mode was used.

SpectroSize 300 was coupled with NEMESYS syringe pumps, by CETONI , automation and micro systems, Low and Medium pressure modules. In this configuration it is possible to measure different samples at the same time, mix them and measure the samples in situ. Both modules are similar however in low pressure module the syringes have to be manually filled. In situ DLS is measured with flows of the different samples mixed.

All fittings and valves used were obtained from BESTA -Technik GmbH and the capillaries were obtained from Optronics.

In order to confirm if crystals were being formed, a Olympus SZX16 microscope was used to examine the samples.

X-Ray diffraction patterns were obtained with Rigaku MicroMax-007 HF.

3.2 Biomolecules Crystallization Experiments

A series of measurements were carried out using Duke Standards Polymer Microspheres with known diameters in order to calibrate the SpectroSize 300.

With the system calibrated and since that the experimental setup was not done before a procedure, for in situ crystallization, had to be created in order to obtain reliable readings.

Procedure:

- Filtration of the samples
- Molecule flow
- Precipitant flow
- Find best flow ratio
- Data acquisition
- Water flow to clean

It is important that all the samples are filtrated in order not to interfere with the readings, 0.10μm filters from Sartorius were used. The molecule that is going to be crystallized has to fill the system first, otherwise the readings will not be able to occur and the system might clog. In order to clean the system it is necessary to flow water through it, this way clogging is prevented. All the fittings should be cleaned through sonication in order to preserve the system.

In figure 3 the setup of NEMESYS syringes pumps, medium pressure module, and SpectroSize 300 detector is shown. Both syringes are connected to a T-piece where solutions mix. A third capillary where the mix solution flows is connected to the detector.

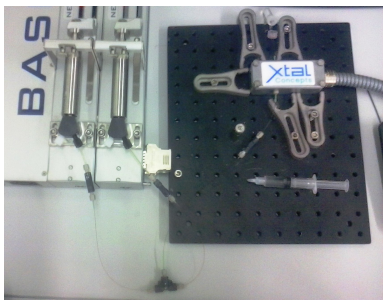

Figure 3: Setup of experiments

4 Results

4.1 Ex situ Crystallization

The low pressure module and 50μm capillaries were used with Duke Polymers Nanospheres and ex situ grown lysozyme crystals. This trials were realized in order to calibrate the system and study the best flow rates for obtain consistent data for in situ crystallization trials.

In figure 4 from left to right, top down, the 0.1μm, 0.4μm, 0.6μm and 1μm Polymer Nanospheres radius distribuition is shown. All runs had duration of 600 seconds and 20μl/min.

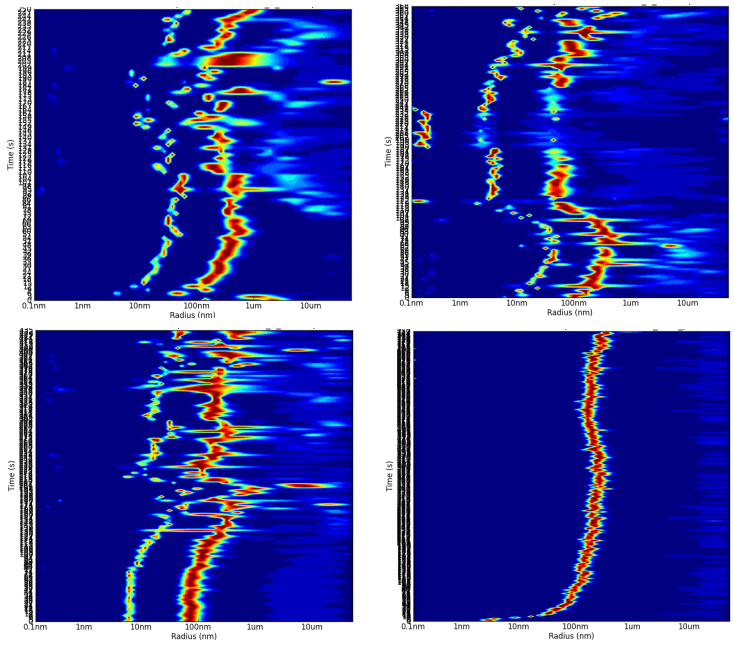

Figure 4: Distribuitions of radius of Polymer Nanospheres

Since it was the best data obtained, the summary 1μm Polymer Nanospheres is presented in figure 5.

For lysozyme a flow of 0.1μm/ml was used the radius distribuition and summary is shown in figure 6 and 7 respectively. The duration of the measures was 300 seconds.

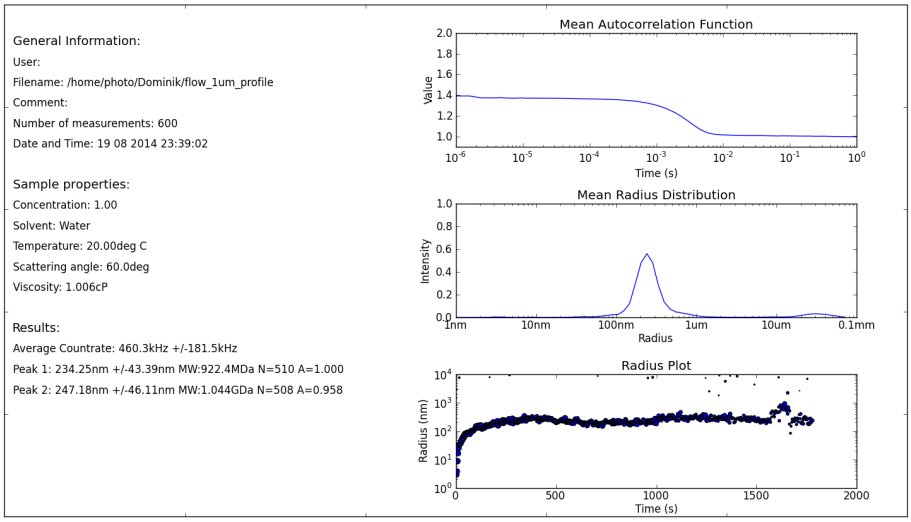

Figure 5: Summary of 1μm Polymer Nanospheres

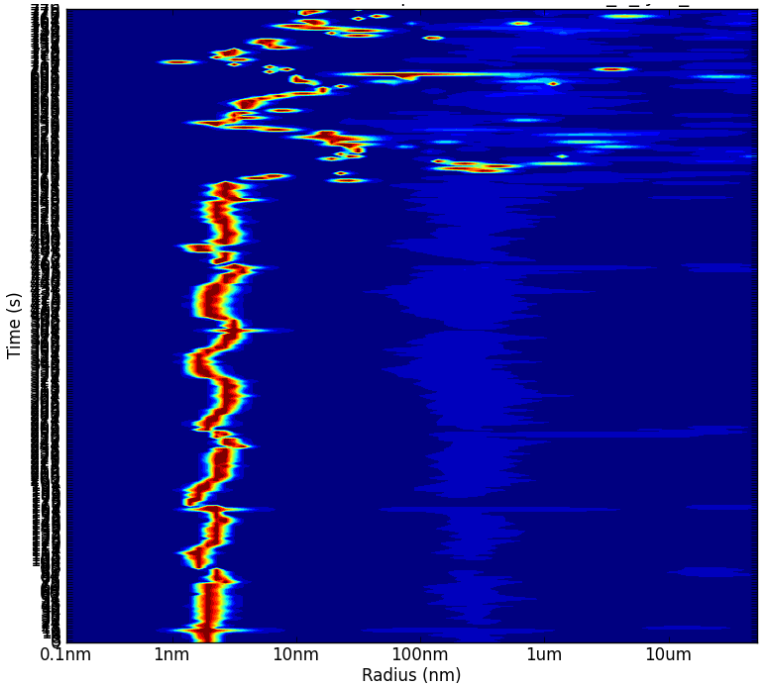

Figure 6: Distribuition of radius of ex situ lysozyme cristals

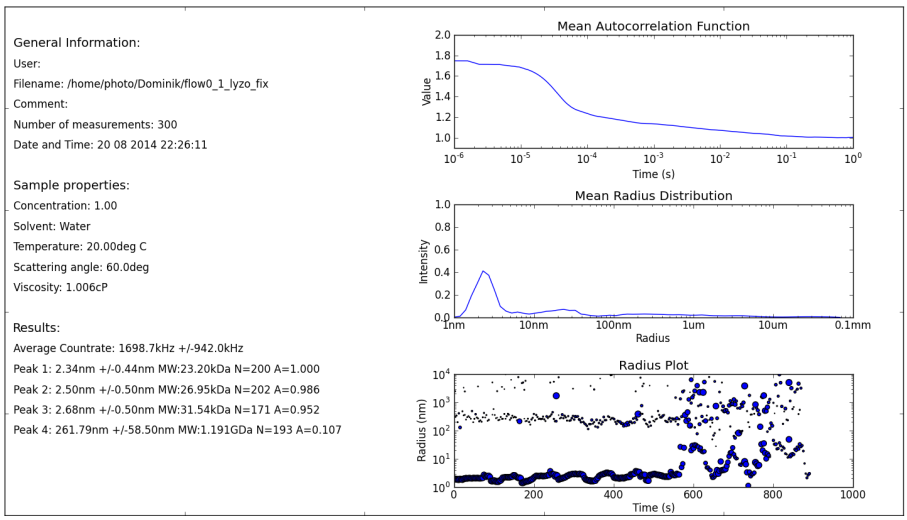

Figure 7: Summary of ex situ lysozyme cristals

4.2 In situ Crystallization

In table 1 the flow rate used for lysozyme runs with the lower pressure module and 50μm capillaries is presented. The protein concentration is 150mg/ml in acetic acid 0.5M. These trials were made using 1M and 0.5M sodium chloride as precipitant solution.

Table 1: Flow rate optimization for Lyzosyme

Flow Rate $[\mu l/min]$	
Lyzozyme [150mg/ml]	Sodium Chloride
10	20
5	20
2,5	20
1	20
5	15

In figure 8 from left to right , top down: 1μl/min ,2.5μl/min, 5μl/min and 10μl/min of protein solution and 0.5M sodium chloride 20μl/min, distribuition ratios. The duration of the readings was 400 seconds for the first two and 500 seconds for the last two.

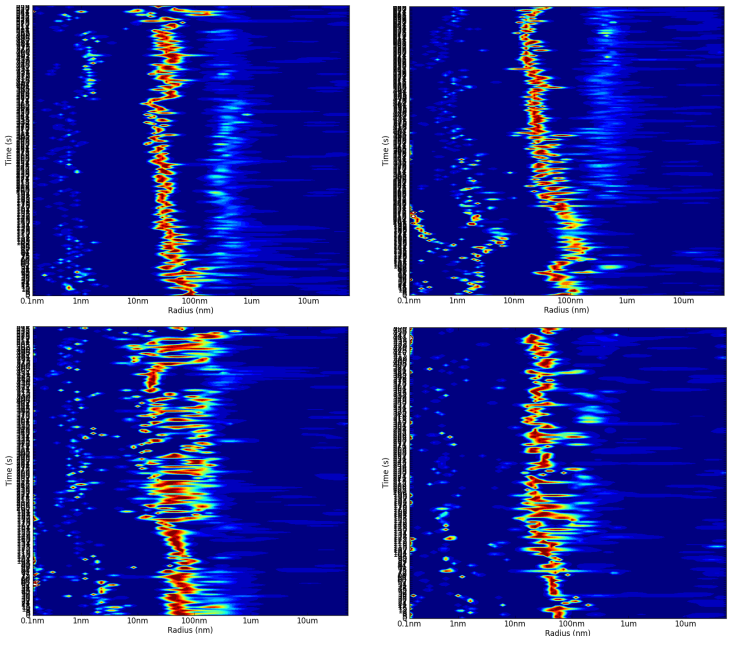

Figure 8: Distribuitions of radius 0.5M sodium chloride

In figure 9 from left to right , top down: 1μl/min ,2.5μl/min, 5μl/min and 10μl/min of protein solution and 1M sodium chloride 20μl/min, distribuition ratios. The duration of the readings was 500 seconds for the first, 1500 seconds for the second, 1000 seconds for the third and 500 seconds for the last.

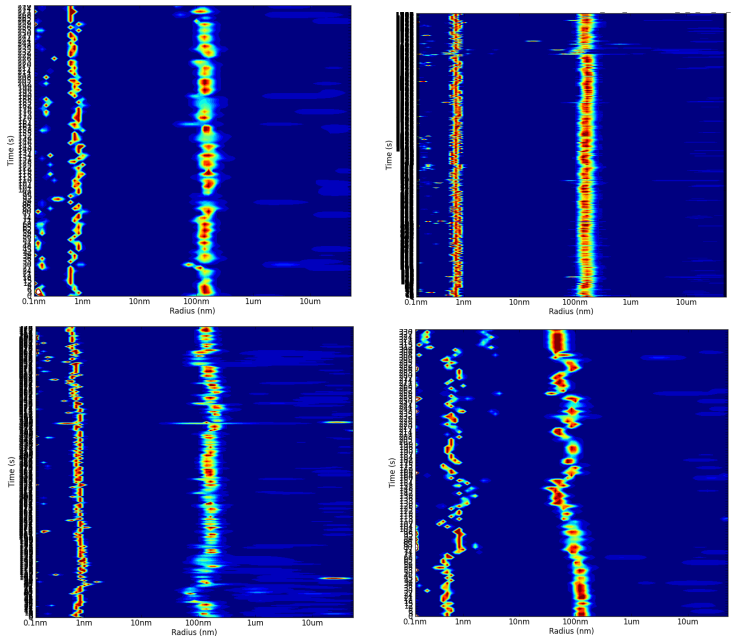

Figure 9: Distribuitions of radius 1M Sodium chloride

The summary of the best run, 2.5μl/min protein and 20μl/min 1M sodium chloride is shown in figure 10.

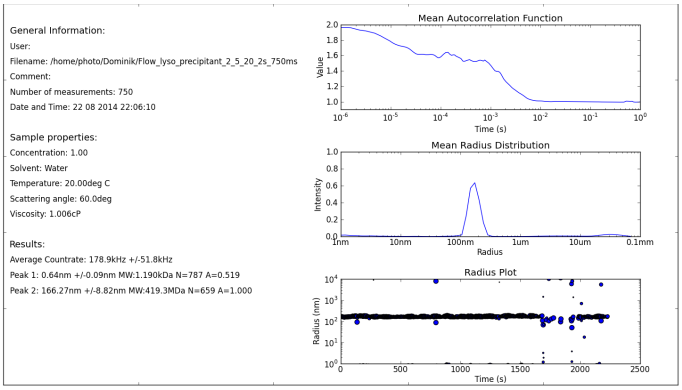

Figure 10: Summary of 2.5μl/min protein and 20μl/min 1M sodium chloride

In figure 11, distribuition radius, and 12 ,summary of the run, is possible to observe the difficulties that arouse in obtaining reliable data with the medium pressure module and

100μm capillaries. The flow rate used for protein and 0.5M sodium chloride was 3μl/min and 40μl/min respectively, the duration of the reading was 840 seconds.

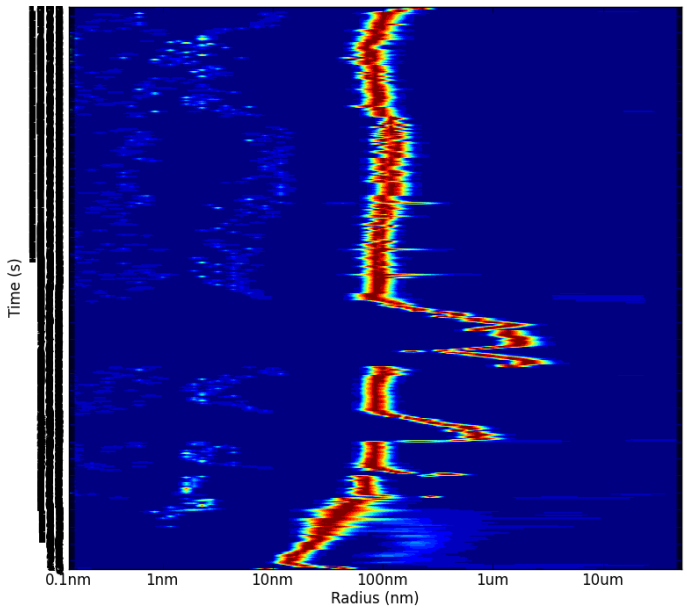

Figure 11: Distribuition of radius 0.5M Sodium chloride

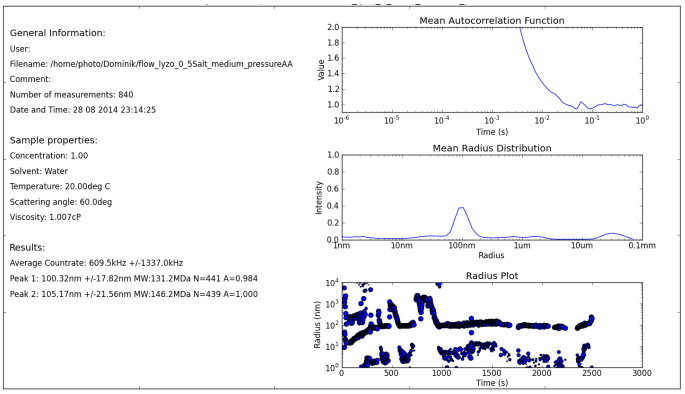

Figure 12: Summary of 2.5μl/min protein and 20μl/min 0.5M sodium chloride

The crystals obtained are shown in figure 13, with average size of 4 μm.

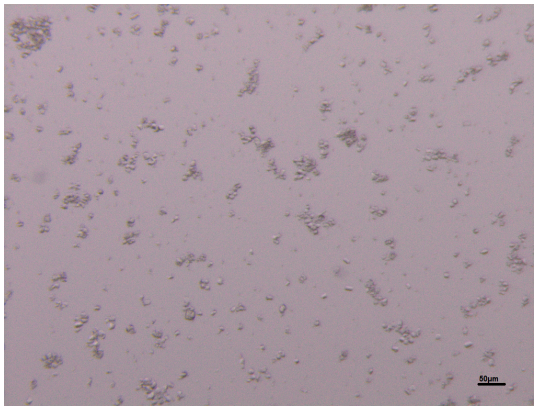

Figure 13: Crystals obtained with medium pressure module

In figure 14 the diffraction pattern obtained for lysozyme crystals is presented, in figure 15 the diffractogram of lysozyme powder and lyzosyme ex situ formed crystals is shown.

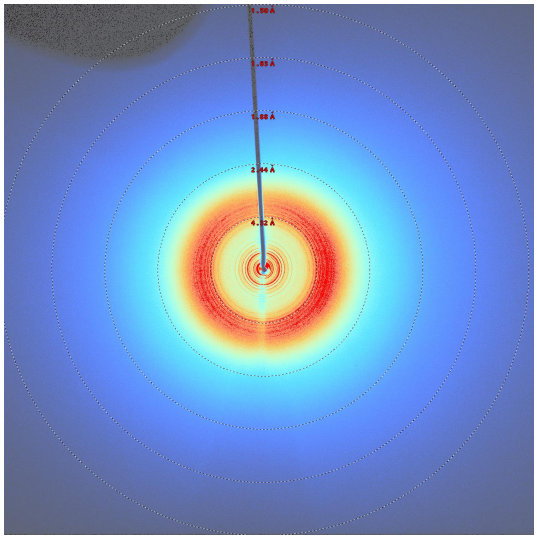

Figure 14: Diffraction pattern of lysozyme crystals obtained

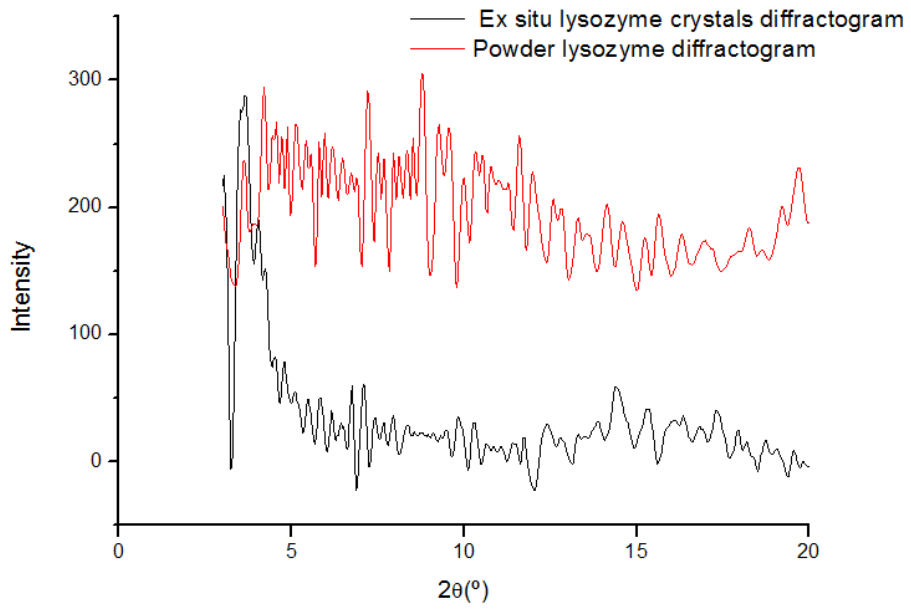

Figure 15: Diffractogram of lyzosyme powder and lyzosyme ex situ grown crystals

For vitamin B12 and acetone as precipitant, due to difficulties with the medium pressure setup and 100μm capillaries it was not possible to retrieve reliable data, of crystallization. However a baseline only with Vitamin B12 was attempted and the summary is presented in figure 15.

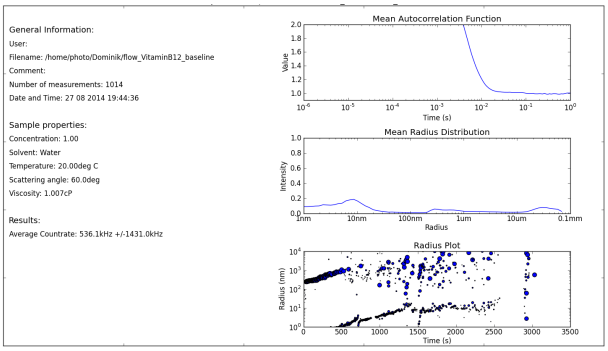

Figure 16: Baseline attempt of Vitamin B12

5 Conclusions

Both the polymer nanospheres and ex situ grown lysozyme crystals gave a good indication of what flows should be used in order to obtain a good signal for the in situ crystallization trials. It was patent that there was no need for high flow rates. The information provided by these runs was fundamental to the in situ crystallization trial.

The best and most stable flow profile for lysozyme was achieved with 2,5μl/min protein and 20 μl/min 1M Sodium Chloride. Low pressure module and 50μm capillaries were used. It was not possible with the time available to try to reproduce these results with the medium pressure module and 100 μm capillaries. Crystals were present in the output solution of medium pressure trials however, due to decalibration of the detector no signal was retrieved.

According to the DLS data presented it was possible to crystalize and measure lysozyme crystals. These had around 165nm hydrodynamic radius, at the flow mentioned above. It is important to understand that the radius presented by the DLS setup presented grows smaller as velocity of the flow increases. There also seems to exist an artifact with a lower radius than the crystals, this can also be the protein monomer, only through DLS it is not possible to determine which one it is. With the use of low pressure module some leakings occured, therefore it is expected that the actual flow of the trials is in fact lower than the actually used.

According to the diffraction patterns acquired, good quality in situ lyzosyme crystals were obtained, it is possible to observe rings up to 3Åand with the diffractograms presented in figure 15 the conclusion is that the crystals are of good quality..

Vitamin B12 revealed to be particularly hard to work with since it jellifies and clogs frequently. The precipitant of vitamin B12, acetone, also requires different tubing since it destroys the capillary system.

Regarding the setup, clogging is a serious issue when conducting the experiments therefore extra attention should be given to the cleaning of the capillaries and making sure all connections are firmly tight. This will also help with the leaking problems that are frequent. The DLS detector is also extremely sensible and requires tuning in order to obtain accurate measurements.

The work conducted serves as a proof of concept that detection of in situ crystallization with this setup is possible. It is also a good starting point for future work to be conducted using this method to serial femtosecond crystalography.

Future work to be conducted :

- Optimization of lysozyme crystallization with medium pressure modules and 100μm capillaries.
- Study of the influence of different flow rates in lysozyme crystals size.
- Vitamin B12 trials should be redone.
- Study of the lenght of capillaries and the effect in the crystalization process.
- Creation of flow profiles.

6 Acknowledgements

I would like to thank the following people:

- My supervisor Dr.Dominik Oberthür for the help and guidance.
- To the Coherent Imaging Division group which welcomed me.
- To all the organizers of the Summer Programme which make it possible.

List of Figures

List of Tables

References

[1] http://desy.cfel.de/cid/research/serial-femtosecond-crystallography/ data accessed 2/08/14

[2] N. E. Chayen, "Turning protein crystallisation from an art into a science.," Curr. Opin. Struct. Biol., vol. 14, no. 5, pp. 577â83, Oct. 2004.

[3] B. J. Berne and R. Pecora, Dynamic Light Scattering: With Applications to Chemistry, Biology, and Physics. Dover Publications, 2013.

[4] W. William Wilson," Light scattering as a diagnostic for protein crystal growth, A practical approach" J. Struct. Biol., vol. 142, no. 1, pp. 56â65, Apr. 2003.