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Pico-litre Sample Introduction and Acoustic Levitation Systems for Time Resolved Protein Crystallography Experiments at XFELS

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Abstract: The system described in this work is a variant from traditional acoustic levitation first described by, Marzo et al [1]. It uses multiple transducers eliminating the requirement for a mirror surface, allowing for an open geometry as the sound from multiple transducers combines to generate the acoustic trap which is configured to catch pico litres of crystal slurries. These acoustic traps also have the significant benefit of eliminating potential beam attenuation due to support structures or microfluidic devices. Additionally they meet the need to eliminate sample environments when experiments are carried out using an X-ray Free Electron Lasers (XFEL) such as the Linac Coherent Light Source (LCLS) as any sample environment would not survive the exposure to the X-Ray beam. XFELs generate Light a billion times brighter than the sun. The application for this system will be to examine turn over in Beta lactamase proteins which is responsible for bacteria developing antibiotic resistance and therefore of significant importance to future world health. The system will allow for diffraction data to be collected before and after turnover allowing for a better understanding of the underling processes. The authors first described this work at Nanotech 2017 [2].

Keywords: XFEL, Pico-litre Sample delivery, Time resolved, Protein crystallography, Beta lactamase, Acoustic levitation.

1. Introduction

With the emergence of X-ray Free Electron Lasers in recent years, with high flux and photon delivery rate, there are extensive opportunities for room temperature investigations into structures of proteins. More so, the delivery rate of these X-rays also make them an ideal candidate for carrying out time resolved experiments. These entail the investigation of a protein's structure and comparing the structure of another crystal of the same protein after it has been exposed to an external stimuli either light or additional biochemistry, gaining its altered structure data. All too often we understand the structural changes of proteins before and after with no interstitial states [3]. As this method matures, more and more of nature's biochemistry will be more fully understood.

The beam from an XFEL has a flux density that is orders of magnitude greater than synchrotron light and when delivered at a frequency of 100 Hz (as it is at the LCLS XFEL), data can be obtained prior to the destruction of the protein crystal due to radiation damage, even at room temperature. In a typical data collection at a synchrotron, over 90 % of the X-rays used to yield diffraction data lead to the sample's destruction. This is the case even with the sample being presented at cryogenic ground state [4].

The perfect sample delivery system would offer a protein crystal of choice surrounded by minimal mother liquor and no support or sample environment. The reasoning behind this is twofold: any sample support or environment would instigate attenuation of the beam and loss of diffraction data due to attenuation and any sample environment with continued X-ray exposure will eventually be destroyed.

From these key requirements for an XFEL sample delivery perspective, we determined a very good candidate would be acoustic levitation initially with traditional acoustic traps [5] and more recently using so called acoustic tractor beams as demonstrated by Marzo *et. al* [1]. This approach would facilitate a sample support with no frame or sample environment. The devices are capable of levitating a single drop with the ability to rotate the sample in order to facilitate multi axis diffraction patterns from the crystals suspended within. In essence these devices can be used as acoustic goniometers capable of multi axis data collection from a single crystal. This will dramatically reduce beam attenuation and eliminates the destruction of the sample environment.

The minimum amount of mother liquor will be dependent on the delivery system to the acoustic trap. The choice candidate for sample and reagent introduction to the trap is an acoustic based pico-litre sample delivery system capable of delivering quantities as small as 10 pico-litres and at a rate of up to 10,000 Hz supplied by PolyPico Technologies Ltd.

The system will operate at room temperature in a helium back filled environment to reduce beam attenuation so it will automatically lend itself to being further developed for time resolved studies. The system as described in the work of Marzo [1] requires significant modification to allow the support of such small samples, and to allow for appropriate sized traps in the helium environment. The PolyPico system can dispense pico-litre volumes of mother liquor containing the beta lactamase crystals and then when appropriate deposit substrate to initiate a reaction. Previous workers have used induction based microfluidics but this limits the drops launched to the nanolitre domain. Description of this technology will however still be described here.

2. The Biology

A significant obstacle to overcome in conducting time resolved studies into beta lactamase is creating the mixing between slurries of the crystals and the substrate that initiates the reaction. Suspending them in front of the beam and also having control of mixing rates is also challenging. In addition to this an optimal system will also co-ordinate the presentation of the crystal, the mixing of the substrate with the crystals and then illuminating the crystal with X-rays. In our system we propose to restrain the crystals with an acoustic trap and 'fire' a drop of substrate solution into the crystal prior to X-ray radiation using the PolyPico system.

The current biological system we intend to explore is the mechanism of the beta-lactamases; several families of proteins which catalyse the hydrolysis of beta-lactam antibiotics reducing the effectiveness of antibiotic treatment. We are currently using this system to aid our understanding of the Stenotrophomonas maltophilia beta-lactamase reaction mechanism which catalyse the hydrolysis of beta-lactam antibiotics [6]. The principle challenge of this work is to reduce the crystal sizes which the system can support, as smaller protein crystals allow for quicker substrate diffusion and therefore more coherent diffraction data. However, enabling the handling of smaller droplets requires increasing operating frequencies. Overcoming these challenges will create a method to facilitate time-resolved nano-crystallography experiments and will enable the unlocking of many of nature's secrets.

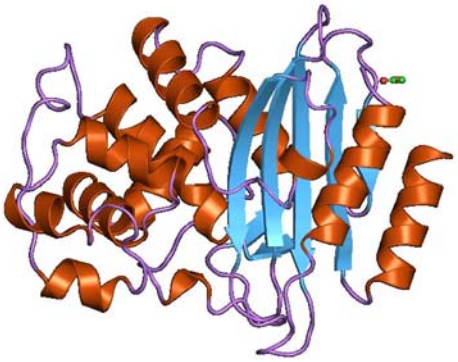

Fig. 1. Protein structure of 'Streptomyces albus beta-lactamase'.

These proteins are ideal for this task, not only because the biological question is exceptionally relevant in a post-antibiotic world, but also several substrates undergo a colorimetric change during catalysis so the X-ray diffraction data can be obtained whilst monitoring the activity of the enzyme.

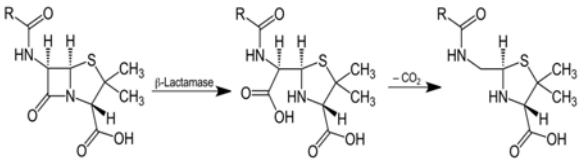

Fig. 2. Action of β-lactamase and decarboxylation of the intermediate.

The same colour change can also be used to study the efficiency of the mixing taking place. This allows for reaction times to be determined using visible light and a spectrometer aiding data collection with X-Rays when such reaction timing data is vital.

3. Acoustic Levitation

The concept of acoustic sample manipulation was first conceived by Kundt leading to the development of the Kundt's tube in 1866, initially to look at the speed of sound in gases [7]. The tube was a transparent horizontal pipe which contained a small amount of a fine powder. At one end of the tube was a source of sound at a single frequency (a pure tone). The other end of the tube was blocked by a movable piston which was used to adjust the length of the tube. Sound waves generated by a speaker at one end, reflected by a movable piston at the other allows for a resonant condition to be achieved. This means that the length of the round-trip path of the sound waves, from one end of the tube to the other and back again, was a multiple of the wavelength λ of the sound waves. Therefore the length of the tube was a multiple of half a wavelength. At this point the sound waves in the tube were in the form of standing waves, and the amplitude of vibrations of air are zero at equally spaced intervals along the tube, called the nodes. The powder was caught up in the moving air and settled in little piles or lines at these nodes, because the air was comparatively still there. The distance between the piles was one half wavelength λ/2 of the sound. By measuring the distance between the piles, the wavelength λ of the sound in air can be found. If the frequency f of the sound is known, multiplying it by the wavelength gives the speed of sound c in air:

$$c = \lambda f \ {\displaystyle c=\lambda f\,}$$

The detailed motion of the powder was in fact actually due to an effect called acoustic streaming caused by the interaction of the sound wave with the boundary layer of air at the surface of the tube.

A bi-product product of such a system however, was the fact that you can manipulate and place particles within the tube. The interest in this technology for this application focuses on using the acoustic energy to do exactly this, thus positioning samples without introducing any material into the beamline. Depending on the frequency there can be a single node or a multiple nodes where there is zero pressure and particles/droplets can be suspended within these zones.

In recent years, other workers have explored using acoustics in this way [8] including multiple transducers and mirrors so that the location and motion of particles can be more specifically manipulated in more than 1 dimension by controlling the relative phase of the acoustic signals.

A slight variant to this technology has been reported in Jan 2015 by Andrade [9] which allows for the distance between the reflector and the transducer to be varied on the fly into non-resonant conditions offering additional flexibility. A schematic of this concept can be seen in Fig. 3.

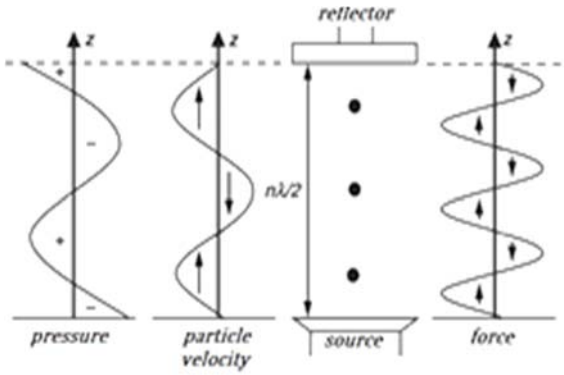

Fig. 3. Acoustic levitation principals [6].

The variant that is the focus of this work however is to build on the work of Marzo et al [1] where by constructing a system with multiple transducers focused to a single spot they can develop an acoustic trap without the need for an acoustic mirror as the configuration uses attractive forces also. With careful control of the phase relationships between multiple transducers, the particle suspended can be moved around, rotated and the whole system can even be inverted against gravity. A rendering of such a system in front of a beamline is shown in Fig. 4.

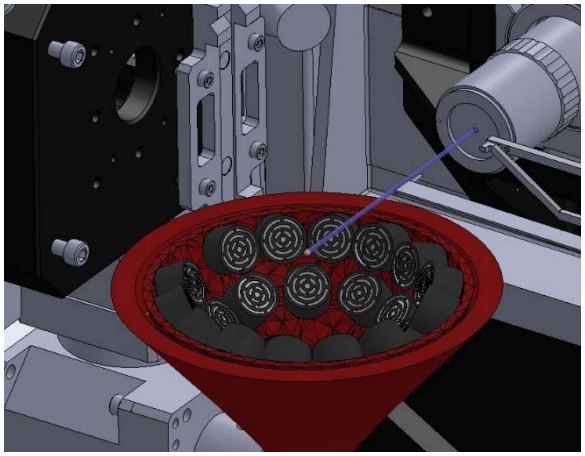

Fig. 4. Rendering of an acoustic pressure system suspending a particle in an X-Ray beam.

4. Sample Introduction

The task of launching the drop into the trap with precision and when the drops themselves are so small is not trivial. Two candidates are a possibility however only one offers the potential to launch Pico-litre quantities. For completeness both are described.

4.1. Induction Based Microfluidics (IBF)

IBF technology can kinetically project drops to targets of all types, and facilitate dynamically directing the liquids in flight to targets (a required, trait for small volumes of liquids). It works by electrically biasing the droplet to be launched and steering it using an electric field to its target, in this case the levitating droplet containing the crystals. Although not currently being used for protein crystallography experiments a relevant example of the technique can be seen in Figs. 5 and 6. The technique has been demonstrated in the literature for instigating time resolved reactions. [10] The suspended drop can be seen before and after a launched sample has been launched to meet it. For reference the launched sample is 400 nL.

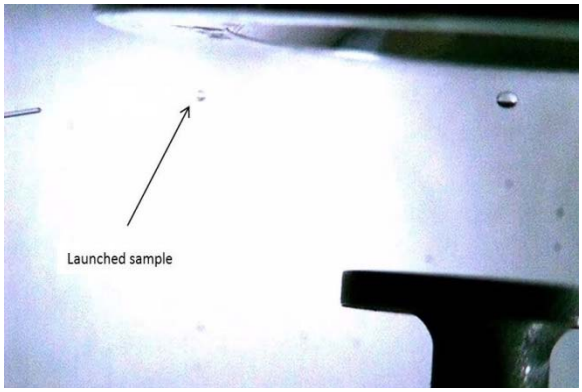

Fig. 5. IBF launching of an additional reagent [9].

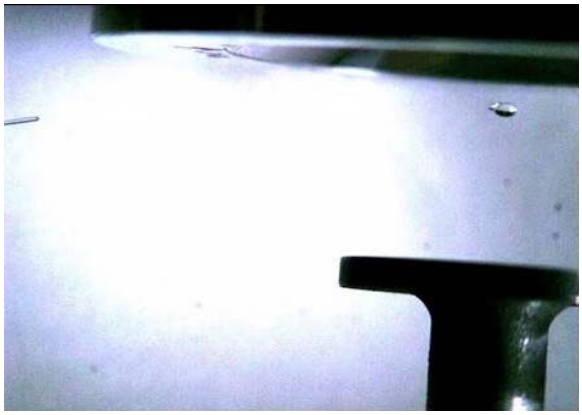

Fig. 6. At the point of mixing [9].

4.2. PolyPico System

PolyPico produce a drop-on-demand liquid handling system which uses acoustic pressure waves to instigate the ejection of micro-droplets of liquid from an orifice at the base of a dispensing cartridge. The system can be used to dispense substances such as: proteins; antibodies; nano-materials; crystals; living cells; bacteria; DNA; and a wide variety of organic and inorganic reagents [11].

Unique to this technology is the use of inexpensive disposable dispensing cartridges, which completely avoid cross-contamination risks and support quick fluid changeover. The volume of the micro-drops can be controlled to be in the range of approximately 10 pl to 120 pl (1pl = 10^{-12} Litres) depending on the fluid properties and dispensing can take place on-demand up to a frequency of 10 kHz. Fig. 7 shows a PolyPico dispensing head on the right and a dispensing cartridge on the left.

Fig. 7. (left) PolyPico dispensing head and (right) dispensing cartridge.

Depending on the characteristics of the fluid being dispensed, and the application, dispensing cartridges can be selected a range of orifice sizes (e.g. 30 um, 50 um, 70 um and 100 um).

The technology works by introducing an acoustic pressure wave into the column of fluid in the dispensing cartridge. This pressure wave then propagates in the fluid and focuses at the orifice of the dispensing cartridge. This focused pressure wave is sufficient to break the surface tension at the orifice and eject a very precisely controlled, and very repeatable, pico-litre volume of fluid from the orifice. Each time an acoustic impulse is introduced into the fluid column a micro-drop is ejected. Fig. 8 gives an example of a micro-drop being dispensed and its volume calculated by the PolyPico systems.

Fig. 8. An example of a micro-drop with a volume of 48.64 pl being dispensed [10].

This system is the only one currently available to actually offer the potential to dispense the pico-litre slurries of crystals required.

6. Current Systems for Sample Presentation to XFELS

Two leading techniques for sample delivery for XFELS are Liquid Jets and Tape drive delivery systems. Both are expensive, the former an order of magnitude more so. Liquid jets also yield a very low data collection rate typically only extracting diffraction data from a small fraction of the sample presented to the beam. As it runs in continuous mode, a lot of sample is wasted for a single experiment which is undesirable for expensive samples.

6.1. Liquid Jets

The majority of the XFEL data collection involves liquid jets as a delivery system. By creating a high pressure sheath around a central channel liquid jets of droplets containing crystals can be ejected and presented to the X-ray Beam [12] such as can be seen in Fig. 9.

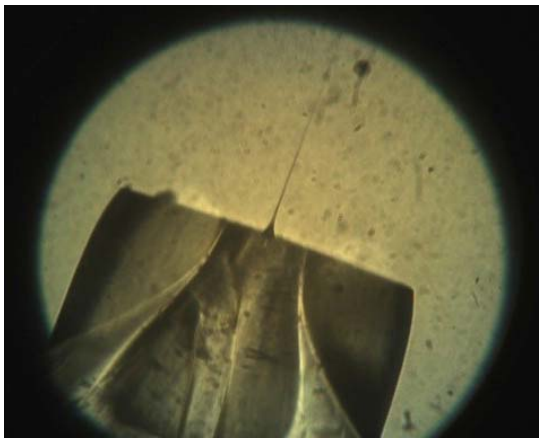

Fig. 9. In vacuum jetting of a capillary with asymmetric bore inside a square ID glass tube. Since the cone tip of the liquid capillary is centered inside the gas aperture, the jet emerges straight. The liquid cone attached to the glass cone tip is visible. Square glass tube OD: 0.6mm (wall to wall). Gas exit aperture diameter ~100 micron. [11].

Typical flow rates of samples can be anywhere between 10 and 20 microliters per minute for a jet diameter which is typically about 6 micrometers. When the liquid jet is running, the vacuum in the experimental chamber is $< 3\times10^{-5}$ Torr. However, although the tip that develops the liquid jet is small the infrastructure to support the jet can is significant (see for example Fig. 10).

Fig. 10. Differential pumping system for the liquid jet injector. It consists of two parts, the catcher shroud and the nozzle shroud, which can be decoupled and retracted via a bayonet coupling. This allows the use of fixed samples without breaking the vacuum.

6.2. Tape Drive Delivery Systems

The tape drive is a methodology for a drop on demand system used at XFELS [13]. It facilitates the ability to build up a queue of droplets that can arrive at the XFEL beam in a desired time frame. The droplets are deposited using an Acoustic Droplet Ejection (ADE) device to deposit drops from a reservoir. Typically they can deposit droplets ranging from 0.8-6 nl routinely. A 250 micron capillary supplies and maintains constant reservoir levels the ADE delivers from allowing ejection conditions to remain stable and maintenance-free for hours. Typically a focused XFEL beam ($< 25\ \mu m^2$) that passes parallel to the belt surface probes the droplet presented on the belt. After X-rays probe the sample, the belt is cleaned before it loops back to the start.

To date this system has been used to explore photosystem two [14], a protein that is responsible for photosynthesis whereby the protein could be activated by light prior to delivery to the beam and diffraction data compared to the same protein without light activation. Drop on drop experiments are planned to also study beta lactamase. The system is large in size and highly complex.

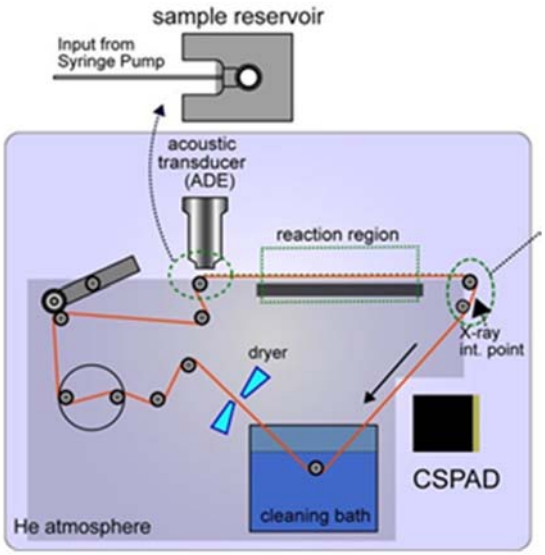

Fig. 11. Schematic of the Tape drive delivery system [12].

7. Challenges

The principal challenge is the development of a methodology for introducing pico-litre droplets to the trap. Although the size of the droplet supported can be reduced as frequency is increased a droplet is still required to be of a nominal size. This is made further complicated by the change in wave speed between air and helium. The phenomenon is described by its Bond number. This is the ratio between the liquids surface tension, density and its own size. If this number is too low the drop will burst. Sound intensity, whilst allowing for the suspension of denser drops, can distort the geometry of the droplet being suspended.

With the Marzo [1] style transducer systems, fully understanding the relationship between surface area, mass and trap configuration in a helium environment will form the most significant challenge.

In addition to these fundamental overarching principles, the system will need to be made to be compatible with X-ray facilities such as the LCLS and Diamond Beamlines. These limitations include physical space and the requirement for the auxiliary hardware to operate in a helium environment.

8. Initial Set Up

The initial set up employed is an adaptation to previous work conducted by Marzo *et. al* [1]. The arrangement employs multiple transducers see Fig. 12 in order to form an acoustic trap which is used to confine the sample in all axes, within the center of the construct owing to a phase difference between the transducers.

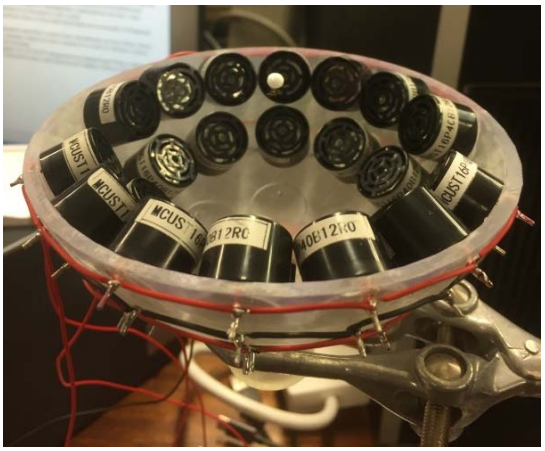

Fig. 12. Reproduction of the Marzo [1] acoustic tractor beam system levitating a polystyrene bead.

By mounting the transducers in a 3D printed dish structure the system can suspend, rotate and grip the suspended drop even when inverted against gravity. A microcontroller is used to generate different phases of the ultrasound frequency which provide the input signal to motor driver chips that in turn drive the ultrasound transducers. Marzo et al. [1] demonstrated that a simple two phase arrangement was adequate to allow the trapped particles to be suspended, utilizing more phases to rotate or otherwise manipulate the sample. In this configuration, the device can be said to be a levitating goniometer for sample presentation to the X ray beam.

The low power offers the potential to maintain the Bond number for the droplet size at a sustainable level. The transducers used in this system are Multicomp MCUST16P40B12RO (see Fig. 13) which are inexpensive. The mounting dish is readily printed using most standard 3D printers. There is a relationship between the maximum mass of object which can be levitated (owing to the transducer power) and the smallest size of object which can be levitated (owing to the wavelength and phase relationship). In order to levitate samples which are most relevant for room temperature analysis in the XFEL beam, these transducers are to be replaced by higher frequency devices to facilitate the entrapment of smaller droplets in the beamline device. This will allow us to levitate nanolitre and pico litre droplets containing nanometre crystals in their mother liquor.

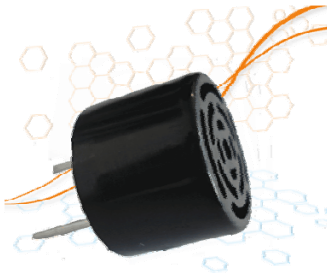

Fig. 13. Multicomp MCUST16P40B12RO transducer (2 cm diameter).

9. Conclusion

This methodology for handling microcrystals in mother liquor for presentation to X-ray beams at both synchrotron and XFEL light sources is showing great promise. In its simplest format it offers an airborne goniometer with unlimited angular access for obtaining multiple angular rotations and their diffraction patterns. With the inclusion of the ability to unite multiple droplets and therefore instigate room temperature mixing, we have an excellent sample environment for time resolved experiments.

As the reaction takes place in free space, there is no contamination between samples which in turn could be dosed with greater amounts of reagents. The other significant benefit for the system is the sample is stationary unlike with liquid jets allowing for reduction in waste and multiple angles and providing the potential to allow a structure being solved from a single crystal. This system particularly lends itself toward the study of Beta Lactamases as it can suspend the protein crystals, add reagents, initiate mixing and

then rotate to collect angular data, all at room temperature. It also offers the potential to reduce handling quantities down to pico-litre slurries of crystals.

10. Further Work

Now the initial system as shown by Marzo has been built and proven to work, adaptations to frequency and environment conditional will be modelled in FEA software. The first work will explore how operating the system in Helium will alter the systems performance. This will be followed by reviewing the parameters to determine new designs in terms of transducers, frequency, power requirement and layout to ensure optimal trapping of the pico-litre drops.

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