Novel sample preparation technique for protein crystal X-ray crystallographic analysis combining microfluidics and acoustic manipulation

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^aInstitute of Mechanical Systems, Department of Mechanical and Process Engineering, ETH Zurich, CH-8092 Zurich, Switzerland, ^bSwiss Light Source at Paul Scherrer Institute, CH-5232 Villigen, Switzerland, and ^cDepartment of Mechanical and Aerospace Engineering, Monash University, VIC 3800, Australia. Correspondence e-mail: dirk.moeller@imes.mavt.ethz.ch

In order to perform X-ray crystallographic analysis, protein crystals are removed from their growing solution by means of a nylon loop, which is then mounted on a goniometer. As this process is repeated for a large number of crystals, there is a need for automation, especially with regard to the placement on the nylon loop. A novel technique involving the use of acoustic radiation forces and a micro-machined fluidic device is introduced here. After insertion into the micro-machined channel, the crystals are positioned in a row along its centre-line by excitation of a high-frequency standing pressure field, and then moved towards an orifice by applying a flow along the channel, which also ensures spatial separation. Once located in a defined orifice, the single crystals can be removed using a nylon loop. X-ray crystallographic analysis showed that application of ultrasound does not influence the diffraction properties of the crystals.

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1. Introduction

X-ray crystallography with synchrotron radiation (Helliwell, 2005; Dauter, 2006) is a commonly applied method for obtaining information about the spatial structure of biological macromolecules at atomic resolution. For this purpose protein crystals (*e.g.* insulin crystals with a size of 20–200 µm) are individually removed from the solution in which they are grown by means of nylon loops, flash-frozen in liquid nitrogen and finally placed onto a goniometer for X-ray crystallographic measurements. As this process is sometimes repeated for a large number of crystals there are efforts to automate it. Whilst the movement and handling of the loops can be performed using robotic actuators, no satisfying solution has yet been proposed to capture the crystals. Furthermore, given their high sensitivity to mechanical damage, the automated process should bypass the problem of stirring of the growth medium, which can be encountered in manual crystal capture. To this purpose it would be advantageous to bring the crystals sequentially into a known position where they can then be collected by the loop, ensuring at the same time spatial separation so that only a single crystal is found in the trapping site. For this, both the vertical and the lateral position have to be controlled, while transport can be achieved using drag forces arising from laminar flow.

Acoustic manipulation has successfully been used for the gentle handling of biological samples such as COS-7 (Hultström *et al.*, 2006), HeLa and MCR10A (Haake *et al.*, 2005)

cells. It has been estimated that the forces generated in an acoustic field are of the order of magnitude of a couple of hundred piconewtons (Evander *et al.*, 2007). This technique is based on the nonlinear interaction of an acoustic wave generated in a fluid by the vibration of a solid object (*e.g.* the walls of the cavity containing the fluid) with a suspended particle (Gröschl, 1998). The second-order momentum terms in the Navier–Stokes equation when time averaged lead to a net force, known as the primary radiation force, acting on the particles, which moves them into predictable locations. The typical frequencies (upper kilohertz to lower megahertz range) and the periodicity of the acoustic field (whose wavelength exceeds the size of the particles) make this technique particularly suited for the handling of micrometre-sized particles within microfluidic systems. In general, standing pressure fields are excited within these devices, which trap particles in single or multiple parallel planes or lines if the field is one-dimensional (Harris *et al.*, 2003; Laurell *et al.*, 2007; Wiklund *et al.*, 2006; Neild *et al.*, 2007). In real systems two-dimensional effects cause aggregation of particles towards the centre of those planes. Even though acoustic manipulation has found application predominantly in the fractionation and separation of particles from the medium in which they are suspended (Harris *et al.*, 2003; Laurell *et al.*, 2007; Wiklund *et al.*, 2006), it has been demonstrated that accurate positioning of single large particles can also be achieved. In particular, within the scope of automating the manipulation process of single particles (*e.g.* microcomponents, biological cells *etc.*), Neild *et al.* (2006) proved that 74 µm particles can be pre-positioned in a row along the centre-line of a micro-machined

[‡] Present address: Novartis Institutes for Biomedical Research, Novartis Pharma AG, 4056 Basel, Switzerland.

channel open at one extremity to aid the removal process by means of a capacitively actuated micro-machined gripper used for further handling. In a recent development of that device (Oberti *et al.*, 2008) the possibility of creating a flow of medium along the channel has been added. By removing fluid in the proximity of the open end a net fluid movement is created, which drags the suspended particles to the region of action of the gripper, thus aiding the removal process.

When dealing with small particles, they have been observed to float in circular motion rather than being significantly affected by the acoustic radiation force. This effect is known under the name of acoustic streaming and has similar origins to the radiation force (Nyborg, 1965). In contrast to the acoustic radiation force, which acts directly on the particles, acoustic streaming arises from a force due to spatial or temporal variations in the pressure field that acts on the fluid,

hence causing a flow, which in turn results in drag forces acting on the suspended particles. Whereas the acoustic radiation force scales with the volume of the particle, the drag force as a consequence of streaming is a function of the particle cross-sectional area. For this reason, the behaviour of small particles can be dominated by the streaming.

Here, a novel technique for sample preparation for spectrometric analysis based on the combined use of microfluidics and acoustics with the aim of bringing the crystal into the standard nylon loop is introduced. In §2, the construction of the system is introduced and its working principle is explained by means of simulations. In §3, experiments are reported where the manipulation of crystals using acoustic radiation forces has been proved. Then, the whole manipulation process is presented and finally, in §4, the X-ray crystallographic analysis of crystals that underwent the process is discussed.

This has been performed in order to exclude the possibility of any detrimental effect on the crystal sample during the process, just as the viability of cells was investigated when manipulation was first attempted.

2. Experimental setup and method

2.1. System description

The setup (Fig. 1) includes a micro-machined acoustic device installed under a microscope (Olympus ZSH) equipped with a camera (Sony SC-M370CE), used for process observation from above, and a three-axis motorized positioning system (Newport MM4600), which moves the nylon loop (Mounted Cryoloop HR4-955, 20 μm , Hampton Research) used to remove the crystals. The acoustic device itself (Fig. 2) consists of a 200 μm -deep, 1 mm-wide and 11 mm-long channel dry etched into a 300 μm -thick silicon wafer and sealed on the top with a 1 mm-thick glass plate using an adhesive. Two holes, manually drilled in the glass plate (diameter 3 and 1 mm), act as a reservoir for the buffer solution and an inlet for the specimen, respectively. An orifice, 300 μm wide, 700 μm long and oval shaped, has been created in the channel bottom surface to provide access for the loop to the crystals (Fig. 1, insert). This is done from beneath so that visual observation from above is not hindered. The fluid surface tension is responsible for avoiding any fluid leakage from the orifice, also during operation of the

Figure 1

Experimental setup consisting of an acoustic device mounted under a microscope, equipped with a CCD camera for process observation from above. The nylon loop is mounted on a stage, controlled by means of a joystick. After the crystals have been aligned by exciting the device using a function generator and an amplifier, they are moved along the channel of the acoustic device using the flow created by a syringe pump and removed from beneath using the loop (see insert).

Figure 2

Acoustic device consisting of a channel (3) etched into a silicon wafer (1) and sealed with a glass plate (2). Crystals are inserted through a dedicated inlet (6) and removed, after manipulation, through an orifice (5) on the bottom channel surface. Buffer solution is inserted in a reservoir (7), flushed through the channel that it leaves (4) through a Teflon tube (10) connected with the syringe pump. Positioning along the channel centre-line is achieved by applying an AC signal to a piezoelectric transducer (9) mounted on the device lower side (12), hence to excite the system to vibration. The Teflon tube is fixed to the device using glue (11) and is used to clamp it in a plastic holder (8).

syringe pump, as the fluid contained in the orifice is in a dead zone less affected by the velocity field caused by the pump as demonstrated by simulations (not reported here). The large volume of buffer solution contained in the reservoir guarantees that the liquid–air interface is always found at the orifice, thus preventing air from entering the channel. A second opening, with a diameter corresponding to the width of the channel, has been etched at one extremity so that a socket to plug in a Teflon tube is created. When connected to a syringe pump (kdScientific) operated in withdrawal mode a flow is created from the buffer reservoir towards the outlet of the channel. On the same lower side of the device, 1 mm away from the orifice, a piezoelectric transducer (PZT26, $3 \times 5 \times 0.5$ mm), which covers almost the entire length between the specimen inlet and the orifice, has been mounted aligned with the channel. The same electrode configuration as that introduced and analysed by Neild *et al.* (2007) has been defined on the lower surface; by applying the electrical AC signal only to a narrow area on the lower surface rather than to the full plate (*i.e.* grounding the remaining part and the counter-electrode in contact with the silicon) the device can be excited to flexural vibrations resulting in the emission of an acoustic wave in a preferred direction when coupled to the fluid. Reflections at the channel walls lead to a standing pressure field if the resonant condition for the whole system is matched, which gathers the crystals in the channel in one or multiple lines parallel to its centre-line. In addition, the use of strip electrodes reduces the heat generation from the piezoelectric transducer. The device is held by clamping the Teflon tube and fixing it with glue into a plastic plate.

2.2. Method

For operation, buffer solution is flushed through the device by loading it in the reservoir and using a syringe pump to withdraw fluid from the other extremity. A small droplet of sample is then introduced by means of a pipette through a second orifice in the glass plate. The use of two distinct inlets ensures that all the injected crystals are exposed to the flow; hence losses of important samples are minimized. Once the crystals have been aligned by actuation of the transducer, they are moved along the channel by means of the fluid flow towards the orifice until a crystal falls into it. Then the loop is introduced from below and moved above the crystal; the crystal is trapped and removed by pulling it towards the bottom, acting against the surface tension (see also §3). A bulk flow is created (through external pumping) in the fluid only for the transport of the crystals and is stopped during positioning and removal. Immediately after removal the crystal is flash-frozen in liquid nitrogen and stored for further analysis. The whole process is currently manually controlled. The stage onto which the loop is mounted is moved using a joystick and its position is monitored directly by the operator through the CCD camera. However, with an image-analysis-based control loop automation can be achieved.

Cubic crystals from porcine insulin were obtained by hanging-drop vapour diffusion crystallization experiments. A

Figure 3 Pressure field (isobars) in the fluid and z displacement (slice) of the silicon and glass plate, as response to an electrical excitation of the piezoelectric transducer at 754.3 kHz. The model consists of the central region of the device, extending 1 mm further than the specimen inlet (6) and the orifice (5).

15 mg ml^{-1} solution of porcine insulin was obtained by dissolving lyophilized insulin in protein buffer ($0.05 \text{ M Na}_2\text{HPO}_4$, $0.01 \text{ M Na}_3\text{EDTA}$, pH 10.8; EDTA is ethylenediaminetetraacetic acid). After mixing $3 \mu\text{l}$ of the insulin solution with $3 \mu\text{l}$ of 25, 28 or 30% (v/v) ethylene glycol the drop was equilibrated at room temperature against $600 \mu\text{l}$ of the appropriate ethylene glycol solution in a hanging-drop plate. Crystals grew within five days.

2.3. Modelling and working principle of the acoustic device

Numerical modelling of the device has been performed in order to simulate its acoustic behaviour as a result of the electrical excitation of the piezoelectric transducer. In this way it can be determined under which conditions a pressure field can be established in the channel, its spatial distribution can be studied and hence conclusions about its suitability for the present application can be drawn. In particular, the frequency range for which a useful pressure field can be obtained is predicted and the influence of the orifice is investigated. The simulation returns the displacement vector $\mathbf{u}$ of the solid structure, as well as the pressure fluctuation p , *i.e.* the augmented pressure when an acoustic standing wave is set up in the fluid. Details about the implementation of the model can be found in the supplementary information.¹

Fig. 3 shows both the pressure field (depicted as isobars) and the vertical displacement of the silicon and glass plate at one extremity of the model (the displacement field being constant along the x axis) at 754.3 kHz. It can be noted that a standing pressure field with a pressure node along the centre-line is formed and that the pressure remains almost invariable

¹ Supplementary information is available from the IUCr electronic archives (Reference: HX5444). Services for accessing this information are described at the back of the journal.

Figure 4

Force potential field (isolines) in the xy plane. Crystals collect at the minima, corresponding to a region confined between the specimen inlet and the orifice. The depicted potential has been calculated assuming spherical copolymer particles with 100 μm diameter

over the channel depth. As a consequence it can be expected that crystals sit on the bottom surface and will be confined in the lateral direction by acoustic radiation forces. This is confirmed by the stuck crystal reported in §3 (note that in general such sticking can be avoided by having only short periods of static bulk fluid). In contrast, variations can be observed along the channel such that the field is not purely one-dimensional. This is mainly due to the presence of the pressure release boundaries and also to the asymmetric actuation.

The locations into which particles are positioned within this field can be analysed by considering the places where the acoustic radiation forces vanish. The particles are pushed there and there they remain. For an arbitrary three-dimensional field in an inviscid medium the time-averaged force acting on a particle of radius r_s can be expressed as the negative gradient of a force potential U as has been defined by Gor'kov (1961):

$$\langle F \rangle = -\nabla U = -\nabla \left[2\pi r_s^3 \rho_F \left(\frac{1}{3} \frac{\langle p^2 \rangle}{\rho_F^2 c_F^2} f_1 - \frac{1}{2} \langle v^2 \rangle f_2 \right) \right], \quad (1)$$

with $f_1 = 1 - \rho_F c_F^2 / (\rho_S c_S^2)$ and $f_2 = 2(\rho_S - \rho_F) / (2\rho_S + \rho_F)$, where ρ is the density and c the speed of sound in the particle (index S) and in the fluid (index F). The $\nabla \langle p^2 \rangle$ term and $\nabla \langle v^2 \rangle$ represent the spatial gradients of the time-averaged pressure and fluid velocity fluctuation and can be interpreted as spatial gradients of the time-averaged kinetic and potential energies. In order for the force to vanish the gradients must vanish. This occurs where the potential energy is minimal and the kinetic energy maximal (velocity is 180° phase shifted with respect to

the pressure). The force potential U is shown in Fig. 4 in the form of isolines, with blue marking the minima, hence the trapping site. The simulation shows that particles are trapped in a region along the centre-line, which is confined between the specimen inlet and the orifice rather than extending over the whole volume between these two boundaries. As a consequence, particles cannot be positioned using acoustic forces along the centre-line right in front of the orifice (in the orifice itself positioning is not possible as the pressure – and in turn the acoustic radiation force – is assumed to be zero there). This limitation is bypassed by the use of the laminar flow regime, which occurs in microchannels and ensures that the lateral position achieved with acoustics is kept down to the level of the orifice. Once the crystal has fallen into the orifice, the flow is stopped. This method prevents the crystals from becoming trapped along the edges of the orifice, which also represent a potential minimum. Finally, the model has been used to study the effect of the orifice on the field. From a parametric study it turned out that the orifice size only shifts the frequency by less than 1%, without significantly affecting the shape of the force potential field. This allows freedom in the design, which can now meet the requirements set by operational utility.

3. Results and discussion

In a set of preliminary experiments the manipulation of crystals using acoustic forces has been proved. To the best of our knowledge acoustic positioning of crystals has not been reported before. In particular it has to be established whether the acoustic manipulation process causes mechanical damage to the crystals. To this end, the effect of ultrasound on the diffraction properties of the crystal has also been investigated and is presented in detail in §4. As shown in Fig. 5, it has been observed that large crystals do indeed move under the effect of acoustic radiation forces, whereas small crystals or crystal fragments (size of the order of magnitude of 1 μm) do not follow the motion but are rather floating as a consequence of streaming. Owing to their size and the poor contrast with silicon, acoustic streaming of small crystals is hard to identify with still images (though clear in videos); it is, however, clear that only large objects are positioned as desired. Even though streaming might be detrimental in most positioning applications, it can be indirectly exploited here to improve the performance of the device, as only large crystals are manipulated.

A sequence showing the whole manipulation process within the channel is shown in Fig. 6 (see also supplementary material). After crystals have been inserted through the specimen inlet (step 1) and transported into the acoustically active zone by applying a flow of 0.05 ml s^{-1} (step 2), the acoustic field is excited at 751 kHz (step 3). All the crystals are so posi-

Figure 5

Positioning of insulin crystals using a 792 kHz signal at the beginning randomly distributed. As a result of their shape, crystals rotate. Positioning is achieved in less than 1 s. The last image, taken after 4.48 s, shows that smaller particles are also attracted towards the trapping site. The white line marks the channel walls, as glue is present.

tioned on the centre-line. One crystal maintained its position, this being due possibly to the fact that it sticks on the bottom surface (it does not move even when fluid is applied; Fig. 6c). A fluid flow is then applied until a crystal has fallen into the orifice (step 4). The loop is at this point inserted from below (as indicated in the insert of Fig. 2, step 5) and the crystal removed (step 6). Owing to the irregular shape of the crystals, some of them might rotate during application of the ultrasound but still keep the position in the middle of the channel in the y-axis direction. Furthermore, as a consequence of the large differences in the size of the crystals, the flow has the beneficial effect of spreading them out along the channel. However, it might be also detrimental, as some crystals traveling faster than others might overtake them, creating a clump or losing lateral position. In such cases, a short appli-

cation of ultrasound would be necessary to position them again on the centre-line, as used previously by Neild *et al.* (2006). In our experiments a combined alternated use of acoustic and fluidics did not seem to be necessary.

A detailed view of the capturing process (step 6) is illustrated in Fig. 7. It can be clearly seen how the loop is inserted from beneath in the orifice once a crystal has fallen into it. The loop is then moved above the crystal and pulled down, acting against the surface tension of the water. During removal a droplet of buffer solution remains trapped in the loop and guarantees a cage from which the crystal cannot fall. The orifice has been so designed that it provides sufficient space to accommodate both the crystal and the loop. From practical experience it became clear that further reduction of the size of the orifice was not feasible without risking damaging the crystal during the insertion of the loop.

Figure 6

Process of crystal manipulation. After having been loaded in the channel (step 1 in the text) through the specimen inlet on the left (not shown) and transported in the acoustically active region by applying a flow of 0.05 ml s^{-1} (a) (step 2), crystals are positioned along the centre-line by exciting the system at 751 kHz (b) (step 3). Next a fluid flow of 0.5 ml s^{-1} (c) (step 4) is applied to move them towards the orifice (on the right). Once a crystal has fallen into it, the fluid is stopped and the nylon loop is inserted from below (d) (step 5) and moved above the crystal to remove it (e) (step 6). During application of ultrasound a crystal (marked by the arrow) remained stuck in the channel and did not change its position.

Figure 7

Process of nylon loop manipulation in the orifice (steps 5 and 6). After a crystal is moved by means of the fluid flow in the orifice (a), the loop is inserted from beneath at the opposite side (b) and moved upwards. Then, the loop is placed above the crystal (c) and pushed down (d) against the surface tension of the water meniscus formed by the orifice. Once this tension is overcome (e) the crystal is trapped in a buffer solution droplet in the loop from which it cannot fall.

4. X-ray crystallographic analysis of acoustically manipulated crystals

After exposing the crystals to the acoustic field they were removed from the device and flash-frozen in liquid nitrogen. In order to judge the effect of the acoustic force field on the integrity of the crystalline lattice, and thus on the diffracting properties of a crystal exposed to acoustic forces, diffraction experiments were carried out at macromolecular crystallography beamlines X06SA and X10SA of the Swiss Light Source (SLS), Villigen, Switzerland. All diffraction data were collected at an X-ray energy of 12.39 keV in 1° increments and recorded by a MAR225 CCD detector. During data collection crystals were maintained at 100 K using an open-flow liquid nitrogen cryostat. As the SLS is operated in TOPUP mode, no corrections for storage-ring-induced decay of the primary beam intensity were required. All data were processed with *XDS* and *XSCALE* (Kabsch, 1993). The quality of data sets obtained from crystals that were exposed to the acoustic was similar to data from unexposed crystals, as judged by comparing the *R* values and resolution limits presented in Table 1, which suggests that the crystalline lattice is unaffected by the acoustic force field applied prior to X-ray data collection.

Table 1

Data collection statistics for cubic insulin crystals.

Data were collected at SLS beamline X06SA. Values for the highest-resolution shell are in parentheses.

	Crystal 1: exposed to acoustic force field	Crystal 2: exposed to acoustic force field	Crystal 3: exposed to acoustic force field	Crystal 4: exposed to acoustic force field	Crystal 5: control
Wavelength (Å)	1.0716	1.0716	1.0716	1.0716	1.0716
Resolution (Å)	50.0–1.4	50.0–1.5	50.0–1.6	50.0–1.6	50.0–1.5
Space group	<i>I</i> ₂ 3	<i>I</i> ₂ 3	<i>I</i> ₂ 3	<i>I</i> ₂ 3	<i>I</i> ₂ 3
Unit-cell parameters <i>a</i> = <i>b</i> = <i>c</i> (Å)	77.75	77.76	77.72	77.75	77.80
No. of reflections	125 276 (14 715)	125 684 (14 665)	107 149 (12 980)	107 674 (13 165)	117 556 (9456)
No. of unique reflections	12 625 (2188)	12 642 (2188)	10 432 (1675)	10 483 (1703)	12 687 (2200)
Redundancy	10.0 (6.7)	9.9 (6.7)	10.3 (7.7)	10.3 (7.7)	9.3 (4.3)
Completeness (%)	99.6 (100.0)	99.7 (100.0)	99.4 (97.8)	99.9 (100.0)	99.8 (99.6)
<i>R</i> _{meas} [†] (%)	4.4 (19.6)	4.0 (25.5)	3.7 (28.7)	4.6 (31.1)	5.6 (26.4)
<i>R</i> _{mrgd-<i>F</i>} [†] (%)	2.9 (15.0)	3.3 (21.3)	3.1 (21.6)	3.6 (23.5)	4.2 (26.2)
<i>I</i> /σ(<i>I</i>)	33.8 (9.6)	34.6 (7.7)	39.0 (7.3)	32.3 (6.8)	25.6 (5.6)

[†] *R*_{meas}: redundancy-independent (multiplicity-weighted) *R* factor. *R*_{mrgd-*F*}: quality indicator for the reduced amplitudes of this data set (Diederichs & Karplus, 1997).

5. Conclusions

The acoustic manipulation of crystals as a method to automate sample preparation for X-ray spectroscopy has been shown. It has been proved that large crystals can be positioned along the centre-line of a channel and moved towards an orifice through which they are removed using a standard nylon loop. By means of X-ray crystallographic analysis it has been established that this process does not change the diffraction properties of the crystals. The setup presented here represents a first step towards a higher degree of automation and shows both the potential and the limitations of this approach. Owing to the differences in size, when moved along the channel using drag forces, crystals travel with different speeds. Therefore, a controlled spatial separation along the channel, so that a constant distance between two crystals is guaranteed, cannot be achieved. Moreover, with the current channel length, separation might sometimes be a problem. A longer channel or two-dimensional positioning [in a similar way as discussed by Oberti *et al.* (2007)] would solve this problem. On the other hand, single crystals are now easily accessible and, provided that sufficient spatial separation is obtained, there is potential for automating the process. In a next step the growth environment can be integrated on the same chip, so that the transfer with the pipette is no longer necessary. Finally it would be possible to exchange the medium, as done by Laurell *et al.* (2007).

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