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Continuous Protein Crystallisation Platform and Process: Case of Lysozyme

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Abstract
In this work, we designed and built a continuous crystallisation oscillatory flow platform. The lysozyme crystallisation behaviours were investigated at concentrations from 30 to 100 mg/mL, under oscillatory conditions with amplitude ($x_0$) from 10 to 25 mm and frequency ($f$) from 0.05 to 0.25 Hz in a batch oscillatory flow crystallisation platform. The nucleation rate increased with increase in concentration of initial lysozyme solution, and was also found to increase with increase in shear rate. By learning the thermodynamics and kinetics of lysozyme crystallisation in batch oscillatory flow, the batch crystallisation process was successfully transferred to a continuous oscillatory flow crystallisation process. The equilibrium state of continuous crystallisation reached at residence time 200 min, and the final product crystals shape and size were consistent during the continuous process. This work demonstrates the feasibility of oscillatory flow based platforms for the development of continuous protein crystallisation as for downstream bioseparation.

Introduction
Crystallisation of protein has presented the potential to be developed as an efficient approach for protein purification (McPherson, 2004; Shah et al., 2012), compared to traditionally used chromatographic methods. Typical downstream purification costs can be up to 80% of the manufacturing cost (Zang et al., 2011). Continuous manufacturing connecting reaction, crystallisation and formulation has been documented to improve the efficiency of manufacturing (Chew et al., 2004; Ni et al., 2004). The steady-state operation of a continuous
crystallisation stabilizes variability in the product, reduce capital costs, and improve control over the CSD of the API (Baxendale et al., 2015). Many research on continuous crystallizers have been investigated in mixed-suspension, mixed-product removal (MSMPR) crystallizer (Su et al., 2015; Yang et al., 2017), i.e. continuous stirred tank reactors (CSTRs) (Lai et al., 2015), plug flow reactor (PFR) (Neugebauer and Khinast, 2015), continuous (baffled) oscillatory flow reactor/crystalliser (OBR)/(COFC) (Chew et al., 2004; Jian and Ni, 2005; Yang et al., 2016).

Much efforts in continuous crystallisation have focused on small organic pharmaceuticals (Baxendale et al., 2015; McGlone et al., 2015). However, knowledge of oscillatory flow crystallisation, like mixing condition (Chew et al., 2004; Yang et al., 2016), nucleation (Yang et al., 2016), growth (Brown and Ni, 2011), process control (Briggs et al., 2015) on small organic pharmaceutical molecules can also help to develop and design protein continuous crystallisation process. However, the differences between crystallisation of proteins with small organic molecules are obvious. Crystallisation of protein is still very difficult primarily due to the complex structures of the proteins (Jacobsen et al., 1998). For instance, lysozyme, a small and simple protein, consists of 129 amino acids involving hundreds of chemical bonds and thousands of degrees of freedom (Malkin et al., 1996). Moreover, due to the impurity of the proteins in solution nucleation and crystallisation from fermentation broths are rarely successful. In addition, the crystallisation of protein is also influenced by pH (Chen et al., 2017), iron strength (McPherson, 2004), concentration of PEG (McPherson, 1976), magnetic fields (Hou and Chang, 2008) and etc.

By high-throughput screening or other micro scale technologies, suitable conditions for crystallisation for many proteins, like lipase, thaumatin, catalase, ferritin (Hebel et al., 2013; Shah et al., 2012), have been determined. However, there are only limited literatures focused on the continuous crystallisation of proteins, for example insulin crystallisation in oscillatory flow glass capillaries (Parambil et al., 2011) and lysozyme crystallisation in meso-oscillatory flow reactor (Castro et al., 2016) and plug flow crystallizer (Neugebauer and Khinast, 2015). There is no continuous oscillatory flow crystallisation research on any protein, neither
comparing, design and developing platform of continuous oscillatory flow crystallisation reported. This work focuses on the crystallisation of lysozyme, a model protein, in several platforms we designed, scaling up from hundreds of μL to hundreds of times larger scale. The thermodynamics and kinetics of lysozyme were investigated at concentrations from 50 to 130 mg/mL, with oscillatory conditions of amplitude ($x_0$) from 5 to 25 mm and frequency ($f$) from 0.05 to 0.25 Hz. The different platforms were compared and the optimizations from batch to continuously protein crystallisation were discussed.

**Experimental**

Lysozyme from chicken egg white (purity ≥90 %, ≥40,000 units/mg protein), sodium acetate (purity > 99%), sodium chloride (purity > 99.5%), and glacial acetic acid (purity > 99.5 %) were purchased from Sigma and used without further purification. Distilled water was used.

Figure 1 Platform of batch (A) /continuous (B) oscillatory flow crystalliser.
Buffer solution was prepared as 0.022 g sodium acetate per mL deionized water (0.1 M) with 200 µL of acetic acid per mL of de-ionized water (pH 4.9, measured with a Mettler Toledo Seven Excellence pH Meter). The lysozyme solutions with concentration of 50 to 130 mg/mL were prepared in buffer solution, and the precipitant solution was 0.064 g NaCl (1.1 M) per mL buffer solution.

The crystallisation experiments were performed in Batch and Continuous Oscillatory Flow Crystalliser (BOFC and COFC). The crystallisation solutions were mixed with 1:1 volume ratio of the lysozyme solution 50 - 130 mg/mL and the precipitant solution at temperature 20 ± 1 °C. A PVC tube of 27 cm length and 3 mm internal diameter was used in BOFC, with volume about 2 mL which is connected to one or two syringe pumps, as shown in Figure 1. The crystallisation solutions in the PVC tube were oscillated with, $x_0$, 5 - 25 mm, and, $f$, 0.05 – 0.25 Hz. In the COFC, crystallisation solutions were mixed by connecting two syringe pumps, filled with the protein and precipitant solution separately. The solutions were pumped with net flow 0.06 mL/min in total to a PVC tube (volume about 20 mL) of about 160 cm length and 3 mm internal diameter. The oscillations were generated by another one or two syringe pumps with, $x_0$, 20 mm, and, $f$, 0.15 or 0.45 Hz. Samples of 2 µL were taken from crystallisation solution every 10 - 30 minutes in batch and continuous crystallisation experiments. Concentrations of the samples were determined by Nanodrop One (Thermo Scientific) with a correlation equation and off-line images were recorded by a microscope (Olympus cx41) connected to a digital camera (Moticam 5.0MP).

**Results**

The velocities of solution with oscillatory during 20 s in BOFC were shown in Figure 2. In each cycle, the solution in crystallizer was infused to peak value, during which stage the velocity is $4x_0f$, i.e. 12 mm/s for $f$ – $x_0$ at 0.15 Hz – 20 mm and 6 mm/s for both 0.15 Hz – 10 mm and 0.1 Hz – 15 mm. In the next stage, the solution was withdrawn to the max negative value, during which stage the velocity is $-4x_0f$. The change of the direction was dependent on the frequency, the direction changed 6 times (3 cycles) and 4 times (2 cycles) for 0.15 Hz and 0.1
Hz, respectively. It is noted that the velocity of the solution generated by syringe pump is
different with that generated by piston pumps, which continuously changes all the time based
on a sine function equation (Yang et al., 2016).

![Figure 2 Velocity of solution in batch oscillatory flow crystalliser with oscillatory conditions at $f - x_0$ at 0.15 Hz- 10 mm, 0.15 Hz – 10 mm and 0.1 Hz – 15 mm.]

Lysozyme solutions with 14 different concentrations were measured by UV absorbance at 280
nm, and the absorbance and lysozyme concentrations ($C$) were correlated by a linear equation.
The typical errors associated with concentration measurements are +/- 0.1 mg/ml. The best
linear correlation with coefficient of determination, $R^2$ of 0.9934 is $C = 0.407 \times \text{Absorbance}$
(supporting information). This correlation was used to estimate the concentrations of the
lysozyme during the crystallisation process.

In the literatures of lysozyme crystallization, buffer as sodium acetate, pH at around 4.7, and
precipitant as sodium chloride (Castro et al., 2016; Durbin and Feher, 1986; Liu et al., 2010;
Roberts et al., 2010) were established and is also used in this work. The concentrations of the
initial lysozyme solution, 50 - 130 mg/mL were investigated in BOFC. Crystallisation solutions
with concentrations >100 mg/mL lysozyme solution in BOFC all nucleated within 30 min, while nucleation of the crystallisation solution with 50 mg/mL lysozyme solution occurred at ~60 min. Figure 3 shows the solution concentration reduced to a relative low level after 200 min in almost all experimental conditions. For crystallisation time less than 100 min, the crystallisation solutions with higher initial lysozyme concentrations had higher concentrations than those solutions with the lower initial lysozyme concentrations, while the order was altered after 100 min. The protein crystallisation is different with normal small organic molecules, which the growth of the protein crystal growth is relative slow (Durbin and Feher, 1986). There are three stages of the changing concentration: (A) induction period (before nucleation), at which stage the decrease of concentration is very low, (B) fast concentration dropping period, i.e. nucleation (homogeneous and secondary) and fast crystal growth at high supersaturation level, (C) slow concentration decrease period, i.e. crystal growth at low supersaturation level. The decrease rate, $R_{con}$, of the concentration during the crystallisation is estimated by successive two samples:

$$R_{con} = \frac{C_n - C_{n+1}}{t_{n+1} - t_n}$$

(1)

Where $n$ is the sample number, $t_n$ is the respective sampling time, and $C_n$ is the respective concentration determined. Correspondingly, the $R_{con}$ increased and decreased during the crystallisation process shown in Figure 4. The crystallisation solution with 130 and 110 mg/mL lysozyme solution had the fastest rate of concentration dropping range ($R_{con}$), ~ 0.7 and ~ 0.6 mg/mL/min, respectively. Comparing with many cases of small organic crystallisation (Mullin, 2001; Yang and Rasmuson, 2012) with a sharp fall of the concentration after nucleation, the concentrations decreased (B-stage) in lysozyme crystallisation at relatively slow rates after nucleation (Neugebauer and Khinast, 2015), due to high packing energy, low attachment frequency and slow kinetics during the crystal growth (Derewenda and Vekilov, 2006). The higher concentration of the initial lysozyme solution was, the maximum $R_{con}$ of the crystallisation solute became, and the shorter time the maximum $R_{con}$ occurred during the
crystallisation process, shown in Figure 4. After 180-200 min, the $R_{con}$ became very similar to each other in most of the experimental conditions.

Figure 3 Lysozyme concentration of crystallisation solutions in batch oscillatory flow crystalliser with $x_0$ 20 mm and $f$ 0.1 Hz. Initial concentrations of lysozyme solution are 50, 60, 70, 110 and 130 mg/mL. Images from bottom to top are lysozyme crystals obtained with 50, 70, 130 mg/mL solution.

Some larger crystals were obtained from the lysozyme solutions of high concentration compared with those obtained from lower concentration solutions, as shown in Figure 3. Because, at this moderate oscillatory condition, the higher concentration induced shorter nucleation time and longer growth time to gain some bigger particles. It is noticed that there were plenty of very fine particles exciting at the same time, due to the higher supersaturation and faster nucleation rate. At more intense oscillation, there were more fine particles without many bigger crystals, due to a higher homogeneous nucleation rate generating larger number of crystals at the B-stage of crystallisation process and the high breakage rate due to the high shear rate (Yang et al., 2018).
Crystallisation solutions with 50 mg/mL lysozyme solution nucleated at ~100 min with $x_0$ at 10 - 15 mm and $f$ at 0.15 Hz, while nucleated at ~50 min with $x_0$ at 20 - 25 mm. With the same initial lysozyme concentration, the larger amplitude was, the faster nucleation occurred, the faster decrease in concentration of crystallisation solution and the smaller crystals were obtained (Figure 5).

Crystallisation solutions with 50 mg/mL lysozyme solution nucleated at about 90 min with $x_0$ at 15 mm and $f$ at all range from 0.05 – 0.25 Hz, shown in Figure 6, while nucleated at only less than 50 min with $x_0$ at 0.5 Hz (Yang et al., 2018). There was not obvious difference between the nucleation and concentration at the range of low frequencies, and the nucleation was much faster when the frequency increased to 0.5 Hz. The crystals looked similar in these experiments performed at low frequency range, 0.05 – 0.25 Hz. Figure 5 and Figure 6 show an overall trend that the nucleation time increased and the $R_{con}$ decreased with the decrease in shear rate, i.e. lower frequency or amplitude, i.e. the mixing was not efficient. Whilst only a limited number of experiments for reproducibility is conducted, the observed effects of higher shear rate and
higher supersaturation on the nucleation and crystallisation of lysozyme are not due to experimental errors. The results are consistent with the literature that the faster diffusion, resulted by faster mixing, leaded to faster crystal growth and therefore the $R_{con}$ is higher (Adachi et al., 2002; Parambil et al., 2011). The difference of the nucleation time was not obvious at very low shear rate range 0.05 - 0.15 Hz in Figure 6. This non uniform mixing could lead to a lower homogeneous nucleation rate or secondary nucleation rate, which is in consistence with the literatures (Ploß and Mersmann, 1989; Yang et al., 2016).

![Figure 5](image.png)

**Figure 5** Lysozyme concentrations in crystallisation solutions with $x_0$ at 10 - 25 mm and $f$ at 0.15 Hz in batch oscillatory flow crystalliser. Initial concentration of lysozyme solutions is 50 mg/mL.

The net flow rate ($Q$) 0.06 mL/min was applied in the COFC, and the residence time of the lysozyme crystallisation solution at the end of the crystallizer in the COFC was 180 min. In experiment with oscillatory condition at 20 mm – 0.15 Hz, the concentrations of crystallisation solutions after 200 min reached an equilibrium state in the COFC, which was close to the concentration in BOFC (Figure 6) at comparable same residence time, indicating the consistence and the similarity of geometry and share rate distributions at same frequency and amplitude.
conditions between BOFC and COFC (Yang et al., 2016). The images of crystals inside tube wall show the crystals attached on the tube wall grew bigger from 200 to 300 min, indicating the fouling became more pronounced with time. It is also observed the fouling is most pronounced at locations near the mixing point. As discussed in literature, an important challenge for the continuous operation of OBR crystallizers is encrustation (McGlone et al., 2015). However, in this work no leaking and blockage inside the crystallizer occurred.

Figure 6 Lysozyme concentrations in crystallisation solutions with $f$ at 0.05 – 0.25 and 0.5 Hz (Yang et al., 2018) and same $x_0$ at 15 mm in batch oscillatory flow crystalliser. Initial concentrations of lysozyme solution are 50 mg/mL.

To achieve a shorter equilibrium time, a higher yield (lower final concentration of the crystallisation solution), and a better suspension of the crystals in the crystallisation solution, higher shear rate (i.e. higher frequency of the oscillatory conditions) can be applied (Yang et al., 2018). In experiments with oscillatory condition at 20 mm – 0.45 Hz, the concentrations of crystallisation solutions are lower than that with oscillatory condition at 20 mm – 0.15 Hz as expected due to the higher frequency and higher shear rate. Figure 7 shows the consistence
between the crystals obtained at 200 min and 300 min, and most of the crystals had good crystalline shape, but there was an extent of agglomerations. The yield estimated from the decrease of the lysozyme concentration was about 50 %, which can be improved by further decrease the temperature or increase the residence time.

![Graph](image)

**Figure 7** Lysozyme concentration in crystallisation solution with $x_0 - f$ 20 mm – 0.15 Hz (photos with red color frame from top left to right: crystal fouling inside tube at 200 and 300 min) and $x_0 - f$ : 60 mm - 0.15 Hz (photos with blue color frame from bottom left to right: crystals obtained at 200 and 300 min) with residence time 180 min in COFC.

**Discussion**

In industrial downstream manufacturing, batch crystallisation is commonly used. However, continuous manufacturing including continuous crystallisation (Chew et al., 2004; Ni et al., 2004) draw more and more attentions from both industrials and academics (Briggs et al., 2015; Brown and Ni, 2011; Chew et al., 2004; Yang et al., 2016). Based on the principle of batch crystallizer, the mixed suspension mixed product removal crystallizer (MSMPR) is designed by
connecting parallel batch crystalliser (Katoh and Katoh, 2000; Kwon et al., 2014). With this platform, the materials can be removed and added at same time, but it is very hard to control residence time. To better control the residence time, a semi-continuous crystallisation process can be operated by three successive steps: filling the material in, crystallization and residency, and removing the product. Continuous stirred tank reactor (CSTR) or multi-stage MSMPR (Alvarez et al., 2011; McGlone et al., 2015) had been introduced in crystallisation of small organic molecules which can achieve a better plug flow with increase of the stages or parallel reactors. These designs are suitable for systems that require longer residence times, but its strong agitation and nonuniform temperature profile may cause problems for particle size and polymorphism control as reported in crystallization of small organic molecules (Yang et al., 2017).

In our previous work and this work, we have tried to develop a new generation of oscillatory flow crystalliser. Previously, we designed glass capillary circulating flow crystalliser (Roberts et al., 2010). This is a simple platform by connecting the glass capillary and a circulating pump, and crystals continuously grew in the circulating flow, by an enhanced mass transferring as well as cooling and heating cycles. Recirculating the protein solution in this platform for 40 h increases the crystal yield to 80 % of the total protein, and it offered promise for growing large protein crystals for use in structure determinations and for the mass preparation of protein therapeutics (Roberts et al., 2010). But the very long residence time and low capacity inside the crystallizer not met the efficient requirement of scaling up. To have better mixing condition, in this work we designed continuous oscillatory flow (tube) crystallizer (Figure 1). It is developed based on our previous platform, we used plastic/polymer tubing instead of glass capillary. This brings us the conveniences for changing the materials of the tubing, adjusting the diameter of the tubing, adding blending curves and etc. Many options of materials can be used in design the crystallizer like PTFE, silicon, Nylon, PVC and etc, shown in supporting information. The different bending curves can be applied to achieve higher shear rate. The tube shape can be adjusted easily, dependent on the space requirement and the capacity of the crystallizer.
The next generation of oscillatory flow crystalliser has been designed to be continuous baffled oscillatory flow tube crystalliser, which is based on the previous platform. Baffles (slightly smaller diameter tubing) inside the larger diameter tubing were added in the oscillatory flow crystallizer for further improving the mixing condition. For example, the segment of PVC tubing connected by smaller PTFE tubing inside, and the PTFE tubing performed as baffles inside this crystallizer (Supporting information). For this platform, it is very easy to assemble and disassemble, there is no leaking when we did the test with water. The next step is to transfer the crystallisation of lysozyme to this designed platform. However, extra jacket or the big incubator is required to control temperature of the crystallisation solution in the further investigations, and the jacket of the PTFE tubing is not easily practicable. A similar but larger scale meso oscillatory flow crystalliser (Castro et al., 2016), with baffles in glass capillaries and the jacket outside, was reported. This is also a small version of the oscillatory baffle flow
crystallizer for small organic compounds reported in literature (Jian and Ni, 2005; Yang et al., 2016). In large scales, tens of mL to hundreds of mL, it is relatively easy to control baffles in the glass tubes. However, making baffles in glass capillary is very difficult, i.e. the baffle shape and size is very hard to uniformly control.

It is also worth to mention a tubular plug flow crystallizer (Neugebauer and Khinast, 2015). In this platform, the shear rates were generated by contacting the gas bubbles with the liquid flow, and the liquid droplet flow were separated into segments to achieve the optimal transport of crystals along the reactor and a narrow residence time distribution. The temperature can be controlled by multiply stage, which is in same principle of CSTR. However, it is very difficult to control the shear rate in the flow and risk for transferring the crystals in the flow.

Developing a continuous protein crystallisation, from nL and μL for the interest of protein structure determination (Pitchayajittipong et al., 2009), to meso-scale (mL) or big scale-L were hardly reported. The residence time, yield, crystal quality, purity and size distribution are all important factors in the design of the continuous crystallisation process (Figure 8), similar as principles in design of platforms for small organic molecules (Brown et al., 2018), despite the scales are different (Roberts et al., 2010) (Castro et al., 2016). The thermodynamics and kinetics are highly determined by supersaturation and share rate, respectively, demonstrated in this work, too. The increase of the supersaturation and share rate can shorten the residence time and, therefore, increase the yield during the same crystallisation time. However, it may lead to poor quality of the crystals, lower purity and wider size distributions, due to high nucleation rate. It also results in high risk to drive the crystallisation solution into a precipitant zone (Chayen and Saridakis, 2008), in which region the salts crystals, instead of protein crystals, nucleate first. On the other side, decrease in the supersaturation and share rate is better for crystal quality but requires longer residence time, which reduce the yield during the same residence time in manufacturing operation. Extending the residence time in the crystallisation process helps to obtain good quality of crystals, but this decreases the efficiency of the manufacturing. Many thermodynamic and kinetic factors affect each other and need to be
tested with the experiments in batch crystallisation before transferring to continuous crystallisation, which would be designed and optimized by design of experiments (Weissman and Anderson, 2014). In addition, factors, including seeding, surface chemistry and surface topography, porous templates (Barros Groß and Kind, 2017; Delmas et al., 2011) (Shah et al., 2015), which can improve crystallisation of protein, can be tested, optimized and applied in developing continuous crystallisation.

Conclusions

This work demonstrates feasibility of a continuous protein crystallisation under oscillatory flow conditions. In the batch oscillatory flow crystallizer, the higher concentration of lysozyme solution was, the faster nucleation occurred. With same initial concentration of lysozyme, the high frequency and amplitude of the oscillatory condition enhanced the nucleation and crystallization, leading to a reduce in the particle size and increase in the number of the crystals. A continuous oscillatory crystallisation process of lysozyme was performed in a designed continuous oscillatory flow crystalliser with a residence time 180 min. The equilibrium state of continuous crystallisation achieved during the crystallisation process, proved by the similar size crystal products obtained and similar final concentration in crystallisation solution. The crystallisation process in continuous oscillatory flow crystallizer was in consistence with the batch oscillatory flow crystallisation process with same oscillatory condition. The process can be further improved by temperature control, enhancing mixing, seeding or adding nano-templates. The next step is to move the continuous crystallisation of lysozyme to baffled oscillatory flow crystallizer, which was designed by comparing several previous continuous crystallisation platforms, like enforced flow, oscillatory flow, plug flow or mixed suspension mixed product removal crystallizer.

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