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Imaging protein crystal growth behaviour in batch cooling crystallisation☆

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ABSTRACT

The temporal and spatial growth behaviour of protein crystals, subject to different cooling strategies in protein crystallisation was investigated. Although the impact of temperature and cooling rate on crystal growth of small molecules was well documented, much less has been reported on their impact on the crystallisation of proteins. In this paper, an experimental set-up is configured to carry out such a study which involves an automatic temperature controlled hot-stage crystalliser fitted with a real-time imaging system. Linbro parallel crystallisation experiments (24-well plate) were also conducted to find the suitable initial conditions to be used in the hot-stage crystallisation experiments, including the initial concentration of HEW lysozyme solutions, precipitate concentration and pH value. It was observed that fast cooling rates at the early stage led to precipitates while slow cooling rates produced crystal nuclei, and very slow cooling rates, much smaller than for small molecules are critical to the growth of the nuclei and the crystals to a desired shape. The interesting results provide valuable insight as well as experimental proof of the feasibility and effectiveness of cooling as a means for achieving controlled protein crystallisation, compared with the evaporation approach which was widely used to grow single large crystals for X-ray diffraction study. Since cooling rate control can be easily achieved and has good repeatability, it suggests that large-scale production of protein crystals can be effectively achieved by manipulating cooling rates.

1. Introduction

Experimental studies of protein crystallisation have focused on obtaining high quality large crystals in order to perform X-ray diffraction analysis with the aim of obtaining the 3-dimensional structure of protein molecules. The experiments [1–11] often used micro-volume protein solutions and were carried out by changing concentration via evaporation. Typical equipment involves high-throughput micro-crystallisers, in which a large number of experiments are conducted and repeated, sometimes by applying external fields such as electric, magnetic, electromagnetic, and micro-gravity [12]. However, it is obviously difficult to achieve large volume production of protein crystals by the method of changing concentration using evaporation. It is also slow and difficult to exercise automatic control. As a contrast, it is a much easier and more efficient technique to alter temperature during a crystallisation process.

In contrast to the large number of publications on cooling crystallisation of small molecules, there are only very limited reports on protein cooling crystallisation in the literature, and few can be considered as comprehensive studies. As a result, as some researchers have stressed, the effect of temperature or cooling on protein crystallisation [13–15] needs more attention. Murai and co-workers [16] also stressed the importance of temperature and cooling in protein crystallisation and developed a laboratory device for such investigation, but the equipment was not able to maintain efficiently the optimal temperature and cooling profile in experiments. Adachi and co-workers [17] reported a high throughput experimental system that could be used to study the effect of temperature on protein crystallisation, known as TACON (temperature at once) system. The system is composed of 45-well (9×5) plates or 84-well (12×7) plates, the temperature of each well in the plate can be controlled to be the same or totally different. However, the original TACON system was found unable to be directly used in protein cooling crystallisation experiments. Murai and co-workers [16] therefore made some improvements to the TACON, and then applied it to controlled cooling crystallisation. Although the HEW lysozyme cooling crystallisation experiment was demonstrated in the new TACON system, much of the discussion in the paper was directed at describing the new instrument; little details were given about the effect of temperature on the cooling protein crystallisation. It is not clear how sensitive TACON can be in controlling the temperature. In this

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study, a hot-stage reactor which manages to execute the temperature profile accurately within a wide range of cooling rates was applied. Repeatable crystallisation condition was achieved and the influence of cooling rate fully investigated.

The purpose of the current study is to carry out an experimental investigation on the temporal and spatial growth behaviour of protein crystals under the influence of cooling in a crystalliser. Crystallisation *via* evaporation often takes a long time, requires small volume, and is difficult to exercise closed-loop control, therefore is not suitable for large scale production of protein crystals. In 2004, Basu and co-workers [18] pointed out that there are many potential advantages of delivering biopharmaceuticals in the form of crystals, and predicted that although there was only one biopharmaceutical that was marketed in the form of crystals at the time which was insulin, more in future would be manufactured in crystalline form. Today there have been more than 150 crystalline form biopharmaceuticals on the market. The efficiency of large scale protein crystallisation can be significantly improved by employing cooling strategies, and research on this subject has been carried out in our previous work using morphological population balance models which demonstrated that operational condition (*e.g.* cooling rate and seed loading) can be optimised and desired shape and size distributions for a population of crystals can be closed-loop controlled [19–23]. The focus of this paper is experimental study on cooling crystallisation of proteins, investigating the temporal and spatial growth behaviour of protein crystals using on-line real-time imaging instrument.

2. Experimental Study of Hen-Egg-White Lysozyme Cooling Crystallisation

2.1. Materials, equipment and instruments

2.1.1. Materials

The materials used in this study include dimethyldichlorosilane (DMDC) (purchased from the Sigma-Aldrich company), 1, 1, 1-trichloroethane (methyl chloroform) written as $C_2H_3Cl_3$ or CH_3CCl_3, Hen-Egg-White Lysozyme, L62971 (purchased from the Sigma-Aldrich company), sodium hydroxide, sodium chloride, sodium azide, hydrochloric acid or acetic acid, and deionised water (>15 MQ·cm^{-1}).

2.1.2. Equipment and instrument

The main experimental rig of HEW lysozyme crystallisation comprises a LTS350 hot-stage reactor and a video imaging system. The LTS350 hot-stage reactor was supplied by the Linkam [24] company, which is combined with the video imaging system to carry out on-line monitoring of the HEW lysozyme crystallisation process. The LTS350 hot-stage reactor consists of a hot-stage reactor, LNP94 Liquid Nitrogen Cooling Pump and CI94 temperature controller, which are shown in Fig. 1. The LTS350 is an easy to use versatile heating or freezing stage, which includes a large area temperature controlled element with a platinum resistor sensor embedded close to the surface for accurate temperature measurements. The details of the hot-stage reactor are listed in Fig. 2. LNP94 Liquid Nitrogen Cooling Pump and CI94 temperature controller are the main components for accurate temperature control of the hot-stage reactor, and the precise control of liquid nitrogen flow enables specific stages to control cooling rates as fast as 130 °C·min^{-1} or as slow as 0.1 °C·min^{-1}.

The video imaging system is made up of a camera interface, computer and Polytec xenon strobe box with a liquid light guide, which is designed to capture digital video using specialised video software (VideoSavant) running on a custom built high specification PC, as shown in Fig. 3. The experiments were carried out on the stage of an optical microscope real-time or time-lapsed and non-invasive.

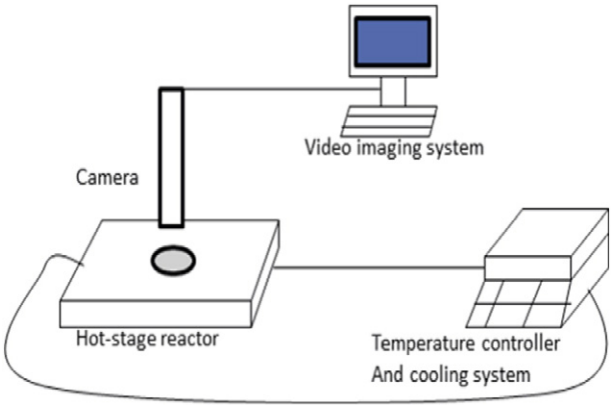

Fig. 1. Set-up of HEWL cooling crystallisation experiment.

Professor Wang and his group [25–32] have carried out much research on small molecular crystallisation processes using the imaging video system.

Other facilities include a vacuum oven, glass or ceramic containers, a pH meter (Mettler Toledo 320 digital meter), a glass stirring rod, a plastic head dropper, Buchner funnel, centrifugal pump, pipette, sitting-drop crystallizer tray (6 × 4 well), and membrane of 0.22 μm, and thermostat (water).

2.2. Experiment

As is known, lysozyme is easily crystallised as the form of dimer or polymer (oligomer) in the condition of lower ionic strength solution and in values of pH higher than 5. However, lysozyme exists as a type of monomer in the form of co-existing aggregates, so the value of pH was set as 4.5 in this study. In addition, lysozyme crystals have polymorphism in their solution, of which lysozyme crystals with tetragonal structure are easily crystallised and more stable. Therefore, lysozyme crystals in tetragonal structure are applied and all data derived from the literature is utilized in the base of tetragonal lysozyme crystals. Silanization [33] process is necessary for all glassware, including the sitting-drop crystallizer. Details about silanization are not given here.

Preparation of HEWL solution with a concentration of 20–50 mg·ml^{-1} is detailed below, where sodium chloride is chosen as precipitate with a concentration of 2.5 wt% and sodium acetate is used as buffer solution with a concentration of 0.1 mol·L^{-1} and pH of 4.5.

Preparation experiments were carried out at a room temperature of 20 °C and involve the following steps:

(1) Preparation of the buffer solution: sodium chloride was dissolved into deionized water to make a sodium chloride solution with a concentration of 0.1 mol·L^{-1}; the value of pH is adjusted by titrating acetate or sodium hydroxide.
(2) Dissolving sodium chloride into solution 1 and making sodium chloride–sodium acetate solution with a concentration of 50 mg·ml^{-1}; if the value of pH needs to be re-calibrated, sodium chloride must be added into solution 1 before calibration.
(3) Triassic nitride solution was made with a concentration of 0.05 wt% and added into the buffer solution to inhibit the interference of bacteria.
(4) Dissolving lysozyme solution into solution 1 (without sodium chloride) and titrating it to be a 40–100 mg·ml^{-1} solution.
(5) Mixing solutions 2 and 4 with the same volume to produce HEWL solution with a concentration of 20–50 mg·ml^{-1} sodium chloride solution with a concentration of 2.5% and sodium acetate solution with a concentration of 0.1 mol·L^{-1} and a pH of 4.5.