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Sensitivity of lysozyme crystallization to minute variations in concentration

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It is well known that the crystallization of proteins is strongly dependent on the crystallization conditions, which are sometimes very sensitive to environmental disturbances. Parameters such as the concentration of precipitants or protein, pH, temperature and many others are known to affect the probability of crystallization, and the task of crystallizing a new protein often involves a trial-and-error test using numerous combinations of crystallization conditions. These crystallization parameters, such as the concentration of either the protein or the precipitant, are important because they directly affect the driving force of crystallization: the supersaturation of the solution. Although it is common sense that the concentration can affect the crystallization process, the sensitivity of the crystallization process to variations in the concentration has seldom been addressed. Owing to the difficulty of directly preparing solutions with very small concentration variations, it is hard to carry out an investigation of their effect on the crystallization process. In this paper, a simple but novel method for studying the effect of minute concentration variations on the success rate of protein crystallization is presented. By evaporating the crystallization droplet, a fine concentration gradient could be created. With this fine-tuned concentration gradient, it was possible to observe the effects of minute variations in the concentration or supersaturation on the crystallization. A very minor change in concentration (as low as 0.13% of the initial concentration, *i.e.* 0.026 mg ml^{-1} for lysozyme and 0.052 mg ml^{-1} for NaCl in the current study) or a very minor change in supersaturation (as small as 0.018) could cause a clear difference in the crystallization success rate, indicating that the crystallization of proteins is very sensitive to the concentration level. Such sensitive behaviour may be one reason for the poor reproducibility of protein crystallization.

1. Introduction

As one important method of obtaining three-dimensional structural information for proteins, X-ray crystallography has been used to solve more than 88% of the protein structures that have been deposited in the PDB (Protein Data Bank; http://www.pdb.org). To obtain the diffraction necessary for this technique, it is essential to obtain protein crystals of high quality (Durbin & Feher, 1996; Chayen & Saridakis, 2002; D'Arcy *et al.*, 2003; McPherson, 2003; Helliwell, 2008; Ochi *et al.*, 2009; Newman, 2011). However, as a trial-and-error and empirical method, it is not an easy task to obtain protein crystals in many cases (McPherson, 2004; Brzozowski & Walton, 2001). One problem that is often observed yet remains unsolved is that protein crystallization suffers from poor reproducibility (Yin *et al.*, 2008).

Protein crystallographers often complain about the poor reproducibility of the crystallization of proteins. Poor reproducibility means that a combination of identical conditions yields different crystallization results (Yin *et al.*, 2008). For example, in some cases a crystallization condition is found during the crystallization screening process, but owing to poor reproducibility it is very difficult to obtain crystals again using the same conditions. This phenomenon is particularly common for proteins that are difficult to crystallize (Chayen, 2004). Poor reproducibility also results in a lower probability of crystallization. For example, a chemical reagent capable of crystallizing the protein may be ignored because it may fail to crystallize the protein during the screening process. If the reproducibility can be improved, the opportunities for obtaining crystals will be increased. Therefore, an investigation of reproducibility (including the phenomena and the mechanisms) is useful for developing new methods for enhancing protein crystallization.

Although not often discussed or investigated, possible reasons for poor reproducibility may be parameters that are not easy to control or are not controlled in a similar fashion to the environmental conditions (*e.g.* room temperature, dust in the air or humidity) or other conditions (*e.g.* concentration, pH or the condition of the container wall) related to crystallization (McPherson, 2004; Stevens, 2000; Neer, 2004). If protein crystallization is very sensitive to these parameters, a minute change in the parameters may cause a large difference in the crystallization results, resulting in poor reproducibility.

The question thus arises: how sensitive is crystallization to a particular crystallization parameter? To answer this question, it is helpful to investigate the sensitivity of protein crystallization to minute variations in crystallization parameters in order to determine the major reasons responsible for poor reproducibility.

As one of the most controlled parameters in protein crystallization, the concentration of either the protein or the precipitant is closely related to the supersaturation, which is the driving force of crystallization (Baird *et al.*, 1999; Narayanan & Liu, 2003). It has been reported that the concentration of the protein or the precipitant is usually not fully sampled in a sparse-matrix screening experiment, and suitable supersaturation conditions for crystallization are therefore often missed (Thakur *et al.*, 2007). Although in protein crystallization experiments a concentration gradient normally exists in the crystallization solution (Mullin & Leci, 1969; Larson & Garside, 1986; Myerson & Lo, 1991; Ohgaki *et al.*, 1991; Yin *et al.*, 2002), the concentration is in a limited range in most cases. An exception is the method of counter-diffusion in capillaries, which is by far the best method of forming a fine gradient of protein and precipitant concentrations over a broad concentration range and thus can enhance the probability of crystallization (Biertümpfel *et al.*, 2002; Garcia-Ruíz *et al.*, 2002; Ng *et al.*, 2003). To increase the probability of crystallization, other methods of increasing the concentration range, such as forming a local concentration or supersaturation in the crystallization solution, have also been utilized. For example, a local supersaturation can be generated by femtosecond laser irradiation, resulting in easier nucleation (Yoshikawa *et al.*, 2009; Nakamura *et al.*, 2007), or the reservoir solution in vapour diffusion can be replaced by desiccant so that the concentration range in the crystallization droplets will be expanded, leading to a higher probability of crystallization (Lu *et al.*, 2010).

Therefore, concentration is a critical parameter for obtaining crystals. In practical crystallization, preparing a solution at an accurate concentration is challenging. A solution prepared by an automated system may exhibit a concentration error (%CV; standard deviation/mean concentration) of less than 10% (Krupka *et al.*, 2002; Azarani *et al.*, 2006). The concentration error is often related to the viscosity of the solution. For some automated systems the concentration error is approximately 5% for solutions of low viscosity (Hiraki *et al.*, 2006; Newman *et al.*, 2005). However, when the viscosity of the solution is higher the %CV can be greater than 10% (Newman *et al.*, 2005). Further errors can be introduced owing to solvent evaporation after dispensing [this is why a humidity chamber (DeLucas *et al.*, 2005) or evaporation shield has been proposed for use in automated systems]. In the case of manual preparation, the solution can exhibit an even larger error in concentration. If protein crystallization is sensitive to variations in the concentration, the errors in the concentration that occur during solution preparation may partially be responsible for the poor reproducibility of crystallization. There are few reports regarding the sensitivity of protein crystallization to variations in the concentration. To obtain qualitative information on this issue, the preparation of solutions at very small concentration intervals is necessary. However, it is usually not an easy task to prepare solutions at accurate concentration levels, especially at small volumes, and it is even more difficult to prepare a series of solutions at very small concentration intervals.

In this paper, we report a method of preparing solutions at very small concentration intervals (as low as $\sim$0.004 mg ml^{-1}) by evaporating the solvent in the solutions for different periods of time, and the effect of minute concentration differences on crystallization was investigated using these solutions.

2. Materials and methods

2.1. Materials

The model protein utilized in this study was hen egg-white lysozyme (HEWL; six times recrystallized; Seikagaku Kogyo Co., Japan). Other chemicals utilized were sodium chloride (NaCl; Chemical Reagent Co. Ltd, China), sodium acetate (Beijing Chemical Factory, China) and acetic acid (HPLC grade; TEDIA Co., USA). All of these chemicals were used directly without further treatment.

96-well microbatch crystallization plates (catalogue No. HR3-267; Hampton Research, USA) were used as the containers for the crystallization droplets. Crystal Clear Sealing Tape (catalogue No. HR4-506; Hampton Research,

USA) was used to seal the crystallization plates after the crystallization solution droplets had been dispensed.

2.2. Experimental procedures

2.2.1. Creation of a fine concentration gradient between the crystallization droplets. An HEWL crystallization solution was prepared by mixing equal volumes (1.5 ml) of protein solution (40 mg ml^{-1} HEWL dissolved in 0.1 *M* sodium acetate buffer pH 4.60) and NaCl solution (80 mg ml^{-1} NaCl dissolved in 0.1 *M* sodium acetate buffer pH 4.60). To minimize the error in concentration, we took measurements as follows: large volumes of the solutions (HEWL and NaCl) of up to 1.5 ml were utilized so that the errors that arise from the amount of HEWL or NaCl could be reduced. All chemicals were measured using a high-precision microbalance (BT125D, Sartorius Scientific Instruments Co., Beijing, China; precision 0.01 $\pm$ 0.06 mg). The estimated concentrations of the final solutions were 20 $\pm$ 0.044 mg ml^{-1} for HEWL and 40 $\pm$ 0.046 mg ml^{-1} for NaCl.

2 µl of the mixed solution (which served as the mother liquor) was then dispensed into each of the 96 wells of the crystallization plate by an automated crystallization robot (Screenmaker 96+8; Innovadyne Technologies, Inc., USA). The total dispensing time from the first well to the 96th well was approximately 28 s. The time difference between each two consecutive wells was approximately 0.24 s between two wells in the same row and approximately 1 s between two consecutive wells in two different rows (*i.e.* when the dispensing needle moved to another row). Fig. 1 shows a schematic of the movement of the dispensing needle above the crystallization plate. The droplets were exposed to the open air during the dispensing process, so that evaporation of the droplets occurred immediately after they were dispensed into the wells. The evaporation time was longer for the droplets dispensed earlier; therefore, a fine concentration gradient among the droplets could be achieved using this method.

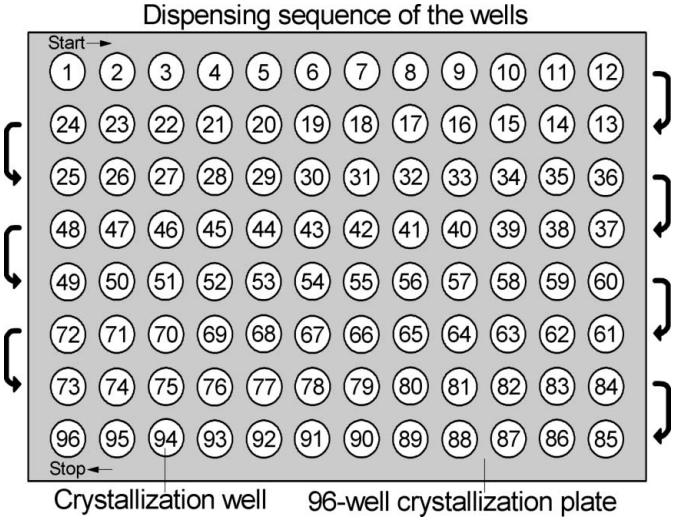

Figure 1
Schematic of the dispensing sequence of the crystallization droplets. The crystallization droplets were dispensed quickly (within 28 s) from well No. 1 to well No. 96 using a protein crystallization robot. The arrows show the direction of movement of the dispensing needle.

2.2.2. Crystallization experiments. After dispensing the solution, we sealed the crystallization plate using Crystal Clear Sealing Tape and placed the crystallization plate into a temperature controller at 293 K. The temperature controller was manufactured specifically for high-precision temperature control by flowing bath water (Polyscience 9712; Polyscience Co., USA) through all six faces of the rectangular chamber (inner dimensions of 300 $\times$ 210 $\times$ 130 mm) so that the temperature in the chamber could be controlled stably and homogeneously within 0.1 K.

The above crystallization experiment was repeated 102 times, *i.e.* 102 $\times$ 96-well plates were utilized.

After incubation in the chamber for 2 d, the crystallization plate was taken out for crystal inspection, which was carried out by capturing images of the droplets using an automated crystal image reader (XtalFinder; XtalQuest Inc., China). In the current study, the crystallization results were simple, *i.e.* only two kinds of results were observed: (i) well defined faceted lysozyme crystals appeared in the droplet and (ii) the droplet remained clear. Therefore, it is easy to distinguish the crystals and the crystallization success rate of each well can be obtained. Here, the crystallization success rate of a well means, for that specific well (*i.e.* any specific one among the wells from Nos. 1 to 96 or from A1 to H12), the ratio of the number of droplets that yielded crystals to the total number of droplets (102 in the current study).

2.2.3. Evaporation test of a crystallization droplet. To obtain information about the evaporation rate of the crystallization droplet, we measured the amount of solvent evaporated against time from a crystallization droplet with the same solution composition as used in the crystallization experiments, *i.e.* 20 mg ml^{-1} HEWL, 40 mg ml^{-1} NaCl in sodium acetate buffer pH 4.60. A drop of 2 µl crystallization solution was pipetted (using an electronic single-channel pipettor; 710521ET; BioHit, Finland) onto a cover slip [silanized using carbon tetrachloride:dimethyldichlorosilane at 9:1(*v*:*v*); Sinopharm Chemical Reagent Co. Ltd, Shanghai, China] and weighed using a microbalance (BT125D; Sartorius Scientific Instruments Co., Beijing, China) every 10 s in the same ambient environment as the dispensing procedure in the crystallization experiment. The total measurement time for one droplet was 23 min. To obtain more accurate results, the same procedure was repeated three times.

3. Results and discussion

3.1. Concentration gradient between the crystallization droplets

The initial concentration of the prepared mother solution was 20 mg ml^{-1} HEWL and 40 mg ml^{-1} NaCl. During the automated dispensing period, the dispensed droplets evaporate, so that a subtle concentration variation occurs in the droplets. The concentrations of the droplets at the moment when the crystallization plate was sealed, which can be

calculated from the evaporation rate of the droplet, differ from each other owing to the differing evaporation times.

We measured the evaporation rate of a droplet of the same volume (2 µl) in the same ambient environment. The amount of evaporation *versus* time is shown in Fig. 2.

From the figure, it can be seen that the evaporation amount in the first 5 min was linear *versus* time. Therefore, the evaporation rate can be calculated using the equation

$$v = \frac{m_0 - m_t}{t}, \quad (1)$$

where m_0 is the initial mass of the crystallization droplet, m_t is the mass of the crystallization droplet at 5 min (300 s) and t is the time period (300 s).

The results showed that the evaporation rate in the first 5 min was approximately 1.8 µg s^{-1}.

According to the evaporation rate, the minor variations in concentration in the droplets owing to evaporation can be calculated. Fig. 3 gives the calculated concentrations of both lysozyme and NaCl when the crystallization plate was sealed.

From the figure and the calculated results, it can be seen that the concentration difference between two consecutive droplets in the same row (*e.g.* from No. 1 to No. 2, from No. 2 to No. 3 *etc.*) was approximately 0.0043 mg ml^{-1} (0.0215% of the initial concentration) for lysozyme and 0.0086 mg ml^{-1} (0.0215% of the initial concentration) for NaCl. The concentration difference between two consecutive droplets in two different rows (*e.g.* from No. 12 to No. 13, from No. 24 to No. 25 *etc.*) was approximately 0.018 mg ml^{-1} (0.09% of the initial concentration) for lysozyme and 0.036 mg ml^{-1} (0.09% of the initial concentration) for NaCl. Thus, a very fine concentration gradient among the droplets was created; this type of gradient is not easy to achieve by direct mixing of different components.

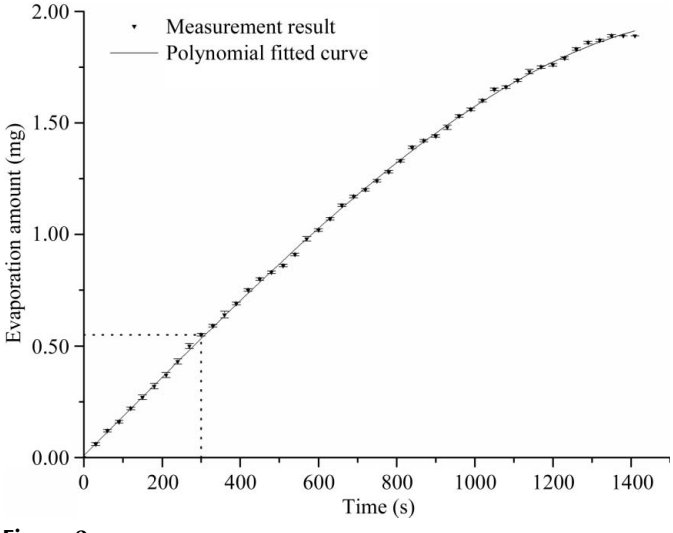

Figure 2
Evolution of the amount of evaporation of solvent *versus* the evaporation time of a crystallization droplet. The initial solution conditions were identical to those used in the crystallization experiments: 20 mg ml^{-1} HEWL and 40 mg ml^{-1} NaCl. The experiment was repeated three times and the amount of evaporation was remarkably reproducible. The initial weight of the droplet was 2.05 ± 0.01 mg and the evaporated amount was 1.89 ± 0.01 mg. The results showed that evaporation was complete after 1380 s and the amount of evaporation was linear *versus* the evaporation time in the first 5 min (300 s).

3.2. The effect of minute variations in concentration on the crystallization success rate

Fig. 4 illustrates the crystallization success rate for each of the specific crystallization wells. In Fig. 4(*a*), the success rate is shown against the sequence number of the wells.

Over the entire range of a dispensed sequence, a decreasing trend in the crystallization success rate can be observed against the sequence number of the wells, indicating that the droplets

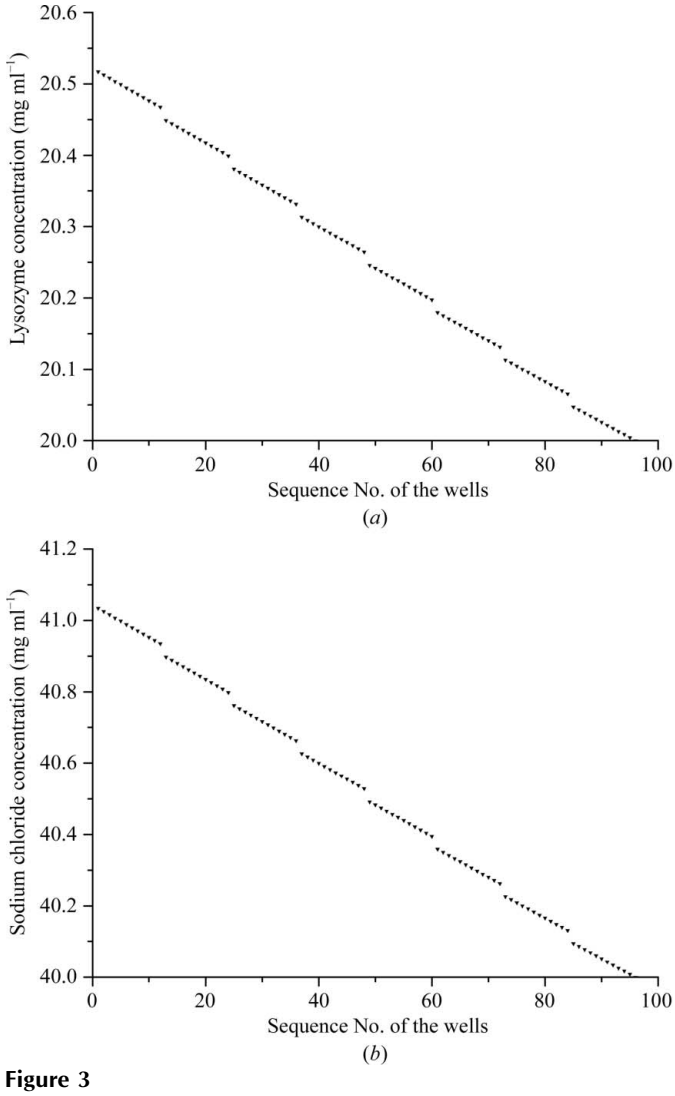

Figure 3
The concentration of lysozyme (*a*) and NaCl (*b*) in different crystallization wells upon sealing. The initial concentration of the crystallization solution before dispensing into the wells was 20 mg ml^{-1} HEWL and 40 mg ml^{-1} NaCl. Owing to solvent evaporation, the concentration in the different crystallization wells is different; thus, upon sealing the crystallization plate fine gradients of concentration could be obtained. The smallest concentration difference between two consecutive droplets was 0.0043 mg ml^{-1} for HEWL and 0.0086 mg ml^{-1} for NaCl. The concentration drops between wells No. 12 and No. 13, No. 24 and No. 25 *etc.* were caused by a longer time interval owing to the change in direction of the dispensing needle.

dispensed earlier exhibited a higher crystallization success rate. However, it is hard to distinguish such a trend over a narrow range of sequence numbers. For instance, when comparing the crystallization success rate between two consecutive droplets (the concentration difference between the two droplets was less than 0.018 mg ml^{-1} for lysozyme and less than 0.036 mg ml^{-1} for NaCl), some droplets that were dispensed earlier exhibited a higher success rate; however, the phenomenon that droplets dispensed later exhibited a higher success rate could also be observed. These contradictory results indicated that the concentration difference between two consecutive droplets is not large enough to induce a clear trend in the crystallization success rate. Therefore, how much of a concentration difference is necessary for observation of a clear trend in the crystallization success rate?

To determine the answer to this question, we carried out a statistical analysis of the obtained data. In Fig. 4(*a*), the crystallization success rate is plotted against the sequence number of the wells. Replacing the sequence number by the concentration calculated according to the evaporation rate allowed us to obtain the crystallization success rate *versus* the concentration of lysozyme in the droplets. Fig. 4(*b*) shows the results. As expected, the slope of the crystallization success rate against the lysozyme concentration is positive over the entire studied concentration range. However, if we examine the success rate within smaller concentration ranges (*i.e.* smaller variations in concentration), the slope can be negative. That is to say, crystallization was insensitive to the variation of concentration within a small concentration range. If we sample a different number of consecutive droplets (from three to 95 droplets) and calculate the slope of the success rate against the concentration, we can obtain the averaged slope of the success rate against the concentration at different sampling numbers.

Fig. 5 shows the averaged slope at different sampling numbers and, indeed, all of the averaged slopes were positive. However, the error bar was very large when the sampling number was small, so that the positive trend was not significant. When the sampling number was seven, the positive trend became significant (P = 0.025 < 0.05) according to a one-sample t-test. This result showed that the crystallization success rate was significantly affected by the concentration when the variation in concentration was greater than 0.026 mg ml^{-1} (0.13% of the initial concentration) for HEWL and 0.052 mg ml^{-1} (0.13% of the initial concentration) for NaCl. In other words, the smallest concentration variation of

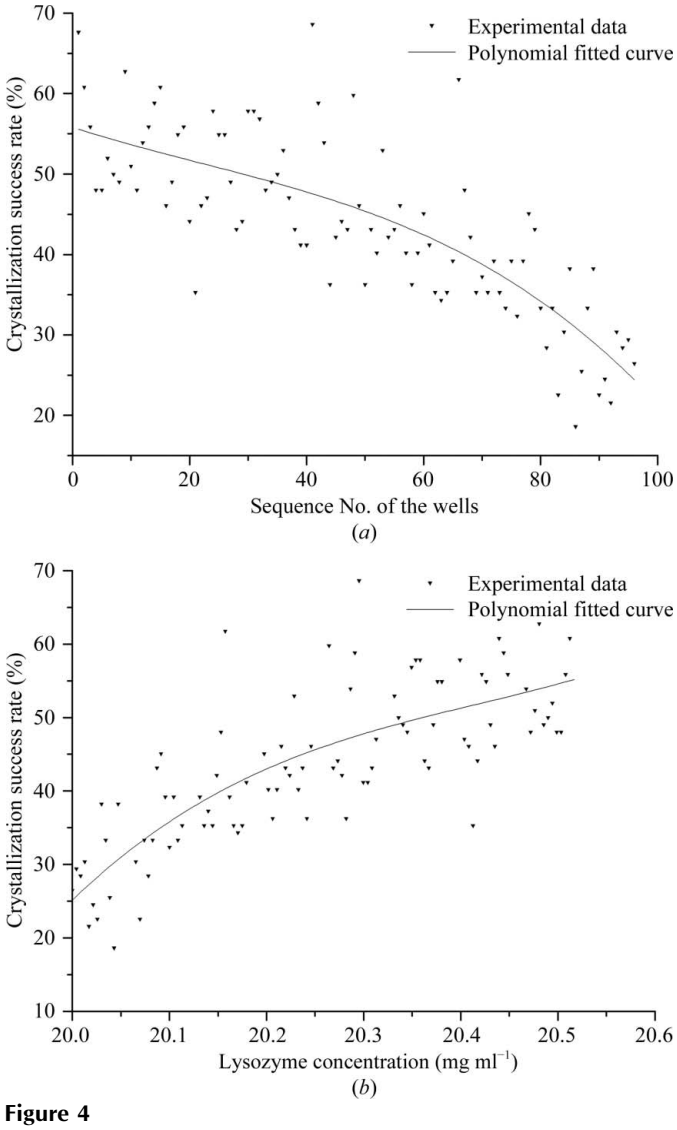

Figure 4
The crystallization success rate for different wells. Since a total of 102 crystallization plates were used, there were 102 droplets for every well. The crystallization success rate of a specific well can be obtained by summing the total number of droplets that yielded crystals in that specific well and dividing by 102. The crystallization success rate is plotted against (*a*) the sequence number of the wells and (*b*) the concentration of lysozyme in different wells. Judging from the overall data, we detected a clear trend in the crystallization success rate against the sequential crystallization wells.

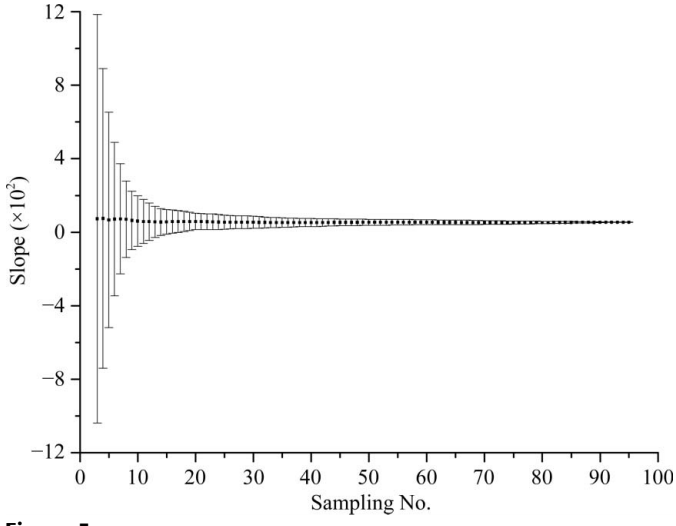

Figure 5
The averaged slope of crystallization success rate against concentration for different sampling numbers of consecutive droplets. All averaged slopes were positive. However, the standard deviations were large when the sampling number was small. When the sampling number was seven, the positive trend could be considered significant (P = 0.025 < 0.05). This result indicated that crystallization of lysozyme is sensitive to minute concentration variations as small as 0.13% (0.026 mg ml^{-1} for lysozyme and 0.052 mg ml^{-1} for NaCl in the current study).

the solution that resulted in a clear effect on the crystallization of lysozyme was 0.13% of the initial concentration (0.026 mg ml^{-1} for HEWL and 0.052 mg ml^{-1} for NaCl).

There is one more point that it is necessary to address: it has been well established that evaporation is faster around the edges of 96-well plates than in the middle, even after the plate has been sealed. However, in our current study all 96 wells were sealed off from each other and the temperature control was very stable and homogeneous. We believe that the effect of differential evaporation around the edges of the plates is not noticeable after 2 d; therefore, this effect was neglected in our analysis.

3.3. Discussion

3.3.1. The smallest variation in supersaturation that can affect the crystallization of lysozyme. As the driving force for protein crystallization, supersaturation is a more fundamental parameter than concentration. The sensitivity of lysozyme crystallization to supersaturation can be obtained from the sensitivity of lysozyme crystallization to concentration variation.

The supersaturation of lysozyme in each droplet when the crystallization plate was sealed can be obtained by dividing the concentration of lysozyme by the solubility. Because evaporation will increase the concentration of both lysozyme and NaCl, the solubility of lysozyme in the solution changes upon evaporation. However, it is easy to estimate the solubility in different droplets because the solubility data for lysozyme (Forsythe *et al.*, 1999) in different conditions have been well documented and can be utilized in our study.

By curve-fitting the published data (Forsythe *et al.*, 1999) using an exponential equation (Ferreira *et al.*, 2011; Crespo *et al.*, 2010), we can obtain the relationship of the lysozyme solubility C_s to the NaCl concentration C_{NaCl} when lysozyme and NaCl are dissolved in sodium acetate buffer pH 4.60 at 293 K (in the range of NaCl concentration between 20 and 50 mg ml^{-1}),

$$C_s = -0.0016C_{NaCl}^3 + 0.2146C_{NaCl}^2 - 9.6437C_{NaCl} + 148.06. \quad (2)$$

The supersaturation σ of each droplet can be thus obtained using the equation

$$\sigma = C/C_s, \quad (3)$$

where C is the concentration of lysozyme in each droplet.

Fig. 6 illustrates the calculated supersaturation in each droplet. The supersaturation of lysozyme differed in different droplets. The droplets dispensed earlier clearly exhibited a higher supersaturation. According to the results of the effect of a minute variation in lysozyme concentration on the crystallization success rate, the smallest variation in supersaturation $\Delta\sigma$ that can affect the crystallization success rate was approximately 0.018 (corresponding to a concentration variation of 0.13%).

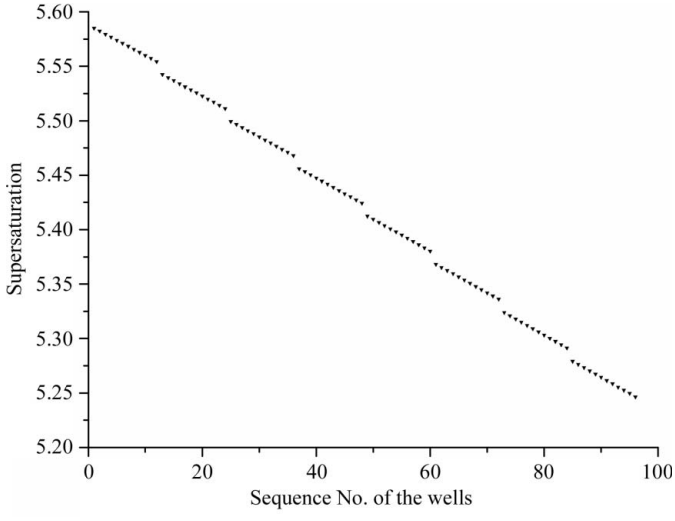

Figure 6
Supersaturation in each droplet. The supersaturation was calculated according to the concentration of lysozyme and NaCl and the solubility data. Clearly, the supersaturation of the droplets dispensed earlier was higher than that of those dispensed later.

3.3.2. The nucleation rate and the crystallization success rate of lysozyme. It is well known that crystallization comprises nucleation and growth processes. The crystallization success rate is determined by nucleation, which is directly affected by supersaturation. The minute variations in concentration affect the supersaturation and further affect the nucleation rate, which finally leads to differences in the crystallization success rate. Therefore, it is meaningful to investigate the relationship between the nucleation rate and the crystallization success rate.

The nucleation rate I can be expressed as follows when the concentration of lysozyme is C (Tavare, 1987; Saikumar *et al.*, 1998; Carbone & Etzel, 2006),

$$I = k_n\left(\frac{C - C_s}{C_s}\right)^a, \quad (4)$$

where k_n and a are constants.

The trend in nucleation against concentration of lysozyme should be similar to the trend in crystallization success rate against concentration of lysozyme. To compare the trends in these two parameters and display them in a single figure, we normalized the success-rate and nucleation-rate data by dividing the success rates or the nucleation rates at different concentrations of lysozyme by those at 20 mg ml^{-1} HEWL. Fig. 7 displays the normalized data of the crystallization success rate and the normalized data of the nucleation rate *versus* the lysozyme concentration according to the equation proposed by Saikumar *et al.* (1998),

$$I = 7.71 \times 10^{-2} \times \left(\frac{C - C_s}{C_s}\right)^3. \quad (5)$$

As expected, judging from the trends in both the crystallization success rate and the nucleation rate, these two parameters showed the same tendency to increase with increasing concentration. However, the slopes of these two parameters against the concentration were clearly not the same. Therefore, the crystallization success rate is not proportional to the

nucleation rate. Nevertheless, the crystallization success rate is certainly closely related to the nucleation rate, so we can assume that the crystallization success rate R is a function of the nucleation rate I,

$$R = f(I). \quad (6)$$

We used a polynomial equation to fit the data. However, owing to the scattered crystallization success-rate data in Fig. 4, we smoothed the data by averaging the crystallization success rate over seven consecutive droplets so that an empirical equation for crystallization success rate against nucleation rate could be obtained,

$$R = 4.8097I^3 - 108.98I^2 + 827.76I - 2053.4. \quad (7)$$

Fig. 8(*a*) shows the fitted curve together with the averaged crystallization success rate against the nucleation rate. A clear trend showing an increase in the crystallization success rate with increasing nucleation rate can be observed.

Because the nucleation rate is determined by the supersaturation, the crystallization success rate is also a function of supersaturation. To further simplify (7), we performed a curve-fitting of the crystallization success rate against the supersaturation. An empirical equation was thus obtained,

$$R = 333.62\sigma^3 - 5665.4\sigma^2 + 32085\sigma - 60541. \quad (8)$$

Fig. 8(*b*) shows the fitted curve together with the averaged crystallization success rate against the supersaturation. The trend was the same as shown in Fig. 8(*a*).

3.3.3. Does the solution preparation affect the reproducibility of protein crystallization?. From the current quantitative study, it is clear that a very small difference in concentration or supersaturation can cause a large difference in the crystallization success rate. In a practical solution preparation, deviation of the concentration from the designated value will be unavoidable owing to the many uncertainties in solution preparation. For example, errors in the concentration can arise from weight or volume measurements of the components; errors may also occur from evaporation of the solvent that alters the concentration. When the volume of the solution is very small (*e.g.* in manual preparations down to a volume of less than 0.5 µl or in automated preparations down to a volume of less than 50 nl), errors in concentration can become much more severe because a minute change in any component can cause a large difference in the overall concentration of the solution. The volume of the crystallization solution is usually very small owing to the difficulty in obtaining large quantities of protein sample. Therefore, large errors can be introduced during preparation of the solution. Deviation of the actual concentration from the expected concentration during preparation of the protein crystallization solution can be widely observed. As pointed out in previous studies, an automated robot can prepare solutions with a CV

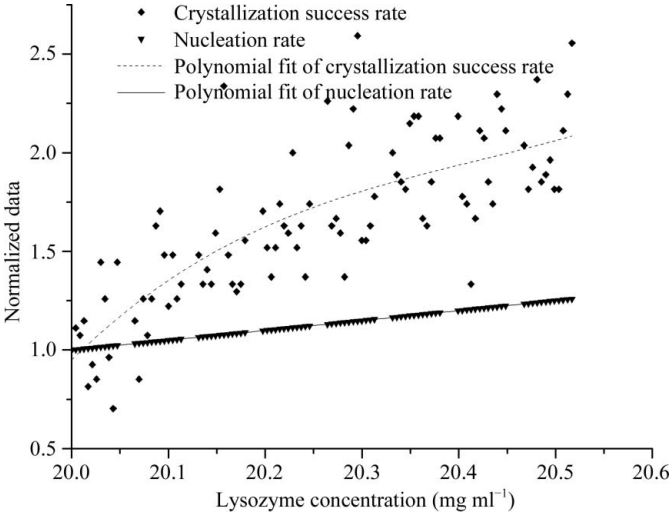

Figure 7
Normalized values of both the crystallization success rate and the nucleation rate *versus* the lysozyme concentration. These two parameters show the same tendency, *i.e.* an increase with increasing concentration. However, the slopes of the two parameters against the concentration were not the same.

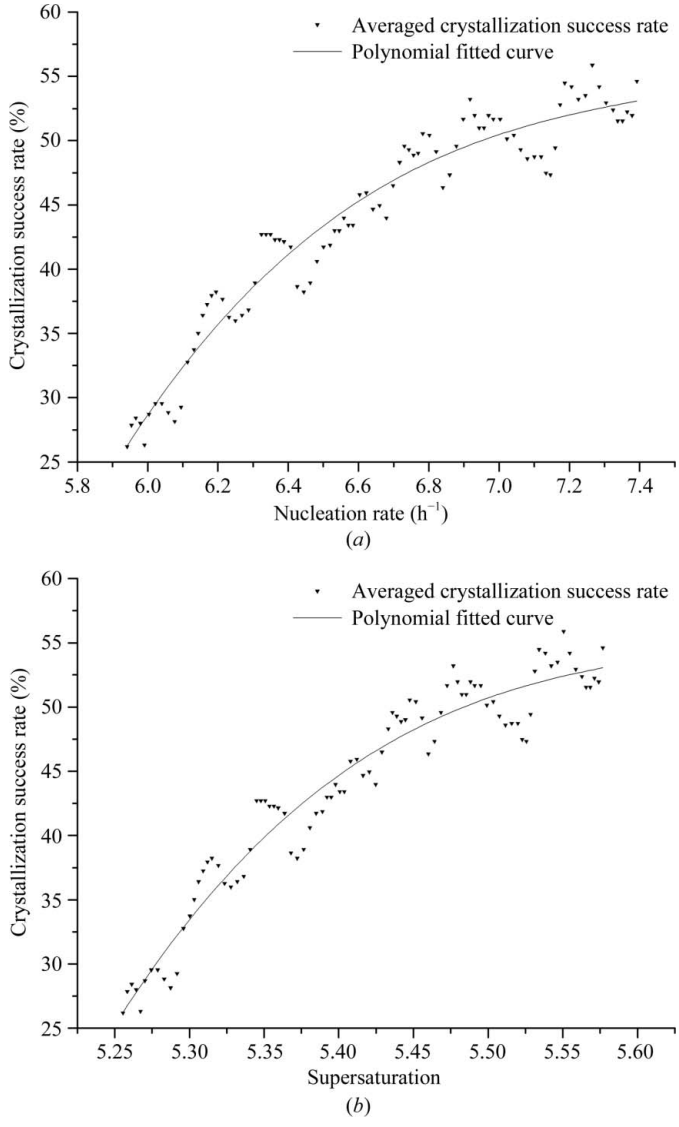

Figure 8
The averaged crystallization success rate of seven consecutive droplets plotted against (*a*) the nucleation rate and (*b*) the supersaturation. Both were curve-fitted using a polynomial equation.

of 5%, with manual preparations having a higher error. If the concentration of the solution is approximately 20 mg ml^{-1}, then the error in the concentration can be 1 mg ml^{-1}. Such an error is much larger than the smallest concentration difference that can cause a difference in the crystallization. Therefore, the routine solution-preparation procedure can play a role in poor reproducibility of protein crystallization.

4. Conclusions

In the present study, we investigated the effect of small concentration variations on the crystallization success rate of lysozyme. By performing a large number of experiments examining the reproducibility of crystallization using the same crystallization conditions repeatedly over one year, a statistically reliable trend was obtained and some conclusions were drawn.

(i) The crystallization of lysozyme can be very sensitive to variation in the concentration of the protein or precipitants. Minute differences in the concentration caused by evaporation can exert a large effect on the crystallization success rate. The smallest concentration difference that caused a clear difference in the crystallization success rate was determined to be approximately 0.13% of the initial concentration (0.026 mg ml^{-1} for lysozyme and 0.052 mg ml^{-1} for NaCl).

(ii) In terms of supersaturation, the smallest supersaturation difference that could induce a clear difference in the crystallization success rate was found to be 0.018.

(iii) The high sensitivity of crystallization to variation in supersaturation can cause very different crystallization results owing to small variations in the supersaturation, which is often encountered and unavoidable; thus, the poor reproducibility of protein crystallization may be related to the uncertainties arising from small deviations in concentration during solution preparation.

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