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Analysis of Kinetic Behavior of Protein Crystallization in Nanodroplets

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This paper demonstrates the possibility of controlling nucleation and crystal growth behavior within a droplet formed in a microspace. The effects of droplet volume and shape were examined by using thaumatin as a model system. The droplet size and shape affected the number and size of the obtained protein crystals.

The combination of protein crystallization and X-ray crystal structure analysis has been becoming a strong tool in drug discovery. In recent years, an automated robot screening system was developed and high-throughput screening was carried out.[1,2] Moreover, an automated system for X-ray crystal structure analysis was also developed.[3] However, these automated systems are independent, thus an integrated system that can continuously conduct experiments both on protein crystallization and X-ray diffraction is strongly desired. An integrated system that carries out protein crystallization within a nanodroplet in a capillary/microchannel has been developed as a connecting tool between crystallization and X-ray diffraction.[4–6] When protein crystal was obtained within a droplet, a capillary was mounted on an X-ray diffractometer followed by in situ X-ray crystal structure analysis. However, kinetic mechanisms of protein crystallization in nanodroplets has not yet been investigated in detail. The nanodroplet has larger surface/volume ratio than those of the macroscale systems. This feature may affect protein crystallization particularly at the nucleation stage. Also, small volume influences growth kinetics. This information needs to be elucidated in order to establish nanodroplets as a protein crystallization tool.

In this paper, we report the kinetic behavior of nucleation and crystal growth of protein crystal within a nanoliter-scale droplet by examining the effects of droplet volume and shape on protein crystallization.

We used thaumatin as a model protein because this protein has been well investigated to analyze growth kinetics in macroscale crystallization.[7,8] We used known crystallization conditions in the experiments (see Table S1 in Supporting Information; SI[16]).

Microfluidic PDMS chips were fabricated by using rapid prototyping and micromachining[9,10] and used as a droplet forming devices (see Figure S1 in SI[16]). After assembly, PDMS chips were subjected to surface modification by treatment with trichloro(1*H*,1*H*,2*H*,2*H*-perfluorooctyl)silane[11,12] to reduce nonspecific adsorption prior to use. Crystallization experiments were performed by mixing the protein solution and precipitants followed by formation of the droplet of this mixture in fluorinated oil (FC40) containing fluorosurfactant (1*H*,1*H*,2*H*,2*H*-perfluoro-1-octanol). Droplets were collected in the Teflon capillary at the outlet of the PDMS chip. We used three types of Teflon capillary with different internal diameter (i.d.: 130, 200, and 360 μm). To use these capillaries, we can control droplet shape.

In the case of nanodroplet generated by using the PDMS chip, microchannel structure and flow rate of continuous phase and dispersed phase were employed for the control of droplet volume. Control of the droplet shape was also achieved by using appropriate diameter Teflon capillary. When the same volume droplet was collected on a small diameter PTFE capillary, the droplet shape became elongated compared to the diameter of a large PTFE capillary. For example, Figure 1 shows photographs of droplets collected in a PTFE capillary. The volume of these droplets was 50 nL, but the droplet shape was clearly different (Figures 1a and 1b). When the capillary diameter was 200 μm, the droplet shape became elongated (Figure 1a). In contrast, when the capillary diameter was 360 μm, the droplet shape became elliptical. Under this condition, only a few thaumatin crystals appeared after incubation for several hours. Figures 1c and 1d show photographs of thaumatin crystals within a nanodroplet. We examined the effects of droplet volume on protein crystallization. Various volumes of droplets were

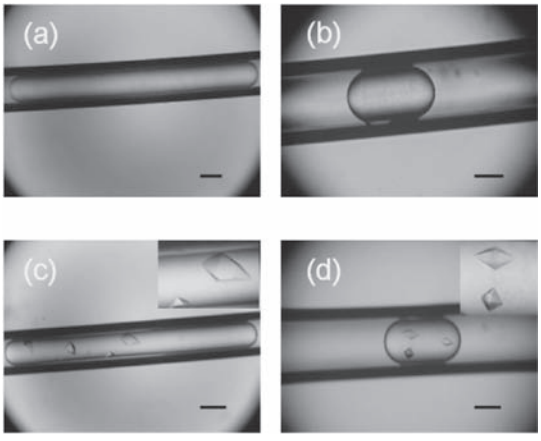

Figure 1. Photographs of 50-nL volume droplets collected on PTFE capillary. (a) The capillary diameter is 200 μm. Droplet shape became elongated. (b) The capillary diameter is 360 μm. The droplet shape becomes elliptical. (c and d) Thaumatin crystal within a nanodroplet after incubation for 24 h. Each scale bar is 200 μm.

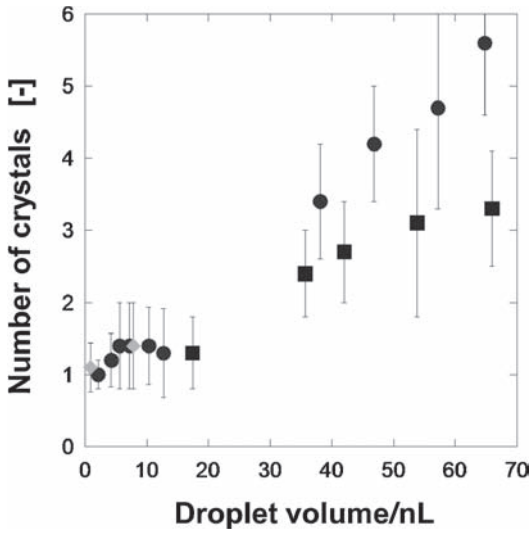

Figure 2. Relationship between droplet volume and number of crystals. The error bar for each plot was calculated from ca. 200 droplets. Each plot represents the number of crystals within a nanodroplet at a capillary diameter of 360 (■), 200 (●), and 130 μm (◆), respectively.

generated by using PDMS chip, and crystallization was carried out. Figure 2 shows the relationship between droplet volume and number of crystals. The number of thaumatin crystals was gradually increased with increasing droplet volume. A large volume of droplet generated a large amount of thaumatin molecules thus, the number of crystals increased with increasing droplet volume. In the conventional micro-batch method, thaumatin crystal appeared in 1–2 h (see Figure S2 in SI[16]). A lot of crystals precipitated within a 3-μL droplet, thus showing that we could not control crystallization of thaumatin in the macroscale and that the number of crystals was dramatically increased compared to nanodroplet.

Next, we investigated the effect of droplet shape on crystallization. As a result, a lot of thaumatin crystals were obtained within a nanodroplet by using a small diameter PTFE capillary (Figure 2). For example, in the case of 50-nL volume droplet and capillary diameter of 200 μm, we often observed 3 to 6 crystals (the average number of crystals was 5) within a nanodroplet. On the other hand, in the case of 50-nL volume droplet and capillary diameter of 360 μm, we often observed 2 to 4 crystals (the average number of crystals was 3) within a nanodroplet. Furthermore, we observed more than 100 sphere-shaped droplets in both capillaries i.e., only one crystal was obtained within a nanodroplet. We expect this technique to offer significant advantages to in situ X-ray crystal structure analysis. Protein crystallization can be controlled by preventing precipitation of bicrystal within a droplet. One of the attractions of this technique is that it can facilitate continuous X-ray diffraction experiment by using PTFE capillary. The crystal size of thaumatin was approximately 60–150 μm, and it could be used in X-ray crystal structure analysis by synchrotron radiation. These results show that by controlling droplet volume and droplet shape, we could control protein crystallization.

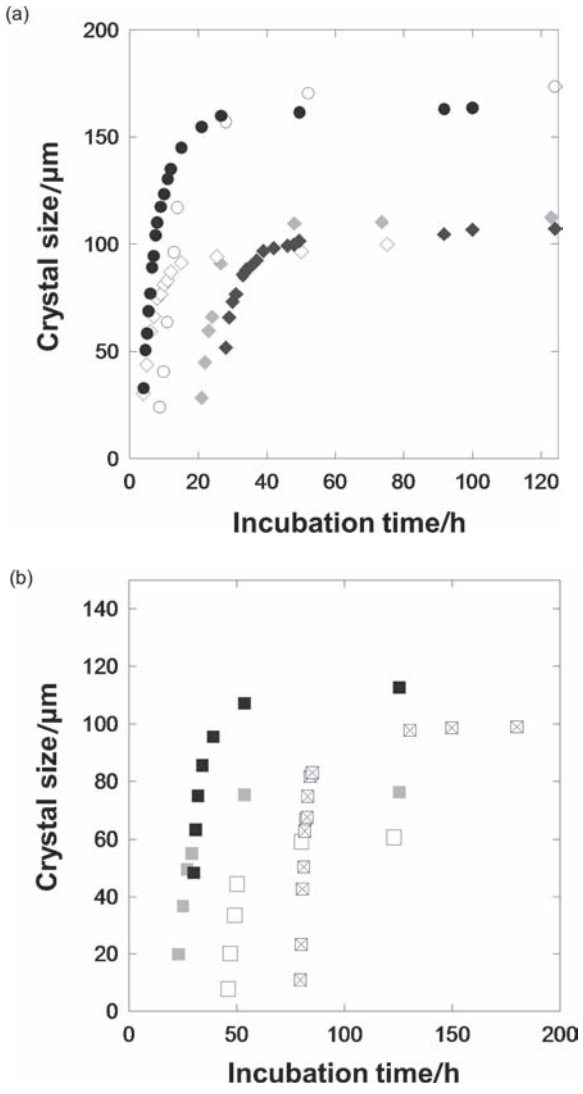

Figure 3. (a) Crystal growth behavior within a nanodroplet. (●, ○) Droplet volume is 14 nL; (◆, ◈, ◇) Droplet volume is 10 nL. Each plot represents different experiments. (b) Crystal growth behavior of thaumatin within a 4 nL droplet. Each plot represents different experiments (■, ▩, □). (■, ⊠) Crystal growth behavior within a different nanodroplet collected in the same PTFE capillary. The crystal size was measured as the long axis of the thaumatin crystal.

Finally, we analyzed the crystal growth of thaumatin. In the case of macroscale micro-batch method, a lot of thaumatin crystals appeared after incubation of 1–2 h; thus, the analysis of crystal growth of thaumatin focusing on one crystal was difficult. However, in nanodroplets formed by microfluidic setup, each droplet formed only one crystal; therefore, we were able to observe crystal growth of thaumatin in detail. We measured the thaumatin crystal growth rate at various droplet volumes within a PTFE capillary (i.d.: 200 μm). Figure 3 shows the behavior of crystal growth within a nanodroplet. Under this condition, thaumatin crystal growth rate was almost constant (10–20 μm h^{-1}), but induction times for nucleation were varied by droplet volume. In the case of 14-nL droplet volume,

thaumatin crystal appeared within droplets after several hours of incubation. In 10-nL droplet, the induction time of nucleation was slightly longer than that of 14-nL droplet with variability (Figure 3a). When droplet volume was reduced to 4 nL, the induction time of nucleation was much longer than the droplet volumes mentioned above with considerable variation (Figure 3b). These results show that the induction time of nucleation becomes longer with decreasing droplet volume. Even with 1-nL droplet, we could obtain thaumatin crystal and its induction time of nucleation increased dramatically. To elucidate this phenomenon, we analyzed the mechanism of thaumatin crystal growth by using Avrami equation. The Avrami equation ($C = 1 - \exp(-kt^n)$) was used to understand the kinetics of crystallization, where C is the percentage of crystallization, k is the crystallization rate constant, t is time, and n is the Avrami exponent.[13,14] As a result, the Avrami exponent was 1.5 for all cases and did not show dependency on droplet volume. The thaumatin crystal growth was three-dimensional sphere-like; thus, this result suggests that thaumatin crystal growth is a diffusion-controlled growth. Protein molecule is generally larger than inorganic materials and is nonsymmetrical. For this reason, in most cases, protein crystal growth is understood as a process with interfacial interaction as the rate-limiting step. We propose two possible reasons for variation of induction time and difference of mechanism of crystal growth. First, we consider absolute surface area (Figure S3[16]) and interface of liquid–liquid phase in droplet system to affect nucleation. Small volume droplet or sphere-shaped droplet has large surface to volume ratio. For example, in this study, surface-to-volume ratios were calculated 23, 30, and 46 mm^{-1} for 10 (i.d.: 200 μm), 4 (i.d.: 200 μm), and 1 nL (i.d.: 130 μm), respectively. Thaumatin molecules congregated in liquid–liquid interface because of the presence of fluorosurfactant at continuous phase to prevent nonspecific adsorption at interface or surface.[11] If the nucleation step is suppressed at liquid–liquid interface, induction time of sphere-shaped droplet that has large surface-to-volume ratio becomes long. The second possible reason is the effect of suppression of free convectional flow in the nanodroplet. The *Ra* number, a dimensionless number for free convection is proportional to the cube of the capillary diameter ($(Ra = g\beta\ (T_w - T_g)L^3/\nu\alpha)$, where g is the gravitational acceleration, β is the coefficient of thermal expansion, T_w is the temperature of the wall, T_g is the fluid temperature, L is the capillary diameter, ν is the kinematic viscosity, and α is the thermal diffusivity). In the microspace, free convection in a droplet would be suppressed because of the small value of *Ra* number. Thus, free convection in a droplet in a microspace would be suppressed, and concentration gradient of thaumatin would be formed around crystal nuclei. This condition is similar to microgravity or convection less environment.[15] Under the microgravity condition, protein crystal growth is characterized as diffusion-controlled growth and crystal growth rate is decreased. This hypothesis is suggested by Figure 2. When droplet shape becomes elongated, the distance of nuclei and nuclei becomes large. Thus, concentration gradient was easily formed around nuclei because of diffusion-control in thaumatin molecules. As a result, a lot of thaumatin crystals were obtained within a droplet in a small diameter PTFE capillary (i.d.: 200 μm) compared with the one having a large diameter (i.d.: 360 μm).

In conclusion, our results show that the kinetic mechanism of protein crystallization could be analyzed by using a nanodroplet system. Droplet volume is related to the number of thaumatin crystals as demonstrated by the gradual decrease in the number of crystals upon reduction in droplet volume. Droplet shape also influences the number of crystals. The induction time of nucleation becomes longer by decreasing the droplet volume. The Avrami analysis gives constant Avrami exponent value despite the change in the volume of nanodroplet. These results indicate the significant effects of droplet volume and droplet shape on protein crystallization.

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16 Supporting Information is available electronically on the CSJ-Journal Web site, http://www.csj.jp/journals/chem-lett/index.html.

