AN APROACH FOR SINGLE CRYSTALLIZATION OF PROTEIN BY USING DROPLET BASED MICROFLUIDICS

Masatoshi Maeki[1], Yuki Teshima[1], Saori Yosizuka[1], Hiroshi Yamaguchi[2], Kenichi Yamashita[1,3], Hideaki Maeda[3], Masaya Miyazaki[1,3]

[1]Kyushu University, Japan, [2]Tokai University, Japan, [3]AIST, Japan

ABSTRACT

This paper reports a method for generating single crystals by using droplet based microfluidics. We studied theoretical background, and estimated the droplet size for obtaining only one crystal within each droplet. We also investigated crystallization behavior of four model proteins to confirm the effect of protein molecular diffusion on crystallization. A single crystal was obtained within a microdroplet which was smaller than the critical size. We consider that single crystallization or decoupling protein nucleation and crystal growth can be easily achieved by controlling droplet size.

KEYWORDS

Protein crystallization, Droplet, Single crystallization, Critical droplet size

INTRODUCTION

Protein crystal structure analysis offer essential information for the field of drug discovery and understanding biochemical mechanism. To determine the three dimensional structure of a protein, preparing a large single crystal is a fundamental procedure for X-ray crystallography. Therefore, controlling nucleation of protein crystal and obtaining a large single crystal is a significant issue for X-ray crystal structure analysis. Herein, we present an approach for single crystallization of protein by using droplet based microfluidics. We focused on the alleviation of supersaturation by controlling the protein concentration gradient in a microdroplet after the first nucleation.

Generally, the concentration of protein and precipitant solution i.e., supersaturation in solution, plays an important role in controlling nucleation of a protein crystal. Nucleation has to overcome energy barrier in order to form a critical nucleus size. To overcome energy barrier for nucleation, the degree of supersaturation is understood as a driving force. Thus, under the too high degree of supersaturation condition, microcrystals or amorphous of protein were obtained due to the rapid nucleation. On the other hand, under the low degree of supersaturation condition, nucleation does not occur. Ideally, controlling protein crystal nucleation and forming one nucleus within a droplet or system in order to obtain a large single crystal is desired. It is usually called mononucleation or single crystallization. A variety of methods including microfluidics technology have been applied to separate nucleation and crystal growth. Microfluidics technology-based platform has already been reported as a convenient tool to explore protein crystallization conditions [1]. Furthermore, another application for protein crystallization, such as *in-situ* X-ray diffraction measurement, decoupling of crystal nucleation and growth, and membrane protein crystallization have been reported [2,3]. We previously reported the effect of droplet volume and shape on protein crystallization [4]. Moreover, we also studied the theoretical background, and estimated the droplet size for obtaining only one single crystal within each droplet [5]. In this paper, we report a potential for controlling nucleation of protein crystal by using droplet-based microfluidics. We also investigated crystallization behavior of four model proteins based on droplet microfluidics to confirm the effect of protein molecular diffusion on crystallization.

EXPERIMENT

We employed lysozyme, thaumatin, glucose isomerase, and ferritin as model proteins in this study. Lysozyme and thaumatin were used without further purification. Glucose isomerase was dialyzed overnight. Ferritin stock solution was centrifuged at 13.2 krpm for 10 minutes and supernatant solution was collected. Both of the protein and precipitant solutions were filtered through 0.22 μm or 0.45 μm filters prior to use in the crystallization experiment. Microfluidic device was made from poly (dimethylsiloxane) (PDMS). PDMS replica was fabricated by soft lithography and replica molding method [6]. After assembly, PDMS chip was subjected to surface modification by treatment with trichloro (*1H, 1H, 2H, 2H*-perfluorooctyl) silane to reduce non-specific 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 (*1H, 1H, 2H, 2H*-perfuluoro-1-octanol). Droplets were collected in the perfluoroalkoxyalkane (PFA) capillary at the outlet of the microfluidic chip. We used three types of PFA capillaries (I.D. 200 μm, 360 μm, and 500 μm) with different internal diameters to control the droplet size. The PFA capillary that contained droplets of protein solution was cut off from the PDMS chip and was sealed with a capillary wax. After setting up crystallization experiment (4 °C), we measured the number of crystals within a droplet every one hour by using an optical microscope.

We also studied the theoretical background and estimated the critical droplet size in radius R_c for obtaining only a single crystal within a droplet as follows:

$$R_c = \sqrt{\frac{6DC_0}{q}} \qquad (1)$$

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The R_c value was derived from Fick's first law and was calculated from diffusion coefficient (D), initial concentration of protein (C_0), and the consumption rate of protein in solution (q) [5].

RESLUTS AND DISCUSSION

We used thaumatin (22 kDa), glucose isomerase (170 kDa), and ferritin (440 kDa) as model proteins to confirm the effect of protein molecule diffusion on crystallization. Fig. 1 shows the concept of our droplet based single crystallization method. This technique is based on the alleviation of supersaturation by controlling the protein concentration gradient in a microdroplet after first nucleation. We employed suitable droplet size based on R_c to generate single crystals each droplets. R_c is, in other words, the maximum distance to which protein can be transported to the center of the droplet by simple diffusion. Therefore, according to equation (1), we considered single crystallization as difficult when we employing large molecular weight protein due to the smaller R_c value. We carried out preliminary protein crystallization experiment within a droplet to calculate R_c. The estimated critical droplet size is summarized in Table 1. The critical droplet size was roughly calculated 600, 600, 450 and 60 ~ 200 μm for lysozyme, thaumatin, glucose isomerase, and ferritin, respectively.

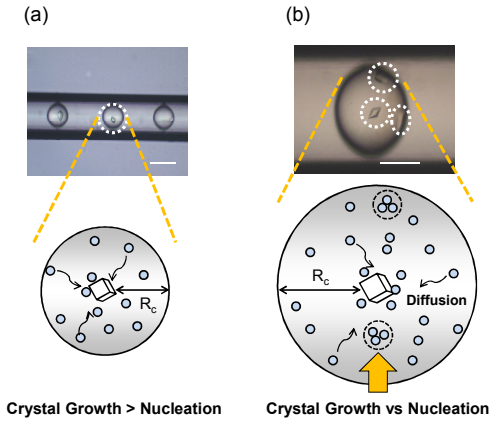

Table 1. The Estimated Value of Critical Droplet size

Protein	Lysozyme	Thaumatin	Glucose isomerase	Ferritin
R_c [μm]	600	600	450	60 ~ 200

Figure 1. The concept of single crystallization based on droplet microfluidics.
(a) Droplet size < Critical size (R_c) (b) Droplet size > R_c. The critical size was estimated diffusion coefficient (D), initial concentration of protein (C_0), and the consumption rate of protein in solution (q). Each scale bar is 200 μm.

The diffusion coefficient of ferritin monomer was reported to be 3.2×10^{-11} m^2/s as measured by dynamic light scattering [7]. In this study, we used 24 subunits ferritin solution thus, we estimated the diffusion coefficient of ferritin to be 1×10^{-12} - 1×10^{-13} m^2/s at 4 °C. In fact, the actual critical size is considered to be smaller than these R_c values, because nucleation does not always occur at the center of the droplet and crystal size at the late stage is not negligible. Therefore, we carried out protein crystallization experiment within droplets based on this slightly smaller droplet size compared to critical size.

Thaumatin crystallization was carried out in 200 μm and 500 μm droplets. These droplet volumes were 4 nL and 50-65 nL, respectively. We employed flow rate and structure of microchannel to obtain the desired droplet volume. Homogeneous droplets were collected from each capillary with the corresponding diameter. The crystallization experiments were carried out at 4 °C. Figure 2 (a) and (b) show the distributions of the number of thaumatin crystals within 200 and 500 μm droplets. The initial thaumatin concentration C_0 was 10 mg/mL and 0.8 M potassium sodium tartrate was contained as a precipitant. Results show that we were able to obtain a single crystal within the 200 μm droplet although nucleation frequency was slightly low. On the other hand, when we generated 500 μm droplets, the nucleation profile showed a larger distribution compared with 200 μm droplets as shown in Figure 2 (b). This result suggests that the estimated critical size roughly agrees with the experimental results and that droplet size also plays an important role in microdroplet-based protein crystallization. In order to enhance nucleation frequency, the initial concentration of thaumatin was increased from 10 mg/mL to 12 and 15 mg/mL. As a result, a single thaumatin crystal also appeared within the droplet even with higher protein concentration. Nucleation frequency was increased to 60 % (C_0=15 mg/mL) when we used 200 μm droplet. Moreover, we previously reported the ability to achieve single crystallization by using 360 μm droplets [4]. In contrast, distribution of the number of thaumatin crystals within 500 μm droplets showed significantly larger distribution than 200 μm droplet. Figure 2 (c) shows distribution of the number of lysozyme crystals within 200 μm droplet. The initial lysozyme concentration C_0 was 40 mg/mL and 0.5 M NaCl was contained as a precipitant. In the case of lysozyme and glucose isomerase crystallization, a single crystal of each protein was obtained within a 200 and 360 μm droplet similar to thaumatin crystal. When we employed 360 μm droplet, lysozyme nucleation frequency was increased to almost 70 % (C_0=40 mg/mL), and 60 % of droplets contained a single crystal. Consequently, these results suggest that we can control the nucleation behavior of a protein by controlling not only the crystallization condition but also by droplet size.

We also carried out ferritin crystallization based on the R_c value above. In the case of ferritin crystallization, the precipitant concentration was changed to control supersaturation within the droplet. As a result, when 360 μm droplets were generated and initial concentration of cadmium sulfate was 30 mM, a single ferritin crystal was contained in almost all droplets as shown in Figure 2 (d). However, under the slightly higher supersaturation condition, the initial concentration of cadmium sulfate was 40 mM, and the number of ferritin crystal that appeared within droplets showed a wide distribution. The R_c value of ferritin was significantly smaller compared with other proteins used in this study as shown in Table 1. Due to the small R_c value, we consider that the microdroplet of ferritin has relatively small effect on the alleviation of supersaturation in a droplet after the first nucleation. Therefore, the region of high supersaturation remained far away from a ferritin crystal contained in a droplet. This high supersaturation made possible the induction of further nucleation. For this reason, the crystal distribution of ferritin showed larger distribution compared with thaumatin crystallization. This result indicates that the diffusion of protein molecule is a significant parameter for controlling nucleation of protein by droplet-based microfluidics.

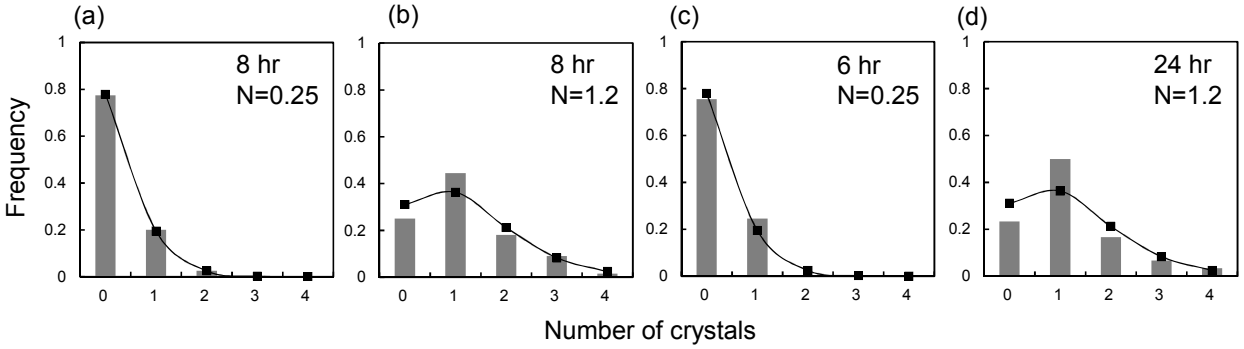

Figure 2. Distributions of the number of protein crystals that appeared within droplets.
(a) and (b) Distribution of the number of thaumatin crystals. Droplet sizes are (a) 200 μm, and (b) 500 μm, respectively. Lines and symbols represent Poisson distribution when mean number of the thaumatin crystals N were 0.25 (200 μm), and 1.2 (500 μm), respectively. (c) Frequency of the number of lysozyme crystals that appeared after 6 hr incubation. Droplet size is 200 μm. (d) Frequency of the number of ferritin crystals that appeared after 24 hr incubation. Droplet size is 360 μm.

CONCLUSIONS

In conclusion, we present an approach for single crystallization of protein by using droplet based microfluidics. In the case of thaumatin, lysozyme and glucose isomerase crystallization, a single crystal was obtained within 200 μm and 360 μm droplets even with higher supersaturation. We also investigated the crystallization behavior of four model proteins to confirm the effect of protein molecular diffusion on crystallization. In the case of ferritin crystallization, the distribution of the number of ferritin crystal was larger compared with thaumatin crystallization. Moreover, when supersaturation is slightly increased, the distribution of the number of ferritin crystals shows larger distribution, too. These results indicate the capability of controlling the nucleation behavior of a protein by controlling not only the crystallization condition but also the droplet size. Consequently, we consider that single crystallization or decoupling protein nucleation and crystal growth are easily achieved by controlling droplet size.

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CONTACT

Masaya Miyazaki 81-942-81-4059 or m.miyazaki@aist.go.jp