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Notes

X-ray Diffraction of Protein Crystal Grown in a Nano-liter Scale Droplet in a Microchannel and Evaluation of Its Applicability

Masatoshi MAEKI,*1 Saori YOSHIZUKA,*1 Hiroshi YAMAGUCHI,*2 Masahide KAWAMOTO,*3 Kenichi YAMASHITA,*2 Hiroyuki NAKAMURA,*2 Masaya MIYAZAKI,*1,*2† and Hideaki MAEDA*1,*2,*4†

*1 *Department of Molecular and Material Sciences, Interdisciplinary Graduate School of Engineering Sciences, Kyushu University, 6-1 Kasuga-Koen, Kasuga, Fukuoka 816–8580, Japan*
*2 *Measurement Solution Research Center, National Institute of Advanced Industrial Science and Technology (AIST), 807-1 Shuku, Tosu, Saga 841–0052, Japan*
*3 *Kyushu Synchrotron Light Research Center, 8-7 Yayoigaoka, Tosu, Saga 841–0051, Japan*
*4 *CREST, JST, 5 Sanban-cho, Chiyoda, Tokyo 102–0075, Japan*

We describe the technical aspects of the *in-situ* X-ray diffraction of a protein crystal prepared by a nanodroplet-based crystallization method. We were able to obtain diffraction patterns from a crystal grown in a capillary without any manipulation. Especially in our experimental approach, the crystals that moved to the nanodroplet interface were fixed strongly enough to carry out X-ray diffraction measurements that could be attributed to the high surface tension of the nanodroplet. The crystal was damaged by an indirect action of the X-rays because our *in-situ* X-ray diffraction measurement was carried out in the liquid phase without freezing the crystal; however, the obtained several diffraction patterns were of sufficiently fine quality for the crystal structure factors to be generated. We consider the technical examination presented in this paper to represent a seamless coupling of crystallization to X-ray analysis.

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Introduction

Protein crystallization and structure determination using X-ray crystallography play an important role in structural biology. The crystallization conditions are typically explored by performing a large number of trials in which variable ratios of solutions of a protein, precipitants, and additives are pipetted together by hand, or with a robotic dispenser.[1] In addition to such batchwise crystallization condition exploration, the nano-liter scale droplet is also a beneficial platform of protein crystallization.[2-6] The nanodroplet is typically prepared by a microfluidic technique, which enables fine size-controlling, monodispersity large-scale preparation, easy handling, and good reproducibility.[7-10] Moreover, a nanodroplet offers the characteristic crystal growth environment, such as free interface diffusion,[2] two-phase fluid flow,[3-5] and vapor-diffusion in the capillary.[6] In particular, it has been reported that proteins could be crystallized by free interface diffusion, a method that was possible only in microgravity environments.[2] We have successfully achieved diffusion-controlled protein crystallization that creates a large crystal, and obtains high crystal lattice perfection by focusing on the similarity between the internal fluid dynamics of a nanodroplet under a ground-based environment and a low-gravity environment. In addition, only one crystal within each "isolated" nanodroplet is obtainable by our method.[11] Such a feature of our method is useful for X-ray crystallography, because it is important to avoid stacking crystals. Obtaining a large crystal is not always required, which could be attributed to the development of a syncrotron source and other apparatuses.

For carrying out X-ray crystallography, manual handling of crystals was previously required for the extraction of crystals from the crystallization device and subsequent mounting. Such manual handling is tedious, and has a potential to damage the crystal. Generally, the protein crystal is fragile because it contains much water. Therefore, *in-situ* X-ray crystallography is desirable so as to avoid manual handling. The nanodroplet platform is compatible with the direct analysis of crystals by X-ray diffraction. Several papers successfully reported *in-situ* X-ray diffraction studies of a crystal in a nanodroplet.[2,5,6,12] Herein, we studied the *in-situ* X-ray diffraction of a protein crystal obtained by our "isolated" nanodroplet crystallization method.[11] The exposure of a crystal to X-rays in the liquid-phase at room temperature is known to damage the crystal by the indirect action of X-rays.[6] We estimated such on influence, and also studied the procedures that are suitable for our *in-situ* X-ray diffraction, such as crystal handling and mounting. We consider a technical examination of a crystal grown in a nanodroplet presented in this paper to represent the successful coupling of protein crystallization to X-ray crystallography, and an advanced technique in the micro/nano-bioanalysis field.

Experimental

Materials, nanodroplet formation and crystallization assay

Lysozyme and thaumatin were employed as model proteins because these proteins have been typically used and well

† To whom correspondence should be addressed.
E-mail: m.miyazaki@aist.go.jp; maeda-h@aist.go.jp

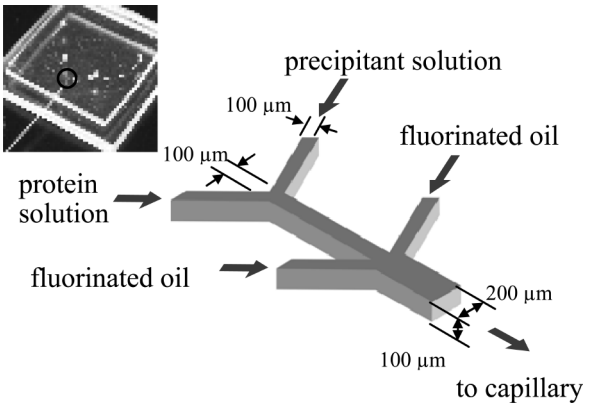

Fig. 1 Photograph of the composite PDMS-Teflon capillary microfluidic device and a schematic illustration of the method for forming microdroplets.

investigated in the analysis of growth kinetics in macroscale crystallization.[13,14] A microfluidic device composed of polydimethylsiloxane (PDMS)-Teflon capillary (o.d., 360 μm; i.d., 200 μm) was used to form microdroplets, as shown in Fig. 1. A protein solution (60 mg/mL lysozyme in an aqueous solution of 100 mM CH_3COOH/CH_3COOK buffer (pH 4.5), or 20 mg/mL thaumatin in an aqueous solution of 100 mM *N*-(2-acetamido) iminodiacetic acid (ADA) buffer (pH 6.5)), precipitant solution (1 M NaCl in an aqueous solution of 100 mM CH_3COOH/CH_3COOK buffer (pH 4.5) for lysozyme, or 1.6 M potassium sodium tartrate in an aqueous solution of 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 7.0) for thaumatin), and fluorinated oil (a mixture of 3M™ Fluorinert™ Electronic Liquid FC-40 and 1*H*,1*H*,2*H*,2*H*-perfluoro-1-octanol (10:1 (v/v)) were loaded as shown in Fig. 1. The protein solution and precipitant solution joined together and formed a single droplet in a microchannel. The protein solution and precipitant solution were loaded at the same flow rate, and two fluorinated oil injections were also pumped at the same flow rate. Each flow rate was finely adjusted to obtain 200 μm-diameter (2 nL) spherical microdroplets. The flow had to be stopped when the capillary was filled with microdroplets, and the capillary was disconnected, sealed with wax, and stored at 4°C. The microdroplets within the capillary were monitored with a video camera until crystal growth was saturated. It took about a few hours for the crystal growth to be completed.

In addition, a part of the crystals grown in a Teflon capillary were transferred to a glass capillary (Hampton Research; o.d., 200 μm; i.d., 180 μm) with nanodroplets. The Teflon capillary and glass capillary were connected together, and the crystals were pushed out from the Teflon capillary to the glass capillary. This was followed by disconnecting these capillaries and sealing with wax.

In-situ X-ray diffraction measurement

Crystals of two proteins grown in nanodroplets were analyzed by X-ray diffraction on a synchrotron source (BL 07 in the SAGA Light Source (SAGA-LS)). The above-mentioned Teflon or glass capillaries, which contained protein crystals, were mounted without further treatment. Diffraction data were corrected at a wavelength of 1 Å, with a 60-s exposure and 1° oscillation. All measurements were carried out at 4°C (lysozyme) or room temperature (thaumatin). The sizes of the crystals used in this measurement were 50 – 100 μm. Three (0, 45 and 90°, lysozyme) or four (0, 30, 60 and 90°, thaumatin) diffraction images were corrected. Structure factors were generated using the program iMosflm from CCP4.[15]

Batchwise crystallization and X-ray diffraction

We also prepared lysozyme and thaumatin crystals, which were crystallized by a batchwise process, and their X-ray diffraction data were corrected as a reference. The same protein solutions and precipitant solutions were used in the microfluidic crystallization assay. Many small crystals appeared after storing at 4°C for several hours. The crystals were extracted by using a CryoLoop (Hampton Research) dipped in a cryoprotectant solution (25% ethylene glycol with mother liquor), and flash-frozen in liquid nitrogen; 90 diffraction images (1° step at a range of 0 – 90°) were collected with a 15-s exposure at 100 K. The size of the crystals used in this measurement was about 300 μm. All other conditions were the same as in the *in-situ* X-ray diffraction measurement.

Results and Discussion

To fix the crystal is the first hurdle for *in-situ* X-ray diffraction measurements. In our experiments, the crystals inside the mounted capillary slowly moved to the interface of the nanodroplet, and stopped. The crystals that moved to the nanodroplet interface were fixed strong enough to carry out X-ray diffraction measurements that could be attributed to the high surface tension of the nanodroplet, *i.e.*, the crystal could not break the nanodroplet interface. Therefore, in our experimental approach, the difficulty of fixing the crystal had been eliminated.

In our *in-situ* X-ray diffraction study, measurements were carried out without freezing the crystal, and such an approach generally causes damage to the crystal due to the indirect action of X-rays. Therefore, it was realistically impossible to obtain a complete diffraction data set necessary for determining the protein's molecular geometry. However, our approach is effective and could be used for obtaining cell parameters, and for evaluating the quality of the crystal and the diffraction images.

Lysozyme and thaumatin were employed in the experiments described in this paper, and it seemed that lysozyme was damaged by an indirect action of X-rays compared to thaumatin. We consider this to have been caused by lysozyme crystal containing much more water than thaumatin. A long time exposure was required, since the crystals grown in the nanodroplets were relatively small, and only three diffraction images were obtainable for lysozyme.

We examined two different wall thicknesses and material capillaries for *in-situ* X-ray diffraction (80 μm-thick Teflon, and 10 μm-thick glass capillary), and no difference was observed in the results obtained from these two kinds of capillaries. Figure 2 shows the X-ray diffraction pattern of lysozyme in the glass capillary as an example. We could also obtain thaumatin's X-ray diffraction patterns, which were sufficiently fine for generating the structure factors. As shown in Fig. 2, we observed scattering patterns from the capillary wall and the mother liquor present around the crystal, but this scattering did not obscure the diffraction of the crystals.

The unit-cell dimension, space group, and crystal system obtained from X-ray diffractions are summarized in Table 1. In both crystals by microfluidic and batchwise crystallization, the same crystal system and space group were confirmed. The unit

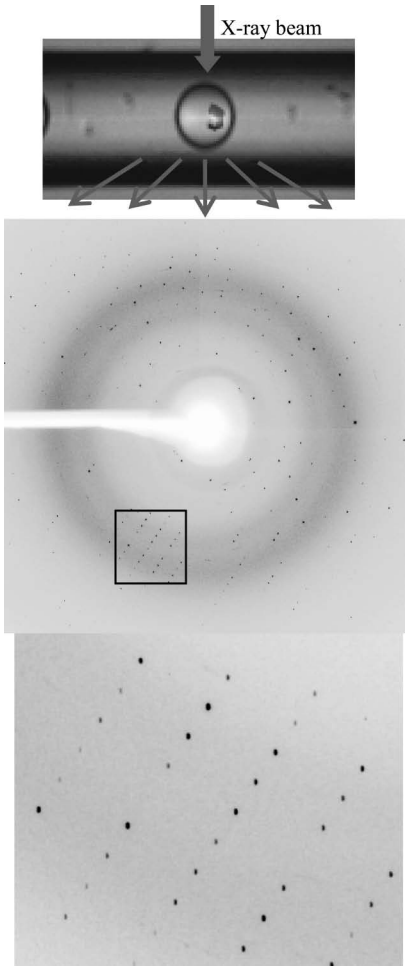

Fig. 2 Lysozyme crystal in capillary (top), which could be directly subjected to X-ray diffraction. The obtained diffraction patterns (middle and bottom) were of sufficiently fine quality to generate crystal structure factors.

Table 1 Unit-cell dimensions, space group, and crystal system obtained from X-ray diffractions

	$a = b$ (Å)	c (Å)	Space group	Crystal system
Thaumatin (nanodroplet)	58.69 ± 0.13	151.53 ± 0.58	$P4_12_12$	Tetragonal ($\alpha = \beta = \gamma = 90^\circ$)
Thaumatin (batchwise)	57.79	150.04		
Lysozyme (nanodroplet)	79.3 ± 0.2	37.7 ± 0.4	$P4_32_12$	Tetragonal ($\alpha = \beta = \gamma = 90^\circ$)
Lysozyme (batchwise)	78.54	36.96		

A tetragonal space group with correct unit cell dimensions could be obtained from a single diffraction image. The diffraction image was refined to unit cell dimensions as above.
Five microfluidic-prepared crystals for each protein were measured, and the results are presented as the average ± standard deviation.

cell of *in-situ* X-ray diffraction was slightly larger than that of the batchwise prepared crystal, which was extracted on CryoLoop. The latter lattice constants agree with the reported values.[16,17] We consider this to be due to the crystal not being frozen, and to the measurement being carried out in the liquid phase in our *in-situ* X-ray diffraction approach.

Conclusions

We herein describe the technical aspects of the *in-situ* X-ray diffraction of a protein crystal prepared by our nanodroplet-based crystallization method. We were able to obtain diffraction patterns from a crystal grown in a capillary without any manipulation. The crystal in a nanodroplet was fixed near to the droplet interface, and there was no difficulty in carrying out the X-ray diffraction measurement without any treatment to fix the crystal. The crystal was damaged by the indirect action of X-rays because our *in-situ* X-ray diffraction measurement was carried out in the liquid phase without freezing the crystal. However, the obtained several diffraction patterns were of sufficiently fine quality for the crystal structure factors to be generated. In addition, due to the same reason, the unit cell of a microfluidic-prepared crystal, which was measured by *in-situ* X-ray diffraction, was slightly larger than that prepared by the batchwise method, extracted on CryoLoop. We consider the technical examination presented in this paper to represent a seamless coupling of crystallization to X-ray analysis.

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