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Protein Cryocrystallography Using Laser-Processed Crystal

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We propose a new procedure in biological cryocrystallography, using the laser-ablation technique. This is the first report on the successful processing of cryo-cooled crystals to create a "protein crystal ball" that is conducive to X-ray diffraction (XRD) data collection. Pulsed UV laser soft ablation (PULSA) modifies protein crystals into a spherical shape and removes surrounding materials, but does not negatively affect crystallinity and may improve diffraction data quality. Additionally, we demonstrate treatment of problematic samples to make them serviceable for XRD analysis. Isolation of single crystals can be performed after flash cooling with the PULSA technique. [DOI: 10.1143/JJAP.44.L54]

KEYWORDS: protein crystal, cryocrystallography, laser ablation, UV laser, X-ray diffraction, structural analysis

X-ray crystallography is a vital tool in determining the three-dimensional structure of materials. It produces best-quality data when the analyzed crystal has an isometric shape and is not accompanied by any surrounding obstacles in the X-ray beam path. Therefore, inorganic material X-ray diffraction (XRD) data are usually collected using mechanically processed spherical crystals. In contrast, macromolecular crystallographers have customarily accepted that minimum handling and processing for as-grown crystals yields reliable XRD data sets because macromolecular crystals are highly sensitive to chemical and mechanical stimulation. The only preferable treatment regularly employed is flash cooling for reducing secondary X-ray radiation damage.[1,2] Few attempts have been made to process cryo-cooled crystals into an ideal condition. Here, we present an innovative procedure in biological macromolecular X-ray crystallography, processing cryo-cooled samples by laser irradiation. Pulsed ultraviolet laser soft ablation (PULSA) enables the effective processing of crystals, cryoprotectants, and nylon loops at cryogenic temperature. These treatments produce ideal samples for XRD data collection: cryo-cooled, damage-free crystals with best shape and without obstacles.

We employed an original, compact solid-state laser at a wavelength of 193 nm, as the processing light source.[3] The output laser pulses (beam quality of $M^2 < 2$, pulse duration of less than 1 ns) are remarkably suited to processing tiny biological targets where precise photoablation with few thermal effects is required. Our preliminary experiments revealed that protein crystals under growth temperature could be processed without affecting the crystallinity, using this deep-ultraviolet laser.[4,5] In this work, the 193-nm light source was placed in XRD equipment in order to realize *in-situ* laser processing of cryo-cooled crystals. The experimental setup is shown in Fig. 1. The output beam was directed to the samples through two galvano mirrors and a focusing lens, with a spot diameter of 15 μm and a fluence of 100 mJ/cm^2. A well-known model protein, hen egg-white lysozyme (HEWL), was employed in the experiments. HEWL crystals were obtained by a vapor-diffusion method. After immersion in a cryoprotectant (Paratone-N), the crystals were collected using nylon loops, mounted on a goniometer head of the XRD equipment, and flash-cooled at 100 K by a cryogenic nitrogen gas stream. The scanning of galvano mirrors and suitable rotation of the goniometer controlled the laser-irradiated position on the target.

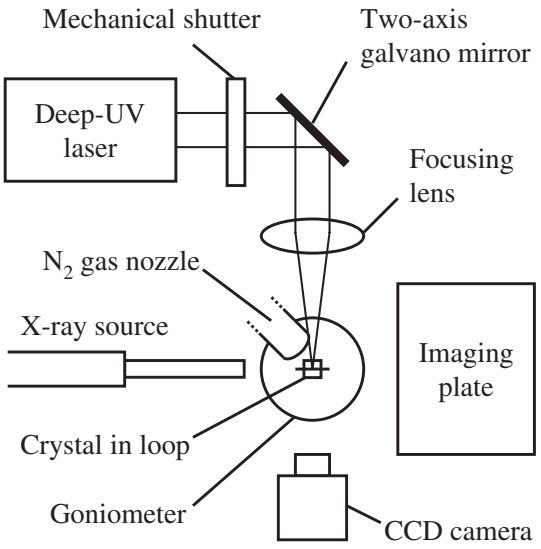

Fig. 1. Experimental setup for processing cryo-cooled protein crystals.

Repetitive irradiation of laser pulses achieved the designed processing (such as a spherical shape, a cylindrical shape, and a simple straight-line ablation) for examined crystals. The non-irradiated part of the crystals did not exhibit any visible sign of crack or denaturation induced by the laser irradiation. Figure 2 presents an example of a "protein crystal ball" sculptured to a damage-free single crystal of HEWL. The loop-mounted, flash-cooled sample before laser processing is shown in Fig. 2(a). XRD data of

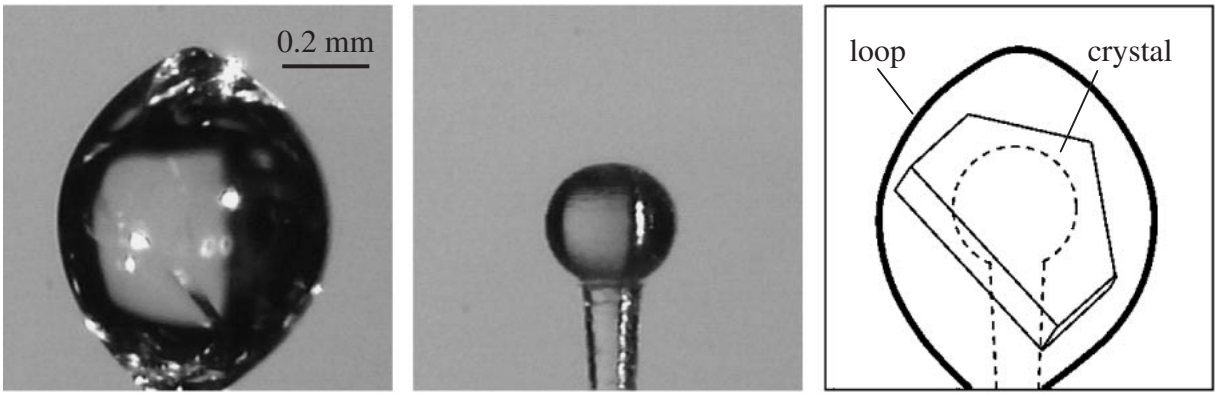

Fig. 2. A protein crystal ball. The HEWL crystal was modified into a spherical shape by laser irradiation. (a) The loop-mounted crystal before laser irradiation. The crystal was flash-cooled after immersion in a cryoprotectant. (b) The laser-processed crystal. A diameter of the spherical part was 0.3 mm. (c) Corresponding illustration of the photographs. The dashed line indicates a contour of the sample after laser irradiation.

the pre-irradiation crystal were collected on a Rigaku R-AXIS IV^{++} imaging plate. Cu Kα radiation was produced using a Rigaku ultraX18 rotating anode generator operated at 50 kV and 100 mA. The detector was positioned 150 mm from the crystal, and the time per image and crystal oscillation angle were set at 10 min and 2°. The beam diameter of the X-ray was adjusted to 0.3 mm. After initial XRD-pattern recording, the crystal was processed using PULSA. With arched-beam scanning and goniometer rotation, 5×10^6 laser shots at a pulse repetition rate of 3.4 kHz shaped the crystal into a sphere, in which the surrounding cryoprotectant and the nylon loop and were also ablated. Figure 2(b) presents a photograph of the laser-processed sample. The spherical part consisted of the HEWL crystal with a diameter of 0.3 mm. Figure 2(c) represents a corresponding illustration; the dashed line indicates a contour of the sample after laser irradiation. The XRD patterns of the post-irradiation crystal were also recorded.

The XRD data were processed by DENZO and SCALEPACK.[6] Table I summarizes the XRD analyses before and after laser processing. The results suggest no negative influence of the laser processing, even taking into account potential accumulated X-ray radiation damage in the laser-processed sample. Unchanged resolution limit and cell dimensions demonstrate that the crystallinity was not degraded. Decreased I/sigma is reasonable because the crystal volume is reduced by the laser ablation. The R-merge in the highest resolution shell (1.97–1.90 Å) is not affected by the PULSA treatment. However, the overall R-merge is slightly improved compared with that before laser irradiation, because the R-merge in low- and middle-resolution shells (30.0–2.5 Å) is substantially improved. Additionally, PULSA treatment slightly reduces crystal mosaicity without laser-induced damage. A more plausible hypothesis is that the mosaicity in the exterior of the HEWL crystal is higher than that in the interior. These results indicate that the PULSA technique has potential to improve data quality in biological cryocrystallography. Modification into the better shape decreases dispersion of the angle-dependent scale factor. Moreover, nylon loops and surrounding cryoprotectants, the scattering of which reduces the signal-to-noise ratio of spots by increasing the background noise, can be removed after flash cooling. This effect would be especially obvious for data collection using high-brightness synchrotron radiation or X-ray sources at a longer wavelength.

Table I. XRD analysis of the laser-irradiated HEWL crystal. The data were processed by DENZO and SCALEPACK. The numbers in parentheses are given for the highest resolution shells. R_{merge}, $\Sigma_h \Sigma_i |I(h,i) - \langle I(h)\rangle| / \Sigma_h \Sigma_i I(h,i)$, where $I(h,i)$ is the intensity value of the i-th measurement of h and $\langle I(h)\rangle$, is the corresponding mean value of $I(h)$ for all i measurements; the summation is over the reflections with I $I\sigma(I)$ larger than 0.0.

	Before laser irradiation	After laser irradiation
Corresponding photograph	Fig. 2(a)	Fig. 2(b)
Number of frame	45	45
Cell dimensions (Å)	$a = 78.45$ $c = 37.04$	$a = 78.46$ $c = 37.06$
Resolution limit (Å)	1.9	1.9
Total number of observations	56,687	57,394
Number of unique observations	9,414	9,430
Completeness (%)	98.1 (96.1)	98.1 (95.5)
R-merge (%) (overall)	3.9	3.5
(30.0–2.5 Å)	3.5	3.1
(1.97–1.90 Å)	7.4	7.4
Estimated mosaicity	0.33	0.29
I/sigma	30.7	29.7

The usefulness of laser processing is not limited to crystal reshaping. Another promising application is treatment of problematic crystals to make them serviceable for XRD analysis. Crystallographers often encounter problems during the process of crystal growth (*e.g.*, poor crystallinity, polycrystal formation, and adhesion to growth vessel). In XRD analysis, single crystals with high quality are isolated before being captured in small loops. The isolating process, however, requires mature skill in manipulating crystals under microscopic observation. Even the experienced manipulator frequently fails in this operation. One solution to this difficulty is isolation after flash cooling. If obstructive crystals are contained in the loop, they can be ablated with

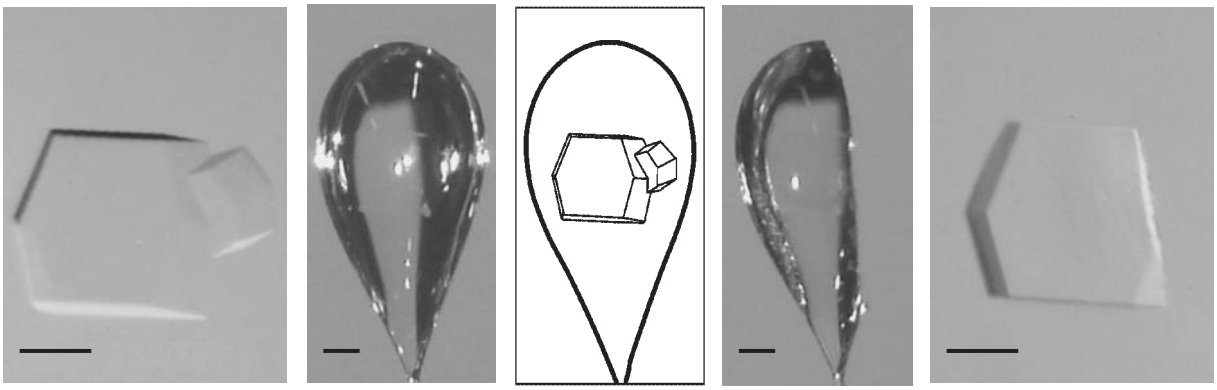

Fig. 3. Treatment of a problematic sample. The scale bar is 0.1 mm. (a) A HEWL polycrystal. (b) The loop-mounted, flash-cooled sample before laser irradiation. (c) Corresponding illustration of (b). (d) The sample after laser irradiation. (e) Magnified image of the laser-processed crystal.

the PULSA technique.

Figure 3(a) shows an as-grown polycrystal of HEWL, and Figs. 3(b) and 3(c) presents a photograph and corresponding illustration of the crystal captured in the nylon loop. This sample is considered unsuitable for XRD analysis because it yields reduplicative diffraction patterns. Although we measured 45 frames of XRD patterns for the sample at cryogenic temperature, it was not successfully processed by DENZO and SCALEPACK. The polycrystal was then partially ablated with 7×10^5 laser shots. Figure 3(d) presents a photograph of the laser-processed sample. The single crystal was obtained by the laser irradiation as shown in Fig. 3(e). The post-irradiation sample diffracted beyond 1.9 Å, and was processed using the same software, thus producing a reasonable analyzed result. We can say the PULSA technique reduces a risk of making target crystals useless or of lesser quality.

Most laser ablation techniques are currently applied to the target at ambient temperature. The cryogenic laser processing we describe here is expected to extend the capability of laser technology. Some biological materials, such as protein crystals, are soft and stable only in liquid at ambient temperature. The biological macromolecular crystals used for XRD measurements are held in the loops by a weak surface tension of the filled liquid. The advantages of laser processing, high accuracy and reproducibility, are enhanced for frozen fixed targets. Our experiment results indicate that appropriate laser parameters for processing protein crystals at cryogenic temperature do not significantly differ from that at growth temperature, while the response of crystals to mechanical processing drastically changes. It is almost impossible to handle frozen crystals with mechanical tools. In recent years, considerable efforts have been made to develop robotic or automated crystallography systems for accelerating structural analysis of biological molecules. The PULSA treatment is well matched to a series of high-through-put crystallography processes. Comparable laser-processing results were also obtained with other protein crystals, as will be reported elsewhere. We believe this technique will change the concept of biological cryocrystallography to accept that most samples should be laser-processed in order to yield high-quality XRD data sets.

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