Chem Soc Rev

TUTORIAL REVIEW

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Laser ablation for protein crystal nucleation and seeding

Cite this: *Chem. Soc. Rev.*, 2014, 43, 2147

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Received 1st July 2013

DOI: 10.1039/c3cs60226e

www.rsc.org/csr

With the recent development in pulsed lasers with ultrashort pulse widths or wavelengths, spatially precise, low-damage processing by femtosecond or deep-UV laser ablation has shown promise for the production of protein single crystals suitable for X-ray crystallography. Femtosecond laser processing of supersaturated solutions can shorten the protein nucleation period or can induce nucleation at low supersaturation, which improves the crystal quality of various proteins including membrane proteins and supra-complexes. In addition to nucleation, processing of protein crystals by femtosecond or deep-UV laser ablation can produce single crystalline micro- or macro-seeds without deterioration of crystal quality. This tutorial review gives an overview of the successful application of laser ablation techniques to nucleation and seeding for the production of protein single crystals, and also describes the advantages from a physico-chemical perspective.

Key learning points

(1) Protein crystal nucleation and seeding by laser ablation enable the production of high-quality protein single crystals that are necessary for X-ray crystallography.

(2) Use of lasers with ultrashort pulse widths or wavelengths allows spatially precise, low-damage processing of protein solutions and crystals *via* specific ablation processes (*e.g.* photomechanical and photochemical).

(3) Processing of supersaturated solutions by femtosecond laser ablation can induce protein nucleation at low supersaturation, which results in the growth of high-quality single crystals.

(4) Processing of protein crystals by femtosecond or deep-UV laser ablation can produce single crystalline micro- or macro-seeds without deterioration of crystal quality.

1. Introduction

The three-dimensional (3D) structures of proteins have been attracting considerable attention because of a fundamental interest in the many biological processes involving proteins, as well as for structure-based drug design.[1] X-ray diffraction (XRD) of protein single crystals is the most widely used technique for determining the 3D structures with atomic resolution. However, protein crystallization, which involves nucleation and crystal growth, remains a major bottleneck in the structural determination process.[2] Although automated instrumentation has been developed to carry out high throughput screening of various crystallization conditions,[3] crystallographers often face difficulty in determining which set of conditions provide high-quality protein single crystals suitable for XRD. There are also many types of recalcitrant proteins (*e.g.*, membrane proteins) which are particularly difficult to crystallize because of their weak interactions and instability. Moreover, to improve the understanding of protein functions, structural determination at higher resolution is needed, even for proteins whose structures have already been deposited in the Protein Data Bank (PDB).

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For example, X-ray resolution better than 1.0 Å may enable the assignment of hydrogen atoms, giving a more detailed view of protein hydration and enzymatic functions. However, a very limited number (~400) of protein structures have been solved beyond 1.0 Å resolution with respect to total number (~80 000) of protein structures in the PDB (as of June 2013). Thus innovation in protein crystallization techniques is necessary for accelerated advancement in the structural biology field.

The most conventional methods for protein crystallization are vapor diffusion (evaporation) and batch techniques, which basically adjust supersaturation of a protein solution by alternations in crystallization parameters such as concentration, temperature, pH value, and precipitant. In these methods, once the initial crystallization conditions are set, researchers usually try to avoid any perturbation of the protein solution, such as mechanical shock and/or temperature fluctuation. Protein crystallization in the microgravity environment of space is an ultimate idea to suppress even convection and sedimentation due to gravity, and allows for the growth of crystals *via* transport by pure diffusion.[4] In contrast to such passive crystallization methods, light has been utilized as a non-contact means for active control of crystallization. Historically, Garetz and Myerson *et al.* pioneered light-induced nucleation from supersaturated solutions in 1996.[5] They reported that nucleation of a small organic molecule (urea) could be induced by non-focused nanosecond laser irradiation, wherein the optical electric field was considered to align molecular units within clusters. It should be noted that optical absorption is not involved in the nucleation mechanism because there is no absorption at the laser wavelength, and the laser fluence (~MW cm^{-2}), which denotes laser energy per unit area, is not sufficient to cause multiphoton absorption. Thus, such a non-absorption technique was considered to be a harmless approach to crystallization. Nevertheless, in the last decade, high energy laser processing by laser ablation has shown promise for the control of protein crystallization. In contrast to the non-absorption technique, laser ablation is in general a process initiated by optical absorption of intense laser pulses that ultimately results in a change in material morphology (*e.g.*, removal of materials from a solid or liquid surface, formation of a void or bubble in the materials, *etc.*). With the recent development in pulsed lasers with ultrashort pulse widths or wavelengths (*e.g.*, femtosecond and deep-UV lasers), spatially precise, low-damage

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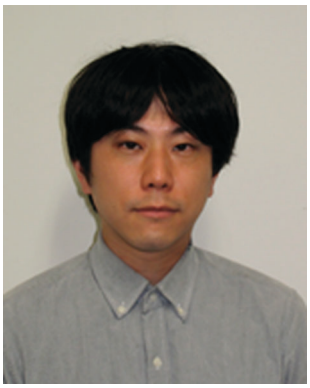

Ryota Murai

Ryota Murai received his PhD from Osaka University in 2010, under the guidance of Prof. Yusuke Mori. Afterwards he worked on protein crystallization in the same group as a postdoctoral fellow. In 2011, he joined the group of Prof. Kazuo Nakajima at Kyoto University as a postdoctoral fellow, where his research is focused on the development of a new technique to produce Si ingots for solar cells.

From right: Kazufumi Takano, Hiroaki Adachi, Shigeru Sugiyama, Tsuyoshi Inoue, Hiroyoshi Matsumura, and Yusuke Mori. Inset (left bottom): Satoshi Murakami

From right: Prof. Kazufumi Takano (Kyoto Prefectural University), Dr Hiroaki Adachi (SOSHO Inc.), Prof. Shigeru Sugiyama (Osaka University), Prof. Tsuyoshi Inoue (Osaka University), Prof. Hiroyoshi Matsumura (Osaka University), and Prof. Yusuke Mori (Osaka University). Inset (left bottom): Prof. Satoshi Murakami (Tokyo Institute of Technology). They all have been working on development of protein crystallization techniques under the interdisciplinary research project called "SOSHO project".

processing of various organic- and bio-materials has been demonstrated. For instance, femtosecond laser ablation techniques have widely been used for fine material processing including nanosurgery of cells and tissues,[6] because they can realize highly localized, low-damage processing *via* multiphoton absorption[6] and photomechanical processes.[7] In fact, the first light-induced nucleation of protein was realized by femtosecond laser processing in 2002.[8] There, protein supersaturated solutions were focused with femtosecond laser pulses with a fluence on the order of PW cm^{-2}, which is high enough to cause laser ablation of even transparent materials *via* multiphoton absorption.[8,9] Note that protein nucleation could not be induced with nanosecond laser processing, which generally causes substantial thermal damage to biological materials. To date, it has been confirmed that processing by femtosecond laser ablation can induce nucleation of various proteins at low supersaturation, thus contributing to the improvement of protein crystal quality.[10] In addition to protein nucleation, processing of protein crystals by femtosecond laser ablation can be applied to the production of single crystalline 'micro-'seeds from polycrystals and cracked crystals that are unstable for XRD studies.[11] Moreover, production of single crystalline 'macro-'seeds was achieved by deep-UV laser processing because of its potential for photochemical processes, which can reduce thermal damage to protein crystals.[12] Hence, processing by femtosecond and deep-UV laser ablation has been increasingly recognized as a promising technique for the production of protein single crystals. This review gives an overview of the successful application of femtosecond and deep-UV laser ablation to protein crystal nucleation and seeding, and also describes the advantages of laser ablation techniques from a physico-chemical perspective.

2. Femtosecond laser ablation for protein crystal nucleation

Nucleation is a first step in the crystallization process and is crucial in determining the size, shape, structure, and quality of the crystals. To induce nucleation, supersaturation should be higher than that in the "metastable" zone, where solutions are supersaturated but provide no nucleation. In general, a primary strategy for obtaining high-quality protein single crystals is to induce nucleation at supersaturation that is just above but as close as possible to the metastable zone. In fact, successful crystallization has been performed by inducing nucleation at such low supersaturation in many cases. A systematic analysis of the correlation between supersaturation and crystal quality was reported for lysozyme[13] which revealed that the crystals grown in a lower supersaturated solution diffracted better with higher signal-to-noise ratios. However, protein nucleation at low supersaturation is generally very challenging. Spontaneous nucleation rarely occurs in low supersaturated solutions (Fig. 1a, top), because the interactions between protein molecules are quite weak due to their complicated structures and large sizes.[4] In addition, the long incubation time of supersaturated solutions often denatures protein molecules in the presence of additives and/or residual protease. Therefore, crystallographers are often forced to induce protein nucleation at very high supersaturation, which results in low-quality crystals and polycrystals (Fig. 1a, bottom). In order to overcome this protein crystallization dilemma, a novel stimulus using femtosecond laser processing based on laser ablation has been utilized to induce nucleation at low supersaturation (Fig. 1b). Herein, representative proteins and small organic molecules for which nucleation can be induced by the femtosecond laser technique are reviewed, and the underlying nucleation mechanism based on laser ablation is also described.

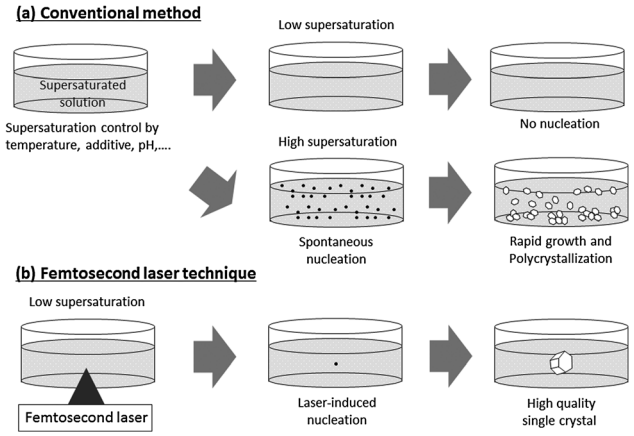

Fig. 1 Schematic illustration of crystallization processes realized by a conventional method (a) and the femtosecond laser technique (b). Reprinted with permission from SPIE, ref. 10, 2007.

2.1. Femtosecond laser-induced nucleation of proteins and small organic molecules

Adachi *et al.* demonstrated for the first time the laser-induced nucleation of proteins using a femtosecond laser.[8,9] As a first sample, they chose hen egg-white lysozyme (HEWL) (14 kDa), which has widely been used for studies of the protein crystallization mechanism. Supersaturated solutions of HEWL were

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irradiated with regeneratively amplified Ti:sapphire laser (average power: 1.0 W, wavelength: 780 nm, pulse width: 120 fs, repetition rate: 1 kHz) pulses through an objective lens (numerical aperture: 0.4). They found that the number of nucleated HEWL crystals increased with the number of laser pulses. It is notable that this HEWL crystallization could not be induced by focused nanosecond laser irradiation, even though many conditions were tested (*e.g.* altering laser energy, exposure time, repetition rate, *etc.*). After the first demonstration with HEWL, the utility of the femtosecond laser technique was tested again using another model protein, glucose isomerase (GI) (tetramer; 173 kDa), having a molecular weight more than 10 times larger than that of HEWL.[9] GI crystals were also obtained by focused femtosecond laser irradiation at low supersaturation where no spontaneous nucleation occurred. This result clearly indicates that focused femtosecond laser irradiation triggered the nucleation of GI in the meta-stable zone. In addition, they found a drastic shortening of the crystallization period of *Trypanosoma brucei* prostaglandin F2α synthase (31 kDa) (TbPGFS) from several months (vapor diffusion method) to a few days (femtosecond laser technique).[9] So far, this nucleation enhancement by the femtosecond laser technique has been confirmed for a variety of proteins such as thaumatin, cytochrome *c*, and ribonuclease H (RNase H).[9,14]

Based on the success of crystallizing water-soluble proteins, the femtosecond laser technique was then applied to the crystallization of high-quality single crystals of membrane proteins, which are particularly difficult to obtain.[15] In fact, the number of membrane protein structures deposited in the PDB is only ~1400 (as of June 2013). Despite the difficulty in crystallizing membrane proteins, detailed structural information is strongly needed for the pharmaceutical sciences, where over 60% of all commercial therapeutic drugs target membrane proteins.[15] In 2004, Adachi *et al.* demonstrated nucleation of the membrane protein, AcrB,[15] which is a major multidrug efflux transporter in *Escherichia coli* and is responsible for exporting various types of antibiotics, antiseptics, anti-cancer drugs and cellular toxins from the cell.[16] AcrB supersaturated solutions with different precipitant concentrations (12, 13, and 15% of polyethylene glycol (PEG) 2000) were prepared and irradiated with focused femtosecond laser pulses (Fig. 2).

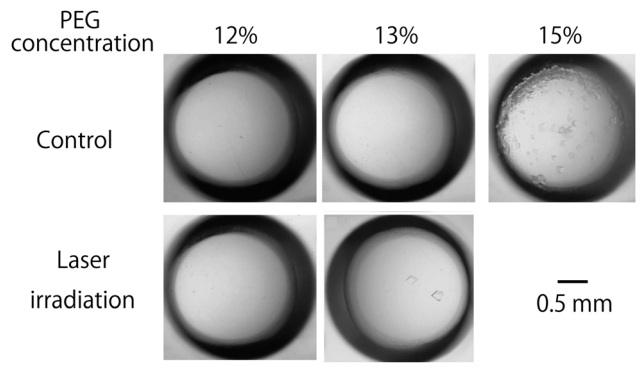

Fig. 2 Bright field images of AcrB solutions (PEG 12, 13, and 15%) irradiated without (control) and with femtosecond laser pulses. No crystallization was observed at PEG 13% without laser irradiation even after weeks. Reprinted with permission from the Japan Society of Applied Physics, ref. 15, 2004.

The images of solutions after several days clearly show that laser irradiation could induce nucleation in the meta-stable zone (13% PEG) where no nucleation occurred without laser irradiation. Large single crystals (~200 μm) were obtained from the meta-stable zone by laser irradiation, while small crystals (≤100 μm) and polycrystals were obtained by spontaneous nucleation at higher PEG concentration (15%). In addition, the AcrB crystals grown from the metastable zone diffracted to 2.3 Å resolution, which is drastically higher than that of the crystals (3.5 Å) grown from a solution with higher supersaturation (15% PEG). The femtosecond laser technique also succeeded in improving the diffraction quality and increasing the size of crystals of protein complexes including a membrane protein complex (TSecDF)[17] and a protein–RNA complex (Mnm-A-tRNA^{Glu}).[18] More recently, high-resolution structures of an enzyme (arabinanase Abnx) at 1.14 Å and its complex with a substrate (arabinobiose, DP2) at 1.04 Å were determined from single crystals grown *via* a combination of the femtosecond laser technique and a solution stirring technique.[19] A detailed study of the 3D structure at such high resolution provided new insights into the substrate-recognition mechanisms of the enzyme.

The successful protein examples clearly indicate that femtosecond laser-induced nucleation offers a viable pathway to obtaining high quality protein crystals for X-ray crystallography. Furthermore, the femtosecond laser technique can be applied not only to biological macromolecules but also to various small organic molecules.[20–24] In fact, the use of focused pulsed laser irradiation was originally proposed to induce nucleation of 4-dimethylamino-*N*-methyl-4-stilbazolium tosylate (DAST) by using a "nanosecond" laser in 2002.[25] DAST crystals are promising materials in organic nonlinear optoelectronics because of their excellent nonlinear properties. To realize high-efficiency production of high-quality DAST single crystals, nanosecond laser pulses (Nd:YAG laser, 1064 nm, 23 ns) were shot into a supersaturated methanol solution of DAST at low supersaturation. Although the efficiency of nucleation was quite low (<3%), nucleation was induced at low supersaturation where no spontaneous nucleation occurs (metastable zone). In 2005, the same research group applied focused femtosecond laser irradiation to the nucleation of DAST. They found that the efficiency of nucleation was significantly improved (~10%).[20] The next year, Yoshikawa *et al.* demonstrated femtosecond laser-induced nucleation of a water-soluble molecule, urea (Fig. 3).[21] Here, the first direct observation of crystallization dynamics initiated by focused femtosecond laser irradiation was achieved, owing to the more rapid crystallization of urea from nucleation to the formation of visibly large crystals (~mm), as compared to biological macromolecules. Another year later, Nakamura *et al.* reported the crystallization of a non-polar molecule, anthracene, from a cyclohexane solution triggered by only a single femtosecond laser pulse.[22]

Materials in which nucleation has been induced by focused femtosecond laser irradiation are listed in Table 1. The enhancement of nucleation by the femtosecond laser technique has been confirmed for various materials having widely differing properties and crystallization conditions (*e.g.*, molecular weight,

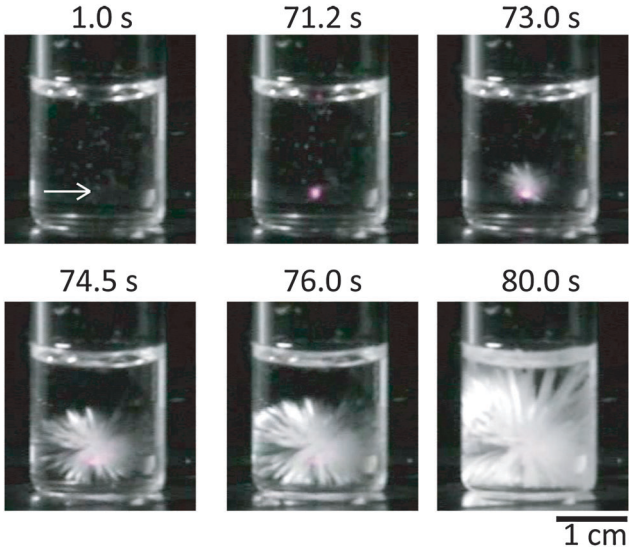

Fig. 3 Urea crystallization induced by focused femtosecond laser irradiation. The focal point of the laser pulse is indicated by an arrow in the image at 1.0 s. The delay time from the onset of laser irradiation is indicated above the images. Reprinted with permission from the Japan Society of Applied Physics, ref. 21, 2006.

solvent, additives, *etc.*). In fact, the high efficiency and versatility of the femtosecond laser technique are noteworthy compared to other light-induced crystallization techniques. For instance, Garetz *et al.* demonstrated the photophysical crystallization of urea using a non-focused nanosecond laser, wherein the optical Kerr effect organized existing prenucleating clusters, which increased the chances of nucleation and growth.[5] However, nucleation could be induced only from a supersaturated solution aged for a few days prior to laser irradiation, because the optical Kerr effect is not sufficient to reduce the entropic contribution to the free energy of activation for the induction of nucleation from a supersaturated solution in the absence of large clusters. On the other hand, the femtosecond laser technique does not require this long aging and can even induce nucleation from supersaturated solutions immediately following preparation.[21] In addition, the femtosecond laser technique can induce nucleation at 11.0 M (the solubility of urea is 10.47 M at 25 °C), while the non-focused nanosecond laser cannot below 12.0 M. As an alternative photophysical approach, the nucleation of HEWL[26] and amino acid[27] was demonstrated using radiation pressure applied by a tightly focused 1064 nm continuous wave (CW) laser. However, the solvent is limited to D_2O with this technique, because the temperature elevation due to 1064 nm photon absorption by the overtone band of the OH stretching mode in H_2O disturbs nucleation. In contrast to the CW laser approach, focused femtosecond laser irradiation can induce nucleation effectively *via* the multiphoton absorption process, which should also result in significant temperature elevation. Femtosecond lasers were typically focused into supersaturated solutions with a fluence of $\sim$PW cm^{-2}, which is extremely higher than that of a focused continuous wave (CW) laser ($\sim$MW cm^{-2}). Although most of the proteins and organic molecules mentioned above do not undergo single-photon absorption at the femtosecond laser wavelength (780–800 nm), higher order multiphoton absorption should be induced at the focal point under such intense excitation conditions. In addition, considering the versatility of the femtosecond laser technique, it seems unlikely that femtosecond laser-induced nucleation is based solely on a single, specific photochemical reaction. In the past several years, the mechanism of femtosecond laser-induced nucleation has been investigated primarily from another perspective, cavitation bubbles induced by femtosecond laser ablation, which is reviewed in the next section.

2.2. Nucleation mechanism based on femtosecond laser ablation

The first question to be answered in order to reveal the nucleation mechanism involves the identity of what is induced in protein solutions by focused femtosecond laser irradiation.

Table 1 List of materials in which nucleation can be induced by femtosecond laser ablation

	Material		Molecular weight	Solvent	Additives	Ref.
Protein	Water-soluble protein	HEWL	14.3 kDa	Water[a]	Salt	9
		Thanumatin	22 kDa		Salt	14
		RNase H	17 kDa		No additives	9
		TbPGFS	31 kDa		Salt, PEG	9
		Gl	173 kDa		Salt, PEG	9
	Membrane protein	AcrB	113.57 kDa	Water[a]	PEG, detergent	15
		TSecDF	80.5 kDa			17
	Protein complex	MnmA-tRNA^{Glu}	66 kDa	Water[a]	Salt, PEG	18
		Abnx-DP2	40 kDa		Alcohol	19
Small organic molecule	Polar molecule	Urea	60.06	Water	No additives	21
		Glycine	75.07	Water		23
		Acetaminophen	151.17	Water		24
		DAST	410.1[b]	Methanol		20
	Nonpolar molecule	Anthracene	178.23	Cyclohexane	No additives	22

[a] pH buffer solution. [b] Molecular weight of the cation–anion pair.

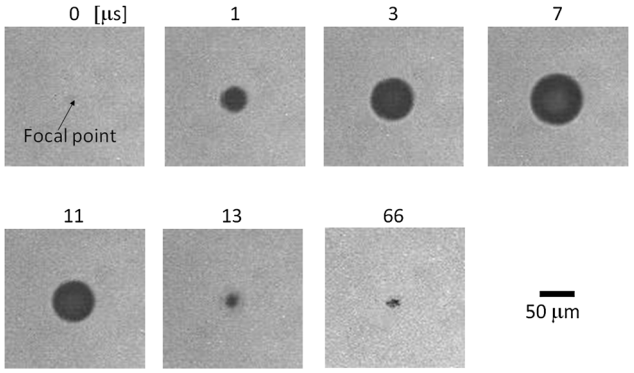

Fig. 4 Bright field images of HEWL supersaturated solutions monitored with a framing camera operating at 1×10^6 frames per s. The solutions were irradiated with a single femtosecond laser pulse. The arrow in the image at 0 μs indicates the laser focal point. Reprinted with permission from Springer, ref. 28, 2008.

To address this, Yoshikawa *et al.* carried out the high-speed imaging of supersaturated solutions of protein (HEWL), as shown in Fig. 4.[28] The round, black object observed at 1 μs is attributed to a cavitation bubble, which is formed by local boiling and/or vaporization of the supersaturated solutions. The single cavitation bubble expands and shrinks symmetrically in a few tens of microseconds. Finally, the cavitation bubble collapses, leaving some small "long-lasting" bubbles (at 66 μs in Fig. 4), which are considered to contain dissolved gases from the supersaturated solution.

On the basis of studies of laser ablation of water and biological materials,[7,29] the mechanism of such cavitation bubble formation can be explained as follows. When a near-infrared femtosecond laser pulse is tightly focused into a transparent liquid such as a protein solution, the intensity in the focal volume can become high enough to cause multi-photon absorption. Then, dense excited and ionized states of the solutes and solvent molecules are formed. The following rapid energy conversion results in an accumulation of heat and thermoelastic pressure within the absorbing volume, which increases the vapor pressure of the solution. Finally, cavitation bubbles are generated when the vapor pressure of the solution exceeds that of the atmosphere. In principle, such cavitation bubble formation can be induced in any liquid by pulsed laser irradiation having an energy above a certain threshold. The threshold energy for 200 fs pulses to induce cavitation bubbles in supersaturated solutions of HEWL was estimated to be 0.3 μJ per pulse with a 10× objective (NA 0.25) from the maximum diameters of the cavitation bubbles *vs.* pulse energy.[28]

For small organic compounds, it was revealed that nucleation occurs only when cavitation bubbles are induced by femtosecond laser ablation.[20–22] In particular, in the case of anthracene, circularly bent crystalline films were formed on a bubble surface (Fig. 5).[22] These results strongly suggest that cavitation bubbles are the key for triggering nucleation. To clarify whether cavitation bubbles are also involved in the mechanism for proteins, the influence of laser energy on the nucleation of HEWL was systematically investigated.[28] The statistical analysis revealed

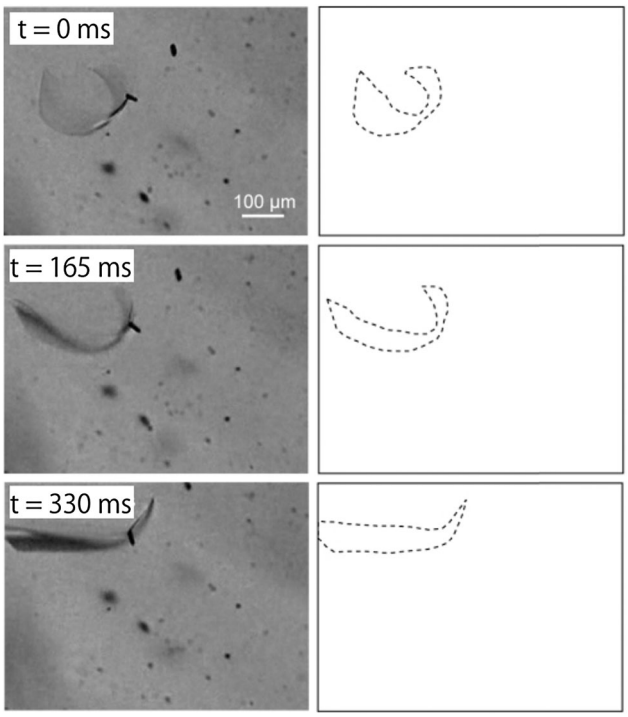

Fig. 5 Bright field images of bent film-like anthracene crystals generated with a single femtosecond laser pulse. The right side illustrations represent outlines of the crystal shown in the left side images. Reprinted with permission from American Chemical Society, ref. 22, 2007.

that the crystallization probability significantly increased beyond a laser energy of 0.4 μJ per pulse, while the crystallization probability below 0.4 μJ per pulse was the same as for the control (without laser irradiation). This threshold for nucleation, 0.4 μJ per pulse, is in good agreement with that for cavitation bubble formation (~0.3 μJ per pulse).

These results imply that cavitation bubbles induced by femtosecond laser ablation act as a trigger for nucleation of proteins as well as small organic compounds. To further clarify a protein nucleation mechanism based on laser-induced cavitation bubbles, fast imaging of protein concentration around cavitation bubbles was carried out using a fluorescent dye-labeled protein (F-lysozyme),[14,30] where the ε-amino group of the N-terminal lysine (Lys1) within each lysozyme molecule was chemically labeled with tetramethylrhodamine-5-isothiocyanate (5-TRITC, M_W 443 g mol^{-1}). Since the molecular weight of 5-TRITC is much less than that of native lysozyme (M_W 14 307 g mol^{-1}), the diffusion of F-lysozyme can be considered to be the same as that of native lysozyme. Fig. 6a shows fluorescence images of a supersaturated HEWL solution containing F-lysozyme and 2% agarose. The bright spot at 0 μs was attributed to plasma emission and scattering by an initial laser ablation process. The cavitation bubble expanded and shrank, and finally collapsed by 30 μs. Interestingly, at 20 μs, a bright spot with a peak intensity approximately three times larger than the average intensity of the surrounding gel medium was detected (Fig. 6b). This result indicates that cavitation bubbles induce the formation of a protein rich-region, which may lead to nucleation. In an alternative

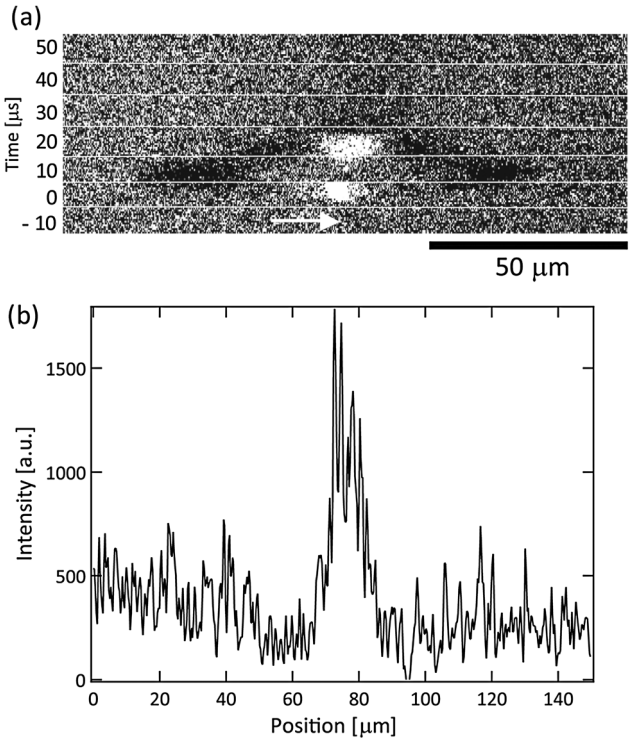

Fig. 6 (a) Fluorescence images of F-lysozyme in agarose gel. The samples were irradiated with a femtosecond laser pulse at 0 μs. The white arrow in the −10 μs image indicates the focal point of the laser. (b) Fluorescence intensity profile of (a) at 20 μs. Reprinted with permission from Springer, ref. 14, 2009.

approach to visualizing protein concentration around cavitation bubbles, Iefuji *et al.* used cytochrome *c*, which in solution is red in color due to the presence of heme groups.[31] They reported a clearer dynamics of formation of a highly concentrated protein region; a bright area (low concentration) was detected at the focal point (center of cavitation), and a dark area appeared around the cavitation bubbles (Fig. 7). Such dark and bright areas were not observed in pure water (absent proteins).

These results indicate that cavitation bubbles possibly induce the local condensation of protein molecules. Actually, an air-water interface is known to be preferable for protein nucleation and crystal growth.[32,33] In addition, evaporation of solvents may condense protein (and precipitants) near the bubble surface and result in nucleation. In fact, generation of the cavitation bubble is mainly due to the vaporization of water, not the other materials in the protein solution such as protein and precipitant, because there was no detectable difference in cavitation bubble size between the protein solution and pure water.[28] Furthermore, the shock wave[34] and the expansion and collapse of cavitation bubbles[35] generate a transient pressure with a magnitude reaching MPa–GPa. Such pressure fluctuations could also act as a nucleation trigger.[36]

In addition to the role of cavitation bubbles in nucleation, the photochemical effects of femtosecond laser-induced nucleation of proteins should be evaluated. Okutsu *et al.* reported that UV light produces photochemical covalently bonded protein dimers, which enhance nucleation.[37,38] The photochemical covalently bonded dimer of HEWL is considered to enhance nucleation because dimer formation *via* weak physical interactions (van der Waals force or hydrogen bonding) is energetically unfavorable. However, Murai *et al.* reported that the dimer contribution to nucleation is rather small in the femtosecond laser case, because the enhancement of nucleation by laser irradiation at λ = 260 nm (producing more dimers) is the same as that at λ = 780 nm.[39] One of the reasons why the contribution is small in the femtosecond laser case may be because femtosecond laser ablation would cause rather protein denaturation than dimer formation in the absorption volume. Murai *et al.* found that focused femtosecond laser irradiation produced not only dimers but also white, visibly large aggregates of denatured HEWL, which did not act as seeds for nucleation.[39] Since a femtosecond laser has a higher peak intensity [∼GW] than a Xe lamp [∼100 W], proteins rather undergo the simultaneous and/or stepwise multiphoton absorption, which can produce the permanently damaged species.[37]

Fig. 8 shows the currently considered mechanism of femtosecond laser-induced nucleation of proteins. Cavitation bubbles formed by femtosecond laser ablation increase the protein (and precipitant) concentration (supersaturation) locally and transiently in supersaturated solutions. Such a condensed region is relaxed due to the spontaneous diffusion of protein molecules. Hence, crystal nuclei which are generated by the transient increase in supersaturation grow slowly at lower supersaturation, and thus

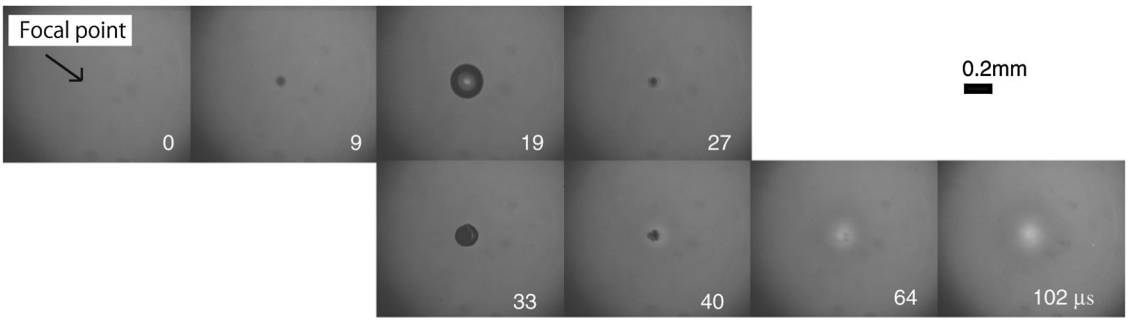

Fig. 7 Bright field images of cavitation bubbles induced by focusing a single femtosecond laser pulse into a cytochrome *c* solution. Reprinted with permission from Springer, ref. 31, 2011.

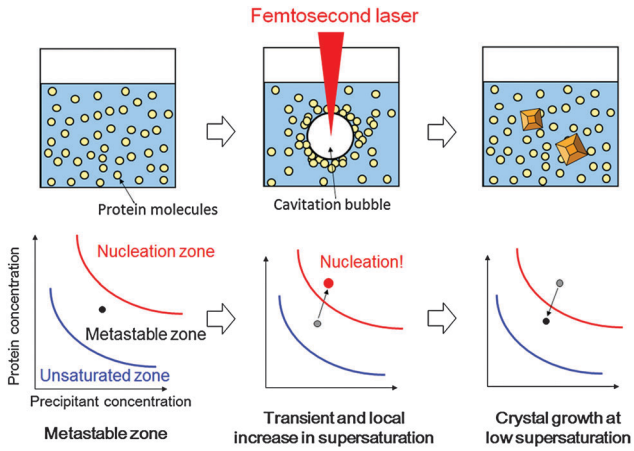

Fig. 8 Mechanism of femtosecond laser-induced protein nucleation.

high-quality crystals can be obtained. This is an ideal approach to overcome the persistent dilemma in protein crystallization. Cavitation bubbles can be generated by ultrasonication instruments, which have been utilized in protein crystallization.[40] However, transducers should be in contact with solutions to focus ultrasonic waves in small droplets (nL–μL, which is the standard volume for protein crystallization). On the other hand, a femtosecond laser can stimulate a tiny region (~μm) of such small droplets in a non-contact manner. Thus, the femtosecond laser technique would be easier for protein crystallization in practice. In addition, femtosecond laser ablation *via* multiphoton absorption[6] and photomechanical processes[7] can induce cavitation bubbles with less heat compared with longer laser pulses (*e.g.*, nanosecond lasers). When a near-infrared femtosecond laser pulse is tightly focused into a protein solution, the intensity in the focal volume can become high enough to cause multiphoton absorption, which then restricts the energy deposition within the absorbing volume and minimizes the collateral thermal damage. Also, the following rapid energy conversion results in an accumulation of heat and thermoelastic pressure within the absorbing volume, which increases the vapor pressure of the solutions. Finally, cavitation bubbles are generated when the vapor pressure of the solution exceeds that of the atmosphere. Thus, in the absence of photochemical decomposition (usually the case for proteins), a cavitation bubble generation process by femtosecond laser ablation of protein solutions is involved in the elevation of not only temperature but also thermoelastic pressure. In fact, there are a significant number of experimental observations suggesting that femtosecond laser ablation can be initiated at energy densities much lower than those required for boiling and vaporization.[7,41] Such energetically efficient cavitation bubble generation by femtosecond laser ablation, of course, is advantageous to the crystallization of proteins whose structures are sensitive to the surrounding temperature.

For protein nucleation, past studies strongly suggest that a cavitation bubble induced by laser ablation is a primary trigger. Nevertheless, other potential triggers such as the photochemically formed dimers[37,38] and the optical Kerr effect[2] should not be ruled out completely, because femtosecond laser pulses anyway provide high photon density and are also absorbed at a focal point. These may have to be taken into account on a case-by-case basis depending on protein properties. However, in other words, this implies that the femtosecond laser technique is a mixed approach which employs elements of other light-induced nucleation techniques as well as cavitation bubbles induced by laser ablation. This may be one of the reasons why the femtosecond laser is effective in inducing nucleation in a variety of materials. In addition, Knott *et al.* recently reported that unfocused laser pulses such as nanosecond lasers can induce bubble nucleation, and that small bubbles can induce crystal nucleation.[42] This suggests that extremely small (possibly nanoscale) and transient bubbles may form in the nucleation experiment by the optical Kerr effect[5] and catalyze crystal nucleation without being observed.[42] Thus, further studies on the mechanism of light-induced nucleation should consider multiple factors.

3. Laser ablation for protein crystal seeding

Crystal growth after nucleation is also a challenging step in obtaining high-quality, protein single crystals for X-ray crystallographic structural studies. Even if the environmental chemical and physical parameters (*e.g.*, temperature, solvent, and cosolutes) are carefully adjusted, irregularly shaped crystals (*e.g.*, polycrystals and cracked crystals) which are not suitable for crystallography are often obtained. One way that irregularly shaped crystals can be used is to produce single crystalline seeds by cutting or crushing the crystals with mechanical tools such as Micro-Tools (Hampton Research, USA). The seeds can be used for macroseeding or microseeding, which usually grows single crystalline seeds with the size of typically 5–50 μm (macroseeding) or much smaller (microseeding) in less supersaturated solutions.[2] However, special handling is required to cut or crush protein crystals with mechanical tools because protein crystals are soft, fragile, sensitive to changes in their surroundings, and in some cases very small (≤100 μm per dimension). To overcome the difficulties in processing protein crystals for macroseeding and microseeding, laser ablation is promising to produce crystal seeds by non-contact means. In this section laser ablation techniques are reviewed involving a pulsed deep-UV laser for macroseeding and a femtosecond laser for microseeding.

3.1. Macroseeding by deep-UV laser ablation

A pulsed deep-UV laser is one of the light sources which can be used for spatially precise, soft processing of protein crystals by laser ablation. Proteins have a broad absorption spectrum below the wavelength of 300 nm. In particular, the absorption coefficient below 200 nm is known to be twenty times larger than that at the 280 nm absorption peak.[43] Deep UV light ($\lambda <$ 200 nm) can cause direct photochemical decomposition of C–C and C–N bonds in the peptide linkage. Thus, the use of deep UV pulses enables laser ablation based on a photochemical decomposition process, which is desirable in order to reduce thermal

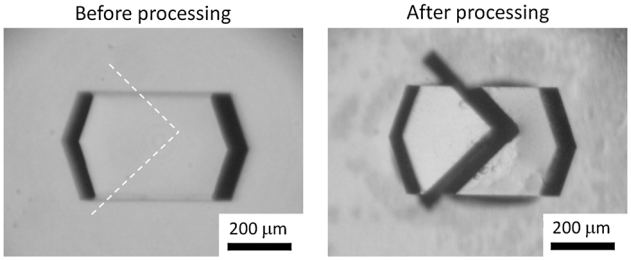

Fig. 9 Bright field images of a HEWL crystal before and after deep-UV laser ablation. The laser was scanned along the white dotted lines. Reprinted with permission from SPIE, ref. 10, 2007.

damage to the crystal. In addition, such photochemical laser ablation restricts the ablated region within the absorbing volume due to high absorbance and low heat generation. Kitano *et al.* demonstrated that deep-UV (λ = 193 nm) laser ablation enables processing of protein crystals of arbitrary shapes.[12] Fig. 9 shows representative examples of a HEWL crystal before and after laser ablation. The original crystal was divided into two parts by scanning the deep-UV laser pulses in *xyz* directions. No visible signs of cracks or denaturation were observed. The cross section was very sharp, and the nonirradiated parts of the crystal retained their original shape. It has also proven possible to obtain other shapes including a rectangle and a doughnut-like crystal.[20] Such shaping of protein crystals is, of course, extremely difficult to accomplish using conventional mechanical tools. To confirm the quality of the laser-irradiated crystal, the XRD pattern of the cut HEWL crystal was measured. The crystal diffracted beyond 1.9 Å resolution, which was the same as that of the as-grown HEWL crystal obtained under identical growth conditions. Fine processing using the deep-UV laser has been applied to various proteins, including GI, human lysozyme, phosphoenolpyruvate carboxylase, and AcrB without deterioration of the XRD data.[44,45]

Crystals processed by deep-UV laser ablation can be used for macroseeding. A HEWL crystal which was successfully laser-processed was seeded in a supersaturated solution and then it grew larger than its original size (Fig. 10).[46] Polarized light microscopy and X-ray diffraction analysis showed that the resulting crystal grown by seeding was a single crystal of suitable quality for X-ray crystallography. Thus, this deep-UV laser ablation seeding technique is useful for the production of seed crystals of adequate size and shape in macroseeding as well as for the elimination of a damaged section in a growing crystal.

3.2. Microseeding by femtosecond laser ablation

Microseeding, which conventionally utilizes small crystals (~μm) produced by crushing original crystals, has been demonstrated by femtosecond laser ablation. The technique was originally developed for the microseeding of urea crystals in supersaturated solutions.[47] A small region (~1 μm^2) of a urea crystal was ablated by irradiation with a near-infrared femtosecond laser pulse. Then a single, newly formed urea crystal appeared near the laser focal spot on the original

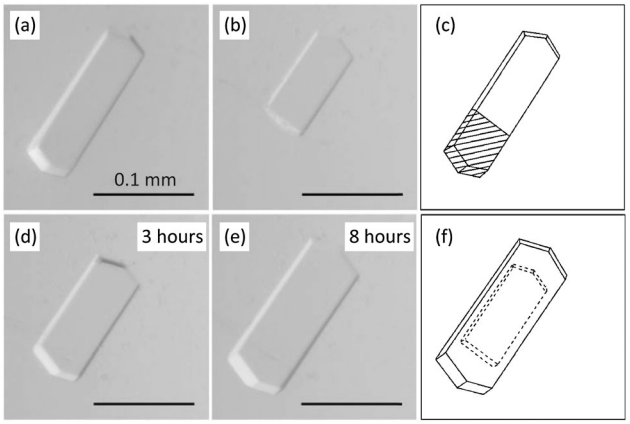

Fig. 10 Macroseeding of protein crystals by deep-UV laser ablation. (a) An as-grown HEWL crystal before laser ablation. (b) A seed crystal produced by laser ablation of the crystal in (a). (c) Outline of the crystal with ablated (hatched) and non-ablated regions shown. (d) and (e) Growth of the seed crystal at 3 and 8 h after laser ablation, respectively. (f) Outlines of the crystals immediately after laser ablation (dotted line) and after 8 h (solid line). Reprinted with permission from the Japan Society of Applied Physics, ref. 46, 2005.

(mother) crystal and grew independently. A single crystal fragment which was of the same size as the laser focal spot was ejected, and this fragment then acted as the seed crystal.

Given the physical characteristics of femtosecond laser ablation listed below, the advantages of the microseeding technique for protein can be explained as follows. (1) Focused irradiation with a near-infrared femtosecond laser induces ablation *via* multiphoton absorption, which should enable the spatially precise isolation of seed crystals from an arbitrary area of the mother crystals while present in a supersaturated solution. Conversely, irradiation with a deep-UV laser cannot reach a protein crystal in a supersaturated solution owing to the high absorption of UV light by the protein solute.[12] The dimension of spatial accuracy of femtosecond laser ablation *via* multiphoton absorption is similar to that of the laser focal spot, ~1 μm, which is small enough to precisely target the mother crystals. (2) Femtosecond laser ablation produces less heat than does ablation by other lasers with longer pulse width. The mechanism of femtosecond laser ablation just above the ablation threshold fluence is ascribed to a photomechanical process;[7] mechanical stress is confined to the excited region of the mother crystals and disrupts the surface resulting in collective ejection of crystal fragments. Hence, photomechanical ablation reduces potential thermal damage to seed crystals. That is, it is a "soft" method for microseeding of protein crystals.

In 2012, the microseeding technique was applied to proteins. Fig. 11a shows snapshots of HEWL crystals produced by femtosecond laser ablation. A tetragonal crystal (daughter crystal) was obtained by ablating a tetragonal HEWL mother crystal with a single 0.2 μJ femtosecond laser pulse.[11] Daughter crystals were not obtained with laser irradiation below the ablation threshold (0.2 μJ), which was confirmed by atomic force microscope (AFM) imaging of the HEWL crystals. XRD proved that the daughter crystal was a single crystal with almost

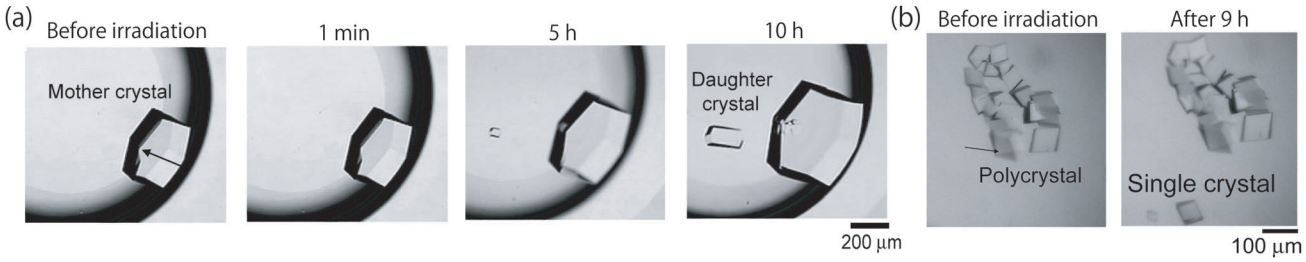

Fig. 11 Microseeding of protein crystals by femtosecond laser ablation. (a) Growth of HEWL microcrystals produced with a single femtosecond laser pulse. (b) Single crystal of the membrane protein, AcrB, isolated from the mother polycrystal by a single femtosecond laser pulse. The arrows point to the positions on the mother crystals where the laser was focused. Reprinted with permission from American Chemical Society, ref. 11, 2012.

the same crystallographic parameters (*e.g.*, the space group and unit-cell parameters) as those of the mother crystals. Notably, the daughter crystal diffracted to nearly the same resolution (1.47 Å) as the mother crystal did (1.46 Å), even though the daughter crystal was less than half the size of the mother crystal, suggesting that the quality of the daughter crystal was similar to that of the mother crystals. Such microseeding by femtosecond laser ablation was also demonstrated with polycrystals of a membrane protein, AcrB (Fig. 11b).[11] When the polycrystal was irradiated with a 0.3 μJ laser pulse (just above the ablation threshold (~0.2 μJ)), AcrB trigonal single crystals were obtained. AcrB crystals contain ~90% water, which made the production of seed crystals more difficult than for the HEWL system. Nevertheless, given the ability to produce AcrB daughter crystals, femtosecond laser ablation has the potential to produce microseeds of other recalcitrant proteins.

To assess the impact of femtosecond multiphoton, photomechanical ablation on the growth of single crystal microseeds, the surface of a HEWL mother crystal was observed using AFM.[11] Fig. 12a shows a representative image of a mother crystal surface etched using an ablation threshold (0.2 μJ per pulse). The etching is ~1 μm in diameter, which is much smaller than the laser spot size estimated by the diffraction limit (3.8 μm). Because HEWL crystals do not absorb 800 nm light, the region of the crystals impinged at the center of the laser spot is instead excited by multiphoton absorption, which decreases the diameter of the etching in comparison with etching formed by linear absorption. The crystal surface etched with a higher energy laser pulse (0.4 μJ per pulse) resulted in surface cracking (Fig. 12b). Notably, swelling was not observed surrounding the etched area. Such sharp etching and surface cracking strongly indicate that the crystal surface is mechanically disrupted *via* a photomechanical process, which should reduce critical thermal damage to HEWL molecules.

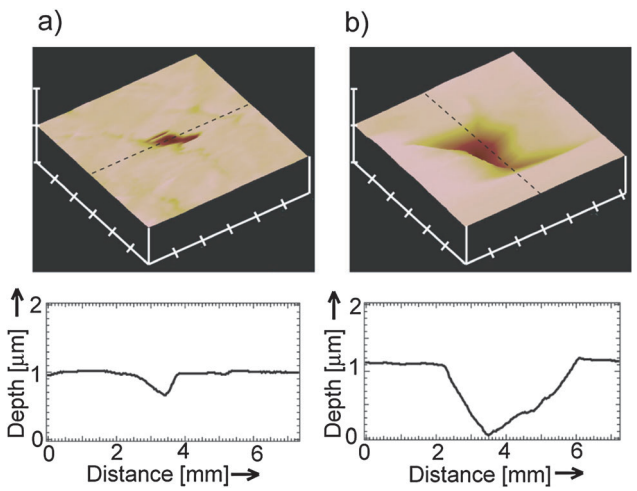

Fig. 12 (top) AFM images of HEWL crystal surfaces that were irradiated with a femtosecond laser pulse. (bottom) Depth profiles along the dotted line in the AFM images. Reprinted with permission from American Chemical Society, ref. 11, 2012.

Photomechanical ablation of protein crystals should result in ejection of bulky crystal fragments. If the surrounding solution is supersaturated and the ejected fragments are not significantly damaged, they can act as seed crystals. The molecular mechanism of the ejection of such bulky crystal fragments by photomechanical ablation has been intensively studied both experimentally and theoretically. Hosokawa *et al.* studied the ablation process on the basis of photoexcitation relaxation dynamics for a copper-phthalocyanine (CuPc) film,[48] where the molecules associate with each other *via* weak cohesion due to Van der Waals force as in the protein crystal case. Here, ultrafast temperature elevation, dT/dt ~100 °C/10 ps > 10^{13} °C s^{-1}, due to the rapid nonradiative relaxation (~20 ps) of highly excited electronic states formed by femtosecond laser excitation was observed. This implies that intramolecular and lattice vibrations are enhanced tremendously in a time scale of 10 ps, while the molecules are unable to change their positions from the equilibrium. As a result, mechanical stress accumulated in the film. If the laser energy is near the threshold of laser ablation, but still below plasma generation, the transient pressure mainly plays a role in causing morphological changes photomechanically, such as sharp surface etching and bulky fragment ejection with little thermal damage.[49] On the other hand, nanosecond laser ablation of the CuPc thin film is considered to involve relatively slow heating due to cyclic excitation,[50] which results in a gradual etching pattern and the ejection of molecularly dispersed ablated fragments. Using a molecular-level simulation technique, Zhigilei *et al.* also reported that photomechanical laser ablation of organic solids and liquids leads to disintegration and ejection of large relatively cold chunks of material.[41]

On the basis of the results, one can expect that femtosecond laser ablation *via* photomechanical processes results in the ejection of bulky crystal fragments with little damage, which can act as high-quality microseeds in supersaturated solutions. In addition, focused femtosecond laser irradiation enables the isolation of single protein microcrystals with high spatial accuracy in supersaturated solutions *via* multiphoton processes. Thus, the microseeding by femtosecond laser ablation will contribute to the production of high-quality protein single crystals from polycrystals or cracked crystals that are not suitable for XRD studies.

4. Conclusions and outlook

The utility and the underlying physico-chemical mechanism of laser ablation for protein crystal nucleation and seeding were reviewed. These successful results clearly show the potential of laser ablation techniques for the production of high-quality single crystals suitable for XRD studies. The key to the successful application of laser ablation to protein crystallization involves the use of lasers with ultrashort pulse widths or wavelengths (*e.g.* a femtosecond or a deep-UV laser), which allows for the spatially precise, low-damage processing of protein solutions and crystals *via* specific ablation processes (*e.g.*, photomechanical or photochemical). Although femtosecond and deep-UV lasers are a bit costly (>$100 000), the integration of laser ablation techniques in structural determination processes such as purification, crystallization, and XRD will accelerate advancement in the structural biology field. Laser ablation techniques are fully non-contact means that do not require significant changes in the conventional protein crystallization setup, and thus their application in automated and high-throughput screening/processing systems for protein crystallization is feasible in practice. In fact, Mori *et al.* have developed a system wherein conventional crystallization plates containing sub micro-liter solutions with sitting-drop or hanging-drop positions can be mounted on a motorized stage and irradiated with femtosecond laser pulses automatically.[10] High-quality protein single crystals produced using such an automated system will enhance the database of knowledge in structural biology and subsequent structure-based drug design.

Acknowledgements

This review is based upon works supported in part by grants from the Japan Society of the Promotion of Science (KAKENHI Nos. 24656006, 24680050, and 24106505 to HYY and 23360011 to YM and HYY).

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