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Enhancement of femtosecond laser-induced nucleation of protein in a gel solution

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We found that the use of a gel solution with agarose enhanced femtosecond laser-induced nucleation and produced hen egg white lysozyme crystals at three to five times lower supersaturation than those by the femtosecond laser or agarose alone. The fast fluorescence imaging of the protein in the gel solution revealed that cavitation bubbles created high-concentration regions at the focal point, which could be the trigger for protein nucleation. The lower diffusions of protein molecules in agarose gel retained the high-concentration regions for a longer time, and facilitated the nucleation. © *2010 American Institute of Physics*. [doi:10.1063/1.3294622]

Light-induced nucleation has been attracting attention as a method for temporal and spatial control of crystallization of organic and biological molecules, and is of fundamental interest in the field of light-matter interactions. To date, an increasing number of studies have demonstrated light-induced nucleation by photo-physical[1–5] and chemical[6–8] methods. A femtosecond laser (peak intensity ~gigawatts) is one of the promising techniques to induce nucleation at low supersaturation, which will improve crystal quality. Its effectiveness has already been confirmed for various kinds of materials including organic molecules, water-soluble proteins, membrane proteins, and their complexes.[9–17]

Our present aim is to realize protein nucleation at lower supersaturation by developing a femtosecond laser technique. Focused irradiation of a femtosecond laser causes multiphoton absorption, leading to the formation of shock-waves and cavitation bubbles, which can be a mechanical stimulation to nucleation. In fact, we found that the rapid shrinkage (microsecond-scale) of a cavitation bubble creates a local high concentration of protein.[17] Since the local high concentration is relaxed due to the diffusion of proteins, the use of viscous solutions would be the key to realizing more effective protein nucleation. In this letter, we report on the enhancement of the femtosecond laser-induced nucleation of protein in an agarose gel solution. The laser irradiation in the gel condition realized the nucleation of hen egg white lysozyme (HEWL) at three to five times lower supersaturation than those by the femtosecond laser or agarose alone. By the fast imaging of cavitation bubbles and fluorescent-labeled lysozyme (F-lysozyme), we discuss how agarose gel facilitates the creation of local high-concentration regions created by the rapid shrinkage of cavitation bubbles.

HEWL was purchased from Seikagaku (six-times recrystallized, Lot No. E05801) and used without further purification. Supersaturated HEWL-agarose gels were prepared with the following protocol. HEWL solutions at a concentration of 12–24 mg/ml in a 0.1 M NaAc buffer (pH 4.5) and precipitating solutions at a concentration of 24 wt % NaCl in a 0.1 M NaAc buffer (pH 4.5) were prepared. Powdered agarose from Sigma–Aldrich (Agarose IX-A, catalog No. A2576) was dissolved in Millipore water at a concentration of 3 wt % at 95 °C, and then cooled to 30 °C, which is still a few degrees above the gelling temperature. The HEWL, precipitating, and agarose solutions were well mixed in a 1:1:1 (v/v) ratio. Then, 6 μl aliquots of the mixture were pipetted onto wells in microbatch plates (Hampton Research, Imp@act plate). The plates sealed with a clear sheet (HTstick, JT-SCIENCE LLC) were incubated at 23 °C for 24 h prior to shooting with a femtosecond laser. Agarose-free supersaturated solutions with the same HEWL and precipitant concentrations were also prepared for the control.

A femtosecond laser (τ=200 fs, λ=780 nm, 1 kHz, IFRIT, Cyber Laser Inc.) was used for the irradiation source. Eighty shots of single-laser pulses with the energy of 5.5 uJ/pulse were focused into the supersaturated gel or solution with an objective lens (10×, NA.0.4, Olympus). Cavitation bubble behavior was monitored with a high-speed imaging camera (HPV-2, Shimadzu Corp.).[16] Fluorescence imaging of F-lysozyme in the crystallization samples was carried out with an EMCCD (Photon MAX 512B, Princeton Instruments). Details of the high-speed fluorescent imaging procedure have already been described in our previous reports.[16,17]

Figure 1 shows the crystallization probabilities (ratio of the crystallized batch numbers to the total batch numbers) of the crystallization samples. In the agarose-free condition,

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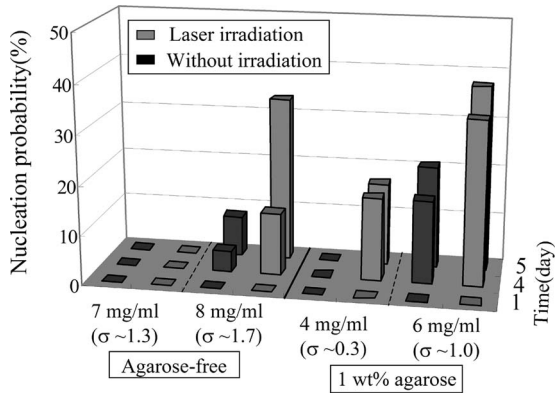

FIG. 1. Crystallization probability of HEWL in agarose-free solution, and in 1 wt % agarose gel. The crystallization probabilities were calculated as ratios of the number of crystallized batches and the total batch numbers (24).

spontaneous nucleation occurred at the HEWL concentration of 8 mg/ml, which corresponds to 1.7 of supersaturation [(σ, defined by (C–Ce)/Ce, where Ce is the solubility at 23 °C (Ref. 18)]. Focused femtosecond laser irradiation increased the nucleation probability from 8.3 to 33 % due to the nucleation enhancement effect,[16] although the laser did not realize nucleation at lower $\sigma \sim 1.3$. On the other hand, in the agarose gel condition, spontaneous nucleation occurred at lower $\sigma \sim 1.0$. Numerous studies have reported such enhancement of nucleation by agarose,[19] and its mechanism was explained by promotion of the association among protein clusters.[19] However, we found that laser irradiation in the agarose gel condition realized the nucleation at the lowest $\sigma \sim 0.3$, which is three times lower than that of the spontaneous nucleation. This clearly indicates that the focused femtosecond laser irradiation in the presence of agarose gel enhances nucleation more than the laser or agarose alone.

In order to evaluate the stimulus by cavitation bubbles in agarose gel, high-speed imaging of cavitation bubbles was carried out at framing rate of 1 μs. In agarose-free solution, cavitation bubbles repeated rapid expansion and shrinkage [Fig. 2(a), 1 ~ 25 μs], and then finally collapsed into small bubbles [Fig. 2(a), 26 μs]. On the other hand, in agarose gel (1 wt %), cavitation bubbles also exhibited rapid expansion and shrinkage, while cavitation bubbles did not collapse and left single long-lasting bubbles [Fig. 2(b), 24 μs]. The long-lasting bubbles gradually became smaller and disappeared in approximately 20 s. Figure 3 shows the time evolution of diameter and velocity of cavitation bubbles. Regardless of being in gel solution or not, the maximum velocity of cavitation expansion was about 60 μm/μs. This result indicates that cavitation bubbles apply almost the same magnitude of mechanical stimulation to agarose gel as to gel-free solution.

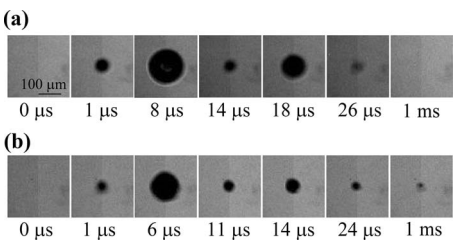

FIG. 2. Microscopic images of cavitation (a) in agarose-free solution, and (b) in 1 wt % agarose. The solutions were irradiated with a 200-fs pulse from a femtosecond laser. The laser energy was 5.5 μJ.

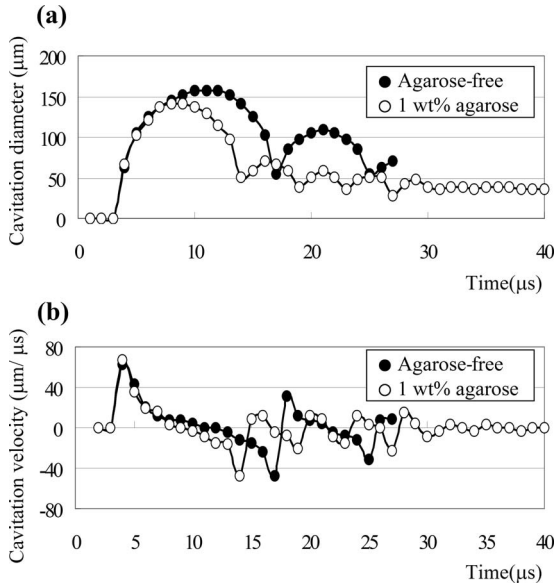

FIG. 3. Time evolution of diameter (a) and velocity (b) of cavitation bubbles induced by laser irradiation in agarose-free solution (●) and in 1 wt % agarose gel (○).

In order to clarify the influence of such a mechanical stimulation by cavitation bubbles on nucleation, we determined the spatial mapping of the HEWL molecules around cavitation bubbles with F-lysozyme (Fig. 4). In the agarose condition (4 wt %), when the cavitation bubbles began shrinking (Fig. 4(b), 20 μs), a bright spot was detected at the focal point. The fluorescence peak intensity of the bright spot was approximately three times greater than the average intensity of the surrounding gel medium. The surface of the cavitation bubbles is known to be a preferred region for the adsorption of protein molecules.[20] Hence, the bright spot can be attributed to the generation of a local high-concentration region of F-lysozyme molecules, which adsorb at the cavitation surface during expansion and then gather to a focal point by the rapid shrinking of the cavitation bubbles. In the

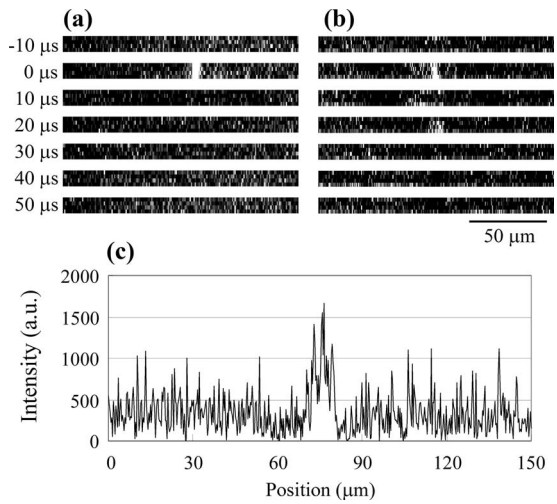

FIG. 4. Fluorescence images of F-lysozyme (a) in agarose-free solution, (b) in 4 wt % agarose gel. (c) Fluorescence intensity profile of the image of (b) at 20 μs. The samples were irradiated with a femtosecond laser pulse at 0 μs. A bright spot, attributed to plasma emission and reflection of the laser, was observed. In this technique, the spatial distribution of HEWL molecules is monitored by the fluorescence from F-lysozymes, and expansion and shrinkage of cavitation bubbles is observed as a black rim (Ref. 17).

agarose-free condition, on the other hand, no bright spot was detected. This result clearly indicates that use of the combination of a femtosecond laser and agarose facilitates the creation of high-concentration regions by cavitation bubbles.

Although the magnitude of mechanical stimulations induced by cavitation bubbles was almost the same in solution and agarose gel, the increment of the local concentration of HEWL became larger in agarose gel. This difference is probably a result of the difference of molecular diffusion. High-concentration regions created by cavitation bubbles were relaxed due to the diffusion of proteins. Thus, the low diffusion in agarose gel will retain high-concentration regions for a longer time, making nucleation more likely. In fact, it was reported that the diffusion coefficient of lysozyme monomers (hydrodynamic radius is about 2 nm) in 1 wt % agarose condition became about 50 % to 80 % that of gel-free condition.[21] In addition, in the supersaturated solution, HEWL clusters grow larger (several tens to hundreds of nanometers) with time.[22] These clusters diffuse more slowly than monomers due to the large interaction with the agarose matrix as well as their large hydrodynamic radius. Pluen *et al.* reported that the diffusion coefficients in agarose gel drastically decrease above a critical hydrodynamic radius (in 2 wt % agarose, the critical hydrodynamic radius is about 30 nm).[23]

Moreover, the suppression of the rapid collapse of cavitation bubbles in agarose gel contributed to the increase of the probability of nucleation, because the collapse of cavitation bubbles induces chemical decomposition and temperature jumps.[24] Alternatively, the longer duration of the bubbles formed at focal points should promote nucleation. However, nucleation was not induced when we made long-lasting bubbles with a nanosecond laser. Thus, the contribution of long-lasting bubbles for nucleation was rather small, and the local increase of protein concentration induced by the generation of cavitation bubbles was the main trigger for nucleation.

In summary, the use of agarose gel was shown to enhance femtosecond laser-induced nucleation, and induced HEWL crystals at three to five times lower supersaturation than those by the femtosecond laser or agarose alone. By the fast imaging of cavitation bubbles and fluorescent-labeled lysozymes (F-lysozyme), we found that higher-concentration regions were induced at the focal point in agarose gel, although the magnitude of mechanical stimulations induced by the cavitation bubbles was almost the same in solution and agarose gel. The low diffusion of protein molecules in agarose gel probably retained the local high concentration for a longer time, which enhanced nucleation. In addition, agarose gel is useful for spatial control of nucleation. In fact, we previously found that crystals were formed around the laser focal point in agarose gel.[17] In a similar example, Duffus *et al.* reported the spatial control of KCl nucleation in agarose gel (0.25 wt %) using the electric field of a laser.[3] These results clearly indicate that use of the combination of a femtosecond laser and agarose gel is a very practical technique for crystallization. We used agarose gel in this study, but it is possible that other gels will enhance the probability of laser-induced nucleation because the diffusion of protein molecules becomes smaller in gels. In our future work, we will focus on producing high-quality drug target protein crystals in various gel solutions.

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