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Protein crystallization induced by strong photons–molecules coupling fields photochemical reaction

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ABSTRACT

Photochemically induced protein crystallization byprotein's multiphoton excitation based on enhanced field of localized surface plasmon resonance (LSPR) of gold nanostructures was investigated. As strong photons–molecules coupling fields, gold nanostructures composed of nanoblocks and gold nanocolloid were used. With gold nanostructures, we could observe enzyme activity decrease by light irradiation that protein does not absorb. Surface observation of gold nanostructure by DFM (non-contact dynamic force microscope) shows that proteins were aggregated at around the nanoblock due to photochemical reaction by enhanced field. Crystallization probability depends on the excitation photon fluence, which indicates 3 photons absorption process occurred.

1. Introduction

An important area of post-genomic research is the determination of 3D protein structures. The main technique used for this purpose is X-ray crystallography, which has become a valuable tool for the analysis of crystals with the use of synchrotron radiation. Techniques for the preparation of high-quality single crystals are being developed continuously. Since protein molecules have highly anisotropism, nucleation often does not occur spontaneously even in supersaturated solution. Fig. 1 shows the solubility curve of hen egg-white lysozyme. Protein crystallization occurs at fairly high supersaturation as indicated by nucleation zone. On the other hand, crystal grows sat low supersaturation which is called as metastable region. It is desirable to obtain a high quality single crystal, nucleation and crystallization are carried out at metastable region. To achieve the condition mentioned above, some researchers have studied protein crystallization, induced by external fields, electric or magnetic [1–3].

Recently, we observed covalently bonded photochemical dimers in an electrophoresis experiment (SDS-PAGE), and the dimers were concluded to play an important role as the smallest stable clusters in early stage of the nucleation process. The enhancement of protein crystallization due to the covalently bonded dimers is illustrated in Scheme 1. Molecules gather to form clusters ($n = 2, 3, 4, \ldots$), which grow into a bulk crystal. When the cluster size is small, the clusters are unstable owing to the surface/volume energy disadvantage. Growth and dissolution take place even in supersaturated solutions. After the cluster size becomes larger than the critical size, the clusters grow into bulk crystals spontaneously. The first step of the normal nucleation process begins from the formation of the smallest cluster $n = 2$. The smallest cluster is unstable because the interaction between the molecules is weak (van der Waals, or hydrogen, bond). Thus, $n = 2$ cluster formation is an important step in an early stage of the nucleation process. In contrast, if the covalently bonded dimers behave similarly to the $n = 2$ cluster, the dimers grow to $n = 3, 4, \ldots$, and onward to the critical size. Therefore, if nucleation starts from a stable cluster, i.e., a covalently bonded dimer, the nucleation frequency becomes higher.

We have demonstrated that photochemically induced crystallization of protein in lysozyme, thaumatin, ribonuclease A [4–7]. However, a problem that has come up too much UV irradiation makes proteins denature and eventually crystallization frequency is inhibited as an opposite effect. UV irradiation should be carried out with least strength. To obtain crystal by photochemically induced technique, only few dimers to grow crystal are required [8]. And we consider the method of irradiation. Weak strength and long time irradiation gave better results than with strong fluence and short time irradiation at the same condition with same photon number.

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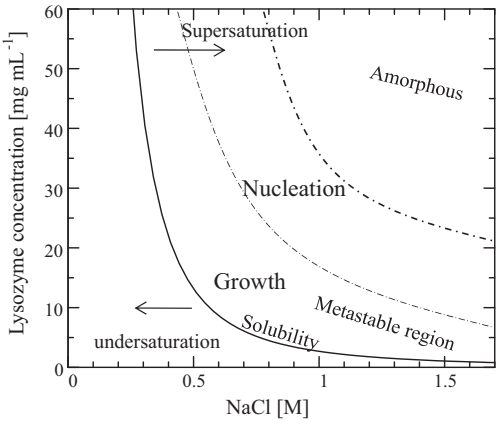


Fig. 1. Solubility curve of lysozyme against NaCl concentration.

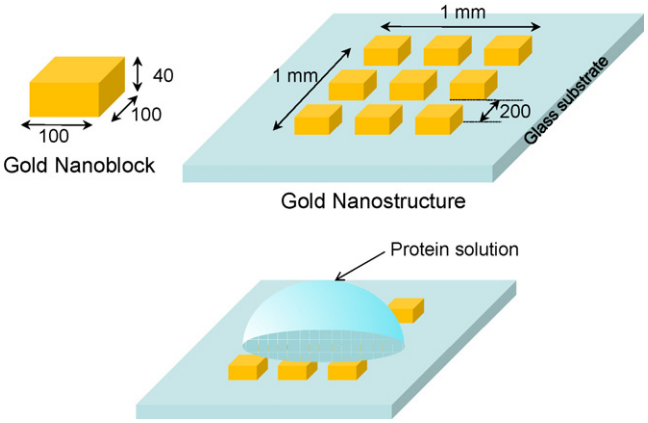
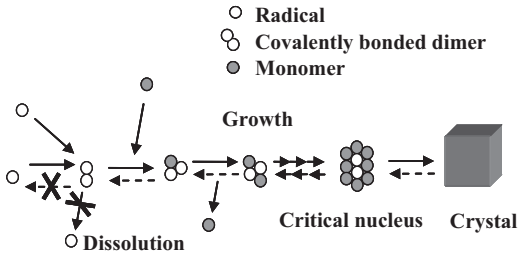


Fig. 2. Gold nanostructure used in this experiment.

Especially, strong intense pulsed laser irradiation results complete failure. This is explained as follows. With strong photon fluence irradiation, intermediate protein radical concentration is high, and then the second order reaction, radical–radical encounter, becomes dominant. With weak photon fluence, intermediate protein radical react with ground state protein molecule through pseudo-first order reaction [7]. The produced dimer by radical–radical reaction has a configuration that binds at ^{53}Trp–^{53}Trp position which has different configuration from the two neighbor molecules in the crystal in the case of lysozyme. This dimer does not grow to crystal. On the other hand, the dimers produced between intermediate protein radical and ground state molecule are expected to show variety of configurations that depending on the position of the ground state molecule, where protein radical attacked. Some of configurations are expected to have specific configuration that is identical to the neighbor molecules in crystal. These dimers are expected to grow to crystal. Thus, the irradiation with low fluence is suitable to the photochemically induced crystallization. Protein crystal growth is a phenomenon of from day to month time order scale. Fast reaction or high efficiency is not required for photochemically induced crystallization.

Nanostructures or nanoparticles of noble metals exhibit characteristic bands of optical attenuation at visible and infrared wavelength due to localized surface plasmons (LSPs) [9,10]. These LSP bands are also associated with enhancement of the electromagnetic field due to its localization. Utilizing these extremely localized enhanced fields, non-linear optical phenomena such as optical harmonic generation, light mixing and multiphoton transition have been real phenomenon. Ueno et al. demonstrated two-photon polymerization of a photoresist polymer (SU-8) on gold nanoblocks arranged regularly on a glass substrate by irradiation with an incoherent CW light [11,12]. They concluded that the two-photon absorption of incoherent light by resist was due to enhancement of the electromagnetic field of the incident light by surface plasmons



Scheme 1. Enhancement of protein crystallization by photochemical reaction. The photochemical product, the protein dimer, behaved as the smallest cluster of nucleation.

localized in the gold nanostructures. Furthermore, other groups have also reported the enhancement of polymerization around metal nanoparticles based on the surface plasmon of the particles. Tsuboi et al. reported that the ring-opening reaction of closed form diarylethene (DE) was promoted by two-photon absorption due to the enhanced electric field by gap mode excitation [13]. These researches were demonstrated that plasmon assisted two-photon absorption was happened. At present applications of multiphoton absorption by weak CW excitation are desired onto practical systems.

In this thesis, we tried photochemically induced crystallization by multiphoton excitation of visible light with strong photons–molecules coupling fields. Efficiency of multiphoton excitation is expected to be very low. However, as mentioned previous, efficiency of excitation is not important. If we use visible light instead of UV (<300 nm) light, we can expect at least two advantages compared with UV light irradiation. First, as most proteins do not absorb visible light, protein denaturation can be negligible. Second, with UV irradiation, apparatus for crystal growth must be made of quartz those are expensive. If we use VIS light, the apparatus could be made of synthetic resins, for example, PMMA or normal glasses that are commercially used as disposable.

2. Experiment

As strong photons–molecules coupling apparatus, gold nanoblocks which were defined on glass substrates (Matsunami Co., Japan) having an area of (24 mm × 24 mm) using a high-resolution electron-beam lithography (EBL) system (ELS-7700H, Elionix Co., Ltd., Japan) at a 100 kV accelerating voltage were used. Layout of the utilized gold nanostructure is illustrated schematically in Fig. 2. For plasmonic field enhancement, gold nanoblocks are measuring 100 nm × 100 nm × 40 nm regulated in a grid pattern at 200 nm spacing. The structures occupied an area of 1 mm × 1 mm. Detailed fabrication method was described in the previous literature [14].

The light source used for the irradiation was Xe-lamp (USHIO UXL-300D, 300 W) whose UV radiation was cut by 390 nm cut-off filter.

Hen egg-white lysozyme was purchased from Seikagaku (6 times recrystallized lot E02Z04) and was used without further purification. Sodium acetate, acetic acid and sodium chloride (NaCl), all of them are GR-grade, were purchased from Wako Pure Chemicals. Sodium acetate and acetic acid were dissolved in ultrapure water (Milli-Q) and used as buffer solution (NaAc buffer, 50 mM, pH 4.3). The prepared solution was centrifuged and filtered through a 0.45 μm single-use membrane (Millipore) before

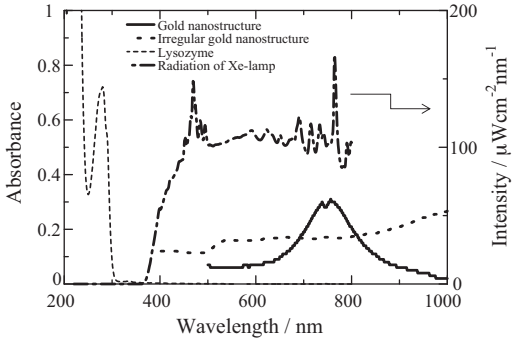


Fig. 3. Absorption spectra of protein (dotted line), gold nanostructure (solid line), irregular gold nanostructure (double dotted line) and radiation spectrum of Xe-lamp cut by UV filter (dashed line).

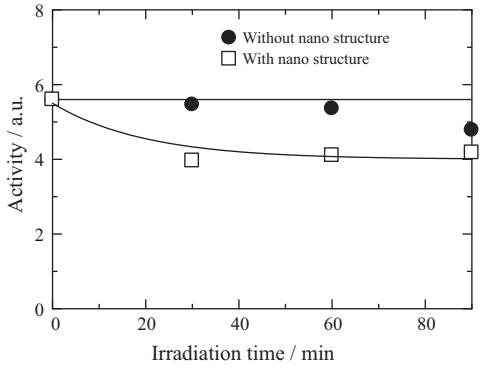


Fig. 4. Enzyme activity change of lysozyme measured by ML method. Activity decreased by irradiation with nanostructure.

the each experiment. The lysozyme concentration was determined by optical absorption at 280 nm using an extinction coefficient of 2.64 mL cm^{-1} mg^{-1}.

Activity of the lysozyme solution was determined by *Micrococcus lysodeikticus* (*luteus*) cell assay test (ML method). This assay is based on the decrease of absorption at 450 nm when the cells are digested. We refer to the experimental procedure described in the literature [15]. *M. lysodeikticus* (lot 052K8618) was purchased from Sigma industry.

Surface observation of gold nanostructure was measured by non-contact dynamic force microscope (DFM) (E-sweep, SII Nano-Technology Inc.).

Crystallization experiment was carried out at metastable condition typically supersaturation $\beta = C/C_e \sim 3$ with batch method. Protein crystallization experiment in this paper is well known procedure among biochemical researchers and described in the textbook written by McPherson [16] and McRee [17].

The absorption spectra of protein and gold nanostructure were measured by Hitachi U-2010 spectrophotometer. Fig. 3 shows the radiation spectrum of Xe-lamp with UV cut filter. The absorption spectra of protein show shorter than 300 nm and the absorption of nanostructure longer than 600 nm. This means that photochemical reaction requires at least 2–3 photons absorption process.

3. Results and discussion

Fig. 4 shows the enzyme activity change of lysozyme against irradiation time with and without gold nanostructure. Without gold nanostructure (round plot), enzyme activity did not change. With irradiation (rectangular plot) enzyme activity decreased by irradiation. This indicates that lysozyme occurred photochemical reaction by strong photons–molecules coupling field.

Then we observed surface change of nanostructure. Fig. 5 shows the pictures of DFM image of gold nanostructure before (a) and after (b) the irradiation. As compared after the irradiation (b) against before (a), gold nanostructures were covered by protein aggregation.

To confirm whether gold nanostructures induce photochemically induced crystallization by multiphoton excitation of lysozyme, we carried out four crystallization experiments with or without gold nanostructures and with or without irradiation. Experiments were carried out by sitting drop batch method with reservoir solution to keep constant salt concentration. Control experiment was intended at metastable condition where spontaneous nucleation hardly happens at $\beta = 2.7$ ($C_p = 15$ mg mL^{-1} lysozyme, NaCl = 0.7 M).

Fig. 6a shows photograph of a droplet prepared after 24 h neither gold nanostructures nor light irradiation as a control experiment. Only one crystal appeared in the droplet. Fig. 6b shows a droplet without gold nanostructures but with irradiation for 30 min. One crystal also appeared in the droplet. Since lysozyme has no absorption this result was expected to show the same result as Fig. 6a. Fig. 6c shows a droplet with gold nanostructures and without irradiation. More than 10 crystals appeared in the droplet. This experiment was also expected as same result as two previous experiment. But, something to induced crystallization would exist in this case. Finally, Fig. 6d shows a droplet with gold nanostructures and with light irradiation. More than 4000 crystals appeared in the droplet. This result exhibit the specific effect when it is used both gold nanostructures and visible lights irradiation. Experiment with gold nanostructure and without irradiation (Fig. 6c) was carried out under fluorescent room light. Crystallization could be induced even by fluorescent room light. Thus, the

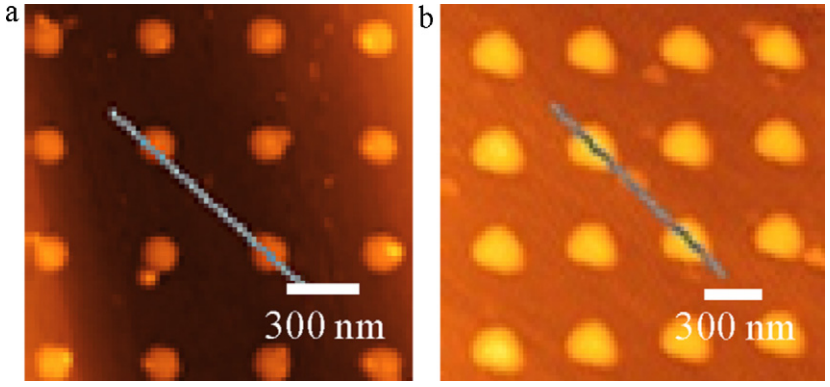


Fig. 5. DFM image of gold nanostructure before (a) and after (b) the irradiation.

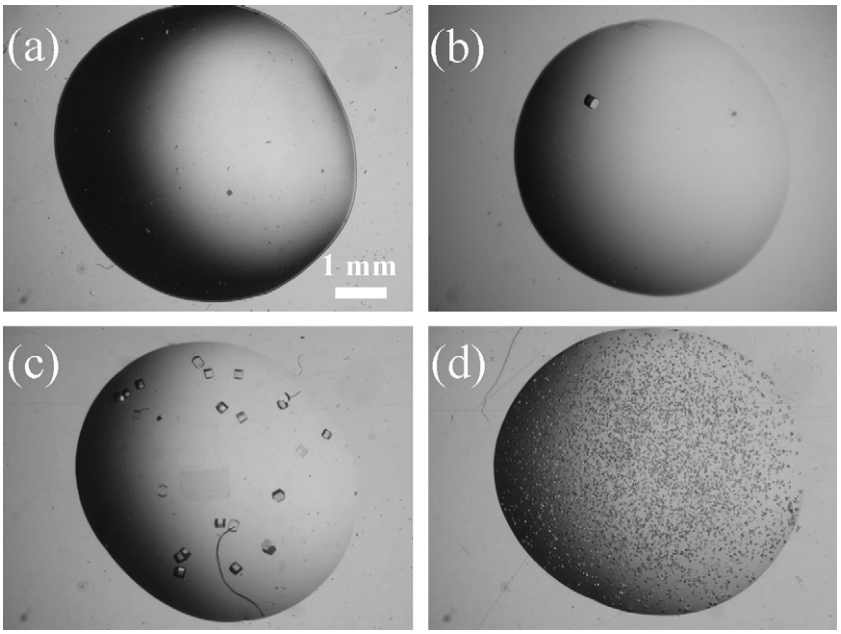


Fig. 6. Photographs of droplets: (a) without gold nanostructures and without irradiation (control experiment), (b) with irradiation, (c) without irradiation and with nanostructures and (d) with irradiation and with gold nanostructures.

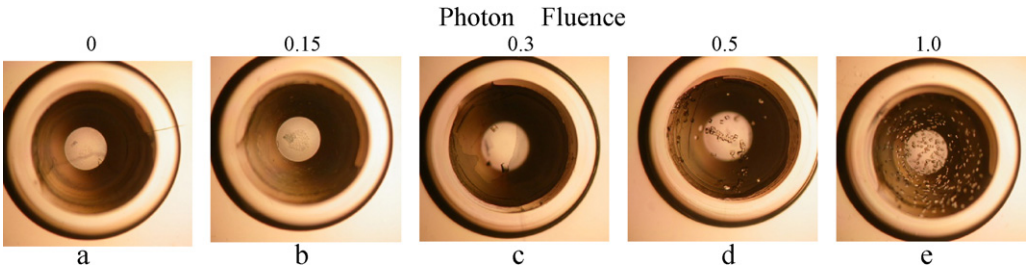


Fig. 7. Photographs of lysozyme solution observed at 24 h after the irradiation with increasing in the excitation fluence. The number of crystal suddenly increased at 0.5 of mother fluence.

result demonstrates that excitation of lysozyme with gold nanostructures induced crystallization.

To probe that light-induced crystallization with strong photons–molecules coupling with visible light excitation is multiphoton process or not, excitation light fluence dependence experiments were carried out. For this purpose, we prepared simplified nanostructure by gold nanoparticles to carry out a lot of experiments simultaneously. 10 μL of solution contains gold nano colloidal particle whose diameter is 40 nm was dropped on a bottom of the well. After drying the solution, irregular gold nanostructure appeared which showed absorption as same wavelength region as aligned gold nanostructure shown in Fig. 2. The excitation light fluence was varied by ND filter from 0, 0.1, 0.15, 0.3, 0.5 and 1.0 against the fluence without using ND filter. Fig. 7 shows the pictures of protein solution against the light fluence. Without irradiation (a) no crystal observed. With increasing in the photon fluence, the number of crystals suddenly increased at 0.5 (d). Without ND filter (e) more than 200 crystals appeared. Logarithmic plot of the number of crystals plotted against the light fluence is shown in Fig. 8. The slope is 2.9 that indicate light-induced crystallization occurred through 3 photons process.

Finally, we discuss photo-thermal effect to temperature increase which induces protein denaturation or dimerization. We calculated photon energy absorbed by gold nano structure. The energy was determined from radiation spectrum of Xe lamp and absorption spectrum of gold nano structure. The energy was 1×10^{-7} W m^{-2}. As the irradiation time was 2×10^{3} s, and the area of gold nano structure was 1×10^{-7} m^{2}, the absorbed energy was determined as 2×10^{-11} J. The volume of the protein solution was 10 μL. The temperature rise of the entire solution is estimated to be 2×10^{-5} K at most. This small value can be negligible.

Consideration of temperature rise in the close vicinity of gold nano structure is also important. We, then, calculated the temperature rise of single island of gold nano structure. The energy of a photon is equivalent to the energy which raises temperature 1×10^{-4} K for single gold nano island. Average interval of incident

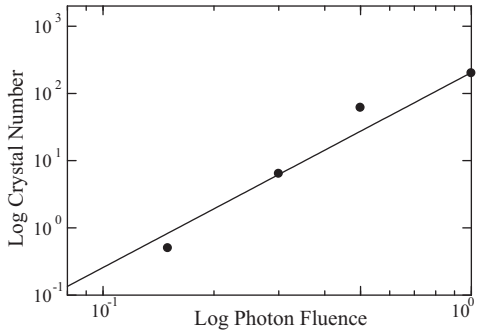


Fig. 8. Logarithm plot of the number of crystal against irradiation fluence. The slope shows 2.9 that indicate 3 photons process for photochemically induced crystallization of protein.

photon is 1×10^{-8} s, which means that 1×10^{-4} s is required to increase 1 K if photon energy is accumulated in a gold island. The heat of the gold island transfers to solvent with some time constant. Though we do not estimate the relaxation time constant from gold island to solvent, this 10^{-4} s is thought to enough for relaxation. Solvent temperature in the close of the gold island might rise steadily at few K at most. Therefore, photo-thermal effect is considered to be negligible.

We summarize that strong photons–molecules coupling device induces multiphoton photochemical reaction, which was demonstrated by photochemically induced crystallization of protein.

Acknowledgements

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