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Evaluation of the smallest protein units in a thin film deposited by IR laser

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Abstract
Silk fibroin (SF) thin films were prepared by pulsed IR laser deposition. Size variation of the smallest protein units (SPU) in the films were evaluated by atomic force microscopy. Average SPU size doubled with increasing the background gas pressure by an order of magnitude. The size of SPU was significantly larger when the film was deposited in Ar atmosphere than in He while the pressure was kept constant at 100Pa. We discussed about the growth of SPU in terms of ionization as a consequence of hydrogen bond rupture by laser irradiation, with the aid of infrared spectra. Effects of the background gas were suspected primarily to be the difference in the states of collision with the fragmented protein units immediately after the laser bombardment of the target, within or near the plume zone. The results suggest the possibility of controlling the nano-structures of the protein thin film by optimizing the background gas condition.

1. Introduction
For many purposes of protein deposition including fabrication of bio-tips and implant surface finishing, homogeneous and continuous coating of the protein units is earnestly desired. As for the biologically significant functions of the protein, it is essential to preserve the primary and higher order structures. While most of the protein film deposition is carried out by adsorption from a solution or via a colloidal route, we recently reported that pulsed laser deposition (PLD) by 1064nm IR beam is versatile for the formation of protein thin films, particularly when proteins are stable and water insoluble like silk fibroin, while mostly preserving its secondary structure [1].

The structure of the PLD film of protein is generally heterogeneous, comprising smallest protein units (SPUs) and larger particles such as debris and droplets [1]. The size of SPU varies with various operation conditions. However, we regard SPUs as protein clusters and are not exclusive for a PLD process, since they are also observed in protein films prepared via a colloidal route [2]. It is conveniently discussed that a certain protein is spherical, elliptical or flat [3]. Such a description is generally based on the molecular structure, without one-to-one correspondence with direct observation. It is also of importance that the change in the shape of the protein units is associated with many different factors including protein – substrate interaction [4,5]. The objective of the present paper is to elucidate the properties of the structural units in the protein thin films to provide answers to the question, how the size of SPU varies by the species and pressure of the background gas.
2. Experimental
Silk fibroin (SF) thin films were deposited by PLD with an Nd:YAG laser (1064nm) by using Nara Laser Ablation System (Nara Machinery). We used hot-pressed refined cocoon powder from *Bombyx mori* (Idemitsu Petrochemical Inc. Protein Powder, average particle size 7.9µm) as a target, without eliminating sericin. Background gases used were He and Ar at pressures 100Pa or 1000Pa. The distance between the target and the substrate was kept constant at 20mm. All the PLD processing was carried out at the constant fluence, 2J/cm², pulse width 5ns, pulse frequency 10Hz and at room temperature, without heating or cooling the target or the substrate.

Morphological analyses were made by atomic force microscopy (AFM, Shimadzu, SPM-9500J3 and Vecco, Nanoscope IV). Reflection-absorption Fourier transform infrared spectra (RAS FT-IR) were obtained from the SF film on Au-sputtered Si substrate by using a spectrometer, equipped with a grazing angle accessory (Jasco, FT/IR-6200).

3. Results
When we observe the SPU size as a function of deposition time or number of laser shots under the fixed fluence (2J/cm²) and background gas condition (100Pa He), the AFM unit size does not change significantly, *i.e.* between 10nm and 13nm, as shown in Figure 1. Another AFM morphological value, the root-mean-square roughness, $R_{\text{rms}}$, remained below 1nm. In contrast, change in the size distribution by the background gas conditions is quite significant. As shown in Figures 2 and 3, increasing the gas pressure from 100Pa to 1000Pa increases the unit size. The gas species also changes the size of the units. Numerical values obtained from the automatic image analysis are given in Table 1. Increase in the pressure of He by an order of magnitude brought about an increase of the average unit size by a factor more than 2. By replacing background gas He with Ar, while keeping the pressure unchanged at 100Pa, the average unit size increases by a factor two.

We further examined the effect of the background gas species by RAS FT-IR for the SF thin films deposited on the Au-sputtered substrate. We observe in Figure 4 that the relative contribution of random coil at around 1650cm⁻¹ becomes larger than that of β-sheet at around 1620cm⁻¹, when we replace He with Ar.
4. Discussion

Growth of the structural units during PLD with increasing the background gas pressure, and hence with increasing number of gaseous molecules is generally explained by collision frequency and associated momentum exchange with the background gaseous molecules [6]. However, we also have to account for the specific properties of the protein species we deal with.

The surface of the protein is rich in NH and C=O groups, capable of forming H-bonds with neighboring species. Thus, the protein unit size is discussed in terms of the interaction with other protein molecules or the substrate [7-11].

As shown in Figure 5, the state of plume is quite different depending on the background gas condition. Increase in the pressure increases the volume of the plume zone (Figures 5(a) and 5(b)). It is also to be noted, that the plume is much larger with Ar than He when compared at the same pressure (Figures 5(b) and 5(c)). We note that the first ionization potential of He is 24.59 eV, and that of Ar, 15.76 eV. Both are by far larger than that of the photon energy of IR laser, i.e. 1.17 eV of the 1064nm beam. Direct ionization of the background gas is, therefore, to be excluded.

On the other hand, H-bond energy is well below the laser photon energy. When H-bond rupture takes place, a part of intramolecular H-bonds can change to intermolecular H-bonds to enhance growth of SPU. We do not exclude the possibility of role of coexisting sericin, whose ability of binding intrinsic fibroin units might be enhanced by partial disruption of its own H-bonds by laser beam.

Possible destruction of protein units is extensively discussed in the interests of MALDI ToF MS [12-14]. Zehl et al [15] showed with their sophisticated laser optical setup, that with increasing laser fluence, the protein intact assembly is gradually replaced by non-assembled subunits with some apparent threshold. They found that disruption of non-covalent bonds takes place very rapidly at around the threshold fluence.

The apparent change of the plume size shown in Figure 5 and eventually the plume color, between He and Ar may thus be associated with the difference in the formation of ionized fragments of fibroin in the plume. We observed that the number of

| Background gas | Threshold height |
|----------------|------------------|
| He 1000 Pa     | 18.0             |
| He 100 Pa      | 9.7              |
| Ar 100 Pa      | 19.1             |

Figure 3 Particle size distribution obtained from AFM images at threshold height, 0.5 nm.

Figure 4 RAS FT-IR Amide I bands of silk fibroin thin film deposited for 0.5 min at 2 J/cm² under varying background gas conditions (a) He, 100 Pa, (b) Ar 100 Pa

Figure 5 Observation of plumes during PLD operation. (a) He, 1000 Pa; (b) He, 100 Pa; (c) Ar 100 Pa
the ionic species increases with increasing the number and mass of gaseous species. At least phenomenologically, growth of the SPU is enhanced with an increasing amount of the ionized fragments indirectly estimated from the size of the plume. Since the ionization energy of the background gases used in this study is orders of magnitude larger than the photon energy of the laser, it is more likely that the effects of background gas is predominantly associated with their physical properties.

5. Conclusion

The average size of the nanostructural units, SPU, of the silk fibroin PLD film doubled by increasing the background gas pressure by an order of magnitude, i.e. from 100Pa to 1000Pa. The size of SPU was significantly larger when the film was deposited in Ar atmosphere than in He while the pressure was kept constant at 100Pa. We discussed about the growth of SPU in terms of ionization as a consequence of hydrogen bond rupture by laser irradiation, with the aid of infrared spectra. Effects of the background gas were suspected primarily to be the difference in the states of collision with the fragmented protein units immediately after the laser bombardment of the target, within or near the plume zone. The present structural studies suggest the unique feasibility of designing microstructures of the fibroin thin films deposited by PLD by controlling the background gas condition.

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