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Pulsed laser deposition of collagen and keratin

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Abstract

Pulsed laser deposition of collagen and keratin, which are representative fibrous proteins, were investigated in terms of laser wavelength and fluence. Chemical structure and surface morphology of thin deposited films were analyzed by means of infrared (IR) spectroscopy and atomic force microscopy (AFM), respectively. For the couple of proteins, IR spectra of the films deposited under some irradiation conditions were almost similar to those of starting targets. This means that a large part of primary structure of the proteins was maintained in the thin deposited films. Also the secondary structures in the thin films were discussed briefly. The random-coil domains increased both for collagen and keratin. Thus we have demonstrated that laser lights are capable of preparing thin films of proteins.

Keywords: Pulsed laser deposition; Collagen and keratin; AFM

1. Introduction

Interests in biomaterials have increased steadily with implication in the field of biomimetics and bioelectronics. Proteins are major materials and have attracted much attention in terms of their various functions. Indeed, novel optical/electrical devices using thin films of proteins have been proposed [1,2]. To extend the applicability of proteins, an excellent as well as convenient tool of processing for proteins seems necessary, and laser is a candidate with high potentials. In the field of inorganic/organic functional materials, lasers have been used in a variety of ways: microfabrication, etching, cleaning, particle formation, thin film deposition, and so on [3].

In the past decade, several studies on interactions between intense laser lights and protein systems have been carried out. Elam and Levy [4] investigated chemical components of ablation plume ejected from a target of a model compound (Ala–Gly) by means of time-of-flight spectroscopy. Fuhr et al. [5] revealed that an integument of an insect (chitin, a glycoprotein in vivo) is accessible to clean-defined etching with KrF laser. Dlott and co-workers [6] developed a novel spectroscopic technique using ablation-shock-wave to detect a structural changes of protein. Ishii and co-workers [7] formed microscopic periodic pattern on collagen films by ArF laser irradiation. It is noteworthy here that laser keratectomy (cornea reprofiling and sculpting) using excimer laser has been widely spread in medical area, and a great part of cornea consists of hydrogel of collagen. In addition to these works, recently we demonstrated that the technique of pulsed laser deposition (PLD), which has various advantages to prepare thin films, is applicable to the formation of thin films of a protein [8]. The target protein was fibroin which is the main component comprising silk and is expected as functional materials. Thus, it would be interesting to apply laser processing to protein systems.

In the present study, to extend the applicability of laser processing to proteins, we investigated PLD of collagen and keratin. In the previous study, we succeeded in the deposition of thin fibroin films [8]. However, the primary structure of fibroin is quite simple (Gly–Ala–Gly–Ala–Gly–Ser), hence it is somewhat difficult to predict whether PLD is applicable or not to other protein systems with complicated structures. Collagen is a main component of biological tissue and has various functions such as excellent fitness to biological tissue. Keratin forms our hairs and nails. Both of the proteins are expected for the applications and have complicated primary structure compared to fibroin. For example, the basic unit of collagen can be roughly described as (Gly–X–Y). Here, X and Y correspond to proline and hydroxyproline, respectively, whose chemical structure is shown in Fig. 1(a).

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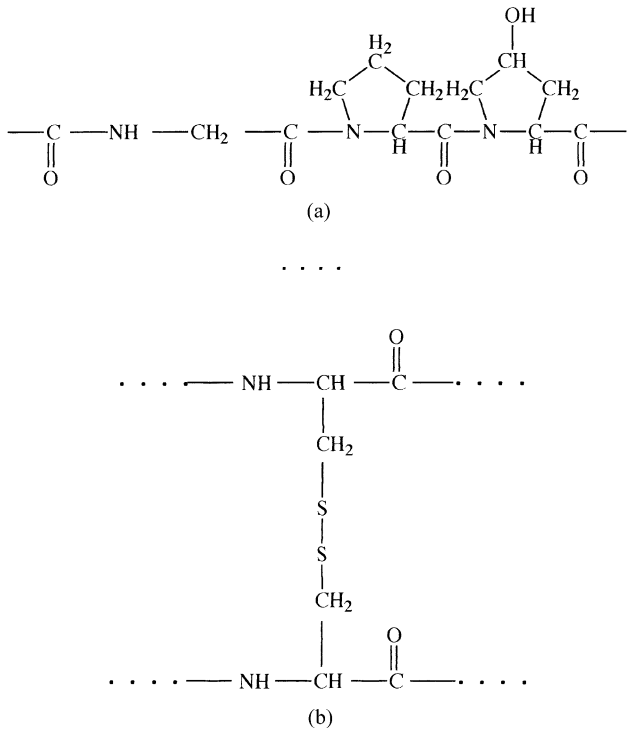

Fig. 1. Chemical structures of: (a) the main basic unit of collagen; glycine–proline–hydroxyproline; (b) the crosslinking part due to cystine in keratin.

The content of these three amino acids to other amino acids involved in the collagen (Bovine Tendon, Type I collagen) is: 30% (glycine), 10% (proline), and 10% (hydroxyproline). The chemical structure of keratin has not completely been understood yet since the sequence of amino acids in keratin is somewhat irregular and various amino acids (about 20 kinds) are contained. The structure of keratin is characterized by the crosslinking due to the cystine bond (–S–S–) as illustrated in Fig. 1(b). We investigated chemical structures and surface morphology of thin deposited films using infrared (IR) spectroscopy and atomic force microscopy (AFM), respectively.

2. Experimental

Commercially supplied collagen from Bovine Achilles Tendon (Tokyo Kasei) and keratin (Tokyo Kasei) were used as received. These proteins were pressed at 8 t/cm^2 to give tablets which were used as ablation targets. The ablation targets were fixed on a rotary stage in a vacuum chamber ($\sim 10^{-3}$ mmHg) with quartz windows. We adopted a couple of pulsed laser systems to induce ablation. Excimer lasers (Lambda Physik EMG 101 for 248 and 351 nm, full width at half maximum (FWHM) ~20 ns, and Lumonics EX500 for 308 nm, FWHM ~15 ns,) and Nd^{3+}:YAG laser (Spectra Physics, PRO-250-30 for 266, 355, and 1064 nm, FWHM ~8 ns,) were used as excitation light source. Repetition rates of irradiation of the excimer lasers and the YAG laser were 1–3 and 30 Hz, respectively. Ablation plume was deposited on ZnSe, KBr, or quartz substrates at room temperature, and the distance between the targets and the substrates was 20 mm. A heat treatment of a sample was done under a vacuum.

Fourier-transform infrared (FT-IR) spectra were measured for films deposited on ZnSe or KBr substrates with Firis-100 (Fuji Electric) or JEOL2000 (Nihon Denshi) spectrometer. An AFM (Digital Instruments, Nanoscope IIIa) was used in the tapping mode for films deposited on quartz substrates.

3. Results and discussion

3.1. Collagen

We initially examined PLD of collagen at various laser wavelengths (1064, 355, and 266 nm). Laser fluences were 60 mJ/cm^2 at 266 nm, 260 mJ/cm^2 at 355 nm, and 1500 mJ/cm^2 at 1064 nm. All these fluences were slightly above ablation thresholds for each laser wavelength. Deposition at these wavelengths gave transparent and colorless thin films with rainbow-type interference patterns. Fig. 2 shows the FT-IR spectra of target collagen and thin deposited films at the three laser wavelengths. The spectrum of target collagen has characteristic absorption peaks peculiar to proteins [9]. The amido I around 1650 cm^{-1}, amido II around 1540 cm^{-1}, and amido III band around 1240 cm^{-1} are observable, and no other bands are observed in a wide wavenumber region of 2800–1800 cm^{-1}. Amido I, II, and III bands are mainly ascribable to the modes of C=O stretching, N–H bending, and C–N stretching, respectively. The broad band around 3400 cm^{-1}, amido A, is due to the Fermi resonance of N–H stretching and the overtone of amido II. The other broad band around 700 cm^{-1}, amido V, is due the mode of N–H bending. Sharp bands around 2900 cm^{-1} are ascribable to the mode of C–H stretching in proline and hydroxyproline. A sharp band centered at 1740 cm^{-1} cannot be assigned to polypeptide, and is probably due to C=O stretching mode of fatty acids involved in the Tendon tissue, since we use the Tendon sample without purification of elimination of the acids.

The spectrum of the film deposited at 1064 nm deviates from the spectrum of the target collagen. The amido II and V bands, both of them are mainly due to N–H bending, almost disappear in the spectrum of the thin deposited film. Instead, a new band around 1100 cm^{-1} become conspicuous. A tentative assignment of this band is due to C–N stretching in amine. These results indicate that chemical structure of collagen was destroyed with a great degree in the PLD process at 1064 nm. A high fluence (~1500 mJ/cm^2) was necessary to induce ablation at this wavelength compared to other two laser wavelengths. At 1064 nm light whose photon energy is relatively low, ablation due to simultaneous multi-photonic electronic excitation would hardly take place. Presumably,

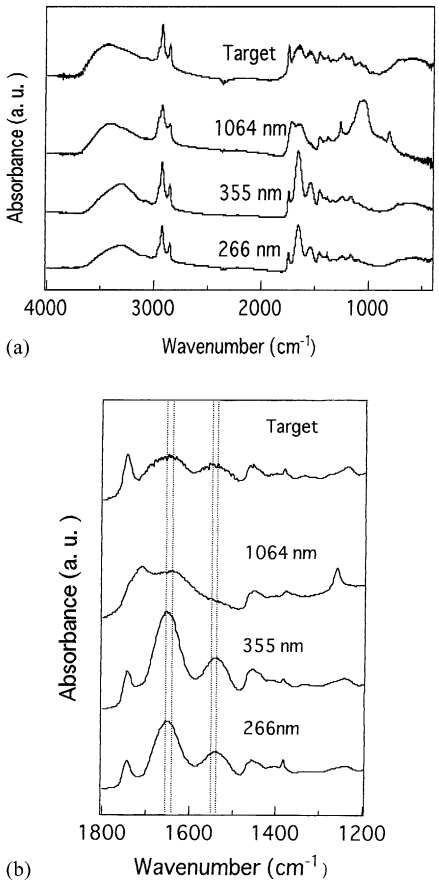

Fig. 2. (a) FT-IR spectra of target collagen and thin films deposited at 1064, 355 and 266 nm. Laser wavelength is given in the figure. (b) Expanded spectra of (a) in a lower wavenumber region. Dotted lines indicate absorption frequency peculiar to the helical structure (1640 and 1550 cm^{-1}) and to random-coil structure (1655 and 1540 cm^{-1}).

ablation is considered to be brought about a plasma generation by 1064 nm irradiation, for we observed a visible white light emission at the irradiation spot of the target. Quite high temperature in plasma destroyed the chemical structure of target collagen.

On the other hand, the spectra of thin films deposited at 355 and 266 nm have the absorption band of collagen, although the relative intensity among the peaks changes from the target to the thin films. For the band of amido A around 3400 cm^{-1}, the peak position in thin deposited films slightly shift to lower wavenumber side, although this shift is not observed for the thin film deposited at 1064 nm. The origin of this shift is not clear, since vibrational mode responsible for this band (N–H vibration) is well recognized in the spectra of the films as described below. In Fig. 2(b), expanded spectra in a lower wavenumber region (1800–1200 cm^{-1}) are shown. A one-to-one correspondence is detectable between absorption peaks in the spectra. In addition to these amido bands, several slight peaks are well reproducible in the spectra of thin deposited films. These results strongly suggest that the primary structure are maintained in PLD processes at 266 and 355 nm. However, the amido I band at 1650 cm^{-1} is sharpened and intensified and the peak at 1740 cm^{-1} is relatively decreased in the spectra of thin films deposited at 266 and 355 nm. Here, we consider the chemical structure of thin deposited films in details on the basis of these results. The basic primary structure of collagen is Gly–X–Y; X is proline and Y is hydroxyproline as illustrated in Fig. 1. The apparent feature that distinguishes collagen from other proteins is that the amido bond in collagen is partly involved in the five-membered ring. It is well known that the peak position of C=O stretching of amido bond (amide I) in IR spectroscopy is affected by the structure of amido bond. The peak position of C=O stretching band in lactam-type five-membered rings is shifted to higher wavenumber. The amido I band of target collagen is somewhat broad compared to that of other proteins. This broadening would be ascribable to the superposition of amido I bands with and without the five-membered rings. The sharpening of the amido I band observed for thin deposited films suggest that the five-membered rings were partly broken into primary amido bonds in PLD processes at 355 and 266 nm. The decrease of the sharp band at 1740 cm^{-1} is interpreted as that the fatty acids were not deposited on substrates or in films due to their low molecular weights. According to our previous studies, a chemical specie (anthracene) with low molecular weight involved in targets was not deposited in a thin film because of its volatile character [10].

Next, the secondary structure in thin deposited films is discussed briefly on the basis of amido I–III bands. It is well established that the IR frequencies of these amido bands are quite sensitive to the secondary structure of proteins [9,11]. Collagen proteins are characterized as their triple helix structures. The helical structure has the absorption maximum at 1640 and 1550 cm^{-1} for amido I and II bands, respectively, while random-coil structure has 1655 and 1540 cm^{-1} for each amido band [12,13]. The amido bands of target collagen are broad and do not exhibit apparent peak maximum as seen in Fig. 2(b). We, therefore, deduce that the secondary structure of target collagen is the mixed type of random-coil type and helical structure. It is noteworthy here that collagen in a dried KBr matrix takes random-coil structure in a large part [12]. In Fig. 2(b), the frequencies peculiar to these secondary structures are noted with dotted lines, the peak positions of amido I and II bands of thin films deposited at 355/266 nm are almost similar to the positions of target collagen. However, peaks due to random-coil type become somewhat prevail. We, therefore, conclude that the content of random-coil structure increased in thin deposited films. That is, the denaturation from collagen to gelatin is involved in PLD processes.

The above results are considered in terms of ablation mechanisms. In addition to the plasma formation, ablation mechanisms can be classified into a couple of types: photothermal and photochemical mechanisms. The photothermal mechanism would be dominant for the present case as has been reported for a variety of organic polymers.

Indeed, Ishii and co-workers [7] reported that photothermal denaturation of collagen into gelatin is the origin of the microstructure formation even at 193 nm. The deviation of structures of thin deposited films from that of target collagen may be ascribed to photothermal processes. To investigate thermal effects, we carried out a heat treatment of target collagen up to 520 K which is sufficient higher than the denaturation temperature of collagen. The spectral shapes of amido I and II bands do not change dramatically and quite slight peak shifts in amido II band suggesting the increment of the domain of random-coil structure became appreciable with increasing in temperature. We, therefore, conclude that the destruction of helical structure in PLD processes at 266/355 nm is ascribable to the photothermal processes. However, the spectral sharpening and enhancement of the amido I band seen in the thin deposited films is not detected even at 520 K at which thermal decomposition could take place. Hence, we deduce that the spectral changes in the amido I band in thin deposited films cannot be ascribed only to a photothermal process. Photochemical decomposition of the five-membered rings is suggested. Amino-acids with π-electronic systems is almost not contained in Type I collagen. Hence, collagen has no significant absorption in the wavelength region longer than ∼250 nm. Therefore, the present ablation at 266/355 nm might be brought about by simultaneous two- or multi-photon absorption. Although the multi-photon absorption induce photothermal ablation, this absorption contribute also to the photochemical decomposition of the five-membered rings due to their high photon energy.

The surface morphology of the thin films deposited at 266/355 nm is briefly described. Representative AFM images of the films are shown in Fig. 3. In both the surfaces, grain-like morphology is observed, and less than 1 μm roughness in height is seen in the horizontal areas of 10 μm × 10 μm. Although the size of grains seems to slightly become smaller as the laser wavelength shifted from 355 to 266 nm, the two surface images resemble each other. On the basis of results obtained by IR spectroscopy and AFM, we consider that the fundamental mechanism of laser ablation of collagen at 266 and 355 nm is almost similar. The correlation between the surface morphology and deposition mechanism again is mentioned in the next part.

3.2. Keratin

Laser fluence was fixed to 1000 mJ/cm^2 for PLD of keratin, which gave transparent and colorless thin films with rainbow type interference patterns. Fig. 4 shows the FT-IR spectra of the thin films deposited at 351, 308, and 248 nm, together with the spectrum of target keratin. Characteristic absorption peaks due to polypeptide, amido A, I–III bands, and several small peaks are detectable for the spectra of the thin deposited films. These results suggest that thin films whose chemical structure is close to that of keratin were deposited at these three laser wavelengths. Differences

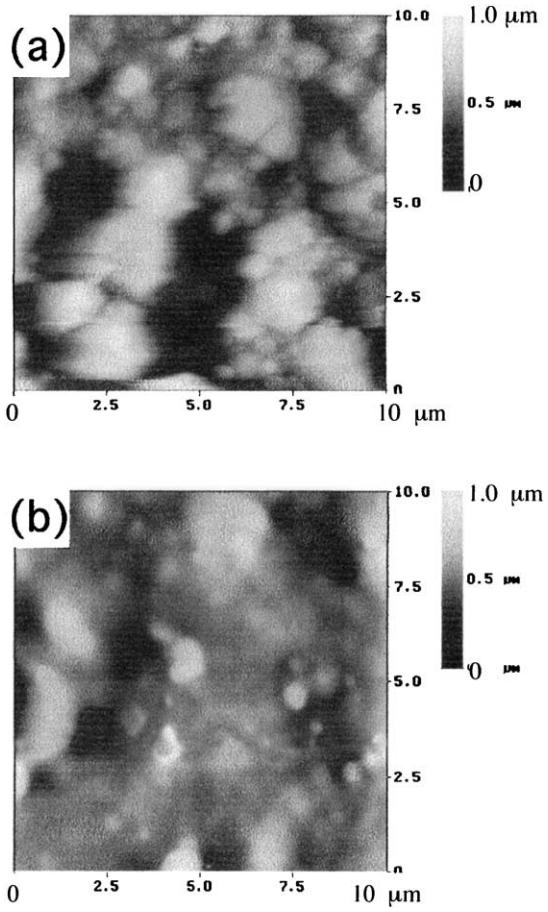

Fig. 3. AFM images of thin collagen film deposited at: (a) 355; (b) 266 nm.

between the spectra of target keratin and these thin deposited films are as follows. For the thin films, the amido A bands at 3300 cm^{-1} are somewhat sharpened, amido II bands at 1530 cm^{-1} is decreased, and amido V bands around 500–800 cm^{-1} become sharp. Since these bands are due to N–H vibration, these results suggest that this chemical bond undergoes chemical reactions through PLD processes.

Expanded spectra of these amido bands are also shown in Fig. 4(b) to discuss secondary structure in the thin films. The main secondary structure of keratin is known to be of β-sheet type in which peptide chains are connected with each other by hydrogen bonds of ≻C=O· · · HN– type. In the spectra, absorption frequencies due to β-sheet and random-coil structures are noted using solid and broken lines, respectively, on the basis of a literature [9]. For target keratin, the peak position of amido III band agrees with that of β-sheet structure. Characteristics of β-sheet structure are detectable also for the amido I and II bands. These results indicate that the target keratin consists mainly of the β-sheet type, including a small amount of the random-coil type. For the thin deposited films, the contribution of the β-sheet type decreases and the spectral shapes of amido I and II bands are sharpened. In addition, peaks due to random-coil become prevail relatively for all the amido bands. We, therefore, deduce that

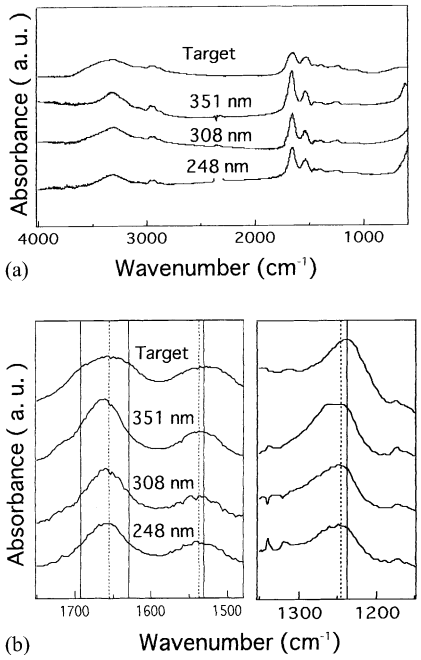

Fig. 4. (a) FT-IR spectra of target keratin and thin films deposited at 351, 308, and 248 nm. Laser fluence is given in the figure. (b) Expanded spectra of (a) in a lower wavenumber region. Solid and broken lines indicate absorption frequency peculiar to the β-sheet structure and the random-coil structure, respectively.

β-sheet structure in the target keratin is destroyed through PLD processes. The destruction of β-sheet structures was also suggested in our previous work for PLD of fibroin [8]. Keratin does not have strong absorption in the longer wavelength than ∼300 nm and laser fluence used here was a high value of 1000 mJ/cm^2. Hence, simultaneous multi-photon absorption should be involved in ablation processes and this multi-photon absorption at peptide bonds would result in the scission of hydrogen bonds to destroy the β-sheet structure. This effect has been suggested also for the PLD of fibroin [8].

The surface morphology of the thin films are displayed in Fig. 5. Grain-like morphology were seen in the AFM images, and the size of grain becomes smaller as the laser wavelength get shorter. This wavelength dependence was slightly found for the PLD of collagen as described above. In addition, this dependence has been reported for PLD of poly(*N*-vinylcarbazole) and fibroin [8,14]. Thin films of poly(*N*-vinylcarbazole) was formed by re-polymerization of monomers (*N*-vinylcarbazole) ejected by ablation, while thin films of fibroin was formed by deposition of ablation fragments. The monomer-re-polymerization mechanism apparently does not hold for collagen, keratin, and fibroin, since the proteins is generated by condensation polymerization. We consider that oligomers are ejected in ablation with subsequent to polymerization in plume or on a substrates to form thin films. This wavelength dependence would be common to PLD of organic materials regardless of deposition mechanisms. The grains seen in the AFM images do not arise from bulk debris directly transferred from targets. They likely correspond to molecular aggregates formed by the polymerization of monomers and oligomers.

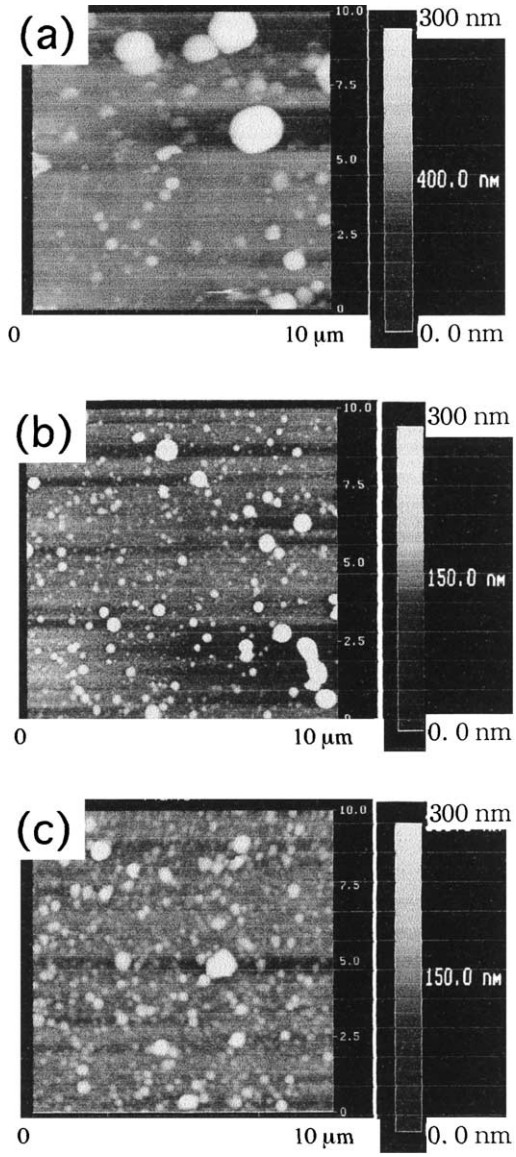

Fig. 5. AFM images of thin keratin film deposited at: (a) 351; (b) 308; (c) 248 nm.

4. Summary

We investigated PLD of collagen and keratin which are representative proteins. FT-IR spectra of thin films have features peculiar to polypeptides, and were almost similar to those of corresponding proteins, although some decomposition were partly suggested. For the secondary structure, random-coil domains increased in the thin deposited films. In order to maintain the original secondary structure (helix and β-sheet) in thin films, photosensitized-PLD which we

have proposed recently would be effective. The surface morphology depended upon laser wavelength. For both of the proteins, thin films were formed by the mechanism of direct deposition of ablation debris and fragments, and the grains seen on the surface would correspond to the debris or aggregates of the fragments. Thus we have demonstrated that laser lights are capable of preparing thin films of proteins.

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