# Irradiation Effect of Infrared Free Electron Laser on Dissociation of Keratin Aggregate

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#### Abstract

A mid-infrared free electron laser (FEL) is a linearly polarized, high-peak powered pulse laser with tunable wavelength within mid-infrared absorption region. We previously found that the FELs tuned to amide bands (I, C=O stretching vibration; II, N-H bending vibration; III, C-N stretching vibration) were effective for dissociation of pathogenic amyloid fibrils into the non-pathogenic monomer form. In this study, we tested the irradiation effect of FEL on keratin aggregate as another model for demonstrating an applicability of the FEL for dissociation of protein aggregates. Both microscopic analyses using scanning-electron microscopy and synchrotron-radiation infrared microscopy showed that the  $\alpha$ -helix-rich aggregate structure was converted into many fragments of  $\beta$ -sheet-dominant monomer form after the FEL irradiation tuned to 6.06 µm (amide I), 6.51 µm (amide II), and 8.06 µm (amide III), while the aggregated solid was largely remained after irradiation at non-absorbance region (5.6 µm). These results evidently indicated that the FEL irradiation targeting amide bands was effective for dissociation of keratin aggregate as well as amyloid fibrils, and we can suggest that the FEL irradiation may be generally applied for dissociation of aggregate structures of proteins.

## 1. Introduction

A mid-infrared free-electron laser (FEL) employs synchrotron radiation as a light source for lasing and provides with a specific pulse profile with complete linear polarization, frequency tunability within infrared region, and high photon density [1]. These characteristics enable us to excite the vibrational states resonantly and selectively and to induce the infrared multi-photon process in the molecules. The FEL has been used for spectroscopic studies of molecules, thermodynamic analyses of biomolecules, and surgical ablation of biomedical tissues [2, 3]. In particular, laser ablation process is effective in removal of tumor tissues in laser-mediated therapeutic strategy, under which the tissue absorbs many photons of laser beam and more than 60% of water is vaporized from the internal space of tissue [4]. These studies imply that the FEL irradiation can induce major conformational changes of peptides, and we have recently found that the FEL tuned to amide bands (amide I: C=O stretching, amide II: N-H bending, and amide III: C-N stretching vibration modes) can promote dissociation of amyloid fibrils of lysozyme and insulin peptide into their monomer structures [5, 6]. Protein aggregates such as amyloid fibrils cause several neurodegenerative diseases in mammals. Although disaggregation of these aggregates is expected to lead to amelioration of pathologies, the aggregated structures are commonly robust under physiological conditions. For the mechanism of dissociation of amyloid fibrils induced by the FEL, it can be proposed that non-covalent bonds such as hydrogen bonds between  $\beta$ -sheets are cleaved by absorption of energy of the FEL into the amide bonds.

For the purpose of demonstrating general usefulness of the FEL for dissociation of protein aggregates, we targeted the keratin aggregate for the FEL irradiation experiment in this study. Keratin is a major structural component of cytoskeleton in both hair and skin tissues, and it tends to aggregate during aging, which in many cases causes hair damage and several skin diseases [7]. It is not easy to disaggregate the keratin polymer as well as amyloid fibrils unless detergents are used which are often toxic for human body. While amyloid peptide is small in molecular size, 1 - 10 kDa, in general, keratin is a comparatively large protein, approximately 50 kDa [8]. We tested the FEL for dissociation of such a large protein aggregate and found that keratin aggregate could be dissociated into the disaggregated form by the similar irradiation method with the case of amyloid fibrils.

# 2. Experimental

## Materials

The reagents were purchased as special-grade chemicals. Dimethyl sulfoxide (DMSO) and phosphate buffered saline (PBS; 10 mM) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Keratin was purchased from Nacalai Tesque, Inc. (Kyoto, Japan).

## FEL-generation system

The experimental setup was described in details in previous studies [5, 6]. In brief, the FEL is generated by using synchrotron radiation (SR) as a seed with a variable wavelength in the mid-infrared region (5.0–14  $\mu$ m; 714–2,000 cm<sup>-1</sup>). The electron beam was generated by a high-radio-frequency (RF) electron gun (2,856 MHz), and injected into an undulator through an  $\alpha$ -magnet and a linear accelerator. SR is amplified between a pair of mirrors positioned at both sides of the undulator through the interaction with the electron beam, producing coherent laser light (FEL). The wavelength of FEL can be tunable by adjusting the space interval of the undulator. Time structure of the FEL is composed of macro- and micro-pulses. The half width of the micro-pulse is 1 to 2 pico-second (ps) and the interval of the consecutive micro-pulses is 350 ps. A pulse train of the micro-pulse form single macro-pulse which has a duration of 2 micro-second (us). A repetition rate of macro-pulse is 5 Hz during operation. The pulse energy per macro-pulse was in the range of 7.0–9.0 mJ, as measured using an energy meter (SOLO2, Gentec-EO Inc., Quebec, Canada). Prior to the irradiation, the FEL beam was focused almost to a restricted area on the sample under the guidance of a He-Ne beam. The spot size of beam line was ca. 0.5 cm in diameter. Accordingly, the power densities of the FEL were estimated to be  $35 - 45 \text{ mJ/cm}^2$ .

## Preparation of keratin aggregate and FEL irradiation method

Keratin powder was dissolved in DMSO to be 10 mg/mL, and the stock solution was diluted by four times volume of PBS containing 2 M NaCl, and it was incubated at 37  $^{\circ}$ C for 1 day. After the aggregate was collected by centrifugation at 14 k rpm for 10 min, it was washed by water and mixed with water (300 µL) for stock solution. The mol number of

keratin was approximately 0.06  $\mu$ mol in the stock solution calculated if molecular weight was approximately 50 kDa. For measuring FEL transmittance, the air-dried aggregate was mixed with KBr powder, and the mini-circle plate consisting of the keratin aggregate and KBr was prepared. The FELs with various wavelengths were irradiated into the plate as describe above, and power energy of FEL was measured in front and at the back of the plate. For microscopic analyses of the irradiated samples, the aggregated material (30  $\mu$ L) was spotted on the glass slide or stainless-steel base and irradiated by the FEL at 37 °C for 1 h. After the irradiation was completed, the sample was air-dried and subjected to the microscopic analyses as described below.

## Scanning electron microscopy (SEM) analysis

Morphologies of keratin aggregates were analyzed using an FE-SEM Supra40 scanning electron microscope (Carl Zeiss). After generating keratin aggregates as described above, the dried material on the glass slide was fixed on a sample holder by using conductive copper tape and injected into the vacuum chamber. The acceleration voltage was set to 5.0 kV and the observation was performed at an oblique position (SE2 mode).

## SR-IRM analysis

The synchrotron-radiation infrared microscopy (SR-IRM) analysis was performed using the IR micro-spectroscopy beam line (SRMS, BL-15) at the SR center of Ritsumeikan University (Kusatsu, Shiga, Japan) as shown in a previous study [9]. The beam line is equipped with Nicolet 6700 and Continuµm XL IR microscopes (Thermo Fisher Scientific Inc.). Measurements were performed in reflection mode with a 32× Cassegrain lens and a 20 µm × 20 µm aperture. Spectra were collected in the mid-IR range of 700–4000 cm<sup>-1</sup> at a resolution of 4 cm<sup>-1</sup> with 64 scans. Smoothing and normalization of spectra were performed on the region containing amide bands (1000-2000 cm<sup>-1</sup>) by using Spectra Manager software Ver. 2 (Jasco International Co., Ltd., Tokyo, Japan). Secondary structure contents were estimated by measuring peak intensity around amide I band by using the attached protein analysis software (IR-SSE; JASCO Co., Ltd.), which was developed in the company for evaluation of protein conformational changes in carcinoma tissue [10].



**Fig. 1** FEL transmittance of the keratin aggregate. The bar represents the estimated error over three measurements. Diamond: KBr only (background), square: KBr + keratin aggregate, triangle: the subtraction (= FEL absorptance).

## 3. Results and Discussion

At first, we measured FEL transmittance of keratin aggregate at 5.0, 5.6, 6.06, 6.51, 7.0, 8.06, and 9.0  $\mu$ m (Fig. 1). As compared to the background transmittance (diamond), the minimum transmittance of keratin aggregate (square) was observed at 6.0  $\mu$ m (amide I) and 6.51  $\mu$ m (amide II), and the medium value was observed at 8.0  $\mu$ m (amide III), while almost FEL energy was transmitted at below 5.6  $\mu$ m. Therefore, near half of the FEL energy was absorbed in the keratin aggregate at amide I and II bands, and little of the FEL was absorbed at 5-5.6  $\mu$ m, which is also appeared in the subtraction (triangle). We next examined how the energy absorption of FEL affected the keratin aggregate structure by using microscopy techniques.

Before aggregation, many particles of keratin monomer with approximately one hundred nanometers in diameter were observed (Fig. 2a). After aggregation, these particles were converted to a large-sized solid, which may be an aggregated structure of keratin (Fig. 2b). The irradiation of FEL at 6.06, 6.51, and 8.06  $\mu$ m promoted dispersion of the solid into many fragments with small particles (Fig. 2c, d, e, respectively), while at 5.6  $\mu$ m a large

solid form unchanged (Fig. 2f). In particular, the irradiation at 6.06  $\mu$ m yielded a number of small particles of which morphology seemed to be similar with that of the monomer keratin. These observations showed that the FEL irradiation at amide bands (I, II, and III) could dissociate the keratin aggregate more effectively than that at non-absorbance region.





Fig. 2 Structure of the keratin sample imaged with scanning electron microscopy; keratin monomer (a), keratin aggregate (b), keratin aggregate after the FEL irradiation at 6.06  $\mu$ m (c), at 6.51  $\mu$ m (d), at 8.06  $\mu$ m (e), at 5.60  $\mu$ m (f). Bar: 200 nm.

SR-IRM improves the spatial resolution with a high signal-to-noise (S/N) ratio compared to IRM using a thermal radiation beam because high-power radiation can be delivered to a limited area in a small sample of several micrometers section [11]. Typical SR-IRM spectra for keratin monomer and aggregate were shown in Fig. 3a, and those for keratin aggregates after the FEL irradiation were shown in Fig. 3b. Frequencies of amide I, II, and III bands in each spectrum were listed in Table 1. Amide I bands for the monomer and aggregate were 1631.5 cm<sup>-1</sup> (black solid line, Fig. 3a) and 1645.0 cm<sup>-1</sup> (black dotted line, Fig. 3a), respectively. According to the previous study using model peptides forming  $\alpha$ -helix [12].

Thus, we suspect that the keratin aggregate is rich in  $\alpha$ -helix or non-ordered structures, while the monomer mainly consists of  $\beta$ -sheet structure. After the FEL irradiation at 6.06 μm (amide I; red dotted line), 6.51 μm (amide II; blue dotted line), and 8.06 μm (amide III; green dotted line), the peak of the amide I band of the aggregate shifted to 1635.3, 1633.4, and 1637.2 cm<sup>-1</sup>, respectively (Table 1, Fig. 3b). In other words, at these FEL wavelengths, the amide I band shifted towards that of the monomer. On the other hand, the location of the amide I band was little changed at 5.60 µm (gray dotted line in Fig. 3b). Compared to the remarkable peak shift of the amide I, either amide II or III band seemed to be not much changed. The large peak shift of the amide I band indicates that the aggregate structure was converted into the monomer structure after the FEL irradiation. By using secondary structural analysis software, the secondary structure content for each spectrum was evaluated as shown in Fig. 3c. In the aggregated form, the contents of  $\alpha$ -helix,  $\beta$ -turn, and non-ordered structures were much higher than those in the monomer state. These contents decreased near to those of the monomer after the FEL irradiation at 6.06 µm. A similar reduction was apparent as well at 6.51  $\mu$ m and 8.06  $\mu$ m, while at 5.60  $\mu$ m little change of those secondary structure contents was recognized. It should be emphasized that 6.06 µm was the most efficient wavelength driving the transformation of the aggregate into the monomer form. These results also support the above electron microscopy observation.

In our previous study, we applied the FEL to dissociate amyloid-like fibrils into the monomer form and observed that FEL irradiation markedly affected the  $\beta$ -sheet content of the fibril structure [5, 6]. In the case of lysozyme fibrils, the resonant excitation at amide bands (I, II, and III) by the FEL was considered to be effective for refolding of lysozyme. In the case of keratin, similar dissociation effect by the FEL targeting amide bands was found. Protein aggregate structure, in general, is formed commonly by non-covalent bonds such as hydrogen bonds and ionic bonds between peptide backbones, and the aggregate structure such as amyloid fibrils can be melted by external heating at over 80 °C [13]. Therefore, it can be estimated that the successive resonant excitation at amide bonds by the pulsed laser irradiation can heat the peptide backbone and drive dissociation of the aggregate into the monomer state.



**Fig. 3** Synchrotron infrared microscopy analyses. (a) Reflection spectra before the FEL irradiation. Solid line: keratin monomer, dotted line: keratin aggregate. (b) Reflection spectra after the FEL irradiation. Red dotted line: 6.06  $\mu$ m, blue dotted line: 6.51  $\mu$ m, green dotted line: 8.06  $\mu$ m, gray dotted line: 5.60  $\mu$ m. (c) Secondary structure analyses of keratin monomer, keratin aggregate, keratin aggregate after the FEL irradiation at 5.60, 6.06, 6.51, and 8.06  $\mu$ m. Black bar:  $\alpha$ -helix, white bar:  $\beta$ -sheet, thick gray:  $\beta$ -turn, thin gray: non-ordered structure.

|                                 | Amide I | Amide II | Amide III (cm <sup>-1</sup> ) |
|---------------------------------|---------|----------|-------------------------------|
| Monomer                         | 1631.5  | 1527.3   | 1209.1                        |
| Aggregate                       | 1645.0  | 1535.0   | 1234.2                        |
| FEL irradiation at 5.60 $\mu m$ | 1641.1  | 1535.0   | 1236.1                        |
| FEL irradiation at 6.06 $\mu m$ | 1635.3  | 1531.2   | 1232.2                        |
| FEL irradiation at 6.51 $\mu m$ | 1633.4  | 1529.3   | 1238.1                        |
| FEL irradiation at 8.06 $\mu$ m | 1637.2  | 1531.2   | 1222.6                        |

Table 1 Amide frequencies of keratin samples before and after FEL irradiation

## 4. Conclusions

Dissociation of aggregate structure of keratin could be promoted by mid-IR FEL irradiation tuned to amide bands. The mid-IR FEL is expected to be useful for dissolving the insoluble aggregates of pathological proteins into their active forms.

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