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# Matrix-assisted laser desorption/ionization of protein samples containing a denaturant at high concentration using a mid-infrared free-electron laser (MIR-FEL)

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**Abstract**

A novel matrix-assisted laser desorption/ionization (MALDI) method was developed for insoluble protein analysis. Solubilizing agents, such as 8 M urea, used for preparation of insoluble proteins do not absorb typical desorption lasers and impede transient laser heating in the conventional MALDI process. A mid-infrared free-electron laser (MIR-FEL) can individually activate various chemicals by tuning the MIR-FEL wavelength to selectively excite a vibrational mode. In the present method designated UV/FEL-MALDI, the same position on a sample deposit containing a denaturant at high concentration is exposed to a nitrogen laser pulse which is absorbed by UV-matrix and a MIR-FEL macropulse which is absorbed by denaturant in the same time frame. This scheme lets a denaturant at high concentration to be used for the MALDI sample preparation of insoluble proteins. The simultaneous irradiation of a nitrogen laser and MIR-FEL achieves spatially and temporally defined desorption, which is essential to TOFMS detection, while specificity and selectivity owing to the MIR-FEL wavelength can be conserved. The ability of UV/FEL-MALDI to detect the analyte which is deeply embedded in denaturant was examined using model protein samples dissolved into 8 M urea solution. In comparison to the conventional UV-MALDI, orders-of-magnitude improvement in signal-to-noise ratio was obtained by UV/FEL-MALDI with the MIR-FEL wavelength tuned around the absorption maximum of urea. The intact protein of human hair keratin, which is extremely insoluble and is not amenable to UV-MALDI and any other ionization methods, was subjected to UV/FEL-MALDI-TOFMS analysis. The molecule-related cluster ions of γ-keratin were detected for the first time.


*Keywords:* Matrix-assisted laser desorption/ionization (MALDI); Time-of-flight mass spectrometry (TOFMS); Infrared; Free-electron laser (FEL); Insoluble protein

## 1. Introduction

Matrix-assisted laser desorption/ionization (MALDI) [1] has become one of the most widely used methods for the mass spectrometric analysis of biological macromolecules [2]. The combination of ultraviolet (UV) lasers and a few kinds of regularly used aromatic matrix compounds, which exhibit strong absorption coefficients in UV wavelengths, is a standard MALDI method. The ionization efficiency of each analyte in the MALDI process depends on a microscopic crystal formation of the matrix–analyte system, whose mixing condition is thus important to gain the signal yield. Although MALDI has a higher tolerance for salts, buffers or solubilizing agents in analyte solutions than other soft ionization methods, such as electrospray ionization (ESI), the MALDI efficiency is lowered by those coexisting materials according to their concentrations.

Pursuing the possibilities of MALDI with non-UV wavelengths, particularly infrared (IR), was already found in early 1990s [3,4], and has been driven by both the fundamental and application concerns. The mechanisms underlying the MALDI process has been investigated by varying the desorp-

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tion wavelength [5]. There are many matrix candidates having richly structured infrared absorption bands [3]. IR-MALDI has gained the information about the correlation between matrix properties and MALDI mechanisms, especially the role of absorption coefficients [5–13]. Because the lower matrix absorption and the corresponding large amount of ablated materials result in a better adiabatic cooling in the plume, the IR-MALDI process has shown to produce cooler gaseous ions. This property makes IR-MALDI feasible to analyze labile macromolecular systems [14–19]. For some applications better results are obtained with mid-IR (MIR) rather than UV wavelengths. Several proteins were analyzed with ice as a matrix [20] or directly from membranes after sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) separation and electroblotting [21] or from polyacrylamide gels themselves [22]. The analysis of large oligonucleotides up to several hundred kilodaltons was also demonstrated [23].

Among laser sources available for IR-MALDI, a free-electron laser (FEL) is particularly interesting because of the tunability in a wide IR range for which no substitutional laser is existing. Cramer et al. have shown the potential of IR-MALDI at wavelengths near the C=O stretch vibration (5.7–5.9 μm) by using a FEL [24]. Despite its versatility and unique properties, the dissemination of IR-MALDI using a FEL has been quite limited due to technical and financial reasons. From the technical view point, the temporal pulse structure as below in detail is the most difficult point when a FEL is applied to IR-MALDI.

In recent years, identifying a large set of proteins has been increasingly emphasized as a global challenge of life science in the post-genome era. The key technology of proteomics is a high throughput identification of proteins based on MS and searching a protein database. MALDI combined with time-of-flight mass spectrometry (TOFMS) is a powerful yet robust tool for protein identification, due to its high sensitivity and theoretically unlimited detectable mass range. A large part of functional proteins, such as membrane proteins, are not amenable to the conventional MALDI-TOFMS analysis yet, because they are insoluble as intact forms in a matrix solution unless using a solubilizing agent at high concentration such as denaturants or surfactants. Aiming at overcoming this difficulty, we have developed a novel MALDI method (UV/FEL-MALDI) [25,26]. The FEL wavelength can be tuned to activate a denaturant, which impedes the conventional MALDI process, while analyte molecules are not heated excessively by the laser. This scheme lets a denaturant at high concentration to be used for the MALDI sample preparation of insoluble proteins. A simultaneous use of a FEL with a 337 nm nitrogen pulse laser for MALDI achieves spatially and temporally defined desorption, which is essential to TOFMS detection unless the orthogonal injection is successfully applied to this case, while specificity and selectivity owing to the FEL wavelength can be conserved.

In this paper, we present that UV/FEL-MALDI-TOFMS was successfully applied to analyze protein samples with a denaturant at high concentration (8 M urea). Model measurements using standard soluble proteins were followed by a trial for an insoluble protein to demonstrate the performance of UV/FEL-MALDI. Hard keratin is a primary component of human hair shaft and known as an extremely insoluble protein having a high content of cysteine residues, which form intermolecular disulfide bonds. Although tryptic peptides of keratin have an enough solubility to coexist in peptide maps of almost any sample and are known to be the most common contaminants in proteome analysis, keratin spectra of the intact protein and its oligomers have never recorded before, particularly from a powdery hair rod sample. The intact molecular mass of hair keratin was measured for the first time by means of UV/FEL-MALDI.

## 2. Experimental

### 2.1. Mid-infrared free-electron laser (MIR-FEL)

The MIR-FEL used in this study is a part of the facilities of Graduate School of Engineering, Osaka University. The emission of a FEL is based on the radiation of a relativistic electron beam moving through a field region referred to as either undulator or wiggler, which is an array of magnetic deflectors. The factors controlling the wavelength of a FEL are either the undulator gap and/or the electron beam energy. Because both of them can be varied smoothly, in principle a FEL can continuously covers a range of wavelength. The mid-infrared range (5–22 μm) covered by the MIR-FEL is rich in vibrational absorption bands of organic molecules. Hence, the MIR-FEL is thought to be quite suitable to excite selectively each molecular vibrational mode of any organic compound. FEL pulses have a characteristic temporal structure consisting of macropulse and micropulse trains. The pulse structure of the MIR-FEL is schematically shown in Fig. 1. The diameter of the MIR-FEL beam delivered to the user's facility was approximately 10 cm. This was reduced

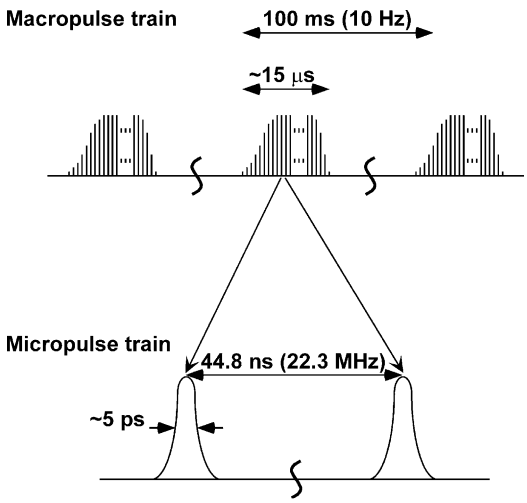


Fig. 1. Temporal pulse structure of the MIR-FEL.

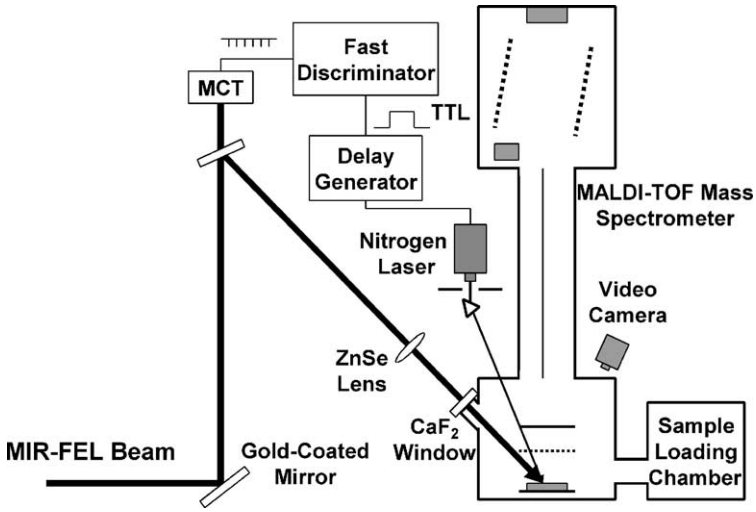


Fig. 2. Experimental setup for UV/FEL-MALDI-TOFMS experiments.

to approximately 5 mm by a confocal demagnification optical system using a parabola mirror and a ZnSe lens. The minified MIR-FEL beam was guided by gold-coated mirrors and focused by a ZnSe lens of 12.7 mm in diameter and 127 mm in focal distance to generate a small exposed spot (0.2 $mm^2$) on a sample. A portion of the MIR-FEL beam was separated by a gold-coated mirror inserted in front of the demagnification optics and received by a mercury-cadmium-telluride (MCT) infrared detector (R005, Vigo System, Warsaw, Poland). The MCT output was converted to a TTL signal by a delay generator (DG535, Stanford Research Systems, Sunnyvale, CA, USA), which also put an appropriate delay (ca. 4 μs) on the TTL signal to discard an early part of the MIR-FEL macropulse suffering from a distortion and a shot-to-shot fluctuation and to keep sufficient intensity and stability of micropulses at firing of the nitrogen laser.

### 2.2. TOF mass spectrometer

The experimental setup used for this study is schematically shown in Fig. 2. A commercially manufactured MALDI-TOF mass spectrometer (Voyager DE Pro, Applied Biosystems, Foster City, CA, USA) was used with minor in-house modifications. The mass spectrometer is equipped with a nitrogen laser (VSL-337ND, Spectra-Physics, Mountain View, CA, USA) for the conventional UV-MALDI method. The nitrogen laser was disconnected from the original control circuit and externally triggered by the TTL signal generated from the MIR-FEL pulse as above described. A $CaF_2$ window mounted on a 1.33 in. mini conflat flange was attached to the vacuum chamber for letting the MIR beam pass into the ion source region. The superposition of the nitrogen laser and MIR-FEL exposure spots on the sample plate was ensured by a liquid crystal film sensitive to a small change of the surface temperature. The TOF mass spectrometer was operated in the linear mode with the delayed extraction throughout this study. The exposure conditions of the nitrogen laser and MIR-FEL are summarized in Table 1.

### 2.3. Sample preparation

The MALDI matrix grade of 3,4-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) was obtained from Fluka (Buchs SG, Switzerland). The re-crystallized MALDI matrices, α-cyano-4-hydroxycinnamic acid (CHCA), was obtained from LaserBio Labs (Sophia-Antipolis Cedex, France). Both the matrix solutions were prepared by dissolving 10 mg of each matrix compound in 1 ml of acetonitrile/water (1:1) with 0.05% (v/v) trifluoroacetic acid. Bovine erythrocytes ubiquitin and bovine serum albumin were purchased from Sigma (St. Louis, MO, USA) to be used as model analytes. They are soluble proteins indeed; however, analytical conditions for insoluble proteins were modeled by dissolving them into 8 M urea solutions. The final analyte concentration in each solution was adjusted to 10 μM. A human hair keratin powder was obtained from Nacalai Tesque (Kyoto, Japan) and used without further purification. The keratin powder was added to a mixture of 50 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol

Table 1
Typical exposure conditions of UV/FEL-MALDI

| | Nitrogen laser | MIR-FEL | |
|---|---|---|---|
| | | Macropulse | Micropulse |
| Wavelength | 337 nm | 5.5–7.0 μm (tunable) | |
| Exposed area ($cm^2$) | $2 \times 10^{-3}$ | $2 \times 10^{-3}$ | |
| Average laser power (W) | $1.6 \times 10^{-3}$ | $1–3 \times 10^{-2}$ | |
| Repetition ratio (Hz) | 10 | 10 | $2.23 \times 10^{7}$ |
| Pulse width | 4 ns | 15 μs | 5 ps |
| Pulse energy (J) | $2 \times 10^{-4}$ | $1–3 \times 10^{-3}$ | $3–9 \times 10^{-6}$ |
| Fluence ($J/cm^2$) | 0.1 | 0.5–1.5 | $2–6 \times 10^{-3}$ |
| Intensity ($W/cm^2$) | $2 \times 10^{7}$ | | $3–9 \times 10^{8}$ |

(TRIS buffer), 8 M urea and 0.1 M 2-mercaptoethanol in the final concentration to be 7 mg/ml. The keratin solution was rigorously vortexed, then the supernatant layer was separated for use. The ubiquitin and albumin samples were mixed with the CHCA and sinapinic acid solutions, respectively, then were applied to the MALDI plate by the dried-droplet method. Deposits of the keratin sample were formed by the crushed-crystal method [27] with sinapinic acid matrix. Firstly, a drop of the sinapinic acid solution (1 μl) was applied to the MALDI plate and dried in air. Then a slide glass was placed on the matrix deposit and turned laterally with a small force down to the surface. Crushed particles were wiped out with a tissue paper and a smeared spot of the matrix was left. The keratin solution was mixed with the same volume of the sinapinic acid solution. A drop of the analyte/matrix mixture solution (1 μl) was applied to the smeared spot on the MALDI plate. Before the deposit was completely dried, water was applied to wash the spot. Finally excess water was removed by blotting with a tissue paper and the deposit was dried in ambient air.

## 3. Results

### 3.1. Bovine ubiquitin with 8 M urea

Fig. 3a is a typical UV-MALDI-TOF mass spectrum obtained from the model sample of ubiquitin dissolved in an aqueous solution of 8 M urea. Because the UV-matrix was diluted by about a factor of 100 in molarity with the denaturant which presumably impedes the activation process of UV-matrix, the conventional UV-MALDI did not show the regular performance for this model sample, i.e., the signal intensity was lowered by about a factor of 100 than that obtained from a usual sample without denaturant. Fig. 3b is an example of the UV/FEL-MALDI-TOFMS results, where the MIR-FEL wavelength was tuned in the IR absorption region of urea. A strong peak at the molecular mass of ubiquitin was found in the latter mass spectrum, which has an almost equal quality to those obtained from a usual sample without denaturant. Both the UV-MALDI and UV/FEL-MALDI measurements always suffered from a severe spot-to-spot variation due to the heterogeneity of the sample deposit. It should be noted that the results shown in Fig. 3 were obtained from the same exposed spot of the sample deposit, i.e., a single spot was exposed to a nitrogen laser for recording the UV-MALDI mass spectrum (Fig. 3a), then was simultaneously exposed to the nitrogen laser and MIR-FEL for recording the UV/FEL-MALDI one (Fig. 3b). In this manner, the sensitivities of UV-MALDI and UV/FEL-MALDI for this model sample can be compared without an influence of the sample heterogeneity. Table 2 summarizes gains in peak intensity and signal-to-noise ratio (S/N) at the molecular mass of ubiqui-

Table 2
Summary of UV-MALDI and UV/FEL-MALDI results for ubiquitin dissolved into 8 M urea

| | Spot no. | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | 4 |
| MIR-FEL wavelength (μm) | 5.8 | | 6.3 | |
| Average MIR-FEL power (mW) | 10 | | 20 | |
| Peak intensity | | | | |
| UV-MALDI | 1889 | 10 | 138 | 32 |
| UV/FEL-MALDI | 7550 | 576 | 18755 | 14235 |
| Gain | 4.0 | 58 | 136 | 449 |
| S/N | | | | |
| UV-MALDI | 4.0 | 0.05 | 0.4 | 0.1 |
| UV/FEL-MALDI | 15.2 | 3.4 | 46.8 | 39.4 |
| Gain | 3.8 | 68 | 117 | 394 |

The peak intensity and S/N were measured at the location of $[M+H]^+$ whether or not a distinct peak was observed.

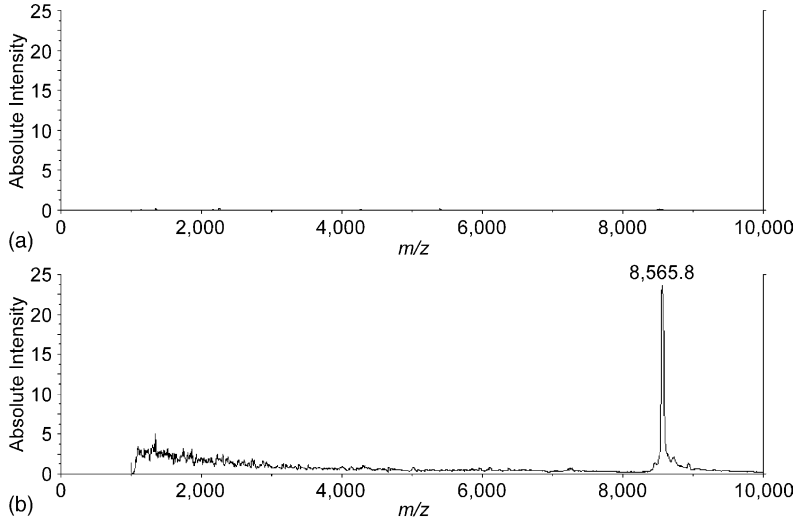


Fig. 3. TOF mass spectra obtained from ubiquitin with 8 M urea. CHCA was used as UV-matrix: (a) UV-MALDI result; (b) UV/FEL-MALDI result. MIR-FEL wavelength was 6.3 μm.

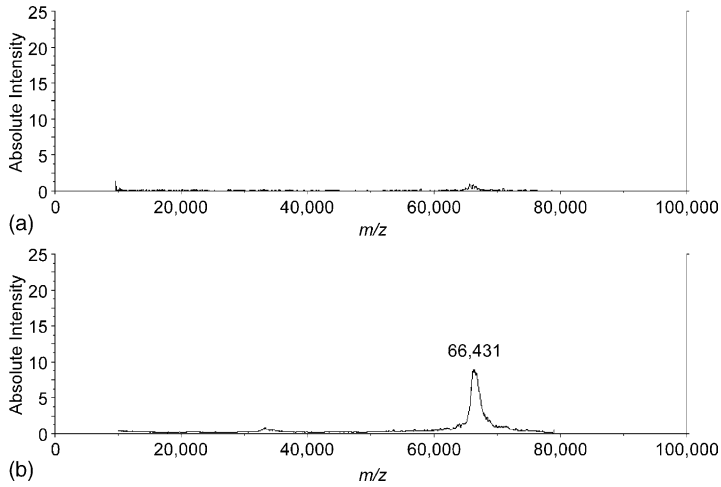


Fig. 4. TOF mass spectra obtained from albumin with 8 M urea. Sinapinic acid was used as UV-matrix: (a) UV-MALDI result; (b) UV/FEL-MALDI result. MIR-FEL wavelength was 6.2 μm.

tin, achieved by applying UV/FEL-MALDI to the same spot from which each control UV-MALDI mass spectrum was recorded. UV/FEL-MALDI achieves order(s)-of-magnitude improvement in S/N for this model sample.

### 3.2. Bovine albumin with 8 M urea

Fig. 4 is a pair of UV-MALDI and UV/FEL-MALDI-TOF mass spectra obtained from a single exposed spot of the model sample of albumin. UV/FEL-MALDI is still effective for the larger protein sample dissolved in an aqueous solution of 8 M urea, although the achieved improvement is found to be less than the case of ubiquitin. Fig. 5 indicates gains in S/N at the molecular mass of albumin as a function of the MIR-FEL wavelength. Orders-of-magnitude improvement in S/N was achieved by setting the MIR-FEL wavelength to 6.1 μm. Despite of being tuned in the IR absorption region of urea, some MIR-FEL wavelengths resulted in no improvement.

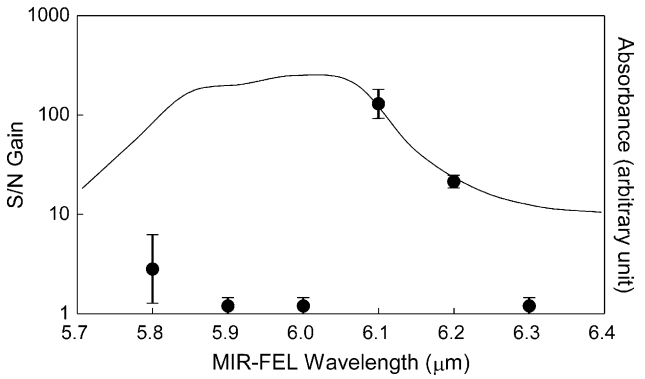


Fig. 5. Gain in signal-to-noise ratio (S/N) of as a function of MIR-FEL wavelength for albumin with 8 M urea, comparing UV-MALDI and UV/FEL-MALDI results. The solid curved line indicates the IR absorption spectrum of urea in the relevant wavelength range.

### 3.3. Human hair keratin (hard keratin)

A typical UV-MALDI-TOF mass spectrum obtained from the insoluble protein sample of hair keratin dissolved in an 8 M urea solution indicates no sign attributable to the protein (Fig. 6a). In contrast to this, the same keratin sample produced remarkable peaks on a UV/FEL-MALDI-TOF mass spectrum (Fig. 6b), where the MIR-FEL wavelength was set to 5.8 μm. Amazingly, ions with extremely high masses up to *m*/*z* 850,000 were observed. Two distinct series of peaks can be found in the mass spectrum. A series in the lower mass region (*m*/*z* 20,000–135,000) exhibits almost an equal mass difference (ca. 10,200 u) between every adjacent peak. The other series (*m*/*z* 180,000–850,000) also appears with equal spacing (ca. 28,800 u). These mass differences are attributable to two types of keratin molecules, presumably γ-keratins, which are amorphous components in hair rod. Native keratin molecules have many intermolecular disulfide bonds to form supramolecular networks. The observation of the peak series is most probably elucidated by the contents of γ-keratin clusters, which could be conserved via the sample preparation and the ionization process of UV/FEL-MALDI, or, could be gas phase adducts.

## 4. Discussion

MALDI using the MIR-FEL alone did not give any analyte peaks throughout this experiment (results not shown). Our previous study revealed that ions could be generated in the gas-phase from a solid target by irradiating the MIR-FEL alone [28]. In the previous study, peaks presumably attributable to carbon, aluminum and fumaric acid were generated by the MIR-FEL tuned at 10.6 μm, where a substantially higher irradiance can be obtained (i.e., macropulse fluence was 10 J/cm$^2$, micropulse fluence was $3 \times 10^{-2}$ J/cm$^2$, micropulse intensity was $6 \times 10^9$ W/cm$^2$,

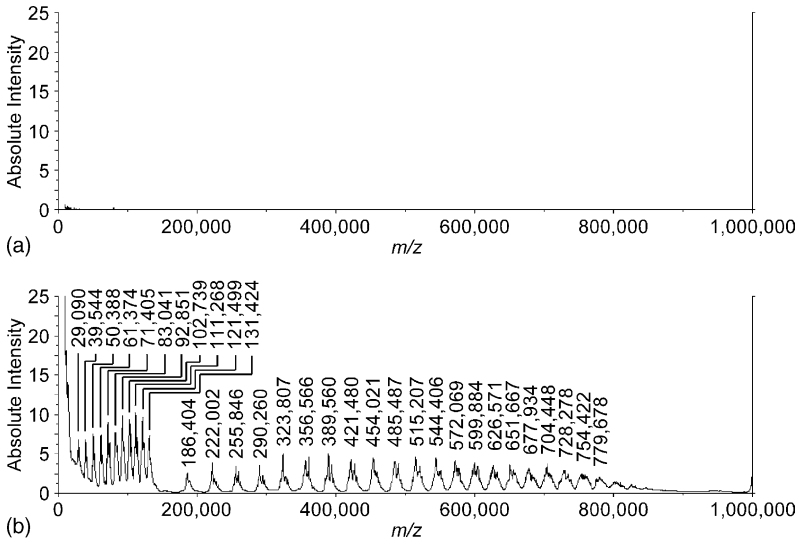


Fig. 6. TOF mass spectra of human hair keratin dissolved in 8 M urea, 50 mM Tris buffer and 0.1 M 2-mercaptoethanol. Sinapinic acid was used as UV-matrix: (a) UV-MALDI result; (b) UV/FEL-MALDI result. MIR-FEL wavelength was 5.8 μm.

respectively) than the present wavelength region, although irradiation of the whole macropulse (ca. 15 μs) had introduced a too wide temporal spread uncorrectable with the delayed extraction technique. It was suggested from the pulse structure of the MIR-FEL that each micropulse exceeding some threshold intensity possesses a desorption ability and the gas-phase ions may be generated either sustainedly or intermittently in the duration of a macropulse. Usually, an adjustment of the length of micropulse train, so-called micropulse-picking, is required to make use of a FEL for MALDI. This is accomplished by using a high-speed broad-band optical switching device, such as an acousto-optic modulator (AOM). Cramer et al. have implemented a micropulse-picking based on a Pockels cell [29] to succeed FEL-MALDI measurements of several protein samples with IR-matrices [24]. In the present study without micropulse-picking, bulk ablation from the target surface was found instead of a sign of analyte ions as increasing the MIR-FEL intensity (micropulse intensity). Apart from the temporal spread issue, the macropulse structure may bring about a cumulative thermal effect which is difficult to be addressed because of thermal diffusion processes in each interval between every successive micropulses. It cannot be concluded whether MALDI using the MIR-FEL alone (FEL-MALDI) will work or not unless implementing a micropulse-picking operation, which is currently being undertaken at this laboratory. There is no certain necessity of that only the combination of UV-laser and MIR-FEL can obtain spectra from the keratin sample, and MALDI with a suitable IR-laser of short pulse width alone is an important alternative still to be tested. UV/FEL-MALDI is, however, found to succeed in eliminating both the temporal spread and unnecessary cumulative heat leading to bulk ablation. Timing of ion generation in UV/FEL-MALDI seems to be defined by the pulse width of the nitrogen laser, no matter the whole macropulse is irradiated. Although the simultaneous irradiation of the MIR-FEL and nitrogen laser in the macropulse time-scale is essential for UV/FEL-MALDI, the precise synchronicity has not been established in the present system, mostly due to the timing jitter of the nitrogen laser. Because the micropulse spacing (44.5 ns) is substantially longer than the UV pulse width, this timing jitter may affect the synergy of UV- and IR-induced effects. Therefore, reduction in the timing jitter may be of benefit to the efficiency and reproducibility of UV/FEL-MALDI. Precisely synchronized experiments may also be required for insight into the ionization mechanism.

A tentative mechanism of UV/FEL-MALDI is proposed as below. In a solid deposit dominated by urea, which is transparent to the UV wavelength, analyte molecules are deeply embedded and the UV matrix is diluted. When the deposit is exposed to the nitrogen laser alone (UV-MALDI), the thermal energy which is converted from the UV photon energy by a matrix molecule diffuses into surrounding “cool” urea quickly, thus the transient heating becomes insufficient to initiate the desorption process. In the case of UV/FEL-MALDI, the bulky solid of urea is directly activated by the IR photons whose wavelength is tuned at the absorption band of the denaturant. This activation process presumably supports the generation of transient heat driven by the UV-absorbed matrix; that is to say UV/FEL-MALDI is basically UV-MALDI and the MIR-FEL acts as an assistant laser. Urea has been examined as a matrix of IR-MALDI using 3 μm region; however, it has shown a relatively poor performance [9]. The remarkable success of UV/FEL-MALDI for urea-containing samples is owing to the use of UV-matrix and simultaneous exposure to UV and IR lasers being engaged in essential steps of the MALDI process, such as a local transient heating of the sample deposit. Little et al. reported two-laser IR/UV-MALDI experiments which aimed at exploring the time evolution of the MALDI process [30]. Their results indicate that

the efficiency of UV-MALDI can be increased by pre-heating a sample consisting of analyte and 2,5-dihydroxybenzoic acid (DHB) matrix with an IR laser pulse. Our findings are basically consistent with the previous IR/UV-MALDI results, additionally, pronounce for the ability of IR/UV-MALDI for the analyte which is deeply embedded in a supporting material.

The observed relationship between the ionization efficiency and the MIR-FEL wavelength is not elucidated in a straightforward way. The IR absorption characteristics of the sample deposit, in which urea concentration is at least two orders of magnitude higher than the UV-matrix, is most likely to follow that of the denaturant. The optimal wavelength of the MIR-FEL for the three samples containing urea was not constant, although it was always positioned around the IR absorption maximum originating from overlapped N–H bending and C=O stretching modes in urea. Particularly, the effective IR wavelength in the analysis for keratin was shorter than those for the other samples. The considerable discrepancy in the optimal MIR-FEL wavelength depending on the analyte proteins is presumably attributable to a different degree of intermolecular hydrogen bonds between urea and analyte molecules. The formation of strong intermolecular hydrogen bonds in a crystalline solid are known to cause a red shift of the IR absorption spectrum and a subsequent blue shift of the wavelength dependency of the threshold fluences around 3 μm, owing to weakened O–H and N–H stretching modes which are recovered by a weakening of hydrogen bonds in the course of the IR-MALDI process [5]. A parallel mechanism may be applicable to the shift of the effective MIR-FEL wavelength as regards the contribution of hydrogen bonds which may depend on properties of the analytes and their preparations. Precisely designed experiments are planed to address the MIR-FEL wavelength dependency in more detail.

## 5. Conclusion

We have developed a novel MALDI method, UV/FEL-MALDI, in which a sample is exposed to a MIR-FEL macropulse and a nitrogen laser pulse simultaneously. Preliminary experiments of UV/FEL-MALDI-TOFMS were carried out for protein samples dissolved into 8 M urea solution to examine the ability to analyze samples prepared under a strong denaturing condition. In comparison to the conventional UV-MALDI, significant gains of the ion yields were obtained by UV/FEL-MALDI with the MIR-FEL wavelength tuned around the absorption maximum of urea. The results indicate that the synergy of UV and IR pulses and the tunability of the MIR-FEL are effective for increasing the ionization efficiency of MALDI for analyte which is deeply embedded in a solid denaturant. The intact form of human hair keratin, which is an extremely insoluble protein, has been analyzed by mass spectrometry for the first time. The molecule-related ion of γ-keratin, i.e., the intact molecular mass, was detected in the UV/FEL-MALDI-TOF mass spectrum along with an evidence of clustering. UV/FEL-MALDI is a highly promising method for approaching to insoluble proteins.

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