Compact Tunable Cr:LiSAF Laser for Infrared Matrix-assisted Laser Desorption/Ionization

Mehrnoosh Sadeghi,[1] Zohra Olumee,[1] Xiaodong Tang,[1] Akos Vertes,[1*] Zhi-Xing Jiang,[2] Angus J. Henderson,[2] Hyo Sang Lee,[2] and Coorg R. Prasad[2]

[1]Department of Chemistry, George Washington University, Washington, D.C. 20052, USA
[2]Science and Engineering Services, Inc. 4032 Blackburn Lane, Burtonsville, MD 20866, USA

SPONSOR REFEREE: Dr. John H. Callahan, Analytical Chemistry Section, Naval Research Laboratory, Washington, DC 20375, USA.

A tunable Cr:LiSAF laser-pumped optical parametric oscillator was used for mid-infrared matrix-assisted laser desorption/ionization (MALDI) mass spectrometry experiments. The mass spectra of substance P, bovine insulin and pd(T)$_{10}$ in the 2.88–2.96 µm range showed excellent single shot signal quality (signal-to-noise-ratio, resolution) but poor shot-to-shot reproducibility. The reproducibility is expected to improve with more stable laser design. No correlation was found between the absorption sepectrum of the matrix and the MALDI response. © 1997 by John Wiley & Sons, Ltd.

Over the past few years ultraviolet matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) has become a well established method for the analysis of large biomolecules. Numerous studies have been conducted on the fundamentals and applications of MALDI with laser sources emitting in the 260–360 nm wavelength range. However, it was not until 1990 that results of the first infrared (IR) MALDI experiments were presented by Overberg *et al.*[1] An Er:YAG laser, emitting at $\lambda = 2.94$ µm, was used to exploit the strong absorption bands of N—H and O—H bonds around 3 µm and MALDI spectra of large biomolecules were obtained.

One of the advantages of IR-MALDI would be the great number of potential matrices. Practically every carboxylic acid (for example, succinic acid) and many amides (e.g. urea) could be used, based on their N—H and O—H absorption bands. As emphasized in a recent article by Hillenkamp and co-workers,[2] in the IR even water ice could be used as a matrix for biomolecules. A very attractive feature of this proposition is the possibility of avoiding one of the questionable and cumbersome steps in UV-MALDI, namely, co-crystallization with a UV absorbing matrix, a step that leads to highly non-physiological guest molecule environments. Water ice as matrix would retain the natural environment of the biomolecules and help preserve their native conformation, as well as any possible non-covalent superunits.

One of the most important limitations in this venture is the lack of appropriate lasers. IR lasers are bulky, cumbersome and expensive. As a consequence, only a handful of groups have conducted IR-MALDI experiments. Currently, there are three benchtop lasers and three wavelength ranges available: Nd:YAG at $\lambda = 1.06$ µm, Er:YAG at $\lambda = 2.94$ µm and TEA-CO$_2$ at $\lambda = 10.6$ µm. Despite multiple efforts, no one has reported successful IR-MALDI experiments using $\lambda = 1.06$ µm radiation from Nd:YAG. The majority of studies related to IR-MALDI have been carried out on

mass spectrometers with Er:YAG laser sources emitting at $\lambda = 2.94$ µm.[3–7]

The utility of IR-MALDI-MS in the molecular weight determination of large molecules is under investigation by several groups. Comparisons of suitable matrices, sample preparation, and the amount of sample removed by a single laser shot in MALDI experiments with UV and IR lasers have been reported.[3,4] IR-MALDI results for large oligonucleotides (pd(T)$_{19-24}$) have shown better quality spectra than UV-MALDI in one of these studies.[3] Specific fragments were observed in IR-MALDI that provided structural data leading to sequence information for low-picomol amounts of oligonucleotides containing < 21 bases.[5] In a recent study, IR-MALDI results were presented for proteins electroblotted onto polymer membranes after sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) separation.[6] Superior signal-to-noise (S/N) ratio was obtained in negative-ion IR-MALDI-MS of diaminoplatinum(II)-oligodeoxyribonucleotide adducts.[7] These studies pointed to the importance of IR-MALDI as a complementary method to UV-MALDI and emphasized the need for affordable and compact IR laser sources in the mid-IR wavelength region.

Based on the observation that most matrices used at 2.94 µm had comparable absorption coefficients at 10.6 µm, the TEA-CO$_2$ laser source has also been applied in IR-MALDI experiments. Using urea as a matrix, in some of these studies mass resolutions superior to those achieved in UV-MALDI were reported.[8] The results obtained with a TEA-CO$_2$ laser were similar to those for the Er:YAG case indicating, however, that the laser irradiance at $\lambda = 10.6$ µm was a more critical factor than at $\lambda = 2.94$ µm.

The first results on tunable IR-MALDI-MS of small organic and biomolecules using the free electron laser at the Vanderbilt Free-Electron Laser (FEL) facility have shown moderate variations in spectrum quality.[9–11] Using succinic acid matrix, successful ion-formation threshold measurements in the 2.94–4.2 µm (O—H stretching mode) and 5.5–6.4 µm (C=O
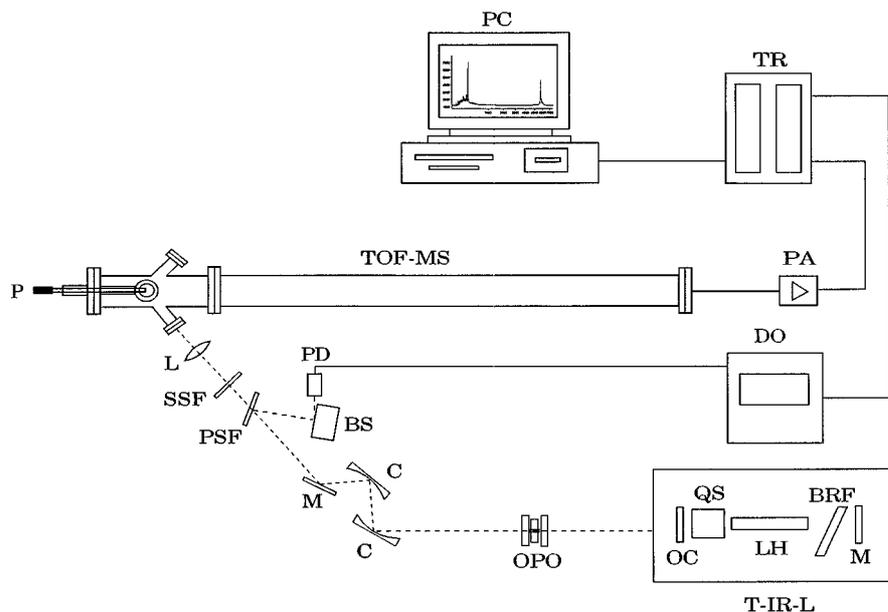
* Correspondence to: A. Vertes

Figure 1. Laser desorption TOF-MS with a tunable mid-IR laser source. T-IR-L: tunable near-IR laser; DO: digital oscilloscope; TR: transient recorder; PA: preamplifier; PC: personal computer; P: probe; BS: beam stop; PD: photodiode; SSF: signal separator filter; PSF: pump separator filter; OPO: optical parametric oscillator; L: IR lens; C: collimator; M: mirror; BRF: birefringent tuner; LH: laser head; QS: Q-switch; OC: output coupler.

stretching mode) showed puzzling differences in wavelength dependence.[11] Whereas no correlation was found between threshold energy and matrix absorption in the O — H absorption region ($\sim 3\ \mu m$), resonance absorption was found to govern the desorption events for the C = O excitation ($\sim 6\ \mu m$).

The appropriate combination of matrix and laser wavelength remains one of the crucial issues in MALDI. A compact tunable IR laser source would enable the exploration of a large number of matrices and to take advantage of the optimal desorption and ionization properties without the photochemical side-effects induced by UV and visible radiation. Considering the difficulties of accessing and operating a FEL, it is obvious that FEL studies can only serve as exploratory tools for IR-MALDI-MS. In the present communication, we introduce the first results using a compact tunable IR laser (Cr:LiSAF) for mid-IR-MALDI. Tuning was achieved between 2.88 μm and 3.4 μm by applying a tunable near-IR source and an optical parametric oscillator (OPO). The feasibility of using this laser source for IR-MALDI-MS was demonstrated on peptide, protein and nucleic acid analytes.

EXPERIMENTAL

Tunable IR laser system

The tunable mid-IR laser system was composed of a tunable solid-state laser-pumped potassium titanyl arsenate (KTA) OPO. The tuning of the OPO in the mid-IR was achieved by the tuning of the pump wavelength of the solid-state laser. A Q-switched, flash lamp pumped Cr:LiSAF laser (Cr^{3+}:LiSrAlF$_6$) (Science and Engineering Services, Inc. (SESI) Burtonsville, MD, USA) served as tunable near-IR source for these experiments. The laser was set up in a two-mirror cavity as shown in the tunable IR-laser section of Fig. 1. The pulse length of the laser was less than 100 ns.

The cylindrical Cr:LiSAF laser rod (VLOC, Tarpon Springs, FL, USA) was doped at 1.5% Cr^{3+} and

antireflection-coated on both surfaces for the wavelength region of 800 nm to 900 nm. Q-switching was achieved by using an RF opto-acoustic switch. The laser was operated at 10 Hz and the temperature of the laser rod was controlled at 14 °C by a water bath. Since the Cr:LiSAF rod is slightly amphoteric, the pH value of the cooling water was frequently monitored and kept at approximately pH 7.1 to enhance the water durability of the crystal. A smooth and continuous tuning range of 830 nm to 883 nm was obtained with a three-plate birefringent (BRF) tuner.

Widely tunable OPO output was achieved in a non-critically phase-matched KTA (Crystal Assoc. Inc., Waldwick, NJ, USA) with low pulse energy pumping (a few mJ) by the tunable laser pump source. The mirrors for the OPO oscillator (VLOC) were dielectric-coated CaF$_2$ substrates. CaF$_2$ was selected for its wide transmission 0.15 to 9 μm) in the mid-IR. The mirror was antireflection-coated for the pump wavelength (780 to 880 nm) and idler wavelength (2.65 to 3.2 μm).

The Cr:LiSAF laser beam was focused to a 400 μm diameter spot with a 0.75 m focal-length lens to pump the KTA OPO. The signal wave in the wavelength region of 1.1 μm oscillated in the singly resonant OPO while the idler was allowed to pass through. Therefore, the signal wave was traveling in both directions and the idler wave only in one direction, i.e. the pump direction. A long-pass filter was used to block the pump but allow the signal and idler to pass.

At the pump wavelength of 860 nm, the idler and signal wavelengths were measured to be 2.94 μm and 1.22 μm, respectively. At a pumping energy of 18 mJ, up to 1.5 mJ total output (idler + signal) and 0.5 mJ idler energy were obtained. The OPO output showed approximately linear dependence on pump energy.

Two filters, a pump-blocking filter to eliminate the pump beam and a second bandpass filter, were used to allow only the idler to pass. The bandpass filter was used to separate the signal and the idler (see Fig. 1). The idler beam was collimated by two concave gold-

coated mirrors. A 0.254 m IR lens made of infrasil quartz was used to focus the idler beam through an uncoated sapphire window onto the MALDI sample in the vacuum chamber.

In the wavelength region of 2.88 to 2.96 µm up to 500 µJ/pulse idler energy was measured before the focusing IR lens. Due to intensity fluctuations in the pumping laser and the OPO, considerable shot-to-shot idler energy fluctuations ($\sim$100%) were encountered. The focal spot size at the sample in the mass spectrometer was estimated by placing a photographic paper on the sample stage. The elliptical burn mark was measured to be 300 µm × 600 µm for 120 shots. Taking into account the 70% and 85% transmission of the IR lens and the sapphire window, respectively, and assuming 300 µJ/pulse normal operation energy, the $\sim$ 0.1 J/cm^2 laser fluence at the sample corresponded to an irradiance of $\sim 10^6$ W/cm^2. This value turned out to be close the IR-MALDI ion generation threshold in succinic acid.

Time-of-flight mass spectrometer

MALDI-MS experiments were carried out on a home-built linear time-of-flight mass spectrometer (TOFMS) described in detail elsewhere.[12] The laser port of the instrument was modified to accommodate the IR laser used in this study. The quartz UV viewport was replaced by an uncoated sapphire window transparent in the mid-IR. The focusing optics were also changed to appropriate IR-grade optical elements that projected the laser beam on to the probe tip at an angle of 45°. A high-voltage power supply (205B-30R, Bertan Associates Inc., Hicksville, NY, USA) provided the ripple-free 30 kV accelerating voltage for the extraction of the generated ions. Field penetration was eliminated by the interlocking design of lens elements which, in addition, provided excellent field homogeneity. At the end of a 215 cm long field-free drift region, the ions were detected by a dual multichannel plate assembly (Galileo Co., Sturbridge, MA, USA) biased to –1900 V. After tenfold preamplification (9305, EG&G ORTEC, Oak Ridge, TN, USA), followed by amplification with a variable gain amplifier module (6103, LeCroy, Albuquerque, NM, USA), the signal was recorded by a fast transient recorder (TR8828D, LeCroy). A custom-made data acquisition package (TOFWARE, Ilys Software, Pittsburgh, PA, USA) running on a 486 personal computer was used for data analysis.

Sample preparation

The matrices succinic acid (1,4-butanedioic acid, > 99% purity), urea and nicotinic acid (pyridine-3-carboxylic acid) were purchased from Sigma Chemical Co. (St. Louis, MO, USA) whereas sinapinic acid (3,5-dimethoxy-4-hydroxy-cinnamic acid) was supplied by Aldrich Chemical Co. (Milwaukee, WI, USA). Succinic acid was recrystallized twice prior to use to minimize sodium contamination. Saturated matrix solutions were prepared fresh daily in 1:1 v/v ethanol + water solvent. The 2×10^{-3} M bovine insulin and 1.2×10^{-4} M substance P analyte solutions were prepared in 0.1% trifluoroacetic acid (TFA) solvent, whereas pd(T)$_{10}$ solution was made in deionized water at 4×10^{-4} M concentration.

The optimum matrix-to-analyte ratio proved to be significantly lower than the values used in UV-MALDI-
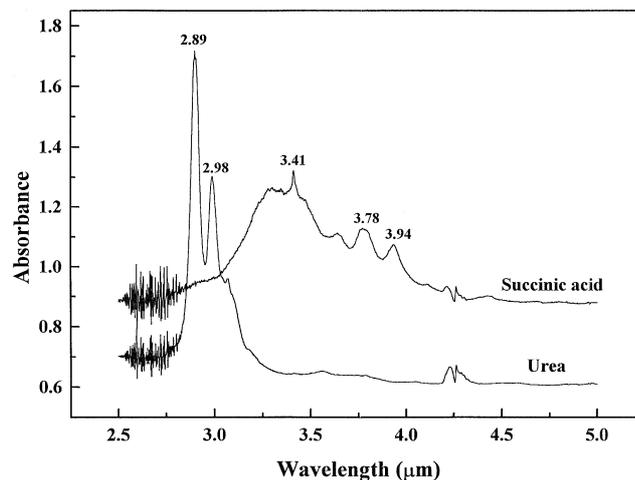

Figure 2. IR spectra of succinic acid and urea (1% concentration in FTIR-grade KBr). The strong absorption bands between 2.89 and 3.94 µm of the two matrices indicate resonance absorption into O — H, N — H and C — H vibrational modes.

MS where 10 000:1 proportions are typical. In contrast, the best analyte signal in the mid-IR was achieved using 1000:1 matrix-to-analyte molar ratio in the solid phase. Thus, at least under the explored circumstances, IR-MALDI seems to need somewhat higher analyte/matrix ratio than UV-MALDI for successful detection (i.e. it is less sensitive). The samples were prepared by mixing equal volumes (3 µL) of the matrix and analyte solutions on a 5 mm diameter stainless steel sample holder. The resulting droplet was dried in a stream of cold air (drop-dry method) prior to insertion into the vacuum system. Crystallization usually occurred on the perimeter of the sample holder, thus the laser was focused to the edge. In all cases, spectra were externally calibrated using corresponding spectra in the UV range. The consistency of mass calibration was also checked on sodium, potassium, and matrix peaks in the IR.

IR spectroscopy

Mid-IR absorbance measurements for succinic acid and urea were carried out on samples embedded into KBr pellets at 1% concentration. The Fourier transform infra-red (FTIR) grade KBr (Aldrich Chemical Co.) was heated prior to the preparation of the pellets for at least 48 hours at 70 °C temperature to eliminate water residues. Mid-IR absorption spectra were recorded on an FTIR instrument (6020 Galaxy, Mattson Instruments, Madison, WI, USA). Each spectrum was obtained in 16 scans with a resolution of 2 cm^{-1} and a mirror velocity of 0.6 cm/s.

RESULTS AND DISCUSSION

In order to explore variations in matrix absorbances, the mid-IR spectra of succinic acid and urea were taken. The absorption spectrum of succinic acid showed a fairly broad and strong O — H stretching absorption band in the 3.2 to 4.0 µm region (see Fig. 2). While the KBr had been thoroughly dehydrated before the FTIR experiments by heat treatment, the succinic acid samples were used directly after recrystallization to ensure similarity with the MALDI sample preparation conditions. As a consequence, the presence of residual water in these crystals could not be excluded. The OH stretching mode of these residual water molecules is
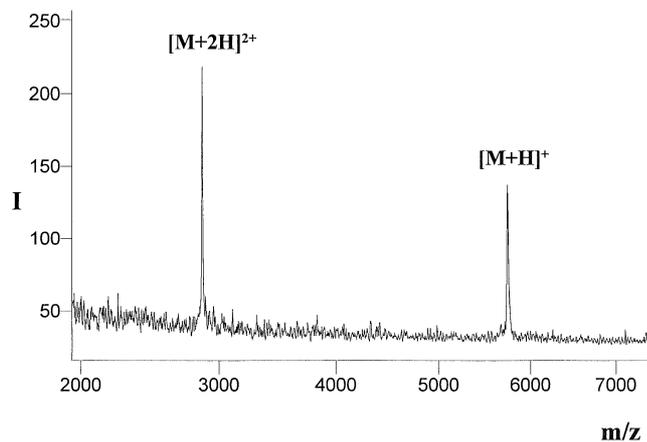
Figure 3. Mid-IR-MALDI mass spectrum of bovine insulin at $\lambda = 2.95\,\mu m$ in succinic acid matrix (average of 2 shots).

Figure 4. Mass spectrum of substance P averaged over 3 shots, obtained at a laser wavelength of $2.94\,\mu m$ in succinic acid matrix.

believed to contribute to the broadness of the major feature in the spectrum. There were several additional peaks ($3.41\,\mu m$, $3.78\,\mu m$ and $3.94\,\mu m$) superimposed on this broad band. It was obvious from the spectra that the $2.94\,\mu m$ radiation did not capitalize on resonance absorption in the succinic acid system. Increased absorption efficiency was expected at $3.41\,\mu m$, a wavelength not readily available with the present laser system. In the case of urea, two pronounced absorption bands were observed at 2.89 and $2.98\,\mu m$. For this matrix the tuning range of our laser pumped KTA OPO system should clearly provide an opportunity to compare the effect resonance and non-resonance absorption on the MALDI-MS signal.

In an attempt to explore the effect of laser wavelength, MALDI-MS experiments were conducted with the idler wavelength tuned in the $2.88\,\mu m$ to $2.96\,\mu m$ region for matrices of succinic acid, nicotinic acid, sinapinic acid and urea, and analytes insulin, substance P, and $pd(T)_{10}$. During these experiments the typical pulse energy was $\sim 0.5\,mJ$. Although urea worked fairly well, succinic acid turned out to be the best matrix for reproducible MALDI spectra. The other two matrices did not provide detectable analyte signal under the described conditions.

Based on their IR spectra (see Fig. 2), we expected to observe no differences in the MALDI spectra from succinic acid but large variations using a urea matrix. To our surprise, the tuning of the laser within the 2.88–$2.96\,\mu m$ range did not significantly affect any of the IR-MALDI spectra. Since shot-to-shot laser energy fluctuations were in the order of 100%, it is possible that the differences in MALDI spectra due to wavelength changes were masked by differences due to energy fluctuations. More precisely, when absorbances are converted to transmission values, the variations in the tuning range correspond to ~ 5–10%. The consequences of these variations are hard to detect on the background of 100% laser intensity fluctuations. Thus, at this point, the effect of wavelength with respect to matrix absorption properties cannot be fully evaluated.

The MALDI spectrum of bovine insulin ($m/z\,5733.5$) using the Cr:LiSAF laser/OPO is presented in Fig. 3. Using the birefringent tuner, $2.95\,\mu m$ idler wavelength was selected. In the spectra, insulin appears as a protonated molecule, i.e. gives the ion $[insulin + H]^+$. This feature was always accompanied by a stronger

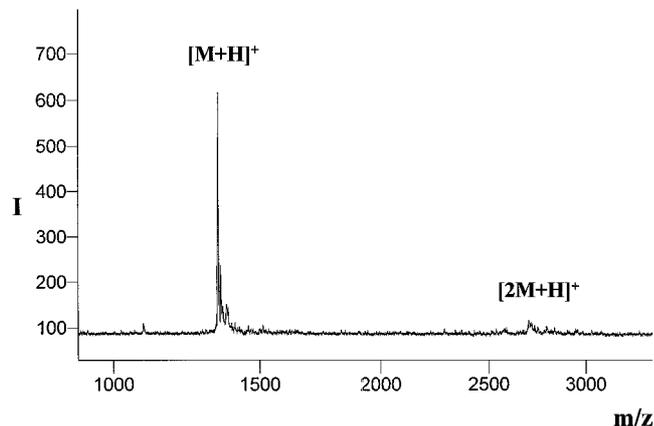doubly charged peak, $[insulin + 2H]^{2+}$, and often a triply protonated insulin peak was also observed.

In accordance with the notion that the amount of material removed in IR-MALDI was greater than in UV-MALDI, it was necessary to change the sample spot by continuously rotating the probe tip during the experiments. Higher ablation rate, in principle, should be accompanied by lower resolution in the IR case. In reality, no deterioration of mass resolution was observed in averaged spectra. This may be explained by reduced ionization efficiency and consequently lower space-charge effects. For example, the 50% resolutions of the m/z 5734.5 and 2866 peaks of insulin were 377 and 129, respectively, which were comparable to the values obtained for the same peaks under similar instrumental conditions by UV-MALDI. It is worth mentioning, however, that single shot IR-MALDI-MS spectra seem to have better mass resolution and better signal-to-noise (S/N) ratio than their UV-MALDI-MS counterparts. This feature may improve the efficiency of data acquisition in future automated IR systems, and may also compensate for the seemingly lower sensitivity.

In the IR-MALDI spectra of substance P, protonated molecules and weak dimer peaks were detected (shown in Fig. 4). Again, remarkably good S/N ratio and resolution ($m/\Delta m = 591$ with 50% definition) were observed in successful single shots. The success rate of shots, however, was significantly lower than in UV-MALDI. A possible explanation of this difference is the large difference in pulse energy fluctuations between the two types of lasers. While pulse energy fluctuations for a typical nitrogen laser (Laser Science, Inc., Newton, MA, USA) are less than 5%, the pulse-to-pulse energy variations for our 'breadboard' Cr:LiSAF laser are approximately 100%. Typically $3\,\mu L$ analyte solution, containing 360 pmol substance P, was used to prepare the sample. This amount is larger than the sample requirement in a UV-MALDI-MS experiment. Further exploration and refinement of the IR sample preparation methods is needed to decide if this limitation is inherent in the IR-MALDI method or can be overcome by method development. Even if lower sensitivity turns out to be inherent, in practical terms it can be compensated for by better the S/N ratio of the single shot IR spectra.

Spectra of the oligonucleotide $pd(T)_{10}$ indicated the presence of a quasi-molecular ion accompanied by several oligonucleotide fragments due to cleavage of
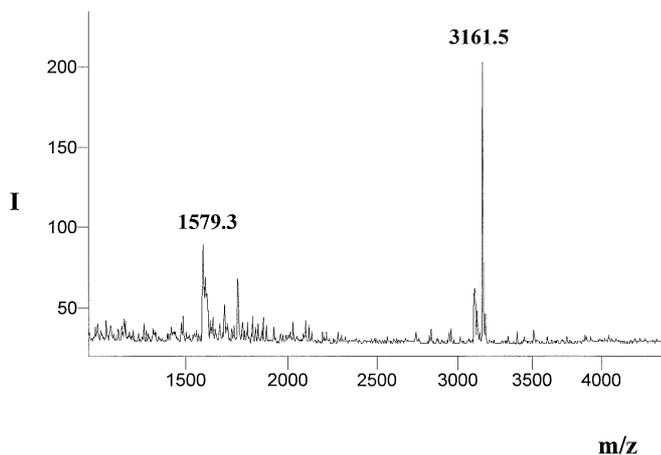
Figure 5. Single shot mass spectrum of pd(T)$_{10}$ in succinic acid matrix at $\lambda = 2.94\ \mu m$ using Cr:LiSAF laser excitation.

the backbone as abundant peaks in the $1000 < m/z < 2000$ region (see Fig. 5). Similarly to the peptide case, both the S/N ratio and the resolution from a single shot were high. The appearance of fragment ions proved that, similar to the UV case, nucleic acids were fragmented during the IR-MALDI process. Compared to proteins, positive nucleic acid ions are thought to undergo more fragmentation because of the relative instability of the phosphodiester backbone.

The presented laser system has additional wavelengths readily available. For example, the pump pulse was tunable in the region of 800 nm to 920 nm and the signal pulse was tunable from 1.1 μm to 1.25 μm. By frequency doubling of the pump pulse, tunable blue laser light was generated and the corresponding MALDI signal was observed. These results will be presented in a separate publication.

By introducing a different OPO crystal, the idler wavelength was also tuned between 3.0 μm and 3.2 μm. At this wavelength, however, the pulse energy of the idler decreased to 250 μJ. Despite our efforts to improve focusing conditions and minimize losses on the transfer optics, the achieved irradiance proved to be below the MALDI ion generation threshold. Although peaks of sodium, potassium and matrix (succinic acid) related ions could be observed, no peaks of analytes were detected. We concluded that, at this wavelength, higher idler pulse energy was necessary to reach the MALDI threshold. A higher energy pump laser is under construction.

In future experiments, we can further extend the tuning range by using an Alexandrite pump laser and other OPO crystals such as rubidium titanyl arsenate (RTA) and potassium titanyl phosphate (KTP). Since IR-MALDI-MS is still an emerging technique, this wide tuning range of the laser/OPO system will be useful to explore new matrix candidates and optimize ion production. In addition, multi-wavelength MALDI schemes are also possible by mixing the beams of different wavelength. The signal beam and/or the pump beam may also prove useful to enhance the ionization process.

We have demonstrated the feasibility of tunable mid-IR-MALDI mass spectrometry for the diagnosis of peptides, proteins and oligonucleotides with a rugged and compact laser system. The MALDI mass spectra show good mass resolution and S/N ratio, even for single shots, thus allowing high throughput operation. MALDI-MS based on the described laser system can become more versatile by combining the advantages of UV, visible and mid-IR-MALDI analysis.

Acknowledgements

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