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A High Repetition Rate (1 kHz) Microcrystal Laser for High Throughput Atmospheric Pressure MALDI-Quadrupole-Time-of-Flight Mass Spectrometry

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Sample throughput has been increased in many areas of proteomics, but the last significant advance in lasers used for matrix-assisted laser desorption/ionization (MALDI) was the introduction of cartridge-type N_2 lasers (337 nm, 4-ns pulse widths, 1−30-Hz repetition rates) more than a decade ago. This report describes the application of a 1-kHz repetition rate Nd:YAG laser (355 nm, <500-ps pulse widths) for atmospheric pressure MALDI-QqTOFMS, and data obtained are compared to a conventional nitrogen laser. For example, the signal intensity for angiotensin II using the 1-kHz laser was in some cases enhanced by a factor of 80 and high-quality data could be obtained in as little as 1 s.

Time-of-flight (TOF) mass analyzers are now considered standard hardware for bioanalytical mass spectrometry (MS),[1] but rapid growth in biological applications of TOFMS did not occur until the introduction of matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI).[2,3] Continuous advances in instrument hardware, especially reflectron TOF, hybrid TOF, TOF/TOF, and ion trap TOF instruments, have greatly enhanced mass accuracy, sensitivity, and resolution, as well as providing unparalleled utility for high throughput proteomic applications.[4−7] In addition, ion mobility coupled with orthogonal TOFMS (IM-o-TOFMS) could potentially increase throughput even further, i.e., ∼1 to 10 ms gas-phase separation of complex biological mixtures, and reduce the need for sample cleanup by more traditional time-consuming separation methods such as HPLC coupled with MS.[8,9] Surface-induced dissociation (SID) has also been incorporated in IM-SID-TOFMS, which significantly increases sample throughput by enabling simultaneous acquisition of MS^1 and MS^2 data.[10,11] Recent developments in atmospheric pressure (AP) MALDI also provide unique capabilities for proteomic-scale research.[12,13]

A major application of MALDI-TOFMS is for the identification of proteins separated by 1-D and 2-D polyacrylamide gel electrophoresis (PAGE). The gel-separated proteins are digested with a protease, mass analyzed, and the experimental masses are searched against a database.[14] Due to the time- and labor intensive nature of PAGE, current advances in throughput are focused on sample handling/preparation, which are based upon microfluidics or robotics to screen and process proteins prior to mass analysis,[15−18] as well as increasing the efficiency of proteases used to digest proteins or protein complexes.[19−21] A further development in sample handling and throughput includes performing MALDI at atmospheric pressure.

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Recently, the utility of AP-MALDI using orthogonal TOFMS instrumentation,[12,22,23] or quadrupole ion trap-MS[24−27] was demonstrated. There are three primary advantages of AP-MALDI-MS in comparison with high-vacuum MALDI-MS. First, it appears to be a softer ionization technique because the abundance of prompt fragment ions is reduced, probably due to collisional cooling at atmospheric pressure. Second, it is relatively straightforward to interface an AP-MALDI source with a conventional ESI-source mass spectrometer. Third, complications arising from the introduction of samples into the high vacuum of the mass spectrometer are eliminated. However, ion transport efficiency is degraded in AP-MALDI compared with traditional high-vacuum MALDI, resulting in poorer sensitivity.[12]

One area in which significant improvements can be made in MALDI-TOFMS is the laser source used for desorption. For example, the UV laser is not much different today than the laser used in the early stages of MALDI development.[28,29] Many commercial MALDI-TOFMS instruments are equipped with a nitrogen laser (337 nm) that can operate at shot-to-shot repetition rates of 1−30 Hz. Although these lasers are advantageous because they are a compact, inexpensive, and easy-to-use source of UV radiation, the low repetition rate limits the duty cycle and overall throughput of MALDI-TOF mass spectrometers. Recent advances in solid-state lasers that are compact, relatively inexpensive, and capable of operating at high repetition rates (1−10 kHz) should have a significant impact on MALDI-TOFMS for proteomics. In an earlier report, Standing and co-workers evaluated a low-power (0.25 μJ/pulse), 10-kHz Nd:YAG laser using both a conventional axial TOFMS and an orthogonal TOF instrument.[30] Their preliminary results indicated that the 10-kHz laser produced data similar to that of a conventional nitrogen laser, but they noted that significant gains in signal intensity by using higher laser power were needed to make the laser practical.

This report describes the use of a moderate-power (21 μJ/pulse) high repetition rate (1-kHz) Nd:YAG laser for use in AP-MALDI coupled with a quadrupole-time-of-flight mass spectrometer (QqTOFMS) for high throughput MALDI-TOFMS. Results obtained with the high repetition rate laser are compared with those of a low repetition rate nitrogen laser in the analysis of several model peptides, peptide sequencing, and peptide mass mapping.

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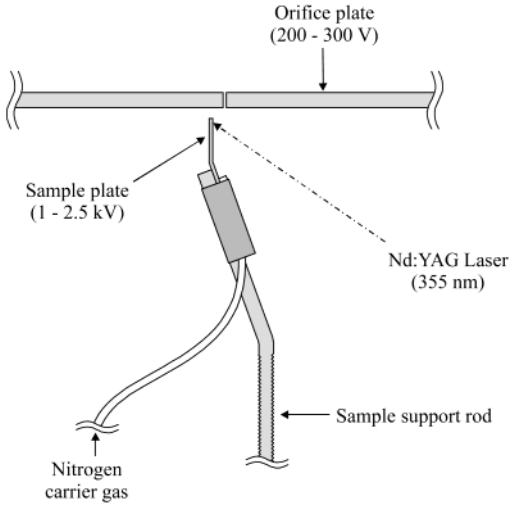

Figure 1. Schematic diagram of the atmospheric pressure MALDI sample probe used in these studies. The stainless steel sample plate (8 mm × 10 mm) is attached to a threaded support rod that screws directly into the electrospray source of the instrument. Nitrogen carrier gas is directed over the face of the sample plate via Teflon tubing ($^1/_{16}$ in. o.d. × 0.8 mm i.d.) held at the back of the plate by heat shrink (shown in dark gray). The nitrogen gas is supplied by the instrument connection used for electrospray operation. The sample plate is held at 1−2.5 kV and the orifice plate (254-μm orifice diameter) is typically held at 200−300 V.

EXPERIMENTAL SECTION

High Repetition Rate Laser and Atmospheric Pressure MALDI Source. The solid-state Nd:YAG laser used in these studies (model PowerChip PNV, JDS Uniphase, San Jose, CA) is a diode-pumped, passively Q-switched laser operated at a repetition rate of 1 kHz. The frequency-tripled output (355 nm) provides pulse widths and energies of <500 ps and ∼21 μJ, respectively. For comparison purposes, a nitrogen laser (model VSL-337ND-S, Thermo Laser Science, Franklin, MA) was also used. The latter provides 337-nm pulse widths and energies of <4 ns and ∼300 μJ, respectively. For both lasers, the beam was attenuated to a threshold level by varying the optical density of a metallic neutral density filter. The beam was then focused onto a MALDI sample plate by means of a 20-cm-focal length achromatic lens. The Gaussian beam profile (∼1-mm diameter) of the Nd:YAG output was focused to a spot ∼200 μm in diameter. The nitrogen laser output (rectangular ∼5 mm × 7 mm) was focused to an ellipse of ∼300 μm × 200 μm.

To interface AP-MALDI with the mass spectrometer, a sample probe was constructed in-house and is illustrated in Figure 1. Briefly, a stainless steel sample plate (8 mm × 10 mm) was welded to a threaded support rod (10−32 threaded stainless steel stud) that screws directly into the ion spray source typically used with this instrument. Positioning of the sample plate relative to the mass spectrometer inlet orifice was accomplished by adjusting the $x-y-z$ linear positioners intrinsic to the electrospray source. Nitrogen carrier gas was directed over the face of the sample plate via Tefzel tubing ($^1/_{16}$ in. o.d. × 0.8 mm i.d.) held in the center, but at the back of the sample plate by heat shrink. Nitrogen gas flow (GS1) rate was computer controlled and varied (arbitrary units), but no significant dependence of flow rate on signal intensity was observed. In these studies, the sample plate was biased at 1−2.8 kV (typically 2 kV) by the electrospray power

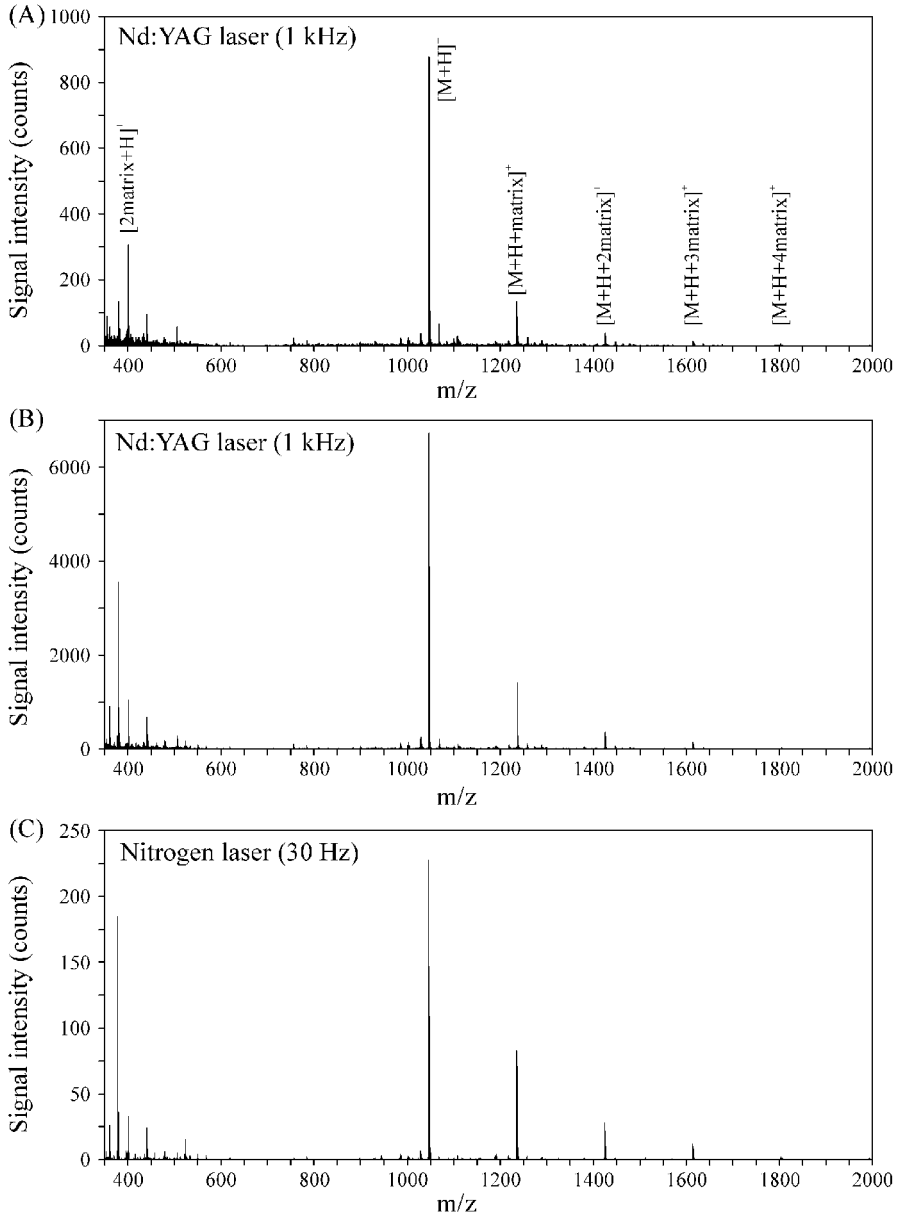

Figure 2. AP-MALDI mass spectra obtained for 5 pmol of angiotensin II (DRVYIHPF) using (A) 1-s scan using a Nd:YAG laser at 1 kHz, (B) summation of scans obtained over 20 s using a Nd:YAG laser at 1 kHz, and (C) summation of scans obtained over 20 s using a nitrogen laser at 30 Hz.

supply and the orifice plate of the instrument was typically held at 200−300 V.

Quadrupole-Time-of-Flight Mass Spectrometer. Mass spectra were acquired using an API QStar Pulsar (MDS Sciex, Concord, ON, Canada) quadrupole-time-of-flight mass spectrometer.[31] For AP-MALDI operation, the curtain gas plate was removed, but no other modifications were made to the QqTOFMS instrument. The AP-MALDI sample plate was positioned 1−2 mm from and perpendicular to the orifice plate of the instrument. The orifice plate aperture is 254 μm in diameter. The laser beam was focused on the sample plate at ~45° relative to the normal of the probe. For comparison in tandem MS analysis, a nanospray source (Protana, Odense, Denmark) was also used. The nanospray needle voltage was set at 900 V. Spectra were obtained in MCA mode with the "enhance all" feature activated, for both AP-MALDI and nanospray.[32]

Samples and Preparation. The following peptides and proteins were used in these studies: angiotensin II (FW 1045.54), bradykinin (FW 1059.55), [des-arg^9]-bradykinin (FW 903.46), cytochrome *c* (horse heart, FW 11 701.55), and lysozyme (chicken egg white, FW 14 313.14) were obtained from Sigma-Aldrich (St. Louis, MO) and used without further purification. Enzymatic digestion of the cytochrome *c* and lysozyme was performed by using sequencing grade modified trypsin (Promega, Madison, WI) with thermal denaturation as described elsewhere.[19,20] Briefly, protein samples were dissolved in 50 mM ammonium bicarbonate

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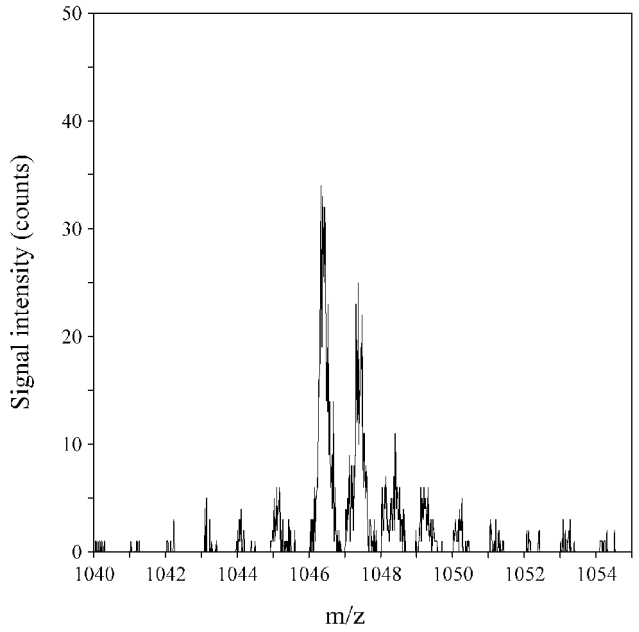

Figure 3. Partial mass spectrum obtained for 50 fmol of angiotensin II. AP-MALDI-QqTOFMS was performed using the Nd:YAG laser operated at 1 kHz.

(Sigma) and thermally denatured at 90 °C for 20 min. Denaturation was then quenched by placing the samples in a freezer and followed by trypsin digestion at 37 °C for 4 h. The concentration of trypsin was maintained at 40:1 (weight of substrate/weight of trypsin). For analysis, samples were deposited within 2 mm of the edge of the sample probe closest to the orifice using the overlayer technique with α-cyano-4-hyroxycinnamic acid as matrix.[33,34]

RESULTS AND DISCUSSION

There have been major technological advances made in TOF instrumentation over the past decade. For example, modern TOF instruments equipped with detectors that operate on the picosecond time scale are more common, and significant advances have been made in the design and construction of multidimensional position-sensitive detectors.[35,36] Gigahertz digitizers with picosecond time measurement accuracy are now commercially available for reasonable cost. Orthogonal TOFMS instruments typically operate at ∼10 kHz, which allows for the detection of a nearly continuous beam of ions from the source, but utilize MALDI laser repetition rates of only 1−30 Hz (i.e., tens of milliseconds from shot to shot). Clearly, the pulse rate of the laser limits the throughput of the MALDI-TOFMS experiment. Thus, to increase sample throughput, we have evaluated a 1-kHz Nd:YAG laser to perform atmospheric pressure-MALDI-QqTOFMS analyses.

Atmospheric Pressure MALDI at High (1 kHz) and Low (30 Hz) Laser Repetition Rates. In coupling atmospheric pressure ion sources with the high-vacuum region necessary for MS, ion transport efficiency is typically limited to <0.1%.[37] This can be largely attributed to two factors: (1) scattering and neutralization losses owing to the short mean free path at atmospheric pressure in air (∼(6−7) × 10^{-8} m), and (2) spatially directing the transport of ions through a small aperture required to isolate the vacuum. In these studies, there are six primary factors affecting the ion transport and ion yield, including the following: the distance between the sample plate and the orifice plate, the radial distance from the orifice to the face of the sample plate, the sample plate and orifice plate voltages, the carrier gas flow rate, the laser wavelength, and the laser repetition rate. For the probe design in Figure 1, these parameters were investigated sequentially to provide maximal signal intensity.

The optimal position of the sample plate with respect to the orifice was found empirically to be approximately 1−1.5 mm from the face of the orifice plate and ∼2 mm radially displaced from the aperture. This position was held constant, and the sample plate voltage was varied from 0 to 2500 V. Signal intensity was severely degraded at voltages below ∼1000 V and was highest between 1500 and 2000 V. Previous reports[12,24] indicated that the position and flow rate of a carrier gas flow provided a significant improvement in sensitivity. In this work, the position and gas flow rate did not appear to have a significant affect on the signal intensities obtained; thus, the nitrogen gas flow rate was reduced to the minimum value (arbitrary units) allowable by the instrument.

The effect of laser wavelength and laser shot repetition rate on the signal intensity obtained in the analysis of angiotensin II is illustrated in Figure 2. The mass spectrum for angiotensin II (Figure 2A) was obtained in 1 s with the Nd:YAG laser operated at 1 kHz. A signal-to-noise ratio greater than 500:1 was obtained, indicating that even faster data acquisition can be used. The throughput advantage of the Nd:YAG (1 kHz) over the nitrogen laser (30 Hz) is also apparent. Where the nitrogen laser takes approximately 20−30 s to produce an equivalent spectrum obtained using the Nd:YAG in less than 1 s, this corresponds to a potential increase of the daily sample output from the mass spectrometer from a couple of thousand to tens of thousands of samples per day.

Panels B and C of Figure 2 are 20-s acquisitions for angiotensin II using the Nd:YAG laser and the nitrogen laser operated at 1 kHz and 30 Hz, respectively. Three primary findings are evident in a comparison of the Nd:YAG laser operated at 1 kHz and the nitrogen laser operated at 30 Hz. First, in both cases, the peaks observed are similar; the dominant ion is $[M + H]^+$ (m/z = 1046.54), followed by matrix dimer $[2\text{matrix} + H]^+$ (m/z = 379.09), and matrix adducts with the protonated peptide $[M + H + \text{matrix}]^+$ (m/z = 1235.62), $[M + H + 2\text{matrix}]^+$ (m/z = 1424.67), $[M + H + 3\text{matrix}]^+$ (m/z = 1613.73), and $[M + H + 4\text{matrix}]^+$ (m/z = 1802.82). Additionally, low-intensity peaks are observed for small molecule loss and alkali metal adduction. Second, the relative abundance of matrix adducts is lower with the high repetition rate Nd:YAG laser in comparison with the nitrogen laser. Finally, the signal intensity observed with the Nd:YAG laser is a factor of ∼20 higher than that obtained with the nitrogen laser and in some cases up to 80-fold higher. This relative

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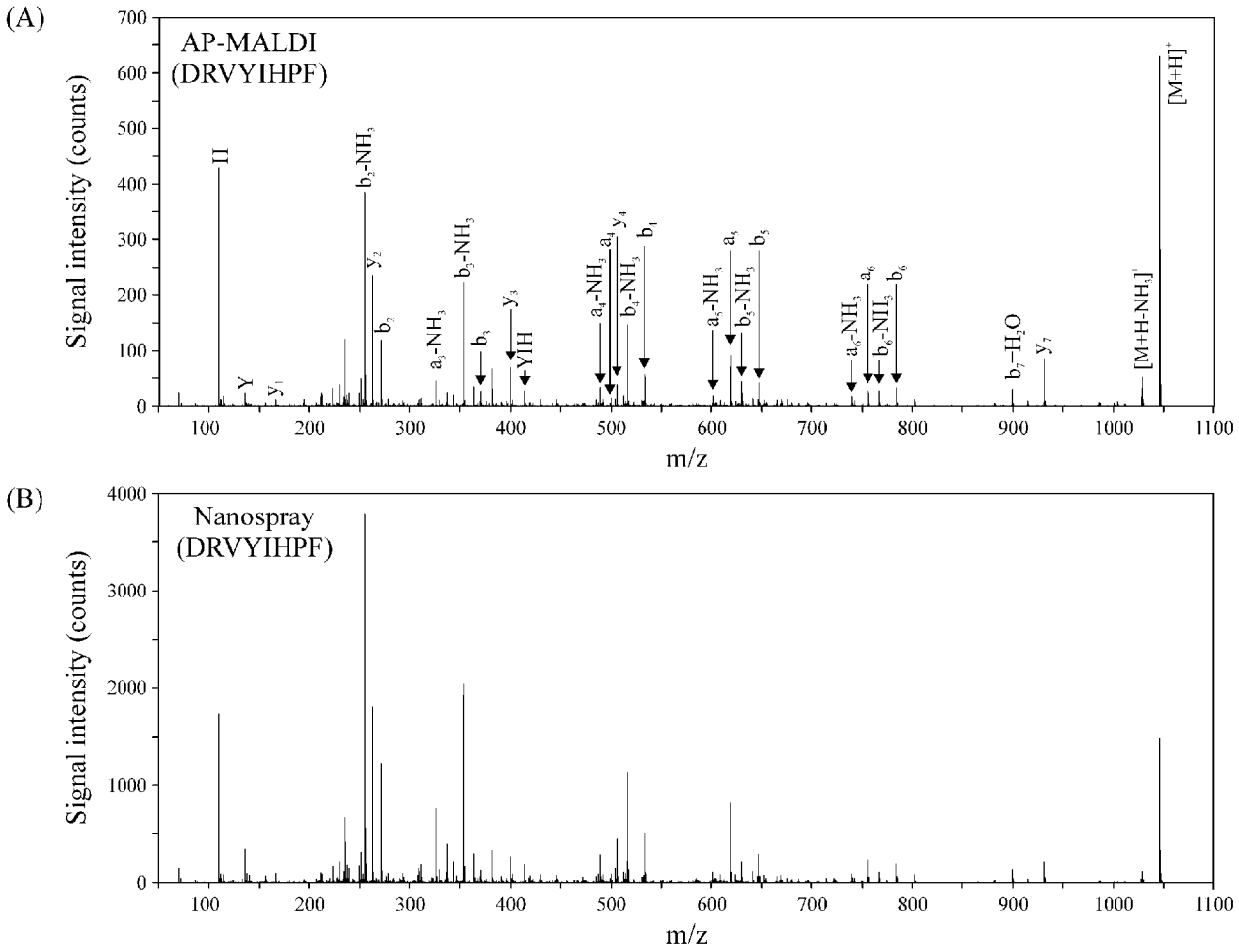

Figure 4. Comparison of the $[M + H]^+$ fragment ion spectra obtained for angiotensin II by (A) AP-MALDI (5 pmol) using the Nd:YAG laser operated at 1 kHz and (B) nanospray (2 ng/μL) operated at 900 V.

enhancement in signal intensity is not a consequence of desorption/ablation of an absolute greater amount of analyte since the entire sample spot is consumed (data not shown), similar to previous AP-MALDI studies.[12,24]

Three factors may contribute to the relative signal intensity enhancement with the high repetition rate Nd:YAG laser: (1) a higher relative ion transmission efficiency from the point of ion production to their introduction into the mass spectrometer, (2) a greater ion yield as a consequence of different laser optical parameters (e.g., wavelength, beam profile, and fluence), and (3) an enhanced ion yield as a consequence of operation at higher shot-to-shot repetition rates. Higher ion transmission efficiency is not likely, because the physical position of the probe was similar and the voltages were identical for both lasers. In turn, a notable difference in the ion yield owing to differences in the optical parameters is also not likely, but difficult to determine empirically. The most significant difference, besides repetition rate, is the wavelength used for desorption/ionization being 355 nm for the Nd:YAG laser and 337 nm for the nitrogen laser. However, the solid-phase absorption coefficient for CHCA matrix is not significantly different over the wavelength range used in these studies (337−355 nm, $\sim(2-3.5) \times 10^5$ cm^{-1}),[38] indicating that the difference in wavelengths should not yield a large difference in the signal intensity obtained. Thus, the relative enhancement in sensitivity is attributable to operation at higher laser repetition rates.

Although the intensity of the signal obtained using the Nd:YAG laser was superior to that of the nitrogen laser, the overall sensitivity of AP-MALDI is lower than conventional high-vacuum MALDI.[12,24,25] Figure 3 shows the signal obtained from spotting 50 fmol of angiotensin II on to the sample stage and performing MALDI at 1 kHz. The signal-to-noise ratio obtained is ~10:1, which is approaching the detection limit. The decrease in sensitivity by using AP-MALDI versus high-vacuum MALDI is attributed to the overall lower ion transport efficiency into the mass spectrometer and to the partitioning of the analyte ion signal intensity into both analyte and analyte/matrix cluster ion channels (Figure 2). The formation of analyte/matrix cluster ions has been observed in several high-pressure MALDI experiments, for example, MALDI-ion mobility-TOFMS,[9] MALDI Fourier transform ion cyclotron resonance-MS,[39] AP-MALDI-TOFMS,[12] and AP-MALDI-ion trap-MS.[24,25,27] The formation of these analyte/matrix clusters is attributed to collisional cooling of ions at high pressure. However, it should be noted that the relative enhancement in signal intensity owing to operation at high laser repetition rates can be readily extended to high-vacuum MALDI applications.

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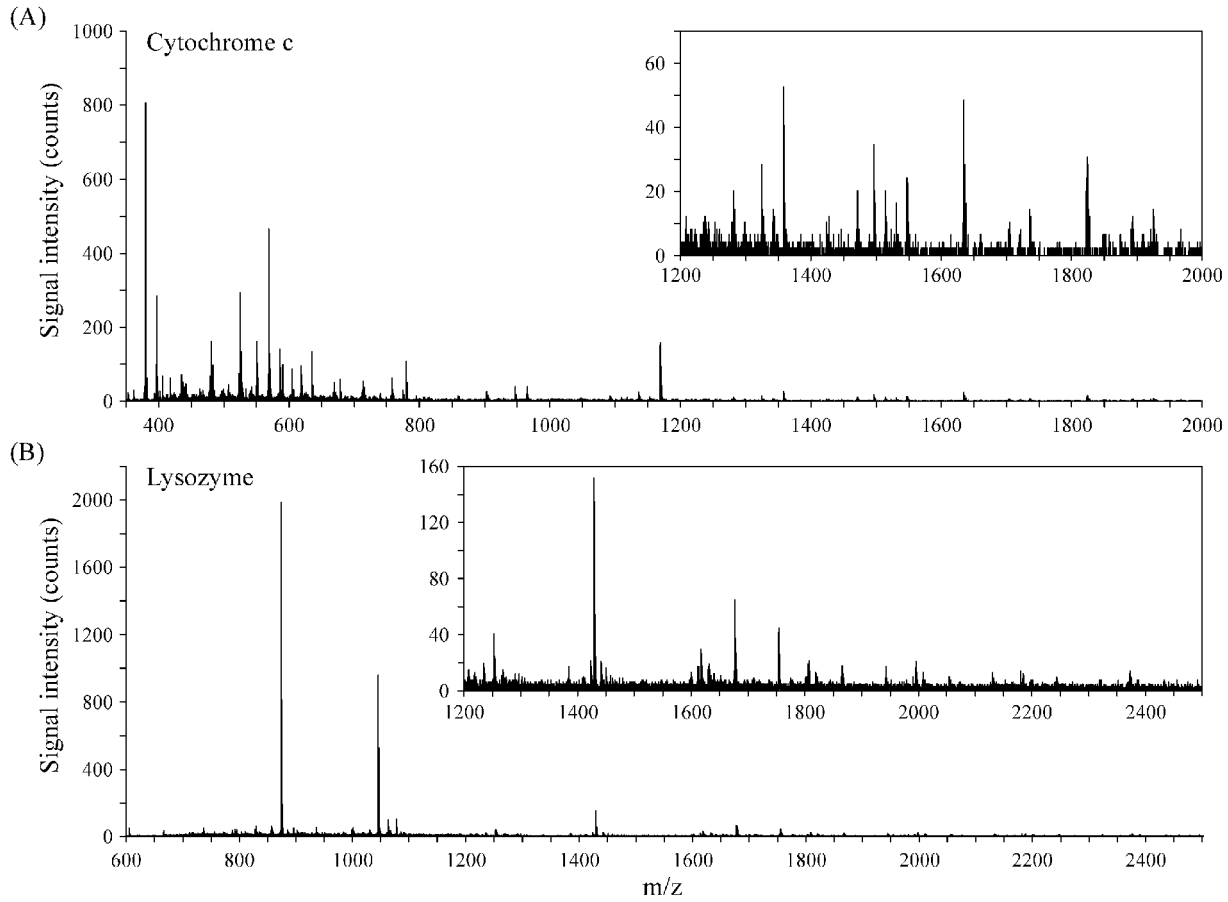

Figure 5. Mass spectra of (A) tryptic digest of cytochrome *c* (horse heart) and (B) tryptic digest of lysozyme (chicken egg white) using the Nd:YAG laser operated at 1 kHz. The positions of observed fragment ions in cytochrome *c* and lysozyme are listed in Tables 1 and 2, respectively. Insets are expanded views of the indicated mass range of the spectrum.

Peptide Sequencing and Peptide Mass Mapping. The utility of high laser repetition rate MALDI for proteomic applications was further evaluated for peptide sequencing and peptide mass mapping. Figure 4 illustrates peptide sequencing performed on $[M + H]^+$ of angiotensin II (FW 1045.54) using tandem MS with both the 1-kHz AP-MALDI technique (Figure 4A) and nanospray (Figure 4B). Both AP-MALDI and nanospray provide similar fragmentation patterns for angiotensin II, where y-, b-, and a-type ions are observed in addition to several internal fragment ions. Although in both cases complete sequence coverage was achieved, the fragment ion intensity in the nanospray mode is a factor of ~10 greater than that observed by using AP-MALDI. However, high repetition rate, high-vacuum MALDI should provide significant enhancements in the fragment ion intensity obtained.

Peptide mass mapping was performed by analyzing tryptic digestion fragments of cytochrome *c* and lysozyme as shown in the spectra of Figure 5. In both cases, a large number of intense fragments are observed which are listed in Table 1 and Table 2 for cytochrome *c* and lysozyme, respectively. For cytochrome *c*, protein sequence coverage of 78.8% was achieved with a mass accuracy of 3.1−32 ppm. Nearly complete coverage is obtained if low-intensity fragment ions are also considered but with a concomitant degradation of mass accuracy for those fragments owing to poorer counting statistics. Somewhat lower sequence coverage (53.5%) was obtained for lysozyme with mass accuracies ranging from 0.54 to 83 ppm. Lower sequence coverage for lysozyme is expected owing to the "harder" nature of the protein; that is, lysozyme contains four disulfide bonds whereas cytochrome *c* contains none. Interestingly, in these peptide mass mapping studies, little matrix/adduct cluster ion formation was evident in comparison with the extensive adduct formation observed for angiotensin II alone (Figure 2) or for bradykinin or [des-arg^9]-bradykinin (data not shown). The mechanism(s) of ionization, cluster formation, and matrix effects in high repetition

Table 1. Listing of Fragments Observed, Experimental Mass, Theoretical Mass, and Mass Accuracy for a Tryptic Digest of Cytochrome *c* (Horse Heart)

	mass (m/z)		
position	experimental	theoretical	error (ppm)
1−5[a]	589.2786	589.2828	−7.1
56−60	604.3477	604.3453	4.0
9−13	634.3901	634.3922	−3.3
74−79	678.3781	678.3821	−5.9
80−86	779.4434	779.4484	−6.4
92−99	964.5260	964.5349	−9.2
28−38	1168.6522	1168.6221	26
40−53	1470.6814	1470.6859	−3.1
61−72	1495.6628	1495.6985	−24
14−22 + heme	1634.6748	1634.6217	32

[a] Modified by acetylation of N-terminus.

Table 2. Listing of Fragments Observed, Experimental Mass, Theoretical Mass, and Mass Accuracy for a Tryptic Digest of Lysozyme (Chicken Egg White)

	mass (m/z)		
position	experimental	theoretical	error (ppm)
19−23	606.3751	606.3722	4.8
33−39	874.4153	874.4166	−1.5
80−86	936.3545	936.3781	−25
32−39	1030.5071	1030.5177	−10
135−143	1045.5400	1045.5425	−2.4
19−31	1423.6363	1423.7548	−83
52−63	1428.6668	1428.6502	11.6
116−130[a]	1676.7800	1676.7809	−0.54
64−79	1753.8296	1753.8351	−3.1

[a] N → D variant at position 121.

rate MALDI at atmospheric pressure are the subject of a separate report.[40]

CONCLUSIONS

The use of high MALDI laser repetition rates provides a significant improvement in sample throughput while a high level of data quality in terms of sensitivity and mass accuracy is retained. Although the potential increase in sample throughput is possibly beyond the needs of most laboratories, the time savings allows for more time to be spent away from routine sample analysis and to focus on more challenging projects. Importantly, one can conceptualize proteomic-scale projects that for practical purposes will require this apparent 80-fold increase in sample throughput. The 1-kHz (or greater) laser repetition rate provides a nearly continuous ion beam making it ideally suited for MALDI sample introduction for orthogonal TOF hybrid instruments. Although these studies have focused on the use of high laser repetition rates for atmospheric pressure MALDI, in principle even better figures of merit should be obtained when used with high-vacuum MALDI systems, a subject of ongoing investigations in our laboratories.

(40) Russell, W. K.; McLean, J. A.; Russell, D. H. *Int. J. Mass Spectrom.*, in preparation.

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