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Miniaturized Ultra High Field Asymmetric Waveform Ion Mobility Spectrometry Combined with Mass Spectrometry for Peptide Analysis

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Miniaturized ultra high field asymmetric waveform ion mobility spectrometry (ultra-FAIMS) combined with mass spectrometry (MS) has been applied to the analysis of standard and tryptic peptides, derived from α-1-acid glycoprotein, using electrospray and nanoelectrospray ion sources. Singly and multiply charged peptide ions were separated in the gas phase using ultra-FAIMS and detected by ion trap and time-of-flight MS. The small compensation voltage (CV) window for the transmission of singly charged ions demonstrates the ability of ultra-FAIMS-MS to generate *pseudo*-peptide mass fingerprints that may be used to simplify spectra and identify proteins by database searching. Multiply charged ions required a higher CV for transmission, and ions with different amino acid sequences may be separated on the basis of their differential ion mobility. A partial separation of conformers was also observed for the doubly charged ion of bradykinin. Selection on the basis of charge state and differential mobility prior to tandem mass spectrometry facilitates peptide and protein identification by allowing precursor ions to be identified with greater selectivity, thus reducing spectral complexity and enhancing MS detection.

The preseparation of ions on the basis of charge state prior to mass spectrometry has been shown to provide additional selectivity by simplifying ion populations. In traditional drift tube ion mobility[1] and traveling wave ion mobility spectrometry[2] systems, ions are separated on the basis of absolute mobility. However, field asymmetric waveform ion mobility spectrometry (FAIMS), or differential mobility spectrometry, separates ions on the basis of compound-dependent differences in ion mobility under high and low electric fields.[3−6] Ions traveling between two electrodes at atmospheric pressure are subjected to alternating high and low field conditions by application of an asymmetric RF waveform, known as the dispersion field (DF). The mobility of an ion at a constant pressure subjected to a high-field electric waveform (K_h) is described by;

$$K_h(E) = K_0(1 + \alpha(E)) \quad (1)$$

where K_0 is the reduced mobility at low electric field, E is the high-field component of the electric waveform, and α is a coefficient for the dependence of mobility of an ion on the electric field strength, described by the following equation, where N is the number density of the buffer gas:

$$\alpha(E/N) = \{1 + \alpha(E/N)^2 + b(E/N)^4 + c(E/N)^6....\} \quad (2)$$

Ions with a $K_h(E)$ value not equal to zero accumulate a net drift toward an electrode eventually resulting in a collision and neutralization. A small dc voltage, the compensation voltage (CV), is applied to one of the electrodes to reverse the drift of the ion so that collision with the electrode walls is avoided, allowing the ion to pass between the electrodes. The CV for ion transmission is compound specific, so ions of only a single differential mobility will be transmitted through the device at a selected CV. FAIMS devices either can be used to obtain a complete representation of all ions generated in the ion source by scanning over the full CV range or can be used as an ion filter, set to allow selected ions through the device. The separation of ions by the difference in high and low field mobility is orthogonal to m/z separation, making the hyphenation of FAIMS with mass spectrometry (MS) a powerful tandem combination.[7]

The use of FAIMS for the preseparation of ions to facilitate the rapid, high throughput analysis of peptides is well documented and has been demonstrated to be a useful means of increasing the selectivity of the analysis and reducing the complexity of samples in order to enhance MS detection. The first report of the application of FAIMS to peptide ions described the selective determination of isobaric leucine enkephalin complexes in mix-

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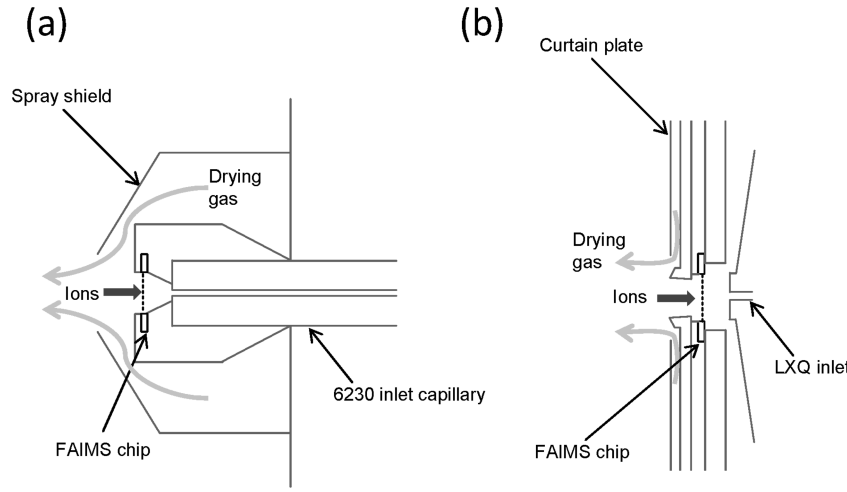

Figure 1. Schematic diagrams of (a) the ultra-FAIMS chip mounted into the chip cartridge and housed within the Thermo "Ion Max" ion source and (b) located within the spray shield of the Agilent Jet Stream source.

tures with a noted improvement in mass spectral quality with background ions no longer masking target analytes.[8] FAIMS has also been able to distinguish peptide[9] and intact protein conformers,[10−13] a feature utilized in the application of FAIMS to the selective transmission of target conformers prior to investigations into electron capture dissociation and detection by Fourier transform ion cyclotron resonance MS.[14]

The first report of the application of a FAIMS-MS method to the analysis of tryptic peptides observed the reduction in chemical background reported in the FAIMS-MS analysis.[15] In addition, charge state separation was reported, previously observed in conventional ion mobility systems.[16] The reduction in background signals in the detection of low-level peptides was later exploited to enhance the quality of MS/MS data.[17] Recently, FAIMS has been interfaced with a hybrid LTQ-Orbitrap mass spectrometer, facilitating the identification of low-abundance peptide ions and increasing the number of assigned MS/MS spectra, contributing to an overall improvement in protein identification and sequence coverage.[18] It was proposed[19] that FAIMS could be successfully interfaced with other analytical techniques such as conventional ion mobility and LC-MS, for added selectivity, as large proportions of multiply charged tryptic peptides could be detected by monitoring a few specific CV values.[16,20,21]

Recently, there has been a move toward the development of FAIMS devices with planar electrodes, rather than the cylindrical electrodes used in earlier devices. Coupled with mass spectrometry and electrospray ionization (ESI), transmitted ions have been introduced into the mass spectrometer by means of a hole in the positive ion detector plate[22] or an ion funnel.[23] Both methods of ion introduction showed an increase in resolving power, which resulted in the discovery of new peptide and protein conformers.[23] Further studies with the latter of these devices have focused on the use of helium/nitrogen gas mixtures which improved the resolution, allowing the separation of unresolved peptide conformers[24] and small molecules derived from complex biological samples such as tryptic digests.[25]

In this work, we report the use of a novel, miniaturized ultra high field FAIMS (ultra-FAIMS) device, made from a silicon microchip mounted onto a printed circuit board,[26−28] for the analysis of peptides. The parallel planar electrode channels are an order of magnitude smaller than previous electrode designs, permitting higher electric field intensities to be applied across the gap without the electrical breakdown of gases at atmospheric pressure. The smaller gap allows fields to be increased from 25 to 60 kV cm^{-1}, allowing analysis on the millisecond time scale[27] without the loss of resolution experienced with earlier FAIMS technologies. The interleaved electrode pairs enable multiple,

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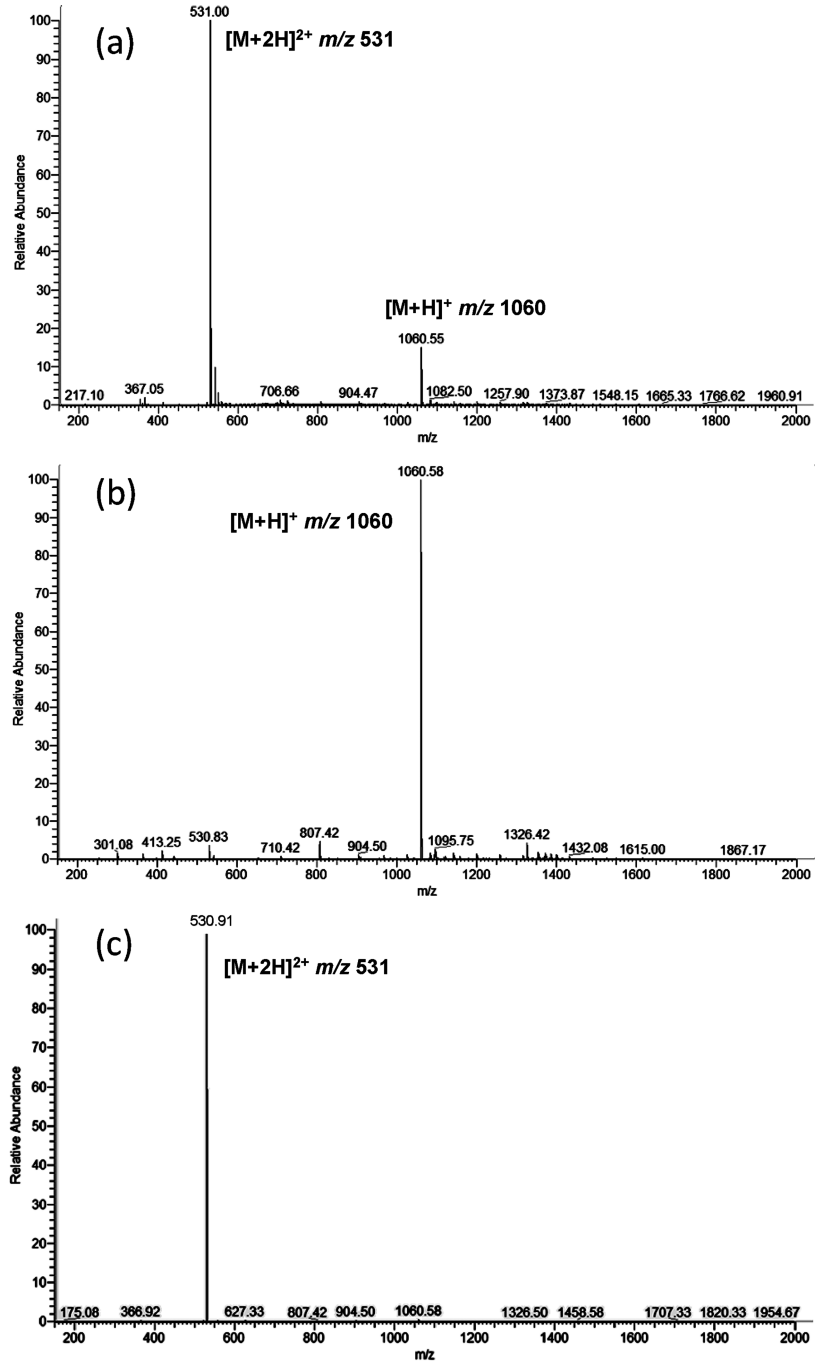

Figure 2. FAIMS-MS charge state separation of bradykinin ions. (a) Ion trap mass spectrum without FAIMS separation (S/N ratio 100:1 (*m*/*z* 1060); 1000:1 (*m*/*z* 531)). (b) Selective enhancement of the $[M + H]^+$ (*m*/*z* 1060) ion at a CV of +1.4 V (S/N ratio 1000:1) and (c) selective enhancement of the $[M + 2H]^{2+}$ (*m*/*z* 531) ion at a CV value of +3.5 V (S/N ratio 10 000:1).

simultaneous ion transmission through the electrodes, reducing the charge capacity constraints previously observed in planar FAIMS devices and potentially increasing the sensitivity. Charge state and conformational separation of model and tryptic peptides is demonstrated using ultra-FAIMS combined with ion trap and time-of-flight mass spectrometry. Preseparation of peptide ions on the basis of their charge state facilitates rapid, high-throughput protein identification by increasing the selectivity of the analysis and reducing the sample complexity.

EXPERIMENTAL METHODS

Instrumentation. ESI-FAIMS-MS analysis was carried out in positive ion mode using a prototype miniaturized ultra-FAIMS device (Owlstone, Cambridge, UK),[26] interfaced to an ion trap (LTQ, Thermo Scientific, Hemel Hempstead, UK) or a time-of-flight (TOF; 6230 series, Agilent, Stockport, UK) mass spectrometer. The ultra-FAIMS chip has been described elsewhere[26] and consists of a serpentine channel, consisting of 47 individual channels with a channel width of 35 μm and a channel length of 300 μm. In both interfaces, the FAIMS chip was connected to a

field generator module, containing the electrical circuitry required to generate field asymmetric waveforms. The frequency of the applied asymmetric waveform was 22.2 MHz for the ion trap interface and 25 MHz for the TOF interface, in a roughly 2:1 ratio of low field to high field. FAIMS separation was carried out at a maximum 60 kV cm^{-1} dispersion field, over a compensation voltage (CV) range of −2 to +5 V, at a sweep rate of 0.5 V s^{-1}.

The ultra-FAIMS device was located within the existing ESI and nanoelectrospray ionization (NSI) source housing of the TOF and ion trap mass spectrometers as shown schematically in Figure 1.[28] In the ultra-FAIMS-ion trap configuration (Figure 1a), standard peptides and tryptic digests were ionized using a 1 μm Picotip emitter (New Objective, Massachusetts, USA) with an applied voltage of 1.1 kV. A 50 V bias was applied to the curtain plate and orifice, and a 30 V bias was applied to the chip, encouraging ions to be drawn into the FAIMS device. The LTQ capillary temperature was held at 60 °C, and the capillary voltage was at 0 V, with the FAIMS high purity nitrogen carrier gas set at a flow rate of 2.3 L min^{-1} and a temperature of 60 °C. The ion trap was scanned in MS mode over the m/z range of 150−2000 using a maximum ion injection time of 100 ms and no scan averaging. Data acquisition under MS^n mode was carried out using data-dependent scanning on the base peak, using a normalized collision energy of 35%.

The electrospray source of the TOF mass spectrometer was modified to accommodate the presence of the ultra-FAIMS chip within the spray shield (Figure 1b). The ESI source was operated at a flow rate of 100 μL min^{-1} and a capillary voltage of 1.5 kV. The drying gas flow was 5 L min^{-1} at a temperature of 150 °C. The nebulizer was operated at 15 psi, and the sheath gas flow was at 12 L min^{-1} at 250 °C. The spray shield was set at 400 V; the ultra-FAIMS chip was at 0 V, and the capillary inlet was at −20 V. The fragmentor voltage was set to 150 V, and the skimmer voltage was set to 65 V. TOF spectra acquisitions were performed at a rate of 50 spectra s^{-1}. Data were processed using Xcalibur version 2.0.7 (Thermo Scientific, Hemel Hempstead, UK), Mass Hunter version B.01.03 (Agilent, Stockport, UK) and Excel 2003 (Microsoft, Seattle, USA).

Sample Preparation. High-performance liquid chromatography (HPLC) grade methanol, water, and formic acid (FA) were purchased from Fisher Scientific (Loughborough, UK). Angiotensin, (DRVYIHPFHLVI) bradykinin (RPPGFSPFR), substance P (RPKPQQFFGLM), bombesin (EQRLGNQWAVGHLM), and gramicidin S (*cyclo*(VOLDFP)$_2$ were obtained from Sigma Aldrich (Gillingham, UK). All peptide standards were prepared at concentrations between 10 and 16 nmol μL^{-1} in 50/50 (v/v) methanol/water containing 0.1% FA.

The tryptic digest was prepared from α-1-acid-glycoprotein (AAG; Athens Research and Technology, Georgia, USA). A 250 μL aliquot of a 1 mg mL^{-1} AAG solution in 25 mM ammonium bicarbonate was added to a 20 μL of 0.1 mg mL^{-1} trypsin Gold Mass Spectrometry grade (Promega, Southampton, UK) in 50 mM acetic acid and digested for 15 min at 55 °C, at 50 W, using a microwave digester (CEM Corporation, North Carolina, USA). The reaction was quenched with 100 μL of 1% trifluoroacetic acid (TFA) solution (Sigma Aldrich, Gillingham, UK), and the sample was cleaned up and concentrated using Supel-Tips C18 Micropipet Tips (Sigma-Aldridge, Gillingham, UK).

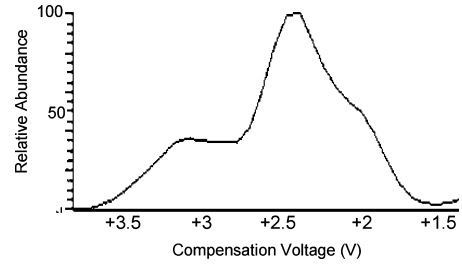

Figure 3. CV scan for the FAIMS-MS ion trap analysis of the bradykinin $[M + 2H]^{2+}$ ion at a DF of 60 kV cm^{-1}.

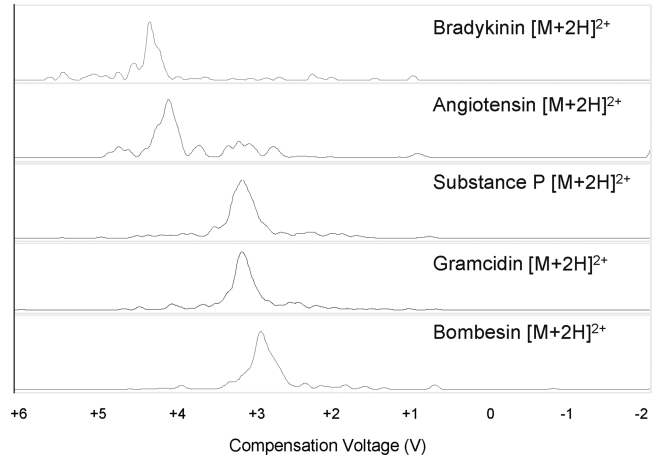

Figure 4. FAIMS-TOF-MS analysis of a mixture containing bradykinin (m/z 530.7), angiotensin (m/z 523.7), substance P (m/z 674.3), gramicidin S (m/z 571.3), and bombesin (m/z 810.4) at a DF of 60 kV cm^{-1}.

Each tip was prepared for binding with five cycles of 50% acetonitrile (ACN) in water and equilibrated with five cycles of 0.1% TFA in water. Twenty-five microliters of digested protein was bound to the tip by cycling the peptide solution 15 times. Salts were washed with seven cycles of 0.1% TFA in water, and the peptides were eluted in 8 μL of 80% ACN in water with 0.1% FA, giving a final concentration of digested AAG material of approximately 70 nmol μL^{-1}, assuming 100% digestion and recovery.

RESULTS AND DISCUSSION

Analysis of Standard Peptides. In order to ascertain effectiveness of the miniaturized ultra-FAIMS device, bradykinin was first directly infused into the source of the ion trap mass spectrometer without FAIMS separation. The $[M + H]^+$ (m/z 1060) and $[M + 2H]^{2+}$ (m/z 531) ions were observed in the resulting mass spectrum (Figure 2a). Data were then acquired while the FAIMS was scanned over a CV range of −2 to +4 V with a 60 kV cm^{-1} DF. The selected ion responses for both $[M + H]^+$ and $[M + 2H]^{2+}$ were extracted from the total ion response, and the optimum CVs for transmission of the ions were determined (CV values of +1.4 and +3.5 V, respectively). The FAIMS device was programmed to the appropriate CV for transmission, determined by the CV sweep, corresponding to the optimum ion counts. Figure 2 shows the selective enhancement with an increase in signal-to-noise ratio of an order of magnitude for both the singly (Figure 2b) and doubly (Figure 2c) charged ions under the appropriate CV conditions.

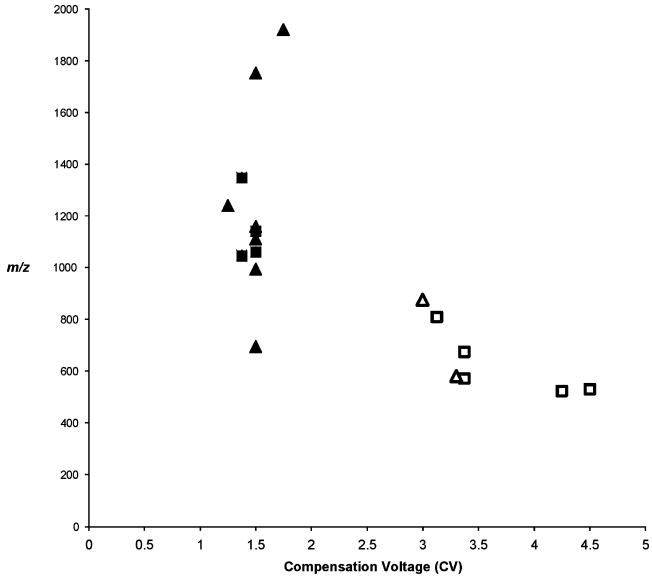

Figure 5. Compensation voltage vs *m/z* plot of singly and doubly charged standard and tryptic peptides ions: ■, singly charged standard peptide ions; ▲, singly charged tryptic peptide ions; □, doubly charged standard peptide ions; △, doubly charged tryptic peptide ions.

The CV scan from the FAIMS-MS ion trap analysis of the doubly charged bradykinin ion is shown in Figure 3. The shape of the trace for the $[M + 2H]^{2+}$ ion (m/z 531) indicates the presence of three partially resolved components with varying differential mobilities. This observed peak shape is very similar to that previously reported by Guevremont and Shvartsburg for the analysis of the $[M + 2H]^{2+}$ ion of bradykinin using a FAIMS interface with cylindrical electrodes,[9] although the shoulder on the main peak at a CV of +1.5 V is better resolved, demonstrating the ability of the ultra-FAIMS system to differentiate between structural conformations.

FAIMS-TOFMS analysis of angiotensin, bradykinin, gramicidin S, substance P, and bombesin was carried out at a DF of 60 kV cm^{-1}. The peptides were analyzed in triplicate both individually and as a mixture. The resulting CV spectra for the $[M + 2H]^{2+}$ ions are shown in Figure 4. The optimum CVs for the transmission of the singly and doubly charged peptide ions were calculated and plotted against their m/z ratio in Figure 5. This plot shows a clear separation of singly and doubly charged peptide ions. This type of plot has been reported previously in ESI-FAIMS-MS studies with cylindrical electrodes, depicting the CV dispersion of singly, doubly, and triply charged peptides.[15] While this reports a much narrower CV dispersion (5 V) for the singly charged peptides compared to multiply charged species (27 V), there is significant overlap of some low mass doubly charged ion in the singly charged region. Using the miniaturized ultra-FAIMS device, the singly charged peptide species all fall within a very narrow CV window for transmission (1.2−1.6 V), clearly resolved from the doubly charged ions, allowing the selective transmission of singly charged ions through the ultra-FAIMS. The CV range for the selective transmission of singly charged ions through the ultra-FAIMS uses 8.3% of the analytical space available, compared to 12.5% using cylindrical electrode FAIMS. Additionally, a scan of the singly charged peptide region using the ultra-FAIMS can be performed in less than a second compared to 5 s at the scan rate described with macroscopic systems.[15]

In contrast to the singly charged ions, separation of $[M + 2H]^{2+}$ peptide ions with different amino acid sequences was observed over a much wider CV range, demonstrating that these peptide ions may be separated on the basis of their differential ion mobility under the appropriate dispersion field conditions. The required CVs for the transmission of the five $[M + 2H]^{2+}$ standard peptide ions are observed in the range +3 to +4.5 V, shown in Figure 5. The wider CV window for the transmission of doubly charged ions limits the simultaneous transmission of all ions of the same charge state. However, this can be achieved by performing the analysis at a lower dispersion field or by scanning over the CV range +3 to +4.5 V which would take 3 s at a 0.5 V sec^{-1} scan speed. This scan rate is compatible with the typical peptide peak widths observed in HPLC analysis. However, faster scan times may be required when combined with ultra-performance liquid chromatography (UPLC) separations.

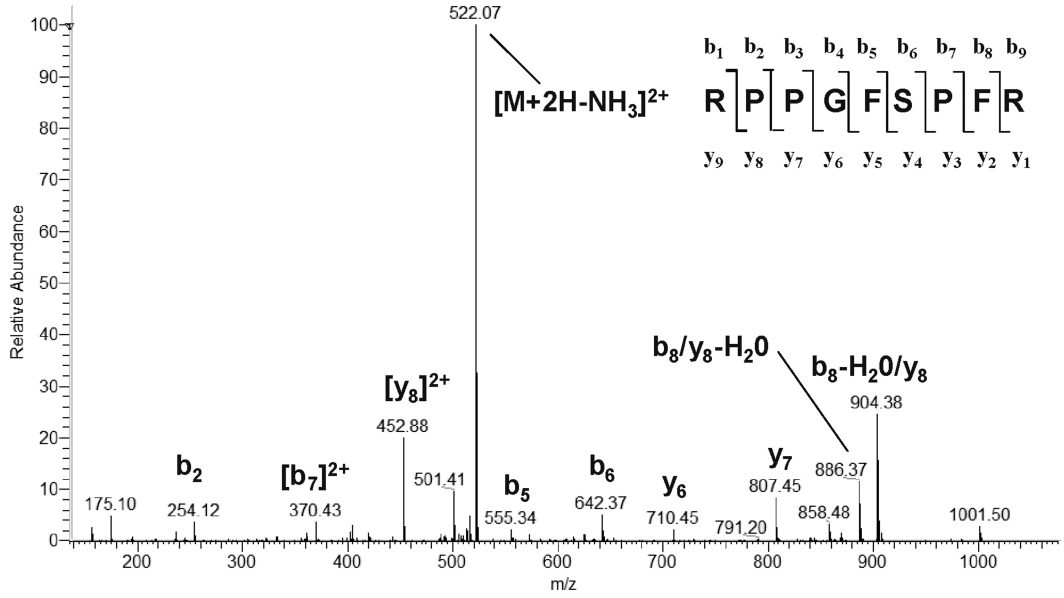

Figure 6. Ion trap tandem mass spectrometry of the ultra-FAIMS selected $[M + 2H]^{2+}$ ion of bradykinin (m/z 531).

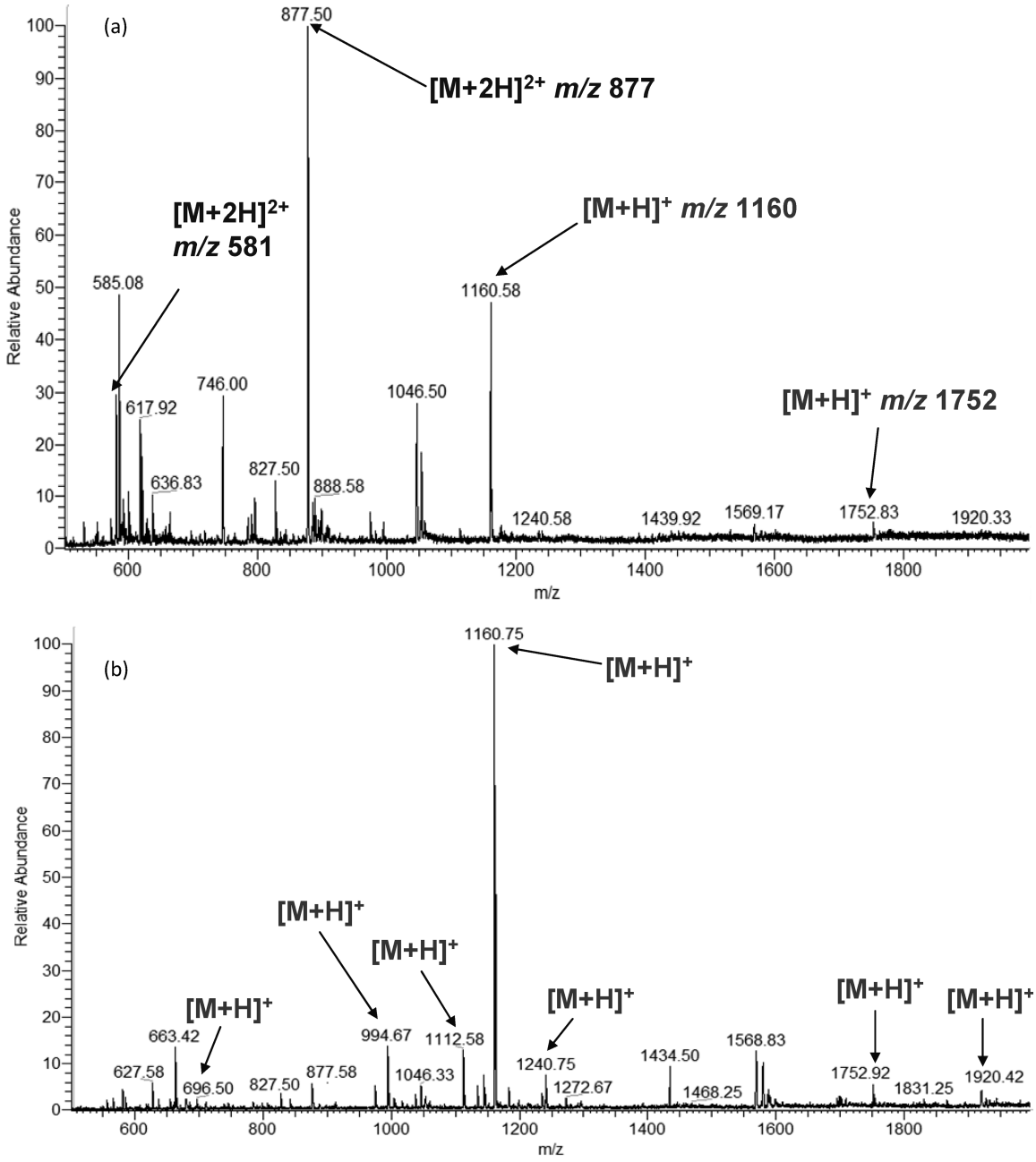

Figure 7. (a) Mass spectrum of the AAG tryptic digest with no FAIMS separation. (b) The mass spectra obtained with the CV window set to +1.5−+1.7 to isolate the singly charged ions.

Alternatively, the FAIMS selectivity can be exploited in the acquisition of tandem MS data, which requires confident selection of the precursor ion prior to fragmentation to avoid isobaric interferences. Separation of ions on the basis of differential mobility prior to tandem mass spectrometry allows a $[M + 2H]^{2+}$ precursor ion to be identified with greater selectivity. The ion trap tandem MS of the differential mobility resolved $[M + 2H]^{2+}$ ion of bradykinin showing b and y fragment ions covering the whole range of the nonapeptide is shown in Figure 6. This demonstrates the proof of principle for the ultra-FAIMS device to simplify complex mixtures by eliminating unwanted peptide responses in combination with MS/MS analyses. These charge state separations were observed for all of the peptide standards analyzed, and the ultra-FAIMS method was evaluated for the simplification of a complex tryptic peptide mixture.

Analysis of Tryptic Peptides. The ability of the ultra-FAIMS device to separate ion populations based on charge state was tested by applying the method to the analysis of singly and multiply charged tryptic peptides derived from the AAG protein. AAG has been identified as a biomarker for differentiating late stage metastatic melanoma patients from healthy controls.[29] The benefit of the hyphenated system is the selectivity afforded by the separation of ions from a complex sample, prior to mass spectrometric detection, allowing peptide responses to be identified with a reduction of noise and spectral interferences resulting in an overall simplification of the resulting mass spectra. The presence of isobaric interferences and multiple charge states in the spectrum, without additional FAIMS separation, may reduce confidence in protein identification.

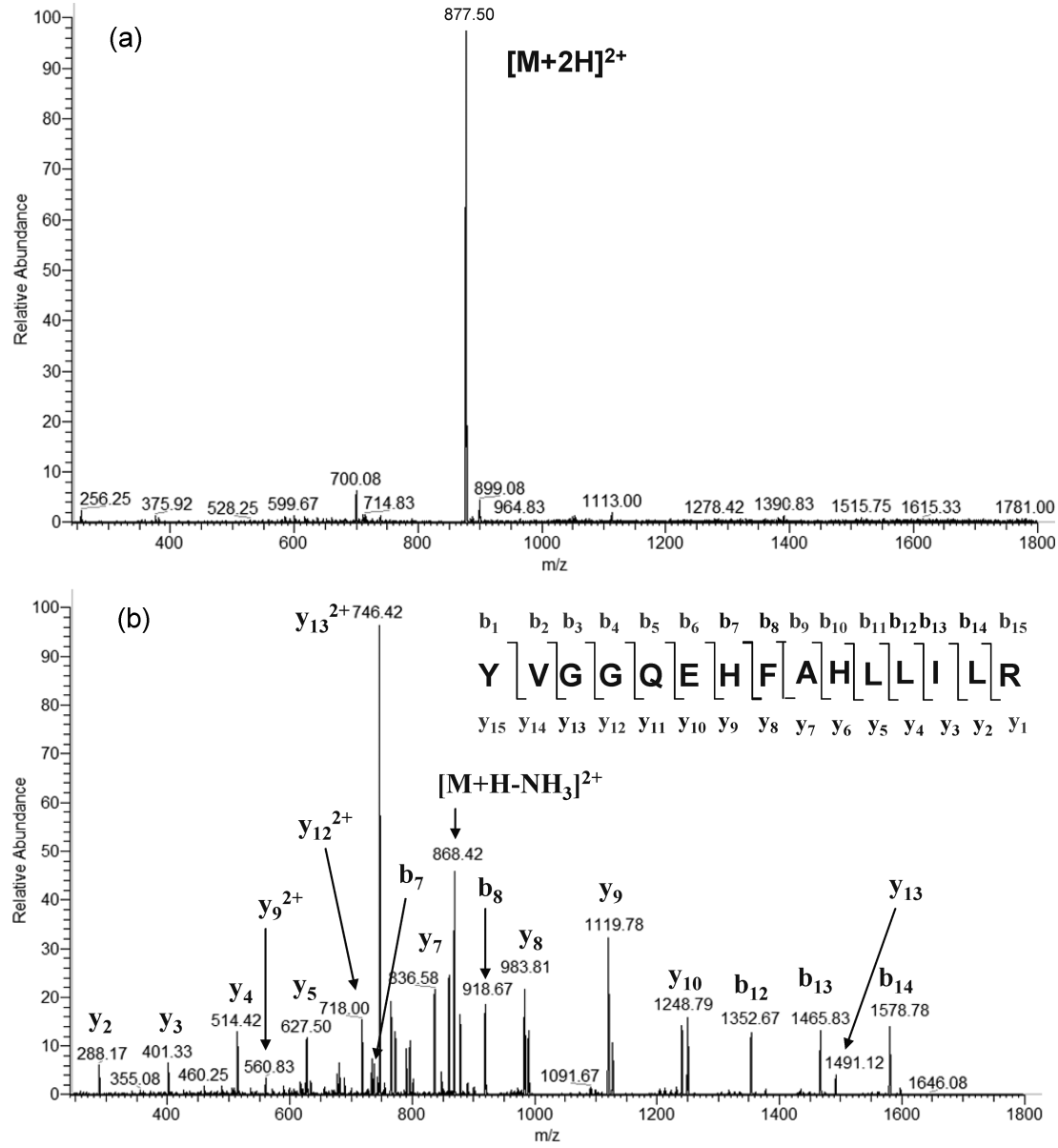

Figure 8. (a) Selective enhancement of the *m*/*z* 877 ion from the AAG tryptic digest and (b) the ion trap MS/MS spectrum of the *m*/*z* 877 ion.

The AAG tryptic digest was initially infused into the nanospray source of the ion trap mass spectrometer without FAIMS separation. The resulting mass spectrum is shown in Figure 7a. Data were then acquired as the CV was scanned over the range −2 to +4 V and the selected ion response for singly and doubly charged ions was extracted from the total ion response. The resulting CVs and *m*/*z* values for the tryptic peptides were added to the standard peptide data in Figure 5, showing the same narrow CV range for the singly charged tryptic peptide ions as the standard peptides. On this basis, a CV window of +1.5 to +1.7 V was applied to allow the selective transmission of the singly charged ions. Figure 7b shows the enhancement of the singly charged peptide ion responses, including the detection of ions previously masked by high intensity multiply charged species or lost in the baseline noise, as a result of the improvements in signal-to-noise ratios. The resulting spectrum demonstrates the ability of the miniaturized FAIMS-MS to generate $[M + H]^+$ data equivalent to a peptide mass fingerprint (PMF) obtained by MALDI ionization and commonly used to identify proteins from PMF databases. The term *pseudo*-peptide mass fingerprint (*p*-PMF) has been used previously in the context of the selective transmission of singly charged ions using drift tube IM-MS to distinguish the data from a true MALDI PMF[30] but not for FAIMS-MS. The ultra-FAIMS generated *p*-PMF for the AAG tryptic digest was searched against the SwissProt protein PMF database using the MASCOT[31] search engine using the top 20 highest intensity ions generated in a spectra list by the Xcalibur software. AAG was identified as the top hit with a significant confidence score of 61 (where 56 or above is deemed statistically significant at a 95% confidence interval). The generation of *p*-PMF data by FAIMS charge state separation allows confident protein identification

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using ESI sample introduction, with results similar to that of well established MALDI-TOF-MS proteomic techniques.

In contrast to the singly charged ions, individual doubly charged ions can be targeted on the basis of their differential mobility by specifying a narrow CV window for the selective transmission of a selected ion. Therefore, an alternative means of identifying the digested protein by performing tandem MS on an ultra-FAIMS selectively transmitted $[M + 2H]^{2+}$ ion to confirm tryptic peptide identity was investigated. The CV was set to +3 V to transmit the doubly charged m/z 877 tryptic peptide ion at its optimum CV. The selectivity of the ultra-FAIMS device for this peptide, displayed in Figure 8a, with a S/N ratio of 500:1 for the m/z 877 ion, contrasts with the spectrum without FAIMS separation, which has a 50:1 S/N ratio (Figure 7a). The ion trap tandem MS spectrum of the selected m/z 877 ion, shown in Figure 8b, was used to confirm the sequence of the tryptic peptide (amino acid sequence YVGGQEHFAHLLILR). The ultra-FAIMS device combined with MS can, therefore, be utilized for the two main areas of proteomics, increasing the selectivity of the analysis prior to conventional peptide and protein identification methods.

Further work is required to evaluate the potential of applying this approach to more complex samples such as tryptic digests of protein mixtures. For example, while some doubly charged peptides are resolved from one another, others overlap, particularly where the presence of multiple conformers increase the CV dispersion. The use of drift gas mixtures, an established means of improving the resolution in FAIMS and drift tube IMS, would be expected to enhance selectivity.[24,32] The effect of the FAIMS separation on the quantitative analysis of peptides in complex mixtures is another area requiring investigation.

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CONCLUSIONS

The hyphenation of a miniaturized ultra-FAIMS device to ion trap and time-of-flight mass spectrometers is demonstrated to yield improvements in peptide analysis for proteomics experiments. The ESI-FAIMS-MS method has been applied to the analysis of peptide standards, and its ability to separate singly and doubly charged peptide ions has been demonstrated, with multiply charged species requiring a higher CV for transmission. The small CV window for the transmission of singly charged ions suggests the applicability of a miniaturized ultra-FAIMS-MS approach for the generation of *pseudo*-peptide mass fingerprints, commonly used to identify proteins from databases. Alternatively, peptide ions with the same 2^+ charge state, but different amino acid sequences, may be separated on the basis of their differential ion mobility under the appropriate dispersion field conditions. In addition, the separation of gas phase conformers has been observed, with results comparable to those generated on commercially available FAIMS systems.

This work demonstrates the applicability of miniaturized FAIMS-MS for the rapid, high-throughput analysis of peptides by enhancing selectivity, reducing the complexity of sample mixtures in order to enhance MS detection.

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