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High-Resolution Differential Ion Mobility Separations Using Helium-Rich Gases

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Abstract

Analyses of complex mixtures and characterization of ions increasingly involve gas-phase separations by ion mobility spectrometry (IMS) and particularly differential or field asymmetric waveform IMS (FAIMS) based on the difference of ion mobility in strong and weak electric fields. The key advantage of FAIMS is substantial orthogonality to mass spectrometry (MS), which makes FAIMS/MS hybrid a powerful analytical platform of broad utility. However, the potential of FAIMS has been constrained by limited resolution. Here, we report that the use of gas mixtures comprising up to 75% He dramatically increases the FAIMS separation capability, with the resolving power for peptides and peak capacity for protein digests reaching and exceeding 100. The resolution gains extend to small molecules, where previously unresolved isomers can now be separated. These performance levels open major new applications of FAIMS in proteomic and other biomolecular analyses.

Mass spectrometry (MS) preceded by separations has become preeminent for the identification and quantification of various chemical species.¹ Separations used increasingly include ion mobility spectrometry (IMS), where ions are sorted based on their gas-phase transport in electric field.^{2–12} In conventional (drift-tube² or traveling-wave⁸) IMS, ions are dispersed by the absolute mobility, K . The field intensity (E) is normally low, and K is governed^{2,3} primarily by the ion-molecule collision cross section, Ω . This quantity may be computed for any ion geometry, which enables characterization of the ion structure. The mobility of ions in gases depends^{2,4} on E , which is exploited in differential or field asymmetric waveform IMS^{4,9,10} (FAIMS) to separate ions by the differences of K values at high and low E . The separation proceeds in a periodic asymmetric field $E(t)$ of some amplitude (E_D) in a gap between two electrodes. Ions, carried through the gap by gas flow, drift toward an electrode and are neutralized on contact. A fixed “compensation field” (E_C) superposed on $E(t)$ can offset this drift for a particular species and let it transit the gap, while others are lost. Scanning E_C provides a spectrum of species entering the gap. While no means to extract ion structures from FAIMS data has yet been devised, the distinctive outcomes of FAIMS and conventional IMS separations are evident from 2-D FAIMS/IMS measurements that also reveal the geometries of species resolved by FAIMS.^{11,12}

Since the emergence of FAIMS, its resolving power R , defined as $E_C/(\text{peak width at half-maximum})$, has been restricted^{9,11} to ~ 10 – 15 vs ~ 100 – 200 for drift-tube IMS.^{13–17} A new planar FAIMS device with the ion residence time of ~ 0.2 s has recently increased^{18–20} R to ~ 30 – 60 for typical species. Despite a lower R , FAIMS often separates isomers or isobars better than conventional IMS and provides greater peak capacity in conjunction with MS,^{11,18,20}

since the $K(E)$ derivative is correlated with the ion mass (and mass/charge) weaker than K . Still, the modest R achievable has limited the FAIMS utility.

IMS has utilized various gases, commonly He or N₂. Conventional IMS using He allows accurate mobility calculations for structural elucidation, but R is independent of the gas composition.²¹ In FAIMS, R is sensitive to the gas properties^{4,9} and improves as the fraction of He in He/N₂ mixtures increases to 50% (v/v). With the planar FAIMS geometry, the gain generally comes from both broader separation space (greater distance between the peaks) and narrowing of the peaks in lighter gases where ions are more mobile.^{4,20} Larger He fractions have not been employed in FAIMS, primarily to avoid electrical breakdown in the gap. For a typical^{9,18–20} gap width of 2 mm and $E_D = 20$ kV/cm, breakdown for dc field at ambient pressure and temperature occurs for He content over ~65%.

Here, we report use of He/N₂ mixtures with up to 75% He for $E_D \sim 21$ kV/cm. This is feasible because the breakdown threshold in a gas increases for rf fields of high frequency w , when ion oscillations are well within the gap, a criterion met in FAIMS.⁴ For present $w = 750$ kHz, we observe breakdown at ~80% He, permitting stable operation up to ~75%. We have found that the higher He content dramatically increases resolving power (e.g., to ~100 and more for peptides) and allows separation of previously unresolved species.

INSTRUMENTATION

Experiments employed a previously described^{18,20} planar FAIMS unit with 1.88 mm gap width, driven by a 2:1 bisinusoidal^{4,9} waveform with the 750 kHz frequency and nominal peak voltage of DV = 4 kV. The DV read-back was ~4.05 kV (creating $E_D = 21.5$ kV/cm) vs ~3.97 kV ($E_D = 21.1$ kV/cm) previously,²⁰ leading to slightly higher E_C and, thus, R values for peptides at the same He fractions. The unit was coupled to an LTQ ion trap MS (Thermo Scientific, Waltham, MA) with an electro-dynamic ion funnel interface in front.²² The FAIMS outlet was ~2.5 mm from the MS inlet capillary held at 30–50 °C (as ion desolvation after FAIMS is redundant). The inlet, FAIMS body, and the curtain plate at the FAIMS entrance were biased at ~120–160 V, ~160–190 V, and 1 kV to the ground, respectively. Ions were produced by electrospray ionization (ESI) in the positive mode. The emitter voltage was 1.5–2.3 kV above the curtain plate, lower at higher He content to avoid electrical discharge and improve the signal stability. The He/N₂ mixtures at room temperature were formulated using digital flow meters with feedback control (MKS Instruments, Andover, MA), filtered by a LC/MS gas purifier for N₂ (Agilent Technologies, Santa Clara, CA), and flowed to the FAIMS unit at 1.8 L/min (for peptides and digests) or 2.3 L/min (for amino acids). Under these conditions, ions traversed the gap in 0.1–0.2 s. The amino acid and peptide standards were dissolved in 50/49/1 water/methanol/acetic acid. The tryptic digest of BSA was prepared as usual, reduced, alkylated, and reconstituted in the 50/49.8/0.2 water/acetonitrile/formic acid. Sample solutions were infused to the emitter at 0.2–0.5 μ L/min. The MS resolution was set to “normal” or (to assign the charge states of features by the isotope pattern) “zoom”. The ion accumulation time in the trap was ~0.2–0.5 s. The FAIMS scan rate was 1–2.5 V/(cm $\times$ min).

We caution that the turbomolecular pumps of LTQ MS lose efficiency at greater He content in the gas, and the pressures measured by the ion and convectron gauges increase from, respectively, 1×10^{-5} and 1.3 Torr at 0% He to 1.7×10^{-5} and 2.0 Torr at 75% He. While we operated in that regime for an extended time, it is above the pressure range normal for the present and other common commercial API/MS platforms that are not designed for aspiration of He-rich mixtures from the inlet. Elevated pressure in the MS manifold may affect the accuracy, resolution, and/or sensitivity of MS analyses and cause hardware failures.

RESULTS

A stringent test of IMS resolution is separating the isomeric leucine (L) and isoleucine (I) amino acids. In conventional IMS, the protonated cations are fully resolved at the highest $R \sim 150$ reached in drift-tube IMS¹⁵ but not by the commercially available traveling-wave IMS.²³ Previously, FAIMS could barely resolve either L and I cations¹⁸ or deprotonated anions.²⁴ For cations, a minimal separation emerged only at 50% He. We find that higher He fractions significantly improve the resolution: r , (peak spacing)/(peak width), changes from 1.0 at 50% to 3.4 at 73% He, providing baseline separation at $\sim 70\%$ He (Figure 1). The gain is mainly due to the peak spacing increasing by 2.7 times (from 1.8 V/cm at 50% to 4.9 V/cm at 73% He), but narrowing of the peaks with increasing K values also contributes. The peak heights reflect the 1:1 L/I ratio in the sample up to $\sim 65\%$ He but deviate in either direction beyond that. The main reasons are that higher He fractions (i) destabilize the ESI source, increasing the temporal fluctuations of ion flux and (ii) reduce ion transmission through the FAIMS gap and, thus, the detected signal, worsening the ion count statistics.

The E_C values for both isomers initially become more negative with added He but minimize at $\sim 25\%$ He and then increase, crossing zero at 65–67% (Figure 1). This signifies the transition from “type A”, where K increases at higher E , to “type C”, where K decreases.^{4,9} This happens because the molecular polarizability is much lower⁴ for He ($0.2 \text{ \AA}^3$) than for N_2 ($1.8 \text{ \AA}^3$) and, thus, the ion–molecule collisions “harden” at higher He fractions; in the limit of hard-shell potential, K decreases^{2,4} with increasing E . The same transition to type C occurs in N_2 at much higher $E_D \sim 55 \text{ kV/cm}$: extreme fields result in energetic collisions where the dynamics essentially samples only the repulsive part of any ion–molecule potential.²⁵

An expanding application of IMS/MS is the conformational characterization of peptides and separation of their mixtures in proteomics. To exemplify FAIMS in He-rich mixtures for peptides, we have chosen the usual model of bradykinin, Bk (RPPGFSPFR, 1060 Da) with the dominant protonated 2+ ion and syntide 2, St (PLARTLSVAGLPGKK, 1508 Da) that exhibits intense ions with the charge states (z) of 2, 3, and 4. Unlike amino acids, peptides of all z behave as C-type ions^{9,20,26,27} even in N_2 . Adding He progressively raises the E_C values and narrows the peaks, thus improving the resolving power.²⁰ For typical peptides, going from N_2 to 1:1 He/ N_2 increases E_C by ~ 1.7 –2 times and R by ~ 2 to 3 times: in the present device,²⁰ from ~ 20 to ~ 50 –60 for Bk²⁺ conformers and from ~ 12 –40 to ~ 40 –70 for St with $z = 2$ –4. As in conventional IMS, the peaks with any gas become narrower for higher z because the (low-field) diffusion coefficients of ions scale as K/z by the Einstein relationship.^{2,4,20}

Here, as we move above 50% He, the E_C values for all features increase further, by ~ 1.5 –1.6 times at 75% He: from ~ 65 –85 V/cm to ~ 105 –135 V/cm for Bk²⁺ and from ~ 50 –105 to ~ 75 –155 V/cm for St ions (Figure 2). The peaks continue narrowing, as seen for all St charge states. (This is less clear for Bk²⁺, because of unresolved isomers created by structural transitions as discussed below.) In the result, the resolving power improves by ~ 1.8 –2.5 times over that at 50%: to ~ 100 for Bk²⁺ and St²⁺ and ~ 180 –200 for St³⁺ and St⁴⁺ (Figure 2). To verify the accuracy and reproducibility of these results, we have scanned a narrow window comprising the base peak of St³⁺ 10 times over 30 min (Figure 3). The mean peak position is 156.52 V/cm with the standard deviation of 0.13 V/cm and maximum deviation of 0.25 V/cm, still $\sim 0.15\%$ in relative terms or $\sim 30\%$ of the peak width. Such a limited E_C drift ought to permit accurate measurements with intermittent calibration. The mean peak width is 0.89 V/cm with the relative standard error of 3%, leading to $R = 177 \pm 11$ at 95% confidence. This value comes close to the maxima achieved in conventional IMS, where high-pressure drift-tubes have provided R up to ~ 170 and ~ 240 for multiply charged ions,^{13,14} and far exceeds the metrics for traveling-wave IMS.^{23,28}

The peaks for peptides of some z that mirror those for $(z + 1)$ at any He content likely result from the proton transfer after FAIMS filtering.^{20,29} Such artifacts were noted²⁰ for St^{2+} and St^{3+} at $\leq 50\%$ He and are likely seen here for $3+$, where minor features coincide with all four St^{4+} peaks (a, b, c, and e), Figure 2. However, a strong peak (b) for St^{3+} at lower E_C has no comparable match for St^{4+} at 60–75% He and, thus, may be not due to charge reduction. The charge transfer appears less intense here than with LCQ MS at the same He fraction,²⁰ possibly because of shorter ion accumulation in the trap or better removal of neutrals in the present MS interface featuring an ion funnel.

For small proteins such as ubiquitin or cytochrome *c* in any charge state,^{12,30} unfolding decreases E_C . Two Bk^{2+} species were resolved by drift-tube IMS,¹⁷ and minor features on the low- E_C side of the base peak (d) in FAIMS were also ascribed to unfolded conformers.^{18,20,31} Those features grew in number and relative intensity²⁰ with the He fraction in He/ N_2 increasing to 50%. As we go to 75% He (Figure 2), the process accelerates with (b) becoming the major peak at $\sim 60\%$ He and further features (grouped under “c”) emerging between the (b) and (d) “book-ends”. Those features have positions and heights sensitive to the solution, source, and/or FAIMS conditions and, hence, likely are intermediates in the isomerization from (d) to (b). Unlike Bk^{2+} that is a zwitterion,³² most peptides exhibit one major conformer in FAIMS at $\leq 50\%$ He.^{20,26} Here, the structures for St ions of any z evolve little between 50% and 75% He. (St^{4+} has lower E_C than the major St^{3+} conformer probably because it is unfolded by Coulomb repulsion whereas St^{3+} with one less proton is not.²⁰) The data for $\sim 10^2$ peptides in a protein digest (below) suggest that this behavior is more common than an extensive isomerization seen for Bk^{2+} .

The greater unfolding in He than in N_2 has been observed for protein ions in drift-tube IMS.³³ The unfolding is induced by field heating (above the gas temperature) by^{2,4}

$$\Delta T = M(KE)^2 / (3k) \quad (1)$$

where M is the gas molecule mass and k is the Boltzmann constant. Thus, ΔT scales³³ as Ω^{-2} . All ions have larger cross sections with N_2 than with He, and the relative difference increases for smaller ions where the size and other properties of colliding partner are more important. For example,³⁴ for peptides at 250 °C, the difference grows from 16% for neurotensin $3+$ (1674 Da) to 24% for kemptide $2+$ (773 Da). Hence, the transition from N_2 to He should augment the field heating more for peptide ions than for larger proteins. As N_2 is more polarizable than He and, thus, forms a deeper potential with any ion,^{4,25} the differences between the Ω values in N_2 and He increase in a colder gas, where attractive interactions contribute to Ω more. For Bk^{2+} , the measured Ω at room temperature in N_2 ($\sim 336 \text{ \AA}^2$)²⁵ exceeds that in He ($\sim 240 \text{ \AA}^2$)^{32,33} by $\sim 40\%$, thus replacing N_2 with He nearly doubles ΔT .

The isomerization in FAIMS appears governed^{35,36} by the maximum heating at peak E . For Bk^{2+} in N_2 at room temperature,²⁵ the reduced mobility is $K_0 = 1.22 \text{ cm}^2/(\text{Vs})$ and the actual mobility is $K = (K_0 \times 298/273) = 1.33 \text{ cm}^2/(\text{Vs})$. Hence, at the present E_D , eq 1 yields $\Delta T = 92$ °C for N_2 and 180 °C for He, for a difference of 88 °C. With mobilities by the Blanc’s law, the difference of ΔT is 57 °C between 0% and 75% He and 24 °C between 50% and 75% He. The real differences may be greater because of the non-Blanc effects in gas mixtures.^{2,4} Based on the drift-tube IMS data,³⁷ heating by >20 °C, leave alone >60 °C, in the >100 °C range may cause major unfolding of peptides during a few milliseconds spent in a capillary. The relevant time scale in the present FAIMS device is longer, allowing further isomerization at equal ΔT . Thus, estimates support the notion that the unfolding of Bk^{2+} seen in Figure 2 is due to stronger field heating upon He addition.

The FAIMS peak capacity, defined as (separation space)/(mean peak width), for peptide mixtures is proportional to the resolving power for representative peptides and, thus, must increase at higher He content. With the commercial cylindrical FAIMS units (Thermo Scientific) operated at $E_D \sim 20$ kV/cm, the R values for most peptides²⁰ are ~ 5 – 12 and the peak capacity for tryptic digests of proteomes²⁷ is ~ 7 – 9 . Thus, the peak capacity for digests is close to the mean R for individual peptides, which makes sense as the peaks densely cover a segment of E_C from near zero to a maximum.^{11,26,27,38–40} Then, the peak capacity of present FAIMS device for digests should range from ~ 50 at 50% to ~ 100 at 75% He.

Allowing for single missed cleavages, the in silico tryptic digest of the dominant blood protein, bovine serum albumin (BSA, 66.4 kDa), comprises 144 peptides with 404–3580 Da. At 50% He, all peptide ions are type C with $E_C \sim 20$ – 100 V/cm for the separation width of ~ 80 V/cm (Figure 4), consistent with the findings^{11,38–40} using cylindrical FAIMS units at the same gas identity and similar E_D . With increasing He content, the spectrum shifts to higher E_C while the separation space broadens and, at 75% He, the spectrum takes up the ~ 27 – 142 V/cm range with the width of ~ 115 V/cm (Figure 4). The increases of E_C and separation width by ~ 1.5 times over those at 50% He follow the observations for Bk and St ions.

Electrospraying tryptic digests typically produces peptide ions with $z = 1$ – 5 . When the results with cylindrical FAIMS units are reproduced,^{11,38–40} at 50% He, the E_C values broadly increase from $z = 1$ to 2 and further to $z = 3$ – 5 (Figure 5). This overall pattern persists to 75% He, but the E_C values for different species depend on the He fraction unequally and the peak sequence is not conserved, even within a charge state (Figure 5). Some peptides undergo dramatic E_C shifts relative to others, e.g., the ions at $m/z = 462$ (2+) and $m/z = 831$ (3+) lie $\sim 1.5\%$ apart and partially overlap at 50% He but are spaced by $\sim 33\%$ (with >10 well-resolved peaks fitting in between) at 75% He. Such shifts could reflect a profound heat-induced isomerization of certain peptides moving them to a different part of the spectrum. Regardless of the cause, these variations allow modifying the separation selectivity by adjusting the He fraction.

With large shifts rare, the E_C values at 50% and 75% He remain strongly correlated (Figure 6). The linear correlation is >0.9 for the full set and $z = 1, 2$, or 4 alone but only ~ 0.4 for 3+ ions. This supports the rationalization of the shifts as the manifestation of unfolding, which under present conditions is particularly common for 3+ peptides.

As should be with planar FAIMS gaps,⁴ the peak widths are independent of E_C and tend to decrease for higher z : on average, from ~ 1.75 V/cm for 1+ to ~ 1.35 V/cm for 2+ to ~ 1.15 V/cm for $z = 3$ – 5 (Figure 5). The weighted mean over z is 1.4 V/cm, versus ~ 10 V/cm with cylindrical FAIMS units.^{20,27} Hence, the total peak capacity is ~ 50 , as was expected from Figure 2. The peaks for all z narrow with increasing He fraction, by $\sim 1/3$ at 75% He with the average widths of 1.2 V/cm for 1+, 1.0 V/cm for 2+, and 0.8 V/cm for $z = 3$ – 5 (Figure 5). Now, the weighted mean is 1.0 V/cm and the peak capacity is ~ 110 , again close to the estimate from Figure 2. The partial peak capacity for each charge state improves by a similar ~ 2 – 2.5 times, e.g., for 1+ from ~ 15 at 50% He (with the E_C range of ~ 20 – 45 V/cm) to ~ 35 at 75% He (with the ~ 30 – 72 V/cm range). To eliminate nonpeptide contaminants such as solvent or matrix clusters, proteomic analyses often focus on multiply charged ions. For $z = 2$ – 5 , the separation width expands from ~ 45 V/cm (from ~ 47 to ~ 92 V/cm) at 50% He to ~ 74 V/cm (from ~ 68 to ~ 142 V/cm) at 75% He, while the weighted mean peak width decreases from ~ 1.25 V/cm to 0.9 V/cm. Thus, the peak capacity for peptides with $z > 1$ increases from ~ 35 at 50% He to ~ 80 at 75% He. Complex proteomes yield more diverse peptides than BSA and, as with any separation method, the total and partial peak capacities would be even higher.

Ions with E_C shifted upon isomerization in FAIMS by more than the peak width are largely removed.^{4,12,35,41} Hence, isomerization may drastically affect the spectra for specific peptides (as seen for Bk²⁺ above) but less so for complex mixtures as the peptides that isomerized are suppressed relative to others. This “self-cleaning” mechanism^{12,35} likely contributes to the paucity of multiple conformers at any He fraction. Some larger multiply charged peptides still exhibit several features, for example, the species at m/z of 632 (4+), 636 (4+), and 801 (5+) with two peaks each (Figure 5). As seen there, those features tend to be separated better at higher He content, and the conformers merged at 50% He are resolved at 75% He. While isomerization in FAIMS may be desired for fundamental studies of ion structure and dynamics, the consequent removal of species impairs sensitivity and, thus, is generally unwelcome from the analytical perspective. One should be able to curb this process via cooling the gas by the greatest ΔT for the set of ions of interest.³⁵

CONCLUSIONS

We have demonstrated differential ion mobility spectrometry (FAIMS) in the He/N₂ mixtures with up to 75% He, improving the resolution by ~2 to 3 times compared to 1:1 He/N₂. This allows full separation of previously unresolved peptide conformers and isomers of small molecules such as leucine and isoleucine. The resolving power for multiply charged peptide ions increases to ~200, producing the peak capacity of ~100 for protein tryptic digests. These parameters compare to those of currently best conventional IMS,^{13–17} but the FAIMS separations are more orthogonal to mass spectrometry.^{26,42}

For deeper fractionation of complex mixtures (e.g., proteome digests) prior to MS and MS/MS analyses, FAIMS has been combined to other separations including conventional MS^{11,12} or liquid chromatography (LC).^{27,38–40} The increased FAIMS resolution achieved in this work will translate into proportional peak capacity gains for multidimensional separation methods involving FAIMS. In particular, the formal 2-D peak capacity of FAIMS/(drift tube IMS) or LC/FAIMS for proteome digests would increase to ~10⁴ and higher, which compares to or exceeds the metrics of the best 2-D condensed-phase approaches such as MudPIT.⁴³

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Figure 1.

Mass-selected FAIMS spectra for the 1:1 Leu/Ile mixture (solid lines) in He/N₂ with $\leq 50\%$ He (left) and $\geq 50\%$ He (right). The values of resolution r are given for separated isomers. Pure Leu or Ile yield single peaks matching “L” and “I”, respectively (dashed lines in the right panel for Ile at 50–70% He).

Figure 2.

Mass-selected FAIMS spectra for bradykinin 2+ (left column) and syntide 2 with $z = 2-4$ (right column) at 50–75% He. Samples were 20 μ M solutions of pure peptides. For well-shaped peaks, we show the widths (V/cm) with the resolving power values underneath.

Figure 3.

Successive replicates of the peak d for Syntide 3+ in Figure 2 at 75% He, measured in a window of 3.2 V/cm width at the scan speed of 1.06 V/(cm $\times$ min). The E_C values and peak widths are given for each case.

Figure 4.

Total FAIMS spectra for the BSA tryptic digest (1.5 μM by the initial protein content) in He/ N_2 with 50–75% He. The separation widths are given in V/cm.

Figure 5.

FAIMS spectra for selected ions extracted from the data in Figure 4 at 50% He (a) and 75% He (b). We color-code the features by the charge state as labeled, giving the m/z values and (where possible) peak widths (V/cm) underneath. These species were chosen to illustrate the separation peak capacity and are not necessarily the most intense ones. The peak heights are arbitrary and adjusted for the picture clarity. We sought to mirror in (a) the most features found in (b), but some did not fit in because of lower peak capacity in (a).

Figure 6.

Correlation of FAIMS separation properties at 50% and 75% He for the peptides in Figure 5b (including those absent from Figure 5a), with the same color coding. The line is the first-order regression for the whole data set, and linear correlation coefficients are listed for the whole set and each z separately.