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High-Resolution Differential Ion Mobility Separations Using Planar Analyzers at Elevated Dispersion Field

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Abstract

The ion mobility spectrometry (IMS) methods are grouped into conventional IMS, based on the absolute ion mobility, and differential or field asymmetric waveform IMS (FAIMS), based on the mobility difference in strong and weak electric fields. A key attraction of FAIMS is substantial orthogonality to mass spectrometry (MS). Although several FAIMS/MS platforms were commercialized, their utility was limited by FAIMS resolving power, typically ~10 - 20. Recently, gas mixtures comprising up to 75% He has enabled resolving power >100 that permits separation of numerous heretofore “co-eluting” isomers. This performance opens major new proteomic and other biological applications. Here, we show that raising the separation field by ~35% over the previous 21 kV/cm provides similar or better resolution (with resolving powers of >200 for multiply-charged peptides) using only 50% He, which avoids problems due to elevated pressure and He content in the mass spectrometer. The heating of ions by the separation field in this regime exceeds that at higher He content but weaker field, inducing greater isomerization of labile species.

Introduction

The dominant approach for characterizing challenging biological or environmental samples is mass spectrometry (MS) preceded by separations.¹ These were traditionally performed prior to ionization, employing chromatographic or electrophoretic methods based on differential affinities to a solid surface or ion properties in solution.^{2,3} While these techniques are powerful and versatile, their speed is limited by slow molecular motion in condensed phases; for example, extensive separations of proteolytic digests often take ~12-24 hours.^{4,5} A need for much higher throughput has motivated interest in replacing or complementing the fractionation in solution with post-ionization separations that rely on rapid ion transport in gases.⁶⁻¹⁰ These methods, called “ion mobility spectrometry” (IMS), have gained popularity over the last decade and are integrated into several commercial IMS/MS platforms.¹¹⁻¹³

IMS methods fall into two categories. Conventional IMS,¹⁴ which may be implemented in drift-tube (DT IMS),¹⁴ traveling-wave (TW IMS),^{13,15} and other modes,^{16,17} is based on the absolute ion mobility (K) in electric fields of moderate strength (E). The newer differential or field asymmetric waveform IMS (FAIMS)¹⁸ exploits that K for any species is a function of E to sort ions by the difference in mobility at two E values. As $K(E)$ dependence is significant only at high E , strong fields are required. Experimentally, an asymmetric oscillatory field, created in the gap between two electrodes by a waveform applied to one, disperses ions by the difference in mobility between high- and low- E segments with opposite polarities.^{11,18} The peak amplitudes of waveform and associated field are termed the “dispersion voltage” (DV) and “dispersion field” (E_D). The resulting unbalanced motion would eventually remove all ions

from the gap via neutralization on electrodes, but may be offset for a given species by a fixed “compensation field” (E_C) produced by “compensation voltage” (CV) also applied to the electrodes. The CV allows a particular moiety to pass the gap to a detector (e.g., MS), and scanning the CV provides a spectrum of the species present.^{11,18}

The K values at low field are easily converted into ion-molecule collision cross sections (Ω) that can be related to ion geometry,¹⁸⁻²⁰ enabling conventional IMS to characterize the ion structure *a priori*.^{21,22} This scheme is not currently feasible with FAIMS, as $K(E)$ variations are usually small and thus far cannot be rigorously related to ion structure. However, this also means that ion size affects FAIMS separation properties less than with conventional IMS, rendering FAIMS more orthogonal to MS.^{23,24} In this respect, FAIMS resembles chromatography,² where the complexity of separation mechanism results in decoupling from molecular mass. To date, the greater independence of dimensions in FAIMS/MS compared to (conventional IMS)/MS has not translated into superior overall 2-D peak capacity because of lower resolving power (R) of FAIMS, typically^{10-12,25} $\sim 10 - 20$ versus up to ~ 170 for singly-charged and ~ 240 for multiply-charged species in DT IMS.^{6,26,27} In FAIMS, R depends on the gap shape, maximizing for planar gaps with homogeneous field.²⁸ In this case, R is roughly proportional to E_D^3 and increasing E_D broadly improves resolution.^{18,29}

Unlike with conventional IMS, the FAIMS separation power is sensitive to the gas nature. Resolution often improves with helium-containing mixtures, particularly He/N₂,³⁰⁻³³ where non-Blanc phenomena expand the separation space³⁴ while higher ion mobility and thus faster diffusion narrow the peaks.^{18,33} Using He/N₂ mixtures in a planar FAIMS device with a gap width (g) of 1.88 mm and DV = 4 kV (leading to $E_D = 21$ kV/cm), we had achieved^{32,33} R up to ~ 80 at 50% He and ~ 180 at 75% He (v/v) - the limit determined by the onset of electrical breakdown across the gap (Fig. 1). As that threshold decreases from ~ 7 kV in N₂ to ~ 1 kV in He,³⁵ a topical question is whether one can optimize separation by trading E_D for the He fraction along the breakdown curve (Fig. 1).

Here we explore FAIMS performance at higher DV and lower He content and demonstrate the separation power equal to or above the best previously reported,³³ without excessive He aspiration into the MS system that poses operational challenges and electrical breakdown risks to hardware. For multiply-charged peptides, $R > 200$ was achieved.

Instrumentation

We employed a planar FAIMS analyzer^{32,33} coupled to a modified LTQ ion trap MS system with an electrospray ionization (ESI) source.³⁶ The 2:1 bisinusoidal waveform^{12,18} of 750 kHz frequency had DV = 5.4 kV to create $E_D = 28.7$ kV/cm. This DV was used for cylindrical FAIMS units,¹² but the previous maximum for planar geometries was 4 kV. Other parameters were per Ref. [33], but the maximum He fraction was reduced to 50% to avoid electrical breakdown at higher DV. (The discharge occurred at $\sim 55/45$ He/N₂, instead of $\sim 80/20$ He/N₂ at DV = 4 kV,³³ Fig. 1). The gas flow to FAIMS was 2.0 L/min (for amino acids, 2.5 L/min), actually equal to those in Ref. [33] where the slightly lower rates given reflected inaccurate meter calibration. Hence the ion residence times in the gap (t) here and in Ref. [33] are same ($\sim 150 - 200$ ms), and the separation power can be compared directly.

The present $E_D = 28.7$ kV/cm is not a record for planar FAIMS analyzers. The Paschen law permits stronger fields in narrower gaps,³⁵ which allows E_D up to 33 kV/cm in micromachined units³⁷⁻³⁹ with $g = 0.5 - 1$ mm and 61 kV/cm in chips^{40,41} with $g = 35$ μ m. However, such gaps necessitate much shorter t of $\sim 1 - 10$ ms for $g = 0.5 - 1$ mm and $\sim 20 - 60$ μ s for $g = 35$ μ m, else ion diffusion would cause devastating signal losses via non-specific neutralization on electrodes. As the resolving power of planar FAIMS analyzers scales^{18,42,43} with $t^{1/2}$, cutting

t by ~ 15 - 10,000 times means decreasing R by ~ 4 - 100 times. This reduction outweighs a gain of R by a factor of ~ 1.5 - 10 (the cube of E_D increase by ~ 1.15 - 2.1 times), and “subscale” devices provide lower R than “full-size” ones with $g \sim 2$ mm. The exceptional resolution here is produced by the relatively high field combined with a “full-length” separation.

Results and Discussion

Analyses of small rigid ions

We first studied reserpine (609 Da), a common MS standard that makes 1+ ions in ESI with a single feature in FAIMS spectra.²⁸ At DV = 4 kV in N₂, this species belongs to “type C”,^{18, 28} meaning that $K(E)$ is a decreasing function of E and thus $E_C > 0$ when $E_D > 0$. A higher He content or E_D shift all ions toward type C,^{33,40,41,44} and hence should make E_C of reserpine more positive. Indeed, with DV = 4 kV (Fig. 2a), the E_C values increase from 14 V/cm in N₂ to 51 V/cm at 77% He. (Operation at $>75\%$ He was unstable because of intermittent electrical breakdown.) Adding He narrows the peaks as usual,^{32,33,39} here by twofold from $w = 2.8$ V/cm in N₂ to 1.4 V/cm at 75 - 77% He, and the resolving power increases from 5 to ~ 35 . With DV = 5.4 kV (Fig. 2b), the peaks at equal He content are transposed to greater E_C values, from 31 V/cm in N₂ to 64 V/cm at 50% He. The highest E_C in Fig. 2b exceeds that in Fig. 2a, although the maximum He fraction is much lower. By theory,^{18,42,43} the peak widths (w) in planar FAIMS should not depend on E_D , and the widths at the same He content in (a) and (b) are essentially equal. However, at higher DV the maximum He fraction is lower and hence the minimum w is greater: 1.9 V/cm in (b) vs. 1.4 V/cm in (a). As a result, the maximum resolving power at two DVs is about the same ~ 35 .

Of more insight is the resolution (r) of two species, defined as (peak spacing)/(mean w). Of most interest are isomers indistinguishable by MS, such as leucine (L) and isoleucine (I).^{28, 33,45} At DV = 4 kV, the resolution of protonated L and I ($m/z = 132$) dramatically improved³³ at higher He fractions, with the peak separation emerging at $\sim 50\%$ He and nearing baseline at $\sim 70\%$ He - about the maximum He content. The results at DV = 5.4 kV are similar (Fig. 3), but now the separation emerges at $\sim 30\%$ He and nears baseline at 50% He. The maximum resolution in two cases is the same ~ 3.3 - 3.4, which is similar to that of L and I in DT IMS²⁷ at the highest achieved $R \sim 170$ and far exceeds that in TW IMS where these species were not separated.⁴⁶

Both L and I are often deemed “type B” ions, for which K goes first up and then down with increasing E , as the scattering is increasingly controlled by the repulsive wall of interaction potential.^{18,47} For such ions, E_C initially drops (from 0) with increasing E_D , then reverses course and eventually crosses to $E_C > 0$. The same sequence is common with increasing He fraction, because He has the lowest polarizability of any molecule ($0.2 \text{ \AA}^3$ vs. $1.8 \text{ \AA}^3$ for N₂) and thus forms the shallowest potentials with ions.¹⁸ Hence, the He fraction that minimizes E_C should decrease at higher E_D , and indeed decreases from $\sim 25\%$ at³³ DV = 4 kV to $\sim 10\%$ here (Fig. 3). At still higher E_D , the minimum vanishes and finally one finds $E_C > 0$ already in N₂; i.e., L and I turn into “type C” ions.⁴¹

Application to peptides and the effect of field heating

Much IMS work has focused on peptides, both in terms of studying conformations for exemplary species and fractionating their mixtures in proteomics. A crucial aspect of FAIMS is the ion isomerization induced by above-thermal collisions in a strong field in the gap.^{18,48, 49} For peptides and proteins, such “field heating” implies unfolding of the secondary or tertiary structure. This may be an unwelcome process that distorts the conformers generated by the ion source that often indicate the structure in solution. A substantial E_C shift upon isomerization in the gap may cause ion elimination, suppressing the signal for certain species.^{48,49} However,

a stronger heating that accelerates isomerization above the FAIMS filtering speed would “anneal” ions rapidly upon injection into the gap, thus reducing signal losses. Annealing could also convert multiple original conformers into fewer low-energy ones, which simplifies separations and is deliberately done in gel-based methods such as SDS-PAGE. Hence, understanding and managing the field heating and its consequences is an important facet of optimizing FAIMS for peptides and other fragile ions.

The magnitude of field heating (ΔT) above the ambient temperature scales^{33,50} as $(E/\Omega)^2$, and the relevant E in FAIMS seems to be^{48,49} the maximum $E = E_D$. Hence, ΔT depends on the gas identity, rising as the fraction of molecules having a smaller cross section with the ion in question increases.^{33,50} Any ion has a lower Ω with He than with N_2 , and adding He promotes unfolding of peptides in both FAIMS³³ and DT IMS.⁵⁰ A common model peptide in IMS is bradykinin (Bk, 1060 Da), which produces intense 2+ ions in ESI. By calculations³³ for Bk^{2+} at room gas temperature, ΔT with DV = 4 kV increases from 92 °C in N_2 to 125 °C at 50% He to 149 °C at 75% He. Raising DV to 5.4 kV increases ΔT values by 82%, to 168 °C in N_2 and 228 °C at 50% He. Thus, with the present DV = 5.4 kV, even minimal heating at 0% He exceeds the maximum with DV = 4 kV, and the heating at 50% He (needed for comparable separation as found above) exceeds that maximum by ~50% or 80 °C. As seen from DT IMS data with thermal heating^{48,51} and FAIMS,⁴⁹ additional heating of that magnitude may drastically unfold proteins.

Known peptides behave as “type C” ions^{23,28,32,33,52} in any He/ N_2 mixture, and unfolding seems to decrease^{28,32,33} E_C . With DV = 4 kV, Bk^{2+} in N_2 exhibited a major compact conformer (d), two moderately intense species (b) and (c) at lower E_C , and one or more trace conformers.^{28,32,33} This picture persisted³³ up to 60% He, with the relative abundances of (b) and (c) increasing at higher He content. At 70 - 75% He, peaks (b) and/or (c) became dominant and split into at least six features total, including a growing peak (a) at yet lower E_C . This pattern suggests progressive unfolding of Bk^{2+} upon He addition. The overall trend at DV = 5.4 kV is same, but shifted to lower He fractions with (c) split already in N_2 and products (c1), (c2), and (c3) emerging at 30% He (Fig. 4). The best agreement between the spectra at two DVs is obtained via offsetting them by ~30 - 35% He, e.g., that at DV = 4 kV and 75% He is closest to that at DV = 5.4 kV and 40% He. Going to 50% He at the higher DV brings more changes: (a) becomes dominant, new features emerge with E_C below that for (a), (b) and (c) split more, and the original feature (d) and the daughter of (c) with greatest E_C disappear (Fig. 4). This evolution is consistent with further unfolding upon stronger heating.

As at DV = 4 kV,³³ the resolving power improves upon He addition, about doubling from ~50 in N_2 to ~100 at 50% He (Fig. 4). Continuing the similarity between increasing the He content and raising DV, the R values at any He fraction (upon transposing the above axis) approximate those at DV = 4 kV.³³ Extensive isomerization and peak broadening due to unresolved conformers render Bk^{2+} inappropriate for the evaluation of absolute R . However, Bk peculiarly has the most basic residue arginine at both termini, which makes Bk^{2+} a zwitterion.^{53,54} Most peptide ions (including tryptic fragments by definition) lack this property, and have produced fewer and often just one major feature in FAIMS.^{10,23,32,33,52} In particular, syntide 2 (St, 1508 Da) exhibits intense well-shaped peaks for the charge states $z = 2, 3$, and 4, which were employed to characterize the resolving power.^{32,33}

The spectra for St ions at DV = 5.4 kV (Fig. 5) follow the trends discussed for Bk^{2+} . First, shifting the He fraction axis up by ~20 - 30% brings the spectral profiles for St^{3+} and St^{4+} (that exhibit minor secondary features) into fair agreement with those at DV = 4 kV (Fig. 5). Second, the R values for all z increase upon He addition (reflecting both higher E_C and narrower peaks). The gain between 0% and 50% He is ~2.5 - 3.5 fold, and an axis shift by ~35% roughly aligns R values with those at the lower DV (Fig. 6). As the highest He content at DV = 4 kV was

75%, the present resolving powers for $z = 3$ and 4 somewhat exceed the previous records.³³ To verify this, the major St^{3+} peak at 50% He was measured 10 times by repeatedly scanning a narrow window (Fig. S1). From these data, we computed $E_C = 242.3$ V/cm with the standard error of the mean $S_E = 0.074$ V/cm and $w = 1.10$ V/cm with $S_E = 0.04$ V/cm. Thus $R = 222 \pm 16$ at 95% confidence, which is greater than $R = 177 \pm 11$ for the same feature³³ at DV = 4 kV with statistical significance. Over the 50-min acquisition time, the relative deviations of E_C from the mean were 0.10% (standard) and 0.17% (maximum). These metrics exceed those of 0.08% and 0.15% measured³³ over 30 min at DV = 4 kV only slightly (if at all), proving excellent waveform stability up to DV = 5.4 kV. The challenge of stabilizing the FAIMS power supplies in this DV range has limited the output of other designs.⁵⁵

The findings for St were confirmed using another common peptide - angiotensin I (An, 1296 Da) that in ESI yields $z = 2$ and 3. The FAIMS spectra for both at DV = 4 kV and 75% He feature one dominant peak and two minor ones at lower E_C that likely are less folded conformers (Fig. 7a). The position and width of the major An^{3+} peak are close to those for St^{3+} , leading to the identical $R = 178 \pm 11$ at 95% confidence (based on 20 replicates acquired over 60 min., Fig. S2a). The spectra at DV = 5.4 kV and 50% He resemble those at the lower DV (Fig. 7b), with the expected displacement to higher E_C values. Again, the major An^{3+} peak is close to that for St^{3+} in position and width, and the R value (from 9 replicates, Fig. S2b) is statistically the same at 216 ± 27 . Hence increasing DV above 4 kV while reducing the He fraction as needed to avoid breakdown can improve the FAIMS resolution for peptides by ~20 - 25%. Unlike Bk^{2+} , St and An do not consistently unfold at higher He content and/or DV: the growth of features at lower E_C is notable (though limited) for St^{4+} , but not for St^{2+} , St^{3+} , An^{2+} , or An^{3+} . Therefore, for some and perhaps majority of peptides, the greater DV benefits resolution without causing isomerization. The degree to which Bk^{2+} is exceptional in this regard is a subject of ongoing research.

The FAIMS resolution may be enhanced by adding water or organic vapors to the gas, with R reaching^{56,57} ~140. The vapor molecules reversibly complex to an ion, decreasing its mobility at low E more than at high E (where stronger field heating causes ion desolvation). This mechanism expands the difference between the K values at low and high E that underlies FAIMS, but *only* when the second exceeds the first. This circumstance restricts the approach to type B ions (typically smaller species up to ~300 Da) such as phthalates (166 Da) or explosives like DNT (182 Da) or TNT (227 Da).⁵⁷ Vapors have not improved the separation of type C ions, including all peptides, and are not expected to as the same mechanism compresses the difference between K at low and high E when the first exceeds the second.

Conclusions

We raised the dispersion voltage in planar FAIMS systems from the previous maximum of 4 kV to 5.4 kV, with stable performance. The best FAIMS resolution is commonly obtained using He/N₂ gas mixtures with the maximum He fraction permitted by electrical breakdown constraints. That fraction decreases at greater DV, in the present design with a 1.88 mm gap from ~75% at 4 kV to ~50% at 5.4 kV. The FAIMS resolving power R normally improves at higher DV and He content, thus the effects of higher DV and lower He content on R largely cancel. However, as either effect scales depending on the ion species, the offset is inexact and raising DV while reducing He content benefits some separations. In particular, R for multiply-charged peptides may increase by up to ~25%, reaching ~220 - the maximum achieved for any analyte. This value compares with the record metrics of conventional IMS, where R of 180 and 240 were reported for 3+ and 4+ peptide ions.⁶ However, because MS is more orthogonal to FAIMS than to conventional IMS in general and especially for peptides,^{23,24} the FAIMS and IMS stages of equal R lead to a greater 2-D peak capacity and specificity for FAIMS/MS than for (conventional IMS)/MS. Unlike DT IMS or chromatography, FAIMS is a scanning

technique that is most suited for targeted analyses (such as selected reaction monitoring) where the duty cycle is not an issue, in parallel to the preferred use of quadrupole filters in MS. Reducing the He fraction to 50% allows ready coupling of FAIMS to unmodified commercial MS platforms and helps with the practical issue of He consumption.

A key aspect of FAIMS is the dissociation or isomerization of ions due to heating in the rf electric field. The effect increases for stronger fields and at higher He content (because of smaller cross sections with He molecules), but the first dependence is generally steeper and ions tend to be hotter at DV = 5.4 kV and 50% He than at DV = 4 kV and 75% He. Small ions and some peptides can tolerate the additional heating, but other peptides (for example, bradykinin 2+) unfold more. Such fragile ions may be best analyzed using a lower DV and higher He content, despite the lower resolution.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.
Operating regimes for various FAIMS systems.

Fig. 2. FAIMS spectra for the protonated reserpine measured at $DV = 4$ kV (a) and 5.4 kV (b), using He/N₂ mixtures with the He fractions as labeled on the top of each panel. The widths (V/cm) and resolving power values (underneath) are given for each peak.

Fig. 3. FAIMS spectra for the protonated leucine (L) and isoleucine (I) derived from the ~1:1 mixture in solution, at DV = 5.4 kV and He fractions as labeled. The peaks are assigned from the data for L or I. The resolution values are given for separated features.

Fig. 4. FAIMS spectra for the $(\text{H}^+)_2$ bradykinin: measured at $\text{DV} = 5.4$ kV and He fractions of 0 - 50% as labeled (left column), with the widths (V/cm) and R values (underneath) given for the major well-shaped peaks (left column); same spectra aligned by scaling the E_C axis such that $E_C = 1$ for the rightmost major peak (right column). The insets reproduce the similarly scaled spectra³³ at $\text{DV} = 4$ kV and 30 - 75% He as marked, selected to best match the present spectral profiles for each He fraction.

Fig. 5. FAIMS spectra for the protonated syntide 2 with $z = 2 - 4$: measured at DV = 5.4 kV and He fractions of 0 - 50% as labeled, with the widths (V/cm) and R values given for the major well-shaped peaks (left column); same spectra aligned as in Fig. 4 for $z = 3$ (middle column) and 4 (right column). The similarly scaled spectra for $z = 3$ and 4 obtained at DV = 4 kV and 50 - 75% He as marked are overlaid in black.

Fig. 6. Resolving power values for the syntide 2 ions with $z = 2 - 4$ at $DV = 4$ kV (solid lines, from the data in refs. [32, 33]) and 5.4 kV (dashed lines). The horizontal axis for the data at 5.4 kV is transposed by 35% up.

Fig. 7.

FAIMS spectra for the protonated angiotensin I with $z = 2$ and 3 , at $DV = 4$ kV and 75% He (a) and $DV = 5.4$ kV and 50% He (b), with the widths (V/cm) and R values given for the major well-shaped peaks.