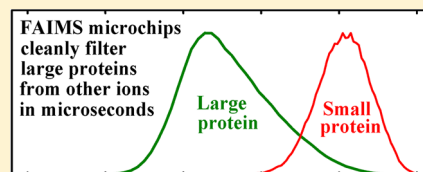


# Protein Analyses Using Differential Ion Mobility Microchips with Mass Spectrometry

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**ABSTRACT:** Differential ion mobility spectrometry (FAIMS) integrated with mass spectrometry (MS) is a powerful new tool for biological and environmental analyses. Large proteins occupy regions of FAIMS spectra distinct from peptides, lipids, or other medium-size biomolecules, likely because strong electric fields align huge dipoles common to macroions. Here we confirm this phenomenon in separations of proteins at extreme fields using FAIMS chips coupled to MS and demonstrate their use to detect even minor amounts of large proteins in complex matrixes of smaller proteins and peptides.



Gas-phase proteins were investigated<sup>1</sup> by ion mobility spectrometry (IMS) in conjunction with electrospray mass spectrometry (ESI/MS) since 1995. The major focus was conformational characterization by conventional IMS, where absolute mobilities  $K$  at moderate electric fields ( $E$ ) may be related to the ion–molecule collision cross sections ( $\Omega$ ) and further to possible ion geometries using *a priori* calculations.<sup>2,3</sup> For protein complexes or aggregates, that approach can help elucidate the global structure and subunit connectivity.<sup>4,5</sup> Differential or field asymmetric waveform IMS (FAIMS) separates ions by  $\Delta K$ , the increment of  $K$  between two  $E$  values.<sup>6,7</sup> While one cannot so far correlate  $\Delta K$  to ion geometries quantitatively, FAIMS shows proteins unfolding for higher charge states and upon field and/or thermal heating in consistency with conventional IMS data.<sup>8–14</sup> Some conformers were separated by FAIMS but not conventional IMS, illustrating substantial orthogonality of these dimensions,<sup>12</sup> and H/D exchange after FAIMS filtering reveals further subpopulations.<sup>15</sup>

In FAIMS, one measures  $\Delta K$  directly employing a periodic asymmetric field  $E(t)$  in a gap between electrodes through which ions are pulled by the gas flow.<sup>6,7</sup> For a given species, the net drift across the gap can be offset utilizing a constant “compensation field” ( $E_C$ ) added to  $E(t)$ . Thus stabilized ions can exit the unit and are detected; hence, scanning  $E_C$  produces a spectrum. The  $E_C$  values depend on the  $E(t)$  amplitude (“dispersion field”,  $E_D$ ) and the gas temperature and composition, and ions are classified based on the  $K(E)$  functional form that determines the  $E_C$  sign.<sup>6,7</sup> With “full-size” FAIMS devices operated at  $E_D \sim 20\text{--}30$  kV/cm (or  $E_D/N \sim 80\text{--}120$  Td at room temperature and pressure, where  $N$  is the gas number density), mobilities in  $N_2$  normally increase at higher  $E$  for ions up to  $\sim 200\text{--}400$  Da (leading to  $E_C < 0$  for  $E_D > 0$ ) and decrease for larger species including all peptides, lipids, and smaller proteins such as ubiquitin,  $\beta_2$ -microglobulin, cytochrome  $c$ , calmodulin, myoglobin, or trypsin (where  $E_C > 0$  for  $E_D > 0$ ).<sup>6–17</sup> These type A and C behaviors, respectively, reflect the contribution of the well of ion–molecule interaction potential to the overall collision process.<sup>7,18</sup> However, proteins

with masses ( $m$ ) of  $>\sim 30$  kDa (carbonic anhydrase, liver alcohol dehydrogenase, bovine serum albumin (BSA), or transferrin) took up<sup>16,17</sup> much wider  $E_C$  segments that spanned  $E_C = 0$ . This has been rationalized in terms of pendency of ion macrodipoles in FAIMS, as detailed below.

The gaps in recently introduced FAIMS microchips<sup>19–22</sup> have  $35\ \mu\text{m}$  width, or  $\sim 60$  times less than the  $2\ \text{mm}$  typical for full-size FAIMS devices used in protein analyses to date. Such narrow gaps permit  $E_D \sim 60$  kV/cm ( $\sim 250$  Td at  $T = 300$  K) without electrical breakdown, which enables ion filtering in  $\sim 20\text{--}50\ \mu\text{s}$ . Here we explore these chips for intact proteins, again encountering the anomaly for larger proteins that supports the pendular hypothesis.<sup>16,17</sup> This effect can be exploited to detect and identify even traces of large proteins in complex samples comprising smaller proteins, peptides, and other substances.

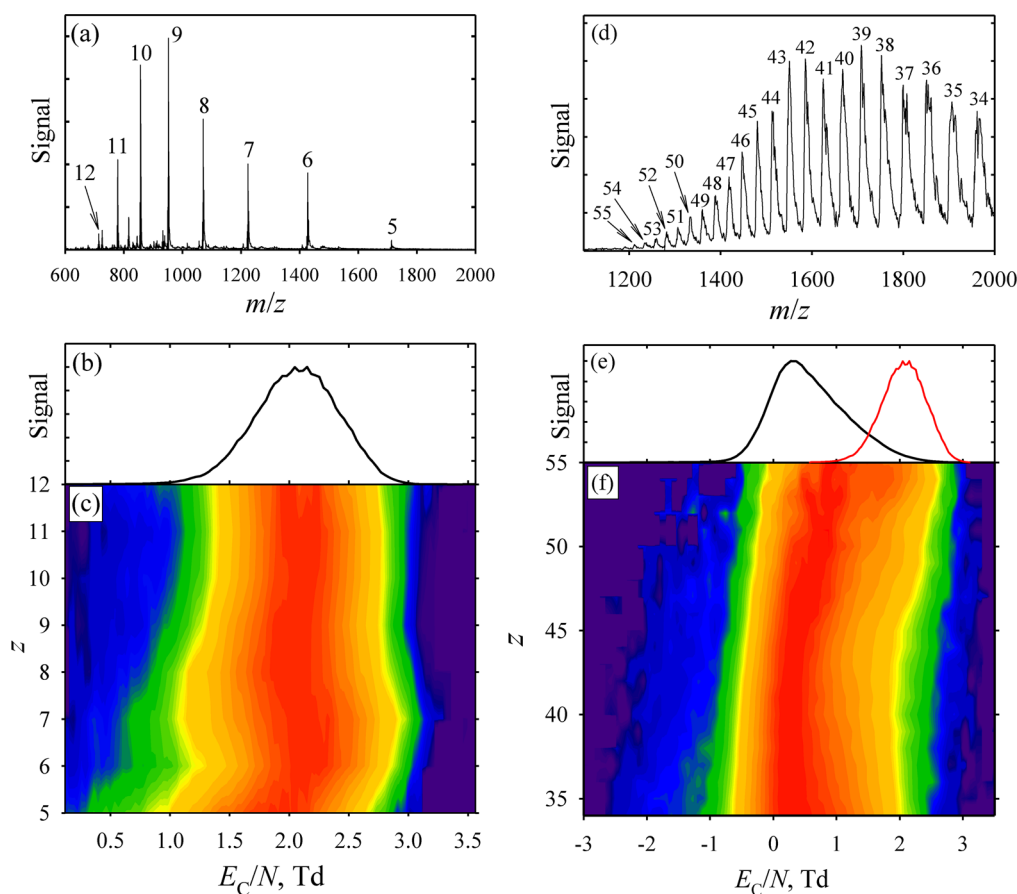
**Fundamentals of Dipole Alignment in Full-Size and Chip FAIMS.** Virtually all macroions have permanent dipole moments ( $p$ ). When the dipole energy in a field (that scales with  $p$  and  $E$ ) at least compares to its rotational energy  $kT_R$  (where  $k$  is the Boltzmann constant and  $T_R$  is the rotational temperature), the dipole becomes materially aligned. The directional  $\Omega$  of aspherical ions in such pendular states<sup>23</sup> at high  $E$  often differ greatly<sup>16</sup> from the orientationally averaged  $\Omega$  of the same ions freely rotating at low  $E$ . That disparity between  $\Omega$  (and thus mobilities) in strong and weak fields would cause a giant FAIMS effect, in line with the findings for large proteins. For neutrals,  $T_R$  equals the gas temperature ( $T$ ) and dipoles with any  $p$  are locked at any  $T$  by a sufficient field:<sup>16,17</sup>

$$E > kT/(2p) \quad (1)$$

Then, with  $E_D = 25$  kV/cm and  $T = 300$  K, alignment requires  $p \sim 250$  D at peak  $E$  and  $p > \sim 390$  D over a considerable part (20%) of the  $E(t)$  cycle.<sup>16</sup> The  $p$  values of proteins increase

Received: July 5, 2012

Accepted: August 9, 2012



**Figure 1.** Data for bovine ubiquitin (a–c) and BSA (d–f) ions: (a, d) mass spectra with the charge states labeled; (b, e) total FAIMS spectra for all charge states (black lines); (c, f) FAIMS/MS spectra drawn as a false-color map (log scale) with interpolation between the discrete  $z$  values provided for viewing convenience. In panel e, the red line recaps the total spectrum for Ub from panel b.

with  $m$  about linearly and statistically exceed 250–390 D at  $m \sim 8$ –20 kDa or somewhat below  $\sim 30$  kDa where the anomalous behavior is first seen.<sup>17</sup>

Electric fields also heat ions in gases, and ensuing rotational excitation impedes the alignment such that species with  $p$  under a critical value ( $p_{\text{crit}}$ ) never align:<sup>17</sup>

$$p_{\text{crit}} = K(kTM/3)^{1/2} \quad (2)$$

where  $M$  is the gas molecule mass. Species with  $p = p_{\text{crit}}$  align at a single  $E$  value of<sup>17</sup>

$$E_{\text{crit}} = kT/p_{\text{crit}} \quad (3)$$

Alignment at other  $E$  requires a larger minimum  $p$ , which we derive from eq 11 in ref 17 as

$$p_{\text{min}} = [M(KE)^2 + 3kT]/(6E) \quad (4)$$

Multiply charged ions generated by ESI for ubiquitin (Ub) and cytochrome  $c$  have  $K = 0.8$ – $1.4$  cm<sup>2</sup>/(V s) in N<sub>2</sub> at  $T = 300$  K,<sup>12,13,24</sup> leading to  $p_{\text{crit}} \sim 190$ – $340$  D by eq 2. The  $p_{\text{min}}$  values for  $E_D = 25$  kV/cm by eq 4 are naturally greater,  $\sim 290$ – $360$  D for alignment at the waveform peak and  $\sim 410$ – $460$  D over 20% of the time. The latter value is, on average, reached by proteins with  $m \sim 20$ – $25$  kDa, in good agreement with the experiment.<sup>17</sup> *Inter alia*, this model explains why Ub ions with  $p = 189$  D for the native geometry and  $p < 250$  D for all simulated conformers with charge states ( $z$ ) of 7, 10, and 12

behave “normally” while BSA ions with  $p \sim 1100$  D (estimated from the native geometry of human albumin) do not.<sup>17</sup>

The FAIMS chips using a much higher  $E_D$  let us further probe the dipole locking theory. By eq 1 where  $p$  is proportional to  $1/E$ , raising  $E_D$  to 60 kV/cm would align ions already for  $p \sim 100$  D at peak  $E$  and  $\sim 160$  D over 20% of the cycle. In particular, a major fraction of Ub conformers would have emerged at  $E_C < 0$  (in parallel to the leucine cations moving from type A at  $E_D \sim 25$  kV/cm to type C at  $\sim 60$  kV/cm).<sup>20</sup> In contrast, eq 2 contains  $E$  only via a weak  $K(E)$  dependence, and  $p_{\text{crit}}$  should change little from that for full-size FAIMS. The  $p_{\text{min}}$  by eq 4 decreases to  $\sim 190$ – $340$  D at peak  $E$  and  $\sim 220$ – $340$  D over 20% of the cycle. These values are equal or close to  $p_{\text{crit}}$  because  $E_{\text{crit}}$  of  $\sim 37$ – $65$  kV/cm<sup>17</sup> by eq 3 is almost entirely within the  $E_D$  range of present chips, which allows alignment at  $p_{\text{crit}}$ . However,<sup>17</sup> the  $p$  values for known Ub conformers are below  $\sim 200$  D, except for rare unfolded geometries at high  $z$  for which<sup>12</sup>  $K > 1.05$  cm<sup>2</sup>/(V s) and  $p_{\text{crit}}$  exceeds 250 Td. Hence Ub ions should still not align at any field. Ions of BSA, where the estimated  $p \sim 1100$  D far exceeds these  $p_{\text{crit}}$  as we mentioned, should firmly align as in full-size FAIMS. The consequent expansion of their spectra to  $E_C < 0$  would then permit separation of albumin from smaller proteins such as ubiquitin and/or peptides or other biomolecules. These predictions are tested below.

## EXPERIMENTAL METHODS

A FAIMS chip by Owlstone (Cambridge, U.K.) was mounted in front of a Thermo LTQ ion trap.<sup>20</sup> For the best separation, we employed the highest enabled  $E_D \sim 60$  kV/cm. For the greatest sensitivity, the filtering time was minimized to  $\sim 20$   $\mu$ s by maximizing the flow through the gap: the gas ( $N_2$ ) was pumped to the unit at 2.8 L/min and evacuated from the FAIMS/MS junction at 2.5 L/min.<sup>20</sup> The scan rate was 3.5 Td/min.

Samples of bovine ubiquitin and BSA were made by dissolving the purchased standards in 50:49:1 water/methanol/acetic acid to  $\sim 10$   $\mu$ M. To gauge the capability to select large proteins from a complex matrix, we mixed these solutions in  $\sim 100$ :1 Ub/BSA ratio, then added a tryptic digest of BSA (exhibiting hundreds of peptide ions even at low sensitivity)<sup>25</sup> and uncharacterized complex lab chemical waste. Samples were infused to the ESI emitter at 0.5  $\mu$ L/min.

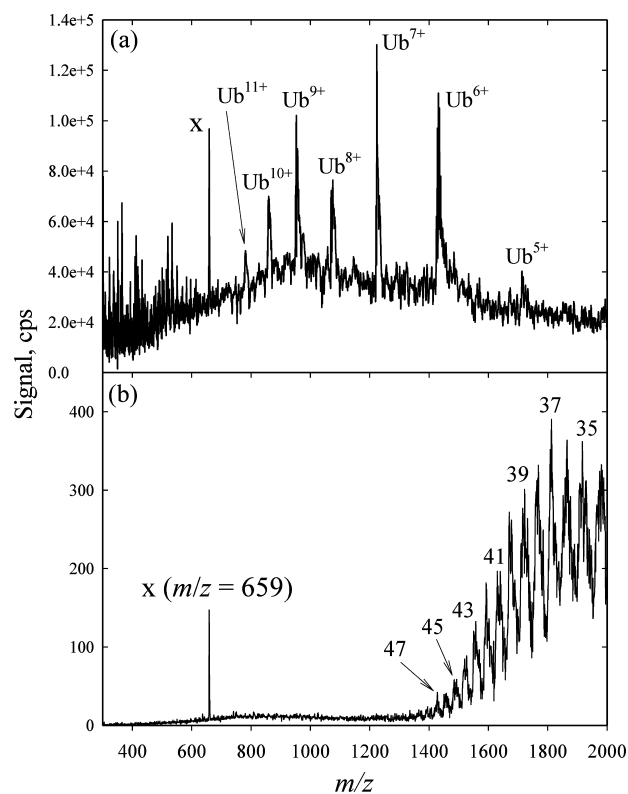
## RESULTS AND DISCUSSION

The Ub ion envelope consists of  $z = 5-12$  as usual (Figure 1a), all of type C (Figure 1b,c). All charge states exhibit single near-Gaussian peaks at similar  $E_C/N = 2.0 \pm 0.1$  Td and widths of  $0.7 \pm 0.1$  Td (fwhm). Besides low resolving power of these chips,<sup>19,20</sup> this uniformity may be attributed to near-complete protein unfolding due to extreme field heating.<sup>12-14,25</sup> At  $E_D/N \sim 80$  Td in full-size FAIMS, isomerization of Ub ions is controlled<sup>13,14</sup> by their maximum (instantaneous) temperature  $T_1$  equal to the gas temperature plus peak magnitude of field heating,  $\Delta T_{\max}$ . In the two-temperature treatment:<sup>2,7</sup>

$$\Delta T_{\max} = M(KE_D)^2 / (3k) \quad (5)$$

In agreement with eq 5, the measured  $\Delta T_{\max}$  value at  $E_D/N \sim 80$  Td was<sup>13,14</sup>  $\sim 50-55$   $^{\circ}$ C. Here the field and heating are 3 and 9 times stronger, respectively, with estimated  $\Delta T_{\max} \sim 470-510$   $^{\circ}$ C and peak  $T_1 \sim 490-540$   $^{\circ}$ C. In heated drift-tube IMS<sup>26</sup> on the  $\sim 50$  ms time scale, ubiquitin ions are folded for  $z < 11$  at  $T = 25$   $^{\circ}$ C but fully unfold at  $\sim 80-132$   $^{\circ}$ C for  $z = 8-10$ . As the Coulomb repulsion of protonated sites aids unfolding, the temperature needed increases for lower  $z$ ; for the lowest reported  $z = 6$ , some folds survived to the highest tried  $T = 132$   $^{\circ}$ C. Little is known about denaturation of protein ions on the present microsecond time scale, but perhaps at  $\sim 500$   $^{\circ}$ C it could be extensive even in such short times. Then the similarity of  $E_C$  for all  $z$  in Figure 2c resembles the findings for unfolded states of  $z = 6-13$  in full-size FAIMS,<sup>12</sup> where  $|E_C/N|$  was  $0.18 \pm 0.02$  Td at the maximum  $E_D/N = 90$  Td. Here, the difference of  $E_C/N = 1.9$  Td for  $z = 5$  from 2.0 to 2.1 Td for  $z = 6-13$  might indicate incomplete unfolding of  $z = 5$ .

The mass spectrum for BSA (Figure 1d) is close to that recorded in ESI/MS previously.<sup>16</sup> As for Ub, the FAIMS spectra for all observed  $z = 34-55$  are similar and  $E_C$  slightly increases for higher  $z$  (Figure 1e,f). However, the peaks for all  $z$  are shifted to much lower  $E_C/N$  values, with the apex of total spectrum at 0.3 Td vs 2.1 Td for Ub (Figure 1e). The BSA ions of all  $z$  include conformers with  $E_C < 0$  (Figure 1f). These findings fit the above model, supporting the pendularity of larger proteins in FAIMS. The separation space for a given  $z$  is  $\sim 50\%$  wider for BSA than for Ub ( $\sim 3.2-3.5$  Td vs  $\sim 2-2.4$  Td), much below the  $\sim 5-10$ -fold expansion in full-size FAIMS.<sup>16,17</sup> At least in part, this reflects the lower resolving power of the present chip, where features are broad regardless of the dipole alignment.



**Figure 2.** Mass spectra for the solution of ubiquitin and trace of BSA in a complex matrix measured (a) without FAIMS (resolved Ub ions are labeled) and (b) with FAIMS at  $E_C = 0$  (charge states of BSA are labeled for odd  $z$ ).

The total FAIMS spectrum for BSA overlaps with that for ubiquitin at the margin ( $E_C/N \sim 1-2.5$  Td), but most ions and the apex in BSA spectra for all charge states lie outside that range (Figure 2e). Further, all peptides and other biological ions analyzed using these chips<sup>20-22</sup> (except smaller single amino acids such as  $H^+Leu$ )<sup>20</sup> come only at significantly positive  $E_C$  when  $E_D/N = 250$  Td. Small ions (such as  $H^+Leu$  with  $m/z = 132$ ) that did not yet clearly cross to type C behavior may appear at  $E_C \leq 0$ , but those species have  $m/z < 300$  and thus are well apart from BSA in the mass spectrum where the lowest detectable  $m/z$  is  $> 1000$  (Figure 2d). Therefore even a minute BSA amount should be separable from light proteins, peptides, and other small-molecule contaminants. The ESI/MS spectrum of a sample comprising Ub, protein digest, and polluted water with added BSA trace (Figure 2a) reveals the Ub charge state envelope, but BSA ions are completely obscured by omnipresent chemical noise. The FAIMS selection at  $E_C = 0$  easily recovers nearly the full BSA charge state distribution by virtually eliminating all other ions except an unassigned species  $x$  at  $m/z = 659$  that is outside the  $m/z$  range for BSA ions (Figure 2b). The non-BSA ions over the whole mass range are suppressed by  $\sim 10^4$  times ( $\sim 10^3$  times for  $x$ ), which is remarkable given the filtering duration of  $\sim 20$   $\mu$ s. As we discussed, this discrimination should broadly apply to other proteins above  $\sim 30$  kDa.

In this aspect, the FAIMS chips may perform better than full-size FAIMS with superior resolving power because many medium-size ions (for example, larger amino acids) that belong to type A at moderate fields<sup>27</sup> convert<sup>20</sup> to type C and thus do not “co-elute” with large proteins.

This separation capability is also unavailable from conventional IMS because larger species tend to have higher charge states and ESI-generated ions of large proteins such as BSA, small proteins such as Ub, and small and large peptides fall into the same mobility range.<sup>5</sup> This preliminary work has employed Generation I microchips,<sup>19</sup> and no detection limits have been quantified. The latest chips with similar  $E_D$  values but 100  $\mu\text{m}$  wide gaps transmit nearly 100% of heavy protein ions at their peak apexes<sup>28</sup> and thus should provide excellent sensitivity far exceeding that of high-resolution FAIMS devices.

## CONCLUSIONS

We report the first analyses of proteins using FAIMS microchips, with small proteins exemplified by ubiquitin and large ones by BSA. For all charge states generated by ESI, ubiquitin ions behave as type C while BSA also exhibit intense type A conformers. These results match findings obtained using full-size FAIMS units and follow the theory for pendular ion states that incorporates rotational heating.<sup>16,17</sup> The presence of type A species for only large proteins and smallest ions with much lower  $m/z$  allows filtering of traces of large proteins (above  $\sim 30$  kDa) from complex biological and environmental matrixes. This capability may be useful to track protein digestion and verify its completion or to prevent lower-mass ions in top-down analyses of proteins and their complexes from entering an MS system (where they take up the limited charge capacity of ion traps or guides and create MS interferences).

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### Notes

The authors declare no competing financial interest.

## ACKNOWLEDGMENTS

We thank Owlstone and specifically Drs. Billy Boyle and Danielle Toutoungi for providing their FAIMS unit, Dr. Keqi Tang for experimental help, and Professor Dudley Herschbach for insights into the pendular states. Portions of this research were supported by the Battelle Independent R&D Program, NIH NCRR (Grant RR 018522), and NIH NIGMS (Grant 8P41GM103493). Work was performed in the Environmental Molecular Sciences Laboratory, a U.S. DoE national scientific user facility at PNNL.

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