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***In situ* analysis of intact proteins by ion mobility mass spectrometry**

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Abstract

We describe ion mobility mass spectrometry techniques for the *in situ* analysis of intact proteins, i.e., the analysis of proteins directly from their biological environment. The benefits of *in situ* analysis include those associated more broadly with analysis of intact proteins, e.g., retention of connectivity between post-translational modifications and direct determination of amino acid substitutions, and those associated with surface sampling, e.g., retention of spatial information. Sampling techniques include liquid extraction surface analysis, continuous-flow liquid-microjunction surface sampling, desorption electrospray ionisation and matrix assisted laser desorption/ionisation. Direct surface sampling is beset by the challenge of inherent sample complexity, a challenge which can be addressed through integration of ion mobility spectrometry. To date, travelling wave ion mobility spectrometry and high field asymmetric waveform ion mobility spectrometry have been applied to the area of *in situ* analysis of proteins. In the case of travelling wave ion mobility spectrometry, information relating to tertiary or quaternary structure can also be obtained.

Keywords: Ion Mobility | Mass Spectrometry | FAIMS | TWIMS | Intact Proteins | *in situ* | LESA | DESI | MALDI | Flowprobe

List of abbreviations

CCS: collision cross section

CF: compensation field

CF-LMJ-SSP: continuous flow liquid microjunction surface sampling probe

DESI: desorption electrospray ionisation

DC: direct current

DF: dispersion field

DMA: differential mobility analyser

DMS: differential mobility spectrometry

DTIMS: drift tube ion mobility spectrometry

FAIMS: high field asymmetric ion mobility mass spectrometry

FT-ICR: Fourier transform ion cyclotron resonance

LC: liquid chromatography

LESA: liquid extraction surface analysis

MALDI: matrix assisted laser desorption ionisation

MS: mass spectrometry

MSI: mass spectrometry imaging

MS/MS: tandem mass spectrometry

m/z : mass to charge

NAMS: Native ambient mass spectrometry

S/N: signal-to-noise

TIMS: trapped ion mobility spectrometry

TWIMS: travelling wave ion mobility mass spectrometry

1.0 Introduction

Proteins play a fundamental role in all biological processes and there is great interest in their characterisation at a molecular level by mass spectrometry. The mass spectrometry analysis of intact proteins offers advantages over approaches in which enzymatic digestion is employed to generate proteolytic peptides. Connectivity between post-translational modifications is maintained and the presence (and location) of amino acid substitutions can be confirmed. Furthermore, higher levels of protein structure (tertiary and quaternary) may be probed. *In situ* analysis of intact proteins, i.e., the analysis of proteins directly from their biological environment, provides further advantages as both spatial information, and correlation of protein structure with molecular environment can be derived. Surface sampling techniques which have been used for *in situ* analysis of intact proteins include liquid extraction surface analysis (LESA) [1], the continuous-flow liquid microjunction surface sampling probe (CF-LMJ-SSP) [2], desorption electrospray ionisation (DESI) [3], matrix-assisted laser desorption/ionisation (MALDI) [4], laserspray ionisation (LSI) [5], matrix assisted ionisation (MAI; also known as matrix assisted ionisation in vacuum, MAIV) [6] and laser ablation electrospray (LAESI) [7]. Schematics of some of these techniques are given in **Figure 1**.

Despite the advantages, *in situ* mass spectrometry analysis of proteins has proved challenging due to the inherent complexity of biological samples, particularly in the case of the ambient surface techniques (LESA, CF-LMJ-SSP and DESI). This challenge can be met through the use of either solution-phase or gas-phase separation techniques. Liquid chromatography is time-consuming, counteracting the benefit of speed of analysis afforded by *in situ* sampling. Gas-phase separation by use of ion mobility spectrometry [8, 9], however, is achieved on the millisecond timescale. In linear ion mobility spectrometry, the reduced mobility (K_0) of an ion is assumed to be independent of the electric field and its transit time is linearly proportional to the field. Linear methods include drift tube ion mobility mass spectrometry (DTIMS) [10, 11], trapped ion mobility spectrometry (TIMS) [12], differential mobility analysers (DMA) [13], and travelling wave ion mobility (TWIMS) [14]. In non-linear ion mobility spectrometry, such as high field asymmetric waveform ion mobility spectrometry (FAIMS) [15] (also known as differential mobility spectrometry, DMS), the mobility of an ion (K) depends on the intensity of the electric field (E). The application of ion mobility mass spectrometry for the analysis of intact proteins is well-established [16]; however, to date, two ion mobility spectrometry techniques have been coupled with *in situ* analysis of intact proteins: FAIMS and TWIMS, see Figure 1. In this review, we describe the integration and applications of these two ion mobility spectrometry techniques with *in situ* surface sampling for the analysis of intact proteins.

2.0 High-field asymmetric waveform ion mobility spectrometry (FAIMS)

High field asymmetric waveform ion mobility spectrometry (FAIMS) (see Figure 1e) separates gas phase ions by exploiting the differences in an ion's mobility in high and low electric fields. FAIMS devices consist of parallel electrodes; these can be of planar or curved geometry. Ions are passed between the electrodes by a carrier gas. An asymmetric waveform is applied to the electrodes such that an alternating high and low electric field is experienced by the ions perpendicular to their trajectory in the carrier gas. The high electric field is also known as the dispersion field (DF). The difference in an ion's mobility in the high and low electric field causes a displacement in the ions trajectory towards one of the electrodes. If the ion collides with the electrode, it is neutralised and therefore not transmitted through the device. To prevent this, a DC voltage can be applied across

the electrodes, creating a compensation field (CF) which counteracts the displacement of the ion towards the electrode. The CF and DF are tuneable parameter in all FAIMS devices and can be used to selectively transmit ions of choice.

FAIMS has been applied to the analysis of both intact proteins and proteolytic peptides, i.e., top-down and bottom-up proteomics [17]. Integration of FAIMS with liquid chromatography tandem mass spectrometry (LC-MS/MS) in bottom-up proteomic workflows has been shown to result in increased numbers of peptide and protein identifications, as well as the separation of isobaric peptide ions [18-20]. FAIMS analysis of intact proteins has demonstrated the ability to separate charge states and conformations of protein standards such as ubiquitin and lysozyme [21, 22]. It is important to note, however, that while FAIMS is capable of separating protein conformations, it is not possible to directly determine the collision cross sections by FAIMS. More recently, FAIMS has been successfully coupled with surface sampling techniques. Galhena *et al.* coupled DESI with FAIMS for the analysis of small molecule drugs [23]. DESI FAIMS was applied to lipid imaging in mouse brain [24] and LESA FAIMS was applied to the separation of isomeric drug metabolites from human kidney and muscle tissue samples [25]. FAIMS has also been coupled with surface sampling techniques (LESA, DESI and CF-LMJ-SSP) for the analysis of intact proteins from complex substrates such as thin tissue sections [26-28], dried blood spots [29] and bacterial colonies growing on agar [26]. In each case, the sampling technique has been coupled with an ultrahigh field chip-based planar FAIMS device (available from Owlstone) on which it is possible to tune both the DF and CF parameters.

2.1 Liquid extraction surface analysis (LESA) FAIMS

Liquid extraction surface analysis (LESA) (see Figure 1a) is an ambient surface sampling technique. The sampling procedure in LESA is as follows: a small volume of solvent (~1-10 μ L) is aspirated from a solvent well and dispensed onto a sample surface using a robotic pipette. A liquid junction is maintained between the surface and the conductive pipette tip for a few seconds (~ 1-30 s) to allow the diffusion of analytes into the solvent. The sample is reaspirated and introduced to the mass spectrometer by nanoelectrospray ionisation. LESA was first described by Van Berkel in 2010 [1] and has since been used to sample a range of biological substrates such as dried blood spots [29], thin tissue sections [30, 31] and bacteria [32]. By tailoring the composition of the solvent system, LESA can be used to extract a range of molecular analytes from small metabolites and lipids [33] to intact proteins and protein complexes [34].

Sarsby *et al.* [26] described a LESA FAIMS MS workflow for the analysis of proteins from thin sections of mouse liver and brain, as well as *E. coli* colonies growing on agar. A two-dimensional FAIMS analysis, in which both the DF and CF are varied, was performed in order to determine the settings for optimum transmission of the ions of interest. At DF = 270 Td, mid to low mass proteins were transmitted in the CF range ~ 1.5 – 3 Td, whereas at lower CF values (i.e., -0.5-1 Td), unresolved higher molecular weight proteins were transmitted. Although the incorporation of FAIMS in the LESA workflow resulted in a drop in overall signal intensity, it also resulted in improved signal to noise (S/N) and therefore much richer mass spectra. For example, at DF = 270 Td and CF = 2.6 Td, 29 individual protein masses were detected compared to 3 proteins detected without FAIMS (see **Figure 2**). Subsequent work demonstrated LESA FAIMS MS of proteins from dried blood spots [29]. LESA mass spectra of dried blood spots are characterised by highly abundant ions corresponding to the α - and β -globin chains of haemoglobin. Integration of FAIMS did not enable separation of the α -globin from the β -globin ions (or the detection of any additional proteins); however, it did enable the

separation of the globin chains and lipids, and consequently the observation of previously undetected lipids.

A major advantage of the incorporation of FAIMS in LESA mass spectrometry is the improved S/N. The increase in S/N allows for a reduced analysis time making it a useful addition in LESA mass spectrometry imaging (MSI) workflows, particularly for intact proteins where longer acquisitions are often required due to the low abundance of the protein ions. The incorporation of FAIMS with LESA MSI, resulted in the detection of 34 proteins across a mouse brain tissue section compared to 15 proteins that were detected using LESA alone [35]. Twenty six of the 34 proteins were unique to the FAIMS dataset. Similar results were obtained from mouse liver with 40 proteins detected with LESA FAIMS (29 unique to FAIMS) compared to 24 proteins detected with LESA alone.

In LESA the sampling process is decoupled from the ionisation process which means that the mass spectrometry data acquisition time can be optimised according to the experiment. This capability allows for greater flexibility in the design of a FAIMS experiment, e.g., the inclusion of multiple FAIMS settings, thereby transmission of different ions, in a single acquisition. Using a multistep static FAIMS approach in the LESA MSI of sections of rat kidney and rat testes, it was possible to increase the number of proteins detected several fold over a single step FAIMS approach [36]. For example, 55 proteins were detected in a multistep FAIMS analysis of fresh frozen rat kidney tissue compared with seven proteins detected in a single-step analysis.

2.2 Flowprobe FAIMS

The continuous flow liquid microjunction surface sampling probe (CF-LMJ-SSP) [2], commercialised as the Flowprobe, is an ambient surface sampling technique which also makes use of a liquid junction between the sample surface and the sampling probe (see Figure 1b). The Flowprobe consists of two coaxial capillaries; the outer capillary carries the extraction solvent towards the substrate with a typical flow rate of 10-60 $\mu\text{L}/\text{min}$. The inner capillary carries the solvent away from the surface to the mass spectrometer where it is introduced by electrospray ionisation. At the surface of the substrate a liquid junction is formed into which analytes diffuse. The size of the microjunction can be controlled by adjusting the flow rates of the solvent in the capillaries. CF-LMJ-SSP, like LESA, has been shown to be suitable for sampling a range of analytes including metabolites [37], lipids [37], drugs [38], and proteins [38, 39]. CF-LMJ-SSP is suitable for extracting proteins from dried blood spots prior to LC separation [38], and extracting proteins (ubiquitin, β -thymosin 4 and haemoglobin) from tissue [39]. Due to the continuous flow of solvent in this sampling method, the sensitivity is much lower compared to LESA as a result of dilution of the analytes in the solvent. To compensate for this loss in sensitivity, Feider *et al.* [27] coupled CF-LMJ-SSP with FAIMS for the sampling of proteins from rat brain. The FAIMS device was optimised to transmit proteins in the mass range 4 - 12 kDa with DF = 230 Td and CF = 2.25 Td. The S/N for the peak corresponding to the 10+ charge state of ubiquitin (m/z 857) was reported to increase from 23 (without FAIMS) to 180 (with FAIMS). Consequently, these researchers were able to detect 84 protein species (of which 66 were unique to FAIMS) compared to the 66 protein species detected using CF-LMJ-SSP alone. CF-LMJ-SSP FAIMS MSI was also applied to the comparison of cancerous human ovarian tissue samples with healthy samples. The resulting ion images show the localisation of proteins such as ubiquitin, β -thymosin 4 and calyculin in tumour regions compared to necrotic and healthy tissue [27].

2.3 Desorption Electrospray Ionisation (DESI) FAIMS

Desorption electrospray ionisation (DESI) [3] is an ambient mass spectrometry technique (see Figure 1c) that has been shown to be suitable for the imaging of metabolites and lipids from tissue samples [40]. DESI sampling is achieved by directing a stream of charged solvent ions at an angle towards the sample surface. When the charged droplets hit the surface, analytes are desorbed and charge is imparted creating analyte ions which are introduced to the mass spectrometer. DESI analysis of purified intact proteins from solid surfaces was first demonstrated over a decade ago [41]. In that work, DESI was coupled with a drift tube ion mobility mass spectrometer for the analysis of cytochrome c and lysozyme. Later work demonstrated DESI of protein standards up to 66 kDa, however limits of detection were found to increase with increasing molecular weight [42]. Douglass *et al.* showed that this mass-dependent drop in sensitivity was due to incomplete dissolution and protein-protein and protein contaminant clustering [43]. It was subsequently demonstrated that the use of solvent additives, such as ammonium bicarbonate, greatly improved the observed S/N of protein standards [44]. Modifications of the DESI set up itself have also resulted in significant improvements. Ambrose *et al.* [45] demonstrated DESI to be capable of sampling the tetradecameric GroEL (~800 kDa) by minimising the length of the sample transfer tube and locating the stage directly under the inlet of the ion source.

Despite these advances, DESI analysis of intact proteins from thin tissue sections remained challenging due to inefficient desorption of larger molecules and the high amount of chemical noise inherent to tissue sampling. This challenge was finally addressed by the integration of ion mobility spectrometry, which enables the separation of protein ions from ions from other molecular classes, and therefore improves S/N. In 2018, two groups independently demonstrated DESI for the analysis of intact proteins from tissue. Garza *et al.* [28] approached this challenge by introducing FAIMS into their workflow, while Towers *et al.* [46] incorporated TWIMS into their workflow. The latter is discussed in more detail below. Garza *et al.* first optimised the DESI set up for proteins by reducing the spray angle to 55°, decreasing the sample-to-inlet distance (to 2.5 mm) and using a solvent system of acetonitrile and water (80:20) with 0.5 % formic acid, before coupling with the FAIMS device. The workflow was optimised for mouse kidney tissue sections. Two-dimensional FAIMS analyses (DF and CF scanning) were performed in order to find the optimal parameters for detection of proteins (DF = 180 Td, CF = 1 Td). An overall decrease in the absolute signal intensities was observed; however, the improved S/N resulted in the detection of 11 proteoforms from kidney. The optimised workflow was subsequently applied to DESI imaging of proteins (α -globin, β -globin, MBP isoform 4, and an unidentified 8 kDa protein) in mouse brain as well as comparison of the distribution of α -globin and S100 A6 in healthy and cancerous human ovarian tissue. The protein S100A6 was observed at higher abundance in cancerous tissue.

3.0 Travelling wave ion mobility spectrometry (TWIMS)

Travelling wave ion mobility spectrometry (TWIMS) (see Figure 1f) utilises a stacked-ring ion guide (SRIG) operated with a travelling wave that separates ions based on their size and shape [47]. The radial confinement of ions is achieved by applying opposite phases of an RF voltage to neighbouring ring electrodes. A pulsed DC voltage is superimposed on the RF voltage, which is applied to each electrode plate pair for a fixed pulse, and stepped from one side of the device to the other in succession. The ion mobility cell is filled with an inert buffer gas, typically nitrogen, in which the ions traverse the device. Alterations in the speed and magnitude of the periodic travelling wave and

collision with the inert buffer gas will lead to the separation of ions according to their mobility. Smaller, more compact ions encounter fewer collisions and are propelled more quickly through the device, whereas larger, more extended ions experience a greater lag and take longer to travel. Since the electric field is neither uniform nor time dependent [47] and the pressure readouts are not accurate, a drift time calibration with protein standards of known collision cross section (CCS) is necessary [48, 49]. It is important that consideration is given to the nature of buffer gas in which the CCS is derived. A systematic approach for reporting collision cross sections obtained from TWIMS and other forms of ion mobility measurements has been recently devised [9]. One of the unparalleled merits of TWIMS is its versatility. It has been integrated with gas phase infrared spectroscopy [50], surface induced dissociation [51] and ultraviolet photodissociation of proteins [52]. Furthermore, TWIMS can be coupled to matrix assisted laser desorption/ionisation (MALDI) and DESI for *in situ* analysis of proteins from tissue.

3.1 DESI TWIMS

The coupling of DESI with FAIMS [28] was discussed above; however Towers *et al.* [46] have also met success imaging intact proteins by using DESI in combination with TWIMS for the analysis of thin sections of rat liver. A modified heated inlet capillary was used, together with solvents comprising high organic content (80% acetonitrile). In the absence of TWIMS, only the 9+ through 22+ charge states of α - and β -globin were observed. Incorporation of TWIMS enabled the DESI imaging (at a spatial resolution of 150 μm) of a number of proteins and peptides by allowing the selection of specific regions of interest from the plot of drift time vs. m/z . Proteins imaged included the haemoglobin subunits, acetylated fatty acid binding protein, 10 kDa heat shock protein and a cytochrome c oxidase subunit 8A, see **Figure 3**.

3.2 Matrix assisted laser desorption/ionisation (MALDI) TWIMS

Matrix assisted laser desorption/ionisation (MALDI) coupled with ion mobility mass spectrometry has been successfully deployed in an array of applications for the analysis of peptides and proteins [53, 54]. MALDI is a surface sampling technique, where the analytes of interest, e.g., the constituents of thin tissue sections, are coated with matrix, placed under vacuum and ablated with a laser (see Figure 1d) to generate predominantly singly- or doubly charged molecular ions [4, 55] for profiling or imaging purposes. MALDI imaging permits the acquisition of a series of multiple mass spectra across the entire tissue section at a defined spatial resolution. These spectra are then combined together to create images, representing the spatial distribution and relative intensity of an ion present within the tissue. Owing to the complexity of tissue environment, *in situ* digestion of proteins, enabling subsequent MS/MS fragmentation and assignments of proteolytic peptides (bottom-up approach), prior to MALDI imaging is often favoured. That is, MALDI MSI of intact proteins is less common than MALDI MSI of proteolytic peptides. Spraggins *et al.* [56] demonstrated MALDI MSI of intact proteins up to ~12 kDa in rat brain using a Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer. Later work showed MALDI FT-ICR MSI of intact proteins up to 20 kDa [57]. To the best of our knowledge, *in situ* MALDI analysis of intact proteins from tissue coupled with TWIMS has not been demonstrated to date.

The addition of TWIMS to the MALDI MSI of proteolytic peptides can enhance selectivity and specificity by minimising peak interferences and distinguishing between isobaric species. Djidja *et al.* applied bottom-up MALDI TWIMS MSI for the *in situ* identification of glucose regulated protein 78 kDa (Grp78) from formalin-fixed, paraffin-embedded pancreatic adenocarcinoma tissue [53].

Integration of TWIMS enabled the separation of isobaric tryptic peptides based on their ion mobility. Similarly, Stauber *et al.* applied bottom-up MALDI TWIMS imaging to formalin fixed paraffin embedded rat brain tissue and frozen human brain tissue [54]. Again, the benefit of ion mobility separation was illustrated by the effective discrimination of proteolytic peptide ions with similar m/z values but different mobility. For example, TWIMS separated two isobaric peptide ions with m/z 1039, enabling the identification of tubulin and ubiquitin. Moreover, ion mobility selection could distinguish between low intensity peptide and lipid fragment ions. Cole *et al.* [58] applied bottom-up MALDI TWIMS MSI to the analysis of tissue from a drug treated mouse fibrosarcoma model. Integration of TWIMS enabled separation of doubly charged peptide ions from interfering singly-charged ions (peptides, matrix, lipids) and thus improved MS/MS data quality and confidence in peptide assignments. Bottom-up MALDI TWIMS MSI revealed the spatial distribution of proteins and peptides from *ex-vivo* human skin [59]. Imaging of peptides from the proteins keratin 1, collagen alpha 1 and keratin sulphate proteoglycan at 30 μm resolution was possible as a result of the separation afforded by TWIMS.

3.3 Laserspray ionisation (LSI) and matrix assisted ionisation (MAI) TWIMS

MALDI typically generates singly-charged ions limiting its usefulness for the analysis of higher molecular weight proteins. LSI is an atmospheric pressure MALDI method which generates multiply-charged ions [5]. The sample and matrix are deposited on a glass slide. Laser ablation via a UV laser directed at the back side (transmission geometry) of the glass slide results in the formation of highly-charged clusters which are desolvated and introduced to the mass spectrometer through a heated capillary. Detection of proteins up to ~ 20 kDa from mouse brain tissue sections, and up to 15 kDa from rat brain sections, by LSI mass spectrometry has been demonstrated [60, 61].

Building on this work, Trimpin and co-workers introduced MAI, in which multiply-charged ions are produced when the sample (analyte and matrix) are produced spontaneously under the vacuum conditions of the mass spectrometer [6]. That is, MAI does not require a laser or high voltages. (LSI is a subset of MAI). MAI was combined with TWIMS for the *in situ* analysis of proteins directly from sections of mouse brain. A challenge for MAI TWIMS of tissue sections is spatial profiling of analytes, but the authors note that by depositing matrix in defined regions of interest of the tissue spatial information could be obtained.

3.4 Native LESA TWIMS

Native mass spectrometry is a rapidly growing field that enables the study of folded proteins and protein assemblies in the gas phase by maintaining weak non-covalent interactions, such as hydrogen bonds and salt bridges, that were present in solution phase [62]. Non-covalent bonds are maintained through the use of carefully tailored electrospray solutions. The growth of native mass spectrometry has been accompanied by development of ion mobility spectrometry techniques which enable the conformation of the folded protein to be probed including DTIMS and TWIMS.

Native ambient mass spectrometry (NAMS) of proteins is an emerging area of research. Native mass spectrometry generally requires the use of electrospray ionisation, and therefore any ambient surface technique which employs electrospray may, in principle, lend itself to NAMS. Note that the acidic and denaturing nature of matrices used in MALDI means that non-covalent interactions are

generally not maintained. Moreover, the intrinsic production of singly charged ions combined with the high masses of native proteins and complexes result in high m/z values that are often out of the range of most mass spectrometers [55, 63]. Nevertheless, liquid MALDI of protein-protein complexes has recently been demonstrated [64].

Native LESA mass spectrometry of the haemoglobin tetramer from blood vessel features in thin tissue sections of mouse liver [65] and large purified protein assemblies, including GroEL (~800 kDa) from glass substrates [66] has been demonstrated. Native DESI of GroEL from glass substrates [45] has also been shown. Recent work in our laboratory has coupled native LESA mass spectrometry of tissue with TWIMS. The native LESA workflow was optimised by control of the pressure in the mass spectrometer source region, resulting in the extraction of folded proteins from the bulk tissue of mouse liver and brain [34] (i.e., away from the vasculature). The benefit of incorporating TWIMS into the workflow was demonstrated through the determination of CCS for proteins extracted from mouse brain. The $^{TW}CCS_{N_2 \rightarrow He}$ of β -thymosin 4 (4+), β -thymosin 10 (4+), and ubiquitin (5+) were calculated to be 733 ± 2 , 796 ± 2 and $1047 \pm 8 \text{ \AA}^2$ respectively (errors represent one standard deviation). The CCS of the ubiquitin 5+ ion from brain was compared against literature CCS values, as well as that of a purified ubiquitin standard, and was found to be in good agreement [34].

4.0 Conclusion

Integration of ion mobility spectrometry in the *in situ* mass spectrometry analysis of intact proteins provides two main benefits. Firstly, sensitivity is increased as a result of improved S/N. Improved S/N has been observed for both FAIMS and TWIMS, resulting in greater numbers of proteins detected and more comprehensive protein imaging. For DESI, the incorporation of ion mobility separation is critical for detection of proteins in tissue. It is likely that as ion mobility spectrometry techniques develop, further improvements for *in situ* protein analysis will be realised, and that additional ion mobility techniques, such as TIMS, will be integrated into these workflows. A challenge associated with intact protein analysis is identification of the protein by top-down mass spectrometry. The greater the number of proteins detected, the more challenging the identification problem, particularly in the case of protein mass spectrometry imaging where there is limited time and sample associated with each pixel. Methods and software for rapid identification need to be developed.

The second benefit of integration of ion mobility spectrometry is that structural information in the form of collision cross sections can be obtained. To date, this feature has only been demonstrated with TWIMS but it is anticipated that as the field develops other ion mobility techniques, such as TIMS, may be applied. An emerging area of research is native ambient mass spectrometry, which focuses on analysis of folded proteins and non-covalent protein assemblies. The ability to combine native ambient mass spectrometry with ion mobility spectrometry will be particularly useful. So far, native mass spectrometry imaging of proteins and protein complexes has been decoupled from measurement of CCS. That is, native mass spectrometry imaging of a thin tissue section is followed by measurement of CCS in an adjacent tissue section. By incorporating ion mobility spectrometry into the imaging experiment, it will be possible to image protein *structures* rather than simply their masses, a truly exciting prospect.

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Figure legends

Figure 1: Surface sampling and ion mobility techniques for *in situ* protein analysis a) Liquid extraction surface analysis (LESA) b) Continuous-flow liquid microjunction surface sampling probe (CF-LMJ-SSP) c) Desorption electrospray ionisation (DESI) d) Matrix assisted laser desorption ionisation (MALDI) e) High field asymmetric waveform ion mobility spectrometry (FAIMS) f) Travelling wave ion mobility spectrometry (TWIMS). Adapted and reproduced from Kocurek, K.I. Griffiths, R.L. and Cooper, H.J. J. Mass Spectrom. (2018) 53:565–578 <https://doi.org/10.1002/jms.4087>. Published by John Wiley & Sons Ltd. under the terms of the Creative Commons Attribution 4.0 International License (<http://creativecommons.org/licenses/by/4.0/>)

Figure 2: Liquid extraction surface analysis MS of mouse brain tissue. Top – LESA mass spectrum obtained with no FAIMS field. Bottom – LESA FAIMS mass spectrum obtained using DF = 270 Td and CF = 2.6 Td. Inserts – Expanded m/z region of each spectrum showing the charge states of proteins detected. Reproduced from Sarsby, J. Griffiths, R.L. et al. Anal Chem (2015) 87: 6794-6800 <https://doi.org/10.1021/acs.analchem.5b01151>. Published by ACS Publications under the terms of the Creative Commons Attribution 4.0 International License (<http://creativecommons.org/licenses/by/4.0/>)

Figure 3: Desorption electrospray ionisation analysis of rat liver tissue. Top Left - Typical drift time plot of liver tissue with three distinct regions highlighted a) high velocity region containing multiply charged proteins and peptides (b) haemoglobin trend line (c) singly charged molecules. Top Right - The corresponding summed mass spectrum for each region highlighted in drift time plot. Bottom – DESI images of proteins showing, from left to right, the distribution of a-globin subunit 1, a-globin subunit 2, b-globin subunit 2, b-globin subunit 2 with an S to T amino acid substitution, cytochrome c oxidase 8A, fatty acid binding protein liver and 10 kDa heat shock protein. Adapted and reproduced from Towers, M.W., Karancsi, T., Jones, E.A. et al. J. Am. Soc. Mass Spectrom. (2018) 29: 2456. <https://doi.org/10.1007/s13361-018-2049-0>, Published by Springer under the terms of the Creative Commons Attribution 4.0 International License (<http://creativecommons.org/licenses/by/4.0/>)

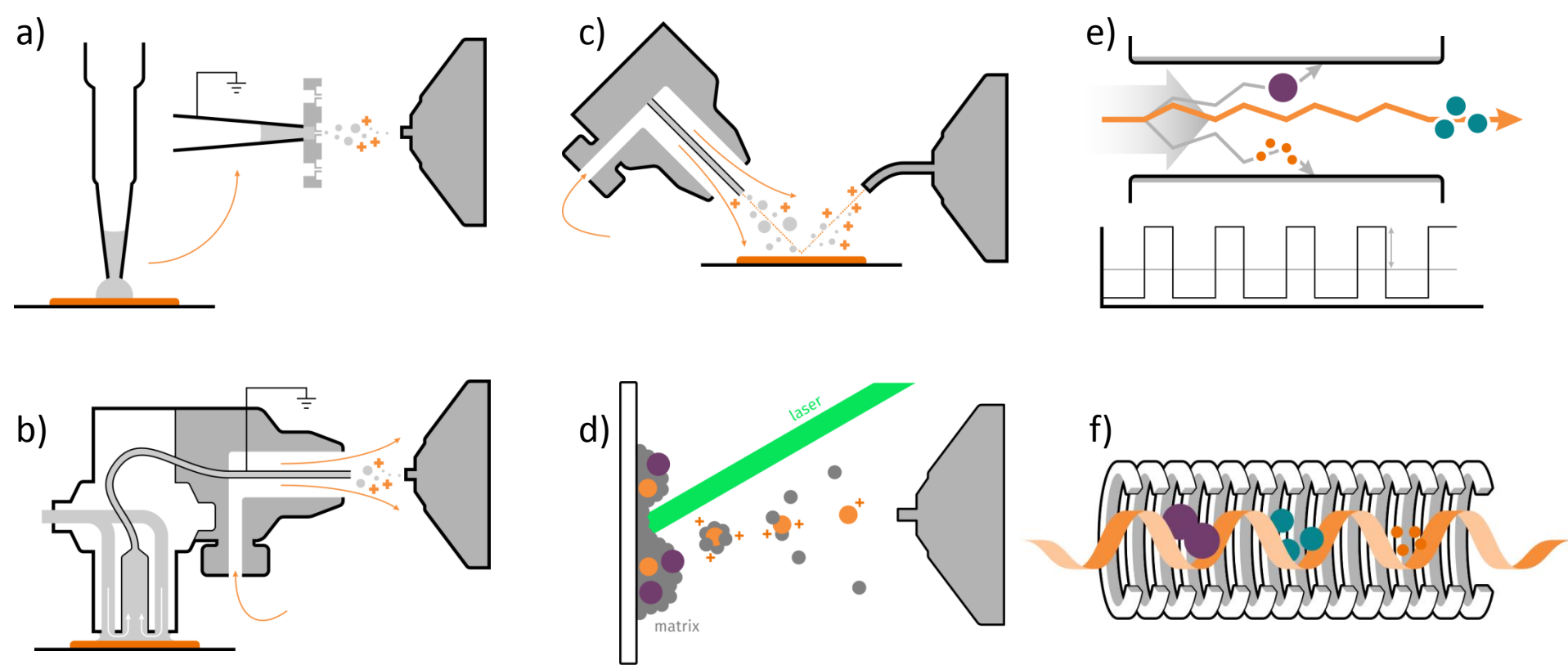


Figure 1

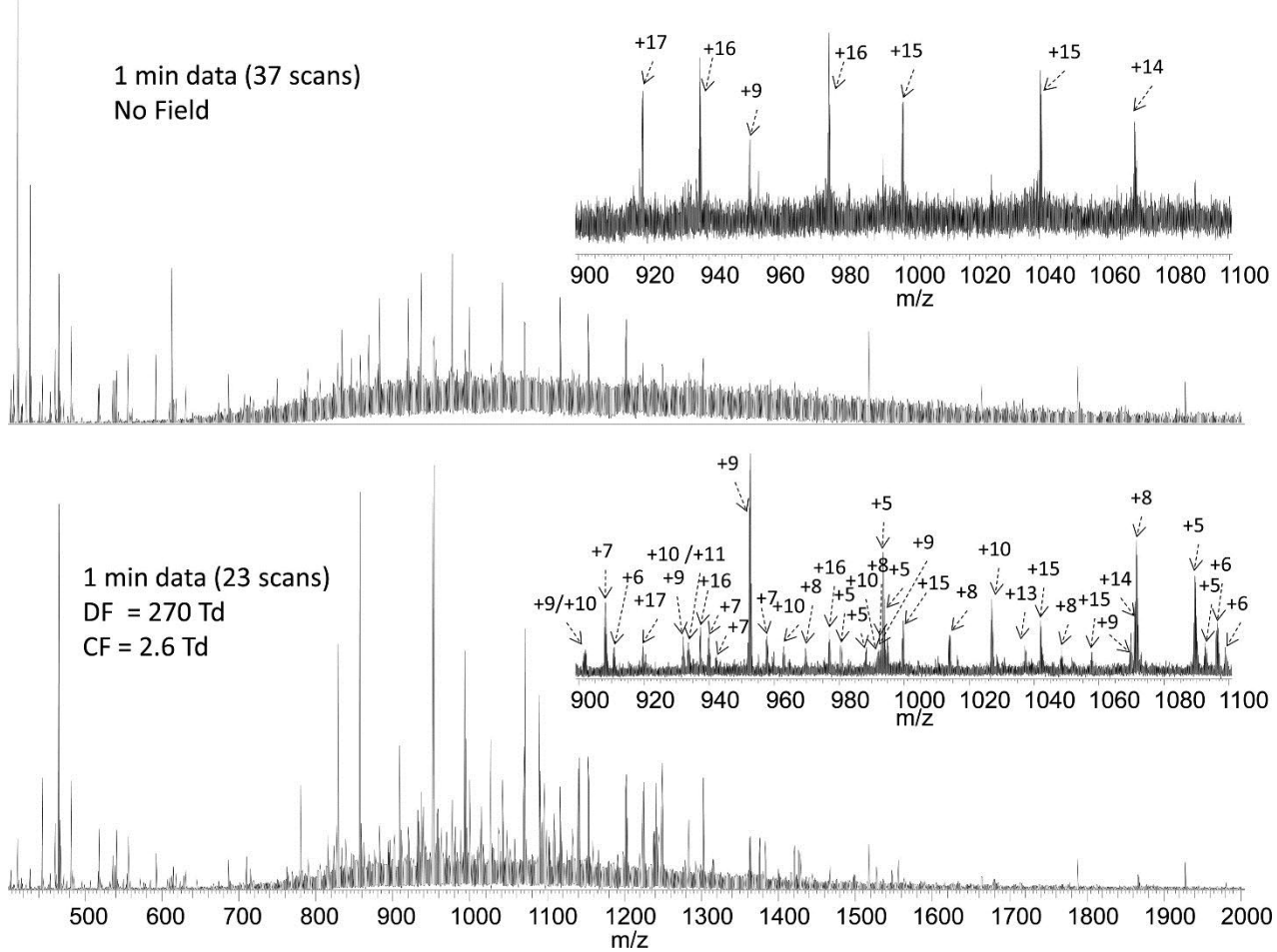


Figure 2

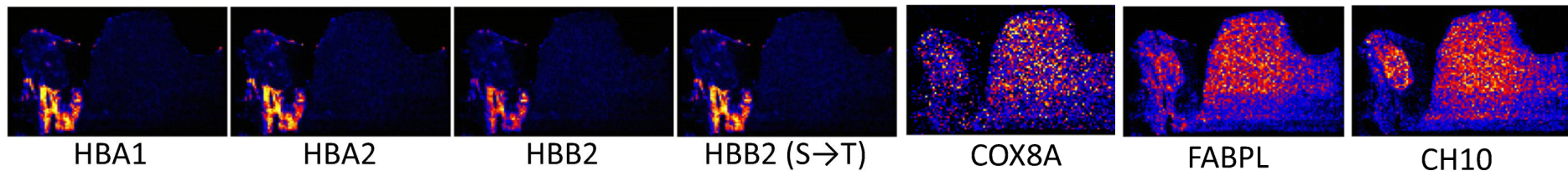
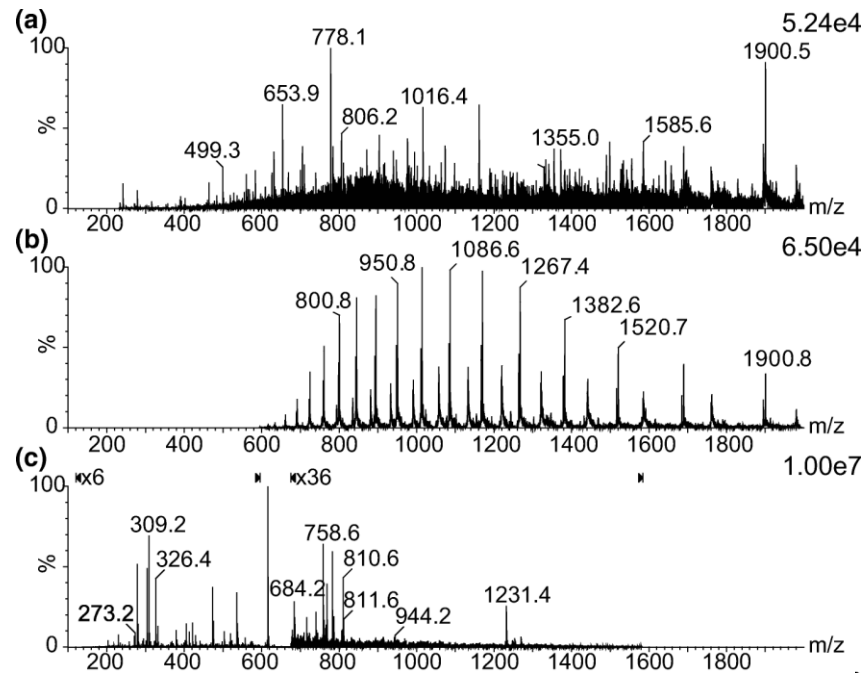
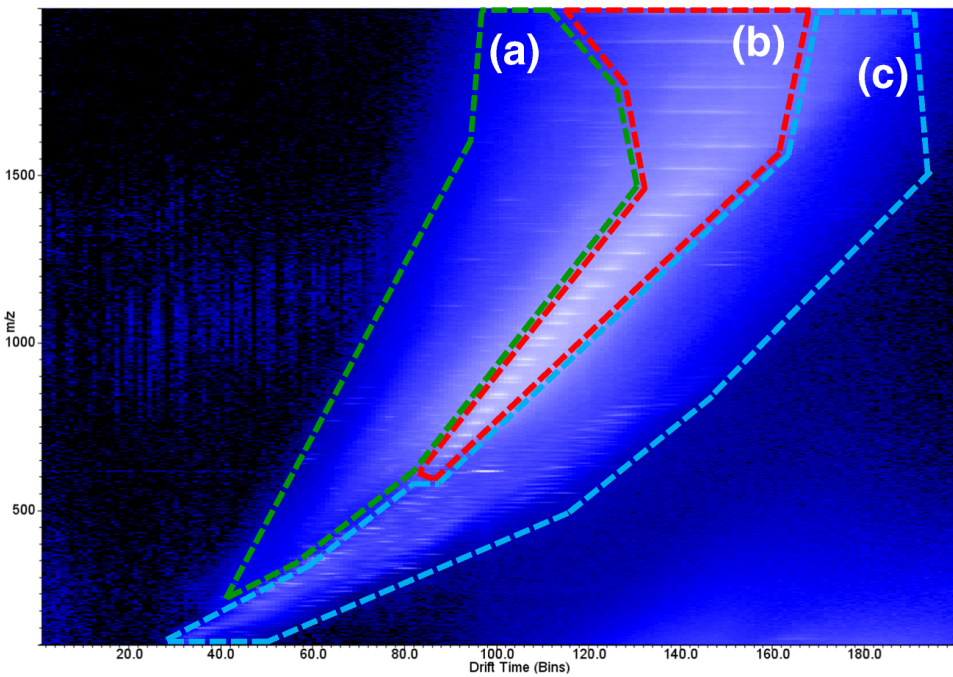


Figure 3