Deep characterisation and quantitative analysis of proteins and post-translational modifications

How TIMS has extended the capabilities of MS and shown considerable potential for improving PTM identification and critical understanding of signalling pathways and abnormal disease states.

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Proteins are the most relevant functional molecules to all diseases; all biological products, drug targets, and diagnostic reagents are proteins, so being able to reliably identify and characterise them is crucial for biomedical research. Detecting the variations that alter protein behaviour, known as post-translational modifications (PTMs), can help transform the way human diseases are understood, diagnosed, and treated. However, comprehensive protein and PTM characterisation raises several challenges, including how to maximise sequence coverage, detect low abundant modified peptides, and manage the fragmentation difficulties encountered by specific modifications like glycosylation or phosphorylation. Consequently, many basic and clinical research laboratories are looking towards advanced analytical tools such as mass spectrometry (MS), which have proven to be powerful solutions for proteome-wide profiling of PTMs at a site-specific level. The sensitivity and speed of MS instruments allow for the measurement of millions of spectra and identification of thousands of proteins and peptides in single experiments, paving the way for deep characterisation of PTMs.

Uncovering new insights into disease

PTMs increase the functional diversity of the proteome and are involved in most cellular processes. The pattern of these modifications can significantly impact almost all aspects of normal cell biology and pathogenesis, such as the protein functionality, activity level, solubility, or its half-life. For instance, proteins may be phosphorylated, glycosylated, acetylated, ubiquitinated, farnesylated, sulphated, or linked to lipid (GPI) anchors. The impact of such modifications on cell signalling and disease pathways have been widely reported and form the focus of clinical and pharmaceutical research into potential drug targets [1-3].

Detecting PTMs of proteins is an important tool for obtaining insights into health and disease states such as cancer, diabetes, and stem cell research, and in facilitating drug discovery. However, direct protein analysis from tissue or biofluids raises a variety of analytical challenges. Protein expression varies depending on the genetic background of an individual, but also depending on time, localisation, and as a physiological response for external stimuli. The dynamic nature of the proteome is further exacerbated by the effects of PTMs, leading to a variety of proteoforms being expressed from a single protein, each with dedicated biological activity.

Despite advancements in high-resolution mass spectrometers, identifying PTMs continues to be significantly more challenging in comparison to unmodified peptides. Often this is because they occur at low abundances and the differences span several orders of magnitude, driving the need for instruments with a higher sensitivity and increased peak capacities. Accurate profiling and characterisation require regular and more rigorous analysis, which can lead to incomplete amino acid sequence coverage and insufficient understanding of the crosstalk and selectivity of different PTMs and their impacts on cellular regulatory networks.

Levelling up MS analysis

Trapped ion mobility spectrometry (TIMS) is a highly efficient gas-phase technique built upon ion mobility spectrometry (IMS) technology, capturing a broad molecular weight range of signals at high scan rates. Separating ions by mobility using TIMS boosts sensitivity and provides further selectivity to protein analysis as the ions are separated by a fourth parameter, their collision cross-section (CCS). CCS is a measure of how likely the ions are to be deflected by a collision with other gas molecules as they drift through an ion tube. Compact ions with a small CCS drift faster than extended ions with a large CCS, essentially allowing ions to be separated by shape, retention time, mass-to-charge (m/z) ratio and intensity.

The combination of IMS with MS is a well-established technique that has shown considerable potential for improving peptide identification and provides structural information that is complementary to liquid chromatography (LC) and MS. In recent years, pairing trapped ion mobility spectrometry (TIMS) with a time-of-flight (TOF) mass analyser has become an increasingly popular method of identifying and characterising proteins that capitalises on its high-speed capabilities.

TIMS are relatively small devices that can be easily integrated into a mass spectrometer without a noticeable loss in ion transmission; adding the ion focusing effect from TIMS to the ultra-high resolution and high-speed of TOF technology enables the discovery of low level biologically relevant proteins which, currently, non-TIMS MS systems cannot detect.

Therefore, this four-dimensional approach to proteomics enables the separation of proteins or peptides with similar molecular weights, which would otherwise show as the same peak on the mass spectra.

Case studies of PTM analysis

1. Ubiquitination is a well-known PTM essential in regulating key biological processes such as protein degradation, immune response, signal transduction and DNA repair. MS-based proteomics platforms have proven to be powerful tools in enabling proteome wide profiling of PTMs at the site-specific level. The characterisation of global ubiquitination, however, is still proving particularly challenging due to its sub-stoichiometric abundance and co-elution of ubiquitinated peptides only differing in the position of the modified site. A characterisation of enriched ubiquitination peptides from HeLa cell lysates, performed by PTM Bio using TIMS and parallel accumulation serial fragmentation (PASEF) (timsTOF Pro, Bruker Daltonics), identified more than 17,000 ubiquitination sites with only 50 min gradients that corresponded to 5093 ubiquitinated proteins from two cell lines *(Figure 1)* [4]. TIMS provided an additional dimension of separation that enabled the identification and quantification of co-eluting isobaric peptides with different modification sites *(Figure 2)*.

TIMS also enables the PASEF, offering superior speed and improved sensitivity to proteomics workflows. The PASEF acquisition method – based on using the position of the quadrupole for selecting peptides by their m/z and moving it in synchronisation with the mobility elution profile – exploits TIMS to achieve up to a 10-fold increase in sequencing speed. The increase in sequencing speed is crucial for delving deeper into complex proteomes to obtain quantitative data in a short amount of time. The combined power of TIMS and PASEF allows for greater proteome and sub-proteome coverage from small sample volumes taken from complex mixtures.

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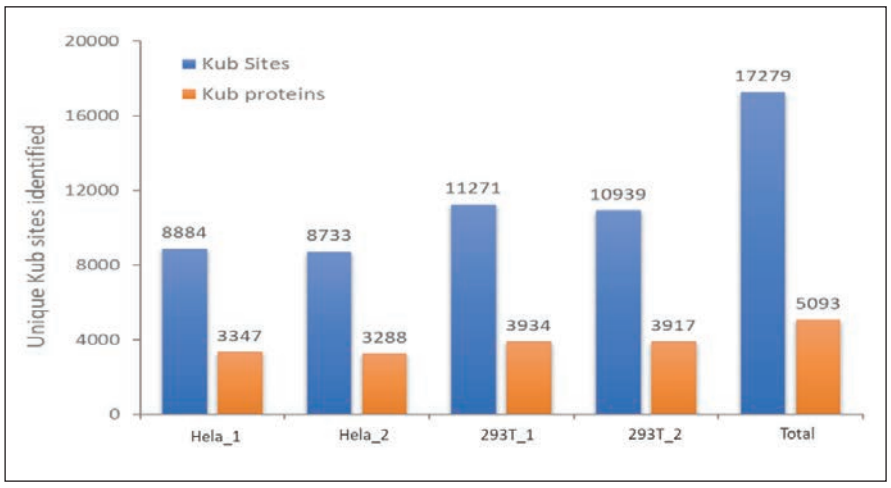

Figure 1. The identification of ubiquitinated sites and proteins from different human cell lines with 50 min gradient. For each cell line, two replicates were performed.

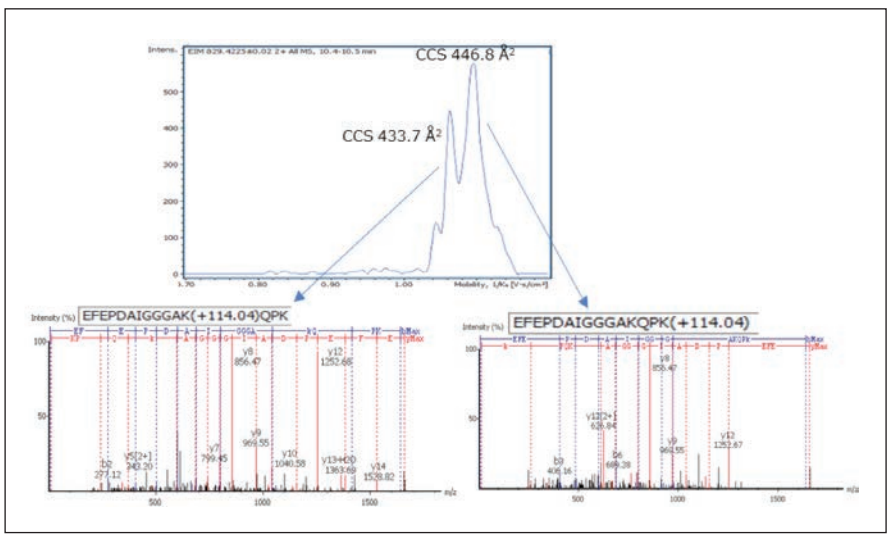

Figure 2. The separation of the two co-eluting isobaric ubiquitinated peptides with different modification sites by ion mobility. The extracted ion mobilogram for 829.4115 m/z at 10.45 min (top). The isomers can be resolved by collisional cross section (CCS) value. Non-chimeric MS/MS spectra of the two site-specific ubiquitinated peptides (bottom).

2. Another of the most common PTMs playing an essential role in cellular processes like transcriptional regulation, cell cycles and apoptosis is acetylation. Thorough MS-based profiling is made particularly challenging by its low abundance and high dynamic range. Using TIMS and PASEF technology, such challenges can be overcome to achieve comprehensive characterisation of acetylation.

In one study, we enriched acetylated peptides from mouse liver and rice leaf samples, resulting in over 1800 acetylated lysine peptides being identified from 100µg of starting material *(Figure 3)* [5]. *Figure 4* shows the high reproducibility of identification made possible with the combination of TIMS and time of flight (TOF)-MS. For rice leaf sample analysis, 10,000 lysine acetylated sites were identified in a single shot over 4800 protein groups (50-min gradient).

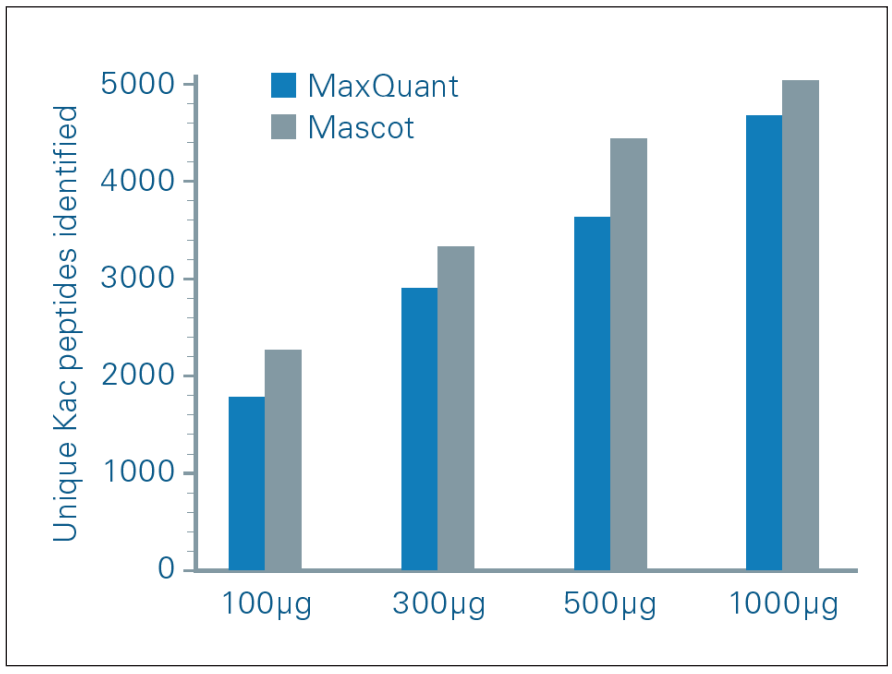

Figure 3. Identification of the acetylated lysine peptides with four different starting amounts (mouse liver).

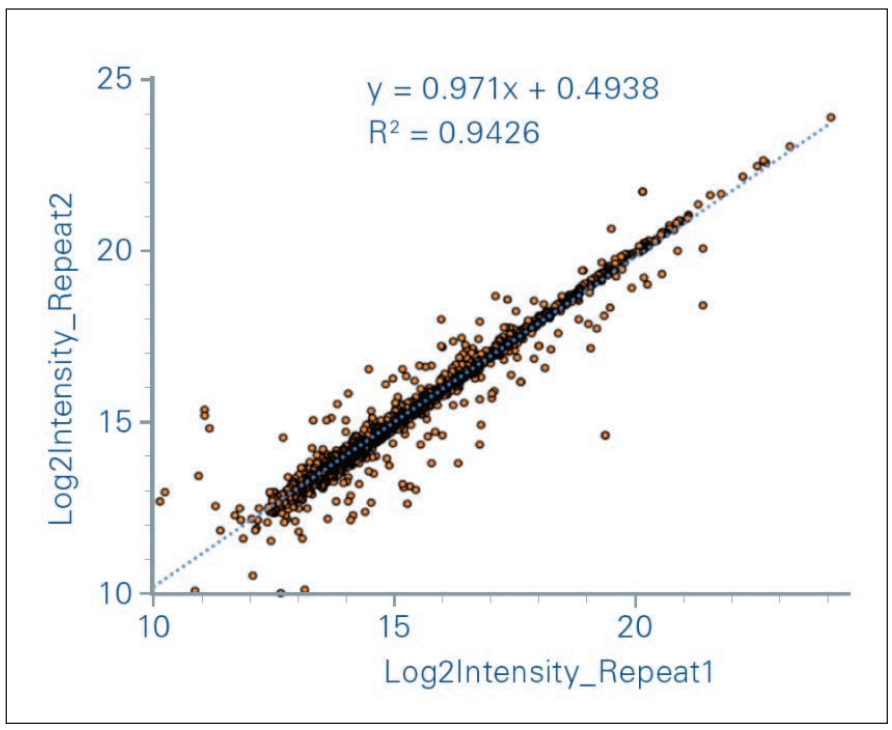

Figure 4. Biological reproducibility of the acetylated lysine peptides (mouse liver).

Conclusion

The high performance provided by TIMS offers a powerful alternative technique for quantification, with the ability to not only identify more proteins than other MS technologies under the same conditions, but also to separate near isobaric peptides with different PTM sites that can't be separated using traditional methods such as HPLC.

The speed and robustness of modern TIMS spectrometers combined with TOF-MS is making considerable contributions to understanding the role of proteomes in health and disease. This is proving vital for analysing large sample cohorts for clinical research where researchers can gather information about individual biological responses to disease with the aim of developing personalised treatment options.

References

1. Cohen, P and Tcherpakov, M. Will the ubiquitin system furnish as many drug targets as protein kinases? Cell 2010, 143, 686–693.

2. Moslehi, JJ. Cardiovascular toxic effects of targeted cancer therapies. N. Engl. J. Med. 2016, 375, 1457–1467.

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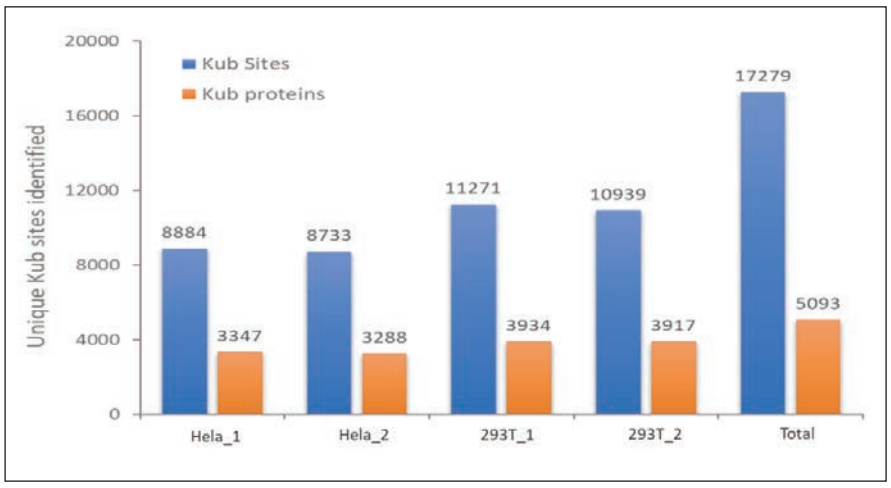

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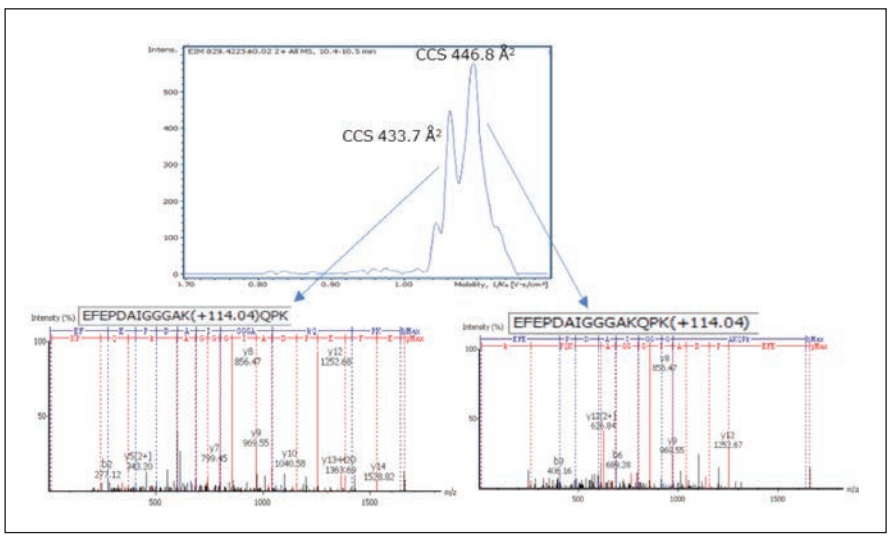

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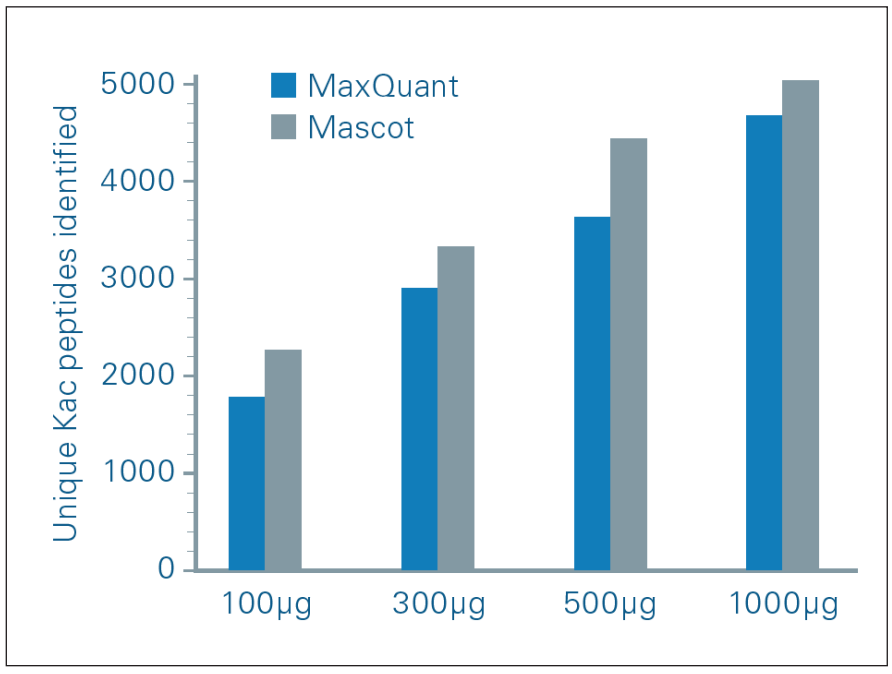

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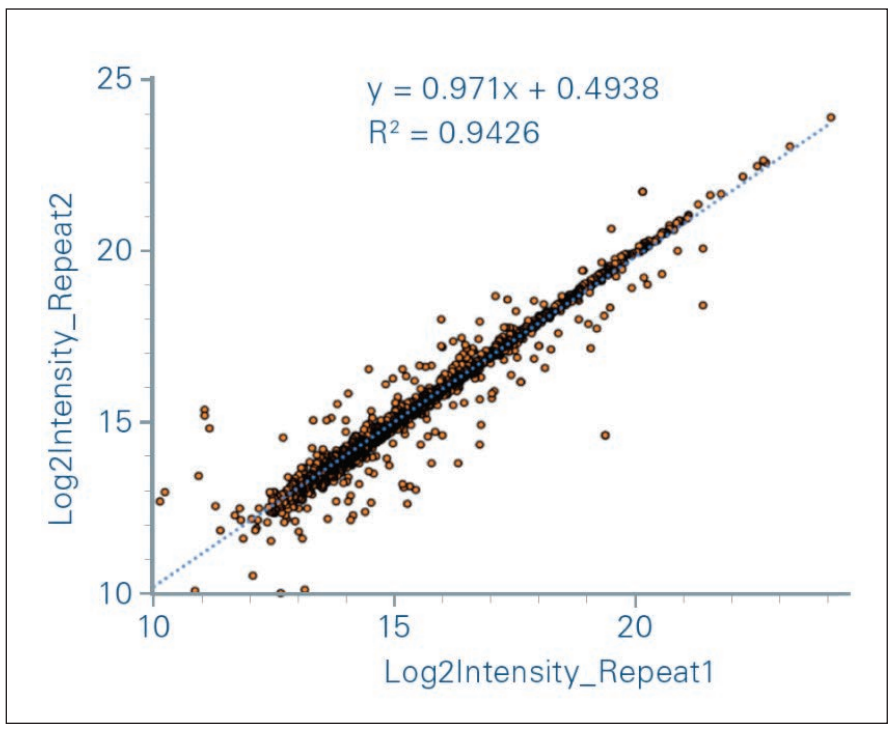

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