**Introduction**

Matrix-assisted laser desorption/ionisation (MALDI) in-source decay (ISD) is a useful technique for N- and C-terminal sequencing of intact proteins\(^1\)\(^-\)\(^3\). MALDI is commonly classified as a soft ionisation technique meaning that, when laser powers close to threshold values are used, intact protonated peptide and protein ions are generated. In the case of peptides, increasing the laser fluence (e.g. 10-20%) results in unimolecular decomposition (fragmentation) in the time-of-flight region between the ion source and the reflectron - a technique termed post-source decay (PSD). However, in the case of proteins, fragmentation can occur in the MALDI plume prior to ion extraction from the ion source i.e. in-source fragmentation (decay).

Typically, protein identification is achieved by subjecting the protein(s) of interest to enzymatic digestion followed by MS/MS sequencing of the resulting peptides in a bottom-up approach. In contrast, MALDI-HSD is referred to as a top-down approach as sequencing is performed on the intact protein, thereby eliminating the lengthy digestion step.

Further benefits of the top-down approach are found in the analysis of post-translational modifications (PTMs) as even labile PTMs (that may be lost or not detected in a bottom-up approach) are retained thereby facilitating localisation during sequencing.

MALDI-ISD does however have a limitation in the lower mass region where ISD fragment ions begin to overlap with chemical noise and matrix-related ions (<800 Da). A solution to this issue is to perform pseudo MS\(^3\) through MS/MS fragmentation of precursor ions created by ISD (known as T\(^3\)-sequencing\(^4\)).

However, using conventional MALDI-TOF-TOF instrumentation, this approach is limited to pseudo MS\(^3\).

In this application, we report ISD\(^n\): the extension of top-down sequencing to maximise sequence coverage of intact modified and unmodified proteins by using the AXIMA Resonance MALDI-QIT-TOF for MALDI-ISD and pseudo MS\(^n\) (up to n=5).
**Experimental**

Tau protein was expressed and purified in-house at Aix-Marseille University. Oxidised Tau protein was prepared by incubating the protein with NaOCl (5 mM) solution (15 min @ 37 °C). Proteins were further purified using C₄ ZipTips (Millipore).

Samples were prepared for MALDI analysis using α-cyano-4-hydroxycinnamic acid (CHCA; 10 mg/mL) and 2,5-dihydroxybenzoic acid (DHB; 60 mg/mL).

Samples were analysed using an AXIMA Resonance (high vacuum MALDI-QIT-TOF mass spectrometer) and an AXIMA Performance (MALDI-TOF-TOF mass spectrometer). Both instruments are equipped with nitrogen lasers (337 nm). In the AXIMA Resonance, ions were cooled in the ion trap and then CID was performed using resonant excitation and pulsed argon gas. Fragment ions were then extracted and measured in the TOF analyser. In the case of the AXIMA Performance, helium was introduced into the collision cell allowing selected precursors to undergo high energy collisions (HE-CID, 20 keV lab-frame-of-reference). The resulting fragments exit the collision cell and are resolved using a curved-field reflectron.

**Results**

The advantages of using ISD⁰ (AXIMA Resonance) over a conventional MALDI-TOF-TOF mass spectrometer for ISD analysis of proteins were demonstrated using recombinant Tau protein (42 kDa).

Tau has been reported to be involved in Alzheimer’s disease(⁵) and is a good in vitro model for protein modification analysis. The unmodified protein, along with the corresponding oxidised form, was used to demonstrate the ability of the described technique for the characterisation of modified proteins.

ISD of Tau protein was induced by increasing the laser power by around 30% above the ionisation threshold value. In the MALDI-QIT-TOF instrument, this increase led to the detection of various fragment ion series: cₙ-series, zₙ₊²-series and more interestingly bₙ-series and yₙ-series also. During ISD on a conventional MALDI-TOF-TOF instrument, it is reported that fragmentation proceeds via a radical pathway, a process resulting in the formation of primarily cₙ and (zₙ₊²) ions, and is therefore not expected to produce b- and/or y-ion fragments⁴,⁷. This was indeed verified to be the case using the AXIMA Performance MALDI-TOF-TOF.

The presence of b- and y-ions in the spectrum obtained using the MALDI-QIT-TOF is possibly a result of CID processes occurring in the ion-trap⁶. In the MALDI-QIT-TOF, the time frame of the experiment (trapping and flight time of the ions) is of the order of milliseconds compared with tens of microseconds for a conventional MALDI-TOF-TOF instrument. The longer residence time in the instrument may permit the formation of additional ion types by means of further fragmentation.

A further advantage of the AXIMA Resonance MALDI-QIT-TOF instrument is the ability to detect isotopically resolved MSⁿ fragment peaks (see insets in Figures 1 & 2) demonstrating fragment ion resolution of >8000 (FWHM) essentially independently of the laser power.
Figure 1 shows the MALDI-QIT-TOF in-source decay spectrum obtained for unmodified Tau. N-terminal c\textsubscript{7−} (c\textsubscript{7} to c\textsubscript{36}) and b\textsubscript{n−} ions (b\textsubscript{8} to b\textsubscript{30}) and C-terminal y\textsubscript{n−} ions (y\textsubscript{12} to y\textsubscript{34}) were detected. To ensure that the detected ions were not a result of proteolytic fragments present in the samples, the same samples were prepared and analysed using CHCA, using lower laser power. The low m/z range did not contain any peaks consistent with the masses of expected proteolytic fragments.

ISD analysis of oxidised Tau using MALDI-QIT-TOF did not show significant losses of sulfenic acid (CH\textsubscript{3}SOH = 64 Da) (see Figure 2) i.e. the modification remained intact during analysis. In addition to N-terminal c\textsubscript{n−} ions, c-ions showing a mass shift of +16 Da (c\textsubscript{n+16}) [oxidised Met\textsubscript{10} (sulfoxide form)] and c-ions showing a mass shift of +32 Da (c\textsubscript{n+32}) [oxidised Met\textsubscript{10} (sulfone form)] were also detected. C-terminal y\textsubscript{n−} ions and y-ions showing a mass shift of +16 Da (y\textsubscript{n+16}) [oxidation of Met\textsubscript{418}] were detected.
Multiple cycles of ISD\textsuperscript{n} analysis were then carried out on the ISD fragments of oxidised Tau. Pseudo MS\textsuperscript{3} was performed on the c\textsubscript{16}\textsuperscript{+} ion (1860.8 m/z) of oxidised Tau (see Figure 3(a)), and oxidation at Met\textsubscript{10} was confirmed. Intense fragment ions were detected in the resulting CID spectra corresponding to the facile neutral loss of CH\textsubscript{3}SOH (-64 Da) from methionine sulfoxide (b\textsubscript{12}\textsuperscript{+} - 64).

The b\textsubscript{12}\textsuperscript{+} ion (1477.6 m/z) from the oxidised Tau sample was selected for pseudo MS\textsuperscript{4} (see Figure 3(b)). Pseudo MS\textsuperscript{5} of the [y\textsubscript{438}\textsuperscript{+}-b\textsubscript{12}] ion (1277.5 m/z) of the oxidised Tau sample is shown in Figure 3(c).

Figure 3: MALDI-QIT-TOF collision induced dissociation (CID) analysis of fragment ions from oxidised Tau: (a) Pseudo MS\textsuperscript{3} of 1860.8 m/z corresponding to the c\textsubscript{16}\textsuperscript{+} fragment ion; (b) Pseudo MS\textsuperscript{4} of 1477.6 m/z (i.e. MS\textsuperscript{3} of 1860.8 m/z) corresponding to the b\textsubscript{12}\textsuperscript{+} fragment ion and (c) Pseudo MS\textsuperscript{5} of the [y\textsubscript{438}\textsuperscript{+}-b\textsubscript{12}] ion (1277.5 m/z).

Conclusion
ISD\textsuperscript{n} performed on the AXIMA Resonance high vacuum MALDI-QIT-TOF mass spectrometer offers several advantages for detailed characterisation of biomolecules compared with ISD performed on conventional MALDI-TOF-TOF instruments. The high performance of the AXIMA Resonance affords good resolution (>8000 (FWHM)) across the various stages of MS (MS\textsuperscript{n}) and provides good mass accuracy on the detected ISD and MS\textsuperscript{n} fragment ions essentially independent of the laser fluence. Using the ion trap, precursors can be efficiently isolated and subsequent CID fragmentation is controllable, allowing up to pseudo MS\textsuperscript{5} to be performed.

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References