Poster Note 64494

A Novel Instrumental Strategy for Parallel Reaction Monitoring of Intact Proteins with an Orbitrap Analyzer

Mikhail Belov[1, 2]; Dmitry Grinfeld[2]; Philip Compton[3]; Neil Kelleher[3]; Alexander Makarov[2]
[1]Spectroglyph LLC, Kennewick, WA; [2]Thermo Fisher Scientific, Bremen, Germany;
[3]Northwestern University, Evanston, IL

Overview

Purpose: Increase sensitivity of analysis by concurrent mass selection of multiple species

Methods: Time-of-flight mass selector is used in place of mass selection quadrupole filter in a quadrupole-Orbitrap hybrid Mass Spectrometer

Results: Viability of the concept is explored using simulation and initial experiments

Introduction

Recent advances in top-down analysis of intact proteins were largely driven by improving of liquid separation techniques, increasing sensitivity of ion sources and mass analyzers, improving the fragmentation methods [1]. Meanwhile, the crucial step of selecting a protein of interest for subsequent fragmentation remained limited to the traditional single-window isolation. While acceptable for low-charge peptides in bottom-up proteomics, this approach results in an order of magnitude loss of sensitivity when applied to intact proteins even of moderate size as their signal appear to be spread over 10-30 different charge states.

A similar problem is encountered in a broader range of pan-omics type experiments, for example in advanced types of targeted analysis such as Multiple Reaction Monitoring (MRM), Data Independent Acquisition (DIA), etc. In all these cases, sequential nature of the precursor ion selection reduces analysis throughput and the number of identifications per unit time.

In this work, we demonstrate a novel approach for parallel, concurrent selection of multiple precursors and multiple charge states of intact protein precursor species followed by higher-energy collision induced dissociation (HCD) and high mass accuracy detection of fragment species, yielding highly confident identifications.

Methods

Sample Preparation

Optimization was carried out using a calibration solution containing n-butylamine, caffeine, the peptide MRFA, and Ultramark, dissolved in a solution of water, acetonitrile, and 0.1% acetic acid. The calibration solution was infused at 0.3 µL/min using a syringe pump. In addition, protein solutions (Ubiquittin) were used in infusion mode.

Instrumentation

All experiments were performed with a modified Thermo Scientific™ Q Exactive™ Orbitrap™ mass spectrometer using H-ESI II ion source.

To implement parallel acquisition of different charge states of the intact protein ions, an orthogonal linear time-of-flight separation capable of selecting precursor ion species at m/z over 20,000 at a resolving power of up to 100 has been developed and introduced between the source and the C-trap regions of the Orbitrap.

Additionally, an inject flatapole mass filter and a quadrupole collision cell were incorporated between the ion source and the TOF separator (Fig. 1). Other important hardware modifications included reduced frequencies on the RF multipoles; an increased gas intake into the HCD cell for higher efficiency trapping, additional custom-made printed circuit boards. Modified Tune program and embedded software were used to control the instrument.

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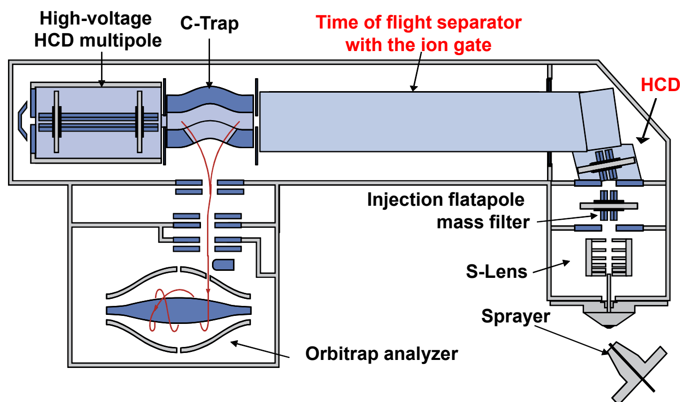
Principle of Operation and Simulations

Layout of the instrument

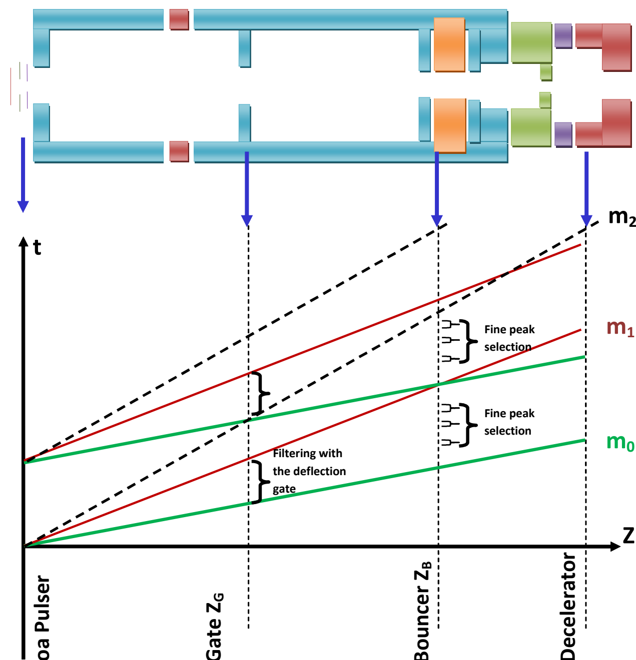
General layout of the modified instrument is shown in Fig. 1. An additional HCD collision cell after the mass filtering injection flatapole was intended for the use in MS^3 experiments which are outside of the scope of this initial stage of work. In the experiments below, the injection flatapole was used as a collision ion guide to provide continuous beam of ions to the time-of-flight separator.

FIGURE 1. General layout of the modified instrument. The original quadrupole mass filter was replaced with a collision cell and a time-of-flight selector.



Simulations and Principle of Operation

FIGURE 2. Graphical representation of the three-stage filtering. m_0 and m_1 are the minimum and maximum mass-to-charge ratios from the range of interest, while m_2 is the lowest m/z of the entire mass range. Pulsing at the gate located at Z_G provides rough pre-selection of the range [m_0, m_1], while pulsing of bouncer at Z_B ensures fine selection of the multiple precursors of interest.



The time-of-flight mass selection was based on an orthogonal acceleration principle and presented a number of challenges, including:

1. Improving duty cycle of continuous beam utilization, a known challenge for orthogonal acceleration scheme.
2. Precursor ion separation, which is realized by kinetic energy modulation at the end of the TOF liner.
3. Deceleration of higher kinetic energy ion packets after TOF separation.
4. Ion refocusing and accumulation in the HCD trap.

Improving duty cycle of continuous beam utilization

A multi-start approach was employed to reduce duty cycle losses to negligible levels. To avoid selection of erroneous m/z from overlapping ion populations, three-stage filtering was developed:

1. Orthogonal injection slit (with energy resolution R= 5 eV / 1 eV = 5) filters out the ions with m/z > m_2, that potentially can pass the deflection gate having one or more injection cycles skipped over.
2. Deflection gate (R = 150 mm / 4mm / 2 ~ 15) selects ions with m/z from the range [m_0, m_1] for which trajectories will not overlap in the bouncer downstream.
3. So-called Bouncer is implemented as a set of thin parallel plates to enable TOF selection resolution from R ~ 25 (all plates are energized) up to R ~ 150 (only one plate is energized).

Figure 2 summarizes graphically operation of this three-stage filtering. This concept intrinsically assumes that mass range of interest is limited to the range [m_0, m_1] wherein the range m_1/m_0 is at least 4 or more. This roughly corresponds to a typical charge distribution of a protein in a denatured state, while proteins in native state are known to have $m_1/m_0 < 2$.

Operation of the Bouncer, Simulations Results

The middle operational voltage of the Pulser is kept below the potential of the exit deceleration electrode, so that most of the ions cannot enter C-trap and are reflected back as shown in Fig. 4A. However, when the ions with a specific m/z arrive at the bouncer, all or some plate electrodes are pulsed up by ~ 100 V. Upon leaving the bouncer, the selected ions gain extra energy from its fringe field, which is sufficient to enter the C-trap as shown in Fig. 4B. The selected ions are then cooled down by the buffer gas collisions in the C-trap and in the HCD.

The Bouncer is located at the temporal focus of the extracted ion packets. The number of pulsed plate electrodes depends on the desirable selection window width, keeping the transmission function as flat as possible.

At the moment t_0, the lightest mass of interest enters the bouncer. $t_1 = t_0 + L/V_0$ - the lightest mass is about to leave the active part (L) with the velocity V_0, and the heaviest mass of interest enters the bouncer. The bouncer electrode voltage is elevated and kept high at least until the heaviest ions leave the bouncer in the moment $t_2 = t_1 + L/V_1$. In the moment $t_3 >= t_2$, the bouncer voltage is reset and next selection pulse can be applied. The latency time is given by the total length L of the bouncer.

FIGURE 3. Construction and operation of the bouncer.

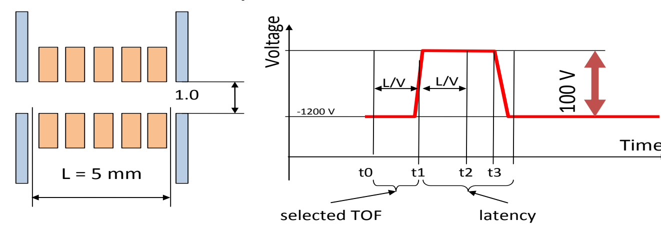


FIGURE 4. Energy distribution at the decelerator entrance with bouncer inactive (A) and activated (B).

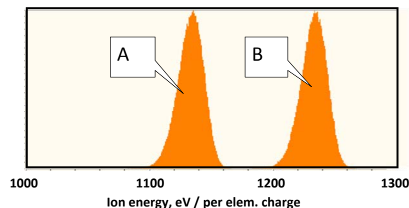


FIGURE 5. Ion trajectories with bouncer inactive (A) and activated (B). The latter view also shows ion focusing in the plane of orthogonal acceleration.

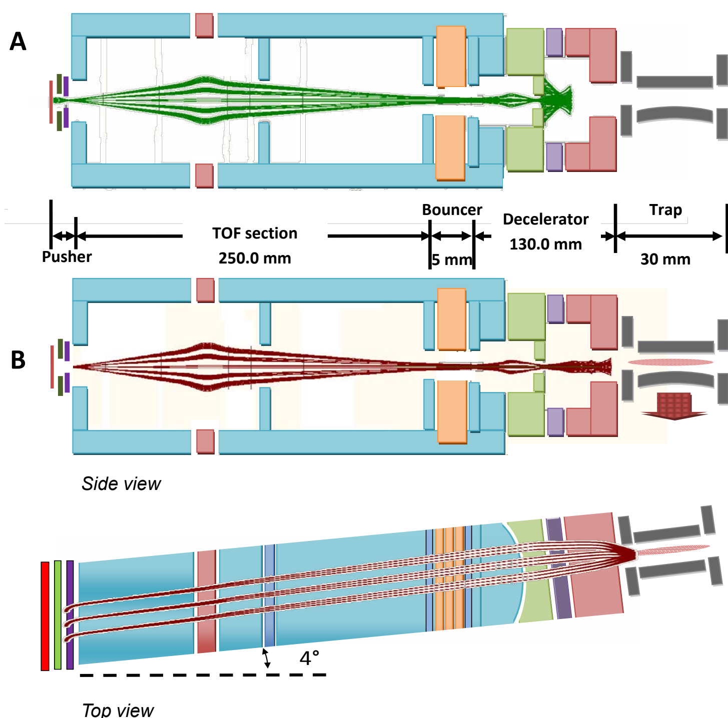
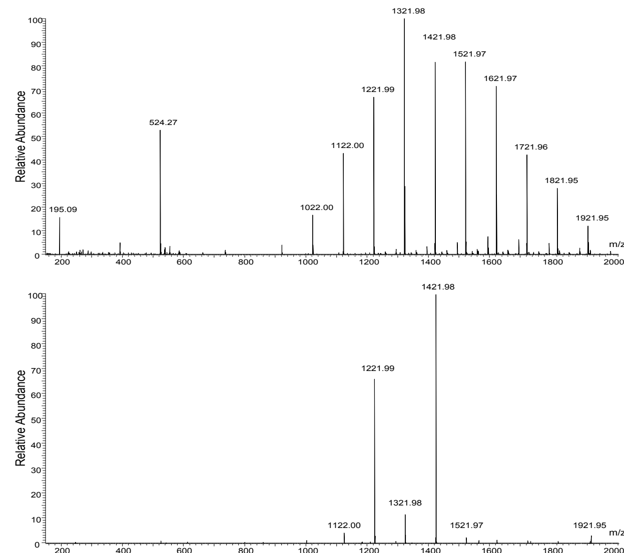
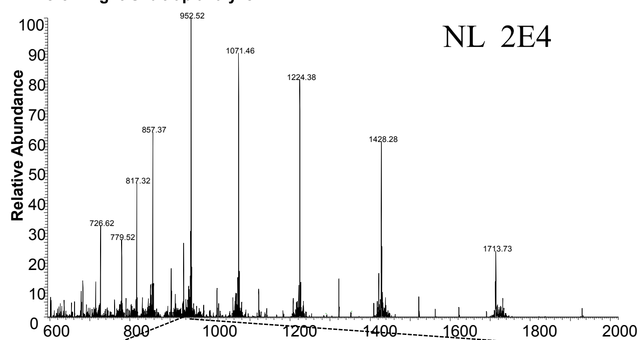


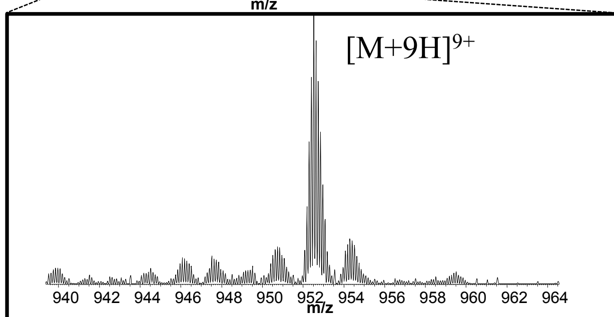
FIGURE 6. Panoramic mass spectrum without mass selection (A) and with parallel TOF selection of two m/z windows (B) for calibration mixture.



Experimental Results

FIGURE 5. Panoramic mass spectrum of 1 uM Ubiquitin solution obtained with Time-of-Flight Orbitrap analyzer





Conclusion

- It is shown that the time-of-flight mass selector has potential to greatly increase sensitivity of top-down and targeted experiments.
- Efforts are currently focused on investigating a large parameter space for lossless ion injection into the TOF extraction region, pulsed selection of multiple m/z windows, effective capture of selected ions in the C-trap and the HCD cell
- The final objective is implementation of MS^3 strategy for intact protein complexes as described in [2]. During the MS2 step, collisional dissociation of the m/z-selected precursor protein complex of interest to the constituent monomer subunits will be performed in the elevated pressure region preceding the TOF separator. In the following MS3 step, the derived monomer subunits will be subjected to m/z selection using the linear TOF separator and further dissociated to the backbone fragment ions in the HCD cell.

References

1. A. Catherman et al, *Biochem. Biophys. Res. Comm.*, 445 (4), 2014, 683-693.
2. M. Belov et al., *Anal Chem.* 2013; 85(23):11163-73.

Acknowledgements

The authors gratefully acknowledge generous support from Northwestern University and the W.M. Keck Foundation.

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