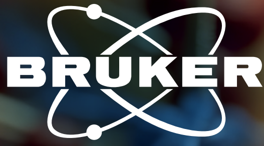

Leveraging online parallel accumulation, serial fragmentation (PASEF) in timsTOF mass spectrometers for high sensitivity, high throughput bottom-up N-glycoproteome analysis with the glyco-PASEF® method

In this application note we describe an advanced set of methods for utilizing the timsTOF Pro by developing and implementing a unique acquisition method, glyco-PASEF®, tailored for glycoproteomics based on work originally carried out by Mukherjee and Jankevics et al. [1] in the laboratory of Dr. Albert Heck at the University of Utrecht.

Abstract

We show that the device can be precisely tuned to sequence glycopeptides by leveraging ion mobility and stepped collision energy (SCE) fragmentation. Furthermore, its rapid scanning capability enhances the quality of the spectra obtained. We demonstrate that this approach yields a high level of glycopeptide detection and can achieve this effectively also with short chromatography gradients.

Keywords:
glyco-PASEF, glycosylation, glycoproteins, glycopeptides, glycobiology, glycomics

Introduction

Glycoproteomics is a rapidly evolving field that focuses on the study of glycoproteins, proteins that have carbohydrates covalently attached to them. This field is gaining recognition for its significant contributions to our understanding of biological processes and disease mechanisms. Many diseases, including cancer and autoimmune disorders, are associated with changes in glycoprotein structures. Glycoproteomics allows for the identification and quantification of these changes, which can be used for disease diagnosis and monitoring. Furthermore, some glycoproteins serve as targets for therapeutic drugs, making glycoproteomics essential in drug discovery and development. Glycoproteins are often used as biomarkers for various diseases. Glycoproteomics enables the discovery of new biomarkers, which can lead to the development of more accurate and reliable diagnostic tests. Personalized medicine aims to tailor medical treatment to the individual characteristics of each patient. Glycoproteomics can contribute to this field by identifying individual variations in glycoprotein structures, which can influence a person's response to certain treatments.

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Despite its well-recognized biological importance, glycobiology has remained, by and large, a niche science. This is primarily due to the significant challenges that the analysis of glycoproteins presents. Glycans exhibit a very high degree of compositional and structural complexity. They can be branched or linear and can vary in length and in the types of sugars and linkages they contain. This complexity makes it difficult to fully characterize glycan structures. Moreover, glycosylation, the process by which glycans are attached to proteins, is not a template-driven process like DNA replication or protein synthesis. Instead, it is a complex, enzyme-driven process that can result in a high degree of heterogeneity, with different glycoforms of the same protein commonly present within a single biological sample. The structural complexity and heterogeneity of glycoproteins can make it difficult to separate and detect them using standard analytical techniques. While methods such as liquid chromatography and conventional mass spectrometry are commonly used, they often require extensive sample preparation and may not fully resolve all glycoforms. In addition, there is currently a lack of standardized methods and tools for glycoprotein analysis. Different laboratories may use different methods, making it difficult to compare results across studies.

In conclusion, glycoproteomics is a powerful tool in the field of biomedical research. Its ability to provide detailed insights into the structure and function of glycoproteins makes it indispensable in our quest to understand, diagnose, and treat diseases. While glycoprotein analysis is challenging, advances in analytical techniques and the development of new tools and methods are poised to enable more detailed and accurate analysis of glycoproteins in the future. Here we present one such advance, the glyco-PASEF method which leverages the power of TIMS and the PASEF method to greatly increase the number of glycoforms that can be analyzed by LC/MS.

Materials and Methods

Glycoprotein standard

Recombinant tissue nonspecific alkaline phosphatase (TNAP) was a gift from Copenhagen Centre of Glycomics.

Proteolytic digestion of human plasma

Human plasma was mixed with SDC buffer, TCEP, CAA, and boiled. After cooling down, it was digested with Lys-C and trypsin, quenched with TFA, and desalted using an HLB plate. Samples were lyophilized and stored for MS analysis.

Detailed information about sample preparation is described in papers by Mukherjee et al. and Bärenfänger *et al*. [1,2].

Data acquisition

Chromatographic separation of tryptic peptides from individual purified glycoproteins and plasma was performed using a Dionex Ultimate 3000 nano-UHPLC with an Aurora column (pre-fitted nanoZero™ connection and integrated emitter tip, 25 cm x 75 μm, 1.6 μm, C18; IonOpticks, Fitzroy, Australia) coupled to a captive spray ionization source on a timsTOF Pro mass spectrometer. Gradient times of 15 min, 30 min, 60 min, 90 min, and 150 min at a flow rate of 400 nL/min were used.

The instrument was operated in PASEF mode starting with default settings used customarily for proteomics, consisting of 10 PASEF ramps of 100 ms each. MS/MS spectra were acquired with a threshold intensity of 2500 and target intensity of 20,000. Charge states from 2-5 were generally included in precursors selection and active exclusion was done for 0.4 mins.

The glyco-PASEF method was optimized for glycopeptide analysis as follows:
TIMS Offsets: Δt5 0 → 75, Δt6 55 → 75, TIMS RF 300 → 350.

Collision energies are dependent on ion mobility values, using a slope of $1/K_0$ 0.6 at 20 eV to $1/K_0$=1.6 at 59 eV. For improved glycopeptide fragmentation SCEs were used: CE1: $1/K_0$ 0.5 at 35 eV to $1/K_0$=1.6 at 54 eV and CE2: $1/K_0$ 0.5 at 40 eV to $1/K_0$=1.6 at 100 eV. The number of PASEF ramps was reduced from 10 to 7 in combination with SCEs.

Optimized ion mobility precursor *m/z* windows are described in the results section.

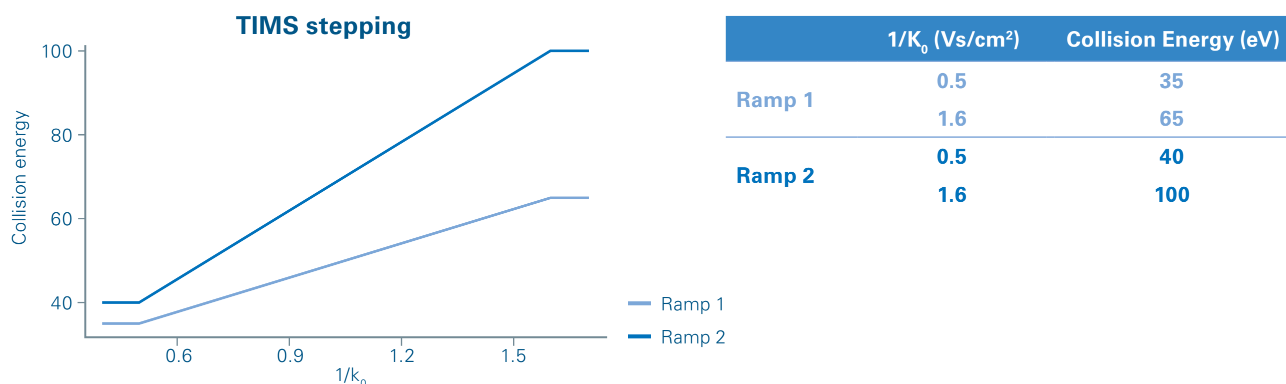
Data analysis

Raw data were processed using the glyco-N-HCD workflow in MSFragger-Glyco [3, see Bruker FlashNote: 1910131_FN-29_MSFragger-Glyco].

Human plasma: Default search parameters were used, where the lower and upper mass limits for the precursor window were set to 400 and 5000 Da, respectively, and the precursor and fragment mass tolerance to ±20 ppm. Trypsin digestion was carried out to completion, with a maximum of two missed cleavages allowed; carbamidomethylation at cysteine residues was set as a fixed modification, and oxidation at methionines and the protein N-terminal acetylation were set as variable modifications. Peptide filtering at a 1% false discovery rate (FDR) was applied using PeptideProphet [4]. Default parameters for N-glycan analysis were set to an FDR of <1% for glycan identification, and to a glycan mass tolerance of 50 ppm.

Results and Discussion

The glyco-PASEF method described here demonstrates the ability to physically separate N-glycopeptides from nonmodified peptides and uses CEs optimized for N-glycopeptides. The separation achieved by glyco-PASEF contributed to excellent analytical depth. The glyco-PASEF method introduces a dedicated glycan-specific polygon within the PASEF mode, coupled with SCE. This combination significantly improves N-glycopeptide identification by enhancing spectrum quality and maximizing analysis time for specific analytes of interest. Glyco-PASEF was optimized based on the analysis of purified single glycoproteins as well as more complex samples such as plasma.

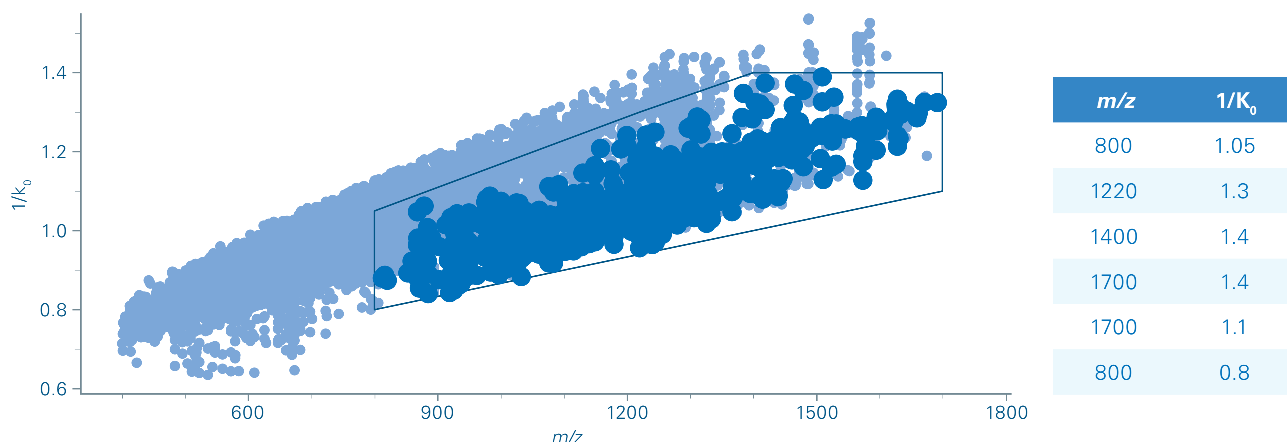


	$1/K_0$ (Vs/cm^2)	Collision Energy (eV)
Ramp 1	0.5	35
	1.6	65
Ramp 2	0.5	40
	1.6	100

Figure 1
TIMS SCEs for fragmentation of glycopeptides.

Collision energies

Applying CID energies customarily used in proteomic applications to N-glycopeptide fragmentation results predominantly in fragmentation of glycans. SCE applies low collision energies (CEs) for resolving the composition of N-glycopeptides, while higher CEs provide information on the glycosylation site, of peptide fragment ions, and the assignment of features related to glycan core structures such as core-fucosylation. Optimization of CEs was performed by applying various constant CEs. Figure 1 shows the optimized CE settings obtained from the fragmentation of two simple glycopeptides from purified tissue-nonspecific alkaline phosphatase (TNAP) with different ion mobilities. These two optimized N-glycopeptide fragmentations were subsequently used for linearly extrapolating the calibration curve from reduced IM ($1/K_0$) 0.5 to 1.6, combining high and low energy frames.



m/z	$1/K_0$
800	1.05
1220	1.3
1400	1.4
1700	1.4
1700	1.1
800	0.8

Figure 2

Identification of N-glycopeptides from human plasma sample.

Physical separation of glycopeptides and non-modified peptides in $1/K_0$ vs. *m/z*. The glycopeptide-selective polygon is outlined in black.

The resulting curve is sensitive for the detection of specific glyco-oxonium ions and led to the successful identification of 28 unique N-glycopeptides originating from purified tissue-nonspecific alkaline phosphatase (TNAP).

Optimized precursor selection

Because of their inherent physical properties, N-glycopeptides have different mobilities compared to nonmodified peptides and therefore cluster in a specific ion mobility region inside the TIMS device. To evaluate the ion mobility region of interest, purified single glycoproteins (sialylglycopeptide [α2,6-SGP]and asialo-SGP) were measured and a region-specific clustering was performed based on diagnostic oxonium ions identified during data analysis. These results were subsequently confirmed for complex glycoprotein samples (Figure 2).

The glyco-PASEF approach using the specific N-glycopeptide-polygon yields a nearly 10-fold increase in N-glycopeptide identifications compared to the default PASEF method for proteomics. This improvement was particularly pronounced for shorter gradients. The workflow described here allows identification of ~400 unique N-glycopeptides from human plasma (Figure 3).

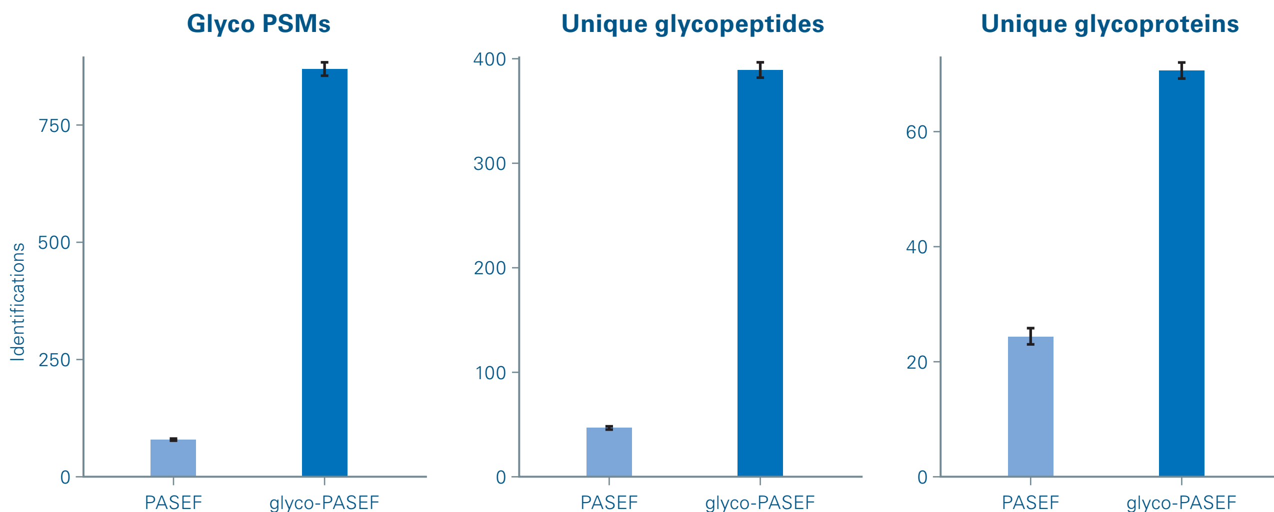


Figure 3

Comparison of PASEF proteomics method and optimized SCE method using a glycopeptide-specific polygon (glyco-PASEF) for the identification of glycopeptides and -proteins from human plasma sample.

These numbers represent substantial improvements of applying the glyco-PASEF method as compared to the standard proteomics PASEF method, using a 150-minute retention time gradient. Notably, the results compare favorably to a recent study that identified 352 unique N-glycopeptides (associated with 89 glycoproteins) in plasma [5]. However, the glyco-PASEF approach using SCE and gating on the glycopeptide-specific polygon consistently outperformed SCE-PASEF alone. For all gradient lengths, a stringent polygon outperformed the normal polygon (Figure 4).

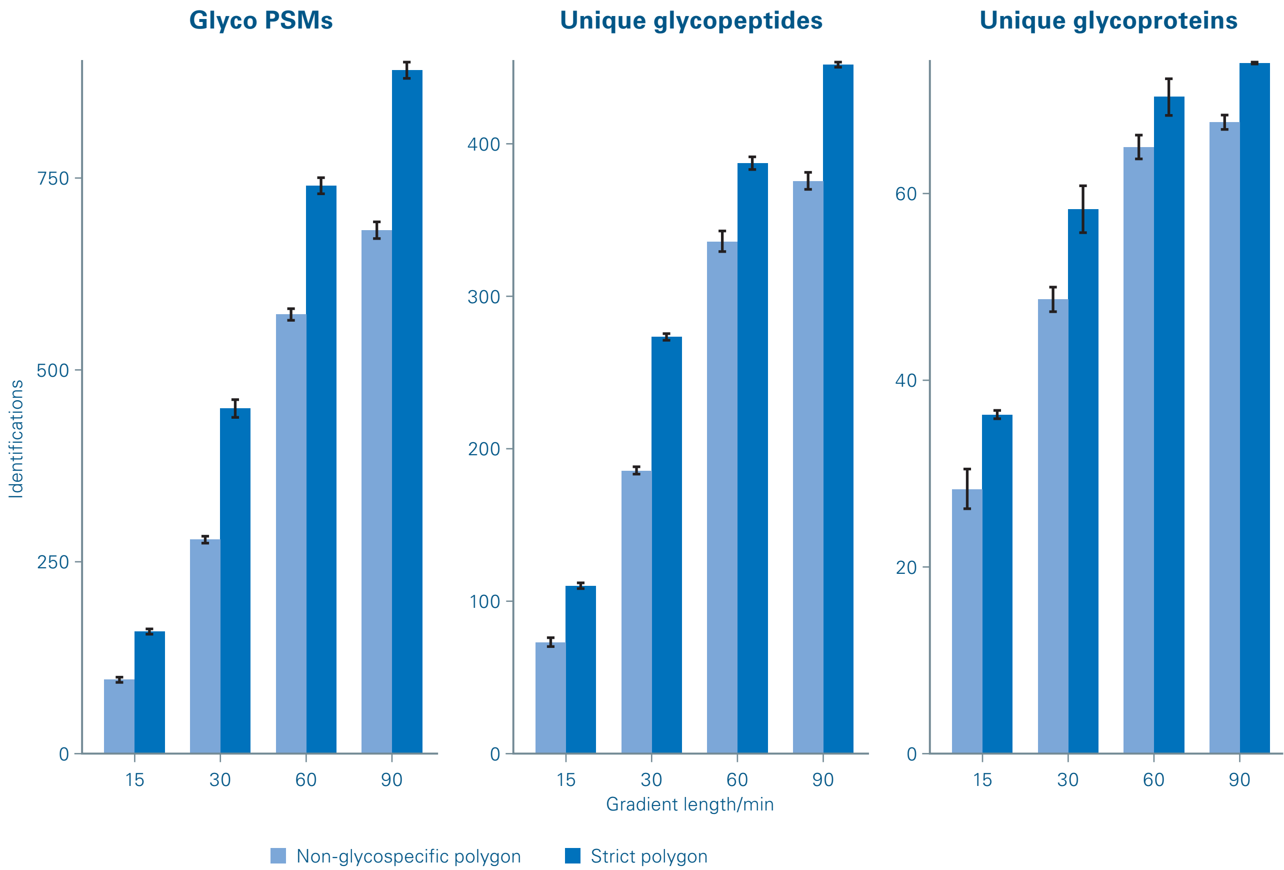


Figure 4
Identification of N-glycopeptides from human plasma by stepped collision energy with standard polygon (non-glycopeptide-specific) and strict polygon.

Conclusion

The Bruker glyco-PASEF method represents a powerful new workflow for N-glycoproteomic analysis with improved depth, speed, and accuracy. This method holds promise for biomarker discovery and clinical applications by:

- Implementing ion mobility as a method for acquiring N-glycoproteomic data on the timsTOF platform.
- Reducing the number of chimeric MS/MS spectra of co-eluting isobaric and near-isobaric glycoforms of the same peptide by ion mobility separation.
- Excluding precursors with m/z < 800, thus directing the focus to the selection of N-glycopeptides and enhancing coverage for glycopeptide identification.
- Fine-tuning collisional energies to optimize the fragmentation of glycopeptides with respect to glycan and peptide backbone fragmentation..
- Concentrating instrument time on glycopeptides through the use of ion mobility.
- Enhancing data quality by merging duplicate spectra produced without throughput penalty due to the speed of the instrument.
- Conducting glycoproteomic analyses on samples with high complexity, such as human plasma.

The glyco-PASEF method can be downloaded as a standard method from Bruker's methods home page here (Default methods).

Dr. Albert Heck

Professor of Chemistry and Pharmaceutical Sciences at Utrecht University and Scientific Director of the Netherlands Proteomics Center, noted:

"I am pleased to see the pioneering work our laboratory carried out, leveraging the unique strengths of the timsTOF platform for glycoproteomics, to now be translated as glyco-PASEF® into a widely applicable tool enabling and accelerating research in the field of glycobiology."

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