Bioanalysis ZONE

Sponsored by

SCIEX
The Power of Precision

The importance of post-translational modification studies in the drug discovery process

fsg

Contents

Foreword

The acceleration of emerging technologies – in today's pharmaceutical and biotechnological industries – has enabled companies to expand operations significantly in the last couple of years. However, the growing market for biopharmaceuticals brings new challenges and expectations for drug developers and manufacturers, which can be mainly attributed to product variability caused by post-translational modifications (PTMs). As a result, new advancements in technology that offer higher sensitivity, accuracy and throughput will be an essential part of a drug developer's toolkit.

In this modern era, biotherapeutics and proteomics have driven the drug discovery process to analyze drug action, toxicity, resistance and efficacy. Mapping and measuring the levels of PTMs is a crucial component of early biologic drug development but is by necessity now starting to move into QC processes too, particularly with the growing requirements of pharmacovigilance. Currently, the gold standard technique is to use LC–MS peptide mapping, carried out during early drug development to dig deep into the product and understand what PTMs exist, how they might affect structure function and start to get an idea of the levels of PTMs. However, limitations in PTM analysis are faced by traditional mass spectrometry methods. As a result, new advancements in LC-HRMS are required to overcome these challenges.

In this eBook, we explore how important PTM studies are in the drug discovery process and how MS is a powerful technique that can provide data on the quality, structural integrity and PTMs of proteins.

We hope you enjoy this eBook!

Naamah Maundrell
Editor-in-chief, Bioanalysis Zone
n.maundrell@future-science-group.com

Review

For reprint orders, please contact: reprints@future-science.com

Bioanalysis

Biopharmaceutical quality control with mass spectrometry

Shulei Liu[1,2] & Benjamin L Schulz*[,1,2]

[1]School of Chemistry & Molecular Biosciences, The University of Queensland, St Lucia, QLD 4072, Australia

[2]Australian Research Council Industrial Transformation Training Centre for Biopharmaceutical Innovation, Australian Institute for Bioengineering & Nanotechnology, The University of Queensland, St Lucia, QLD 4072, Australia

*Author for correspondence: b.schulz@uq.edu.au

Mass spectrometry (MS) is a powerful technique for protein identification, quantification and characterization that is widely applied in biochemical studies, and which can provide data on the quantity, structural integrity and post-translational modifications of proteins. It is therefore a versatile and widely used analytic tool for quality control of biopharmaceuticals, especially in quantifying host-cell protein impurities, identifying post-translation modifications and structural characterization of biopharmaceutical proteins. Here, we summarize recent advances in MS-based analyses of these key quality attributes of the biopharmaceutical development and manufacturing processes.

Tweetable abstract: MS is powerful for biopharmaceutical quality control. We review the status and opportunities of data independent acquisition, glycoproteomics, top-down MS and hydrogen-deuterium exchange MS for measuring host-cell protein contamination, post-translational modifications and protein structure.

First draft submitted: 3 June 2021; Accepted for publication: 17 August 2021; Published online: 31 August 2021

Keywords: biopharmaceutical quality control • host-cell proteins • mass spectrometry • protein post-translational modifications • protein structure

Biopharmaceuticals

Biopharmaceuticals (also known as biologics) are a category of medical products composed of nucleic acids, proteins or living cells that are produced through biotechnology. Most commonly, recombinant DNA technology is used to heterologously express protein biopharmaceuticals from mammalian cell lines [1]. Today, they are broadly used to treat disease indications including cancer, inflammatory and infectious diseases, wound healing, fertility, supplementation of hormone or cytokine deficiencies, modulation of immune function and replacement of enzymes. The general categories of biopharmaceuticals with corresponding examples and estimated current market values are shown in Table 1.

Biopharmaceuticals are safe and effective high-molecular weight drugs with few side effects compared with small-molecule drugs [10]. The chemical structure of many small-molecule drugs cannot be found in the human body, while the structures of biopharmaceuticals are often very similar to native human compounds because they are derived from a biological source [11]. This high complexity and structural similarity give them high specificity with few side effects, as well as the potential capacity to cure diseases instead of just treating the symptoms. However, this structural diversity and complexity, along with their high molecular mass, makes the manufacture of biopharmaceuticals comparatively complex [12]. Thus, quality control is extremely important during the whole manufacturing process including production, purification and packaging. This quality control includes assessment of the identity, purity and potency of the product. To precisely control biopharmaceutical quality, MS is a front-line tool for protein identification and characterization.

newlands press

Table 1. Classification of biopharmaceuticals with examples and market values.

Types	Examples	Annual revenue (US$)	Ref.
Coagulation factors	Factor VIII and IX	8.5 billion in 2017	[2]
Thrombolytic agents	Tissue plasminogen activator	5 million in 2012	[3]
Hormones	Insulin, growth hormone and gonadotropins	8 billion in 2019	[4]
Growth factors	Erythropoietin and colony stimulating factors	6 million in 2019	[5]
Interferon	IFN-α, -β and -γ	9 billion in 2019	[6]
Interleukin-based products	IL-2	6 billion by 2026	[7]
Vaccines	Hepatitis B surface antigen, varicella and HPV	61 billion by 2020	[8]
Monoclonal antibodies	Herceptin, alemtuzumab and rituximab	115.2 billion in 2018	[9]
Additional products	Tumor necrosis factor and therapeutic enzymes	–	

HPV: Human papillomavirus.

Overview of current biopharmaceutical markets

The global biopharmaceutical market has grown continuously in recent decades with increasing demand from geriatric populations, and investment in related research as well as manufacturing processes has effectively expanded the market by providing customers greater choice and biopharmaceuticals with improved potency. At present, biopharmaceuticals occupy nearly a quarter of newly introduced drugs to the market, and demonstrate high competitiveness and large potential partly because of their ability to treat previously incurable diseases.

In 1990, the total annual revenue from biopharmaceuticals was only around US$4.4 billion, while it has significantly increased to over US$275 billion at present with a growth rate of 61.5%, and is expected to continue to maintain an annual increase rate around 12–13% [13]. The year 2019 was an outstanding year for global biopharmaceutical markets with 7.32% of compound annual growth rate, which is well positioned for further stable increases [14].

Biopharmaceutical manufacturing process

The key conceptual steps in the biopharmaceutical manufacturing process are cell-line development, upstream processes and downstream processes (Figure 1). Microbial systems (bacteria, yeast, filamentous fungi and unicellular algae) and mammalian systems (CHO, NS0 and HEK 293 cells) are both widely used as host cells to produce protein-based biopharmaceuticals. *Escherichia coli* is a common and inexpensive bacterial system with fast growth that is used to produce first-generation biopharmaceuticals like insulin and growth hormone [15]. However, not all biopharmaceuticals can be produced in bacterial systems as these are not natively able to modify proteins after translation, which can lead to production of misfolded or inactive proteins [16]. Most therapeutic protein drugs require complex post-translational modifications (PTMs) such as glycosylation, acetylation, disulfide bonds or phosphorylation for desired drug stability and efficacy [17]. Thus, mammalian cells are widely used to produce the protein products that must be modified, such as monoclonal antibodies (mAbs) [18].

Upstream process can be divided into cell culture optimization, fermentation process optimization and application in large-scale bioreactors. Batch, fed-batch and continuous perfusion fermentation are common types of fermentation processes, with batch fermentation currently the most common with a 90% usage rate in industry [19]. During the fermentation process, fermentation conditions involving temperature, pH and oxygen concentration need to be monitored and regulated to guarantee optimal yield and production efficiency [20]. Additionally, sterile techniques or antibiotics can be used to protect the bioreactor environment from contamination.

Downstream processes refer to the process from after cell culture to the final biopharmaceutical product, and involve clarification, purification, polishing and viral inactivation to collect biomolecules of interest and remove impurities such as host cell debris and endotoxin [21]. Purified proteins can then be optionally modified by enzymatic conversion [22] or other methods depending on the specific biopharmaceutical, followed by formulation and/or lyophilization. Before packaging, product quality is controlled through a series of analyses to ensure the identity, purity, and quantity of the biopharmaceutical. MS is one of the most powerful analytical techniques for these purposes.

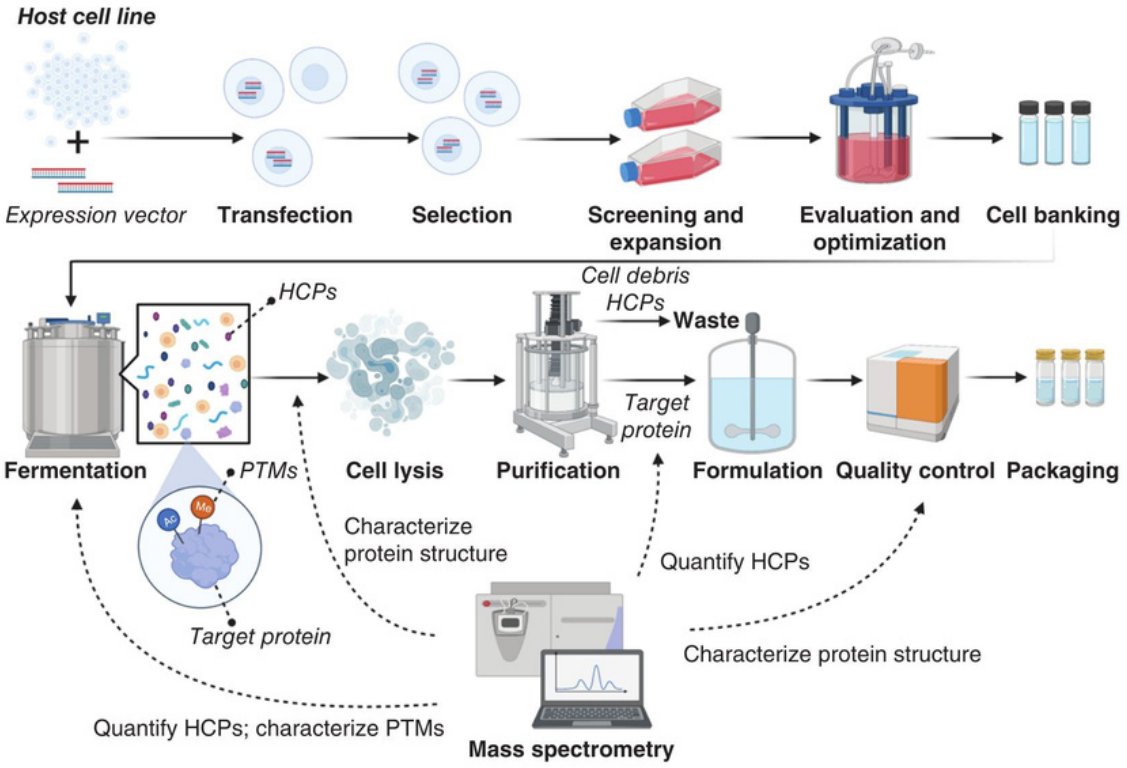


Figure 1. Biopharmaceutical manufacturing processes with MS applications. HCPs are contaminating proteins secreted from host cells along with the target protein. PTMs refer to the chemical modification of proteins through the removal or addition of functional groups that affect the structure and function of proteins. Created with BioRender.com.
HCP: Host-cell protein; PTM: Post-translational modification.

Mass spectrometry

MS is an indispensable analytical technique that is widely used in chemistry, biochemistry and pharmacy. It plays an essential role in the biopharmaceutical industry to identify, quantify and characterize proteins during production, purification and packaging processes to ensure the final biopharmaceutical products are pure, correctly folded and active proteins.

MS analysis of complex samples is commonly enabled by online or offline fractionation by various separation techniques. LC is particularly powerful and popular for this purpose. Separation of peptides or proteins typically uses reversed-phase LC. In addition to simplifying samples before MS analysis, LC of glycans or glycopeptides can separate different structures or isomers to improve identification [23]. Ion-exchange chromatography is suitable for fractionation and purification of charged compounds, including peptides and proteins [24]. Size-exclusion chromatography can separate biomolecules according to size, and can be combined with MS to characterize protein structural diversity or protein–protein interactions [25]. Hydrophobic interaction chromatography is a nondenaturing separation technique based on the hydrophobicity of the native analyte, and is becoming more popular to characterize the hydrophobicity, heterogeneity, sequence and structure of mAbs [26].

The three key components of an MS instrument are an ion source, a mass analyzer and a detector [27]. However, MS instruments can have many different and varied configurations. The basic theory of MS is to produce ions by one of the various ionization methods (depending on the characteristics of the sample), to separate these ions by virtue of their m/z, and to detect the ions to determine their m/z and abundance [28].

ESI and MALDI are the two most common methods used for analysis of proteins and peptides [29]. ESI ionizes analytes from a solution, and so is easily coupled to techniques that apply liquid-based separation such as LC. Integrated LC ESI–MS systems (LC–MS) are therefore commonly used to analyze complex samples. In contrast, MALDI uses laser pulses to sublimate and ionize molecules in samples from a dry crystalline matrix, and is usually used to analyze relatively simple peptide mixtures [30].

The mass analyzer is the central component of an MS instrument, and separates the ionized molecules based on their m/z ratios [31]. Mass analyzers are sensitive, high-resolution and capable of generating information-rich ion

mass spectra from proteins, peptides and their fragments. Quadrupole, TOF and Orbitrap are the most common types of analyzer, and these analyzers can be used singly or in combination to take advantage of the strengths of each [32].

MS/MS is a popular and powerful option in biomolecular analysis. In MS/MS, the ionized proteins or peptides are separated by the first mass analyzer (MS1) by their m/z ratio, and then ions of a certain m/z-ratio are selected to be further fragmented. After fragmentation, the smaller fragment ions are introduced into the second mass analyzer (MS2) which separates the fragments by their m/z ratio again and detects them [33]. As such, MS/MS can provide information on the composition or structure of complex molecules. Different fragmentation methods can provide complementary structural information, and are suitable for different types of biomolecules. For example, Collision Induced Dissociation (CID) or Higher Energy Collision Dissociation (HCD) are commonly used for analysis of peptides, as they result in efficient and predictable fragmentation at peptide bonds. Electron Transfer Dissociation (ETD) or Electron Capture Dissociation (ECD) provide complementary fragmentation patterns, and are particularly useful for assigning the site of modification in glycopeptides.

Other gas phase separation techniques can be combined with MS, including ion mobility spectrometry (IMS), which is increasing in popularity in modern MS instruments. The basic principle of IMS is that ionized molecules are separated through a cell filled with an inert 'drift gas' on a millisecond timescale according to their ion mobility, which is related to their mass, shape and charge [34]. IMS can be used between LC and MS as an additional intermediate fractionation technique for complex samples, and can also be used to obtain structural information by separating isomeric ions, revealing primary conformations and tracking dynamic changes in structure [35]. IM–MS can also be combined with complementary strategies such as fragmentation with ETD to obtain insights into protein conformation and modifications, or collision-induced unfolding to characterize protein dynamic structure and stability [36].

With the rapid development of MS instrumentation in recent years improving speed, accuracy, sensitivity and robustness, and offering diverse fragmentation options, this technology has become one of the most powerful analytical techniques for analysis and quantification in proteomics, glycoproteomics and detailed protein characterization. MS is therefore clearly a useful and versatile tool for many aspects of biopharmaceutical quality control which can quantify host-cell proteins (HCPs), identify PTMs and characterize the structure of biopharmaceutical proteins to guarantee the purity, safety and potency of biopharmaceuticals.

Recent advances

Quantification of HCPs

HCPs are contaminating proteins expressed and secreted from host cells that accompany the production of intended recombinant biopharmaceutical proteins [37]. The presence of HCPs is what necessitates additional purification steps to obtain pure biopharmaceutical protein product. HCPs must be removed during the purification process, as if some of them still remain as impurities in the final products they may result in reduction of biopharmaceutical efficacy or unintended immunogenic responses. The general guideline for acceptable levels of HCPs in biopharmaceutical products is less than 100 ng/ml (100 p.p.m.), and products with higher levels are generally not accepted by regulatory agencies [38]. Thus, quantification of residual HCPs in biopharmaceutical products is critical to ensure their adequate removal during the manufacturing process.

Traditionally, ELISA are commonly used to detect and measure HCPs during the biopharmaceutical manufacturing process [39]. ELISAs typically provide both high sensitivity and selectivity. However, ELISAs are only available for around 70% of all HCPs in typical samples [40], and development of new anti-HCP antibodies for use in ELISAs can be difficult and time consuming. In addition, HCPs may sometimes not be successfully detected even though the reagent contains the corresponding antibodies due to incompatible binding conditions or the accessibility of the relevant HCP epitopes [41].

As it is not possible for ELISA to identify all possible HCP contaminants, MS has emerged as an alternative technique for HCP analysis, as MS can monitor and identify multiple HCPs in a sample in one unbiased analysis. Moreover, even low quantities of HCPs are still able to be detected by MS; this is crucial for biopharmaceutical manufacture because even low levels of impurities can lead to adverse effects such as provoking immunogenicity.

HCP analysis requires both identification and quantification. MS can identify and quantify proteins, using either label-free or various chemical labeling strategies. In either approach, LC–MS/MS with rapid measuring speed as well as high sensitivity and selectivity has been widely applied to quantify HCPs in bottom-up proteomics workflows. In this method, proteins are digested with specific proteases, and the resulting peptides are desalted and analyzed

by LC–MS/MS. Proteins are identified by matching experimental MS/MS spectra to theoretical fragmentation patterns from predicted peptides. LC–MS/MS data can also be used for quantification of peptides and the proteins from which they originate. MS labeling strategies have been widely applied and demonstrate high accuracy. Such strategies include metabolic labeling such as stable isotope labeling with amino acids in culture [42] and chemical labeling of proteins such as 2D-difference fluorescence gel electrophoresis [43], or of peptides such as with isobaric tag for relative and absolute quantitation [44] or Tandem Mass Tag [45] systems. However, there are some drawbacks of labeling approaches, as they involve extra sample processing and are not possible for all sample types. In recent years, label-free quantification strategies have become an alternative popular and effective method used in MS proteomics [46–50]. Label-free quantification can use spectral counting or intensity-based measures. Spectral count is derived from identification from MS/MS spectra, or the total number of MS/MS spectra that correspond to a particular protein [51]. Generally, proteins with higher abundance in a sample will have more detectable peptides present after protease digestion and will therefore subsequently be represented by more MS/MS spectra [52]. Label-free quantification can also be based on peptide-ion intensity derived from LC–MS/MS data. Because the signal intensity of peptide ions is related to the peptide concentration, peptide abundance can be measured based on ion intensity through AUC or peak height. Data independent acquisition (DIA) LC–MS/MS workflows such as sequential window acquisition of all theoretical ions mass spectrometry (SWATH–MS) are powerful label-free approaches for deep, proteome-wide profiling with high-throughput and reproducible analysis [53]. Additionally, absolute quantification of proteins is an effective label-free strategy, in which stable isotopes are incorporated into synthetic peptides, imitating native peptides generated through proteolysis, and are added as internal standards to allow absolute quantification of targeted proteins [54,55].

A key challenge of HCP analysis is that the HCPs may be present at very low concentrations in the presence of a very high concentration of the biopharmaceutical product of interest. The analytical challenges posed by this difficulty can be overcome in several ways.

Sample preparation is key for all LC–MS/MS workflows, and can be used strategically to increase HCP detection. ProteoMiner technology has been used to increase detection of low abundance HCPs by reducing the dynamic range of peptides after proteolysis [56,57]. Depletion of the biopharmaceutical product before proteolysis has also been achieved with denatured HILIC fractionation [58]. The speed of sample preparation can be critical for the overall efficiency of HCP measurement. For example, sodium deoxycholate is a protein denaturant that does not need to be removed before trypsin digestion, and which can be easily removed by acidification after digestion to enable LC–MS/MS analysis [59].

After sample preparation, LC–MS/MS workflows can be tailored for HCP quantification. Targeted detection of known HCPs at very high sensitivity can be performed with multiple reaction monitoring [60,61]. However, multiple reaction monitoring relies on previous identification of HCPs which may be present. In contrast, DIA analysis can measure previously unpredicted proteins, and LC–MS/MS DIA–MS workflows also have excellent quantitative dynamic range and have been used for HCP quantification [62]. In a recent study that predicted yield and quality of the purified coagulation factor IX product through analysis of bioreactor supernatant, a set of LC–MS/MS DIA/SWATH workflows were established and used to quantify the factor IX product and HCPs, both during cell culture in bioreactors and after purification (Figure 2) [63]. In another study, a data independent liquid chromatography/mass spectrometry platform (2D-LC/MS^E) with Hi3 quantitation was used to measure HCPs in purified mAb samples to evaluate the impact of elution buffer choice for downstream purification, cell culture harvest time and additional downstream purification steps [64]. The high dynamic range of these DIA workflows allowed detection and quantification of low abundant HCPs in the presence of abundant biopharmaceutical product (Factor IX or mAb) without enrichment or depletion [65]. Another approach for increasing dynamic range, but using data-dependent acquisition (DDA), is the recently reported HCP-automated iterative MS workflow for identification and quantification of HCPs at extremely low levels (10 p.p.m.) without enrichment or pretreatment, in which samples were analyzed by LC–MS/MS multiple times, with precursor ions automatically excluded for selection for MS/MS in iterative replicates [66].

Recent years have seen the addition of IMS capabilities to MS instruments from several vendors. The additional online fractionation provided by IMS can allow deeper proteome profiling to increase the dynamic range of LC–MS/MS experiments. This is exemplified by the use of high-field asymmetric waveform ion mobility spectrometry on a Orbitrap Fusion Lumos Tribrid MS instrument, which increased the depth of HCP measurement [67].

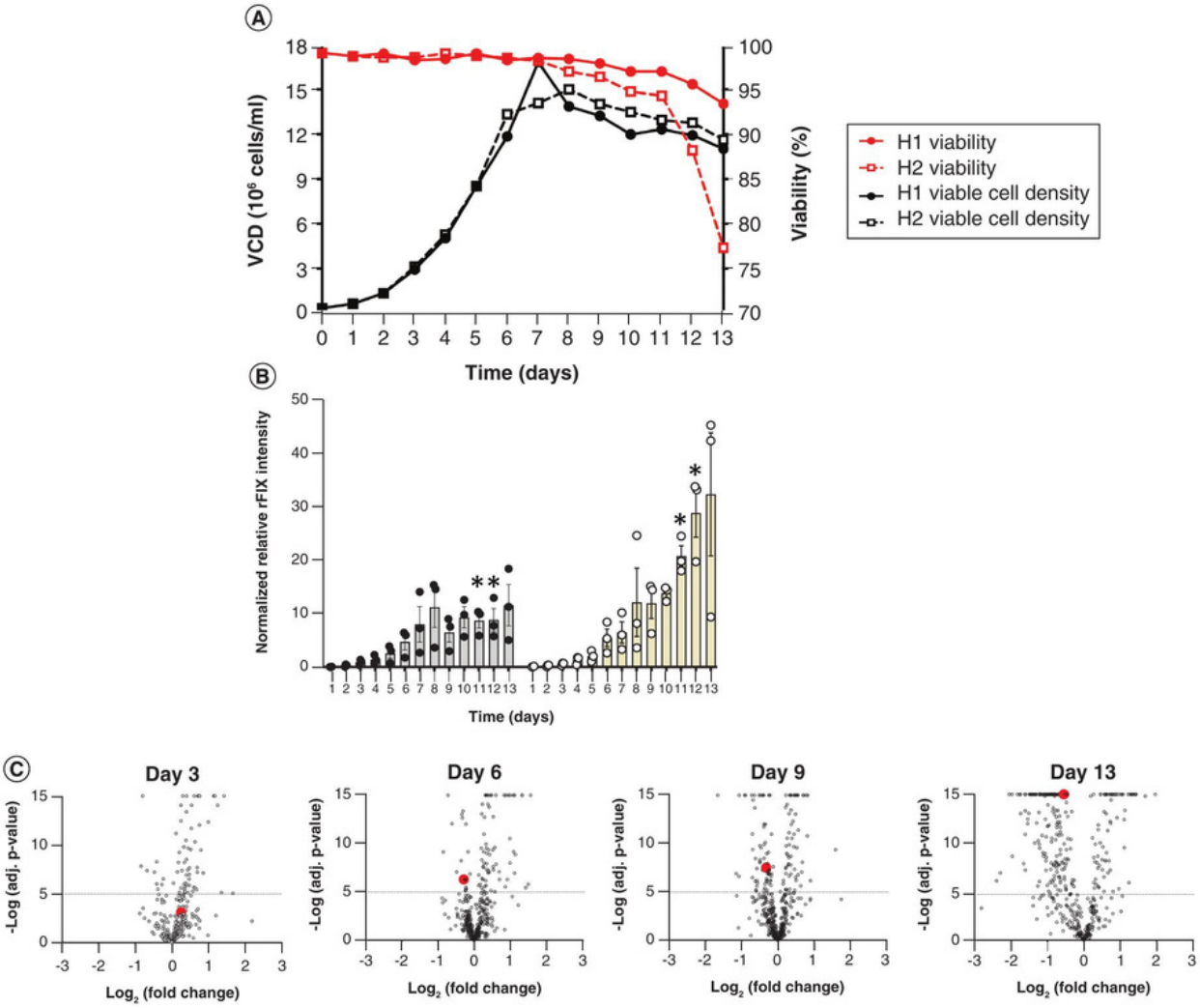


Figure 2. Viability, productivity and host cell protein profile of CHO cells expressing FIX in fed-batch conditions.
CHO cells co-expressing FIX and PACE/Furin were grown in fed-batch bioreactor mode with either EfficientyFeed A (H1) or EfficientFeed B (H2) as feeds. **(A)** Viability (red line) and (VCD; black line) in H1 (solid line, closed circle) and H2 (dotted line, open square); n = 1. **(B)** Relative FIX abundance (normalized to trypsin) in the bioreactor supernatants during operation (Mean ± SEM; multiple *t* test, n = 3 independent technical replicates; *p = 0.0072 and p = 0.0166 for day 11 and day 12 in H1 vs H2, respectively). Individual data points are indicated in black (H1 bioreactor, gray bars) or white circles (H2 bioreactor, yellow bars). **(C)** Volcano plots depicting log2 of the fold change in protein abundance versus -log10 of adjusted p-value for comparisons of culture media of bioreactor H1 versus H2 at days 3, 6, 9 and 13. The dotted horizontal line indicates the value above which the comparisons were significant (MSstats, $p < 10^{-5}$, n = 3 independent technical replicates). The red dots indicate rFIX at day 3 (adjusted p = 0.00078), day 6 (adjusted $p = 5.02 \times 10^{-7}$), day 9 (adjusted $p = 3.1 \times 10^{-8}$) and day 13 (adjusted p = 0) in H1 versus H2. Each open circle is a unique protein.
SEM: Standard error of the mean; VCD: Viable cell density.
Reproduced with permission from [63] © Zacchi *et al.* (2021).

The increased sensitivity and speed of modern MS instruments is enabling their use with rapid LC systems, while still maintaining deep proteome coverage. For instance, the Evosep ONE LC system can allow rapid robust online LC separation, for up to 60 samples per day [68].

HCPs are a major process-related impurity, and their sufficient removal (<100 p.p.m.) is crucial to obtain high-quality biopharmaceutical products. Thus, quantification of HCPs is necessary during and after purification. ELISA is an effective method for quantification of HCPs, but it has largely been replaced by LC–MS/MS due to the latter's rapid analysis time, high sensitivity and ability to measure all detectable HCPs in a sample in an unbiased manner. A variety of quantitative LC–MS/MS workflows are possible for this purpose, including labeled and label-free methods, depending on the precise experimental questions at hand.

Characterization of PTMs

Proteins can be modified with a highly diverse range of PTMs, including glycosylation, phosphorylation, proteolysis, acetylation, formylation, methylation, ubiquitination, carboxylation and many more. These PTMs increase the structural and functional diversity of proteomes [69]. Most PTMs are catalyzed by enzymes, allowing tight regulation of these functionally important features of proteins. As the correct presence and structure of PTMs are often critical for protein function, their detailed characterization is a necessary step in the quality control of biopharmaceuticals. For instance, antibodies, blood factors, erythropoietin, some IFNs and some hormones are glycosylated, which is important for their folding, stability, function, half-life and immunogenicity [70,71]. However, the diversity and structural complexity of PTMs on biopharmaceuticals can make their analysis complex and time-consuming.

Although PTMs are critical for biopharmaceutical quality, the measurement of various PTMs is challenging. Conventionally, Edman degradation, isotopic labelling, immunochemistry and amino acid analysis were commonly used techniques used to measure PTMs [72]. These approaches can be very sensitive, and are effective in single-site PTM detection, but their low-throughput makes them inappropriate for large-scale measurement of PTMs. This is a critical flaw, as many biopharmaceuticals are modified with multiple PTMs at many different sites. MS has emerged as the technique of choice for identifying and measuring PTMs. It has high sensitivity, and can identify specific-site PTMs, novel or unexpected PTMs, and PTMs in complex mixtures of proteins. None of the traditional methods has all of these abilities.

Generally, approaches for PTM identification by MS can be divided into bottom-up, middle-up and top-down strategies. Bottom-up analysis works at the peptide level, which means that the studied proteins are digested by proteases such as trypsin to produce peptides generally in the range of 500–3000 Da [73]. These proteolytically cleaved peptides tend to have few PTMs, which substantially simplifies their analysis. Bottom-up analyses are the most popular due to their high throughput and sensitivity, but they also have limitations. Specifically, not all proteolytic peptides resulting from digest with a given protease are normally able to be detected by MS, because some will be too large or too small [74]. This deficiency can be overcome, at least in part, by the use of independent treatment with different protease enzymes with complementary specificities. However, bottom-up analyses also lose any connectivity between sites of PTMs on the same protein molecule. To overcome these limitations, top-down approaches can be performed. In this approach, intact proteins are directly analyzed by LC–MS/MS without prior proteolytic digestion. This strategy is especially effective for characterization of essentially pure samples of small proteins without extensive or overly heterogeneous PTMs [75], and is inappropriate for high-throughput analyses owing to its low sensitivity and time-consuming data evaluation and interpretation [76]. Middle-down analyses, with restricted or limited proteolytic digestion, combine some of the benefits of top-down and bottom-up proteomics. These approaches aim to analyze protein fragments around 5–20 kDa in size, with intermediate PTM diversity [77].

Coagulation factor IX (FIX) is a biopharmaceutical with a very high number and diversity of PTMs, many of which are critical to its function. In particular, FIX is modified with many glycosylation events and γ-carboxylation of its N-terminal GLA domain. γ-carboxylation is a PTM mediated by γ-glutamyl carboxylase during protein biosynthesis, and complete γ-carboxylation is a key quality determinant of recombinant FIX [78]. The study mentioned above developed DIA LC–ESI–MS/MS methods to measure site-specific PTMs across FIX during bioreactor operation and after purification [63]. It was found that it is difficult to detect and identify fully γ-carboxylated GLA peptides in positive ion mode LC–ESI–MS/MS owing to the negative charge of the γ-carboxyl groups, and neutral loss of CO_2 upon CID fragmentation. However, standard bottom-up DIA LC–ESI–MS/MS could detect uncarboxylated or partially γ-carboxylated GLA peptides in positive ion mode, and could be used to infer the extent of site-specific γ-carboxylation. Derivatization of γ-carboxyl groups also allowed measurement of fully modified peptides, although this increased the complexity of the procedure [79]. These DIA–MS methods were used to monitor γ-carboxylation throughout bioreactor operation and compare differences in the extent of modification in the finished product with varied bioreactor operation. FIX is also modified by other heterogeneous PTMs such as proteolysis, *N*- and *O*-glycosylation, sulfation, phosphorylation, β-hydroxylation and disulfide bonds. To test the occupancy and structure of these PTMs, in-depth DDA–MS analysis was performed to identify and characterize PTMs, which was then used as the basis for DIA–MS quantification. The majority of the known PTMs on rFIX and several new PTMs (Figure 3) were observed and monitored in this study, highlighting the benefits of DIA–MS for PTM profiling of biopharmaceuticals.

Glycosylation is one of the most widespread, important and analytically challenging PTMs present on biopharmaceutical products. Glycoproteomic workflows are powerful and commonly used approaches for profiling the

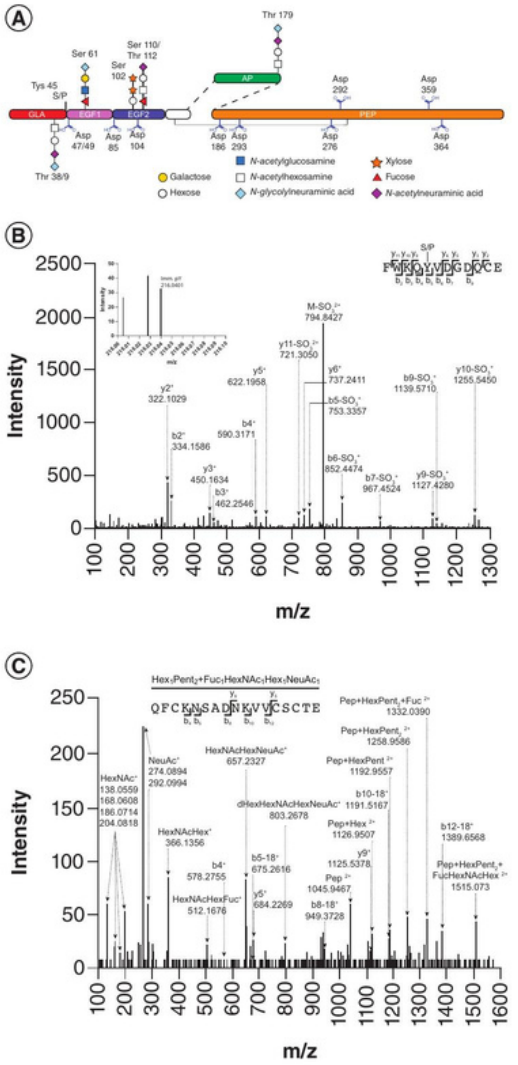


Figure 3. New post-translational modifications identified by DDA LC–MS/MS on recombinant Factor IX. (A) Schematics of FIX containing the new PTMs identified on rFIX. **(a, b)** CID fragmentation of select GluC FIX peptides. **(B)** $F^{41}WKQYVDGDQCE^{54}$ peptide with sulfation/phosphorylation (S/P) at Y^{45} (observed precursor m/z 834.8189^{2+}, Δ2.4 p.p.m.). The inset shows the phosphotyrosine immonium ion (pY, 216.0401 m/z, Δ2.9 p.p.m.). **(C)** $Q^{97}FCKN(+1)SADN(+1)KVVCSCTE^{113}$ glycopeptide with Hex_1Xyl_2 and $Fuc_1HexNAc_1Hex_1NeuAc_1$ *O*-glycans attached to $S^{102/110}$ and T^{112} (observed precursor m/z 1107.1067^{3+}, Δ7.56 p.p.m.). Pep, peptide.
CID: Collision-induced dissociation; PTM: Post-translational modification.
Reproduced with permission from [63] © Zacchi *et al.* (2021).

site-specific glycosylation of biopharmaceuticals, as they use relatively standard proteomic bottom-up LC–MS/MS techniques [80–82]. CID or HCD fragmentation can provide substantial information on the peptide identity and glycan monosaccharide composition of glycopeptides [83], while ETD or ETD with HCD supplemental activation (EThcD) fragmentation is typically required for unambiguous localization of the site of modification, especially for *O*-glycans [84]. Data analysis pipelines must consider the additional structural complexity of glycopeptides compared with peptides, and many informatics solutions are currently available and under further development [85]. Glycoproteomic workflows can also be complemented with identification of the sites of *N*-glycosylation by deglycosylating biopharmaceuticals with enzymes such as peptide-N-glycosidase F (PNGase F), which leave a 'chemical scar' of deamidation of asparagine to aspartate at previous sites of glycosylation, prior to LC–MS/MS analysis [86,87]. Enzymatically released glycans can also be analyzed using glycomics workflows, which can provide detailed structural information about the glycans which can be difficult to obtain with glycoproteomic LC–MS/MS workflows alone [88]. Analysis of both released glycans and intact glycopeptides can also benefit from complementary separation methods such as LC, capillary electrophoresis and IMS [23,89], which can allow separation of glycan structural isomers [90].

A recent study characterizing the SARS-CoV-2 glycan shield demonstrated the power of mass spectrometric glycoproteomics to reveal the site-specific glycosylation of a recombinant SARS-CoV-2 S immunogen, including site-specific *N*-linked glycan composition and occupancy [91]. To maximize the coverage of the many *N*-glycosylation sites present on the S glycoprotein, three different proteases were used – trypsin, chymotrypsin and alpha-lytic protease. LC–MS/MS with high-energy HCD fragmentation was then used to analyze the glycopeptide pools and determine the glycan composition. Different glycosylation sites were found to vary in their site-specific *N*-glycosylation profiles (Figure 4). More specifically, three sites, N234, N709 and N801, were mainly oligomannose-type glycosylation; several sites, especially N657, possessed diverse hybrid-type glycans; and sites N61, N122, N165, N603, N657 and N1074 were occupied by a mixture of oligomannose- and complex-type glycans. The high confidence characterization of site-specific *N*-glycosylation structures and occupancy across the many modified sites of the S protein achieved by this analytical approach demonstrates its utility for glycoprotein biopharmaceutical quality control.

The previous examples highlight the power of bottom-up analysis for PTM identification and quantification at the peptide level. However, the peptide-centric focus of this approach means that most analyzed peptides have single PTMs, which hinders overall profiling of the entire protein. A recent study used an integrated strategy combining high-resolution native MS and middle-down proteomics to characterize co-occurring PTMs of human erythropoietin and human plasma properdin, enabling profiling of the structural micro-heterogeneity that often affects the functionality of biopharmaceuticals [92]. Native MS could measure the relative abundance and overall PTM composition of different proteoforms that could be distinguished by mass [93]. Middle-down analyses were then applied to characterize the site-specificity of these PTMs. The data from both approaches were then combined and compared to assess the completeness and reliability of PTM assignments. This combined integrated MS strategy provided a very complete profile of the measured glycoproteins and also discovered unexpected heterogeneity in three C-glycosylation sites on properdin. In theory, this integrative workflow could be used to quantitatively profile the site-specific molecular heterogeneity of PTMs on any protein, only limited by the resolution of the MS and the PTM heterogeneity of the protein.

Most biopharmaceuticals are proteins with diverse and complex PTMs that play important roles in their stability, function, half-life or immunogenicity. Detailed characterization of PTMs is therefore critical to guarantee high quality and effective potency of biopharmaceuticals. LC–MS/MS is a powerful technique for identifying and quantifying site-specific PTM structure and occupancy, particularly with a combination of bottom-up, middle-up, top-down or integrated analytical strategies.

Structural characterization of proteins

Protein function depends on correct folding and structure. Unfolding or misfolding can lead to unstable proteins with partial or total loss of function. Additionally, and of particular importance for biopharmaceuticals, disordered or misfolded proteins may aggregate, decreasing the effectiveness of the biopharmaceutical products and leading to other risks such as increased immunogenicity [94]. As correct protein structure is crucial for therapeutic proteins, the structural characterization of biopharmaceuticals is therefore necessary to ensure product quality by avoiding unfolded, misfolded or aggregated proteins.

X-ray crystallography and NMR are both classical tools for protein structural analysis, while hydrogen-deuterium exchange mass spectrometry (HDX–MS) has emerged as a highly complementary technique for mapping protein folding, protein–protein and protein–ligand interactions, and dynamic conformational changes in proteins. Additionally, HDX–MS is versatile and can be used to explore other systems including highly dynamic proteins, large biomolecular complexes and membrane-associated species [95]. In a typical HDX–MS analysis, proteins in H_2O-based solvent are diluted into D_2O-based solvent, which induces the liable hydrogens on the protein to exchange with deuterium in the solvents, with the exchange rate largely determined by surface accessibility, protein structure and dynamics, as limited solvent access and hydrogen bonding can protect hydrogens from exchange. Data on the extent of exchange are typically collected at several intervals, providing a profile of deuterium exchange versus time which reflects protein conformation and dynamics [96].

Bottom-up and top-down workflows are both available for HDX–MS analysis, with the former more common as it can be used for any protein without limitations on protein size. In this approach, proteins are rapidly digested with pepsin and LC–MS/MS data are collected, which can measure the extent of HDX at a peptide- or even amino acid-level. However, approximately 10–50% deuterium label loss can occur in this approach during enzymatic digestion and HPLC separation of the peptides [97]. In contrast, in top-down workflows, intact protein is directly analyzed

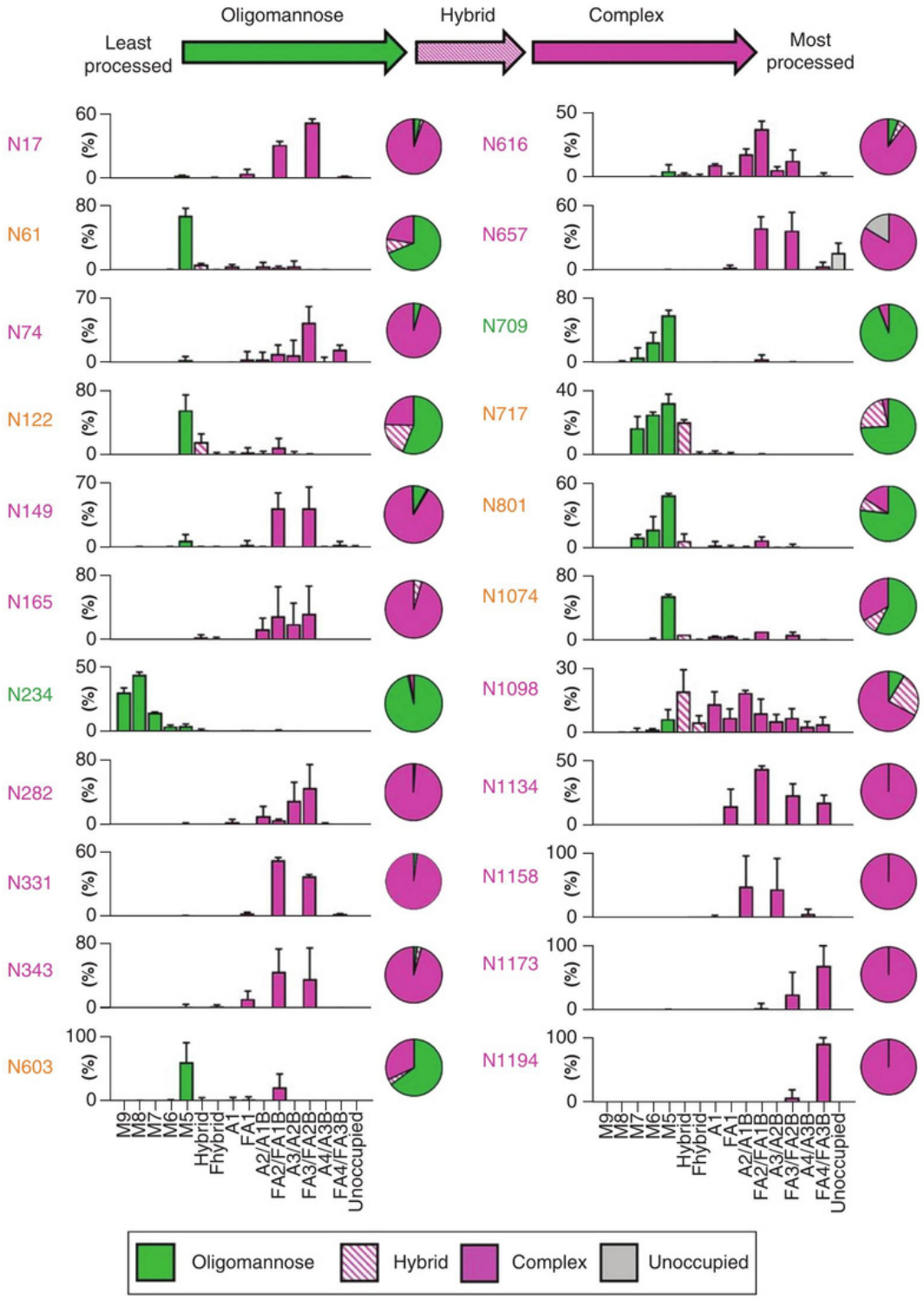


Figure 4. Site-specific *N*-linked glycosylation of the SARS-CoV-2 S glycoprotein. The schematic illustrates the color code for the principal glycan types that can arise along the maturation pathway from oligomannose- to hybrid- to complex-type glycans. The graphs summarize quantitative mass spectrometric analysis of the glycan population present at individual *N*-linked glycosylation sites simplified into categories of glycans. The oligomannose-type glycan series (M9 to M5; Man9GlcNAc2 to Man5GlcNAc2) is colored green, afucosylated and fucosylated hybrid-type glycans (hybrid and F hybrid) are dashed pink, and complex glycans are grouped according to the number of antennae and presence of core fucosylation (A1 to FA4) and are colored pink. Unoccupancy of an *N*-linked glycan site is represented in gray. The pie charts summarize the quantification of these glycans. Glycan sites are colored according to oligomannose-type glycan content, with the glycan sites labeled in green (80–100%), orange (30–79%) and pink (0–29%). The bar graphs represent the mean quantities of three biological replicates, with error bars representing the standard error of the mean.
Reproduced with permission from [91] © Watanabe *et al.* (2020).

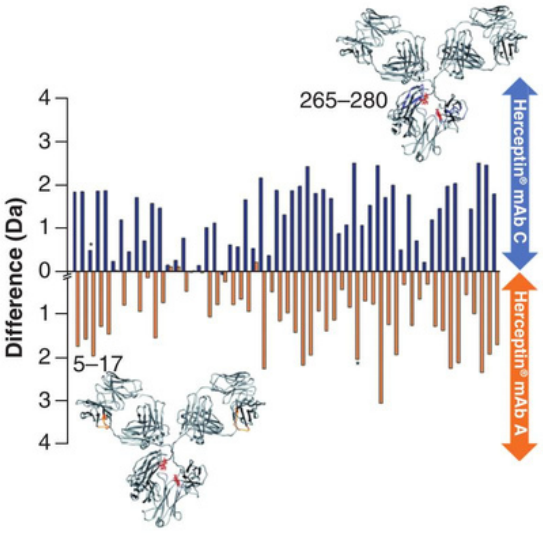


Figure 5. Deuterium uptake difference plots for heavy chain intact versus endoS2 treated Herceptin®; endoS2–intact. Each bar represents a different peptide (HC sequence coverage = 70.2%, 55 peptides). Blue bars represent the deuterium uptake differences for Herceptin lot C; orange bars represent deuterium uptake differences for Herceptin lot A. Labeled peptides 5–17 (VESGGGLVQPGGS) and 265–280 (VVVDVSHEDPEVKFNW) are those with significant uptake differences between the two Herceptin lots, i.e., a difference of >1 Da. The peptide locations for the two peptides with uptake differences >1 Da are highlighted on the mAb structures in blue and orange for lot C and lot A, respectively (PDB: 1IGY). A representation of endoS2 treated glycans are shown in red. Subtraction of intact mAb data from enzyme treated mAb data for individual lots, means that the intact data serves as a control to counteract any day-to-day variations in the HDX setup.
*Corresponds to the equivalent peptide in the other sample.
HC: Heavy chain; HDX: Hydrogen-deuterium exchange; mAb: Monoclonal antibody; PDB: Protein data bank.
Reproduced with permission from [111] © The Royal Society of Chemistry (2019).

by LC–MS/MS, involving ionization and fragmentation. This minimizes loss of deuterium and potentially allows true site-specific HDX measurement [98]. However, the success of this method decreases with increasing protein size, and is only currently feasible with proteins less than 30 kDa [99,100]. Additionally, deuterium scrambling can occur during MS/MS fragmentation, where hydrogen or deuterium migrate along the peptide backbone leading to distortion or loss of the original labeling pattern [96].

A recent study combined the complementary approaches of bottom-up and top-down HDX-MS to characterize and compare the higher-order structure of an originator antibody drug and two batches of biosimilars [101]. Although the same samples were used in the two approaches, it is somewhat difficult to directly compare the results due to the different back-exchange rates and spatial resolution achieved with the two methods. Nonetheless, the structural data from the two approaches were consistent and complementary, with both approaches finding no structural differences between the three drug samples. Moreover, the sequence coverage for heavy chain and light chain was 87 and 74%, respectively with bottom-up analysis, and 50 and 100% with the top-down approach. This highlights the consistency and complementarity of the two methods. Overall, the combination of both methods provided high-quality complete structural information for the whole antibody without any missed regions or residues. HDX–MS is an effective technique to rapidly map binding epitopes in the early stage of biosimilar development, providing data to assess similarity [102–104]. Compared with more traditional HDX–MS, time-resolved ESI hydrogen-deuterium exchange MS (TRESI–HDX–MS) with ms time-scale deuterium labeling can detect more subtle changes in protein conformation and interactions [105–109]. This powerful tool was used in a recent study to compare the interactions of a commercial Avastin and its biosimilar ApoBev with their biological target, VEGF-A. Clear epitope mapping was obtained through TRESI–HDX–MS, which showed that the binding epitopes of Avastin and ApoBev for VEGF-A are very similar, but with subtle differences in VEGF dynamics [110]. Combinations of techniques can provide particularly informative descriptions of protein structure and dynamics. For instance, HDX–MS has been used together with IM–MS to identify batch-to-batch signatures of the mAb Herceptin that correspond with the impact of *N*-glycosylation on protein structure and dynamics (Figure 5) [111]. IM–MS can be particularly informative in combination with collision-induced unfolding, to characterize the structure, dynamics and interactions of proteins [36,112,113].

The potency of biopharmaceuticals depends on them having correct structures, so correct folding is a key quality requirement. HDX–MS can be applied as a rapid and unbiased technique to monitor the folding or aggregation status of diverse proteins, with bottom-up, top-down or integrated analytical workflows, while TRESI–HDX–MS is capable to achieve faster and more unambiguous detection. IM–MS also shows exciting potential for rapid and informative structural profiling of biopharmaceuticals.

Conclusion

MS is a mature and powerful technique that is applicable to many aspects of biopharmaceutical quality control. In particular, it is useful for identification and quantification of HCP contaminants, characterisation of complex PTMs and monitoring the structural integrity of biopharmaceutical products. Diverse MS workflows enable this wide range of applications, including DIA LC-MS/MS, DDA LC-MS/MS with diverse fragmentation techniques, IM-MS, HDX-MS and native MS. Future developments in sample preparation, instrumentation and data analysis will undoubtedly further extend the capabilities and utility of MS analyses in biopharmaceutical quality control.

Future perspective

LC–MS/MS technology and applications are expected to rapidly progress in coming years, with wider and more frequent application in biopharmaceutical process and product quality control. Modern MS instruments have incredible performance in sensitivity and resolution, and the amount of biopharmaceutical product required for analysis is not generally limiting. Instead, it is analytical through-put which limits the usefulness of MS for many applications. Current MS workflows including sample preparation, analysis, and data processing typically take 1–2 days, limiting their utility in time-sensitive applications such as process monitoring. We therefore see improvements in the speed, through-put, automation and robustness of LC–MS/MS analytic workflows as a critical opportunity, with rapid automated digestion and sample preparation for bottom-up strategies, or improved technology for top-down strategies. For instance, with such improvements it may prove feasible to use LC–MS/MS during fermentation to monitor desired or unwanted product PTMs, or to monitor the purification process for residual HCP impurities in real-time, increasing product quality and purification efficiency of target biopharmaceutical proteins.

Executive summary

- MS has become one of the key methods used in the characterization and quantitation of proteins in biopharmaceutical quality control during the past two decades owing to improvements in instrument sensitivity, resolution, specificity and selectivity.
- Detailed information is needed for quality control of biopharmaceuticals, including residual host-cell proteins (HCPs), site-specific post-translational modifications (PTMs) and protein folding status.
- LC–MS/MS can identify and quantify HCPs with high selectivity and sensitivity, as even low quantities of HCPs are detectable by MS, and many HCPs can be identified in one analysis.
- For characterization of PTMs, MS strategies include bottom-up, top-down and middle-down. Bottom-up analyses are the most common, providing high sensitivity and high-throughput peptide level measurements, but can be limited by incomplete coverage of a protein's sequence. Top-down analyses are suitable for analysis of small proteins with modest PTM heterogeneity, although data interpretation can be time-consuming. Middle-down approaches, or an integrated combination of all three strategies, have emerged as an effective approach for detailed global, site-specific PTM analysis.
- Hydrogen-deuterium exchange MS allows structural characterization of the folding and aggregation status of proteins with bottom-up or top-down analyses, with bottom-up approaches being applicable for proteins of all sizes.
- Improvements in the speed, automation and throughput of bottom-up LC–MS/MS, and of the resolution and data analysis workflows of top-down LC–MS/MS are expected to allow these techniques to be useful for real-time monitoring of product quality during fermentation, or of HCP impurities during purification, improving process efficiency and product quality.

Financial & competing interests disclosure

This work was funded by an Australian Research Council Industrial Transformation Training Centre IC160100027 to BL Schulz. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

References

Papers of special note have been highlighted as: • of interest; •• of considerable interest

1. Introductory chapter: biopharmaceuticals. In: *Biopharmaceuticals* Chen YC, Yeh MK (Eds). IntechOpen, London, UK (2018).
2. Top O, Geisen U, Decker EL, Reski R. Critical evaluation of strategies for the production of blood coagulation factors in plant-based systems. *Front. Plant Sci.* 10, 261 (2019).

3. Adivitiya Khasa YP. The evolution of recombinant thrombolytics: current status and future directions. *Bioengineered* 8(4), 331–358 (2017).
4. Grand View Research. Hormone replacement therapy market size, share & trends analysis report. https://www.grandviewresearch.com/industry-analysis/hormone-replacement-therapy-market
5. Global market study on growth factors - 'reinforcing' the life sciences research. https://www.persistencemarketresearch.com/market-research/growth-factors-market.asp
6. Interferons global market report. https://www.thebusinessresearchcompany.com/report/interferons-market-global-report-2020-2030-covid-19-implications-and-growth
7. Interleukin inhibitors market. https://www.bloomberg.com/press-releases/2019-09-11/interleukin-inhibitors-market-worth-74-6-billion-by-2026-cagr-17-4-grand-view-research-inc
8. Big pharma and big profits: the multibillion dollar vaccine market. https://www.globalresearch.ca/big-pharma-and-big-profits-the-multibillion-dollar-vaccine-market/5503945
9. Global Monoclonal Antibody Industry 2019–2025. https://apnews.com/press-release/pr-businesswire/af15cf4cf54e4698a93689afb4a906e8
10. Takahashi H, Letourneur D, Grainger DW. Delivery of large biopharmaceuticals from cardiovascular stents: a review. *Biomacromolecules* 8(11), 3281–3293 (2007).
11. Craik DJ, Fairlie DP, Liras S, Price D. The future of peptide-based drugs. *Chem. Biol. Drug Des.* 81(1), 136–147 (2013).
12. Kesik-Brodacka M. Progress in biopharmaceutical development. *Biotechnol. Appl. Biochem.* 65(3), 306–322 (2018).
13. Rader RA. Langer ES. Biopharma market: an inside look. https://www.pharmamanufacturing.com/articles/2018/biopharma-market-an-inside-look/
14. Mordor Intelligence LLP. Biopharmaceuticals market-growth, trends, and forecast. https://www.reportlinker.com/p05815028/Biopharmaceuticals-Market-Growth-Trends-and-Forecast.html
15. Baeshen NA, Baeshen MN, Sheikh A *et al.* Cell factories for insulin production. *Microb. Cell. Fact.* 13(1), 141 (2014).
16. Tripathi NK, Shrivastava A. Recent developments in bioprocessing of recombinant proteins: expression hosts and process development. *Front. Bioeng. Biotechnol.* 7, 420 (2019).
17. Jenkins N. Modifications of therapeutic proteins: challenges and prospects. *Cytotechnology* 53(1–3), 121–125 (2007).
18. Li F, Shen A, Amanullah A. Cell culture processes in monoclonal antibody production. In: *Pharmaceutical Sciences Encyclopedia: Drug Discovery, Development, and Manufacturing.* Gad SC (Ed.). John Wiley & Sons, NJ, USA (2010).
19. Molowa DT, Mazanet R. The state of biopharmaceutical manufacturing. *Biotechnol. Annu. Rev.* 9, 285–302 (2003).
20. Patel PK, King CR, Feldman SR. Biologics and biosimilars. *J. Dermatolog. Treat.* 26(4), 299–302 (2015).
21. Jozala AF, Geraldes DC, Tundisi LL *et al.* Biopharmaceuticals from microorganisms: from production to purification. *Braz. J. Microbiol.* 47, 51–63 (2016).
22. Braun AC, Gutmann M, Lühmann T, Meinel L. Bioorthogonal strategies for site-directed decoration of biomaterials with therapeutic proteins. *J. Control. Release.* 273, 68–85 (2018).
23. Pallister EG, Choo MS, Walsh I *et al.* Utility of ion-mobility spectrometry for deducing branching of multiply charged glycans and glycopeptides in a high-throughput positive ion LC-FLR-IMS-MS workflow. *Anal. Chem.* 92(23), 15323–15335 (2020).
24. Alia KB, Nadeem H, Rasul I *et al.* Separation and purification of amino acids. In: *Applications of Ion Exchange Materials in Biomedical Industries.* Inamuddin (Ed.). Springer, NY, USA, 1–11 (2019).
25. Ehkirch A, Hernandez-Alba O, Colas O, Beck A, Guillarme D, Cianférani S. Hyphenation of size exclusion chromatography to native ion mobility mass spectrometry for the analytical characterization of therapeutic antibodies and related products. *J. Chromatogr. B* 1086, 176–183 (2018).
26. Chen B, Lin Z, Alpert AJ *et al.* Online hydrophobic interaction chromatography–mass spectrometry for the analysis of intact monoclonal antibodies. *Anal. Chem.* 90(12), 7135–7138 (2018).
27. Parker C, Warren M, Mocanu V. Mass spectrometry for proteomics. In: *Neuroproteomics.* Alzate O (Ed.). CRC Press, FL, USA, 71–92 (2010).
28. Field ionization and field desorption. In: *Mass Spectrometry: A Text Book.* Gross JH (Ed.). Springer, NY, USA, 518 (2004).
29. Zhang Y, Wang J, Liu JA *et al.* Combination of ESI and MALDI mass spectrometry for qualitative, semi-quantitative and *in situ* analysis of gangliosides in brain. *Sci. Rep.* 6, 25289 (2016).
30. Clark AE, Kaleta EJ, Arora A, Wolk DM. Matrix-assisted laser desorption ionization–time of flight mass spectrometry: a fundamental shift in the routine practice of clinical microbiology. *Clin. Microbiol. Rev.* 26(3), 547–603 (2013).
31. Yates JR III. Mass spectrometry: from genomics to proteomics. *Trends Genet.* 16(1), 5–8 (2000).

32. Abushareeda W, Tienstra M, Lommen A *et al.* Comparison of gas chromatography/quadrupole time-of-flight and quadrupole Orbitrap mass spectrometry in anti-doping analysis: i. Detection of anabolic-androgenic steroids. *Rapid Commun. Mass Spectrom.* 32(23), 2055–2064 (2018).
33. Nesvizhskii AI, Keller A, Kolker E, Aebersold R. A statistical model for identifying proteins by tandem mass spectrometry. *Anal. Chem.* 75(17), 4646–4658 (2003).
34. D'atri V, Causon T, Hernandez-Alba O *et al.* Adding a new separation dimension to MS and LC–MS: what is the utility of ion mobility spectrometry? *J. Sep. Sci.* 41(1), 20–67 (2018).
35. Mu Y, Schulz BL, Ferro V. Applications of ion mobility-mass spectrometry in carbohydrate chemistry and glycobiology. *Molecules* 23(10), 2557 (2018).
36. Botzanowski T, Hernandez-Alba O, Malissard M *et al.* Middle level IM–MS and CIU experiments for improved therapeutic immunoglobulin subclass fingerprinting. *Anal. Chem.* 92(13), 8827–8835 (2020).
37. Wang X, Hunter AK, Mozier NM. Host cell proteins in biologics development: identification, quantitation and risk assessment. *Biotechnol. Bioeng.* 103(3), 446–458 (2009).
38. Chon JH, Zarbis-Papastoitsis G. Advances in the production and downstream processing of antibodies. *N. Biotechnol.* 28(5), 458–463 (2011).
39. Hogwood CE, Bracewell DG, Smales CM. Measurement and control of host cell proteins (HCPs) in CHO cell bioprocesses. *Curr. Opin. Biotechnol.* 30, 153–160 (2014).
40. Thompson JH, Chung WK, Zhu M *et al.* Improved detection of host cell proteins (HCPs) in a mammalian cell-derived antibody drug using liquid chromatography/mass spectrometry in conjunction with an HCP-enrichment strategy. *Rapid Commun. Mass Spectrom.* 28(8), 855–860 (2014).
41. Zhu-Shimoni J, Yu C, Nishihara J *et al.* Host cell protein testing by ELISAs and the use of orthogonal methods. *Biotechnol. Bioeng.* 111(12), 2367–2379 (2014).
42. Ma W, Jia J, Huang X *et al.* Stable isotope labelling by amino acids in cell culture (SILAC) applied to quantitative proteomics of Edwardsiella tarda ATCC 15947 under prolonged cold stress. *Microb. Pathog.* 125, 12–19 (2018).
43. Pasquali M, Serchi T, Planchon S, Renaut J. 2D-DIGE in proteomics. In: *Functional Genomics* Kaufman M, Klinger C, Savelsbergh A (Eds). Springer, NY, USA, 245–254 (2017).
44. Ding W, Qiu B, Cram DS *et al.* Isobaric tag for relative and absolute quantitation based quantitative proteomics reveals unique urinary protein profiles in patients with preeclampsia. *J. Cell. Mol. Med.* 23(8), 5822–5826 (2019).
45. Bo C, Geng X, Zhang J *et al.* Comparative proteomic analysis of silica-induced pulmonary fibrosis in rats based on tandem mass tag (TMT) quantitation technology. *PLoS ONE* 15(10), e0241310 (2020).
46. Ryu S, Gallis B, Goo YA, Shaffer SA, Radulovic D, Goodlett DR. Comparison of a label-free quantitative proteomic method based on peptide ion current area to the isotope coded affinity tag method. *Cancer Inform.* 6, doi: 10.4137/cin.s385 (2008) (Epub ahead of print).
47. Couto N, Al-Majdoub ZM, Achour B, Wright PC, Rostami-Hodjegan A, Barber J. Quantification of proteins involved in drug metabolism and disposition in the human liver using label-free global proteomics. *Mol. Pharm.* 16(2), 632–647 (2019).
48. Distler U, Kuharev J, Navarro P, Tenzer S. Label-free quantification in ion mobility–enhanced data-independent acquisition proteomics. *Nat. Protoc.* 11(4), 795–812 (2016).
49. Arike L, Peil L. Spectral counting label-free proteomics. In: *Shotgun Proteomics.* Martins-de-Souza D (Ed.). Humana Press, NY, USA, 213–222 (2014).
50. He B, Shi J, Wang X, Jiang H, Zhu H-J. Label-free absolute protein quantification with data-independent acquisition. *J. Proteomics* 200, 51–59 (2019).
51. Milac TI, Randolph TW, Wang P. Analyzing LC-MS/MS data by spectral count and ion abundance: two case studies. *Stat. Interface.* 5(1), 75 (2012).
52. Washburn MP, Wolters D, Yates JR. Large-scale analysis of the yeast proteome by multidimensional protein identification technology. *Nat. Biotechnol.* 19(3), 242–247 (2001).
53. Gupta S, Rost H. Automated workflow for peptide-level quantitation from DIA/SWATH-MS data. In: *Quantitative Methods for Proteomics.* Marcus K, Eisenacher M, Sitek B (Eds). Humana Press, NY, USA (2021).
54. Gerber SA, Rush J, Stemman O, Kirschner MW, Gygi SP. Absolute quantification of proteins and phosphoproteins from cell lysates by tandem MS. *Proc. Natl Acad. Sci. USA* 100(12), 6940–6945 (2003).
55. Szymkowicz L, Wilson DJ, James DA. Development of a targeted nanoLC-MS/MS method for quantitation of residual toxins from Bordetella pertussis. *J. Pharm. Biomed. Anal.* 188, 113395 (2020).
56. Chen I-H, Xiao H, Li N. Improved host cell protein analysis in monoclonal antibody products through ProteoMiner. *Anal. Biochem.* 610, 113972 (2020).
57. Mörtstedt H, Makower Å, Edlund P-O, Sjöberg K, Tjernberg A. Improved identification of host cell proteins in a protein biopharmaceutical by LC–MS/MS using the ProteoMiner™ Enrichment Kit. *J. Pharm. Biomed. Anal.* 185, 113256 (2020).

58. Wang Q, Slaney TR, Wu W, Ludwig R, Tao L, Leone A. Enhancing host-cell protein detection in protein therapeutics using HILIC enrichment and proteomic analysis. *Anal. Chem.* 92(15), 10327–10335 (2020).

59. Li D, Farchone A, Zhu Q *et al.* Fast, robust, and sensitive identification of residual host cell proteins in recombinant monoclonal antibodies using sodium deoxycholate assisted digestion. *Anal. Chem.* 92(17), 11888–11894 (2020).

60. Gao X, Rawal B, Wang Y *et al.* Targeted host cell protein quantification by LC–MRM enables biologics processing and product characterization. *Anal. Chem.* 92(1), 1007–1015 (2019).

61. Chen I-H, Xiao H, Daly T, Li N. Improved host cell protein analysis in monoclonal antibody products through molecular weight cutoff enrichment. *Anal. Chem.* 92(5), 3751–3757 (2020).

62. Pythoud N, Bons J, Mijola G, Beck A, Cianférani S, Carapito C. Optimized sample preparation and data processing of data-independent acquisition methods for the robust quantification of trace-level host cell protein impurities in antibody drug products. *J. Proteome Res.* 20(1), 923–931 (2020).

63. Zacchi LF, Roche-Recinos D, Pegg CL *et al.* Coagulation factor IX analysis in bioreactor cell culture supernatant predicts quality of the purified product. *Commun. Biol.* 4(1), 1–19 (2021).

•• **LC–MS/MS data independent acquisition workflow was performed to quantify host-cell proteins (HCPs) and measure post-translational modifications both during cell culture process in bioreactors and after purification.**

64. Farrell A, Mittermayr S, Morrissey B *et al.* Quantitative host cell protein analysis using two dimensional data independent LC–MSE. *Anal. Chem.* 87(18), 9186–9193 (2015).

65. Levin Y, Hradetzky E, Bahn S. Quantification of proteins using data-independent analysis (MSE) in simple and complex samples: a systematic evaluation. *Proteomics* 11(16), 3273–3287 (2011).

66. Huang Y, Molden R, Hu M, Qiu H, Li N. Toward unbiased identification and comparative quantification of host cell protein impurities by automated iterative LC–MS/MS (HCP-AIMS) for therapeutic protein development. *J. Phar. Biomed. Anal.* 200, 114069 (2021).

• **HCP–automated iterative MS workflow was applied for HCP identification and quantification.**

67. Johnson ROB, Greer T, Cejkov M, Zheng X, Li N. Combination of FAIMS, protein a depletion, and native digest conditions enables deep proteomic profiling of host cell proteins in monoclonal antibodies. *Anal. Chem.* 92(15), 10478–10484 (2020).

68. Ma J, Kilby GW. Sensitive, rapid, robust, and reproducible workflow for host cell protein profiling in biopharmaceutical process development. *J. Proteome Res.* 19(8), 3396–3404 (2020).

69. Parker CE, Mocanu V, Mocanu M, Dicheva N, Warren MR. Mass spectrometry for post-translational modifications. *Neuroproteomics* Chapter 5, 2010 (2010).

70. Jefferis R. Posttranslational modifications and the immunogenicity of biotherapeutics. *J Immunol. Res.* 2016, 5358272 (2016).

71. Jenkins N, Murphy L, Tyther R. Post-translational modifications of recombinant proteins: significance for biopharmaceuticals. *Mol. Biotechnol.* 39(2), 113–118 (2008).

72. Larsen MR, Trelle MB, Thingholm TE, Jensen ON. Analysis of posttranslational modifications of proteins by tandem mass spectrometry: mass spectrometry for proteomics analysis. *BioTechniques* 40(6), 790–798 (2006).

73. Wolters DA, Washburn MP, Yates JR. An automated multidimensional protein identification technology for shotgun proteomics. *Anal. Chem.* 73(23), 5683–5690 (2001).

74. Moradian A, Kalli A, Sweredoski MJ, Hess S. The top-down, middle-down, and bottom-up mass spectrometry approaches for characterization of histone variants and their post-translational modifications. *Proteomics* 14(4–5), 489–497 (2014).

75. Tran JC, Zamdborg L, Ahlf DR *et al.* Mapping intact protein isoforms in discovery mode using top-down proteomics. *Nature* 480(7376), 254–258 (2011).

76. Durbin KR, Fornelli L, Fellers RT, Doubleday PF, Narita M, Kelleher NL. Quantitation and identification of thousands of human proteoforms below 30 kDa. *J. Proteome Res.* 15(3), 976–982 (2016).

77. Zhang Y, Fonslow BR, Shan B, Baek M-C, Yates JR III. Protein analysis by shotgun/bottom-up proteomics. *Chem. Rev.* 113(4), 2343–2394 (2013).

78. Lindberg I, Peinado J. Posttranslational modifications: key players in health and disease. *Encyclopedia Cell Biol.* 1, 84–90 (2015).

79. Hallgren K, Zhang D, Kinter M, Willard B, Berkner K. Methylation of γ-carboxylated Glu (Gla) allows detection by liquid chromatography–mass spectrometry and the identification of gla residues in the γ-glutamyl carboxylase. *J. Proteome Res.* 12(6), 2365–2374 (2013).

80. Thaysen-Andersen M, Packer NH, Schulz BL. Maturing glycoproteomics technologies provide unique structural insights into the N-glycoproteome and its regulation in health and disease. *Mol. Cell. Proteomics* 15(6), 1773–1790 (2016).

81. Chang D, Zaia J. Methods to improve quantitative glycoprotein coverage from bottom-up LC-MS data. *Mass Spectrom. Rev.* (2021) doi: 10.1002/mas.21692 (Epub ahead of print).

82. Ye Z, Vakhrushev SY. The role of data-independent acquisition for glycoproteomics. *Mol. Cell. Proteomics* 20, 100042 (2021).

83. Nilsson J. Liquid chromatography-tandem mass spectrometry-based fragmentation analysis of glycopeptides. *Glycoconj. J.* 33(3), 261–272 (2016).

84. Riley NM, Malaker SA, Driessen MD, Bertozzi CR. Optimal dissociation methods differ for N-and O-glycopeptides. *J. Proteome Res.* 19(8), 3286–3301 (2020).

85. Kawahara R, Alagesan K, Bern M *et al.* Community evaluation of glycoproteomics informatics solutions reveals high-performance search strategies of glycopeptide data. *bioRxiv* (2021). https://doi.org/10.1101/2021.03.14.435332

86. Bodnar J, Szekrenyes A, Szigeti M *et al.* Enzymatic removal of N-glycans by PNGase F coated magnetic microparticles. *Electrophoresis* 37(10), 1264–1269 (2016).

87. Xu Y, Bailey UM, Schulz BL. Automated measurement of site-specific N-glycosylation occupancy with SWATH-MS. *Proteomics* 15(13), 2177–2186 (2015).

88. West CM, Malzl D, Hykollari A, Wilson IB. Glycomics, glycoproteomics and glycogenomics: an inter-taxa evolutionary perspective. *Mol. Cell. Proteomics* 100024 (2021).

89. Quaranta A, Spasova M, Passarini E *et al.* N-Glycosylation profiling of intact target proteins by high-resolution mass spectrometry (MS) and glycan analysis using ion mobility-MS/MS. *Analyst* 145(5), 1737–1748 (2020).

90. Peng W, Reyes CDG, Gautam S *et al.* MS-based glycomics and glycoproteomics methods enabling isomeric characterization. *Mass Spectrom. Rev.* 10982787 (2021). doi: 10.1002/mas.21713

91. Watanabe Y, Allen JD, Wrapp D, McLellan JS, Crispin M. Site-specific glycan analysis of the SARS-CoV-2 spike. *Science* 369(6501), 330–333 (2020).

- **Bottom-up LC–MS used in characterization of glycosylation on SARS-CoV-2 spike protein.**

92. Yang Y, Liu F, Franc V, Halim LA, Schellekens H, Heck AJ. Hybrid mass spectrometry approaches in glycoprotein analysis and their usage in scoring biosimilarity. *Nat. Commun.* 7(1), 1–10 (2016).

93. Rosati S, Rose RJ, Thompson NJ *et al.* Exploring an orbitrap analyzer for the characterization of intact antibodies by native mass spectrometry. *Angew. Chem. Int. Ed.* 51(52), 12992–12996 (2012).

94. Zidar M, Kuzman D, Ravnik M. Characterisation of protein aggregation with the Smoluchowski coagulation approach for use in biopharmaceuticals. *Soft matter* 14(29), 6001–6012 (2018).

95. Trabjerg E, Nazari ZE, Rand KD. Conformational analysis of complex protein states by hydrogen/deuterium exchange mass spectrometry (HDX-MS): challenges and emerging solutions. *TrAC, Trends Anal. Chem.* 106, 125–138 (2018).

96. Narang D, Lento C, Wilson DJ. HDX-MS: an analytical tool to capture protein motion in action. *Biomedicines* 8(7), 224 (2020).

97. Kaltashov IA, Bobst CE, Abzalimov RR. H/D exchange and mass spectrometry in the studies of protein conformation and dynamics: is there a need for a top-down approach? *Anal. Chem.* 81(19), 7892–7899 (2009).

98. Zehl M, Rand KD, Jensen ON, Jørgensen TJ. Electron transfer dissociation facilitates the measurement of deuterium incorporation into selectively labeled peptides with single residue resolution. *J. Am. Chem. Soc.* 130(51), 17453–17459 (2008).

99. Wang G, Kaltashov IA. Approach to characterization of the higher order structure of disulfide-containing proteins using hydrogen/deuterium exchange and top-down mass spectrometry. *Anal. Chem.* 86(15), 7293–7298 (2014).

100. Pan J, Zhang S, Parker CE, Borchers CH. Subzero temperature chromatography and top-down mass spectrometry for protein higher-order structure characterization: method validation and application to therapeutic antibodies. *J. Am. Chem. Soc.* 136(37), 13065–13071 (2014).

101. Pan J, Zhang S, Borchers CH. Comparative higher-order structure analysis of antibody biosimilars using combined bottom-up and top-down hydrogen-deuterium exchange mass spectrometry. *Biochim. Biophys. Acta Proteins. Proteom.* 1864(12), 1801–1808 (2016).

102. Prądzińska M, Behrendt I, Astorga-Wells J *et al.* Application of amide hydrogen/deuterium exchange mass spectrometry for epitope mapping in human cystatin C. *Amino Acids* 48(12), 2809–2820 (2016).

103. Opuni KF, Al-Majdoub M, Yefremova Y, El-Kased RF, Koy C, Glocker MO. Mass spectrometric epitope mapping. *Mass Spectrom. Rev.* 37(2), 229–241 (2018).

104. Comamala G, Wagner C, De La Torre PS *et al.* Hydrogen/deuterium exchange mass spectrometry with improved electrochemical reduction enables comprehensive epitope mapping of a therapeutic antibody to the cysteine-knot containing vascular endothelial growth factor. *Anal. Chim. Acta* 1115, 41–51 (2020).

105. Rob T, Wilson DJ. A versatile microfluidic chip for millisecond time-scale kinetic studies by electrospray mass spectrometry. *J. Am. Soc. Mass Spectrom.* 20(1), 124–130 (2009).

106. Rob T, Gill PK, Golemi-Kotra D, Wilson DJ. An electrospray ms-coupled microfluidic device for sub-second hydrogen/deuterium exchange pulse-labelling reveals allosteric effects in enzyme inhibition. *Lab Chip* 13(13), 2528–2532 (2013).

107. Deng B, Zhu S, Macklin AM *et al.* Suppressing allostery in epitope mapping experiments using millisecond hydrogen/deuterium exchange mass spectrometry. *MAbs* 9(8), 1327–1336 (2017).

108. Resetca D, Wilson DJ. Characterizing rapid, activity-linked conformational transitions in proteins via sub-second hydrogen deuterium exchange mass spectrometry. *FEBS J.* 280(22), 5616–5625 (2013).

109. Resetca D, Haftchenary S, Gunning PT, Wilson DJ. Changes in signal transducer and activator of transcription 3 (STAT3) dynamics induced by complexation with pharmacological inhibitors of Src homology 2 (SH2) domain dimerization. *J. Biol. Chem.* 289(47), 32538–32547 (2014).

110. Brown KA, Lento C, Rajendran S, Dowd J, Wilson DJ. Epitope mapping for a preclinical bevacizumab (Avastin) biosimilar on an extended construct of vascular endothelial growth factor a using millisecond hydrogen–deuterium exchange mass spectrometry. *Biochemistry* 59(30), 2776–2781 (2020).

•• **Time-resolved ESI hydrogen-deuterium exchange MS workflow was used to compare epitopes between commercial Avastin and its biosimilar.**

111. Upton R, Migas LG, Pacholarz KJ *et al.* Hybrid mass spectrometry methods reveal lot-to-lot differences and delineate the effects of glycosylation on the tertiary structure of Herceptin®. *Chem. Sci.* 10(9), 2811–2820 (2019).

• **Combination of hydrogen-deuterium exchange MS and IM–MS workflow was used to characterize protein structure and dynamics.**

112. Dixit SM, Polasky DA, Ruotolo BT. Collision induced unfolding of isolated proteins in the gas phase: past, present, and future. *Curr. Opin. Chem. Biol.* 42, 93–100 (2018).

113. Zheng X, Kurulugama RT, Laganowsky A, Russell DH. Collision-induced unfolding studies of proteins and protein complexes using drift tube ion mobility-mass spectrometer. *Anal. Chem.* 92(10), 7218–7225 (2020).

PTM site localization and isomer differentiation of phosphorylated peptides

Tunable electron activated dissociation (EAD) MS/MS using the SCIEX ZenoTOF 7600 system

Joanna Bons[1], Jason Cason[2], Birgit Schilling[1], Christie Hunter[3]
[1]Buck Institute, USA, [2]SCIEX, Canada, [3]SCIEX, USA

Protein phosphorylation is an important post-translational modification (PTM) as it is involved in a large variety of dynamic cellular processes. However, PTM site localization and quantification of phosphopeptides by collision induced dissociation (CID) MS/MS can be challenging, and phospho-peptides can exhibit a partial neutral loss of the phospho group (-98 Th). Phospho-isomer differentiation and subsequent precise PTM site localization can be achieved by measuring isomer-specific ions containing the actual modification (direct evidence), or by measuring differentiating fragment ions that do not contain the modification (indirect evidence). Depending on the peptide sequence, detecting near complete fragment ion series, and more particularly the challenging fragment ions that would define the peptide C- and N- termini, can be necessary for PTM-site localization, such as for pS-56 and pS-59 of the NDUFA10 subunit of mitochondrial Complex I[1] (Figure 1). The benefits of electron activated dissociation (EAD) versus CID were first explored for malonylation PTM.[2]

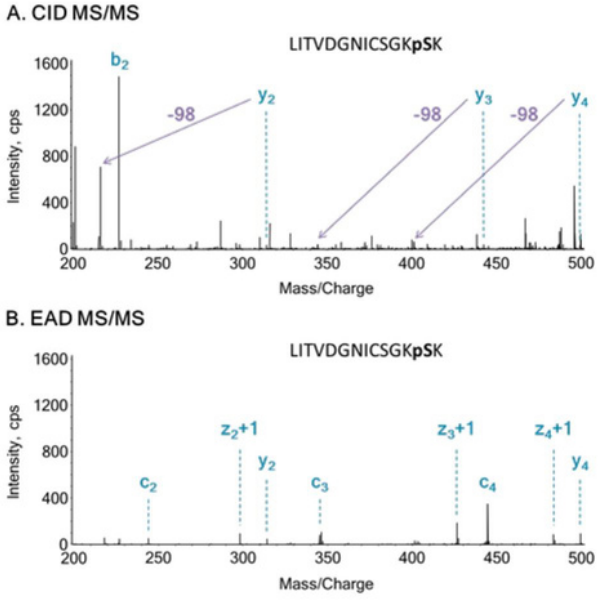


Figure 1. MS/MS spectra of LITVDGNICSGKpSK peptide analyzed with CID and EAD fragmentation. The phosphorylated peptide at m/z 505.58 (z=3) was analyzed in (A) CID mode and (B) EAD mode (kinetic energy, KE = 2). CID fragmentation resulted in low abundant and noisy PTM-specific differentiating ions (y and prominent y-98, no detected b ions). However, comprehensive EAD MS/MS generated distinct z and c fragment ions that provided evidence for definitive PTM site localization.

In this study, the use and benefits of EAD fragmentation for phosphopeptide analysis, site-localization and differentiation, and MS/MS-based phosphopeptide quantification were evaluated.

Key features of EAD for phosphoproteomics

- Efficient electron activated dissociation (EAD)[3] generates strong and distinct PTM site localization ions, enabling phospho-isomer differentiation
- Tunable kinetic energy (KE) for EAD MS/MS allows for selection of KE that provides the highest fragment ion abundance, while not inducing neutral loss from the phosphoryl group (-98 Th)
  - Generation of strong PTM-containing site localization ions, even small z+1 ions (z_2+1, z_3+1 and z_4+1) and high c ions (c_{10}, c_{11} and c_{12})[4]
  - The optimal KE values are different between different types of modifications, phosphorylation and malonylation[2]
- Using the Zeno trap gives up to ~10x increase in intensity for key site-localizing fragment ions (small z+1 ions and high c ions)

- Preliminary quantification using EAD high-resolution MRM (MRM^{HR})[5] indicates good linearity over concentrations interrogated
- Detailed characterization and MS/MS-based quantification of the modified peptides analyzed by EAD MS/MS using Skyline.

Methods

Sample preparation: Phosphorylated synthetic peptides were obtained from Princeton Biomolecules Corporation and diluted in simple matrix for analysis.

Chromatography: A NanoLC 425 system plumbed for microflow chromatography (5 µL/min) was used and operated in direct injection mode. The analytical column used was a 0.3 mm x 150 mm (2.6 µm particle size) Phenomenex Omega Polar column. Column temperature was controlled at 30°C. Short gradients of 8 minutes were used.

Mass spectrometry: All data was acquired using a SCIEX ZenoTOF 7600 system and an OptiFlow Turbo V ion source[6] equipped with the microflow probe and 25 µm electrodes. The system is equipped with the electron activated dissociation (EAD) cell which enabled hot electron capture dissociation (hot ECD) to be performed in a targeted manner on the phosphorylated peptides. MRM^{HR} data were collected using a TOF MS scan of 250 msec and MS/MS accumulation times of 150 msec both in CID and EAD fragmentation modes. Electron current was also ramped to optimize and a final value of 5000 nA was used throughout study. Kinetic energies were ramped and optimized per analyte.

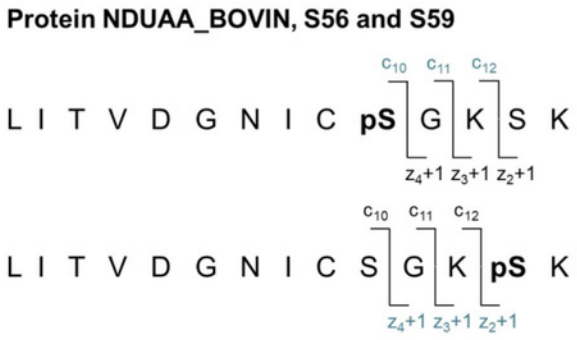


Figure 2. Sequence of the investigated phosphorylated isomeric peptides. Peptides at position 47-60 in bovine NDUFA10 subunit of Complex I (P34942) and carrying phospho-groups on serine S-56 (top) and S-59 (bottom) were investigated. PTM site-specific ions are highlighted in blue.

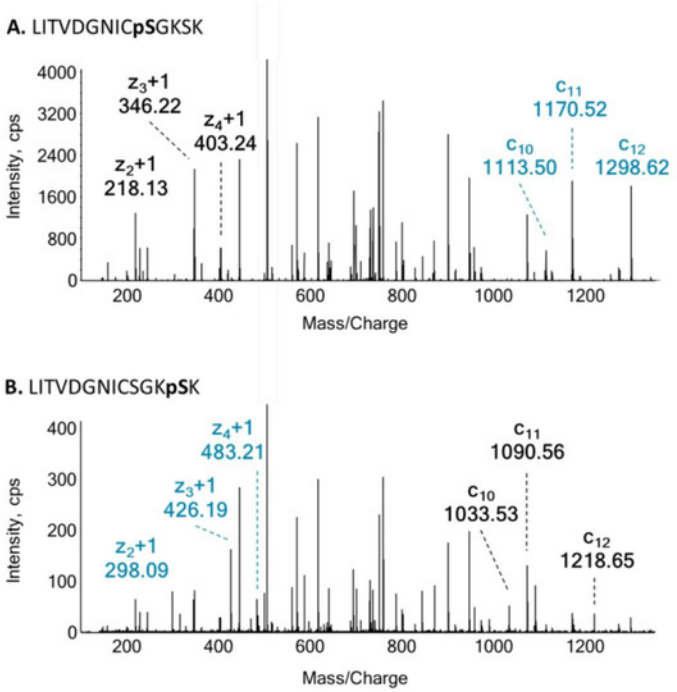


Figure 3. EAD fragmentation enables isomer differentiation. MS/MS spectra of (A) LITVDGNIC**pS**GKSK and (B) LITVDGNICSGK**pS**K at m/z 505.58 (z=3) analyzed in EAD mode (KE = 2). PTM-containing site-specific ions are indicated in blue, additional differentiating ions in black.

Data processing: MRM^{HR} data were processed using SCIEX OS software 2.1 using both Explorer and Analytics modules, and Skyline (version 3.6).[7]

EAD fragmentation enables direct PTM site localization

Two isomeric phosphorylated peptides from bovine mitochondrial NDUFA10 subunit of Complex I (P34942), LITVDGNIC**pS**GKSK and LITVDGNICSGK**pS**K, were synthetized with a phospho-serine at position S-56 and S-59 respectively (Fig. 2) (hereafter referred to as LITV-pS-56 and LITV-pS-59 respectively).
In order to effectively characterize PTM peptides, it is important to detect the 'intact' fragment ion(s) containing the modification to confidently determine its type and localization site. Here, the modification sites of the two isomers are two amino acids apart, close to the peptide c-terminus, respectively at positions pS-56 and pS-59 on the 14 aa-long peptides making these modifications difficult to characterize. Therefore, near complete coverage of the ion series in both directions is required to localize the PTM site.

However, in CID small y-ions and large b-ions are often challenging to detect, and in addition CID MS/MS induces some neutral loss of H_3PO_4 (-98 m/z) from the labile phosphorylation group (Figure 1 and 4B-D). Fortunately, MRM^{HR} analysis using EAD MS/MS enabled detection of PTM-containing, site-specific ions, such as c_{10} to c_{12} for LITV-pS-56 and z_2+1 to z_4+1 for LITV-pS-59, in blue (Figure 3) with very good intensity, providing direct evidence for the phosphorylation site on each peptide (Figure 4A-C). Additional differentiating, non-PTM containing ions were also observed, such as z_2+1 to z_4+1 for LITV-pS-56 and c_{10} to c_{12} for LITV-pS-59, in black (Figure 3). Altogether, these results allow confident discrimination of both isomers.

MRM^{HR} assays using EAD MS/MS uniquely provided quantification of phosphopeptides using PTM-site specific ions (Figure 4A and C) which have very strong signal. Whereas, when using CID MS/MS, the required differentiating ions are very low abundance (Figure 4B and D) which negatively impacts quantification sensitivity in addition to not providing definitive discrimination between the two peptide isomers.

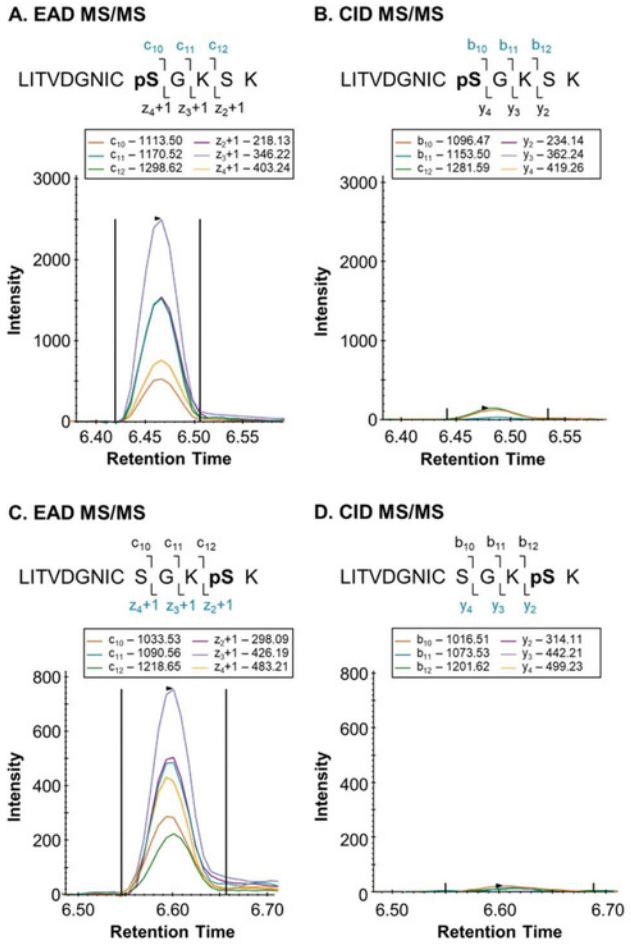


Figure 4. EAD fragmentation enables preserving the labile phospho-group. Differentiating ions of phosphorylated isomers (A, B) LITVDGNIC**pS**GKSK and (C, D) LITVDGNICSGK**pS**K analyzed in (A, C) EAD (KE = 2) and (B, D) CID modes were extracted in Skyline. In EAD mode, the labile phospho-group is preserved.

Targeted EAD MRM^{HR} for detailed characterization

MRM^{HR} is a MS/MS-based targeted acquisition strategy for accurate quantification that offers the possibility to process and refine data post-acquisition using dedicated tools as Skyline. First, near complete c- and z+1-ion series were extracted for the two isomeric peptides in Skyline as shown in Figure 5A and B. When extracting all possible fragment ions (PTM-site specific as well as ions that are in common between the two phospho-site isomers) two chromatographic peaks are detected that correspond to the two isomers that were obtained. However, when only PTM site specific fragment ions are extracted for the corresponding peptide isomers in each case, for pS-56 (Figure 5C) and for pS-59 in (Figure 5D), it is possible to unambiguously differentiate these isoforms with the pS-56 isomer eluting at 6.45 min and the pS-59 isomer eluting at 6.6 min.

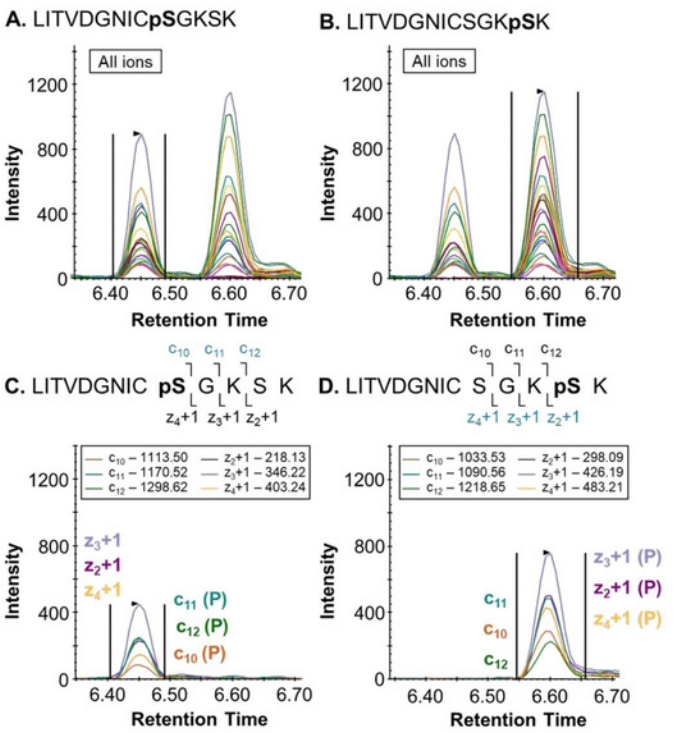


Figure 5. The extraction of site-specific fragment ions in Skyline from EAD data enables PTM localization sites. (A, B) Near complete c- and z+1-ion series were obtained with the EAD dissociation for the phosphorylated isomers (A) LITVDGNICpSGKSK and (B) LITVDGNICSGKpSK. Post-acquisition data refinement in Skyline enables differentiating the two isomeric peptides (C) LITVDGNICpSGKSK and (D) LITVDGNICSGKpSK using only discriminating ions.

Targeted EAD MRM^{HR} data processing in Skyline offers a detailed characterization of the modified peptides of interest. Currently, Skyline computes c- and z+1-ions that can be visualized and used for precise MS/MS quantification.

Tunable kinetic energy for EAD MS/MS preserves labile phospho-groups

The kinetic energy during EAD MS/MS can be tuned on the ZenoTOF 7600 system so that the fragmentation parameters can be customized to favor both the preservation of the labile phospho-group and to generate optimal sensitivity. In this experiment, kinetic energies (KE) were ramped from 0 to 11. For the two phosphopeptide isomers, the optimal KE value was 2, which generated high intensity differentiating and site-specific fragment ions that contained the intact PTM, while generating very limited background noise (Figure 6). Increased KE values, above 7, resulted in some neutral loss for the differentiating ions for LITV-pS-56. To note, the KE-dependent abundance patterns vary slightly for the illustrated, site specific ions between the isomeric peptides. The neutral loss of -98 Th is only observed on the c-ions when using the very high KE values. Thus, the tunable KE allows for the generation of methods that will both preserve the site-specific fragment ions (KE 2) and induce neutral loss (KE 11) for complete characterization of a modified peptide.

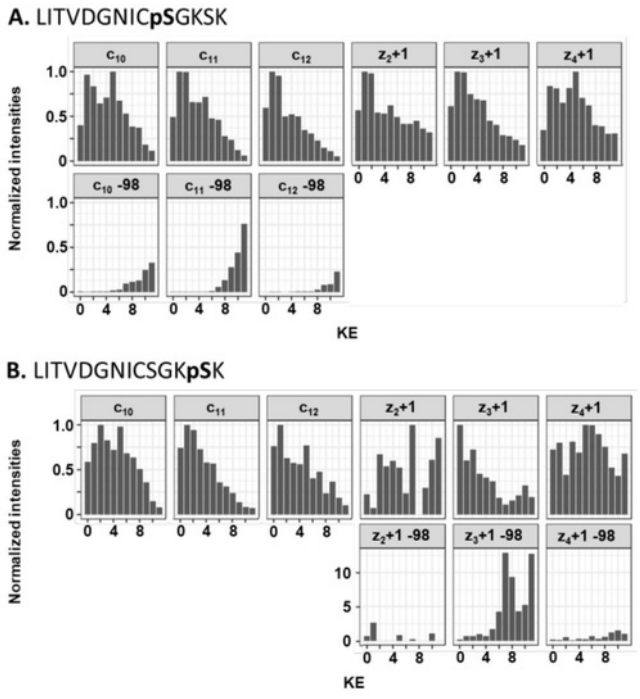


Figure 6. Kinetic energy ramping for EAD MS/MS. Phosphorylated isomeric peptides (A) LITVDGNIC**pS**GKSK and (B) LITVDGNICSGK**pS**K were analyzed in EAD mode with KE values ranging from 0 to 11. Chromatographic peaks were extracted for 12 fragment ions. For the intact ions (first row), peak area values were normalized to the highest area. For the neutral loss ions (second row), peak area values were normalized to their respective intact ion.

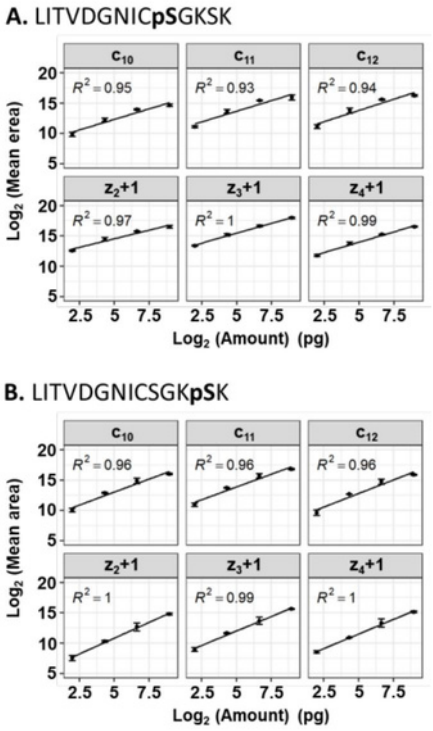


Figure 7. Linear response of fragment ions during EAD MRM^{HR}. Peptides (A) LITVDGNIC**pS**GKSK and (B) LITVDGNICSGK**pS**K were analyzed in EAD mode (KE = 2) in triplicate and at various loading amounts (4, 20, 100 and 500 pg). Six fragment ions are displayed. The R^2 coefficient for determination of the linear regression is displayed for each ion.

Preliminary linear response for quantification

To get an initial appreciation of the quantitative performances of MRM^{HR} assays using EAD MS/MS, 4-point concentration curves were designed (4, 20, 100 and 500 pg on-column), and each point was injected in triplicate (Figure 7). Six differentiating ions were investigated for each peptide, and each showed good linear response ($R^2 \geq 0.93$). Although the explored dynamic range is limited (2.1 orders of magnitude), this first assessment suggests utility of EAD MS/MS for quantification.

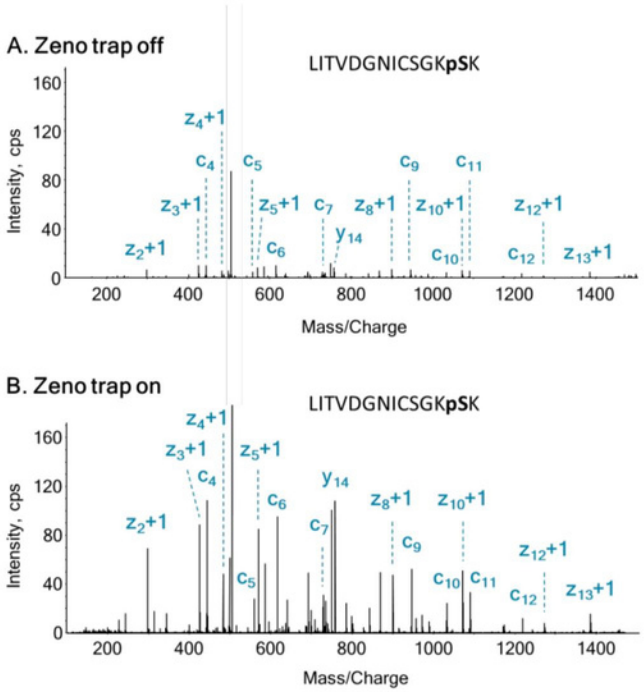


Figure 8. Increased sensitivity using the Zeno trap. MS/MS spectra of LITVDGNICSGK**pS**K peptide (m/z 505.58, z=3) analyzed in EAD mode (KE = 2) (A) without and (B) with the Zeno trap activated. Significant improvements in signal intensity and thus spectral quality was observed while using Zeno MS/MS in combination with EAD, enabling more confident PTM site localization.

Zeno trap improves sensitivity

Finally, to explore the impact of the Zeno trap on quality of EAD MS/MS spectra and sensitivity performances, EAD MS/MS analyses were performed with and without the Zeno trap activated. Analysis with Zeno trap on significantly increases sensitivity and thus generates higher intensity c and z+1 ions (Figure 8), strengthening the confidence for PTM site localization and isomer differentiation. Differentiating fragment ion peaks were extracted, and the ratios of the areas obtained with Zeno trap on and off were determined (Table 1). Sensitivity gains ranging between 7.4 and 14.9 were achieved for the various fragment ions. All the ions benefited from the activation of the Zeno trap, and more particularly the smaller ones, as the average gain was 12.7 for the three investigated small z+1 ions and 9.0 for the high c ions.

Conclusions

In this work, the performance of electron activated dissociation (EAD) on the SCIEX ZenoTOF 7600 system was investigated for the characterization and the quantification of phosphorylated isomeric peptides.

- Specifically tuning the kinetic energy (KE) for the phosphorylated peptide isomers is an added value for determination of PTM site localization, for differentiating isomers, and for improving quantification accuracy
- For the two phosphorylated isomers investigated, an optimal kinetic energy value of 2eV allowed preservation of the labile group and generated fragment ions with site localization evidence
- Activation of the Zeno trap provided large improvements in sensitivity, leading to highly confident PTM characterization
- Using MRM^{HR} and collecting full scan EAD MS/MS fragmentation offers the possibility to refine the extracted chromatographic peaks post-acquisition, using dedicated processing tools such as Skyline
- Preliminary quantitative assessment of EAD MRM^{HR} workflows shows promising performances for the robust and accurate quantification of labile PTMs

Table 1. Gain of MS/MS sensitivity using the Zeno trap. LITVDGNIC**pS**GKSK and LITVDGNICSGK**pS**K peptides were analyzed in EAD mode (KE = 2) with and without the Zeno trap activated, loading various amounts (4 and 20 pg on column). Chromatographic peak areas were extracted and sensitivity changes between Zeno trap on and Zeno trap off were determined.

Ions	LITVDGNIC**pS**GKSK			LITVDGNICSGK**pS**K		
	m/z	Zeno trap on/off 4 pg	Zeno trap on/off 20 pg	m/z	Zeno trap on/off 4 pg	Zeno trap on/off 20 pg
z_2+1	218.13	*14.5*	*12.7*	*298.09*	*10.4*	*12.1*
z_3+1	346.22	*14.9*	*10.8*	*426.19*	*12.7*	*9.2*
z_4+1	403.24	*13.6*	*11.8*	*483.21*	*19.9*	*10.1*
c_{10}	*1,113.50*	*7.4*	*10.6*	*1,033.53*	*8.8*	*9.4*
c_{11}	*1,170.52*	*9.2*	*10.1*	*1,090.56*	*9.9*	*8.5*
c_{12}	*1,298.62*	*8.4*	*9.6*	*1,218.65*	*6.8*	*8.8*

References

1. Schilling B, Aggeler R et al. (2005) Mass spectrometric identification of a novel phosphorylation site in subunit NDUFA10 of bovine mitochondrial complex I. *FEBS Lett*, 579(11):2485-90.
2. Tunable electron assisted dissociation (EAD) MS/MS to preserve particularly labile post-translational modifications. SCIEX technical note RUO-MKT-02-13006-A.
3. Qualitative flexibility combined with quantitative power - Using the ZenoTOF 7600 system, powered by SCIEX OS software. SCIEX technical note RUO-MKT-02-13053-A.
4. Peptide fragmentation using electron activated dissociation (EAD). SCIEX community post RUO-MKT-18-13296-A.
5. Schilling B, Hunter CL et al. (2015) Multiplexed, scheduled, high-resolution parallel reaction monitoring on a full scan QqTOF instrument with integrated data-dependent and targeted mass spectrometric workflows. *Anal Chem*, 87(20): 10222-9.
6. Single source solution for low flow chromatography – OptiFlow® Turbo V source – always working in the sweet spot of sensitivity and robustness. SCIEX technical note RUO-MKT-02-9701-A.
7. MacLean B, Tomazela DM et al. (2010) Skyline: an open source document editor for creating and analyzing targeted proteomics experiments. *Bioinformatics*, 26(7):966-8.

Headquarters
500 Old Connecticut Path | Framingham, MA 01701 USA
Phone 508-383-7700
sciex.com

International Sales
For our office locations please call the division headquarters or refer to our website at
sciex.com/offices

60 seconds with Katherine Tran: the importance of post-translational modification studies in the drug discovery process

Katherine Tran is the Senior Manager for Global Strategic Marketing in Life Science Research at SCIEX (Ontario, Canada), a Danaher company. Katherine has recently joined the SCIEX team with a strong desire to further with the company's mission of delivering solutions to advance human wellness. Her main area of interest is within proteomics using mass spectrometry from which she has accumulated a wealth of knowledge and experience in this space over the past 10 years. Katherine has a degree in Biochemistry from the University of Waterloo (Ontario, Canada) and worked in Prof Dr Praveen Nekkar's lab at the School of Pharmacy investigating novel drug candidates for Alzheimer's Disease using NMR and LC–MS.

How important are post-translational modification (PTM) studies in the drug discovery process?

Post-translational modifications (PTMs) are a major playing factor in the drug discovery process. PTMs play an essential role in various cellular processes by chemically modifying proteins, which ultimately effects their molecular function, half-life and localization within cells and tissues. Since PTMs influence almost all aspects of normal cell biology and pathogenesis, it is crucial to have the ability to identify, characterize and quantify PTMs in the drug discovery process. For example, glycosylation can have wide-ranging effects on the potency and immunogenicity of antibodies, influencing their function and binding ability. Further, biopharmaceuticals deliver their therapeutic effects through one or more PTM(s). Therefore, understanding PTMs to generate a product PTM profile is a crucial step in the drug discovery/development process.

What are the typical challenges to overcome with PTMs in drug discovery?

One of the key challenges in drug discovery is determining how to mitigate changes of PTM activity that occur over time. To do so, we need to use mass spectrometry (MS) to identify, characterize and quantify the PTMs patterns. However, PTM profiling is a challenging task because most PTMs are low in abundance and some PTMs are labile using traditional MS techniques. In addition, unexpected PTMs may arise from sample handling and purification steps. PTMs can also occur at multiple sites on a peptide/protein and may generate very complicated MS data sets that are difficult to localize and quantify. Furthermore, certain PTMs will reduce the ionization and detection efficiency in MS. Overall, characterizing PTM patterns is a complex and time-consuming part of the biopharmaceutical process.

60 seconds with Katherine Tran: the importance of post-translational modification studies in the drug discovery process

What are the advantages of LC-HRMS for these applications?

Today's high resolution accurate mass spectrometry (LC-HRMS) is central to drug discovery, development and quality control. LC-HRMS offers higher sensitivity, higher throughput and a larger dynamic range for more accurate, reproducible protein analyses.

For PTM profiling in particular, challenges faced with traditional MS techniques can now be overcome using new technology offered in LC-HRMS systems. For example, the SCIEX ZenoTOF 7600 mass spectrometer comprises of an EAD cell to offer the flexibility of multiple fragmentation options to preserve labile PTMs, such as glycosylation and phosphorylation, and differentiate isomeric PTMs, such as aspartic acid and isoaspartic acid. Unlike other electron-based fragmentation techniques, this can be achieved in conjunction with fast scan speeds and in a reproducible, consistent manner. In addition to PTM analysis, the EAD cell offers biopharmaceutical characterization by producing differential c and z fragment ions of large multiply-charged biomolecules and isobaric moieties. When combined with the Zeno trap, the ZenoTOF 7600 can drive down the lower limits of quantification when the trap is enabled in MRMHR mode for both small molecules and peptides.

Tunable electron activated dissociation (EAD) MS/MS to preserve particularly labile post-translational modifications

Site-localization of malonylated peptides using the SCIEX ZenoTOF 7600 system

Joanna Bons[1], Jason Cason[2], Birgit Schilling[1], Christie Hunter[3]
[1]Buck Institute, USA, [2]SCIEX, Canada, [3]SCIEX, USA

Post-translational modifications (PTMs) are important players in a diverse group of functions that include protein conformation and signaling. Lysine acylation, such as malonylation, is one such PTM, and is regulated in part by lysine deacylases, which are members of the sirtuin (SIRT) protein family. In a previous study investigating SIRT5-regulated lysine malonylome, it was shown that 183 malonyllysine sites (from 120 proteins) out of the 1,137 identified sites (from 430 proteins) were significantly increased in *Sirt5*$^{-/-}$ KO versus wild-type mice.[1] Specifically, it revealed that malonylation regulated GADPH activity. Malonylated peptides are however traditionally difficult to characterize using mass spectrometry and CID because the modification is extremely labile.

In this work, that involves using EAD technology with tunable kinetic energy, the effects of kinetic energy ramping on the preservation of highly labile PTMs (malonylation, for example) was studied, focusing on one previously identified malonyllysine site from GADPH (K-192). Two orthogonal fragmentation modes were compared (EAD vs. CID), to investigate the utility of each for PTM site localization. In addition, samples were measured using MRM^{HR} mode to investigate the use of EAD for quantitative PTM characterization of labile modifications.[2] MS/MS data were acquired with the Zeno trap activated, which provides significant sensitivity increases and enhances the quality of EAD MS/MS spectra.

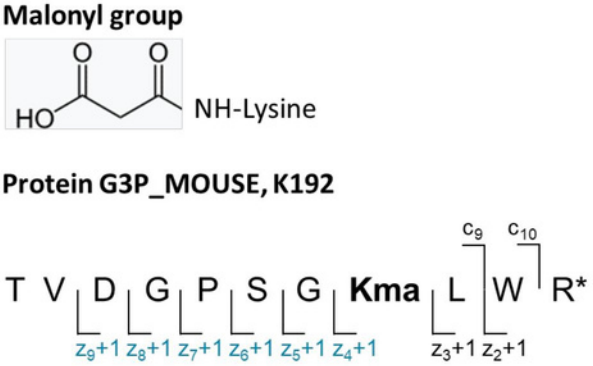


Figure 1. Sequence of the investigated malonylated peptide. Peptide at position 185-195 in mouse glyceraldehyde-3-phosphate dehydrogenase (P16858) and carrying a malonyl group on lysine **K-192** was investigated. PTM site-specific ions are highlighted in blue.

Key features of SCIEX ZenoTOF 7600 system for PTM characterization

- ZenoTOF 7600 system enables acquisition of high-resolution MS and MS/MS spectra at high acquisition rates (up to 130Hz)
- Multiple fragmentation capabilities for flexibility: collision induced dissociation (CID) and electron activated dissociation (EAD)[3]
- Use of the Zeno trap for 5-10x increase in signal intensity on MS/MS fragment ions[3]
- Prevention of neutral loss from labile PTMs (no CO_2-loss from malonyl modifications) using EAD MS/MS
- Tunable kinetic energy for EAD MS/MS using the EAD cell can be optimized and tailored for specific PTMs
- Efficient EAD product ion fragmentation generated strong ions for PTM site localization
- Preliminary assessment of EAD fragment ion quantification shows quantitative performance using high-resolution MRM (MRM^{HR}) workflows

Methods

Sample preparation: Malonylated synthetic peptides were obtained from Thermo Fisher Scientific and diluted in simple matrix for analysis.

Chromatography: A NanoLC 425 system plumbed for microflow chromatography (5 µL/min) was used and operated in direct inject mode. The analytical column used was a 0.3 mm x 150 mm (2.6 µm particle size) Phenomenex Omega Polar column. Column temperature was controlled at 30°C. Short gradients of 8 minutes were used.

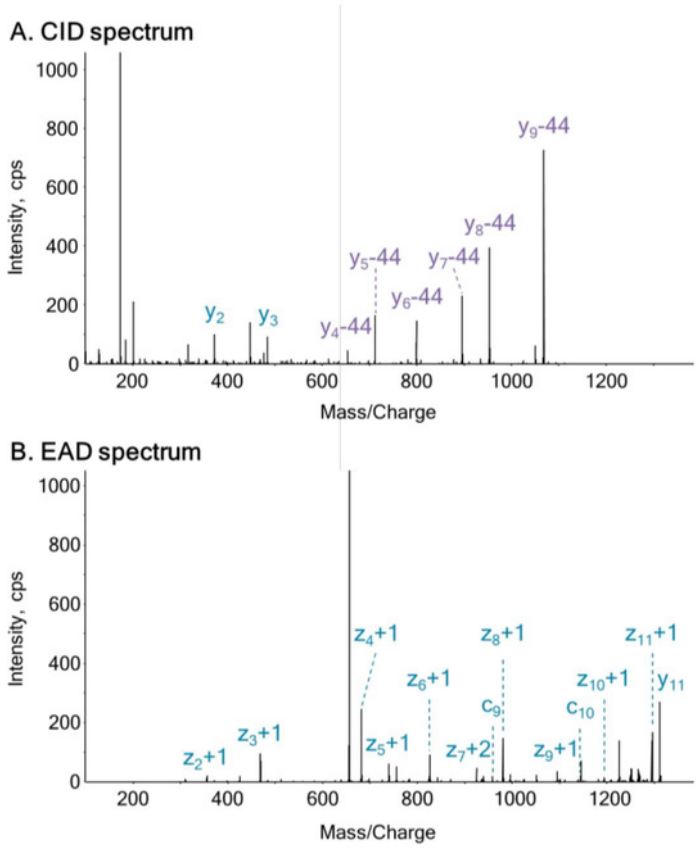
Mass spectrometry: All data were acquired using a SCIEX ZenoTOF 7600 system and an OptiFlow Turbo V ion source[4] equipped with the micro probe and 25 µm electrodes. The system is equipped with the electron activated dissociation (EAD) cell which enabled electron-based fragmentation to be performed in a targeted manner on the malonyl peptides. MRM^{HR} Acquisition data were collected using a TOF MS scan of 250 msec and MS/MS accumulation times of 150 msec both in CID and EAD mode. Kinetic energies were ramped and optimized per analyte. Electron current was also ramped to optimize and a final value of 5000 nA was used throughout study.

Data processing: The MRM^{HR} data was processed using SCIEX OS software 2.1 using both Explorer and Analytics modules.

EAD preserves labile PTMs preventing neutral losses

Malonylated peptide TVDGPSG**Kma**LWR from mouse glyceraldehyde-3-phosphate dehydrogenase (P16858) was synthetized with a malonylated lysine at position K-192 (Figure 1). MRM^{HR} analysis using CID fragmentation mode caused significant neutral loss of CO_2 (-44 m/z) from the labile malonyl group for the y-ion series (Figure 2A). However, EAD fragmentation generated intact z+1-ion and c-ion series, that were easily detectable in the MS/MS spectra (Figure 2B).[5] Specifically, fragment ions were observed, ranging from z_4+1 to z_{11}+1, or also c_9 and c_{10} that contained the labile PTM, that did not undergo a significant neutral loss, enabling confident PTM site localization.

Using the Peptide fragment pane in Bio Tool Kit, the fragment ion coverage is easily visualized between the two dissociation techniques (Figure 2C).



C. Fragment ion coverage for EAD spectrum

Symbol	Res. Mass	# (N)	b	c	y	z	z + 1	z + 2	# (C)
T	101.04768	1	102.05495	119.08150	*1311.65671*	1294.63016	1295.63799	1296.64581	11
V	99.06841	2	201.12337	218.14992	1210.60903	1193.58248	1194.59031	1195.59813	10
D	115.02694	3	316.15031	333.17686	1111.54062	1094.51407	1095.52189	1096.52972	9
G	57.02146	4	373.17178	390.19832	996.51367	979.48713	980.49495	981.50278	8
P	97.05276	5	470.22454	487.25109	*939.49221*	922.46566	923.47349	924.48131	7
S	87.03203	6	557.25657	574.28312	842.43945	825.41290	826.42072	827.42855	6
G	57.02146	7	614.27803	631.30458	755.40742	738.38087	739.38869	740.39652	5
K[_Mal]	214.09536	8	828.37339	*845.39994*	698.38595	681.35941	682.36723	683.37506	4
L	113.08406	9	941.45745	958.48400	484.29060	467.26405	468.27187	469.27970	3
W	186.07931	10	1127.53677	1144.56331	371.20653	354.17998	355.18781	356.19563	2
R[+10]	166.10938	11	1293.64615		185.12722	168.10067	169.10850	170.11632	1

Figure 2. MS/MS spectra of TVDGPSGKmaLWR peptide analyzed in CID and EAD modes. The malonylated peptide (m/z 656.3320) was analyzed in (A) CID mode and (B) EAD mode (KE = 5 eV). CID fragmentation resulted in neutral loss of -CO_2 (-44), and no "intact" PTM-specific differentiating ions were detectable. Comprehensive EAD fragmentation generated high-intensity fragment ions that provided evidence for straightforward PTM site localization. (C) Near complete sequence characterization was achieved with the EAD dissociation.

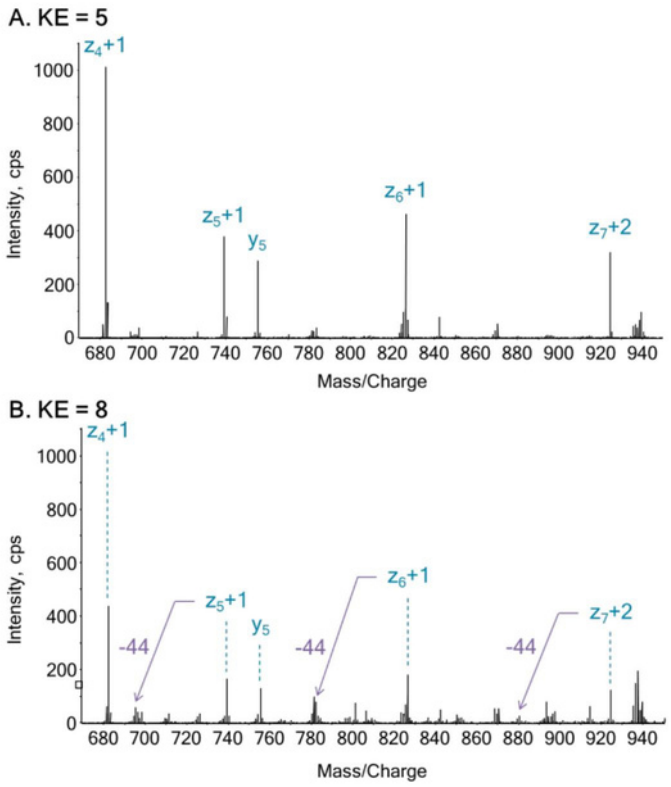


Figure 3. Impact of the kinetic energy (KE) value in EAD on fragmentation pattern. MS/MS spectra of malonylated peptide TVDGPSG**Kma**LWR (m/z 656.3320) analyzed in EAD mode with a kinetic energy value of (A) 5 eV and (B) 8 eV.

Kinetic energy ramping for EAD MS/MS

EAD fragmentation was performed across a range of kinetic energies, taking advantage of the tunable nature of EAD on the ZenoTOF 7600 system. This allowed determination of the optimal fragmentation parameters to preserve labile modifications on PTM modified peptides, while achieving best sensitivity. Kinetic energies were ramped from 0 eV to 11 eV. In this study, it was observed that a KE value of 5 eV generated high intensity, intact PTM site-specific fragment ions with very little background noise. When increasing KE to 8 eV or higher, MS/MS spectra became more complex, and neutral losses from fragment ions containing the PTM modification emerged (Figure 3).

Interestingly, different dissociation patterns were observed for fragment ions across the different kinetic energies during EAD MS/MS fragmentation (Figure 4). The peak area for most fragment ions containing the modified residue and the labile malonyl modification was highest at a KE value of 5 eV. With higher KEs some neutral PTM losses were observed, but a KE value of 5 eV maintained the integrity of the post-translational modification and maximized peak area intensity for site specific fragment ions.

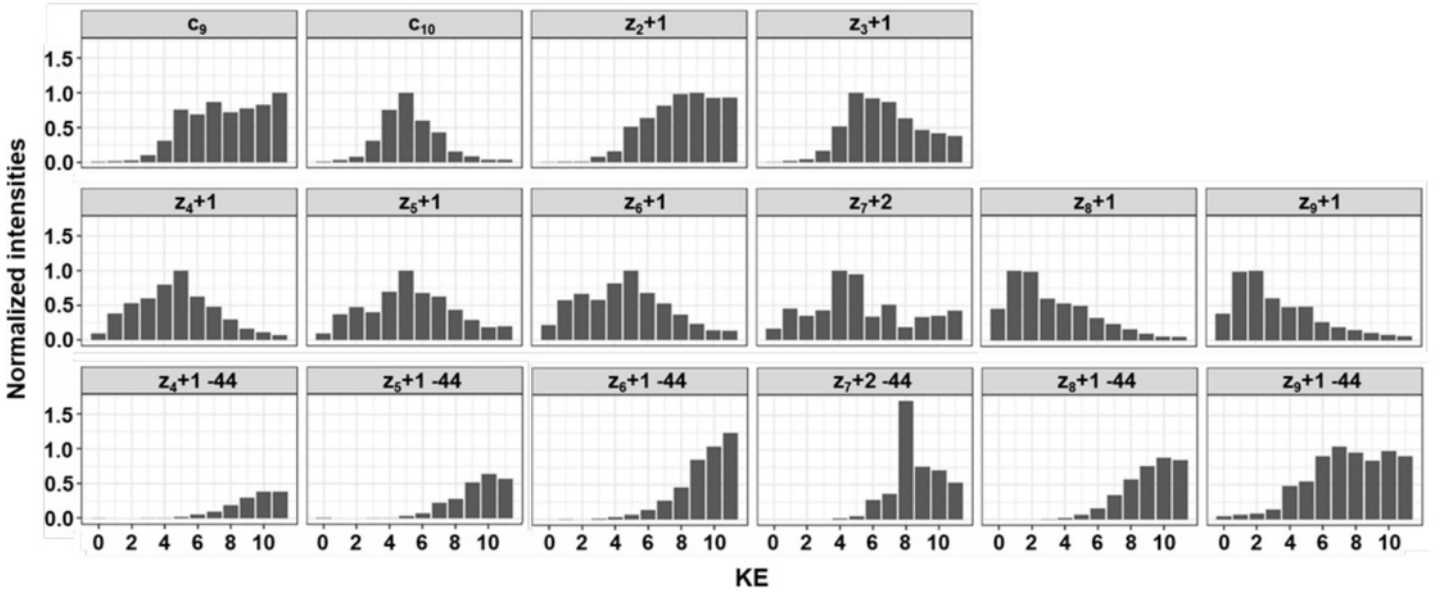


Figure 4. Kinetic energy ramping for EAD MS/MS. Malonylated peptide TVDGPSG**Kma**LWR was analyzed in EAD mode with kinetic energy (KE) values varying from 0 eV to 11 eV. Chromatographic peaks were extracted for 16 ions. For the intact ions (two first lines), peak area values were normalized on the highest area. For the ions with a neutral loss (third line), area values were normalized to their respective intact ion.

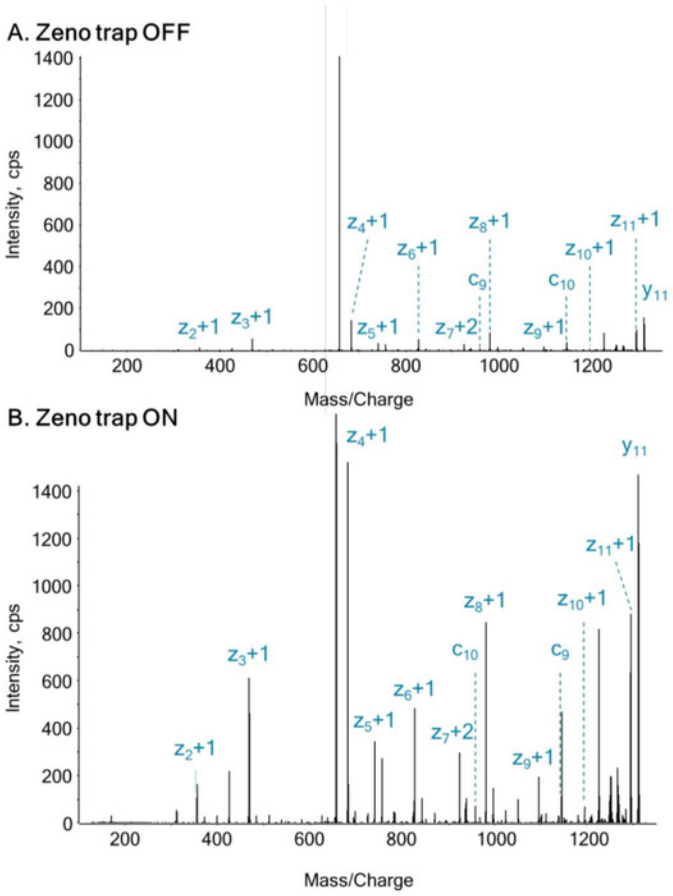


Figure 5. Increased sensitivity when the Zeno trap is activated. MS/MS spectra of TVDGPSG**Kma**LWR peptide (m/z 656.3320) analyzed in EAD mode (KE = 5 eV) (A) without and (B) with Zeno trap activated. Significant improvements in signal intensity and thus spectral quality was observed while using the Zeno trap in combination with EAD, enabling more confident PTM localization.

Zeno trap boosts MS/MS sensitivity for fragment ions

The impact on MS/MS spectral quality and sensitivity when using the Zeno trap was also investigated. The Zeno trap is located in the back half of the collision cell and is used to reduce ion losses between the collision cell and the accelerator. Ions are captured in the Zeno trap and then ejected in order of high *m/z* to low *m/z,* such that each ion reaches the center of the TOF accelerator simultaneously. This increases the duty cycle to greater than 90% through this region and greatly increases the sensitivity for MS/MS acquisition.[3]

EAD MS/MS spectra, collected with and without the Zeno trap activated, illustrate the clear gain in sensitivity provided by this feature (Figure 5). The ratios of extracted fragment ion peak areas, obtained with and without the Zeno trap activated, were determined for PTM site-specific ions at two different amounts loaded (Table 1). The sensitivity gain ranged between 3.5 and 9.6-fold for the various fragment ions, with an average value of 6.4. This highlights the added value of the Zeno trap for investigating very low-abundance ions.

Table 1. Gain of MS/MS sensitivity using the Zeno trap. TVDGPSG**Kma**LWR peptide was analyzed in EAD mode (kinetic energy = 5 eV) with and without using the Zeno trap at various amounts (16 and 80 fmol) on column. Chromatographic peak areas were extracted and sensitivity changes between having the Zeno trap on and off were determined.

Ions	m/z	Zeno trap on/off 16 fmol	Zeno trap on/off 80 fmol
z_4+1	682.3672	7.5	7.8
z_5+1	739.3887	9.6*	5.6
z_6+1	826.4207	8.0	8.0
z_7+2	924.4813	5.2	4.0
z_8+1	980.4950	7.0	6.8
z_9+1	1095.5219	4.0	6.2
c_9	958.4840	9.2*	4.1
c_{10}	1144.5633	3.5	6.8

** Weak signal without using the Zeno trap*

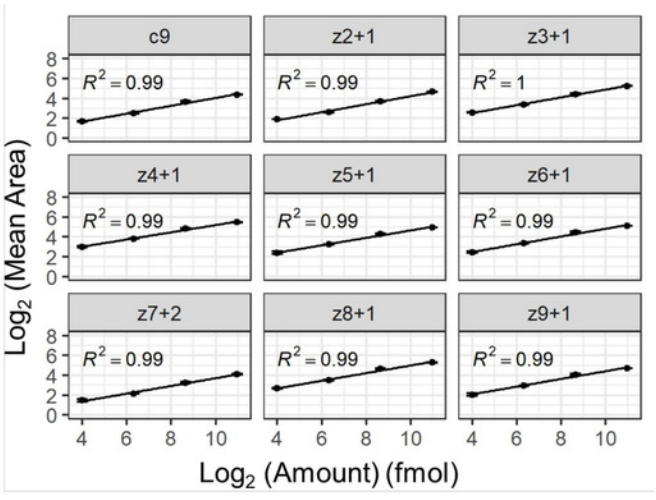


Figure 6. Linear response of fragment ions during EAD MRM-HR. Peptide TVDGPSG**Kma**LWR was analyzed in EAD mode (KE = 5 eV), in triplicate, loading various amounts (16, 80, 400 and 2000 fmol). 9 fragment ions are displayed. The R^2 coefficient for determination of the linear regression is displayed for each ion.

Preliminary assessment of quantitative response using targeted MRM^{HR}

To gain first insights into the quantitative performances of EAD MS/MS, initial dilution curves were generated by performing triplicate injections of 4 different peptide concentrations (16, 80, 400 and 2000 fmol on column) (Figure 6).

Good linearity performances were achieved for 9 investigated fragment ions ($R^2 \geq 0.99$) in these initial assessments.

Conclusions

In this work, the utility of electron activated dissociation on the SCIEX ZenoTOF 7600 system was investigated for the characterization and quantification of labile post translational protein modifications.

- Kinetic energy for EAD can be adjusted and exactly tailored to the different analyte necessities
- For this extremely labile PTM, malonyl, it appeared that a KE value of 5 eV was a good kinetic energy impact to preserve the PTM and to achieve optimal sensitivity
- The ability to optimize the kinetic energy for a specific PTM should enable accurate site-localization of labile PTM-containing peptides and maximize/improve quantification accuracy
- In addition, use of the Zeno trap provides significant gains in sensitivity, further improving the utility of this approach for characterizing PTMs
- Preliminary results using MRM^{HR} with EAD fragmentation suggests this approach should provide robust quantification of labile PTMs, but work is planned to explore this further.

References

1. Nishida Y, Rardin MJ et al. (2015) SIRT5 regulates both cytosolic and mitochondrial protein malonylation with glycolysis as a major target. *Mol Cell*, **59**:321-32.
2. Schilling B, Hunter CL et al. (2015) Multiplexed, scheduled, high-resolution parallel reaction monitoring on a full scan QqTOF instrument with integrated data-dependent and targeted mass spectrometric workflows. *Anal Chem*, **87**: 10222-9.
3. Qualitative flexibility combined with quantitative power - Using the SCIEX ZenoTOF 7600 LC-MS/MS system, powered by SCIEX OS software. SCIEX technical note RUO-MKT-02-13053-A.
4. Single source solution for low flow chromatography – OptiFlow Turbo V Source – always working in the sweet spot of sensitivity and robustness. SCIEX technical note. RUO-MKT-02-9701-A.
5. PTM site localization and isomer differentiation of phosphorylated peptides - Tunable electron activated dissociation (EAD) MS/MS using the ZenoTOF 7600 system. SCIEX technical note RUO-MKT-02-13174-A.

Headquarters
500 Old Connecticut Path | Framingham, MA 01701 USA
Phone 508-383-7700
sciex.com

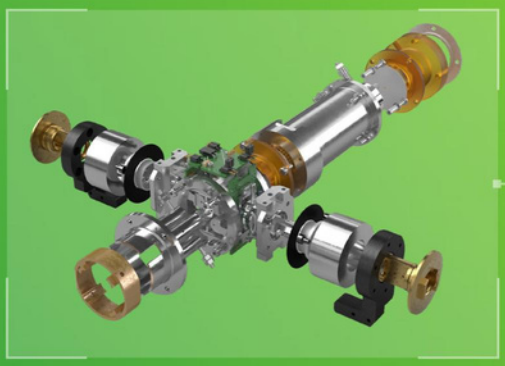
International Sales
For our office locations please call the division headquarters or refer to our website at
sciex.com/offices

BE **EXTRAORDINARY**

The Zeno revolution is now...

Introducing the SCIEX ZenoTOF 7600 system

Driven by the power of the Zeno trap coupled with EAD fragmentation technology.



This high-resolution accurate mass system encompasses the power of the Zeno trap with electron activated dissociation (EAD) or Collision Induced Dissociation (CID) technologies, allowing you to uncover structural information that was previously inaccessible.

- Reach MS/MS scan rates of up to 133Hz without sacrificing resolution
- Achieve sensitivity gains of up to 5-20x with Zeno trap pulsing
- Overcome duty cycle deficiencies injected to TOF
- Characterize large molecules including post-translation modification (PTMs)
- Understand molecular structures of entire compound classes

Now, you can be extraordinary.

Learn more at sciex.com/zenorevolution

SCIEX
The Power of Precision

Review

For reprint orders, please contact reprints@future-science.com

Bioanalysis

Quantitation of protein post-translational modifications using isobaric tandem mass tags

Post-translational modifications (PTMs) of proteins are known to modulate many cellular processes and their qualitative and quantitative evaluation is fundamental for understanding the mechanisms of biological events. Over the past decade, improvements in sample preparation techniques and enrichment strategies, the development of quantitative labeling strategies, the launch of a new generation of mass spectrometers and the creation of bioinformatics tools for the interrogation of ever larger datasets has established MS-based quantitative proteomics as a powerful workflow for global proteomics, PTM analysis and the elucidation of key biological mechanisms. With the advantage of their multiplexing capacity and the flexibility of an ever-growing family of different peptide-reactive groups, isobaric tandem mass tags facilitate quantitative proteomics and PTM experiments and enable higher sample throughput. In this review, we focus on the technical concept and utility of the isobaric tandem mass tag labeling approach to PTM analysis, including phosphorylation, glycosylation and *S*-nitrosylation.

Hui-Chung Liang[1], Emma Lahert[1], Ian Pike[1] & Malcolm Ward*,[1]

[1]Proteome Sciences plc, Coveham House, Downside Bridge Road, Cobham, KT11 3EP, UK

*Author for correspondence:
Tel.: +44 207 848 5112
Fax: +44 207 848 5114
malcolm.ward@proteomics.com

Quantitative MS-based analysis of protein post-translational modifications

In the postgenomic era, the study of proteins and respective post-translational modifications (PTM) on a global, proteome-wide scale has become extremely important owing to their key role in the regulation of cell signaling pathways. There are more than 200 types of PTMs among eukaryotes and prokaryotes, including phosphorylation, glycosylation, uniquitinylation, SUMOylation and *S*-nitrosylation (SNO) [1]. PTMs are capable of regulating specific biological function, subcellular location or activity of a protein, ordered assembly/disassembly of protein interactions and cell signaling [1,2]. For example, ubiquitinylation and phosphorylation or SUMOylation interact frequently to coordinate the regulation of cellular signaling process [1]. Additionally, abnormal changes in the PTM status of particular proteins have been shown to be a key driver in disease mechanisms [2]. The diverse nature of PTMs, and their occurrence in various forms at either a single site or multiple sites, complicates PTM analysis.

MS is an extremely powerful tool for the detection, quantification and characterization of protein PTMs. Improvements in the sensitivity and resolution of mass spectrometers along with developments in sample preparation protocols (e.g., chromatographic fractionation and antibody-based affinity enrichment) have enabled scientists to dig ever deeper into the proteome, which has enabled the identification and quantification of large numbers of protein PTMs on a global, proteome-wide scale, with a high degree of sensitivity and resolution provided by MS [3–5]. Qualitative and quantitative PTM analysis can now be achieved through the technological advancements in MS design, and via superior combination of ESI or MALDI ion sources with ion trap, quadrupole (Q) or TOF mass analyzers [5–7]. In particular, the introduction of high-resolution, high mass accuracy mass spectrometers including the

FUTURE SCIENCE part of fsg

Key terms

Post-translational modification: Step in protein biosynthesis. Proteins are created by ribosomes translating mRNA into polypeptide chains. These polypeptide chains undergo post-translational modification (e.g., folding, cutting and other processes) before becoming the mature protein product.

Multiplexing: Ability to simultaneously identify and quantify signals from several samples in a single experiment.

Quantitative proteomics: Powerful approach used for both discovery and targeted proteomic analyses to understand global proteomic dynamics in a cell, tissue or organism. Rather than simply a qualitative assessment of the content of a sample, these quantitative experiments provide information pertaining to the amount of protein present and this level can be measured relative to another sample or calibrated using an absolute amount of an internal standard.

Isobaric interference: Occurs when a molecule is coselected and cofragmented with the analyte of interest. The interfering molecule has a precursor *m/z* within the selection window tolerance of the analyte of interest. . With each level of selection that is undertaken the level of inference is reduced, therefore MS/MS/MS has less chance of interference than MS/MS.

Orbitrap™ have shown outstanding performance for the analysis of PTMs [5]. This new generation of mass spectrometers has enabled different fragmentation approaches including collision-induced dissociation (CID), higher-energy C-trap dissociation (HCD), electron transfer dissociation (ETD) and electron capture dissociation (ECD) to be utilized to facilitate PTM analysis and to provide detailed structural characterization of complex PTMs including glycosylation [5]. The recent launch of Orbitrap Fusion™ by Thermo Fisher provides a promising avenue for global PTM discovery. Its exceptional high resolution (up to 450,000) and state-of-the-art combinational analyzers – quadrupole, Orbitrap and ion trap – leads to faster, more comprehensive and more precise analysis of low-abundance, high-complexity and large-scale samples [8]. The incorporation of quantitative techniques (label free, metabolic labeling and chemical tagging) into MS-based proteomics workflows [9,10] and their application to a wide range of samples including tissues, microorganisms and cancer cell lines has enabled the study of significant roles of PTMs in disease by comparing, for example, the proteome of disease versus control samples or the signal transduction pathways that are activated or deactivated when particular cancer therapies are applied to cancer-derived cell lines [11–13].

Chemical isobaric tags, such as isotope-coded affinity tag (ICAT), cleavable ICAT (cICAT), isobaric tags for relative and absolute quantitation (iTRAQ) and tandem mass tags (TMTs), can all be used for protein and peptide labeling for quantitative purposes. Owing to their **multiplexing** ability (up to ten samples in a single experiment), TMT (Thermo Fisher, Waltham, MA, USA) [14] and iTRAQ (Applied Biosystems) [15] isobaric tags have gained popularity and have proven to be essential tools for **quantitative proteomics** [16–24], as it reduces overall analytical run time and technical variance between samples. Both TMT and iTRAQ are applicable to a wide range of MS platforms, for example QTOF, ion trap and Fourier transform (Orbitrap). The application of isobaric tag labeling at both the protein and peptide level has been applied to various samples and this has been extensively reviewed elsewhere [25–27]. Recently, Dephoure and Gygi developed a hyperplexing technique to increase the multiplexing capacity of isobaric tagging. They performed a 3 × 6 experiment using three MS1-separable SILAC (stable isotope labeling by/with amino acids in cell culture) metabolic labels and six MS/MS-distinguishable TMT isobaric labels, which allows the simultaneous quantification of 18 samples in a single run and provides a map of dynamic response to rapamycin in yeast [28].

Not only do TMT and iTRAQ serve as isobaric TMTs, but N,*N*-dimethyl leucine (diLeu) reagent is also synthesized for the use of quantitative proteomics [29]. DiLeu reagents, such as TMT and iTRAQ, are applicable across various MS platforms, for example ESI-Q-TOF, MALDI-TOFTOF and MALDI-Fourier transform ion cyclotron resonance [29]. Most recently, diLeu-labeled proteins have been analyzed by ion mobility (IM) QTOF-MS (Waters, Milford, MA, USA) to increase quantitation accuracy [30].

TMT and iTRAQ are also incorporated in SRM, also known as multiple reaction monitoring (MRM). SRM/MRM, performed by QQQ MS, provides a targeted approach with high selectivity and sensitivity to detect relative and absolute peptide levels in a large number of samples (see [31–33]). SRM/MRM has been developed to identify PTMs, including phosphorylation [34], ubiquitinylation [35], methylation [36], acetylation [37] and glycosylation [38,39]. The combination of iTRAQ/TMT isobaric tags and the SRM/MRM-targeted proteomics method has shown promise in clinical studies. For example, specific histone acetylations in both Alzheimer's Disease (AD) and controls have been reliably quantified using TMT6plex and SRM analysis [40]. This quantitative targeted proteomics strategy will be a powerful tool for identification and verification of candidate protein biomarkers.

This review will focus on the applications of isobaric mass tags, with a particular emphasis on TMT, in the

analysis of PTMs. Furthermore, we will also discuss and explore how the different peptide-reactive groups that are now available within the TMT family are uniquely suited to the analysis of PTMs on proteins on a global, proteome-wide scale.

Structure of isobaric mass tags

The isobaric mass tags TMT and iTRAQ have the same basic structure consisting of a reporter group, a mass-balancing group, a cleavable linker and a peptide-reactive group (see Figure 1A). The distributions of ^{13}C and ^{15}N isotopes across the mass reporter and mass normalization groups of the reagents results in each tag variant within a particular TMT family (TMTzero, Figure 1F; TMTduplex, Figure 1G; TMT-6plex Figure 1H; TMT8plex and TMT10plex [structures not shown]) having the same overall mass and identical chemical structure. Since the label tags within each family are isobaric, the MS signal is the sum of the peptide contribution from all samples, so there is a gain in sensitivity. During MS/MS analysis, the cleavable linker preferentially fragments to release the mass reporter groups. The signal intensities of the reporter ions shown in the low mass range of the MS/MS spectra are utilized to achieve relative quantification of the peptides, and thus proteins, in different samples.

The iTRAQ and amine-reactive TMT tags are the standard repertoires for quantitative proteomics techniques. TMT and iTRAQ are based on a similar principle. The peptide-reactive group for the amine-reactive TMT tag (Figure 1B) covalently binds to the *N*-terminal α-amino groups and ε-amino groups of lysine residues within the peptides/proteins using *N*-hydroxysuccinimide ester. As such, theoretically all tryptic peptides within the samples are labeled by amine-reactive TMT. The peptide-reactive group of Cys-reactive TMT (Figure 1C), however, selectively targets and specifically labels sulfhydryl groups of Cys using a thiol-reactive peptide-reactive group [41,42]. By using the TMT antibody, CysTMT-labeled peptides are effectively captured and purified. Owing to the high chemical reactivity and relatively low occurrence in the proteome, Cys has frequently been targeted as one of the common amino acids for chemical tagging [43], such as in ICAT [44], metal-coded affinity tag [45] and C3T [46]. Typically an average protein only contains two or three Cys-containing peptides, which leads to a significant reduction of sample complexity by reducing the number of peptides per protein that are analyzed [47]. Consequently, although fewer peptides are identified per protein, this enrichment method increases the total number of proteins identified and facilitates the detection and quantitation of low-abundance proteins [48].

Recently, Hahne *et al.* have introduced carbonyl-reactive TMTs (glyco-TMTs) as a novel approach for the quantitation of *N*-glycans [49]. This is based on the previous studies of glycan quantitation using isotope-coded labeled hydrazide/aminooxy reagents via hydrazone/oxime formation [50,51]. TMT reagents containing hydrazide or aminooxy reactive groups are pioneering isobaric labelling reagents for quantitation of glycans and glyco-conjugates. (Figure 1D & E). Figure 2 shows that glyco-TMTs are labeled at the reducing end of carbohydrates. Hydrazone and oxime formation normally do not require further sample clean-up but do require acid stabilization via methyl esterification [52] prior to MS analysis.

Although isobaric TMTs have many advantages, particularly their multiplexing capability, there are issues concerning their use in quantitative analysis. One issue is that since the intensity of the isobaric precursor ion will be split across all labeling channels, when increasing multiplex channels, the individual reporter ion signals will be reduced without higher sample loading [53]. Another drawback of isobaric TMTs is that coisolation and cofragmentation of multiple labeled precursor ions, termed **isobaric interference**, can result in chimeric MS/MS spectra, which leads to underestimation of the fold-change expression of each peptide [25,54–55]. Several methods have been reported to improve accuracy of isobaric tag-based quantification, such as prefractionation [56], delayed peptide fragmentation and narrow isolation width [57], gas-phase purification (QuantMode) [58] and triple-stage MS (MS/MS/MS or MS3) [59]. Most recently, two methods have been shown to efficiently eliminate isobaric interference and increase quantitation accuracy. One method is to further fragment ions created during MS/MS. This process is called MS3. Additionally, MultiNotch MS3 developed by McAlister *et al.* uses isolation waveforms with multiple frequency notches to coisolate and cofragment multiple MS2 fragments, which improved sensitivity by increasing the number of of reporter ions that can be detected. This method exhibits a tenfold increase in TMT reporter ion intensities and reduces reporter ion signal variance so that more high-quality quantitative measurements can be made [60]. Isobaric interference can also be eliminated using IM-MS. During IM, ions are separated based on charge and m/z [30]. IM has the potential to mitigate chimeric MS/MS spectra since IM-MS is able to separate ions based on charge and m/z [30].

Analysis of phosphorylation

Phosphorylation, the addition of phosphate to serine, threonine and tyrosine residues, is a fundamental

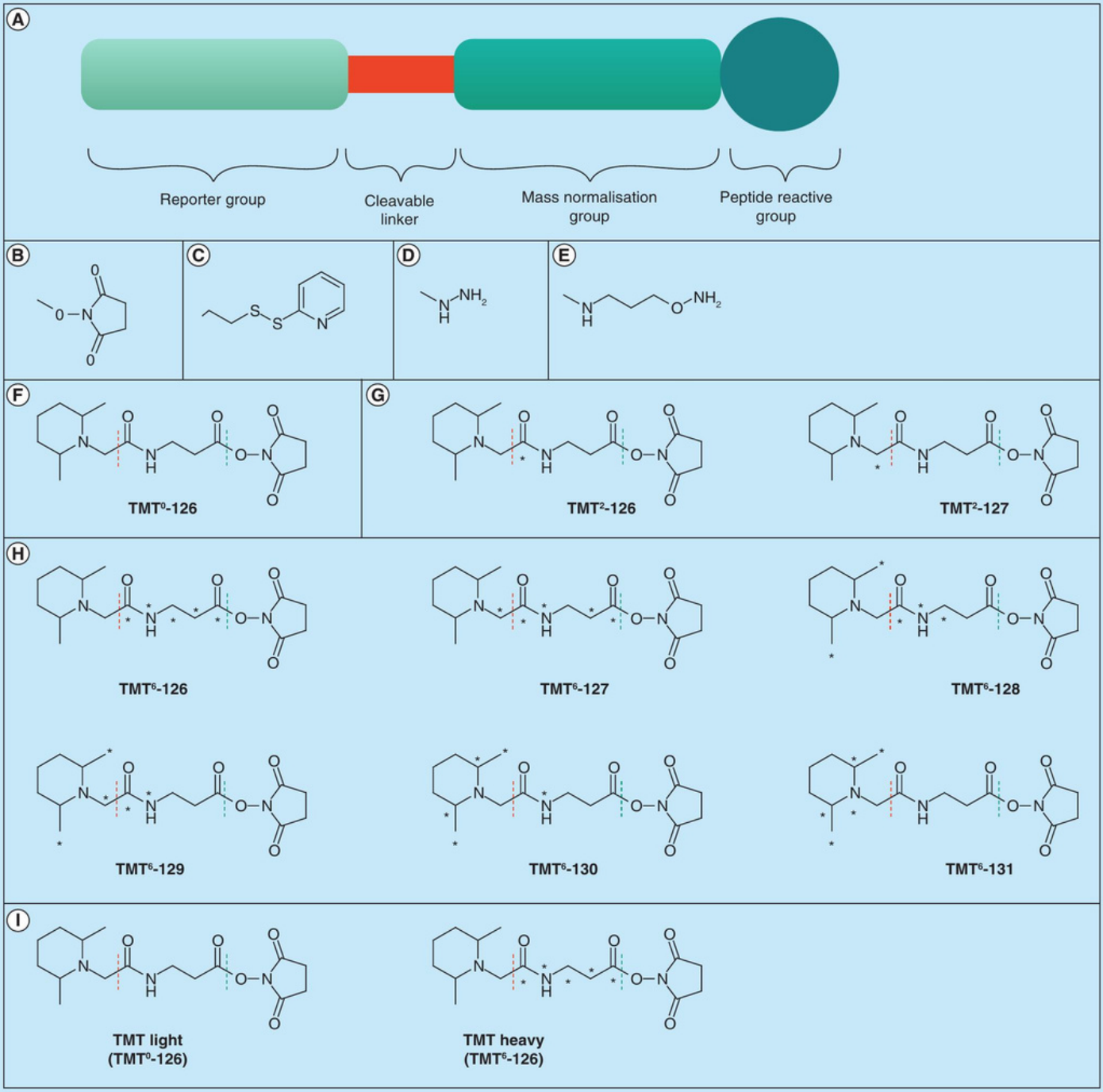


Figure 1. Structure of Tandem Mass Tags®. (A) Basic structure of isobaric mass tags. There are currently four different peptide/carbohydrate reactive group variants within the TMT family of isobaric mass tags: **(B)** amine specific (reactive *N*-hydroxysuccinimide ester); **(C)** cysteine (Cys)-specific (thiol-reactive pyridyldithiol); and **(D & E)** carbonyl-specific (reactive hydrazide and aminooxy group, respectively). For amine- and Cys-reactive TMT, there are currently three different family groups that enable discovery-based projects to multiplex from one up to six samples [14]. **(F)** TMT zero is a nonisotopically substituted core structure that is ideal for method development. **(G)** TMT duplex can be applied in discovery studies for the comparison of two samples. **(H)** TMT6plex™ can be applied in discovery studies, for example time-course, dose-response and replicate analyses for the comparison of up to six or eight samples respectively. **(I)** For the amine-specific TMT, light and heavy versions are also available for TMT-SRM verification projects. TMT8plex™ and TMT10plex™ have also been developed, which increases the plexing rate up to eight and ten samples, respectively [22,23].
TMT: Tandem mass tag.

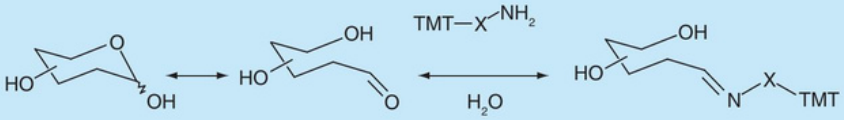


Figure 2. Reaction scheme of glycan labeling using glyco-tandem mass tags. X represents nitrogen for hydrazone formation or oxygen for oxime formation.
TMT: Tandem mass tag.
Reproduced with permission from [49] © American Chemical Society (2012).

PTM involved in several biological processes. Phosphorylation status is a highly dynamic process with phosphorylation events regulated by protein kinases and phosphatases.

A change in status of an individual phosphorylation site on a particular protein may render that protein as active or inactive, which may have downstream effects on several cell signaling pathways. As such the ability to be able to identify and quantify changes in phosphorylation status at an individual phosphorylation site on a proteome-wide scale is key to understanding normal biological function and disease mechanisms. The highly dynamic nature of phosphorylation, and the relatively low stoichiometry of phosphorylated peptides compared with unphosphorylated peptides has, in the past, hindered the ability to assess phosphorylation status on a global, proteome-wide scale. However, in recent years, MS-based workflows have become the technology of choice for the analysis of the phosphoproteome owing, in part, to the fact that the addition of phosphate to serine, threonine and tyrosine residues gives rise to unique masses (167, 181 and 243 Da), which enables the sequence of a phosphopeptide to be derived from an MS/MS spectrum. Today, owing to many of the improvements in sample preparation techniques and MS already discussed, we are able to identify and quantify tens of thousands of phosphorylation sites in a single experiment.

The application of MS/MS strategies for phosphoproteome analysis has been extensively reviewed elsewhere and the authors point readers to the following reviews [61,62–66]. Notably, Verano-Braga *et al.*, for example, recently identified 1288 unique phospho-sites on 699 phosphoproteins with 99% confidence in the phosphopeptide assignments in angiotensin-(1–7)-stimulated human aortic endothelial cells. Using a combination of iTRAQ labeling and phosphopeptide enrichment using titanium dioxide (TiO_2), the authors were able to identify 121 phosphorylation sites on 79 proteins whose phosphorylation status was affected by angiotensin-(1–7) [66]. In another example, Nilsson *et al.* employed a multiple enrichment workflow to assess the STAT3/IL-6/HIF1α signaling network in GSC11 glioblastoma stem cells treated with either STAT3 phosphorylation inhibitor or WP1193 plus IL-6 stimulation. The workflow for this publication involved initial enrichment of phosphoproteins with phosphoprotein, digestion of the enriched phosphoproteins, isobaric labeling with TMT6plex tags, hydrophilic interaction LC fractionation into 24 fractions to reduce sample complexity and final enrichment of phosphopeptide using the TiO_2 method prior to MS analysis. The authors reported the identification of 3414 proteins, which enabled them to gain significant insight into the IL-6 and STAT3 signaling pathways [63].

A key feature to the success of global phosphoproteomics has been the development of phosphopeptide-enrichment strategies including immobilized metal affinity chromatography [67,68], TiO_2 [69,70] and enrichment of phosphotyrosine peptides by immunoprecipitation with global phosphotyrosine antibodies such as 4G10 [71]. These strategies are compatible with all quantitative proteomics techniques including label-free, isotopic and isobaric quantitation and have been extensively reviewed elsewhere [72–74]. For this review, authors aim to discuss the key advantages that we believe sets isobaric tagging technology apart from other quantitative proteome techniques such as SILAC and label free for the analysis of phosphorylation status on a global, proteome-wide scale. In particular, we will explore how the combination of amine-reactive isobaric mass tagging reagents and HCD fragmentation in the Orbitrap Velos™ and more recently Orbitrap Fusion instruments (both Thermo Scientific) have truly revolutionized quantitative phosphoproteomic analysis such that it is now possible to investigate alterations in signaling pathways on a proteome-wide scale [75].

Advantages of isobaric tagging for quantitative phosphorylation analysis

Proteomic/phosphoproteomic workflows involve digestion of proteins into peptides typically with trypsin. Tryptic peptides are ideally suited for analysis by MS as the presence of either an arginine or lysine residue means all peptides have a charge at the C-terminal end of the peptide. This charge results in a strong y ion fragmentation series under CID, the most established form of fragmentation in the proteomics field. CID fragmentation

induces fragmentation of labile amine bonds along the peptide backbone to produce b- and y-type ions and is typically used in QTOF, QQQ, TOF/TOF and ion trap instruments. In the analysis of phosphopeptides, however, the phosphate itself is preferentially labile and therefore CID MS/MS spectra of phosphopeptides often predominately contains fragment ions relating to the neutral losses of phosphoric acid and meta-phosphoric acid. This preferential fragmentation of the phosphate can mean that the identification of the exact location of the phosphate on the phosphopeptide can be a challenge; however, the fragmentation patterns that are produced by CID fragmentation of phosphopeptides have been well studied and are now well established [76,77].

Owing to the production of c- and z-type fragment ions, ECD and ETD fragmentation techniques provide potential advantages over CID for the analysis of phosphopeptides as the phosphate group remains on the serine, threonine or tyrosine residue to which it is associated with. However, they are largely limited to the analysis of multiply charge precursor ions. Despite some application to phosphoproteomics, ECD fragmentation is limited to Fourier transform ion cyclotron resonance instruments and as such it is ETD that shows the greatest potential for the future with regard to phosphorylation analysis owing to its utility in quadrupole ion trap instruments.

In quantitative proteomics, amine-reactive isobaric tags theoretically label all tryptic peptides at the peptide *N*-terminus. This feature is particularly useful in CID fragmentation of phosphopeptides as it helps provide more confidence in the identification as the presence of the tag at the *N*-terminus of the peptide induces a strong b ion series as well as a y ion series, which enables the peptide to be sequenced from both ends of the phosphopeptide.

One drawback, however, of applying CID fragmentation to labeled phosphopeptides is that the generation of isobaric tag reporter ions also competes with peptide backbone fragmentation, which often leads to poor intensity reporter ions being produced, resulting in less confidence in quantitation results. Furthermore, isobaric tags are not suitable for the quantitation of phosphopeptides in ion trap instruments, which have a low mass cut-off. Quantitative phosphoproteomics can be achieved, however, using isobaric tags under CID fragmentation in QTOF instruments, which do not suffer from a low mass cut-off and the authors point readers to the following example [78]. It should also be noted that while TMT6plex and iTRAQ8plex can be used on lower resolution instruments, the use of TMT8plex and TMT10plex is limited to high-resolution, high mass accuracy instruments.

HCD fragmentation, which can be achieved in Orbitrap Velos and Orbitrap Fusion instruments, enables high-resolution, high mass accuracy identification and quantitation of (phospho)peptides with strong reporter ions intensities as the Orbitrap does not have a low mass cut-off. Although HCD fragmentation produces strong reporter ion intensities allowing for good quantitation statistics, it can be at the expense of phosphopeptide identifications, as the higher energy breaks the peptide backbone into too smaller pieces. Linke *et al.* recently evaluated the optimized fragmentation conditions for iTRAQ-labeled phosphopeptides and concluded that parallel acquisition of CID/multistage activation and HCD spectra give the optimal fragmentation parameters to provide confident phosphopeptides identification and accurate quantitation of isobaric reporter ions [79].

One innovative quantitative phosphoproteomics workflow that has demonstrated utility in monitoring the cell signaling response of cancer drugs in cultured tumor-derived cell lines, analysis of fresh/frozen xenograft tumors and frozen clinical biopsy material is the SysQuant® global phosphorylation assay, which is currently being developed for clinical and commercial applications by Proteome Sciences plc [75]. SysQuant warrants inclusion in this article because it represents a pioneering example to illustrate how the use of specific enrichment strategies, together with isobaric mass tags, high-performance MS and extensive bioinformatics, can be streamlined to deliver an effective global analysis of signal transduction mechanisms. Briefly, SysQuant is a multiplexed quantitative MS-based workflow for the simultaneous measurements of multiple phosphoproteins that provides a rapid measurement of signaling pathway activity in various sample types, including cultured tumor-derived cells, xenograft tumor tissue and frozen clinical material. To date, over 3000 phosphorylation events have been identified and quantified, including phosphoproteins known to play a key role in cancer-related pathways including EGF receptor, HER2, ERK1/2, PI3K, AKT, Jun, p53 and Rb protein.

Quantitation of phosphorylation sites using the SysQuant workflow is achieved by isobaric amine-reactive TMT tags with a comprehensive analysis of phosphorylation events achieved by a combination of immobilized metal affinity chromatography and TiO_2 enrichment. The identification and quantitation of not just the enriched phosphopeptides, but also the unphosphorylated fraction is achieved by HCD fragmentation using an Orbitrap Velos. By analyzing the unphosphorylated fraction as well as the phosphopeptide fraction not only does SysQuant provide comprehensive information as to which phosphoproteins within the sample(s) are activated or deactivated (by

analysis of the phosphorylation status of individual phosphorylation sites), but it also provides information as to whether the overall levels of the phosphoprotein has increased or decreased within the samples(s) by quantitation of both the phosphopeptides and the unphosphorylated peptides within that protein. This information is then cross-referenced to known cancer signaling pathways, using pathway maps and software tools such as KEGG and DAVID bioinformatics resources, to obtain a comprehensive picture of those signaling pathways being either activated or deactivated within the study. As such, the SysQuant global phosphorylation workflow is likely to become an extremely valuable tool in preclinical drug discovery, as well as prediction of patient response to therapies.

Over the past decade, global analysis of the phosphoproteome has perhaps led the way within the field of quantitative PTM analysis and as discussed above, standard workflows are now yielding exciting information about how changes in phosphorylation status can drive cell signaling pathways. In more recent years, however, analysis of more complex PTMs, including glycosylation, has started to emerge.

Analysis of glycosylation

Glycosylation is one of the most common PTMs of proteins. The addition and removal of carbohydrate moieties plays a significant role in many essential cellular processes, including cell–cell interactions, cellular recognition and proliferation, and immune signaling pathways [80,81]. Moreover, the structure of individual glycans can exhibit a prevalent influence on glycoprotein properties, such as fine-tuning of protein folding, solubility, maintenance of stability [82,83], and the determination of protein localization and function [84,85]. Aberrant protein glycosylation may play a crucial role in disease mechanisms, indicating that altered glycan structures may represent novel prognostic and/or diagnostic biomarkers [86].

Within cells, a variety of glycan structures can be attached to proteins and lipids to produce numerous polymeric forms of glycosylated molecules. Compared with the linear amino acid sequence of core proteins, glycans are complex, heterogeneous structures that can be highly branched molecules, and can be attached with many different linkage types connecting each monomeric sugar. This heterogeneity and complexity hinders the analysis of glycosylation events and as such quantitative glycoproteomics on a global scale is not as well established as phosphoproteomics. Recent advances in analytical methods and sample preparation techniques mean it is now highly possible to perform the characterization of glycan structure and the mapping of glycosylation sites on a global 'glycoproteomic' scale [87,88].

N- and *O*-linked glycans are the most common glycosylation events in eukaryotes. Typically, glycans are released from the protein/peptide with PNGase F being the most commonly used enzyme to cleave *N*-glycans from glycoproteins. Analysis of released glycans enables the detailed structural characterization of glycans including sugar sequence, composition, branching and linkage; however, site-specific glycosylation and glycan distribution are overlooked by this technique. In contrast to glycan analysis, glycosylation site mapping and varying degrees of site occupancy can be obtained by analysis of intact glycopeptides; however, the complete structural information of carbohydrate components may be lost owing to different analytical characters between peptides and glycans. For example, the peptide bond is prone to breakage by ETD, a soft fragmentation method, but higher energy fragmentation is required for the cleavage of glycosidic bonds.

Many of the recent studies in the field of MS-based glycomics/glycoproteomics have focused on enrichment strategies aimed at improving the sensitivity and relative abundance of glycans and glycoproteins within complex proteomes, enabling more detailed structural characterization [87–89]. In general, the most common MS-based methods of studying protein glycosylation involve: enzymatic or chemical cleavage of glycans from peptide/protein mixtures; derivatization of glycans by permethylation or glycan labeling with 2-aminobenzamide, 2-aminobenzoic acid and 2-aminopyridine via reductive amination; and purification of glycan or glycoproteins using SPE, size exclusion chromatography or affinity chromatography. The above methods are mainly designed for reduction of sample complexity and improvement of sample purity prior to MS analysis. Quantitative glycoproteomics analysis is an emerging approach in glycobiology research and it plays a pivotal role for the understanding of the regulation of glycosylation in physiological and pathological processes.

Quantitative analysis of glycosylation

Traditionally, glycan quantitation was achieved using the ratio of MS signal intensities between heavy- and light-labeled native and derivatized glycans (e.g., permethylation and reductive amination) [90–95]. Several isobaric methyl iodide reagents have been employed during permethylation, for example $^{12}CH_3$ (light), $^{12}CDH_2$, $^{12}CD_2H$, $^{12}CD_3$ and $^{13}CH_3$ (heavy) [90–92]. However, as only a small mass shift is observed, this approach is limited to very high resolution mass instruments. In addition, low permethylation efficiency affects the accuracy of quantitation.

During reductive amination, deuterated or ^{13}C-isotope-coded aromatic amines (i.e., aniline) are utilized to convert the free aldehyde at the reducing

end of the glycans into a Schiff base, followed by stabilization using sodium borohydride [93–95]. Unlike permethylation, reductive amination gives a fixed mass increment for any glycan and allows multiplex quantitation through a tetraplex set of stable isotope-coded tags (D0, D4, D8 and D12) [94].

More recently, isobaric tags (iTRAQ and TMT) have been developed for the quantitation of glycosylation. Although this review will focus on how the different peptide-reactive groups that are available in the TMT family have been specifically utilized to assess glycosylation events, iTRAQ tags in combination with affinity chromatography enrichment strategies have also been used to identify differential plasma glycoproteins from various diseased samples [96,97]. Furthermore, iTRAQ technology has also been applied to the identification of *N*-glycosylation sites with divergent carbohydrate moieties [98,99] as well as released *N*-glycans using iTRAQ with ^{18}O [100].

Amine-reactive TMT

Amine-reactive TMT has successfully been applied to quantify post-translationally modified peptides, including glycosylation [20,101]. The utility of TMT tags in the workflow for the quantitative analysis of glycopeptides has significant advantage over the analysis of unlabeled glycopeptides. This is because the sensitivity of MS-based analysis of glycopeptides can be significantly reduced by negative-charged sugars, such as sialic acids and uronic acids, as these acidic sugar residues significantly reduce the precursor charge state. The addition of TMT tags has been found to increase precursor charge state resulting in an improvement in glycopeptide fragmentation [49,102], especially those containing acidic sugars.

Here, we demonstrate that amine-reactive TMT can be used to label both an intact glycoprotein and trypsin-digested glycopeptides. Furthermore, we will also demonstrate glycosylation site mapping and glycan profiling. Ribonuclease B (RNase B) was utilized as our glycoprotein standard of choice in order to compare TMT labeling at the intact glycoprotein (i.e., before trypsin digestion) and at a glycopeptide level (i.e., after trypsin digestion).

Figure 3 represents the ESI-Q-TOF (Micromass) MS profile of RNase B that has been labeled with amine-reactive TMT at the intact glycoprotein level and then digested with trypsin. It is important to note that when preforming TMT labeling at the intact protein

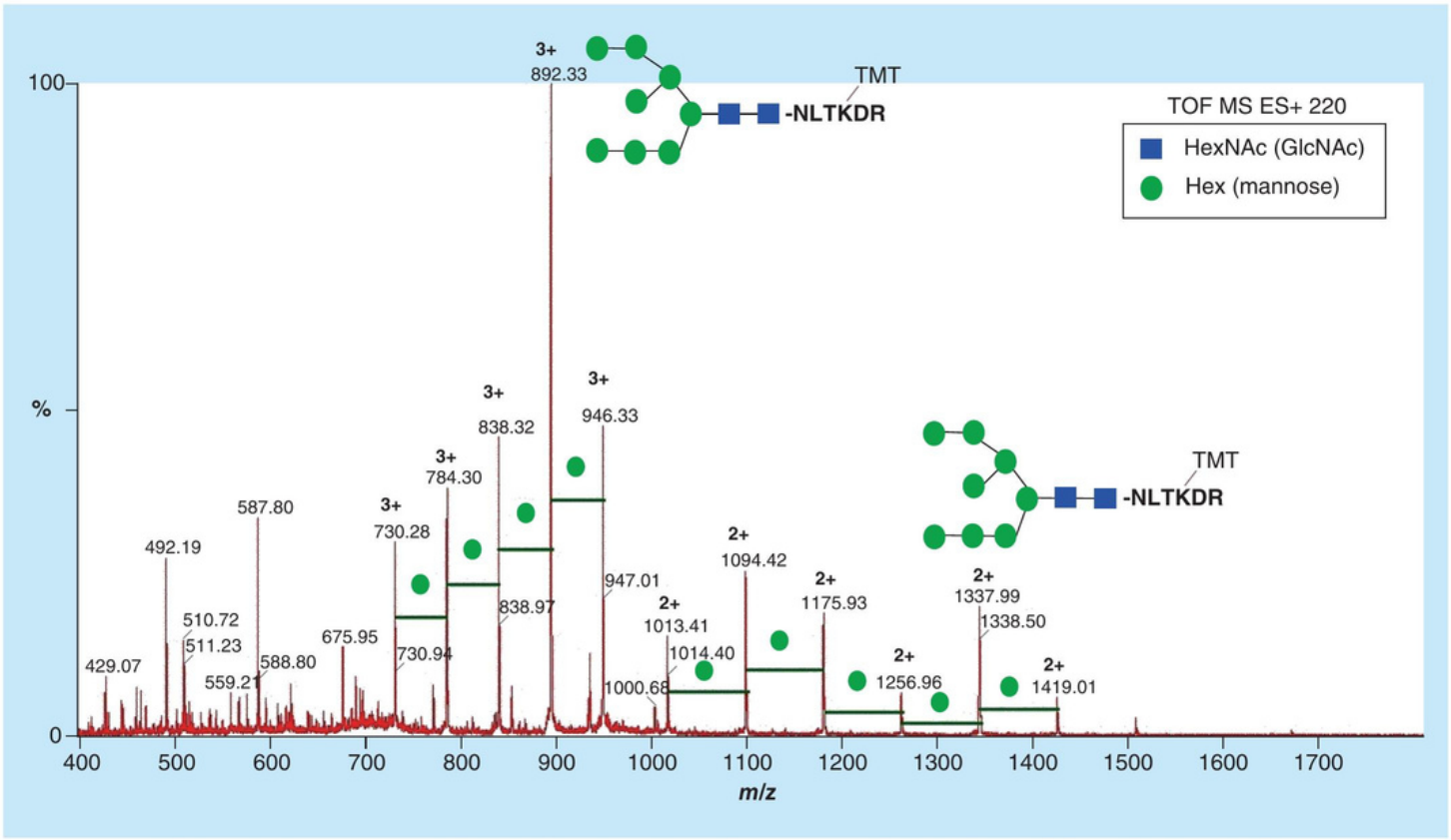


Figure 3. MS of intact tandem mass tag-labeled RNase B, with tandem mass tag labeling being followed by tryptic digestion. The most intense peak observed at *m/z* 892.33^{3+} represented the structure of the glycosylated peptide, $Man_8GlcNAc_2$-NLTKDR, which was also observed as a doubly charged ion at *m/z* 1337.99^{2+}. Other glycoforms, $Man_{5-9}GlcNAc_2$ of doubly charged ions and triply charged ions, were also present within the same peptide sequence, NLTKDR.
TMT: Tandem mass tag.

level, TMT tags label the *N*-terminal end of the protein as well as all lysine residues. As such, during trypsin digestion, the trypsin enzyme is unable to cleave the protein at the labeled lysine residues, resulting in Arg-C peptides. The most intense peak observed in Figure 3 at *m/z* 892.33^{3+} represented the structure of the glycosylated peptide, $Man_8GlcNAc_2$-NLTKDR, which was also observed as a doubly charged ion at *m/z* 1337.99^{2+}. Bovine pancreatic RNase B, containing only *N*-glycans at one glycosylation site, exists as a five high mannose *N*-glycan variant. As seen in Figure 3, other glycoforms ($Man_5GlcNAc_2$, $Man_6GlcNAc_2$, $Man_7GlcNAc_2$ and $Man_9GlcNAc_2$) of doubly charged ions (*m/z* 1013.41, 1094.42, 1175.93, 1256.96 and 1419.01, respectively) and triply charged ions (*m/z* 730.28, 784.30, 838.32 and 946.33, respectively) were also present within the same peptide sequence, NLTKDR. All of the glycosylated peptides were TMT labeled at the lysine residue, suggesting a 100% TMT labeling efficiency.

The structural information of TMT-labeled glycopeptides carrying $Man_8GlcNAc_2$ and the detailed fragmentation patterns of tryptic-labeled *N*-glycopeptides were determined by MS/MS. Distinct MS/MS spectra were exhibited by TMT-labeled glycopeptides derived from TMT labeling at the protein and peptide level as indicated in Figure 4 and Figure 5, respectively.

The *N*-glycan structure of RNase B was characterized by LC–MS/MS using CID fragmentation in a QTOF. CID fragmentation generated fragment ions predominantly from the cleavage of glycosidic bonds without breaking the peptide bonds. The glycosylated site was confirmed by the presence of $[TMT\text{-peptide+HexNAc+H/2H}]^{2+}$ fragment ions. As an example, the dominant doubly charge ion at *m/z* 587.80^{2+} was identified as GlcNAc-NLTKDR-TMT when labeling was carried out prior to trypsin digestion. Meanwhile, the peak at *m/z* 1128.66^{+},characterized as GlcNAc-NLTK-TMT_2,was observed with peptide level TMT labeling. Crucially, these data demonstrated that RNase B contains a single glycosylation site at Asn^{34} at which five glycosylated variants ($Man_5GlcNAc_2$, $Man_6GlcNAc_2$, $Man_7GlcNAc_2$, $Man_8GlcNAc_2$ and $Man_9GlcNAc_2$) are attached. Furthermore, our inhouse data showed that amine-reactive TMT can be used as a qualitative and quantitative approach for direct characterization of glycoproteins and glycopeptides.

In another example, TMT, in combination with an LTQ Orbitrap XL ETD hybrid MS, was used to demonstrate an extensive quantification of labile O-glycosylation modifications as well as other labile PTMs including acetylation, glycation and phosphorylation [20]. Compared with CID, ETD is a soft fragmentation process and is therefore capable of preserving labile PTMs, allowing direct mapping of protein modifications [103,104]. Furthermore, owing to low-energy fragmentation, ETD yields c and z ions instead of b and y ions, which are produced by high-energy dissociation (e.g., CID). Accordingly, the accuracy and precision of quantitation of TMT-labeled peptides by ETD fragmentation are similar to those obtained by CID.

To further explore the use of different fragmentation techniques (CID, HCD and ETD), another model glycoprotein, fetuin, has also been comprehensively characterized by multiplexed isobaric TMT labeling and an LTQ Orbitrap XL ETD hybrid LC–MS [105]. In this study, TMT-labeled fetuin glycopeptides were shown to be well characterized using ETD. Herein, the glycosylation site was identified with the associated glycan structure remaining intact and the peptide sequence was also determined with a series of c and z ions. In contrast to ETD, CID is a stronger dissociation method and hence labile glycans are typically fragmented to provide sugar sequence information. This is achieved primarily by fragmentation of the glycosidic bonds. Finally, HCD yields additional structural information for peptides and glycans with its higher dissociation energy. As a consequence, it was reported that glycan sequencing, glycosylation site localization, peptide sequence identification and quantification of glycopeptides could be accomplished by a combinational CID/HCD/ETD analysis [105].

Carbonyl-reactive TMT (glyco-TMT)

In contrast to amine-reactive TMTs, which label glycoproteins/glycopeptides at the *N*-terminus of the peptide and lysine residues, glyco-TMTs are applied to quantify released glycans. As the analysis of released glycans provides detailed structural characterization of carbohydrate components, sequence and linkage, which leads to valuable information about the biosynthesis and function of glycans, the capability of glyco-TMT reagents to tag and quantify these molecules in an isobaric multiplex workflow is valuable to glycobiology research. Using glycans prepared from standard glycoproteins and standard oligosaccharides, Hahne *et al.* compared the two different glyco-TMT peptide-reactive groups for labeling efficiency, and glycan quantitation accuracy and dynamic range [49]. The authors demonstrated that both aminooxy-reactive and hydrazide-reactive TMTs achieved a high labeling efficiency; however, aminooxy-reactive TMT reagents outperformed hydrazide-reactive TMTs under different experimental conditions. Notably, a labeling efficiency of greater than 95% can be obtained for aminooxy TMTs at a submicromolar TMT concentration. Additionally, using the isotopic versions of the TMT tags, light (TMT^0) and heavy (TMT^6) forms of both hydrazide and aminooxy TMTs

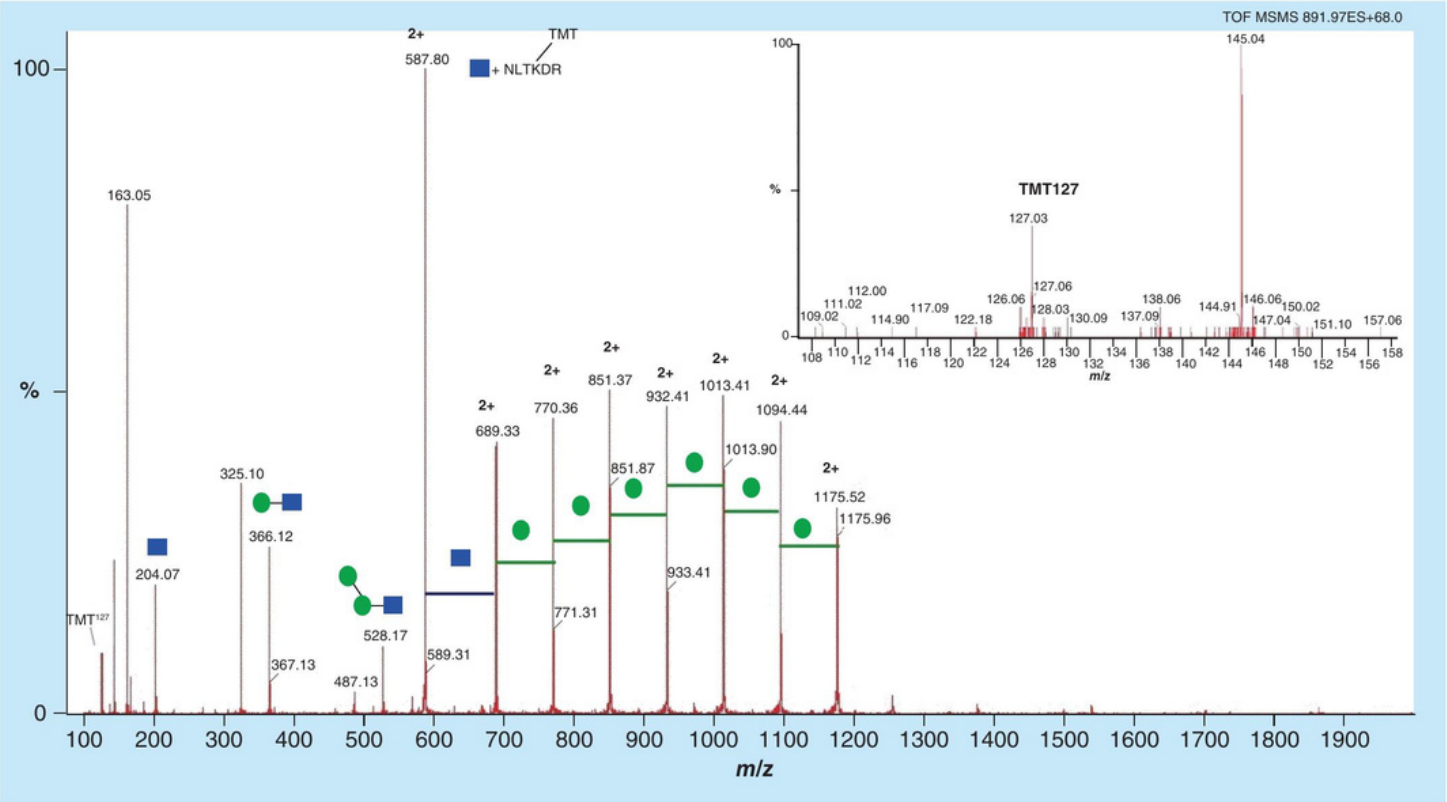


Figure 4. MS/MS of tandem mass tag-labeled intact RNase B, with tandem mass tag labeling being followed by tryptic digestion.
The spectrum reveals the TMT label remained visible. The presence of [TMT-peptided+HexNAc+2H]$^{2+}$ at *m/z* 587.80 represented Asn^{34} as a glycosylation site. The glycan sequence was identified with a series of monomeric sugar units. The insert is an expanded view of the spectrum showing the low mass region and detection of the *m/z* 127 reporter ion.
TMT: Tandem mass tag.

achieved CVs for the labeled ovalbumin *N*-glycans (1:1) of 3% and 1%, respectively. However, increasing the mixing ratio leads to a reduction in the quantitative accuracy of the tags, with CVs of 36% and 15% for hydrazide and aminooxy TMT quantification, respectively, with 1:10 mixtures. The authors also assessed isobaric glyco-TMT versions of the tags against the quantitation of *N*-glycans from ovalbumin. Despite a good quantitation accuracy for isobaric aminooxy-reactive TMTs (CV is less than 10% from the 1:1 mixtures), hydrazide-reactive isobaric TMTs showed higher CVs at more than 20%. The authors suggested that this might be due to the low recovery of TMT reporter ions, which also resulted in a limited dynamic range (up to 1:5) for both aminooxy-reactive and hydrazide-reactive TMTs. However, further studies using these tags are required to confirm these findings.

In a practical usage of glyco-TMT technology, the authors of the above publications applied glyco-TMTs to the analysis of *N*-glycans released from the isogenic human colon carcinoma cell lines SW480 (primary tumor) and SW620 (metastatic tumor) [49]. The *N*-glycosylation profile indicated significant differences in the quantities of high-mannose and fucosylated glycans between the nonmetastatic and metastatic cell lines. In this regard, glyco-TMTs represent a promising tool for quantitative glycan profiling and provide great potential for carbohydrate-based biomarker discovery.

Aldehyde-reactive TMT

Aldehyde-reactive alkoxyamino TMT reagents are the latest addition to the TMT family. In contrast to the previous described glyco-TMT tags, aldehyde-reactive alkoxyamino TMTs can be used for quantitation of *N*-glycans at both native and derivatized levels. Since derivatization is key to glycan analysis, for example permethylation is frequently used to enhance detection of glycans, aldehyde-reactive TMTs are of significant importance in glycomics research and have the ability to add a multiplex component to quantitation. Snovida *et al.* recently presented a comprehensive evaluation of aldehyde-reactive TMTs for MS-based quantitative glycomics at the ASMS (American Society for Mass Spectrometry, 2013) conference [106]. In this study, PNGase F-released *N*-glycans from several standard glycoproteins were labeled with alkoxyamine TMT reagents. Subsequently, half of the labeled *N*-glycans were subjected to permethylation to generate derivatized and nonderivatized samples. The authors established an optimized protocol for *N*-glycan labeling by assessing various reac-

tion times, experimental conditions and the ratio of substrate to TMT reagent, as well as establishing a suitable postlabeling clean-up method. The results of this study demonstrated that the use of aldehyde-reactive TMTs provides a considerable increase in sensitivity of permethylated glycans, with little improvement in the sensitivity of native glycans. Using the ABI 4700 Proteomics Analyzer MALDI TOF/TOF (Applied Biosystems by Life Technologies) and Orbitrap Velos, both ionization efficiency and quantification accuracy after labeling were investigated. Intriguingly, similar results were observed using the different fragmentation techniques from the two instruments (CID on TOF/TOF instruments and HCD on Orbitrap). In this context, the preliminary data presented in this abstract show that aldehyde-reactive TMTs enable the quantification of native and permethylated glycans. This method provides a promising new tool for the quantitative analysis of *N*-glycans.

Analysis of SNO

Protein SNO is a thiol-based covalent modification of the Cys sulfhydryl group with nitric oxide, protecting Cys residues from oxidation [107,108]. Recent studies have addressed the increasing prominence of the biological importance of protein SNO, which serves as a crucial regulatory mechanism that modulates protein activities, and regulates diverse cellular processes and biochemical pathways [109]. In addition, aberrant SNO has been reported to be involved in a wide range of human diseases including cancer [110]. In order to investigate the role of protein SNO in disease, advanced analytical techniques are required to identify and quantify changes in SNO status under various physiological and pathological conditions [111,112].

Quantitative analysis of SNO

MS-based quantitative analysis of SNO remains a challenging task owing to the low abundance of SNO proteins and the labile nature of SNO modifications. Furthermore, much less is known about SNO sites and which specific Cys residues are able to undergo SNO. Forrester *et al.* first demonstrated the quantitative analysis of SNO using iTRAQ, revealing protein denitrosylation is likely to take place at a global scale [113]. Recently, Cys-reactive TMTs have been utilized to identify and quantify the SNO sites [42,114], and to measure SNO occupancy [115]. In another example, Murray *et al.* measured SNO in human pulmonary arterial endothelial cells using Cys-reactive TMTs, followed by an affinity-based capture procedure and MS analysis. The study identified 220 SNO-modified Cys residues on 179 proteins [42]. In a novel

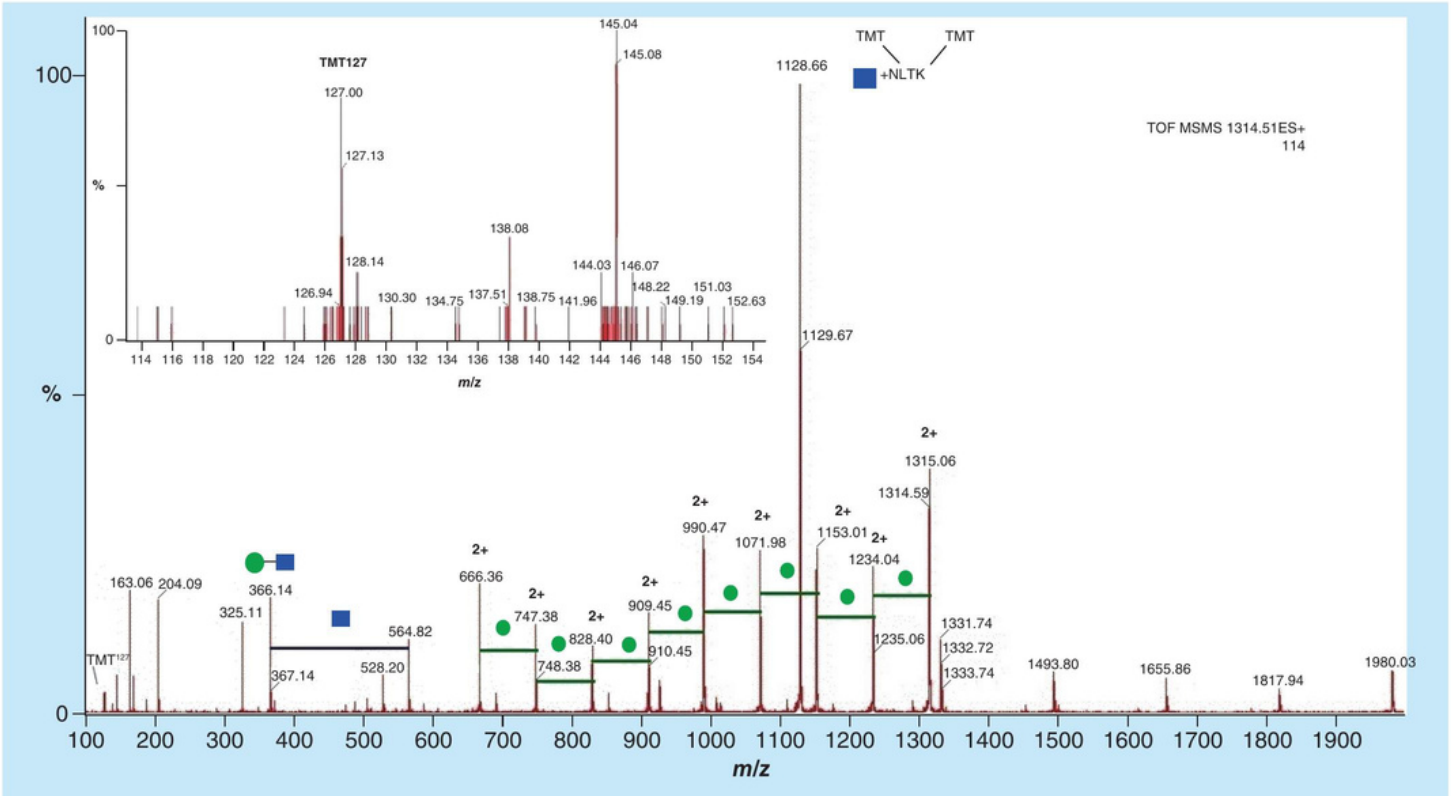


Figure 5. MS/MS of tandem mass tag-labeled RNase B peptides, with tryptic digestion being followed by tandem mass tag labeling. The inserted spectrum reveals the tandem mass tag (TMT) label remained visible. The presence of $[2TMT\text{-}peptided+HexNAc+2H]^{+}$ at *m/z* 1128.66 represented a glycosylated peptide at Asn^{34}. The glycan sequence was identified with a series of monomeric sugar units. TMT: Tandem mass tag.

approach using heavy/light Cys-reactive TMTs the extent of SNO occupancy at each Cys residue has been determined by measuring the ratio of SNO modified residues to unmodified residues [115]. The application of Cys PTM quantification is likely to be particularly important to assess site occupancy as it may reveal the biological roles of SNO and the impact of SNO on protein activities. This validated method of Cys-reactive TMT quantitative analysis of SNO site occupancy has been applied to cardioprotection studies and is likely to be able to identify other Cys oxidative modifications, such as *S*-glytathionylation, *S*-acylation and *S*-sulfenylation [116]. Although one attractive attribute of iTRAQ and Cys-reactive TMTs is their design, which allows multiple samples to be analyzed in one experiment, the specificity of the commercial antibody resin used for the enrichment of labeled peptides is low. This most likely leads to the analysis of mainly non-SNO-modified peptides [117,118].

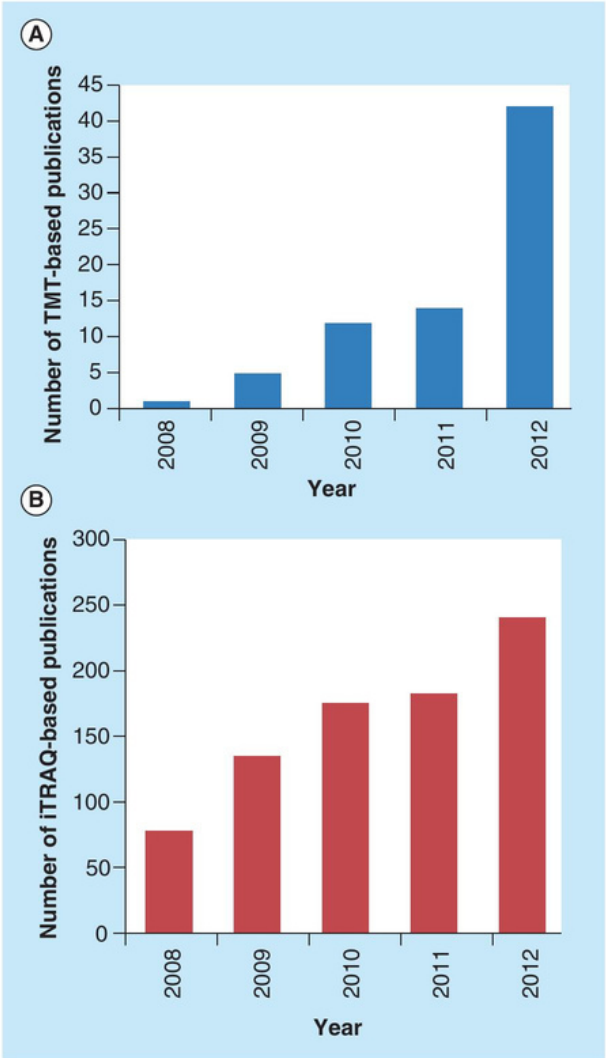


Figure 6. The number of publications using tandem mass tag and isobaric tags for relative and absolute quantitation for quantitative proteomics from 2008 to 2012. TMT: the NCBI database PubMed and Google Scholar were searched using 'tandem mass tag' 'TMT' 'not iTRAQ' as the query term. iTRAQ: the NCBI database PubMed and Google Scholar were searched using 'isobaric tags for relative and absolute quantification' 'iTRAQ' 'not TMT' as the query term. iTRAQ: Isobaric tags for relative and absolute quantitation; TMT: Tandem mass tag.

Conclusion & future perspective

In this review, the application of isobaric mass tag labeling technology to the characterization of PTMs has been reviewed and discussed. Owing to their multiplexing capability, isobaric mass tags are a valuable technology for the characterization and quantification of multiple samples in a single experiment. Furthermore, the extension of the TMT family to include several different peptide-reactive groups (amine reactive, carbonyl reactive, Cys reactive and aldehyde reactive) opens up comprehensive MS-based quantitative analysis to groups of PTM modifications that were not previously amenable to standard isobaric tagging techniques, for example analysis of released glycans using glyco-TMTs. In addition to phosphorylation, glycosylation and SNO, which have been addressed in this paper, isobaric mass tags have also been applied to other PTMs, including SUMOylation, acetylation and methylation [20,119–120]. Most recently, hydrazide-reactive TMTs have been applied to profile steroid metabolites in urine [121]. As such, it is perhaps not surprising that the number of peer-reviewed publications that utilize TMT technology has dramatically increased since 2012 (Figure 6).

A challenging attribute for quantitative PTM analysis is the low abundance, chemical diversity, dynamic complexity and tremendous heterogeneity of PTMs. Published studies as well as inhouse data have been used in this review to demonstrate that isobaric mass tags facilitate the identification and quantitation of PTM-modified peptides and proteins on a global, proteome-wide scale. To date, only quantitative analysis of phosphorylation and SNO status has been demonstrated using isobaric tagging in complex biological samples, whereas quantitation of glycosylation has been mostly applied to standard recombinant proteins, such as RNase B and fetuin, with their well-defined sequence and glycan structure.

Owing to the coexistence of multiple PTMs on the same proteins, the future challenge lies with the ability to be able to probe multiple PTM events simultaneously and resolving the extent of PTM crosstalk if we are to truly reveal the intricate nature of PTM-mediated regulatory networks. A recent study reported the identification of proteins comodified by phosphorylation and ubiquitinylation [122]. This demonstrates that sophisticated MS-based quantitative

PTM analysis is capable of probing complex biological scenario's involving multiple PTM events.

Recently, isobaric TMTs have been demonstrated to show the highest level of quantitative precision and reproducibility compared with label-free (spectral counting), $^{14}N/^{15}N$ metabolic labeling, iTRAQ and TMT using a bacterial sample and a LTQ Orbitrap Velos mass spectrometer [123]. Although spectral counting label-free quantification, based on the number of MS/MS spectra in a LC–MS/MS run, is rather simpler to practice, it is problematic when the number of identifications per protein is small, leading to significant uncertainties. Li *et al.* found that the label-free method provides the deepest proteome coverage and generated the largest number of unique protein identification compared with other labeling-based methods. However, compared with labeling-based approaches, the label-free quantification is less accurate and reproducible, especially for low-abundance proteins [123].

While isobaric TMTs are able to provide PTM quantitative analysis across a large number of biological samples, there is a need for standardization of the experimental design and data analysis. In addition, with the increasing data size generated in each experiment, it is important that MS/MS-based software tools effectively extract reliable information, accurately annotate PTMs and precisely localize modification sites. Various search tools have been developed in recent years for the quantitation of isobarically labeled PTM experiments [124], such as PeaksPTM [125], Byonic™ [126] and IsobarPTM [127]. These software tools have been shown to identify PTMs, search for unknown PTMs and validate PTM localization. Meanwhile, several improvements have been employed to simultaneous search for multiple PTMs without the exponential growth of the search space, reduce false discoveries and accurately localize PTM sites.

With immense progress in bioinformatics tools, an area of great promise is integrative omics profiling – genomics, proteomics, metabolomics, glycomics, lipidomics and transcriptomics. Multi-omics technologies are essential for the study of systems biology by linking genotype to phenotype, and aiding our understanding of the molecular and cellular processes mediated by signaling pathways and interaction networks. For example, multi-omics methods have demonstrated that cancer-related pathways exhibit extremely nonlinear dynamics [128]. Therefore, these advances in technology will facilitate the discovery of the molecular causes of disease, as well as enable biomarker development including the identification of protein biomarkers with prognostic or diagnostic value.

Although the challenges of global quantitative proteomics of PTM-modified proteins and peptides still remain, the developments in sample preparation and chromatographic enrichment techniques, the advances in MS instrumentation including the utility of multiple fragmentation techniques such as CID, HCD and ETD, the targeted SRM approach and the commercialization of isobaric tagging technologies have enabled huge advances in this field. Using workflows such as SysQuant, phosphorylation status can now be determined at a site-specific level on a global, proteome-wide scale and detailed structural characterization of glycosylation can now be achieved in a multiplex experiment. In conclusion, owing to the high throughput, highly sensitive, accurate and robust measurements provided by MS-based quantitative PTM analysis using isobaric TMTs, the investigation of the biological roles of PTMs in a wide variety of biological and diseases mechanisms is now possible. In the future, mechanistic biology will continue to benefit from quantitative PTM proteomics,

Executive summary

- Post-translational modifications (PTMs) play a pivotal role in numerous biological processes by affecting protein structure, function and interaction within global protein networks.
- Owing to their often rapid turnover, relative low abundance, and substantial diversity and heterogeneity, the qualitative and quantitative determination of PTMs remains analytically challenging.
- MS-based quantitative proteomics has developed over the past decade to become an extremely powerful tool for the relative and absolute quantification of proteins from cells, tissues and body fluids, enabling the study of a variety of physiological and pathological processes.
- Isobaric tandem mass tags are widely used for quantitative proteomics. They consist of a reporter group, a mass-balancing group, a cleavable linker and a peptide-reactive group, and can be used to perform at the MS/MS level.
- With the flexibility of multiplexing up to ten samples and the recent addition of several different peptide-reactive groups, tandem mass tag technology is an extremely valuable tool for MS-based quantitative proteomics, and the study of PTMs in the context of fundamental biological processes and disease processes.
- The isobaric tandem mass tags have been extensively applied to several PTM events, including phosphorylation, glycosylation and *S*-nitrosylation.

particularly owing to the multiplex capabilities of isobaric TMTs.

Acknowledgements

The authors would like to formally acknowledge the scientific contribution of our colleagues at Proteome Sciences plc, particularly D Britton, V Mitra, K Kuhn and G Boehm among others involved in the development of SysQuant®. We are also grateful to C Russell for critical review of this manuscript prior to submission.

References

Papers of special note have been highlighted as:
•• of considerable interest

1 Scott JD, Pawson T. Cell signaling in space and time: where proteins come together and when they are apart. *Science* 326(5957), 1220–1224 (2009).

2 Karve TM, Cheema AK. Small changes huge impact: the role of protein posttranslational modifications in cellular homeostasis and disease. *J. Amino Acids* 2011, 207691 (2011).

3 Angel TE, Aryal UK, Hengel SM *et al.* Mass spectrometry-based proteomics: existing capabilities and future directions. *Chem. Soc. Rev.* 41(10), 3912–3928 (2012).

4 Choudhary C, Mann M. Decoding signaling networks by mass spectrometry-based proteomics. *Nat. Rev. Mol. Cell Biol.* 11(6), 427–439 (2010).

5 Olsen JV, Mann M. Status of large-scale analysis of post-translational modifications by mass spectrometry. *Mol. Cell. Proteomics* 12(12), 3444–3452 (2013).

•• **Provides an overview of where MS-based proteomics stands for large-scale analysis of post-translational modification (PTM), what progress has already been made in the past 10 years and what are the future challenges in the field.**

6 Han X, Aslanian A, Yates JR 3rd. Mass spectrometry for proteomics. *Curr. Opin. Chem. Biol.* 12(5), 483–490 (2008).

7 Domon B, Aebersold R. Mass spectrometry and protein analysis. *Science* 312(5771), 212–217 (2006).

8 Hebert AS, Richards AL, Bailey DJ *et al.* The one hour yeast proteome. *Mol. Cell. Proteomics* 13(1), 339–347 (2013).

9 Ong SE, Mann M. Mass spectrometry-based proteomics turns quantitative. *Nat. Chem. Biol.* 1(5), 252–262 (2005).

10 Bantscheff M, Lemeer S, Savitski MM, Kuster B. Quantitative mass spectrometry in proteomics: critical review update from 2007 to the present. *Anal. Bioanal. Chem.* 404(4), 939–965 (2012).

•• **Good review article that discusses the developments and applications of quantitative MS-based proteomics since 2007, and gives readers some ideas of how to choose appropriate technical approaches for their particular applications.**

11 Craft GE, Chen A, Nairn AC. Recent advances in quantitative neuroproteomics. *Methods* 61(3), 186–218 (2013).

12 Gajadhar AS, White FM. System level dynamics of post-translational modifications. *Curr. Opin. Biotechnol.* 228C, 83–87 (2014).

Financial & competing interests disclosure

Financial support for this work was provided by Proteome Sciences plc. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

•• **Excellent article that discusses recent approaches and techniques for understanding system level dynamics of PTMs in biological processes.**

13 Skorobogatko YV, Deuso J, Adolf-Bryfogle J *et al.* Human Alzheimer's disease synaptic O-GlcNAc site mapping and iTRAQ expression proteomics with ion trap mass spectrometry. *Amino Acids* 40(3), 765–779 (2011).

14 Thompson A, Schäfer J, Kuhn K *et al.* Tandem mass tags: a novel quantification strategy for comparative analysis of complex protein mixtures by MS/MS. *Anal. Chem.* 75(8), 1895–1904 (2003).

15 Ross PL, Huang YN, Marchese JN *et al.* Multiplexed protein quantitation in *Saccharomyces cerevisiae* using amine-reactive isobaric tagging reagents. *Mol. Cell. Proteomics* 3(12), 1154–1169 (2004).

16 Wiese S, Reidegeld KA, Meyer HE, Warscheid B. Protein labeling by iTRAQ: a new tool for quantitative mass spectrometry in proteome research. *Proteomics* 7(3), 340–350 (2007).

17 Choe L, D'Ascenzo M, Relkin NR *et al.* 8-plex quantitation of changes in cerebrospinal fluid protein expression in subjects undergoing intravenous immunoglobulin treatment for Alzheimer's disease. *Proteomics* 7(20), 3651–3660 (2007).

18 Dayon L, Hainard A, Licker V *et al.* Relative quantification of proteins in human cerebrospinal fluids by MS/MS using 6-plex isobaric tags. *Anal. Chem.* 80(8), 2921–2931 (2008).

19 Bantscheff M, Boesche M, Eberhard D, Matthieson T, Sweetman G, Kuster B. Robust and sensitive iTRAQ quantification on an LTQ Orbitrap mass spectrometer. *Mol. Cell. Proteomics* 7(9), 1702–1713 (2008).

20 Viner RI, Zhang T, Second T, Zabrouskov V. Quantification of post-translationally modified peptides of bovine alpha-crystallin using tandem mass tags and electron transfer dissociation. *J. Proteomics* 72(5), 874–885 (2009).

21 Ye H, Hill J, Kauffman J, Han X. Qualitative and quantitative comparison of brand name and generic protein pharmaceuticals using isotope tags for relative and absolute quantification and matrix-assisted laser desorption/ionization tandem time-of-flight mass spectrometry. *Anal. Biochem.* 400(1), 46–55 (2010).

22 Werner T, Becher I, Sweetman G, Doce C, Savitski MM, Bantscheff M. High-resolution enabled TMT 8-plexing. *Anal. Chem.* 84(16), 7188–7194 (2012).

23 McAlister GC, Huttlin EL, Haas W *et al.* Increasing the multiplexing capacity of TMTs using reporter ion isotopologues with isobaric masses. *Anal. Chem.* 84(17), 7469–7478 (2012).

24 Rauniyar N, Gao B, McClatchy DB, Yates JR 3rd. Comparison of protein expression ratios observed by sixplex and duplex TMT labeling method. *J. Proteome Res.* 12(2), 1031–1039 (2013).

25 Christoforou AL, Lilley KS. Isobaric tagging approaches in quantitative proteomics: the ups and downs. *Anal. Bioanal. Chem.* 404(4), 1029–1037 (2012).

•• **Good review article that discusses issues associated with the use of isobaric tagging approaches in quantitative proteomics, and offers possible solutions to increase their precision and accuracy.**

26 Evans C, Noirel J, Ow SY *et al.* An insight into iTRAQ: where do we stand now? *Anal. Bioanal. Chem.* 404, 1011–1027 (2012).

27 Pachl F, Fellenberg K, Wagner C, Kuster B. Ultra-high intra-spectrum mass accuracy enables unambiguous identification of fragment reporter ions in isobaric multiplexed quantitative proteomics. *Proteomics* 12(9), 1328–1332 (2012).

28 Dephoure N, Steven P. Gygi: Hyperplexing: a method for higher-order multiplexed quantitative proteomics provides a map of the dynamic response to rapamycin in yeast. *Sci. Signal.* 5(217), rs2 (2012).

29 Xiang F, Ye H, Chen R, Fu Q, Li L. N,*N*-dimethyl leucines as novel isobaric tandem mass tags for quantitative proteomics and peptidomics. *Anal. Chem.* 82(7), 2817–2825 (2010).

30 Sturm RM, Lietz CB, Li L. Improved isobaric tandem mass tag quantification by ion mobility mass spectrometry. *Rapid Commun. Mass Spectrom.* 28(9), 1051–1060 (2014).

31 Kitteringham NR, Jenkins RE, Lane CS, Elliott VL, Park BK. Multiple reaction monitoring for quantitative biomarker analysis in proteomics and metabolomics. *J. Chromatogr. B* 877(13), 1229–1239 (2009).

32 Lange V, Picotti P, Domon B, Aebersold R. Selected reaction monitoring for quantitative proteomics: a tutorial. *Mol. Syst. Biol.* 4, 222 (2008).

33 Picotti P, Aebersold R. Selected reaction monitoring-based proteomics: workflows, potential, pitfalls and future directions. *Nat. Methods* 9(6), 555–566 (2012).

34 Chang CY, Picotti P, Huttenhain R *et al.* Protein significance analysis in selected reaction monitoring (SRM) measurements. *Mol. Cell. Proteomics* 11(4), M111.014662 (2012).

35 Mollah S, Wertz IE, Phung Q, Arnott D, Dixit VM, Lill JR. Targeted mass spectrometric strategy for global mapping of ubiquitinylation on proteins. *Rapid Commun. Mass Spectrom.* 21(20), 3357–3364 (2007).

36 McClure M, DeLucas LJ, Wilson L *et al.* Purification of CFTR for mass spectrometry analysis: identification of palmitoylation and other post-translational modifications. *Protein Eng. Des. Sel.* 25(1), 7–14 (2012).

37 Evans CA, Griffiths JR, Unwin RD, Whetton AD, Corfe BM. Application of the MIDAS approach for analysis of lysine acetylation sites. *Methods Mol. Biol.* 981, 25–36 (2013).

38 Maury JJ, Ng D, Bi X, Bardor M, Choo AB. Multiple reaction monitoring mass spectrometry for the discovery and quantification of O-GlcNAc-modified proteins. *Anal. Chem.* 86(1), 395–402 (2014).

39 Ossola R, Schiess R, Picotti P, Rinner O, Reiter L, Aebersold R. Biomarker validation in blood specimens by selected reaction monitoring mass spectrometry of *N*-glycosites. *Methods Mol. Biol.* 728, 179–194 (2011).

40 Zhang K, Schrag M, Crofton A, Trivedi R, Vinters H, Kirsch W. Targeted proteomics for quantification of histone acetylation in Alzheimer's disease. *Proteomics* 12(8), 1261–1268 (2012).

41 Giron P, Dayon L, Turck N, Hoogland C, Sanchez JC. Quantitative analysis of human cerebrospinal fluid proteins using a combination of cysteine tagging and amine-reactive isobaric labeling. *J. Proteome Res.* 10(1), 249–258 (2011).

42 Murray CI, Uhrigshardt H, O'Meally RN, Cole RN, Van Eyk JE. Identification and quantification of *S*-nitrosylation by cysteine reactive tandem mass tag switch assay. *Mol. Cell. Proteomics* 11(2), M111.013441 (2012).

43 Giron P, Dayon L, Sanchez JC. Cysteine tagging for MS-based proteomics. *Mass Spectrom. Rev.* 30(3), 366–395 (2011).

44 Gygi SP, Rist B, Gerber SA, Turecek F, Gelb MH, Aebersold R. Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nat. Biotechnol.* 17(10), 994–999 (1999).

45 Ahrends R, Pieper S, Kühn A *et al.* A metal-coded affinity tag approach to quantitative proteomics. *Mol. Cell. Proteomics* 6(11), 1907–1916 (2007).

46 Giron P, Dayon L, Mihala N, Sanchez JC, Rose K. Cysteine-reactive covalent capture tags for enrichment of cysteine-containing peptides. *Rapid Commun. Mass Spectrom.* 23(21), 3377–3386 (2009).

47 Wang S, Zhang X, Regnier FE. Quantitative proteomics strategy involving the selection of peptides containing both cysteine and histidine from tryptic digests of *Cell.* lysates. *J. Chromatogr. A* 949(1–2), 153–162 (2002).

48 Parker J, Zhu N, Zhu M, Chen S. Profiling thiol redox proteome using isotope tagging mass spectrometry. *J. Vis Exp.* 61, 3766 (2012).

49 Hahne H, Neubert P, Kuhn K *et al.* Carbonyl-reactive tandem mass tags for the proteome-wide quantification of N-linked glycans. *Anal. Chem.* 84(8), 3716–3724 (2012).

•• **First demonstration of carbonyl-reactive tandem mass tags for the quantitative analysis of N-linked glycosylation.**

50 Walker SH, Budhathoki-Uprety J, Novak BM, Muddiman D. Stable-isotope labeled hydrophobic hydrazide reagents for the relative quantification of N-linked glycans by electrospray ionization mass spectrometry. *Anal. Chem.* 83(17), 6738–6745 (2011).

51 Uematsu R, Furukawa J, Nakagawa H *et al.* High throughput quantitative glycomics and glycoform-focused proteomics of murine dermis and epidermis. *Mol. Cell. Proteomics* 4(12), 1977–1989 (2005).

52 Powell AK, Harvey DJ. Stabilization of sialic acids in N-linked oligosaccharides and gangliosides for analysis by positive ion matrix-assisted laser desorption/ionization mass spectrometry. *Rapid Commun. Mass Spectrom.* 10(9), 1027–1032 (1996).

53 Pichler P, Köcher T, Holzmann J *et al.* Peptide labeling with isobaric tags yields higher identification rates using iTRAQ 4-plex compared with TMT 6-plex and iTRAQ 8-plex on LTQ Orbitrap. *Anal. Chem.* 82(15), 6549–6558 (2010).

54 Ow SY, Salim M, Noirel J, Evans C, Rehman I, Wright PC. iTRAQ underestimation in simple and complex mixtures: "the good, the bad and the ugly". *J. Proteome Res.* 8(11), 5347–5355 (2009).

55 Karp NA, Huber W, Sadowski PG, Charles PD, Hester SV, Lilley KS. Addressing accuracy and precision issues in iTRAQ quantitation. *Mol. Cell. Proteomics* 9(9), 1885–1897 (2010).

56 Ow SY, Salim M, Noirel J, Evans C, Wright PC. Minimizing iTRAQ ratio compression through understanding LC–MS elution dependence and high-resolution HILIC fractionation. *Proteomics* 11(11), 2341–2346 (2011).

57 Savitski MM, Sweetman G, Askenazi M *et al.* Delayed fragmentation and optimized isolation width settings for improvement of protein identification and accuracy of isobaric mass tag quantification on Orbitrap-type mass spectrometers. *Anal. Chem.* 83(23), 8959–8967 (2011).

58 Wenger CD, Lee MV, Hebert AS *et al.* Gas-phase purification enables accurate, multiplexed proteome quantification with isobaric tagging. *Nat. Methods* 8(11), 933–935 (2011).

59 Ting L, Rad R, Gygi SP, Haas W. MS3 eliminates ratio distortion in isobaric multiplexed quantitative proteomics. *Nat. Methods* 8(11), 937–940 (2011).

60 McAlister GC, Nusinow DP, Jedrychowski MP *et al.* MultiNotch MS3 enables accurate, sensitive, and multiplexed detection of differential expression across cancer cellline proteomes. *Anal. Chem.* 86(14), 7150–7158 (2014).

61 Palumbo AM, Smith SA, Kalcic CL, Dantus M, Stemmer PM, Reid GE. Tandem mass spectrometry strategies for phosphoproteome analysis. *Mass Spectrom. Rev.* 30(4), 600–625 (2011).

62 Hoffert JD, Pisitkun T, Saeed F, Song JH, Chou CL, Knepper MA. Dynamics of the G-protein-coupled vasopressin V2 receptor signaling network revealed by quantitative phosphoproteomics. *Mol. Cell. Proteomics* 11(2), M111.014613 (2012).

63 Nilsson CL, Dillon R, Devakumar A *et al.* Quantitative phosphoproteomic analysis of the STAT3/IL-6/HIF1alpha signaling network: an initial study in GSC11 glioblastoma stem cells. *J. Proteome Res.* 9(1), 430–443 (2010).

64 Ruperez P, Gago-Martinez A, Burlingame AL, Oses-Prieto JA. Quantitative phosphoproteomic analysis reveals a role for serine and threonine kinases in the cytoskeletal reorganization in early T Cell receptor activation in human primary T cells. *Mol. Cell. Proteomics* 11(5), 171–186 (2012).

65 Narumi R, Murakami T, Kuga T. A strategy for large-scale phosphoproteomics and SRM-based validation of human breast cancer tissue samples. *J. Proteome Res.* 11(11), 5311–5322 (2012).

66 Verano-Braga T, Schwämmle V, Sylvester M *et al.* Time-resolved quantitative phosphoproteomics: new insights into angiotensin-(1–7) signaling networks in human endothelial cells. *J. Proteome Res.* 11, 3370–3381 (2012).

67 Beltran L, Cutillas PR. Advances in phosphopeptide enrichment techniques for phosphoproteomics *Amino Acids* 43(3), 1009–1024 (2012).

68 Andersson L, Porath J. Isolation of phosphoproteins by immobilized metal (Fe^{3+}) affinity chromatography. *Anal. Biochem.* 154(1), 250–254 (1986).

69 Larsen MR, Thingholm TE, Jensen ON, Roepstorff P, Jørgensen TJ. Highly selective enrichment of phosphorylated peptides from peptide mixtures using titanium dioxide microcolumns. *Mol. Cell. Proteomics* 4(7), 873–886 (2005).

70 Thingholm TE, JØrgensen TJ, Jensen ON, Larsen MR. Highly selective enrichment of phosphorylated peptides using titanium dioxide. *Nat. Protoc.* 1(4), 1929–1935 (2006).

71 Bergström Lind S, Molin M, Savitski MM *et al.* Immunoaffinity enrichments followed by mass spectrometric detection for studying global protein tyrosine phosphorylation. *J. Proteome Res.* 7(7), 2897–2910 (2008).

72 Eyrich B, Sickmann A, Zahedi RP. Catch me if you can: mass spectrometry-based phosphoprotemics and quantification strategies. *Proteomics* 11(4), 554–570 (2011).

73 Loroch S, Dickhut C, Zahedi RP, Sickmann A. Phosphoproteomics – more than meets the eye. *Electrophoresis* 34(11), 1483–1492 (2013).

74 Johnson H, White FM. Toward quantitative phosphotyrosine profiling *in vivo*. *Semin. Cell Dev. Biol.* 23(8), 854–862 (2012).

75 Britton D, Zen Y, Quaglia A *et al.* Quantification of pancreatic cancer proteome and phosphorylome: indicates molecular events likely contributing to cancer and activity of drug targets. *PLoS ONE* 9(3), e90948 (2014).

•• **Provides a global phospho-proteomic workflow for the determination of expression and activity of signaling proteinsas drug targets in pancreatic cancer tissue.**

76 Carr SA, Huddleston MJ, Annan RS. Selective detection and sequencing of phosphopeptides at the fetomole level by mass spectrometry. *Anal. Biochem.* 239(2), 180–192 (1996).

77 Tholey A, Reed J, Lehmann WD. Electrospray tandem mass spectrometric studies of phosphopeptides and phosphopeptides analogues. *J. Mass Spectrom.* 34(2), 117–123 (1999).

78 Sachon E, Mohammed S, Bache N, Jensen ON. Phosphopeptide quantitation using amine-reactive isobaric tagging reagents and tandem mass spectrometry: application to proteins isolated by gel electrophoresis. *Rapid Commun. Mass Spectrom.* 20(7), 1127–1134 (2006).

79 Linke D, Hung CW, Cassidy L, Tholey A. Optimised fragmentation conditions for iTRAQ-labeled phosphopeptides. *J. Proteome Res.* 12(6), 2755–2763 (2013).

80 Dwek RA. Glycobiology: toward understanding the function of sugars. *Chem. Rev.* 96(2), 683–720 (1996).

81 Varki A, Lowe JB. Biological roles of glycans. In: *Essentials of Glycobiology* (*2nd Edition*). Varki A *et al.* (Eds). Cold Spring Harbor Laboratory Press, NY, USA (2009).

82 Woodward HD, Ringler NJ, Selvakumar R, Simet IM, Bhavanandan VP, Davidson EA. Deglycosylation studies on tracheal mucin glycoproteins. *Biochemistry* 26(17), 5315–5322 (1987).

83 Williamson G, Belshaw NJ, Noel TR, Ring SG, Williamson MP. O-glycosylation and stability. Unfolding of glucoamylase induced by heat and guanidine hydrochloride. *Eur. J. Biochem.* 207(2), 661–670 (1992).

84 Jensen LJ, Gupta R, Blom N *et al.* Prediction of human protein function from post-translational modifications and localization features. *J. Mol. Biol.* 319(5), 1257–1265 (2002).

85 Spiro RG. Protein glycosylation: nature, distribution, enzymatic formation, and disease implications of glycopeptide bonds. *Glycobiology* 12(4), r43–r56 (2002).

86 Packer NH, von der Lieth CW, Aoki-Kinoshita KF *et al.* Frontiers in glycomics: bioinformatics and biomarkers in disease. An NIH white paper prepared from discussions by the focus groups at a workshop on the NIH campus, Bethesda MD. *Proteomics* 8(1), 8–20 (2008).

87 Rakus JF, Mahal LK. New technologies for glycomic analysis: toward a systematic understanding of the glycome. *Annu. Rev. Anal. Chem. (Palo Alto Calif.)* 4, 367–392 (2011).

88 Pan S, Chen R, Aebersold R, Brentnall TA. Mass spectrometry based glycoproteomics –from a proteomics perspective. *Mol. Cell. Proteomics* 10(1), doi:R110.003251 (2011).

89 Zaia J. Mass spectrometry and the emerging field of glycomics. *Chem. Biol.* 15(9), 881–892 (2008).

90 Alvarez-Manilla G, Warren NL, Abney T. Tools for glycomics: relative quantitation of glycans by isotopic permethylation using 13CH3I. *Glycobiology* 17(7), 677–687 (2007).

91 Kang P, Mechref Y, Kyselova Z, Goetz JA, Novotny MV. Comparative glycomic mapping through quantitative permethylation and stable-isotope labeling. *Anal. Chem.* 79(16), 6064–6073 (2007).

92 Atwood JA 3rd, Cheng L, Alvarez-Manilla G, Warren NL, York WS, Orlando R. Quantitation by isobaric labeling: applications to glycomics. *J. Proteome Res.* 7(1), 367–374 (2008).

93 Bowman MJ, Zaia J. Tags for the stable isotopic labeling of carbohydrates and quantitative analysis by mass spectrometry. *Anal. Chem.* 79(15), 5777–5784 (2007).

94 Bowman MJ, Zaia J. Comparative glycomics using a tetraplex stable-isotope coded tag. *Anal. Chem.* 82(7), 3023–3031 (2010).

95 Xia B, Feasley CL, Sachdev GP, Smith DF, Cummings RD. Glycan reductive isotope labeling for quantitative glycomics. *Anal. Biochem.* 387(2), 162–170 (2009).

96 Jung K, Cho W, Regnier FE. Glycoproteomics of plasma based on narrow selectivity lectin affinity chromatography. *J. Proteome Res.* 8(2), 643–650 (2009).

97 Lee HJ, Na K, Choi EY, Kim KS, Kim H, Paik YK. Simple method for quantitative analysis of N-linked glycoproteins in hepatocellular carcinoma specimens. *J. Proteome Res.* 9(1), 308–318 (2010).

98 Lei Z, Beuerman RW, Chew AP *et al.* Quantitative analysis of N-linked glycoproteins in tear fluid of climatic droplet keratopathy by glycopeptide capture and iTRAQ. *J. Proteome Res.* 8(4), 1992–2003 (2009).

99 Ueda K, Takami S, Saichi N *et al.* Development of serum glycoproteomic profiling technique; simultaneous identification of glycosylation sites and site-specific quantification of glycan structure changes. *Mol. Cell. Proteomics* 9(9), 1819–1828 (2010).

100 Zhang S, Liu X, Kang X *et al.* iTRAQ plus 18O: a new technique for target glycoprotein analysis. *Talanta* 91, 122–127 (2012).

101 Hung CW, Tholey A. Tandem mass tag protein labeling for top-down identification and quantification. *Anal. Chem.* 84(1), 161–170 (2012).

102 Larsen MR, Trelle MB, Thingholm TE, Jensen ON. Analysis of posttranslational modifications of proteins by tandem mass spectrometry. *Biotechniques* 40(6), 790–798 (2006).

103 Saba J, Dutta S, Hemenway E, Viner R. Increasing the productivity of glycopeptides analysis by using higher-energy collision dissociation-accurate mass-product-dependent electron transfer dissociation. *Int. J. Proteomics* 2012, 560391 (2012).

104 Singh C, Zampronio CG, Creese AJ, Cooper HJ. Higher energy collision dissociation (HCD) product ion-triggered electron transfer dissociation (ETD) mass spectrometry for the analysis of N-linked glycoproteins. *J. Proteome Res.* 11(9), 4517–4525 (2012).

105 Ye H, Boyne MT 2nd, Buhse LF, Hill J. Direct approach for qualitative and quantitative characterization of glycoproteins using tandem mass tags and an LTQ Orbitrap XL electron transfer dissociation hybrid mass spectrometer. *Anal. Chem.* 85(3), 1531–1539 (2013).

106 Snovida S, Kuhn K, Etienne C, Rogers JC. Applications of Aldehyde-Reactive Thermo ScientificTandem Mass Tag (TMT) Reagents for Mass Spectrometry-based QuantitativeGlycomics. 2013. *American Society of Mass Spectrometry Conference proceedings.*8–13th June Minneapolis, Minnesota, USA. (2013).

107 Kohr MJ, Sun J, Aponte A *et al.* Simultaneous measurement of protein oxidation and *S*-nitrosylation during preconditioning and ischemia/reperfusion injury with resin-assisted capture. *Circ. Res.* 108(4), 418–426 (2011).

108 Chen YY, Chu HM, Pan KT *et al.* Cysteine *S*-nitrosylation protects protein-tyrosine phosphatase 1B against oxidation-induced permanent inactivation. *J. Biol. Chem.* 283(50), 35265–35272 (2008).

109 Hess DT, Matsumoto A, Kim SO, Marshall HE, Stamler JS. Protein *S*-nitrosylation: purview and parameters. *Nat. Rev. Mol. Cell Biol.* 6(2), 150–166 (2005).

110 Foster MW, Hess DT, Stamler JS. Protein *S*-nitrosylation in health and disease: a current perspective. *Trends Mol. Med.* 15(9), 391–404 (2009).

111 Forrester MT, Foster MW, Benhar M, Stamler JS. Detection of protein *S*-nitrosylation with the biotin-switch technique. *Free Radic. Biol. Med.* 46(2), 119–126 (2009).

112 Liu M, Talmadge JE, Ding SJ. Development and application of site-specific proteomic approach for study protein *S*-nitrosylation. *Amino Acids* 42(5), 1541–1551 (2012).

113 Forrester MT, Thompson JW, Foster MW, Nogueira L, Moseley MA, Stamler JS. Proteomic analysis of

S-nitrosylation and denitrosylation by resin-assisted capture. *Nat. Biotechnol.* 27(6), 557–559 (2009).

114 Foster MW. Methodologies for the characterization, identification and quantification of *S*-nitrosylated proteins. *Biochim. Biophys. Acta* 1820(6), 675–683 (2012).

115 Kohr MJ, Aponte A, Sun J, Gucek M, Steenbergen C, Murphy E. Measurement of *S*-nitrosylation occupancy in the myocardium with cysteine-reactive tandem mass tags: short communication. *Circ. Res.* 111(10), 1308–1312 (2012).

116 Murray CI, Van Eyk JE. Chasing cysteine oxidative modifications: proteomic tools for characterizing cysteine redox status. *Circ. Cardiovasc. Genet.* 5(5), 591 (2012).

117 Wu C, Parrott AM, Liu T, Beuve A, Li H. Functional proteomics approaches for the identification of transnitrosylase and denitrosylase targets. *Methods* 62(2), 151–160 (2013).

118 Thompson JW, Forrester MT, Moseley MA, Foster MW. Solid-phase capture for the detection and relative quantification of *S*-nitroproteins by mass spectrometry. *Methods* 62(2), 130–137 (2013).

119 Miller MJ, Scalf M, Rytz TC, Hubler SL, Smith LM, Vierstra RD. Quantitative proteomics reveals factors regulating RNA biology as dynamic targets of stress-induced SUMOylation in Arabidopsis. *Mol. Cell. Proteomics* 12(2), 449–463 (2013).

120 Rosenzweig D, Smith D, Myler PJ, Olafson RW, Zilberstein D. Post-translational modification of cellular proteins during Leishmania donovani differentiation. *Proteomics* 8(9), 1843–1850, (2008).

121 Rigdova K, Wang Y, Ward M, Griffiths WJ. A new derivative for oxosteroid analysis by mass spectrometry. *Biochem. Biophys. Res. Commun.* 446(3), 762–767 (2014).

122 Swaney DL, Beltrao P, Starita L *et al.* Global analysis of phosphorylation and ubiquitinylation cross-talk in protein degradation. *Nat. Methods* 10(7), 676–682 (2013).

•• **Excellent article thatprovides a comprehensive MS-based analysis of PTM crosstalk and novel methods for enrichment of comodified peptides.**

123 Li Z, Adams RM, Chourey K, Hurst GB, Hettich RL, Pan C. Systematic comparison of label-free, metabolic labeling, and isobaric chemical labeling for quantitative proteomics on LTQ Orbitrap Velos. *J. Proteome Res.* 11(3), 1582–1590 (2012).

•• **Compares each labeling method (label free, metabolic labeling and isobaric chemical labeling), and provides guidance for choosing a suitable approach for quantitative proteomics research.**

124 Na S, Paek E. Software eyes for protein post-translational modifications. *Mass Spectrom. Rev.* 9999, 1–15 (2014).

125 Han X, He L, Xin L, Shan B, Ma B. PeaksPTM: mass spectrometry based identification of peptides with unspecified modifications. *J. Proteome Res.* 10(7), 2930–2936 (2011).

126 Bern M, Kil YJ, Becker C. Byonic: advanced peptide and protein identification software. *Curr. Protoc. Bioinformatics* Chapter 13, Unit 13.20 (2012).

127 Breitwieser FP, Colinge J. Isobar(PTM): a software tool for the quantitative analysis of post-translationally modified proteins. *J. Proteomics* 90, 77–84 (2013).

128 Kreeger PK, Lauffenburger DA. Cancer systems biology: a network modeling perspective. *Carcinogenesis* 31(1), 2–8 (2010).

A new electron activated dissociation (EAD) approach for comprehensive glycopeptide analysis of therapeutic proteins

Featuring the SCIEX ZenoTOF 7600 system using Zeno EAD and Protein Metrics Inc. software

Fang Wang[1], Jenny Albernese[1], Zoe Zhang[1], Pavel Ryumin[2], Takashi Baba[2], Jason Causon[2], Kerstin Pohl[1]
[1]SCIEX, US; [2]SCIEX, Canada

The data presented here demonstrate the advantage of the novel electron activated dissociation (EAD)[1,2] over traditionally used collision induced dissociation (CID) with regards to glycopeptide characterization and localization in a peptide mapping workflow. In addition, streamlined, advanced characterization in one injection is being offered through high speed, highly reproducible, alternative fragmentation.[3-6] This solution takes peptide mapping experiments to a new level.

As biotherapeutics are becoming more complex, challenges for in-depth characterization increase simultaneously. Their characterization involves a myriad of analytical methods which include, but are not limited to, amino acid sequence confirmation and identification and localization of post-translational modifications (PTMs).[3,6] Glycosylations in particular are PTMs frequently considered critical quality attributes, as their composition and levels can affect the effector functions and the in vivo half-life of a biotherapeutic product. Glycosylations tend to be present in a highly heterogenous manner in terms of structure and abundancies, increasing the complexity of their analysis.[7-8] A robust glycopeptide mapping solution can assign the glycosylation sites, determine the compositions of the attached glycans and estimate their relative abundances.

Figure 1. The SCIEX ZenoTOF 7600 system.

EAD, a newly developed dissociation approach which is unique to the SCIEX ZenoTOF 7600 system, allows for a tunable electron energy that produces varied fragmentation patterns for a wide range of peptides in a peptide mapping workflow.[1,2] The resulting MS/MS fragment ions from glycopeptides showed peptide backbone fragment ions with glycosylation remaining intact. This allows for accurate localization of the linked glycans along with confident identification of the peptide through high MS/MS sequence coverage of the peptide backbone. Zeno EAD (Figure 1) enables fast and sensitive data dependent acquisition (DDA). This approach overcomes challenges of alternative fragmentation such as long reaction times and low sensitivity. Trastuzumab was used as an example to show how users of all levels can apply this technology for their streamlined characterization of glycopeptides, improving efficiency and understanding of their biotherapeutics.

Key features of the SCIEX ZenoTOF 7600 system

- **New depths of peptide mapping analysis:** EAD with fast DDA enables alternative fragmentation for routine, in-depth analysis of next generation protein therapeutics and standard mAbs
- **Higher levels of structural information:** Changing the mechanism of fragmentation by tuning the electron energy may provide a higher level of structural information, particularly well-suited for glycopeptide characterization
- **Higher MS/MS sensitivity:** Increased detection of fragments (5 to 10-fold) using the Zeno trap enables higher confidence in data assignment
- **High reproducibility:** Reproducible fragmentation with EAD for singly, doubly, and multiply charged ions enables analysis of more precursors than other alternative and low reproducibility fragmentation techniques
- **Streamlined and easy-to-use:** Fully automated data acquisition in DDA mode using EAD with SCIEX OS software, and automated data interpretation with Byos software (Protein Metrics Inc.) simplifies the entire user experience

Sample preparation: A sample of trastuzumab was denaturated with 7.2 M guanidine hydrochloride, 100 mM Tris buffer pH 7.2, followed by reduction with 10 mM DL-dithiothreitol and alkylation with 30 mM iodoacetamide. Digestion was performed with trypsin/Lys-C enzyme at 37 °C for 16 h.

Chromatography: 10 µl (4 µg) of the trypsin/Lys-C digest were separated with a CSH C18 column (1.7 µm particle size, 130 Å, 2.1×100 mm, Waters) using an ExionLC system. The mobile phase A consisted of water with 0.1% formic acid, while the organic phase B was acetonitrile 0.1% formic acid. A gradient profile was used at a flow rate of 300 µL/min (Table 1). The column temperature was maintained at 50°C.

Mass spectrometry: Data were acquired with an information dependent acquisition (IDA) method using the SCIEX ZenoTOF 7600 system. General method parameters were kept the same and are summarized in Table 2. Parameters specific for EAD or CID can be found in Table 3.

Table 1. Chromatography for peptide mapping analysis.

Time [min]	Mobile phase A [%]	Mobile phase B [%]
Initial	*98*	*2*
5	*98*	*2*
6	*90*	*10*
40	*55*	*45*
44	*10*	*90*
46	*10*	*90*
47	*98*	*2*
50	*98*	*2*
51	*10*	*90*
54	*10*	*90*
55	*98*	*2*
60	*98*	*2*

Data processing: Data were processed in Byos software (Protein Metrics Inc.). To achieve side by side comparison, the standard PTM workflow was modified to include two MS/MS Id Byonic processing nodes, one for CID data processing, one for EAD data processing. All other processing parameters were kept the same. Peptide identification and fragments mass tolerance were set 6 ppm and 20 ppm, respectively. The processed results were filtered to eliminate results with MS/MS scores lower than 100.

Table 2. General MS parameters.

Parameter	MS	MS/MS
Scan mode	*TOF-MS*	*IDA dependent*
Gas 1	*50 psi*	
Gas 2	*50 psi*	
Curtain gas	*35 psi*	
Source temperature	*450 °C*	
Ion spray voltage	*5500 V*	
Declustering potential	*80 V*	
Collision energy	*12 V*	*
CAD gas	*7*	
Maximum candidate ion	*15*	
Intensity threshold	*125 cps*	
Charge states	*1 to 10*	
Exclusion time	*6 s after 2 occurrences*	
Start mass	*200 m/z*	*100 m/z*
Stop mass	*2,000 m/z*	*3,000 m/z*
Accumulation time	*0.25 s*	*
Time bins to sum	*8*	*10*

*specific for EAD/CID.

Table 3. MS parameters for CID and EAD.

Parameter	CID	EAD
Collision energy	*rolling*	*12 V*
Electron KE	*NA*	*7 eV*
Electron beam current	*NA*	*5500 nA*
ETC	*NA*	*100*
Zeno trap	*ON*	*ON*
Accumulation time	*0.05 s*	*0.09 s*

Glycopeptide ID

In biotherapeutics characterization, glycosylations are usually being classified as a critical quality attribute and therefore closely monitored. Liquid chromatography mass spectrometry (LC-MS) based peptide mapping is considered to be a versatile tool for characterization of protein glycosylation, since it eliminates the need to remove the glycan from the protein, while providing very comprehensive information about the molecule sequence and other PTMs.[3,6] However, traditional CID approaches can either provide fragment information of the fragile glycans when applying low collision energies or of the peptide backbone when higher collision energies are used. Achieving both at the same time and at high quality, along with a general peptide mapping approach, remains a challenge with CID. In addition, the high energies used for CID usually result in the dissociation of the glycan structures from the peptide backbone. Therefore, identification of the peptide and exact localization of the glycan is limited, especially in the case of multiple potential modification sites in a given peptide. On the other hand, in addition to diagnostic oxonium ions of the glycans, the tunable electron energy in the SCIEX ZenoTOF 7600 system produces rich peptide backbone fragment ions and fragments with the intact glycan attached, simultaneously. These data allow for confidence in the correct identification of peptides and the accurate localization and identification of the attached glycans. Using the Zeno trap in combination with EAD allows for accurate and detailed identification of even low abundant glycopeptides due to a boost in the sensitivity of the fragments.

The most intense glycopeptides for each glycan type found in the trastuzumab digest contained one miscleavage site after R304 due to the steric hindrance introduced by glycans at N300 (Figures 2, 3 and 5). Figure 2 shows an example of a glycopeptide carrying G0F. The precursor ion and fragment ion spectra from both CID and EAD were compared side-by-side. MS data were matched with a tolerance of maximal 6 ppm. Subsequent data interpretation of MS/MS spectra included the identification of peptide fragments, oxonium ions and peptide-glycan fragments (Figure 2, top right). As seen in the spectrum, the dominant ions were oxonium ions, in the case of CID, and low abundant *b*- and *y*-ions. It should be noted that the default parameters for the rolling collision energy (CID) can be adjusted to increase the coverage of the peptide backbone as shown previously[9], however this approach usually also limits the overall MS/MS sequence coverage for other peptides. Furthermore no peptide fragments with intact glycans were detected in the case of CID. On the contrary, EAD did not only provide very comprehensive fragmentation of the peptide backbone with 100% MS/MS sequence coverage being superior to CID, but the

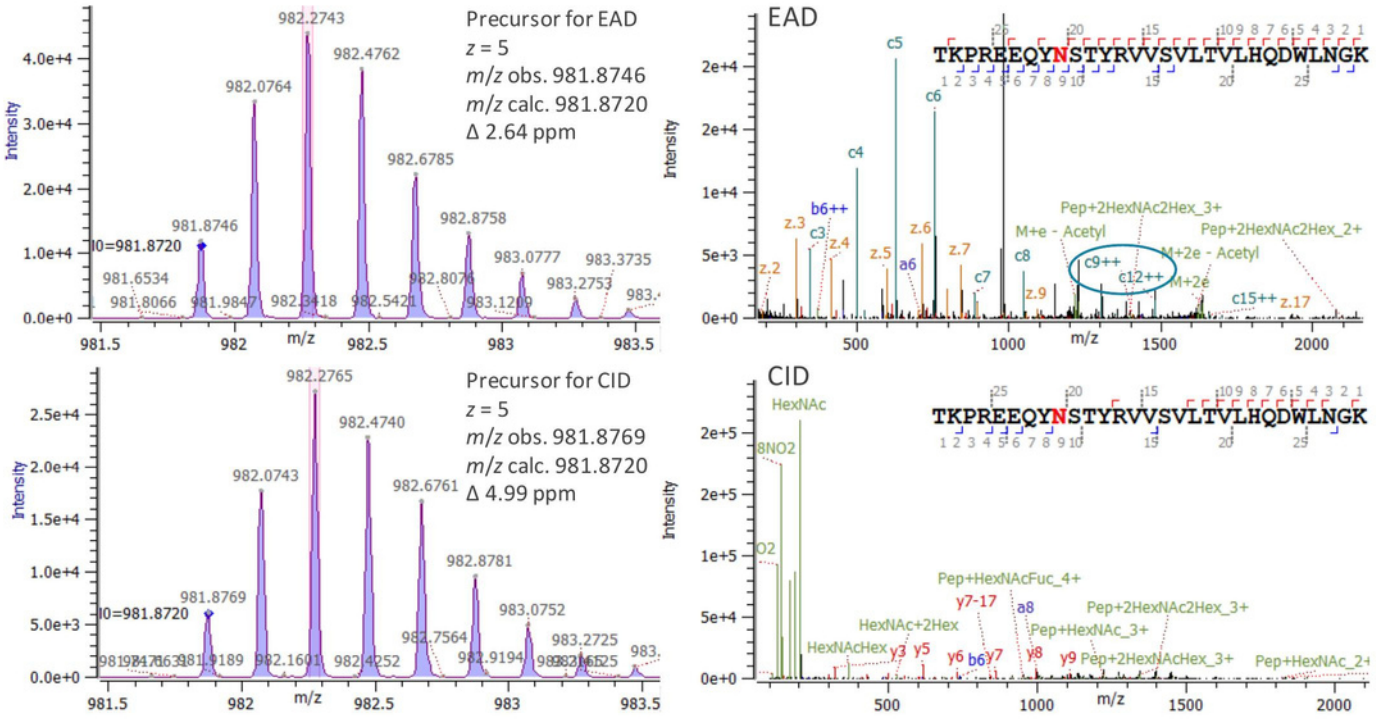


Figure 2. Side-by-side comparison of a glycopeptide fragmented using EAD and CID. Precursor ion spectra (left panel) and respective MS/MS spectra (right panel) are shown for a glycopeptide from trastuzumab carrying G0F. Blue and red hash marks depict fragment ion coverage. EAD resulted in a higher fragment coverage and better S/N for peptide backbone fragments than CID. In addition, diagnostic fragment ions confirm the localization of the glycosylation (encircled ions) in the case of EAD; whereas CID does not provide this information.

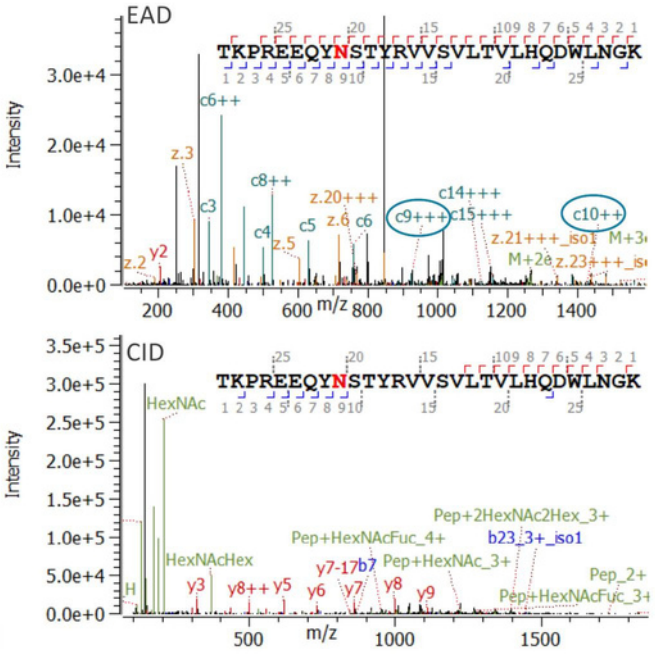


Figure 3. Comparison of glycopeptide, fragmented using EAD and CID. MS/MS spectra are shown for a glycopeptide from trastuzumab carrying G1F (precursor *z* = +6). Blue and red hash marks depict fragment ion coverage. EAD resulted in a higher fragment coverage and better S/N for peptide backbone fragments than CID. In addition, diagnostic fragment ions confirm the localization of the glycosylation (encircled ions) in the case of EAD; whereas CID does not provide this information.

doubly charged *c*9 and *c*12 ion with intact glycan also provided accurate localization of the site of modification (encircled ions in Figure 2, top right).

For the peptide with a G1F modification, a similar behavior was observed (Figure 3). Comprehensive fragmentation coverage was achieved with EAD compared to CID and ions proving the localization of the fragile modification could be detected (*c*9+++ and *c*10++ etc.). Apart from the high abundant glycosylation forms of G0F and G1F, lower abundant forms were reproducibly identified (Figure 4). One example is a high mannose species (Man5 at ~ 3%) in Figure 5. Despite its low abundance (more than 10x lower in relative abundance than the G0F-containing peptide, see Figure 4), high-quality fragment ion spectra were achieved, demonstrating the high sensitivity of Zeno EAD and Zeno CID. In addition, a full series of *z*1- *z*21 ions together with a series of *c* ions allowed for 96-100% fragment coverage for EAD, while CID only achieved 61-68% fragment coverage depending on the glycopeptide (Figures 2, 3 and 5).

For an easy review of the data, a glycan profiling report was generated. The template was formatted to sum and report the glycol forms detected in different peptide sequences (including tryptic cleaved peptides and missed cleavages) and filtered to show peptides with N-linked glycan as a single modification. The automated color coding heat map facilitates a quick understanding of which glycoforms are present in relative high, medium or low abundance, ranging from 44% to 0.2%. All N-linked glycosylations found to be present were in alignment with those previously reported (Figure 4).[10] The results demonstrate great repeatability of the Zeno EAD technology for glycopeptide analysis across different abundancies.

				MS Alias name ←	Trastuzumab EAD r(1) (%)	Trastuzumab EAD r(2) (%)	Trastuzumab EAD r(3) (%)
Protein name ↑	NGlycan ↑	Glycans ↑	Glycan Short Name ↑				
Anti-HER2 Heavy chain (1 and 2)	300	HexNAc(2)Hex(5)	Man5		3.5	3.3	3.1
		HexNAc(2)Hex(6)	Man6		0.4	0.4	0.4
		HexNAc(3)Hex(3)	G0-GlcNAc		1.4	1.4	1.6
		HexNAc(3)Hex(3)Fuc(1)	G0F-GlcNAc		6.9	6.5	7.4
		HexNAc(3)Hex(4)Fuc(1)	G1F-GlcNAc		1.5	1.3	1.7
		HexNAc(4)Hex(3)	G0		3.8	3.8	3.8
		HexNAc(4)Hex(3)Fuc(1)	G0F		44.3	44.1	42.8
		HexNAc(4)Hex(4)			1.2	1.2	1.2
		HexNAc(4)Hex(4)Fuc(1)	G1F		32.7	33.4	33.6
		HexNAc(4)Hex(5)Fuc(1)	G2F		4.0	4.1	4.2
		HexNAc(4)Hex(5)Fuc(1)NeuAc(2)	G2FS2		0.3	0.4	0.2

Figure 4. Identification of N-linked glycosylations in trastuzumab at N300. The table summarizes the identified glycan species based on MS/MS with EAD and the relative abundance based on the XIC of the MS1 for three replicate injections. The color coding indicates the abundances from high abundant (dark red) to low abundant (pastel).

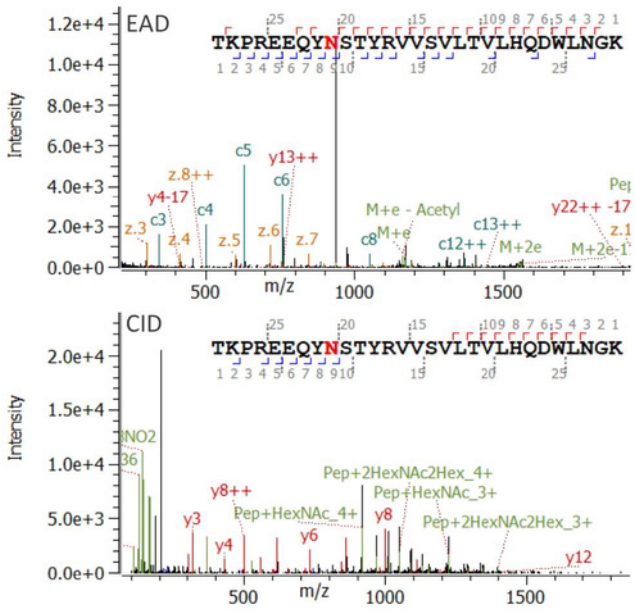


Figure 5. Comparison of glycopeptide, fragmented using EAD and CID. MS/MS spectra are shown for a glycopeptide from trastuzumab carrying Man5 (precursor *z* = +5). EAD resulted in a higher fragment coverage and better S/N for peptide backbone fragments than CID. In addition, diagnostic fragment ions confirm the localization of the glycosylation in the case of EAD; whereas CID does not provide this information.

Conclusions

- The robust, reproducible and easy-to-use alternative fragmentation mechanism EAD enables users to identify, fully characterize, and relatively quantify glycopeptides along with a general peptide mapping analysis in one single injection
- Excellent fragment coverage and localization of fragile modifications can be achieved with Zeno EAD with very high reproducibility, allowing for full confidence in peptide ID
- MS/MS fragment detection was significantly enhanced compared to traditional MS/MS analyses, enabling great data quality for confident fragment assignment even for precursors with medium or very low intensities such as modified peptides utilizing Zeno EAD
- Automatic data processing enables the routine and advanced characterization of complex biotherapeutics and standard mAbs in a reproducible manner using Protein Metrics Inc. software

References

1. Baba T et al. (2015) Electron capture dissociation in a branched radio-frequency ion trap, *Anal Chem*, **87**, 785−792.
2. Baba T et al. (2021) Dissociation of biomolecules by an intense low-energy electron beam in a high sensitivity time-of-flight mass spectrometer. *Accepted by JASMS.*
3. Comprehensive peptide mapping of biopharmaceuticals utilizing electron activated dissociation (EAD). SCIEX technical note, RUO-MKT-02-12639-B.
4. Characterization of an antibody-drug-conjugate (ADC) using electron activated dissociation (EAD). SCIEX technical note, RUO-MKT-02-12834-B.
5. Evaluation of biotherapeutic sequence coverage using electron activated dissociation (EAD). SCIEX technical note, RUO-MKT-02-1290-B.
6. A comprehensive peptide level characterization of a multispecific monoclonal antibody (mAb). SCIEX technical note, RUO-MKT-02-12975-B.
7. Gerardo Alvarez-Manilla et al (2006) Tools for glycoproteomic analysis: size exclusion chromatography facilitates identification of tryptic glycopeptides with N-linked glycosylation sites. *J. Proteome Res. 2006, 5, 701.*
8. Zhongqi Zhang et al. (2014) LC-MS/MS peptide mapping with automated data processing for routine profiling of N-glycans in immunoglobulins. *J Am Soc Mass Spectrom. 2014, 25, 999.*
9. Peptide mapping analysis of etanercept – a highly glycosylated dimeric fusion protein. SCIEX technical note, RUO-MKT-02-12653-A.
10. Ivan Sanchez-De Meloa et al. *(2015)* N-glycosylation profile analysis of trastuzumab biosimilar candidates by normal phase liquid chromatography and MALDI-TOF MS approaches. *Journal of proteomics,* **125**, 225.

Headquarters
500 Old Connecticut Path | Framingham, MA 01701 USA
Phone 508-383-7700
sciex.com

International Sales
For our office locations please call the division headquarters or refer to our website at
sciex.com/offices

Contact us

Editorial Department

Editor-in-Chief
Naamah Maundrell
n.maundrell@bioanalysis-zone.com

Business Development and Support

Head of Advertising
Dionne Murray
d.murrary@future-science-group.com

This supplement is brought to you by Bioanalysis Zone in association with

SCIEX
The Power of Precision