METHOD DEVELOPMENT FOR THE COMPREHENSIVE ANALYSIS OF POST TRANSLATIONAL MODIFICATIONS BY MASS SPECTROMETRY

by

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ABSTRACT

Signal Transduction is mediated by protein complexes whose spatial- and temporal- distribution, composition and function within cells are often regulated by different post- translational modifications (PTM). As PTMs add or subtract a specific mass difference to a protein, mass spectrometry becomes very amenable for modification analysis. These modifications have conventionally been monitored by fragmenting the modified protein or peptide by collision induced dissociation (CID) within the mass spectrometer, and then screening for the characteristic neutral fragment or fragment ion (marker ion), which is particular to the modification in question. Unfortunately, there are two major issues with respect to the traditional mass spectrometric analysis of PTMs: (1) as there are over 300 known types of modifications, the characteristic fragmentation of only a fraction of these modifications has been studied and (2) the traditional mass spectrometric approaches can only monitor these modifications sequentially, and thus comprehensive modification analysis would be unfeasible considering the breadth of PTMs. The following work aims to address these issues by (1) analyzing PTMs that have never been characterized mass spectrometrically and (2) developing a multiplexed technique for comprehensive PTM monitoring by simultaneously screening for all known characteristic fragments. With respect to the first issue, the characteristic fragmentation of lipid modifications and HNO-induced modifications was investigated. The most prevalent indicator(s) of the modification within the mass spectra are as follows: fragmentation of N-terminal myristoylated peptides produced marker ions at 240 and 268 Th, fragmentation of cysteine farnesylated peptides produced a marker ion at 205 Th and a neutral fragment of 204 Da, and fragmentation of cysteine palmitoylated peptides produced a neutral fragment of 272 Th. For HNO-induced
modifications, fragmentation of the sulfinamide- and sulfinic acid-modified peptides produced a neutral fragment of 65 Da and 66 Da, respectively. With respect to the second issue, a multiplexed technique for monitoring modifications that fragment as neutral losses, termed Multiple Neutral Loss Monitoring (MNM), has been developed, successfully validated, and then shown to be the most sensitive approach for PTM analysis. MNM, combined with a second multiplexed approach, targeted Multiple Precursor Ion Monitoring, has been used to provide a comprehensive PTM analysis.
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<td>Phosphotyrosine Binding Domain</td>
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<td>SUMO</td>
<td>Small Ubiquitin-like Modifier</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming Growth Factor Beta</td>
</tr>
<tr>
<td>TIS</td>
<td>Timed Ion Selector</td>
</tr>
<tr>
<td>tMPM</td>
<td>targeted Multiple Precursor Ion Monitoring</td>
</tr>
<tr>
<td>TOF</td>
<td>Time-of-Flight</td>
</tr>
<tr>
<td>Th</td>
<td>Thompson</td>
</tr>
<tr>
<td>TW</td>
<td>Transmission Window</td>
</tr>
<tr>
<td>UCRP</td>
<td>Ubquitin Cross-Reacting Protein</td>
</tr>
<tr>
<td>V</td>
<td>Volt</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

I would like to start off by thanking my supervisor, Dr. Juergen Kast, for all of your guidance and encouragement throughout my research. Without your input, patience, encouragement and support, this study would not have been possible. I would also like to thank Jason Rogalski for training me to use all of the mass spectrometers, ensuring that the mass spectrometers were performing at my unrealistically high standards, as well as all of your valuable insight and discussions regarding my research, as well as this thesis. It was all greatly appreciated.

I would like to thank Matt Sniatynski for all of his computational analysis of my MNM data and Geraldine Walsh for all of the biological samples/preparation. You two always made our collaborations run incredibly smoothly and always put up with my annoying requests/frustrated rants, but without all of your hard work, my research would not have been possible and for that I am truly thankful. To all of the other members of the Kast lab, both past and present, I would like to thank you all for all of your help and suggestions over the years.

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CO-AUTHORSHIP STATEMENT

The following publications have been published in support of this thesis:


The following manuscripts have been submitted for publication in support of this thesis:

The following manuscripts will be submitted for publication in support of this thesis:


Identification and design of research, performing the research, data analyses and manuscript preparation for all chapters of this thesis were performed by Michael Hoffman, unless mentioned otherwise. Biological sample preparation and purification of human platelet proteins were performed by Geraldine Walsh, as was design of the research and manuscript preparation regarding these aspects of the research and the implications of the results. Matt Sniatynski is responsible for all correlation and convolution mapping computational analysis of MNM mass spectra, as well as the design and development of these computational algorithms. Matt is also responsible for the manuscript preparation detailing exactly how the algorithm functions, as well as the discussion of other computational data mining strategies. Yves Le Blanc is responsible for writing a computational script, providing instrument control for both the QTRAP and QSTAR instruments, allowing them to perform Multiple Neutral Loss Monitoring. Yves and Eva Duchoslav also developed a computational tool for MNM data analysis. Jason Rogalski was involved in the design of many research experiments and the critical analysis of many of the chapters of this thesis. Lastly, Juergen Kast was also involved in the identification and design of this thesis, as well as all manuscript preparation.
Chapter 1: Overview

1 OVERVIEW

1.1 Proteins and Post-Translational Modifications

Innovations in genomic sequencing technologies have led to the unveiling of the human genome, generating a database toolset that has allowed for the profiling of gene expression at the mRNA level as a means of studying the cell. However, mRNA levels do not indicate changes in protein expression, degradation or modification, a task that is essential to understanding cellular functions as proteins are most often the actual biological effector molecules within a cell. Proteomics, the global analysis of proteins within the cell, is capable of such a task and, like genomics, has also been going through a technological revolution. Developments such as MALDI\(^1,2\) and ESI\(^3\) techniques for mass spectrometers, as well as other newly developed proteomics technologies, have allowed for the study of many aspects of cellular function such as signal transduction.

Signal transduction embodies the transfer of biological information through a cellular system, regulating all cellular functions, including proliferation, metabolism, gene expression, cytoskeletal organization, and apoptosis\(^4\). This intricate network of molecular interactions is mediated by protein complexes whose spatial and temporal distribution, composition and function within cells are often regulated by different post-translational modifications (PTMs), which are post-synthesis covalent modifications of the protein. More specifically, a PTM, by definition, must add or subtract a specific mass difference and not be

---

particular to any one protein (e.g. the proteolytic removal of an initiation sequence to form the final protein).

These post-translational modifications change a protein’s mass and can also change the charge, structure and conformation, which can result in changes to the protein’s enzyme activity, binding affinity and hydrophobicity. One example of a PTM that changes enzyme catalytic activity is found in receptor kinases, which catalyze the transfer of a phosphate group onto substrate proteins. These substrate proteins may be enzymes whose activities are directly affected by phosphorylation. For instance, from the interaction of TGF-β with its receptor complex, the receptor complex can then phosphorylate SMAD family proteins, activating these proteins which can now regulate target gene expression. With respect to binding affinity, modified residues within a protein can act as binding sites for a specific recognition domain on other proteins. A noteworthy example of this is that phosphorylated tyrosine residues can bind to SH2 or PTB domains. Lastly, regarding changes to hydrophobicity, protein acylation greatly increases a protein’s hydrophobicity, targeting the protein to membrane surfaces, as is the case with the myristoylation of Src kinase.

The aforementioned examples come from some of the more frequently studied types of modifications, which represent only a small fraction of the entire scope of PTMs. In the past, analysis generally focused on phosphorylation as it is heavily involved in signal transduction, but recent reviews are beginning to stress the importance of other modifications. The range of PTM’s found within the cell is vast, and varies with respect to specificity and abundance. Some types of PTMs are universally employed, can have a large number of target proteins, and can be very abundant; whereas other PTMs are very specific in their function, may only have a few target proteins (or one target protein),
and may be of very low abundance. Moreover, some PTMs occur on only one specific amino acid residue, whereas others are associated with numerous different residues. The diversity in potential modification state arises due to the fact that proteins can undergo a single modification or that they can undergo multiple and different types of modification. An individual polypeptide chain can be modified at several sites, thereby producing the possibility of many isoforms, each of which can have a distinct biological activity\textsuperscript{18}. Table 1.1 summarizes the most commonly studied post-translational modifications and their (most common) biological functions. For a more comprehensive discussion of each type of modification, the annotated references are recommended. Furthermore, this table contains only the most commonly studied PTMs, and thus for a complete list of the over 300 known PTMs\textsuperscript{16,17}, the reader is referred to those references.

A cell is not a static entity. It continuously responds to cues from its external and internal environments such as growth factors, ligands on the surface of adjacent cells, the extracellular matrix, electrical excitation, shear stress, cell-cycle checkpoints, DNA damage, oxygen tension and nutrient status\textsuperscript{18}. As many of the modifications described within Table 1.1 are reversible, they are essential for mediating cellular responses to changing conditions. Post-translational modifications are thus essential for a cell's very survival, and it should not be surprising that signaling malfunctions have been linked to a number of diseases\textsuperscript{4}. In particular, PTMs are key to understanding cancer biology as many of the critical signaling events occurring during neoplastic transformation involve protein modification. These modifications are of prime importance for studying the diagnosis and treatment of critical illness\textsuperscript{11}. In diagnosis, PTMs can be used as "biochemical footprints" for tracking and
<table>
<thead>
<tr>
<th>Post-translational modification</th>
<th>Site of PTM</th>
<th>Biological Function(s) / Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetylation(^{12, 15, 19})</td>
<td>K, N-term S</td>
<td>protein stability through the protection of the N-terminus (irreversible modification) from proteolytic degradation; reversible regulation of protein-DNA interactions through histone acetylation controlling epigenetic signaling (e.g. chromatin remodeling, changes in gene expression); regulates protein-protein and protein-ligand interactions, as well as protein function; reversible modification</td>
</tr>
<tr>
<td>Acylation(^{70})</td>
<td>C, (S,T,K)</td>
<td>reversible; cellular localization/targeting to membrane; membrane tethering, mediator of protein-protein and protein-membrane interactions; directs cytosolic proteins to membranes and simultaneously activates them (affects the activities of nuclear lamins and several regulatory proteins that interact with the inner side of cellular membranes).</td>
</tr>
<tr>
<td>• palmitoyl</td>
<td>C, (S,T,K)</td>
<td></td>
</tr>
<tr>
<td>• prenyl (farnesyl &amp; geranyl)(^{19})</td>
<td>C</td>
<td></td>
</tr>
<tr>
<td>• myristoyl</td>
<td>K, R, N-term G</td>
<td></td>
</tr>
<tr>
<td>ADP ribosylation(^{21})</td>
<td>R, (K, E)</td>
<td>• mono(ADP-ribosyl)ation involved in regulating intracellular signal transduction and immune response (reversible reaction) • poly(ADP-ribosyl)ation - regulates DNA repair and replication, transcription and apoptosis (reversible reaction)</td>
</tr>
<tr>
<td>Amidation(^{19})</td>
<td>C-term</td>
<td>protein stability through the protection of the C-terminus; key determinant in ligand-receptor interactions</td>
</tr>
<tr>
<td>(\gamma)-Carboxylation(^{19})</td>
<td>E</td>
<td>blood coagulation (e.g. carboxylation increases strength of prothrombin chelation to Ca(^{2+}))</td>
</tr>
<tr>
<td>Deamidation(^{12})</td>
<td>N(\rightarrow)D, Q(\rightarrow)E</td>
<td>possible regulator of protein-protein and protein-ligand interactions; a common chemical artifact; associated with aging by producing abnormal forms of enzymes</td>
</tr>
<tr>
<td>Glycosylation (simple)(^{22, 23})</td>
<td>S, T</td>
<td>O-HexNAc - addition and removal of the modification is key to histone remodeling, cell growth and division, gene transcription, apoptosis, proteosomal degradation; also regulates cellular responses to hormones (e.g. insulin), initiates a protective response to stress, acts as a nutrient sensor and preventing protein phosphorylation.</td>
</tr>
<tr>
<td>Glycosylation (complex)(^{24, 25})</td>
<td>S, T, N, (K, W, Y)</td>
<td>promote or inhibit intra- or intermolecular protein interactions involved in protein folding (affecting local protein secondary structure) and intracellular trafficking (participating in the sorting/directing of a protein to its final destination); heavily involved in cell-cell communication which is modulated by cell adhesion and repulsion, molecular and cellular homeostasis (e.g. the ability to recognize glycans from foreign agents such as bacteria), receptor activation (e.g. through receptor dimerization), signal transduction and endocytosis (e.g. inducing the turnover of cell-surface molecules through receptor endocytosis).</td>
</tr>
<tr>
<td>GPI (glycosylphosphatidylinositol) anchor(^{26})</td>
<td>C-term</td>
<td>GPI dependent changes in antigenicity/antibody binding; modulates protein-membrane interactions (e.g. many pathogen surface proteins are GPI anchored)</td>
</tr>
</tbody>
</table>

**Table 1.1. Commonly studied post-translational modifications and their biological functions\(^a\)**
<table>
<thead>
<tr>
<th>Post-translational modification</th>
<th>Site of PTM</th>
<th>Biological Function(s) / Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>β-Hydroxylation</strong>&lt;sup&gt;19, 25, 27&lt;/sup&gt;</td>
<td>P,K, (D,N)</td>
<td>structural stability (e.g. hydroxylation of Collagen), hypoxic sensing, blood coagulation (essential for functioning of blood factors and binding to Ca&lt;sup&gt;2+&lt;/sup&gt;)</td>
</tr>
<tr>
<td>Methylation&lt;sup&gt;26&lt;/sup&gt;</td>
<td>K,R, (H,P,D, E,C-term)</td>
<td>• Lysine Methylation - regulates chromatin structure (between heterochromatic and euchromatic regions of chromatin compaction) through specific lysine histone methylation; regulates the activation (euchromatin compaction) and repression (heterochromatin compaction) of gene transcription through specific lysine histone methylation; • Arginine Methylation - function of arginine methylation has, for the most part, yet to be determined, but has been implicated in transcriptional regulation and signal transduction reversibility of methylation is an open question (Is it an unknown enzyme, clipping or proteolysis?)</td>
</tr>
<tr>
<td>Nitric Oxide mediated PTMs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Nitration&lt;sup&gt;29&lt;/sup&gt;</td>
<td>Y,(W)</td>
<td>• nitration signifies the increased formation of reactive nitrogen species during the disease process; co-localization of nitrated proteins with apoptotic markers may play a role in the apoptosis process; possible roles of being involved in signal transduction, protein turnover and losing/gaining protein function; reversible</td>
</tr>
<tr>
<td>• nitrosylation&lt;sup&gt;30-32&lt;/sup&gt;</td>
<td>C</td>
<td>• redox based signaling and may control the activity of a variety of enzymes and regulatory proteins (transcription factors, G-proteins, ion channels, receptors and kinases), modulate energy metabolism and can inhibit or promote apoptosis; signaling molecule in the cardiovascular system; may be associated with protein damage</td>
</tr>
<tr>
<td>Nucleotidylation&lt;sup&gt;19&lt;/sup&gt;</td>
<td>Y</td>
<td>modulates enzyme activity (e.g. glutamine synthetase activity is regulated by adding/removing specific nucleotides)</td>
</tr>
<tr>
<td>Oxidation</td>
<td></td>
<td>- oxidative modifications can also be associated with protein damage - sulphenic (-RSOH) acid is a transient modification during oxidative stress; while sulphinic (-RSOOH) and sulphonic (-RSO3H) acids are generally irreversible and are associated with oxidative damage</td>
</tr>
<tr>
<td>• Amino Acid&lt;sup&gt;31, 33&lt;/sup&gt;</td>
<td>M</td>
<td>• chemical artifact; oxidative stress; a possible antioxidant as the modification is reversible; involved in ion transport processes. • associated with aging by producing abnormal forms of proteins</td>
</tr>
<tr>
<td>• Cys-Cys disulfide bond formation&lt;sup&gt;34, 35&lt;/sup&gt;</td>
<td>C</td>
<td>• reversibly modifies the protein structure; defense against oxidative damage; redox signaling of enzymes, transcription factors and transporters; excessive disulfide bond formation can produce protein misfolding and aggregation leading to cytotoxicity, both an intra- and inter-protein modification</td>
</tr>
<tr>
<td>• glutathionylation&lt;sup&gt;34, 35&lt;/sup&gt;</td>
<td>C</td>
<td>• mitochondrial and ER defense against oxidative damage; redox signaling by affecting the activity of enzymes, transcription factors and transporters; reversible</td>
</tr>
<tr>
<td>• cysteaminylation&lt;sup&gt;36&lt;/sup&gt;</td>
<td>C</td>
<td>• defense against oxidative damage; redox signaling by affecting the activity of enzymes, transcription factors &amp; transporters; reversible</td>
</tr>
</tbody>
</table>
Phosphorylation is the most common and most highly-studied method of modulating signal transduction and can be linked to virtually every type of protein function within a cell (e.g., regulating cell growth, survival, apoptosis, receptor activation and the corresponding signaling, regulating metabolic pathways and enzyme activity, cytoskeletal organization and chromatin compaction); reversible activation/inactivation of protein activity; modulates protein-protein and protein-ligand interactions.

Pyroglutamic acid is modulated protein-ligand and protein-protein interactions (e.g., involved in leukocyte adhesion, hemostasis and hemophilia, and chemokine signaling) (believed to be an irreversible modification in vivo; stoichiometric and substoichiometric degree of modification).

Sulfation involves tyrosine sulfation - modulates protein-ligand and protein-protein interactions (e.g., involved in leukocyte adhesion, hemostasis and hemophilia, and chemokine signaling).

Ubiquitylation and Ubiquitin-like protein modifiers (e.g., SUMO, NEDD, UCRP, etc.) (see ref. 30, 40) are involved in signal transduction, the mechanism for histone methylation, and the endocytosis and endosomal sorting of activated receptors. Ubiquitin-like proteins are indirectly associated with proteasomal degradation by modifying cullins, signal transmission, cell differentiation and DNA repair (NEDD); regulation of ligand-protein activity in transcription and DNA repair, nuclear localization, and inhibits ubiquityl-mediated proteasomal degradation (SUMO); involved in inflammatory and immune responses (UCRP); (see ref. 9 for more)

Ubiquitin and Ubiquitin-like proteins are reversible modifications.

a when referring to site of modification, brackets are used to indicate the less common sites of modification.
verifying key pathways and mechanisms in human disease processes. Such changes in the modification profiles of specific proteins have already been recognized as disease markers. In researching treatments, PTMs are important avenues of potential therapeutic intervention and drug discovery, with many promising agents targeting PTMs. Furthermore, the majority of protein-based pharmaceuticals currently approved or in clinical trials bear some form of post-translational modification, which can profoundly affect protein properties relevant to their therapeutic application.

1.2 Traditional Methods of PTM Analysis

Since post-translational modifications play a role in such a myriad of protein functions within the cell, comprehensive modification analysis is imperative for a complete understanding of a cell’s biology. Traditional methods of analyzing post-translational modifications include 1) immuno-based approaches, 2) modification-specific gel stains or 3) selective mass spectrometric scan strategies. Immuno-based approaches (ie. immunoblot) use an antibody which can selectively bind to the modification and/or a consensus sequence, which can then be visualized using western blot analysis. Approaches employing modification-specific stains apply a stain directly to a polyacrylamide gel where it interacts with the modification of interest, allowing for the visualization of the modified protein(s) via fluorescence. The selective tandem mass spectrometric scan strategies, both precursor ion and neutral loss scans, are generally performed on triple quadrupole instruments and rely on the fact that when a modified protein or peptide fragments during collision induced dissociation (CID) within the instrument, the modification commonly separates from the precursor ion as a characteristic marker ion or a neutral loss. Precursor ion scans on a triple
Chapter 1: Overview

quadrupole instrument are performed by scanning the first quadrupole (Q1), followed by CID in the second quadrupole (Q2), with the third quadrupole (Q3) fixed to the m/z of the characteristic marker ion; only this fragment ion is transmitted to the detector. Alternatively, neutral loss scans are performed with Q1 and Q2 configured similarly to the previous example, but with Q3 scanned with a fixed offset to Q1 — with the offset being specific to the modification and the charge state of the precursor ion. Thus, Q1 transmits the precursor ion to the collision cell and Q3 transmits the product ion of the neutral loss to the detector. Table 1.2 summarizes a selection of characteristic marker ions and neutral loss fragments of selected post-translational modifications.

The first two approaches (immunoblot analysis and modification specific stains) identify the presence of modified proteins and indirectly determine the protein’s approximate molecular weight; however, these approaches cannot determine the identity of the modified protein. In contrast, the mass spectrometric methods identify the modification as well as the exact molecular weight of the associated peptide or protein; thus they can also be used for sequence determination in the same experiment. All of these methods take a targeted approach to PTM analysis, and thus only analyze one particular type of modification. For the first two approaches, a sufficiently wide range of antibodies and/or stains is not available to allow for complete modification analysis. If this constraint were eliminated, the analysis would still be impractical as the cost, both in time and in reagents, would limit the feasibility of this approach. Furthermore, antibodies cannot be manufactured with predictable specificity, making false positive signals inevitable. Although very selective, the mass spectrometric analysis by neutral loss or precursor ion scan is also only capable of screening for one particular modification at a time, and in the case of the neutral loss scan only one
<table>
<thead>
<tr>
<th>Modification</th>
<th>Site of PTM</th>
<th>Mass Change (Da)</th>
<th>Neutral Loss</th>
<th>Marker Ion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphorylation</td>
<td>Ser, Thr</td>
<td>+80</td>
<td>80</td>
<td>-63, -97, -99</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>98</td>
<td>-99</td>
</tr>
<tr>
<td></td>
<td>Tyr</td>
<td>+80</td>
<td>80</td>
<td>-63, -97, -99</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>98</td>
<td>-99</td>
</tr>
<tr>
<td>Glycosylation:</td>
<td>S, T (O-link); N-Acetylated Hex (HexNAc)</td>
<td>+203</td>
<td>203</td>
<td>204</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-99, 55-58</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>168</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>186</td>
</tr>
<tr>
<td></td>
<td>Hexose (Hex)</td>
<td></td>
<td>+162</td>
<td>162</td>
</tr>
<tr>
<td></td>
<td>Hexoyl-N-acetyl-hexosamine (HexHexNAc)</td>
<td>+365</td>
<td>365</td>
<td></td>
</tr>
<tr>
<td></td>
<td>N-Acetylated Neu (NeuNAc)</td>
<td>+291</td>
<td>291</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Deoxyhexose (dHex)</td>
<td></td>
<td>+146</td>
<td>146</td>
</tr>
<tr>
<td></td>
<td>Complex sugar moiety</td>
<td></td>
<td>&gt;400</td>
<td></td>
</tr>
<tr>
<td>Sulfation</td>
<td>Y (S, T, C)</td>
<td>+80</td>
<td>80</td>
<td>-80</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-96, -99, 216</td>
</tr>
<tr>
<td>Farnesylation</td>
<td>C</td>
<td>+204</td>
<td>204</td>
<td>135, 149, 205</td>
</tr>
<tr>
<td>Myristoylation</td>
<td>N-term G</td>
<td>+210</td>
<td>210</td>
<td>211</td>
</tr>
<tr>
<td></td>
<td>K, R</td>
<td>+210</td>
<td>210</td>
<td></td>
</tr>
<tr>
<td>Palmitoylation</td>
<td>C</td>
<td>+238</td>
<td>238, 272</td>
<td></td>
</tr>
<tr>
<td></td>
<td>K</td>
<td>+238</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ubiquitination*</td>
<td>K</td>
<td>+113</td>
<td>114</td>
<td></td>
</tr>
<tr>
<td>Acetylation</td>
<td>K (N-term, S)</td>
<td>+42</td>
<td>126, 143</td>
<td></td>
</tr>
<tr>
<td>Methylation:</td>
<td>K</td>
<td>+14</td>
<td>98</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>+28</td>
<td>31, 70</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>+28</td>
<td>46</td>
<td></td>
</tr>
<tr>
<td></td>
<td>K</td>
<td>+42</td>
<td>59</td>
<td></td>
</tr>
</tbody>
</table>

Bold values are commonly the most intense marker ion or neutral loss, based on the literature and previous experience.

* As a whole, the ubiquitin modification is ~ 8 kDa with no characteristic neutral loss or marker ions. The marker ion is the Gly-Gly residue attached to the lysine after tryptic digestion.

Table 1.2. Characteristic marker ions and neutral loss fragments of selected post-translational modifications.
particular charge state thereof. A comprehensive characterization of each component would thus require a series of precursor ion and neutral loss scans in order to obtain sufficiently detailed modification information, which is costly in time and materials, and often impractical for the study of dynamic signal transduction events.

Traditional methods of PTM analysis are incapable of comprehensively monitoring for post-translational modifications, a task which is a necessity for truly understanding a cell's biology. As well, it is important to realize that PTMs are often transient, substoichiometric, and time- and location-specific, thus adequate speed, selectivity and sensitivity are all required for the analysis. Methods are beginning to appear within the literature which attempt to address the issues concerning both sensitivity and the breadth of PTM analysis. These methods are outlined in Illustration 1.1.

1.3 Multiplexed Modification Analysis

1.3.1 Non Mass Spectrometric Multiplexed Approaches

Within the last few years, studies involving multiplexed PTM detection methods have begun to appear in the literature, which utilize aspects of the traditional PTM analysis approaches. The first approach is essentially an immunoassay which employs either forward phase or reverse phase protein arrays (Illustration 1.2). In a forward phase array, each spot on the array contains one type of immobilized protein or antibody. The array is then incubated with a sample, such as a cell lysate, and multiple analytes from the sample are analyzed in parallel. Researchers have used this approach with immobilized phosphotyrosine, ubiquitin and acetyl lysine antibodies on a solid support, which was then incubated with fluorescently-labeled immunoprecipitated proteins, allowing for the
Illustration 1.1. Schematic of the general approaches for global PTM discovery and analysis. References that utilize these approaches are given in square brackets.
Illustration 1.2. Classes of protein microarray technology. Reverse phase arrays (top) immobilize the test sample analytes (e.g. cell lysate) on the solid phase and are then incubated with an analyte-specific ligand (e.g. antibody). Bound antibodies are detected by secondary tagging and signal amplification. Forward phase arrays (bottom) immobilize a bait molecule such as an antibody designed to capture specific analytes with a mixture of test sample proteins. The bound analytes are detected by a second sandwich antibody or by labeling the analyte directly.
simultaneous profiling of these 3 modifications in mammalian cells under different conditions. Alternatively, the reverse phase array system immobilizes an individual test sample, such as a cell lysate, to each array spot, producing an array composed of hundreds of different patient samples or cell lysates, which is then probed by antibodies for detection. This particular configuration has thus far only been used for phosphorylation, but a multiplexed PTM approach could also be performed with this approach.

Although immuno-based multiplexed strategies would help address the time issue associated with traditional immunoassays, there are still a number of challenges associated with these immuno-array approaches. The specificity of individual antibodies remains an open question, making false positive signals inevitable; antibodies cannot be manufactured with predictable affinity, making it difficult to quantitatively compare different types of modifications captured by different antibodies; lastly, large comprehensive libraries of fully characterized, specific antibodies are required for a comprehensive PTM analysis in this manner.

The second major approach employs two modification specific stains allowing for multiplexed fluorescence detection. The strategy utilizes multicolour fluorescent stains to identify phosphorylated and glycosylated proteins in a single gel, which begins to address the time issue associated with the traditional stain approach. Unfortunately, only two modification specific stains are currently available, and even if numerous different modification specific stains were available, a comprehensive PTM analysis would still be time- and sample- expensive. As well, multiplexed approaches would lose efficacy because the use of multiple stains would require the use of multiple gels, so as to minimize the possibility of interference from molecular fluorescence from the other stains.
1.3.2 Mass Spectrometric Multiplexed PTM Approaches

As described earlier, a post-translational modification adds or subtracts a specific mass difference to a protein. This aspect makes post-translational modifications very amenable to study by mass spectrometry. Precursor ion and neutral loss scans (Illustration 1.3(a-b)) provide a very selective method of analyzing a single modification; however, due to the time constraints associated with the logistics of the scans, it is infeasible to monitor a large number of different modifications. Fortunately, mass spectrometers can be operated in other configurations which allow for multiplexed modification screening. A mass spectrometer can be operated to perform an information- or data-dependent analysis (IDA or DDA) experiment whereby an MS scan is performed, and the subsequent product ion analyses to be performed depend on the intensities of the precursors in the MS scan. This configuration generally analyses the most abundant peptides within the sample and requires computational approaches to search for modifications.

Alternatively, other approaches have recently been described which rely less on computational approaches and more on novel mass spectrometric configurations that utilize either characteristic neutral losses, marker ions or both. These techniques generally operate the first mass analyzer as a high mass filter (or fully open), followed by collision induced dissociation in a collision cell, and then the second mass analyzer is scanned. An example of such an operation is given with a triple quadrupole instrument (Illustration 1.3(c)), allowing for the comparison of this configuration to the precursor ion and neutral loss scans (Illustration 1.3(a-b)).

The following techniques utilize quadrupole time-of-flight instruments to monitor for modifications: Bateman and coworkers\textsuperscript{82} alternated collecting mass spectra with and without
Illustration 1.3. The mass analyzer configurations for a) the precursor ion scan, b) the neutral loss scan and c) the general strategy of the multiplexed PTM discovery approaches.
fragmentation by using a filled collision cell with high and low collision energy, while operating the quadrupole as a high mass filter. They simultaneously identified phosphorylated peptides by means of neutral losses (Ser, Thr) and marker ions (Tyr). Once a marker ion had been detected, the mass spectrometer automatically collected product ion spectra for all of the precursor ions observed in the low collision energy spectrum, thus identifying the modified precursor ion. Alternatively, for neutral loss detection, the software made a list of suspected precursor ions minus the predefined neutral loss from the precursor ions identified in the lower collision energy spectrum. When a suspected product ion was identified in a high collision energy spectrum, a product ion analysis of the associated precursor ion was performed to confirm the neutral loss. Wrona and coworkers\textsuperscript{83} modified this technique by performing only low- and high- collision energy scans, with no data dependent product ion analysis, in their analysis of verapamil metabolites. Post-acquisition analysis looked for 3 types of verapamil metabolites: demethylation, hydroxylation and demethylation with concomitant glucuronidation, through the use of extracted ion chromatograms (XICs) at the m/z of the expected precursor masses. An ultrahigh resolution LC separation\textsuperscript{84} ensured that generally only 1 metabolite eluted at a time and thus the high collision energy scan could also be used to determine the identity of the modified metabolites. Niggeweg and others\textsuperscript{85} operated a quadrupole time-of flight instrument in MS mode using dynamic switching of the collision energy for their global precursor ion scanning mode, which allowed for the multiplexed analysis of all marker ion-generating modifications on peptides present in the MS. The higher collision energy spectra were searched for specific fragment marker ions or pattern of marker ions, and once a marker ion had been identified, the precursor ion was identified through time course correlation of the low and high energy
elution profiles, allowing for the correlation of precursor and fragment ion profiles\textsuperscript{86}. The high mass accuracy of the quadrupole-TOF instruments employed in all of these approaches largely conveyed the specificity needed for the successful identification of the characteristic marker ions and neutral losses.

Researchers have also investigated comprehensive PTM monitoring with other mass spectrometric configurations. Sullivan and others\textsuperscript{87} describe a selective method for monitoring glycopeptides by performing in-source CID followed by a low mass MS scan on a 3D ion trap. Extracted ion chromatograms of HexNAc\textsuperscript{+}, NeuAc-H\textsubscript{2}O\textsuperscript{+}, NeuAc\textsuperscript{+} and Hex-HexNAc\textsuperscript{+} were used to identify the presence of glycosylated proteins. Two issues with this approach are that it is very difficult to control fragmentation using in-source CID, and also that low mass chemical noise could interfere with the detection of these marker ions. A similar method to monitor for multiple marker ions termed Multiple Precursor Ion Monitoring (MPM)\textsuperscript{88}, was developed using a triple quadrupole instrument where Q3 has linear ion trapping capabilities\textsuperscript{89}. In this configuration, the first quadrupole was operated as a high pass filter to transmit all of the peptides into the collision cell where they were fragmented. The low mass fragment ions were then trapped in the third quadrupole and scanned out to identify the modification. MPM identifies the marker ion, but not the associated precursor ion, and consequently targeted MPM was developed to identify the associated precursor ion by using an exponentially decreasing m/z transmission window, thus transmitting a narrower m/z range of precursor ions to the collision cell for fragmentation and production of the marker ion when performing MPM\textsuperscript{90}. The reduced selectivity caused by the transmission of all precursor ions through Q1 was offset by using patterns of more than one
marker ion per modification, which was shown to significantly increase the specificity of this approach\textsuperscript{58,87}.

Operating the mass analyzer in a similar way to MPM, but instead trapping the high mass fragment ions in the third quadrupole of a triple quadrupole instrument before scanning the ions out, has recently been introduced. It is termed Multiple Neutral Loss Monitoring (MNM)\textsuperscript{91} and is a complementary method to MPM to monitor for multiple neutral losses in a single scan. The loss in selectivity due to the use of Q1 as a high pass filter was overcome by the use of several collision energies and by computational processing of the resulting data to reveal shifts in m/z values characteristic of neutral losses, thus localizing the associated peptide. The cause of false positive identifications is currently under investigation, leading to refinements in the MNM method\textsuperscript{90,92}. Due to their similar workflows, MPM and MNM can be combined in a single experiment to screen for all possible post-translational modifications (Figure1.1). As more and more neutral losses and marker ions are reported in the literature for various different modifications (see ref\textsuperscript{93} for a comprehensive summary of PTM reporter fragmentation), the importance of the fast multiplexed methods will continue to increase.

As a result of these new approaches, the comprehensive analysis of post-translational modifications on a protein now requires only two scans in an experiment: one survey scan to monitor for all modifications and to identify the modified precursor ion(s), followed by a product ion analysis to sequence the modified peptide(s). In following experiments, the analysis of modified peptides is given priority over unmodified ones, which reflects the fact that modification information is typically found in single peptides that may be of low abundance but of higher value, while unmodified peptides that are often more abundant usually convey redundant information that is only used for protein identification, unless they
Chapter 1: Overview

Figure 1.1. Comprehensive modification screening of a 1 picomole mixture of modified peptides in 250 femtomoles of a BSA digest using MPM and MNM scans with a collision energy gradient. a) Total Ion Chromatogram, b) Extracted Ion Chromatogram of the MPM analysis, c) corresponding precursor ions that would be identified by Targeted MPM, d) extracted correlation coefficients of the mass shifts corresponding to neutral losses, and e) convolution mapping analysis identifying the precursor ions.
contain information on protein truncations or splice variants. The application of these approaches for the comprehensive analysis of individual protein complex components could lead to a more complete picture of a protein's function; however, as these LC-coupled approaches require that the first quadrupole be operated as a high pass filter, the number of different ions entering the mass analyzer at any given time must be low. These methods thus require that the sample be fairly simple, and they benefit greatly from a high resolution separation and mass analysis. Conversely, novel approaches for the analysis of post-translational modifications on high-resolution FT-ICR instruments that employ electron capture dissociation (ECD), which have been shown to leave the modification site intact, are likely limited in these multiplexed analyses. Since they only allow monitoring for modifications in fragment spectra, they may again be biased towards unmodified peptides.

1.3.3 Computational comprehensive modification approaches

Alternative software-based methods have proved to be useful for comprehensive post-translational modification analysis, as no specialized instrumentation or analytical workup is necessary, and, in principle, almost any modification can be identified given high quality, high resolution MS and MS/MS data. In terms of the identification of post-translational modifications by computational methods, there are two principal methodologies underlying the most common strategies. The first methodology is an adaptation of common database search methods, in which modification-specific information is added to the database, enabling modification discovery via normal database matching. This procedure can make the databases to be searched impractically large – as a result, the techniques involved often filter the raw databases before PTM expansion to limit the final size of the database, and to accelerate the process of finding a match. The majority of computational PTM identification
strategies use this database-centric approach, and the majority of the discussion below deals with techniques and implementations from this domain. The second methodology is broader in terms of implementation, as it involves examining only the MS and MS/MS spectra themselves for evidence of modification, independent of any database. Few techniques exist at the moment which adhere to this methodology, but those that do show considerable future promise, and merit a brief discussion. This area of computational research is still very young, and as a result the techniques involved have very diverse origins and goals – some implementations of computational PTM discovery are generally targeted but poorly tested, whereas other applications grew from very specific and well-tested analytical software. This can make objective comparison in terms of utility and performance quite difficult. The following discussion aims to highlight some of the more prevalent technologies, describe their functioning in general terms, and discuss their overall suitability for use in comprehensive post-translational modification analysis.

Database search strategies are the simplest in terms of implementation, as they consist of a relatively straightforward modification of the standard database search commonly used in proteomic research to identify peptides. For this reason they were the first computational approaches used for PTM discovery, and they remain the most popular and widely deployed. Standard software-based protein identification relies on precursor mass information (from MS acquisition), fragment mass information (from MS/MS acquisition), and databases containing known or predicted peptide data. To make an identification, database sequences are processed computationally to generate theoretical fragmentation patterns (in silico fragmentation), and these theoretical fragmentation patterns are then compared to experimentally derived fragmentation spectra to identify matches, using probability-based
scoring methods or signal correlation. The proteins and peptides that can be identified using this approach are limited to the identities of the proteins and peptides stored in the database used. Occurrences of post-translational modification will usually involve a protein which has previously been identified, and the specific chemical makeup of the modifications will often also be known, or will be assumed to come from a small, restricted subset. It is therefore relatively straightforward to augment the database search space to include potential modifications by adding the expected masses of modifications to the set of peptides on which modifications might be expected. For example, adding 79.97 Da (HPO$_3^-$) to a given peptide precursor mass would represent a phosphorylated version of that peptide. An implementation of this straightforward approach is available in existing search tools such as Mascot$^{95}$, SEQUEST$^{96}$ and X!Tandem$^{97}$, which enables searching for peptides that contain fixed or variable modifications, and missed cleavage sites — necessary when dealing with modified peptides, as modifications can block enzymatic cleavage sites. Recent versions of this software can also use single and multiple neutral loss information present in the MS/MS spectrum to aid in identifying PTMs. Other expanded database approaches tailored for use in PTM analyses exist as well, such as ProSight PTM$^{98,99}$ and the VEMS tool$^{100}$. All these identification tools are based primarily on the database expansion procedure described above.

Strategies that attempt to identify post-translational modifications using an unmodified database search approach rapidly encounter problems in terms of quantity of information that must be processed, and more importantly in terms of the processing time needed to run the search routines. This occurs as a result of the combinatorial explosion of the search space that occurs when a database of protein/peptide sequences is augmented with possible modification information. In the naïve database expansion case the possible number
of peptides to consider when identifying a peptide with a single, fixed-location modification will be almost doubled in the worst case, as each existing sequence containing the modifiable residue in the database will spawn a hypothetically modified copy, with corresponding precursor and MS/MS fragment ion information. Though even a very basic database search will perform some database filtering in order to avoid carrying out *in silico* fragmentation and MS/MS spectrum comparison for each database peptide — usually by selecting peptides for analysis that have similar precursor masses to the unidentified peptide — database expansion will still bring many more peptides into consideration. Modifications with a variable location will necessitate further expansion and computation when comparing fragment ions, as the *in silico* fragmentation spectrum generated depends on the location of the modification. Considering multiple instances of such variable modifications explodes the search space even further, this causes the runtime of the search algorithms to grow exponentially, and the rate of false positives to climb to unacceptable levels. These simple database search techniques may give good results for very specific, targeted PTM searches when the identity of the modification and potential modification sites are known, as these techniques are a straightforward extension of proven computational identification approaches familiar to all proteomics researchers. However, for comprehensive PTM analysis involving many modifications, these strategies may not be appropriate.

To combat this problem, several implementations attempt to filter the databases to be used before any modified sequences are generated and added to the search space, using filtering criteria more rigorous than the precursor mass selection window typically used in database searches. These filtering criteria often make use of sequence information that can be extracted from the MS/MS spectrum by itself, such as peptide sequence tag masses, or short
stretches of sequence discovered using de novo sequencing. Peptide sequence tags are characteristic combinations of residue masses that can be extracted easily from a peptide MS/MS spectrum, and used to pare down the number of potential database peptide sequences that may match, and can even uniquely identify a peptide. One approach that uses this type of database filtering uses sequence tags associated with specific peptide sequences and specific PTM types, implemented in software as an *in silico* precursor ion scan. Another popular tool uses extracted sequence tags merely to reduce the size of the database to be expanded and searched, and then speeds up these latter steps by using specialized data structures for searching and matching, and custom scoring algorithms that incorporate information about characteristic sequence-specific fragments to create more robust and accurate matches.

The lack of successful de novo sequencing applications capable of identifying proteins and peptides on par with database searches attests to the difficulty of the de novo sequencing problem. However, these de novo approaches work well for identifying short stretches of sequence within peptides, similar to characteristic sequence tag masses. The database to be searched may then be filtered so that only peptides containing these short sequence stretches are augmented with modification information, and subsequently searched. This type of database/de novo fusion is found in applications such as SPIDER, which uses an improved algorithmic approach to compare de novo-identified sequence stretches with database peptides. This approach is also used in the well known OpenSea alignment algorithm. This algorithm constructs short stretches of peptide sequence de novo, and then aligns them against database sequences. This alignment procedure is conceptually similar to that employed when aligning genomic sequences of organisms against each other, only in
this case the differences between matching sections of peptide sequence represent the shift in mass produced by a chemical modification, rather than a substitution or deletion of a DNA base pair over evolutionary time.

One further method by which databases may be filtered to enable searches for modifications involves making the assumption that peptide modification occurs sub-stoichiometrically – meaning that if a modified peptide is present in a proteomics experiment, it is assumed that its unmodified counterpart is also present. The database search can then use a two pass strategy, where an initial pass selects database peptides that match unmodified peptides in the analyzed sample. This much smaller filtered group may then be augmented with PTM information, and further matching may determine whether the modified forms of these peptides are also present. The modificomb tool takes this approach\textsuperscript{106}, referring to the modified/unmodified peptide pair as a “base/modified” pair. This tool can incorporate elution time differences between the base/modified peptides to gain additional identification confidence.

The second principal methodology introduced above involves focusing on the MS and MS/MS spectra themselves, and examining them for evidence of modification independently of sequence databases. There are fewer published techniques that take this approach, and in general these approaches are of recent development and not yet widely used. The best characterized of these involves aligning mass spectra to each other (Illustration 1.4(a)) – not only does this enable the search for any combination of PTMs simultaneously (even previously unknown ones), but it also scales linearly with the length of the peptide, allowing very large numbers of PTMs to be included in each search. Elegant instantiations of this approach\textsuperscript{107,108} have shown promise in accurately identifying large
Illustration 1.4. a) Spectral alignment and b) spectral correlation can be used to identify mass shifts present between spectra that indicate the presence of post-translational modifications. In spectral alignment, corresponding peak pairs from each spectrum are aligned with each other, and the mass of modifications is revealed by the length of broken sections (dotted line) that join sections of perfect alignment (solid diagonal lines). In contrast, spectral correlation shifts both entire spectra relative to each other, and looks for shift values at which the calculated correlation coefficient is maximized. These offset correlation maxima may be due to modifications.
numbers of modifications in biological samples, and can be used for exploratory work, since the identities of the potential modifications need not be known \textit{a priori}. This approach has recently been used to annotate the proteome of \textit{Shewanella oneidensis} MR-1\textsuperscript{109}.

Since many modifications are present in mass spectrometry data in both modified and unmodified forms, characteristic information may be present in the form of a mass shift signal. A unique approach to PTM detection filters MS spectra for the presence of a given mass shift, highlighting peaks in a spectrum that belong to a modified/unmodified pair or a modified/modified - neutral loss pair\textsuperscript{110}, and is illustrated in Illustration 1.4b. This approach requires only a few consecutive spectra correlation scans, allowing it to be implemented as a spectrum-level filter in many automated data processing pipelines. Such an approach is used in concert with specialized quadrupole operation in the Multiple Neutral Loss Monitoring technique discussed above.

The performance of computational PTM discovery tools is heavily dependent on the quality of the data used as input. As the resolution and reproducibility of mass spectrometry data continues to improve, and as availability of computing power continues to grow, these tools will have an increasingly important role to play, especially those capable of performing full scale comprehensive analyses that are costly or impossible to implement in hardware.

1.4 Thesis Objectives

The following work aims to make contributions to this research field by addressing two aspects of the mass spectrometric analysis of post-translational modifications: (1) analyzing PTMs that have not been fully characterized mass spectrometrically and (2) developing a multiplexed technique for comprehensive PTM monitoring that can simultaneously screen for all known characteristic neutral losses and marker ions.
1.5 References

(24) Ohtsubo, K.; Marth, J. D. Cell. 2006, 126, 855-867.
2 MASS SPECTROMETRIC CHARACTERIZATION OF LIPID-MODIFIED PEPTIDES FOR THE ANALYSIS OF ACYLATED AND PRENYLATED PROTEINS (MS CHARACTERIZATION OF LIPID-MODIFIED PEPTIDES)\textsuperscript{a}

2.1 Introduction

Lipid modifications of proteins have been implicated in a wide range of functions within the cell ranging from cellular localization and targeting signals to membrane tethering to being a mediator of protein-protein interactions\textsuperscript{1}. Currently, there are three main different types of lipid groups that have been shown to be attached to proteins within the cell: myristoylation (C\textsubscript{14}H\textsubscript{28}O), palmitoylation (C\textsubscript{16}H\textsubscript{30}O) and S-prenylation (farnesyl (C\textsubscript{15}H\textsubscript{25}) or geranylgeranyl (C\textsubscript{20}H\textsubscript{33}) groups). There are two different types of naturally occurring myristoylated proteins: the more common N-terminal myristoylation of glycine and the rare myristoylation of lysine or arginine. The N-terminal myristoylation of glycine occurs in vivo via a two step process in proteins containing the sequence Met-Gly-X. The first step involves the enzymatic removal of the methionine by methionine amino-peptidase, which is then followed by the addition of the myristoyl moiety to glycine through an amide bond, catalyzed by the enzyme N-myristoyltransferase\textsuperscript{2}. Protein myristoylation has been shown to be responsible for a number of functions within the cell including stabilizing three-dimensional protein conformation\textsuperscript{3,4}, membrane binding which mediates virus particle formation\textsuperscript{5-8} as well as membrane targeting which selectively directs proteins to the membrane surface\textsuperscript{9-12}. Furthermore, myristoylation has a central role in oncogenesis as a number of myristoylated proteins are oncogene products\textsuperscript{12}.

Protein prenylation, both farnesylation (a 15-carbon isoprenoid) and geranylgeranylation (a 20-carbon isoprenoid), occurs at the carboxyl terminal cysteine residues of proteins and is involved in membrane interactions. The CAAX sequence at the carboxyl end of proteins (where A is an aliphatic amino acid and X is any amino acid) is generally the substrate for protein farnesyltransferase which catalyzes the reaction of farnesyl pyrophosphate with cysteine leading to the addition of a farnesyl moiety via a thioether bond, followed by the subsequent removal of the AAX motif. Farnesylation plays an important role in membrane association as well as mediating protein-protein interactions. Since the oncogenic transformation activity of H-Ras (as well as N-Ras and K-Ras), a small GTPase that is involved in many different cell signaling networks and has been implicated as an oncogene in a large percentage of carcinomas, is dependent on its farnesylation, the development of farnesyl transferase inhibitors has been of particular interest.

Cysteine palmitoylation is believed to generally occur either by palmitoyl-CoA spontaneously S-acylating cysteinyll thiols or through an enzymatic mechanism that utilizes a protein acyltransferase which attaches the palmitoyl moiety to the cysteine. Palmitoylation of proteins is a contributing factor to a protein’s association with membrane surfaces, as well as playing an important role in subcellular trafficking of proteins between, and within, membrane organelles, and modulating protein-protein interactions. As protein palmitoylation is reversible, it also provides mechanisms for regulating the functional activities of integral and functional membrane proteins.

Research into the analysis of lipid-modified peptides by mass spectrometry in proteomics has largely been overshadowed by MS research into glycosylation and phosphorylation. For the analysis of myristoylated peptides, a number of investigators have
Chapter 2: MS characterization of lipid modified peptides

previously used MALDI\textsuperscript{24} or FAB\textsuperscript{25,26} Mass Spectrometry; however, neither neutral losses nor marker ions for this modification were reported. Chen and coworkers\textsuperscript{27} described the neutral loss of 210 Da (C\textsubscript{14}H\textsubscript{26}O) for the rare type of myristoylation; however, they showed that, in most cases, the neutral loss was formed in low abundance as the peak corresponding to the product ion was of very low intensity. Jedrzejewski and Lehmann\textsuperscript{28} listed three fragment ions containing the myristoyl group from the fragmentation of an N-terminal glycine myristoylated peptide in their study of modified peptides, but did not show the corresponding product ion spectra. In the analysis of farnesylation, Anderegg and others\textsuperscript{29} found a mass shift of 204 in their FAB MS/MS analysis of mating hormone a-factor; however, no marker ions were identified. A more recent study attributed a mass shift of 204 Da observed on prominent fragment ions upon low energy CID of a farnesylated protein to the elimination of the farnesyl moiety from the molecule. As well, they identified the charged fragment ion of m/z 205 corresponding to a farnesyl moiety with a proton\textsuperscript{30}. Lastly, in the analysis of palmitoylation, researchers have used the mass shift in the MS spectra, identifying the modified from the unmodified protein or peptide, as well as the mass shift in the MS/MS spectra to identify the cysteine palmitoylation\textsuperscript{31} and the rare N-terminal glycine palmitoylation\textsuperscript{32}. As well, a marker ion was found for the rare lysine palmitoylation, although it is specific only for lysine palmitoylation\textsuperscript{33}. Other marker ions have been identified for triply-palmitoylated cysteine\textsuperscript{34}; however, as the connectivity is different from a singly-palmitoylated cysteine, these ions cannot be used as a general means of identifying the modification. Here, we investigate the formation of both marker ions and neutral losses in the following singly-modified peptides: N-terminal glycine-myristoylated, cysteine-
farnesylated and cysteine-palmitoylated peptides using different ionization sources in order to determine the most efficient method for the identification of these modifications.

2.2 Experimental

2.2.1 Reagents

The peptides GAPVPYPDPLEPR and MHRQETVDCLK-NH$_2$ were provided as a gift from the Biomedical Research Centre. Peptide EKPLQNFTLCFR-NH$_2$ was purchased from Bachem (Bubendorf, Switzerland). Myristoyl chloride, palmitoyl chloride, farnesyl bromide, $\alpha$-cyano-4-hydroxy cinnamic acid, and pyridine were purchased from Sigma Aldrich (St Louis, MO. USA). Formic acid, acetonitrile, ethanol, and trifluoroacetic acid (TFA) were purchased from Fisher Scientific (Whitby, Ont., Canada).

2.2.2 Peptide Synthesis

The synthesis of the myristoyl-modified peptide was based on the methods of Towler and Glaser (1986)$^{35}$ and Chen and others (2004)$^{27}$ and modified as follows: myristoyl chloride was diluted in pyridine to give a $10^{-3}$ M solution which was mixed with the lyophilized peptide (GAPVPYPDPLEPR) in a 1:50 molar ratio (peptide to myristoyl chloride). The reaction mixture was shaken at 300 rpm for 18 hours in the dark at room temperature. The sample was then evaporated to dryness and resuspended in 50% acetonitrile/5% formic acid. The method used to synthesize a palmitoylated peptide is based on Yousefi-Salakdeh and others (1999)$^{36}$: Palmitoyl chloride was diluted in TFA to a final concentration of $2 \times 10^{-2}$ M. A 100-fold molar excess of palmitoyl chloride solution was added to the lyophilized peptide (MHRQETVDCLK-NH$_2$ and EKPLQNFTLCFR-NH$_2$). After 10 minutes, the reaction was quenched with 80% ethanol. The sample was then
evaporated to dryness and resuspended in 50% acetonitrile/5% formic acid. The same process was carried out to synthesize the farnesylated peptide (MHRQETVDCLK-NH₂ and EKPLQNFLTLCFR-NH₂) using farnesyl bromide, except the mixture was kept at 0°C for 2 hours. The reaction was quenched with 80% ethanol. The sample was then evaporated to dryness and resuspended in 50% acetonitrile/5% formic acid. All solvents were anhydrous in order to minimize the hydrolytic degradation of the acid chlorides/alkyl bromides.

### 2.2.3 Mass Spectrometry

Analysis of the lipid-modified peptides was performed on a electrospray ionization hybrid triple quadrupole/linear ion trap (2000 Q TRAP) instrument, a electrospray ionization quadrupole time-of-flight (Q STAR-XL) mass spectrometer, both in nanospray mode, and on a MALDI-TOF-TOF 4700 Proteomics Analyzer (all Applied Biosystems/MDS Sciex, Concord, Ont., Canada). All analyses were performed with 1-5 μM solutions of the peptides. Electrospray experiments were performed using collision energies between 30 and 180 eV and collision gas pressures of 3-4 x10⁻⁵ torr with nitrogen as the collision gas. MALDI-TOF-TOF experiments were performed with a collision energy of 1000 eV at collision gas pressures of 1-2x10⁻⁶ torr (CID on mode) and 2x10⁻⁸ torr (CID off mode) using atmospheric gas as the collision gas.

### 2.3 Results and Discussion

#### 2.3.1 Synthesis

Three different types of lipid modifications have been synthesized, with the structure of these modifications displayed in Illustration 2.1. The N-terminal glycine myristoylation (Illustration 2.1(a)) reaction produced the singly-modified product with no side products,
Illustration 2.1. Chemical structure of lipid modified peptides: a) N-terminal glycine-myristoylated, b) cysteine-farnesylated, and c) cysteine palmitoylated peptides.
although higher reactant concentrations were necessary to increase the target product yield compared to the method by Chen and others. For cysteine farnesylation (Illustration 2.1(b)), the less reactive farnesyl chloride was initially used with a variety of different reaction temperatures, reaction times, and solvents; however, the target product was formed in a very low yield with a plethora of side reactions occurring. For this reason, farnesyl bromide was used as it is far more reactive, thus allowing for the use of very mild reaction conditions, which provided a very selective synthesis. Cysteine palmitoylation (Illustration 2.1(c)) resulted in a low yield of the singly-modified peptide (where the modification was on the cysteine) and a very minor side product of doubly-palmitoylated peptide which was the result of palmitoylated cysteine and threonine. A variety of reaction temperatures, reaction times, solvents, and concentrations of palmitoyl chloride were looked into in order to try to achieve the most efficient synthesis. The yield was found to be influenced by the peptide as MHRQETVDCLK-NH$_2$ reacted with a 75-100% yield while EKPLQNFILCNR-NH$_2$ reacted with a 10-50% yield, while being synthesized under the same conditions.

2.3.2 Myristoylation

As seen previously, as well as within our research, the formation of a neutral loss of 210 Da from a peptide that is N-terminally myristoylated on the glycine tends to be very rare in ESI mass spectra. The product ion mass spectrum of the doubly-charged N-terminally myristoylated peptide shows no neutral losses from the intact precursor ion or any of the doubly-charged fragment ions (Figure 2.1(a)). Furthermore, there are no singly charged b-ions that show a mass shift of 210 Da. Since the N-terminal glycine myristoylation is the most common form of the modification, a neutral loss scan would not identify the majority of myristoylated peptides. However, in our analysis of myr-GAPVPYPDPLEPR, we have
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Figure 2.1. Product ion analysis of peptide myristoylation myr-GAPVPYPDPLEPR: a) ESI QqQLT Enhanced Product Ion Spectrum of doubly charged precursor ion taken at a CE of 60 eV. b) low mass region of doubly charged precursor ion enhanced product ion mass spectrum, c) low mass region of the Enhanced Product Ion Spectrum of the unmodified peptide taken at a CE of 60 eV, d) MALDI-TOF-TOF product ion spectra of the modified precursor ion with the corresponding enlarged e) low mass region taken at a collision gas pressure of $2 \times 10^{-7}$ torr.
found two marker ions: \( a_1 = 240.2 \), \( [\text{CH}_3(\text{CH}_2)_{12}\text{CONHCH}_2]^+ \) and \( b_1 = 268.2 \), \( [\text{CH}_3(\text{CH}_2)_{12}\text{CONHCH}_2\text{CO}]^+ \) in the low mass region of the product ion mass spectrum of the modified peptide (Figure 2.1(b)) that are absent in the same region in the product ion mass spectrum of the unmodified peptide (Figure 2.1(c)). Jedrzejewski and Lehmann had previously listed these ions, as well as an ion corresponding to a protonated myristoyl fragment ion\(^{28}\); however, the intensity of the 211 Th ion was less than 5% that of the intensity of the \( a_1 \) and \( b_1 \) ions in the Q-TOF analysis (data not shown) and could not be identified when analyzed by the triple quadrupole/hybrid linear ion trap (Figure 2.1(c)).

Identification of all of the low mass ions in Figures 2.1(b) and 2.1(c) were confirmed by MS/MS/MS analysis (data not shown). When combined in the analysis, the \( a_1 \) and \( b_1 \) marker ions provide a very selective as well as sensitive method for the analysis of N-terminal glycine myristoylation, as there are a few amino acid combinations that would generate fragment ions for one, but none that would generate fragment ions for both m/z values (Table 2.1 and 2.2). It was expected that a triply-charged precursor ion would fragment to produce a greater abundance of these marker ions; however, the MS spectra (data not shown) indicated that the peptide was predominantly doubly-charged and thus, this could not be verified.

The MALDI-TOF-TOF product ion spectrum (Figure 2.1(d)) of the singly charged peptide illustrates the effect of proline on the fragmentation. The \( y_2 \)-and \( y_5 \)-ions are enriched in the spectra, as are the internal fragments that contain proline, as they fragment to produce an ion with an N-terminal proline. This phenomenon was also seen previously in the electrospray analysis (Figure 2.1(a)) with the \( y_2, y_5, y_7, y_9 \) and \( y_{11} \) ions being enriched in the mass spectrum and this has been rationalized previously\(^ {41}\). The neutral loss from the
### Fragment Ions

<table>
<thead>
<tr>
<th>Fragment Ions</th>
<th>Elemental Composition</th>
<th>m/z (Da)</th>
</tr>
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<tr>
<td>b$_2$-H$_2$O (QE); b$_2$-NH$_3$ (QQ)</td>
<td>C$<em>{16}$H$</em>{14}$N$_4$O$_4$</td>
<td>240.098</td>
</tr>
<tr>
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<td>C$<em>{14}$H$</em>{14}$N$_4$O$_3$</td>
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<tr>
<td>b$_2$-H$_2$O (KE); b$_2$-NH$_3$ (QK), b type (APA); a type - NH$_3$ (QGV, L/ING, NVA); a type - H$_2$O (VEG, L/IDG, ADV, SPT)</td>
<td>C$<em>{14}$H$</em>{16}$N$_2$O$_3$</td>
<td>240.134</td>
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<tr>
<td>b$_2$-H$_2$O (TR), a$_2$-H$_2$O (ER), a$_2$-NH$_3$ (QR), a type - NH$_3$ (RAG)</td>
<td>C$<em>{14}$H$</em>{16}$N$_2$O$_2$</td>
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<tr>
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<tr>
<td>b$_2$-NH$_3$ (KK); a type (GL/IP, AVP); a type - NH$_3$ (KGV); a type - H$_2$O (TL/IA, VVS)</td>
<td>C$<em>{12}$H$</em>{22}$N$_2$O$_2$</td>
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<td>myr-G a$_4$</td>
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Table 2.1. Fragment Ions, Elemental Composition and exact masses of all possible a-, b- and y- type ions of unmodified peptides with a nominal mass of 240 Da.
### Table 2.2

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<td>b₂-NH₃ (HF)</td>
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<td>b type - H₂O (GMP)</td>
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<tr>
<td>b type - NH₃ (QGV, L/ING, NVA); b type - H₂O (VEG, L/IDG, ADV, SPT); a type - NH₃ (QPS, PNT); a type - H₂O (EPS, DTP)</td>
<td>C₁₂H₁₆N₄O₄⁺</td>
<td>268.129</td>
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<td>b₂-NH₃ (QR); b₂-H₂O (ER); b type - NH₃ (RAG); a type (GHT, SHA, NQA); a type - H₂O (GQQ)</td>
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<tr>
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<tr>
<td>b type (GL/IP, AVP); b type - NH₃ (KGV); b type - H₂O (TL/IAA, VVS); a type (PPT); a type - NH₃ (QL/IA, KPS, NVV); a type - H₂O (EL/IA, VDV)</td>
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<tr>
<td>myr-G b₁</td>
<td>C₁₆H₁₆NO₂⁺</td>
<td>268.227</td>
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Table 2.2. Fragment Ions, Elemental Composition and exact masses of all possible a-, b- and y- type ions of unmodified peptides with a nominal mass of 268 Da
Chapter 2: MS characterization of lipid modified peptides

precursor ion at a m/z of 1408.0 Th is present with a signal to noise ratio of 3, while it was absent in the electrospray mass spectra. The $a_1$ and $b_1$ marker ions decreased in intensity, while the protonated myristoyl fragment ion at 211 Th is now formed (Figure 2.1(e)). The $a_1$ and $b_1$ ions are observed in both the ESI and MALDI analysis because it is believed that these ions sequester the charge on the Nitrogen, producing the corresponding iminium ion and oxazolonium ion respectively.\textsuperscript{39,42} The protonated myristoyl fragment ion is only seen in the MALDI analysis as a much greater collision energy is required to form the less stable acylium ion. These results may be used to argue that low collision energies favour the formation of the oxazolonium ion over the acylium ion for b-ions.

As three marker ions are now observed, the selectivity of the analysis increases; although the relative intensity of the three marker ions is lower in the MALDI analysis compared to the ESI analysis. In the product ion spectra collected at a collision gas pressure of 2x10\textsuperscript{-8} torr, the neutral loss becomes more intense with a signal to noise ratio of 6, while none of the marker ions were observed (data not shown). The decrease in intensity of the marker ions in the MALDI-TOF-TOF analysis, compared to the ESI analysis, results from the low charge state of the ionized peptide that is characteristic of MALDI spectra. This results in competition for the single charge and since the C-terminal arginine has a much higher gas phase proton affinity, the proton will be sequestered on the arginine containing fragment ions (as well as on the proline fragment ions), which was observed in the spectra.

An in-depth analysis of the two marker ions observed in Figure 2.1(b) was then completed to confirm their identity. Figures 2.2(a) and 2.2(b) are high mass accuracy product ion mass spectra from a quadrupole TOF that are used to resolve nominal mass interfering ions. The $b_1$ marker ion was found at a m/z of 268.181 Th compared to the
Figure 2.2. Characterization of the N-terminal glycine-myristoylated marker ions. Q-TOF Product ion spectra of the doubly-charged myr-GAPVPYPDPLEPR peptide with a) the \( b_1 \)-ion (268 Th), and b) the \( a_1 \)-ion (240 Th) taken at a collision energy of 60 eV. MS/MS/MS analysis of the doubly charged myr-GAPVPYPDPLEPR c) \( b_1 \)-ion (collision energy of 60 eV for MS/MS, excitation energy of 50 V for MS/MS/MS) and d) \( a_1 \)-ion (collision energy of 60 eV for MS/MS, excitation energy 125 V for MS/MS/MS), e) collision energy profiling of the \( a_1 \)- (x) and \( b_1 \)- (o) marker ions produced from the enhanced product ion spectra of the doubly-charged myr-GAPVPYPDPLEPR. Collision energies of 50 to 180 eV in 10 eV increments were used.
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theoretical monoisotopic mass of 268.227 Da (Figure 2.2(a)). The \( a_1 \) marker ion was found at a m/z of 240.187 Th compared to the theoretical monoisotopic mass of 240.232 Da (Figure 2.2(b)). As seen from these spectra, the majority of theoretical nominal mass interferences can be resolved from the two marker ions. Figures 2.2(c) and 2.2(d) are MS/MS/MS spectra on a triple quadrupole hybrid linear ion trap instrument which are used to provide structural information on the marker ions. The MS/MS/MS experiment on the 268 Th fragment (Figure 2.2(c)) confirms that the peak at m/z 268 is the \( b_1 \) fragment ion as it fragments to almost exclusively produce the \( a_1 \) fragment ion at a m/z of 240 Th. The fragmentation seen in the MS/MS/MS analysis of the \( a_1 \) ion (Figure 2.2(d)) is more difficult to interpret, with the two major products most likely being \((\text{CH}_2)_8(\text{C}=\text{O})\text{NHCH}_2^+\) at a m/z of 169.2 Th and \((\text{CH}_2)_6(\text{C}=\text{O})\text{NHCH}_2^+\) at a m/z of 141.1 Th, due to fragmentation within the alkane chain. This is reasonable as the requirement of a much higher excitation energy to fragment the ion will lead to the formation of some less stable products.

Collision energy profiling of the two marker ions was then performed by collecting product ion spectra of the doubly charged precursor ion at collision energies of 50 to 180 eV in 10 eV increments (Figure 2.2(e)). Five cycles of the collision energy profiling were performed. The intensities from each cycle were normalized to the most intense data value in the set, the five data sets were averaged, then plotted. Collision energy profiling was performed in order to determine the conditions necessary for monitoring for the marker ions as well as to understand the formation and degradation of the marker ions. As the collision energy increased from 50 to 70 eV, there is a drastic increase in the formation of the marker ions as the precursor ion begins to fragment. As the collision energy increased from 80 to 110 eV, the \( a_1 \)-ion reached a maximum in intensity and then slowly began to fragment, while
the b$_1$-ion continues to be formed; albeit the rate of its formation begins to decrease. It is surprising that the a$_1$-ion reached a maximum intensity prior to the b$_1$-ion as the a$_1$-ion is formed from the fragmentation of the b$_1$-ion. This can be rationalized as the a$_1$-ion has been shown to be produced through alternative fragmentation pathways, such as b$_2$ to a$_1$ fragmentation$^{42}$. As well, the oxazolonium ion is a very stable species. The greater abundance of the b$_1$ (compared to the a$_1$) ion can be rationalized in that b-ions are generally more readily formed as the amide bond of the peptide backbone is generally the most labile bond, and thus the b$_1$-ion is formed with a greater abundance. Continuing to increase the collision energy from 120 to 180 eV, both the a$_1$ and b$_1$ intensities decrease with the b$_1$ ion fragmenting at a higher rate. The a$_1$ ion degrades at a slower rate as the b$_1$ ion fragments to create the a$_1$ ion (shown previously in Figure 2.2(c)). Lastly, it should be noted that although b$_1$ and a$_1$ ions are generally absent as backbone fragmentation occurs through the oxazolone mechanism, these ions are sufficiently enriched in product ion spectra of peptides that are modified at the N-terminus as they contain the structural element necessary for b$_1$-ion formation via five membered ring attack$^{39,40}$. With the enrichment of both the a$_1$ and b$_1$ ions in N-terminally glycine-myristoylated peptides, we propose the use of both the a$_1$ (240.2) and b$_1$ (268.2) marker ions for the detection of this modification when using electrospray as the ionization source.

### 2.3.3 Farnesylation

A recent study attributed a mass shift of 204 Da on prominent fragment ions upon low-energy CID of a multiply-charged farnesylated protein generated by electrospray ionization to the elimination of the farnesyl moiety from the molecule. They also noted that in the low mass region of the mass spectrum there was an ion of m/z 205.2 characteristic of
the farnesyl moiety, as well as attributing peaks at m/z 137.1 and m/z 149.1 to isoprenyl fragment ions. Fragmentation of the singly- (Figure 2.3(a-b)), doubly- (Figure 2.3(c-d)) and triply-charged (Figure 2.3(e-f)) precursor ions can be seen in the product ion spectrum. It is evident that all three peptide charge states fragment to produce the marker ion at m/z 205 as well as show a neutral loss of 204 Da from the precursor ion; however, the formation of these characteristic fragments does depend on the charge state of the precursor ion. The MALDI-TOF-TOF analysis (Figure 2.3(a-b)) clearly shows the formation of the neutral loss of 204 Da from the singly-charged precursor ion as well as from the b_{12} fragment ion in EKPLQNFTLC(far)FR-NH$_2$ (Figure 2.3(a)). Similarly, a very intense neutral loss is observed in the MALDI-TOF-TOF spectra of MHRQETVDC(far)LK-NH$_2$ (Figure 2.3(b)). The marker ion at m/z 205 is barely visible in the analysis of EKPLQNFTLC(far)FR-NH$_2$ (Figure 2.3(a)) as the peak has an intensity with a signal/noise ratio of 3, while it is more prominent in the analysis of MHRQETVDC(far)LK-NH$_2$ (Figure 2.3(b)). Furthermore, no additional modification-specific fragment ions were observed. In the product ion spectra collected at a 100-fold higher collision gas pressure of 2x10$^{-6}$ torr (data not shown), similar intensities of both the marker ions and neutral losses were obtained. The neutral loss is favoured in the MALDI spectra as a result of the low availability of protons due to the single charge and the modification’s low proton affinity.

The doubly-charged precursor ions generated by electrospray ionization (Figure 2.3(c-d)) fragment to produce more of the farnesyl marker ion and less of the neutral loss (compared to the MALDI analysis). Unfortunately, there are two possible charge distributions as the farnesyl moiety can fall off of the precursor ion without a charge, leaving a doubly-charged product ion as seen with the [M+2H-far]$^{2+}$. Alternatively, the precursor ion
Figure 2.3. Characterization of the fragmentation of different charge states of farnesylated peptides. MALDI-TOF-TOF product ion spectrum of singly-charged a) EKPLQNFTLC(far)FR-NH₂ and b) MHRQETVDC(far)LK-NH₂ taken at a collision gas pressure of $2 \times 10^{-8}$, ESI QqQ LT Enhanced Product Ion Spectrum of doubly- and triply charged c), e) EKPLQNFTLC(far)FR-NH₂ and d), f) MHRQETVDC(far)LK-NH₂ taken at a CE of 60 eV.
can fragment to produce a fragment ion pair where the farnesyl moiety retains one charge and the residual product ion [M+H-far]$^+$ retains the remaining proton. As the ratio of [M+H-far]$^+$ and [M+2H-far]$^{2+}$ is approximately 1:1, both fragmentation pathways appear to be equally favourable in the fragmentation of the doubly-charged peptide.

The triply-charged precursor ion (Figure 2.3(e-f)) obtained by electrospray ionization fragments with a similar alternative charge distribution that was seen for the doubly-charged precursor ion. The major fragmentation products appear to be the formation of the farnesyl marker ion and the associated ion pair [M+2H-far]$^{2+}$, while the neutral loss of the product ion [M+3H-far]$^{3+}$ is of lower intensity. The intense farnesyl marker ion is favoured over the neutral loss due to the stability of the allylic cation in the ESI spectrum and the increased availability of protons due to the higher charge state.

There are a number of peaks in the low mass range that might be associated with the farnesyl moiety (Figure 2.4(a)), but unambiguous assignment was not possible. For this reason, an MS/MS/MS analysis of the triply charged precursor ion was performed as it allowed for the fragmentation of the m/z 205 marker ion (Figure 2.4(b)), and the identification of modification-derived secondary fragment ions. The C$_{15}$H$_{25}^+$ marker ion fragmented to produce C$_{10}$H$_{15}^+$ (m/z of 135.2), C$_{11}$H$_{17}^+$ (m/z of 149.2) and C$_{12}$H$_{19}^+$ (m/z of 163.2) isoprenyl fragments. The ions at 135.2 Th and 149.2 Th can be rationalized as being formed from the breakage of one C-C bond and an H rearrangement producing a conjugated system that is resonance-stabilized. All three of these ions produce an associated fragment that is a stable low mass neutral by a mechanism that is similar to other terpene fragmentation analyses$^{43,44}$. All three of these fragment ions were also present in the product ion spectrum of the farnesylated peptide (Figure 2.4(a)). Furthermore, as each fragment
Figure 2.4. Characterization of the farnesylated-cysteine marker ions. a) low mass region of ESI QqQLT Enhanced Product Ion Spectrum of triply charged EKPLQNFTLCFarFR-NH₂ taken at a CE of 60 eV, b) MS/MS/MS analysis of EKPLQNFTLCFarFR-NH₂ (collision energy 60 eV, excitation energy 175 V), c) Q TOF product ion spectra of the 149 farnesyl fragment ion and d) 205 farnesyl marker ion.
differs by the mass of a CH₂ unit, other ions seen in the product ion spectra that are a multiple of 14 Da lower than C_{10}H_{15}^+ or 14 Da higher than C_{12}H_{19}^+ may be associated farnesyl fragment ions. Only one of these 3 fragments was previously identified by Kassai and others\textsuperscript{30} as a fragment of the farnesyl moiety (149.1 Th), while the other fragment that they attributed to being a farnesyl fragment (137.1 Th) seems erroneous. A high mass accuracy product ion analysis with a quadrupole TOF was then completed to resolve interfering ions with the same nominal mass as the farnesyl marker ion and associated fragment ions. A m/z of 149.143 Th was determined compared to a theoretical monoisotopic m/z of 149.133 Th (Figure 2.4(c)) and the farnesyl marker ion was located at 205.200 Th compared to a theoretical monoisotopic m/z of 205.208 Th (Figure 2.4(d)). Other farnesyl fragment ions were located at m/z of 135.117 Th and 163.149 Th compared to the theoretical monoisotopic m/z of 135.125 Th and 163.170 Th (data not shown).

Collision energy profiling of marker ions, associated fragment ions and the neutral loss fragment [M+nH-far]\textsuperscript{n+} was performed by collecting product ion spectra of the triply-charged farnesylated peptides at collision energies of 30 to 195 eV in 15 eV increments (Figure 2.5(a-b)), and the doubly charged farnesylated peptides at collision energies of 30 to 140 eV in 10 eV increments (Figure 2.5(c-d)). Five cycles of the collision energy profiling were performed. The intensities from each cycle were normalized to the most intense data value in the set, the five data sets were averaged, then plotted. Collision energy profiling was performed in order to determine the conditions necessary for monitoring for the marker ion, associated fragment ions and the neutral loss fragment, as well as to understand their formation and degradation.
Figure 2.5. Collision energy profiling of farnesyl marker ions and neutral losses. CE profile of the triply-charged a) EKPLQNLTC(far)FR-NH$_2$ and b) MHRQETVDC(far)LK-NH$_2$ peptides and doubly-charged c) EKPLQNLTC(far)FR-NH$_2$ and d) MHRQETVDC(far)LK-NH$_2$. The fragment ions are denoted as 135.2 (x), 149.2 (o), 163.2 (■), 205.2 (▲) and neutral loss (●). The triply-charged collision energy profile was collected from 15 eV to 195 eV in 15 eV increments and the doubly-charged collision energy profile was collected from 50 to 140 eV in 10 eV increments.
With the triply-charged product ion of EKPLQNFTLC(far)FR-NH₂ (Figure 2.5(a)), as the collision energy increases from 30 to 60 eV, the farnesyl marker ion and associated fragments increase in intensity, while the intensity of the neutral loss fragment ion decreases. At 60 eV, the farnesyl marker ion reaches its maximum intensity and begins to degrade to form the farnesyl fragment ions. As the collision energy increases from 60 to 90 eV, the farnesyl marker ion continues to degrade, as does the neutral loss fragment ion, while the farnesyl fragment ions continue to increase in intensity. Fragment ions at a m/z of 163 and 149 Th reach a maximum at approximately 80 eV, while the ion at a m/z of 135 Th reaches a maximum at approximately 100 eV. Continuing to increase the collision energy, all of the fragment ions and the marker ions are fragmented. The collision energy profile of the triply charged peptide MHRQETVDC(far)LK-NH₂ (Figure 2.5(b)) shows a similar trend to that seen in Figure 2.5(a). The neutral loss fragment is formed with a similar relative abundance at low collision energies, and the farnesyl marker ion is also formed with the greatest relative abundance at low collision energies. As the MHRQETVDC(far)LK-NH₂ ion is smaller than EKPLQNFTLC(far)FR-NH₂, a lower collision energy is required to produce the protonated farnesyl moiety (205 Th); however, the size of the peptide seems to have less of an effect on the production of the neutral fragment. Furthermore, the farnesyl fragment ions all have very similar maxima. The farnesyl fragment ions at 163 and 149 Th reach a maximum at approximately 85 eV, while the ion at a m/z of 135 Th reaches a maximum at approximately 100 eV. The profiles of the farnesyl fragment ions are very similar to those seen in Figure 2.5a because they are both produced from the same precursor ion, the protonated farnesyl moiety (205 Th). As well, the difference in the maxima of the protonated farnesyl moiety
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(205 Th), and the fragment ions at 163, 149 and 135 Th, can be attributed to the larger farnesyl fragments dissociating to produce the smaller farnesyl fragments.

The collision energy profile of the doubly-charged farnesylated peptides (Figure 2.5(c-d)) looks remarkably similar to that of the triply-charged collision energy profile, with only one major difference, the neutral loss is now produced in higher abundance compared to the production of the protonated farnesyl moiety. As the peptide has a lower charge state, the farnesyl modification is more likely to fragment as a neutral loss fragment as opposed to a marker ion. As well, one other difference between the triply- and doubly- protonated profiles can be seen in that the maximum intensities of the protonated farnesyl moiety and the neutral loss fragment are shifted to lower collision energies compared to the doubly-charged profile. This is believed to be due to a positive charge being more readily available for the farnesyl moiety which would support the fragmentation at a lower collision energy. The plots of the three farnesyl fragment ions from the fragmentation of MHRQETVDC(far)LK-NH$_2$ (Figure 2.5(d)) is fairly noisy as a result of the peptide being predominantly present as the triply-charged ion and that the modification tended to fragment as a neutral loss with the doubly charged peptide. As seen from the collision energy profiles (Figures 2.5(a-d)), the 149.2 Th and 135.2 Th fragment ions are relatively intense compared to the farnesyl marker ion and thus may be used as additional constraints in the identification of a farnesylated peptide or protein. As there is no nominal mass interference at 135.1 Th and very limited interferences at 149.2 Th (Table 2.3) and 205.2 Th (Table 2.4) that would be resolved by a quadrupole time-of-flight analysis, the use of these three marker ions should provide a very selective analysis for farnesylation.
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<table>
<thead>
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<th>Fragment Ions</th>
<th>Elemental Composition</th>
<th>m/z (Da)</th>
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<td>C$_9$H$_11$N$_2^+$</td>
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<tr>
<td>Far+H$^+$ (fragment)</td>
<td>C$<em>{11}$H$</em>{11}$+$^+$</td>
<td>149.133</td>
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Table 2.3. Fragment Ions, Elemental Composition and exact masses of all possible a-, b- and y- type ions of unmodified peptides with a nominal mass of 149
## Table 2.4

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<td>$a_2$ - $H_2O$ (CF)</td>
<td>$C_{11}H_{13}N_2S^+$</td>
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<td>$b_2$ (GF); $y_1$ (W); $a_2$ - $H_2O$ (SY)</td>
<td>$C_{13}H_{17}N_2O_2^+$</td>
<td>205.097</td>
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<td>$a_2$ (MT)</td>
<td>$C_8H_{17}N_2O_2S^+$</td>
<td>205.101</td>
</tr>
<tr>
<td>$a_2$ - $H_2O$ (HX)</td>
<td>$C_{13}H_{17}N_4^+$</td>
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<tr>
<td>Far+H$^+$</td>
<td>$C_{14}H_{15}^+$</td>
<td>205.195</td>
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Table 2.4. Fragment Ions, Elemental Composition and exact masses of all possible a-, b- and y- type ions of unmodified peptides with a nominal mass of 205.
2.3.4 Palmitoylation

Cysteine palmitoylation was found to be a more stable modification than both the myristoyl and farnesyl modifications. The palmitoyl modification does not tend to fragment to produce a marker ion and the formation of a neutral loss from the precursor ion is relatively low, as backbone fragmentation appears to be the primary means of peptide decomposition. Fragmentation of the singly- (Figure 2.6(a-b)), doubly- (Figure 2.6(c-d)) and triply-charged (Figure 2.6(e-f)) precursor ions can be seen in the product ion spectra (Figure 2.6(a-c)). The MALDI-TOF-TOF analysis of the singly-charged precursor ion EKPLQNFTLC(pal)FR-NH₂ (Figure 2.6(a)) shows a partial y-series of ions as well as a neutral loss of the precursor ion of $\text{CH}_3(\text{CH}_2)_{14}\text{COSH}$ (272 Da). Similarly, the product ion spectra of MHRQETVDC(pal)LK-NH₂ (Figure 2.6(b)) shows a partial b-ion series as well as the 272 Da neutral loss. The neutral losses were still observed when the product ion spectrum was taken at a higher collision gas pressure of $2 \times 10^{-6}$ torr, although it was less intense (data not shown). The neutral loss is believed to be the result of the sulphur attacking the $\alpha$-hydrogen, leading to the elimination of the palmitoyl moiety as a thioacid. A similar neutral loss of palmitic acid (256 Da) was observed in the MS/MS analysis of phospholipids.55

Fragmentation of the doubly-charged precursor ion of EKPLQNFTLC(pal)FR-NH₂ generated by electrospray ionization (Figure 2.6(c)) led to the formation of a complete y-series of ions; however, there were no marker ions, neutral losses from the precursor ion or fragment ions, or a mass shift corresponding to ions that have lost a palmitoyl group; nevertheless, it is still possible to identify this modification. Since this modification tends to occur near the C-terminus of a protein, and therefore also of any peptide after enzymatic
Figure 2.6. Characterization of the fragmentation of different charge states of palmitoylated peptides. MALDI-TOF-TOF analysis of the singly-charged a) EKPLQNFTLC(pal)FR-NH₂ and b) MHRQETVDC(pal)LK-NH₂ collected a collision gas pressure of 2x10⁻⁸ torr, ESI QqQAT Enhanced Product Ion Spectrum of doubly-charged c) EKPLQNFTLC(pal)FR-NH₂ and d) MHRQETVDC(pal)LK-NH₂ taken at a CE of 100 eV and ESI QqQLIT Enhanced Product Ion Spectrum of triply-charged e) EKPLQNFTLC(pal)FR-NH₂ and f) MHRQETVDC(pal)LK-NH₂ taken at a CE of 90 eV.
digestion, the y-series of ions tends to produce strong peaks, which would allow the palmitoyl modification to be identified by the mass shift of these modification-containing fragment ions when sequencing the peptide or protein. The fragmentation of the doubly charged MHRQETVDC(pal)LK-NH₂ (Figure 2.6(d)) has lead to a more favourable situation as both neutral losses of 238 Da from the b₉²⁺ fragment ion and mass shifts corresponding to the loss of 238 Da were identified, allowing for a simpler identification of the modified peptide. The mass spectrum also shows a partial b-ion series that could aid in the identification of the palmitoyl modification.

Fragmentation of the ESI generated triply-charged precursor ion EKPLQNFNTLC(pal)FR-NH₂ (Figure 2.6(e)) is very rich in fragment ions. The complete y-series of ions as well as a number of b-ions are visible. As well, there are a number of y-ions that show a mass shift of 238 corresponding to a neutral loss of the palmitoyl residue due to the breakage of the thioester bond. At this high collision energy, a neutral loss from the triply-charged precursor ion could not be found; however, when it was fragmented at a lower collision energy, a very weak neutral loss corresponding to the loss of the palmitoyl group (238 Da) was observed (data not shown). Similarly, the product ion analysis of the MHRQETVDC(pal)LK-NH₂ ion (Figure 2.6(f)) shows a partial b-ion series of ions, a neutral loss from the precursor ion and a mass shift corresponding to ions that have lost a palmitoyl group from both singly- and doubly-charged fragment ions. As this neutral loss is formed in a very low yield, utilizing the mass shift of 238 Da seen in the fragment ions would be the preferential method for the analysis of palmitoylation with electrospray ionization.

Whether the modification falls off as a neutral loss of 238 Da, which was seen in the ESI analysis, or 272 Da, which was seen in the MALDI analysis, may be dependent on the
collision energy. It requires a low amount of energy to break the thioester bond; however, the corresponding palmitoyl fragment of 238 Da is not a very stable neutral fragment. Alternatively, the loss of the palmitoyl group as a thioacid requires the breakage of two bonds, although the product is more stable. These neutral losses are much more stable products when compared to possible fragment ions that could represent a marker ion since CH₃(CH₂)₁₄CO⁺ (239 Th) is a rather unstable secondary carbocation. Bean and others did observe this ion at 239 Th and sited it as being the most selective marker ion for the palmitoyl modification; however, this ion was not observed in the MALDI or ESI hybrid triple quadrupole/linear ion trap analyses. It was observed in the fragmentation of the triply-charged precursor ion on the Q-TOF, albeit at very low intensity. The intensity of the 239 Th ion was lower than the associated neutral loss fragment ion. Bean and others also observed other fragment ions specific to different palmitoyl linkages to the peptide and thus were not observed in our analysis. In a similar fashion, one might expect to see a protonated palmitoyl thioacid (273 Th); however, this ion was not observed. Thus, the use of either the neutral loss from the precursor ion or mass shift corresponding to the loss of the palmitoyl residue would be the preferred method of analysis.

2.4 Conclusion

Analysis of the 3 major lipid modifications by MALDI and ESI mass spectrometry has allowed us to determine the most efficient methods to analyze these modifications. These results are summarized in Table 2.5. The decrease in intensity of the marker ions results from the low charge state of the ionized peptide that is characteristic of MALDI spectra. This results in competition for the single charge and since the C-terminal arginine and lysine, as well as other amino acid residues, have a much higher gas phase proton
## Table 2.5. Marker Ions and / or Neutral Losses that are characteristic of lipid modifications using various ionization sources (the most efficient method of identification are in bold).

<table>
<thead>
<tr>
<th>Modification</th>
<th>MALDI</th>
<th>ESI</th>
</tr>
</thead>
<tbody>
<tr>
<td>myristoyl</td>
<td>Neutral Loss (210 Da)</td>
<td>Marker Ions (a₁ 240 Th and b₁ 268 Th)</td>
</tr>
<tr>
<td></td>
<td>Marker Ions (myr-H⁻ 211 Th, a₁ 240 Th, b₁ 268 Th)</td>
<td></td>
</tr>
<tr>
<td>farnesyl</td>
<td>Neutral loss (204 Da)</td>
<td>Marker Ion (205 Th and 149 Th)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Neutral Loss (204 Da)</td>
</tr>
<tr>
<td>palmitoyl</td>
<td>Neutral Loss (272 Da)</td>
<td>mass shift of 238 Da</td>
</tr>
</tbody>
</table>

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affinity, the proton will not be available for the modification. Thus, upon collision induced
dissociation in a MALDI-TOF-TOF, the modification tends to be more readily observed as a
neutral loss of the precursor ion rather than modification specific marker ions. The trend
seems to be that monitoring of the neutral loss would thus be the preferential method for the
analysis with MALDI, while the marker ions would be the preferential method for the
analysis with electrospray ionization. In the case of palmitoylation, as it does not fragment to
produce any modification-specific fragment ions when using electrospray ionization,
utilization of the mass shift would be the preferred method for identifying this modification.
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2.5 References

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Chapter 3: Cysteine Nitroxylation Analysis by Mass Spectrometry

3 CYSTEINE NITROXYLATION IN PLATELETS: DEVELOPMENT OF A NOVEL MASS SPECTROMETRIC DETECTION METHOD FOR DRUG-INDUCED MODIFICATIONS

3.1 Introduction

The biological reactions of nitric oxide (NO) have garnered much attention over the last 15 years, culminating with the 1998 Nobel Prize in physiology which was awarded for discoveries concerning NO as a signaling molecule in the cardiovascular system. NO regulates many processes including platelet function, leukocyte recruitment, vascular tone and cardiac function mainly through the cGMP second messenger system. As well, nitric oxide mediated S-nitrosylation has been linked to a number of diseases such as diabetes, multiple sclerosis, cystic fibrosis, asthma, etc. More detailed biological studies have shown nitric oxide to react with a number of different biological species including metal centers of proteins, nucleophilic amino acid residues (cysteine nitrosation/nitrosylation) and aromatic amino acid residues (tyrosine nitration). The products of these reactions have been analyzed by mass spectrometry.

Nitroxyl (HNO), an alternative redox form of NO, has only recently begun to draw attention in the biomedical research community, and it has been argued that “we’re at the same level now with HNO as at the beginning of the NO field 15 years ago”. The significant increase in interest associated with HNO is due to its novel and important biological activity. There has been particular interest in the effect of HNO on failing hearts as it has been shown to increase left ventricular contractibility and, at the same time, to lower

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cardiac preload and diastolic pressure without increasing arterial resistance\textsuperscript{11,12}; effects which indicate HNO's potential to be developed as a treatment for heart failure\textsuperscript{13}. As well, treatment of platelets with micromolar concentrations of the HNO donor, Angeli's Salt (AS), leads to an inhibition of platelet aggregation which was found to be both time and dose dependent\textsuperscript{14}. Other pharmacological studies have shown that HNO can be protective against the excitotoxicity of the NMDA receptor\textsuperscript{15,16}, it can inhibit aldehyde dehydrogenase which could be used as an anti-alcoholic treatment\textsuperscript{17-19} and pretreatment of ischemic (oxygen-depleted) tissues with HNO has been shown to protect against ischemia-reperfusion toxicity\textsuperscript{20}. Alternatively, HNO has also been shown to be cytotoxic at high concentrations (2-5 mM) by eliciting DNA strand breaks and GSH depletion, causing cellular toxicity due to oxidative protein damage\textsuperscript{21}. However, this toxicological effect is only relevant if physiological HNO levels are high, and it has thus far not been demonstrated to have any \textit{in vivo} relevance\textsuperscript{22}.

These HNO mediated pharmacological effects are dramatically different from those of NO\textsuperscript{12}, most likely because HNO tends to be much more thiophilic, with cysteines being the major site of biochemical reactivity\textsuperscript{23-25}. Therefore, it is no surprise that NO and HNO tend to have different targets. For example, in the vascular system, HNO can act through a cAMP signal transduction pathway, while NO's vascular activity is primarily due to an elevation in cGMP\textsuperscript{26}.

Although a number of pharmacological and toxicological effects have been shown for HNO, a large number of questions still surround its exact mechanism of action. It is known that HNO and cysteine react to produce non-crosslinked sulfinamides and cause disulfide formation. HNO can also react with metals/metalloproteins, oxygen and participate in
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reduction/oxidation reactions\textsuperscript{22,24,25}. However, most of the pharmacological and toxicological studies of HNO have not linked these effects to the molecular targets of HNO: the proteins that are modified, the sites of modification and the type of modification.

Here we describe a mass spectrometric-based method for the analysis of the major type of biologically relevant HNO reaction; a reaction with the thiol on cysteines to produce non-crosslinked sulfinamides as well as disulfide linkages. Although disulfides are produced through many different pathways, non-crosslinked sulfinamides are exclusively produced by HNO and can thus be used to analyze for the presence of HNO and its effects on cysteine containing proteins. As well, the sulfinamide modification imparts a specific mass change to cysteines making sulfinamide analysis, and indirectly HNO analysis, very amenable to investigation by mass spectrometry. The sulfinamide modification has been observed in MS spectra\textsuperscript{27} and in a more recent mass spectrometric analysis, Shen and English\textsuperscript{28} attributed a mass shift of 65 Da on prominent y-ions upon low-energy CID to the elimination of the sulfinamide moiety from the molecule in their mass spectrometric comparison of nitroxyl products formed with free and protein-based cysteines. Here we thoroughly investigate this mass shift and the formation of a previously unstudied neutral loss to determine an efficient method for the identification of the sulfinamide modification, and demonstrate its utility on a sample generated by \textit{ex vivo} treatment of platelets with HNO.

3.2 Experimental

3.2.1 Reagents

The peptide MHRQETVDCLK-NH$_2$ was provided as a gift from the Biomedical Research Centre and peptide EKPLQNFTLCFR-NH$_2$ was purchased from Bachem.
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(Bubendorf, Switzerland). Angeli’s Salt (AS) (Na₂[ONNO₂]) was purchased from Cayman Chemicals (Ann Arbor, MI, USA) and sequencing grade trypsin, formic acid and acetonitrile were purchased from Fisher Scientific (Waltham, MA). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

3.2.2 Mass spectrometry of test peptides

Analysis of the nitroxyl-modified peptides was performed using the following instruments: a nano-electrospray ionization (nESI) interfaced with a triple quadrupole instrument where Q3 has linear ion trapping capabilities (2000 Q TRAP), a quadrupole time-of-flight (Q STAR-XL) mass spectrometer, and a MALDI-TOF/TOF 4700 Proteomics Analyzer (all Applied Biosystems/MDS Sciex, Concord, Ont., Canada). Alternatively, a nESI linear ion trap coupled to a Fourier Transform Ion Cyclotron Resonance mass spectrometer (LTQ FT-ICR) with electron capture dissociation (ECD) capabilities and an nESI LTQ Orbitrap instrument were used (Thermo Electron Corp., Bremen, Germany). All analyses were performed with 1-5 μM solutions of the modified peptides.

3.2.3 Nitroxylation of test peptides

A stock solution of 100 mM AS was prepared in 10mM NaOH. Nitroxylation was performed on 10 nmol of either MHRQETVDCLK-NI or EKPLQNFTLCFR-NI, 1 μl of AS stock solution and 99 μl of 15 mM tris-HCl (pH 7.4) buffer, producing a final AS concentration of 1 mM, and was reacted for 25 min at room temperature. The peptides were then separated from the reactants and purified with C18 ZipTips (Millipore, Billerica, MA, USA) and then reconstituted in 50% acetonitrile/5% formic acid when analyzed by electrospray mass spectrometry, or were spotted onto a MALDI target using the dried droplet method and α-cyano-4-hydroxy cinnamic acid (CHCA) matrix.
3.2.4 Time Course analysis

The kinetics of the reaction of peptides MHRQETVDCLK-NH$_2$ and EKPLQNFTLCFR-NH$_2$ with HNO was investigated on a Q STAR XL employing an ion accumulation time of 1s, leading to the acquisition of 1 kinetics measurement/second. For all kinetics experiments, a 40:1 molar ratio of AS to peptide was used with a final AS concentration of 2 mM. Two different setups were required in order to monitor the kinetics of the reaction from 1.7 seconds to 15 minutes. For the longer time points, the reaction solution was prepared by adding AS to 2.5 nmol of either MHRQETVDCLK-NH$_2$ or EKPLQNFTLCFR-NH$_2$, in 12.5 mM tris-HCl (pH 7.4)/20% methanol, inserted into a nanospray emitter and then analyzed on the mass spectrometer. For such a process, it took between 1-1.08 minutes from AS mixing into the reaction solution to data collection. Three data sets were collected, averaged and a 5 point moving average was applied to the data. For the shorter time points, kinetics data in the range of 1.7 to 90 seconds was collected based on a continuous flow setup, as previously described by Konermann et al (1997)$^{29}$. Two syringes were operated simultaneously with syringe 1 (flow rate of 32 µl/min) containing the buffered peptide mixture and an internal standard (osteocalcin fragment 7-19), and syringe 2 (flow rate of 1 µl/min) containing the AS solution. Each syringe was connected to a fused silica capillary (100 µm i.d., Polymicro Technologies, Phoenix, AZ) which were then connected together by a splitter on the electrospray source (MDS Sciex) to a third reaction capillary (also 100 µm i.d. fused silica) which led the sample to the tip of the emitter. The length of the third capillary was varied from 627 cm to 36 cm corresponding to reaction times from 89.5 to 5.1 seconds$^{29}$. In order to reach reaction times as low as 1.7 seconds, the flow rate of
the 2 syringes were increased proportionately to obtain total flow rates up to 100 μl/min. An accumulation time of 1s per mass spectrum with a total acquisition time of 1 minute was collected for one data set. The internal standard, Osteocalcin fragment 7-19, was used to normalize the intensities of the precursor, intermediates and final product, as they may vary with the flow rate.

3.2.5 Protein Nitroxylation

Ethical approval for this study was granted by the University of British Columbia Research Ethics Board and informed consent was granted by the donors. Whole blood was drawn from the antecubital vein of healthy human volunteers into 0.15% (v/v) acid-citrate-dextrose (ACD) anticoagulant. Platelets were isolated by centrifugation, washed twice in physiological buffer (10mM trisodium citrate, 30mM dextrose, 1U/mL apyrase) and re-suspended at physiological concentration (200-350x10^9/L) in Tris-buffered saline containing 5mM EDTA. Platelets were treated with AS (0, 10μM, 100μM, 1mM and 10mM) from a stock of 1M AS in 10mM NaOH for 2 minutes at room temperature. Samples were spun at 1000 x g for 3 minutes at room temperature to pellet the platelets. Platelet pellets were re-suspended in 1X lysis buffer (20mM Tris pH 7.4, 150mM NaCl, 1mM EDTA, 1% TX-100, 2.5mM Na pyrophosphate, 1mM β-glycerolphosphate, 1mM Na3VO4, 1X protease inhibitor cocktail), and lysates were immediately snap-frozen in liquid N2.

3.2.6 Electrophoresis and Proteolytic Digestion

Samples were thawed slowly on ice, mixed with non-reducing sample buffer and separated by 1-dimensional SDS-polyacrylamide gel electrophoresis (1D SDS-PAGE). Gels were stained with Coomassie Brilliant Blue and bands of interest were excised. Tryptic in-
gel digestions were performed overnight at 37°C, without reducing or alkylating the sample. Following digestion, peptides were extracted for MS analysis.

### 3.2.7 Liquid Chromatography-Tandem Mass Spectrometry

Identification of nitroxylated peptides from the in-gel digests of samples treated with 10 mM AS were performed by nano-HPLC MS/MS on an Agilent 1100 (Agilent, Santa Clara, CA) coupled to an LTQ-Orbitrap using a 15 cm long, 75 μm I.D. fused silica column packed with 3 μm particle size reverse phase (C18) beads (Dr Maisch GmbH, Germany) using water:acetonitrile:formic acid as the mobile phase with gradient elution. Identification of HNO-modified peptides was performed by extracting the Mascot generic format files from the MS data using DTA Super Charge, part of the MSQuant open source project (http://msquant.sourceforge.net) and then searching them against the human SwissProt Database (v.54.5; 589,473 sequences) using Mascot v. 2.1. All MS/MS spectra of peptides that were identified as HNO-modified by Mascot were inspected manually to confirm the peptide assignment. These candidate modified peptides were then selected for Multiple Reaction Monitoring (MRM) analysis for the dose response experiment, with the product ion spectra from the LTQ of the Orbitrap being used to determine the MRM transitions.

The dose response analysis was completed with MRM experiments performed by nano-HPLC MS/MS on an Ultimate (LC Packings, Sunnyvale, CA, USA) using a 15 cm long, 75 μm I.D., 3 μm particle size reverse phase (C18) column (LC Packings) coupled to an ESI 2000 QTRAP. For each suspected modified protein identified by the Orbitrap, the modified peptide was monitored by four MRM transitions (see Table 3.1): one to monitor the precursor ion with no fragmentation in the collision cell and three MRM transitions to monitor three different fragment ions produced from fragmentation of the precursor in the...
collision cell. Peak areas from these three fragment ions were collected from the extracted ion chromatograms and then averaged (A) to increase selectivity as well as to allow for relative quantitation of the amount of modification at different doses.

Two non-modified peptides from the same protein, each with two MRM transitions, were monitored to serve as internal standards: one MRM transition monitored the precursor ion (no fragmentation) and one MRM transition monitored an intense fragment ion. Peak areas from the two precursor ions and two fragment ions were collected from the extracted ion chromatograms, and normalized to the most intense peak area of the 5 dose response samples for each transition. These four values were averaged to obtain a correction factor (CF) for the variation in protein quantity from gel band to gel band. The average fragment peak area (A) of the modified precursor was divided by the CF to calculate the corrected average fragment peak area (A/CF).

Three dose response data sets were collected by reacting three sets of platelets, collected from different donors, on different days using different batches of stock AS solution. In order to account for instrumental variation from dataset to dataset, the corrected average fragment peak areas (A/CF) for each data set were normalized to the most intense A/CF of the 5 dose response samples of that data set, with the maximal A/CF being set to 1. Three data sets were averaged and plotted, along with the standard error of the mean.

3.3 Results and Discussion

3.3.1 Examination of the products of the reaction between HNO and cysteine

In the initial experiment, the major products of the reaction of HNO with cysteine-containing peptides and the relative abundance and stability of these species were
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investigated. To this end, the peptides MHRQETVDCLK-NH₂ and EKPLQNFTLCFR-NH₂ were reacted with AS and analyzed by mass spectrometry. As previously reported\textsuperscript{24}, both the sulfinamide and disulfide modification were produced upon cysteine reaction with HNO (Figure 3.1). The sulfinamide modification was found to be very stable as the relative abundance of the products in the MS spectra did not change with either periodic freeze/thawing, storage of the peptide for 4 months at -20°C or heating of the peptide to 60°C for 1 hour, proving that it is amenable to mass spectrometric analysis using this system.

3.3.2 Nitroxylation of cysteine-containing peptides: time course analysis

According to Illustration 3.1, reaction of the cysteine (Figure 3.1(a)) with HNO generates both the sulfinamide and disulfide modifications (products 4 and 5, respectively) through intermediates (Figure 3.1(b))\textsuperscript{24}. As these products and intermediates have different masses, they can be distinguished by mass spectrometry. If one or more of these intermediates are long-lived, it could lead to a decrease in the final sulfinamide or disulfide yield, making the modification analysis more difficult. Therefore, the kinetics of the HNO/cysteine reaction was monitored mass spectrometrically on a Q STAR XL (Figure 3.1) by examining the reaction of HNO with the cysteine containing peptide, MHRQETVDCLK-NH₂. HNO is produced from the decomposition of AS upon its addition to the buffered peptide reaction solution with a half life of 4.4 min at pH 7.4 and 22°C\textsuperscript{32, 33}. As there was a 40 times excess of AS to peptide in the reaction mixture, within 10 seconds of the reaction, HNO was in excess (at least equivalence) compared to the peptide concentration.

In order to monitor the kinetics of the cysteine/HNO reaction from 3 seconds to 15 minutes, two different setups were used. Figure 3.1(c) displays kinetics data for the first 90 seconds of the reaction while Figure 3.1(d) tracks the reaction from 1 to 14 minutes. Within
Illustration 3.1. Reaction pathway of HNO-induced modifications from the reaction of HNO with cysteine.
Figure 3.1. Mass Spectrometric time course monitoring of the HNO – cysteine reaction. MS spectra of the reaction at a) t=1.7 seconds showing the unreacted (0 Da) doubly charged MHRQETVCLK-NH$_2$ and b) t=39 seconds showing the unreacted (0 Da) doubly-charged MHRQETVCLK-NH$_2$ and the reaction products, Intermediate 2 (+14 Da) and Sulfinamide 4 (+31 Da). Time monitored kinetics from c) 1.7 s to 90 s and d) 1 to 15 min. The degradation of the unreacted peptide ( ), production and subsequent degradation of the +14 Da intermediate 2 ( — — — ) and the production of the sulfinamide 4 ( — ) and disulfide 5 ( — — — ) products with time are shown. e) Determination of the presence of Intermediate 3 by monitoring the ratio of the +31 Da to +32 Da peaks.
one minute, the intensity of the unmodified peptide had dropped dramatically (Figure 3.1(c)). From as early as 5 seconds, significantly detectable quantities of the + 14 Da intermediate 2 and the final sulfinamide product 4 were produced. As the reaction proceeded, the intensities of both the + 14 Da intermediate 2, the final sulfinamide 4 and the disulfide 5 increased. At approximately 85 seconds, the sulfinamide 4 began to appear at a higher intensity than the + 14 Da intermediate 2 as the intermediate was consumed to produce the final product.

The longer time points in the HNO reaction can then be followed in Figure 3.1(d), which displays kinetic data for the consumption of the unmodified peptide MHRQETVDCLK-NH2, the production and subsequent consumption of the + 14 Da intermediate 2, and the production of the disulfide 4 and sulfinamide 5 final products. Within approximately 1 minute, the majority of the unmodified peptide had reacted as seen by its low intensity, and was converted to intermediate 2, the sulfinamide 4 and the disulfide 5. By 1.2 minutes into the reaction, intermediate 2 reached a maximum intensity and was thereafter converted to the sulfinamide 4 final product at a higher rate than it was produced. Within a time span of approximately 10 minutes, the intensities of the unmodified peptide and intermediate 2 (+ 14 Da) had dropped considerably, while the final disulfide 5 and sulfinamide 4 products had reached a plateau. As the +14 Da peak was consumed in the reaction, it could not have been an intramolecular sulfinamide, but instead an intermediate that was converted to the sulfinamide.

Intermediate 1, the N-hydroxsulfenamide, could not be monitored as the mass shift is equal to the sulfinamide. This species has been implicated in both disulfide and sulfinamide formation, but has never previously been isolated or characterized.24,34
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With respect to intermediate 3 (+32 Da), it is 1 Da heavier than the sulfinamide 4 (+31 Da) (Figure 3.1(b)). This causes a nominal mass interference as the isotopes of the sulfinamide 4 modification interfere with the detection of intermediate 3, inhibiting its direct analysis. Therefore, the ratio of the +31 to +32 Da peaks was monitored as a change in the +31/+32 ratio would be indicative of the formation of intermediate 3. The ratio was plotted to roughly determine the abundance and longevity of this intermediate (Figure 3.1(c)). The ratio of the +31/+32 Da sulfinamide 4 isotopes stays constant throughout the experiment indicating the peaks are the 1st and 2nd isotopes of the sulfinamide 4, rather than the sulfinamide and intermediate 3. Thus, there was no significant build up of intermediate 3, and it must be fairly short lived. The above results indicate that the sulfinamide 4 is the final product in the reaction, as none of the intermediates are seen past 12-14 minutes of the reaction, which is advantageous for monitoring the sulfinamide 4 modification.

3.3.3 Determination of the site of sulfinamide modification on nitroxylated peptides using tandem mass spectrometry

A recent study attributed a mass shift of 65 Da on prominent y-ions upon low-energy CID of a multiply charged peptide to the elimination of the sulfinamide moiety from the molecule. In order to determine if, along with this mass shift, the sulfinamide modification fragmented to produce characteristic marker ions or neutral loss fragments which would allow for identification of the modification, product ion analyses of peptides EKPLQNF*LC*FR-NH₂ and MHRQETV*D*LK-NH₂ (C* denoting sulfinamide modification) were performed. Product ion spectra of the singly- (Figure 3.2(a),(b)), doubly- (Figure 3.2(c),(d)) and triply- (Figure 3.2(e),(f)) charged precursor ions were collected.
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Figure 3.2. Product ion analysis of the different charge states of the sulfinamide-modified peptides. MALDI MS/MS (PSD) of a), singly-charged and ESI QqToF of c), doubly- e) and triply-charged EKPLQNFTLC*FR-NH₂ and MALDI MS/MS (PSD) of b) singly-charged, and ESI QqTOF of d) doubly-, e) triply-charged MHRQETVDC*LK-NH₂.
Fragmentation of the singly charged precursors (Figure 3.2(a),(b)), produced from MALDI TOF/TOF post-source decay (PSD), led to a low yield of y- and b- sequence ions. The most prevalent species tended to be the 65 Da neutral loss of HS(O)NH$_2$ from the sulfinamide on the precursor ion, believed to be produced from the sulfur mediated abstraction of the $\alpha$-hydrogen.

Fragmentation of the doubly charged precursors (Figure 3.2(c),(d)), obtained from a Q STAR XL, led to a greater production of y- and b- sequence ions. In each of the mass spectra, signals were also observed corresponding to the precursor ion minus HS(O)NH$_2$ as well as fragment ions with the 65 Da mass shift. Fragmentation of the triply charged precursors (Figure 3.2(e),(f)), also obtained from a Q STAR XL, led to an even greater production of y- and b- sequence ions. The 65 Da neutral loss from the sulfinamide precursor ion could only be observed from the MHRQETVDC*LK-NH$_2$ peptide, although mass shifts corresponding to losses of HS(O)NH$_2$ from fragment ions could be seen for both peptides. The neutral losses from the precursor ions, 65 Da sulfinamide mass shifts on the fragment ions and y- and b- fragment ions from the doubly- and triply- charged precursor ions were all reproduced when analyzed by CID on the linear ion trap of a LTQ-FT (data not shown). Furthermore, when the singly-, doubly-, and triply- charged forms of the unmodified precursor was analyzed, no 65 Da neutral loss or mass shift was observed (data not shown).

The inverse correlation between the prevalence of the neutral loss and the charge state of the precursor ion has previously been observed for lipid modifications.\textsuperscript{35} If the modification has a low proton affinity and the molecular ion is in a low charge state, the probability that the modification fragments as a neutral fragment increases. The generation
of the neutral fragment will also depend on the kinetics of the fragmentation mechanism and the thermodynamic stability of the neutral fragment. Overall, the neutral loss of HS(O)NH$_2$ and the 65 Da mass shift on the fragment ions has been shown to be very prevalent, especially for low-charge state precursors, making it a preferred method of detection for HNO induced modification.

As the CID product ion spectra lacked a complete b- / y- ion series, electron capture dissociation (ECD) was employed on a FT-ICR mass spectrometer to verify that the cysteine is in fact the modified residue. Since post-translational modifications often remain intact after peptide backbone fragmentation by ECD, it is a very useful fragmentation technique for the localization of modification sites. ECD product ion spectra were collected for triply-charged MHRQETVDC*LK-NH$_2$ (Figure 3.3(a)), doubly-charged EKPLQNFTLC*FR-NH$_2$ (Figure 3.3(b)), and doubly-charged MHRQETVDC*LK-NH$_2$ (Figure 3.3(c)). A virtually complete c- ion series was observed for doubly- and triply charged MHRQETVDC*LK-NH$_2$ (Figure 3.3(a,c)). All three precursor ions fragmented to produce c- ions on both sides of the cysteines (c$_9$/c$_9$ in Figure 3.3(a),(c) and c$_9$/c$_{10}$ in Figure 3.3(b)), with a mass difference of 134.01 Da between c-ions, which corresponds to the mass of cysteine plus HNO, or alternatively, the mass of a sulfinamide-modified cysteine.

In order to unambiguously determine the source of the 65 Da neutral loss, a comparative analysis of the fragmentation pattern of the intact precursor ion ([M+2H]$^{2+}$) and the product ion of the neutral loss ([M+2H-HS(O)NH$_2$]$^{2+}$) of peptide EKPLQNFTLC*FR-NH$_2$ was performed. This was sufficient to localize the neutral loss to the cysteine amino acid based on the y-, b- and other fragment ions (Figure 3.3(d)).
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Figure 3.3. Determination of the site of modification and the mass increment resulting from the modification. ECD Fragmentation (FT-ICR) of a) triply-charged MHRQETVDC*LK-NH$_2$, b) doubly-charged EKPLQNFTLC*FR-NH$_2$, c) doubly-charged MHRQETVDC*LK-NH$_2$ and d) MS/MS/MS fragmentation (Q TRAP) of the product ion of the neutral loss EKPLQNFTLC*FR-NH$_2$. 

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To determine the modification's fragmentation characteristics, collision energy profiling of the neutral loss fragment ion \([\text{M}+n\text{H}-\text{HS(O)NH}_2]^{++}\) and precursor ion was performed on a Q STAR XL. The neutral loss began to be produced at relatively low collision energies (~30 eV) with maximum production occurring between 60-70 eV (Figure 3.4). Initial neutral loss production at this low collision energy indicates that the characteristic fragmentation is amenable to any type of MS instrument as it is available by low and medium energy conditions.

### 3.3.4 Analysis of nitroxylated GAPDH from platelets: Targeted proteomic study for method validation

The treatment of platelets with AS and subsequent analysis of platelet proteins to determine the presence of HNO-induced modifications was the system of choice for examining HNO-induced modification in an \textit{ex-vivo} setting. This approach requires separation of platelet lysates on 1D SDS PAGE and in gel tryptic digestion to obtain modified peptides in a peptide mixture of limited complexities. As both the separation and digestion typically involve reduction of the sample to break the disulfide bonds, in order to determine whether the modification could withstand the reducing conditions, the standard sulfinamide modified peptides were subjected to reduction at dithiothreitol (DTT) concentrations ranging from 0.1 mM to 1 M for 1 hour at 56°C. Although the sulfinamide was found to be more stable than the disulfide modification, as little as 0.1 mM DTT was shown to drastically reduce the abundance of the sulfinamide modification to less than 50% of its original intensity while fully reducing the disulfide bonds (data not shown). It is for this reason that all preparatory steps were performed under non-reducing conditions and the gel bands were digested without reduction or alkylation.
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Figure 3.4. Determination of the stability and maximal production of the sulfinamide neutral loss. Neutral loss collision energy profiling (Q STAR XL) of a) doubly-charged MHRQETVDC*LK-NH₂, b) doubly-charged EKPLQNFTLC*FR-NH₂ and c) triply-charged MHRQETVDC*LK-NH₂.
We wished to validate this approach using a known nitroxylated protein. Due to extensive previous work on its modifications, particularly by mass spectrometry, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was chosen. GAPDH, a key enzyme in the carbohydrate metabolism pathway, has been shown to be potently inhibited by HNO both \textit{in vitro} and \textit{in vivo}, an effect thought to occur through the direct modification of its active site cysteine by HNO. Subsequent mass spectrometric analysis of the \textit{in vitro} nitroxylation of rabbit GAPDH revealed the formation of an intrasubunit disulfide between the active site cysteine (Cys 149) and the nearby Cys 153 upon treatment with HNO. The remaining two cysteines, Cys 244 and Cys 281, were converted to sulfinamides.

To examine GAPDH nitroxylation in a physiologically-relevant setting, platelets were treated with 10mM AS, lysed, separated by 1D SDS PAGE and the GAPDH region (approximately 37 kDa) was excised and trypsin digested. Analysis of the GAPDH peptides was by an information dependent acquisition (IDA) whereby an MS scan was followed by a product ion analysis on a Q STAR XL, where the most abundant doubly to quadruply charged ions present in the MS scan were selected for fragmentation in the product ion scan. Two of the three cysteines in human GAPDH (Cys-152 and Cys-156) were identified as being disulfide linked in the tryptic fragment 146-162, similar to earlier studies. The Cys-247 containing tryptic peptide (235-248) could not be found, either as a disulfide or in sulfinamide-modified or unmodified form. However, a peptide 1 Da heavier than the sulfinamide-containing form was consistently observed. Fragmentation of this peptide showed a neutral loss of 66 Da and a fragment ion series matching tryptic peptide 235-248 (Figure 3.5). To determine whether this neutral loss was linked to the sulfinamide modification, the modified peptide, MHRQETVDC*LK-NH$_2$, was subjected
Figure 3.5. High Dose HNO (10 mM AS) reacted platelet lysate separated by SDS PAGE and associated product ion spectra of HNO-modified GAPDH tryptic fragment 235-248 showing the loss of sulfinic acid. Proteins identified by the database search as modified by HNO are listed, with * indicating the peptide fragmented to produce the sulfinic acid neutral loss. Proteins in bold showed a dose dependent response to HNO.
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to the entire sample preparation procedure, which resulted in the peptide’s mass increasing by 1 Da and showing a neutral loss of 66 Da (rather than 65 Da). This experiment also confirmed that the modification was not removed as a result of the high temperature from boiling the sample at 99°C prior to SDS PAGE separation.

This neutral loss was hypothesized to be the loss of HS(O)OH rather than HS(O)NH₂ upon conversion of the sulfinamide into sulfinic acid by a deamidation reaction. This deamidation reaction has previously been observed on other amines such as asparagine and glutamine\textsuperscript{38, 39} and this reaction has previously been proposed for the sulfinamide\textsuperscript{24} (Illustration 3.2). The deamidation reaction is a likely mechanism (Illustration 3.2) as carbon and sulfur have very similar electronegativities, the sulfinic acid produced is a thermodynamically stable product and an intermediate forming a low energy intramolecular five-member ring transition state, similar to the succinimide intermediate seen with asparagine and glutamine deamidation, can be obtained. This low energy transition state lowers the reaction’s activation energy increasing the rate of reaction. Furthermore, combined with the high temperature from boiling the sample at 99°C prior to SDS PAGE separation, which has been shown to increase the rate of deamidation\textsuperscript{39}, and the vast excess of water in the reaction solution, these conditions yield a high rate constant that would lead to the quantitative conversion of the sulfinamide to sulfinic acid.

3.3.5 Global proteome analysis for discovery of nitroxylated platelet proteins

These findings in human GAPDH corroborate the results of the previous mass spectrometry study in rabbit GAPDH\textsuperscript{28}. Furthermore, the effect of the sample preparation method on the sulfinamide modification has been determined, the approach has been adjusted accordingly, and its ability to identify nitroxylated proteins in a physiologically-relevant
Illustration 3.2. Proposed mechanism for the conversion of the sulfinamide modification to sulfinic acid.
setting has been validated.

To further elucidate the role of this modification in human platelets, a global proteome analysis to identify nitroxylated proteins was employed. Initially, platelet samples treated with the highest dose of AS (10mM) were lysed, separated by 1D SDS-PAGE, and in gel tryptically digested (Figure 3.5). The resulting peptides were analyzed by an LTQ-Orbitrap mass spectrometer and searched using Mascot to determine nitroxylated peptides and the corresponding modified platelet proteins. Modified peptides determined from the database searches were verified by manual inspection of the MS/MS spectra, with modified peptides identified in 21 proteins (Table 3.1). Fourteen of the 22 modified peptides also showed the characteristic neutral loss of 66 Da.

Based on these findings, the 21 proteins were further examined to determine the dose-dependency of the modification. Platelets were treated with doses of AS ranging from 10μM to 10mM. The concentrations of AS were chosen to encompass those previously shown to cause inhibition of platelet function (between 1 and 40μM\textsuperscript{14}), as well as those used in other biological systems, such as rat cardiac muscle (50-1000 μM\textsuperscript{40}, 100-1000 μM\textsuperscript{41}). Treatment with AS was for 2 minutes, corresponding to the time at which inhibition of platelet aggregation by AS was previously observed\textsuperscript{14}. These parameters were used to identify those proteins whose modification may be involved in the inhibition of platelet aggregation by HNO. Following treatment, the samples were lysed and the proteins separated by 1D SDS-PAGE and in gel tryptically digested. The modified peptides that had been identified in the initial high-dose study were further analyzed by Multiple Reaction Monitoring (MRM) transitions (Table 3.1), set up to monitor the modified peptides at each dose of AS.
### Table 3.1. Modified peptides identified by the Orbitrap and the associated MRM transitions for the dose response experiment.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Peptides</th>
<th>MRM transition modified precursor</th>
<th>Internal standards</th>
<th>Neutral Loss</th>
</tr>
</thead>
<tbody>
<tr>
<td>Talin</td>
<td>VVAPTTSSPVCQEQLVL EAGR (722-741)</td>
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<td>415.26 → 415.26</td>
<td>Yes</td>
</tr>
<tr>
<td>Glycoprotein 1β</td>
<td>LTQNLNDRCELT (73-85)</td>
<td>526.94 → 526.94</td>
<td>591.82 → 591.82</td>
<td>Yes</td>
</tr>
<tr>
<td>Vinculin</td>
<td>REILGTCK (319-326)</td>
<td>476.25 → 476.25</td>
<td>553.31 → 553.31</td>
<td>Yes</td>
</tr>
<tr>
<td>Integrin β3</td>
<td>IGGFAVDKPVSPYMY ISPEALENCPYDMKT TCLP MFGVK(177-217) LFACSNK (669-675)</td>
<td>937.64 → 937.64, 937.64 → 1348.59, 937.64 → 1348.59, 937.64 → 1348.59</td>
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<td>No</td>
</tr>
<tr>
<td>Gelsolin</td>
<td>NTGIICTIGPASR (44-56)</td>
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<td>597.33 → 597.33</td>
<td>Yes</td>
</tr>
<tr>
<td>Pyruvate Kinase Isozyme M1/M2</td>
<td>NSLDCEIVSAK (423-443)</td>
<td>605.79 → 605.79</td>
<td>436.27 → 436.27</td>
<td>Yes</td>
</tr>
<tr>
<td>Adenylyl-cyclase associated protein 1</td>
<td>VNIQGSVTESLQACK (344-358)</td>
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<td>572.30 → 572.30</td>
<td>Yes</td>
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<tr>
<td>Actin</td>
<td>EKLCYVALDFEQEMA TAASSSSLEK (214-238)</td>
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<td>1172.09 → 1172.09</td>
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<tr>
<td>Actin-like protein 2</td>
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<td>GAPDH</td>
<td>VPTAVSVVDLCRLE KPAK (235-254)</td>
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<td>Fructose bisphosphate aldolase A</td>
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</table>
## Chapter 3: Cysteine Nitroxylation Analysis by Mass Spectrometry

### Table: Protein Peptides MRM transition • Neutral Loss

<table>
<thead>
<tr>
<th>Protein</th>
<th>Peptides</th>
<th>MRM transition modified precursor</th>
<th>Internal standards</th>
<th>Neutral Loss</th>
</tr>
</thead>
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<tr>
<td>Malate dehydrogenase</td>
<td>VIVVGNPANTNCLTAS K (126-142)</td>
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<td></td>
<td></td>
<td>866.95 → 582.36</td>
<td>617.36 → 817.48</td>
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<td>866.95 → 1151.54</td>
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<td></td>
<td></td>
<td>866.95 → 1214.58</td>
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<td>Twinfilin-2</td>
<td>HLSSCAAPAPLTSAR (137-152)</td>
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<td>572.27 → 572.27</td>
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<tr>
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<td>548.27 → 941.51</td>
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<td>548.27 → 773.42</td>
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<td></td>
<td>954.89 → 1762.67</td>
<td>774.86 → 774.86</td>
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<td>954.89 → 571.22</td>
<td>774.86 → 634.28</td>
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<td>Calpain</td>
<td>TDGFGIDTCR(136-145)</td>
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<td>435.22 → 435.22</td>
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<td>889.40 → 699.33</td>
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<tr>
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<td>914.96 → 1425.69</td>
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<tr>
<td>GTP-binding protein SAR1a</td>
<td>FLNARPMEVFMCSVLR KR (167-183)</td>
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<td>573.83 → 573.83</td>
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<tr>
<td></td>
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<td>694.34 → 1425.69</td>
<td>573.83 → 674.35</td>
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<td>696.34 → 517.31</td>
<td>709.90 → 709.90</td>
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<td>696.34 → 404.23</td>
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<td>Rab-1a</td>
<td>QVEVDCQQCMLEILD TAGTEQFTAMR (42-68)</td>
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<td>833.46 → 833.46</td>
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<td>1003.12 → 1056.48</td>
<td>833.46 → 1194.96</td>
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<tr>
<td>Profilin</td>
<td>CSVIRDSLLQDGEFSM DLR (71-89)</td>
<td>744.68 → 744.68</td>
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</tr>
<tr>
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<td>416.90 → 539.80</td>
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<td>744.68 → 660.32</td>
<td>628.85 → 628.85</td>
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<tr>
<td></td>
<td></td>
<td>744.68 → 637.30</td>
<td>628.85 → 950.49</td>
<td></td>
</tr>
</tbody>
</table>
Absolute quantitation of the degree of sulfinic acid modification was not possible as the unmodified form of the peptides could not always be identified because a portion of the free cysteine containing peptides were lost during sample preparation. Relative quantitation was thus employed to analyze the dose responses; however, plotting the average fragment peak areas of the modified precursor does not account for experimental variability from differing amounts of protein in the gel bands and differing amounts of peptide produced from the digestion. In order to correct for the variability from the quantity of protein from gel band to gel band, two non-modified peptides from the same protein were monitored and used to determine a correction factor (CF). The average fragment ion peak area (A) was then divided by this correction factor (A/CF). In the case of the variability in the production of the modified and two non-modified peptides from the same protein by tryptic digestion, the assumption that using similar digestion conditions from gel band to gel band would not produce statistically significant quantities of the peptides from experiment to experiment was made. This is a common assumption that underlies all quantitative proteomics experiments, certainly those that rely on label-free quantitation eg. spectral counting\textsuperscript{42}. Lastly, in order to account for instrumental variation from dataset to dataset, the corrected peak areas (A/CF) of each dataset were normalized (Figure 3.6). Taken together, these normalization steps allowed for the comparison of the responses of different proteins.

This approach led to the identification of 10 peptides, from 10 proteins, which were found to be modified by AS in a dose-dependent manner (Table 3.2 and Figure 3.6). None of the other suspected modified peptides found by the high-dose study could be identified in the MRM transitions, as the fragment ions of the modified peptides were not observed. Interestingly, of the 10 modified proteins identified in the MRM transitions, 9 had previously
Figure 3.6. Dose-dependency of protein modification by HNO. Relative quantitation of the MRM transitions of the modified peptides from doses of AS from 0 to 10mM: a) integrin β3, b) platelet glycoprotein 1ba, c) pyruvate kinase isozyme M1/M2 (△) and adenyl cyclase associated protein 1 (--; GAPDH (▲) and vinculin (--;), and e) actin 214-238 (--; α-enolase (--;), and gelsolin (--;). Data has been corrected for internal standard intensities, and normalized to the most intense value of each dose response. Graphs show mean ± standard error of the mean from n=3 separate experiments.
Table 3.2. Protein function for the sulfinic acid containing peptides that showed a dose response to HNO.
been found in the LTQ-Orbitrap analysis to contain a sulfinamide-modified peptide that fragmented to produce the characteristic neutral loss of 66 Da. The observation that the majority of the modified proteins identified in the MRM transitions were previously found to produce the neutral loss indicates that it is a characteristic marker of this modification. Furthermore, the one remaining protein that did not produce the neutral loss had its modified precursor ion in a highly charged state (5+) which, as discussed in the analysis of the test peptides, is not conducive to neutral loss formation. This also indicates that monitoring this neutral loss is a more accurate and specific method for discovering HNO-modified proteins than solely monitoring for the sulfinamide-modified peptide by mass shift of unmodified to modified peptide, which may generate false positives through incorrect peptide assignments.

The ten proteins identified as modified all show a dose-dependent increase in the level of sulfinamide modification with increasing AS concentration, as measured by the area of the corresponding fragment ion peaks from the MRM transitions (Figure 3.6). These proteins are involved in a variety of platelet processes, including cytoskeletal and cytoskeletal-associated proteins (actin, adenylyl cyclase associated protein 1, gelsolin and vinculin), metabolic enzymes (α-enolase, fructose bisphosphate aldolase A, GAPDH, pyruvate kinase isozyme M1/M2) and platelet surface receptors (integrin β3 and platelet glycoprotein 1bα). Indeed, many of the proteins identified play key roles in the regulation of platelet activation and while the responses of each of the proteins to AS vary, the modification is clearly seen in all proteins at higher doses of AS (1mM and 10mM).

The dose response curves of the various proteins were grouped together based on similar response trends (Figure 3.6), in order to gain some insight into the specificity and selectivity of the HNO-induced modification, about which little is currently known.
Interestingly, the most striking dose response curves are seen in the two platelet surface receptors, integrin β3 and glycoprotein 1ba (Figure 3.6(a,b)), which show increasing modification with increasing AS dose, even at concentrations as low as 0.1 mM in the case of β3 and 1 mM in the case of glycoprotein 1ba. As the HNO donor was added to intact platelets, the extracellular receptors would have encountered HNO relatively early in its concentration gradient, thus explaining, in part, the differences in response to AS seen from protein to protein.

As both of the platelet surface receptors mentioned above play key roles in platelet activation, their modification by HNO may be an important element of the inhibition of platelet aggregation by AS. Platelet glycoprotein 1ba and integrin β3 are involved in platelet adhesion and aggregation respectively. Glycoprotein 1ba, as part of the glycoprotein 1b-IX-V complex, binds von Willebrand Factor (vWF) as its primary ligand, and plays a key role in platelet adhesion and subsequent platelet activation. β3 exists in platelets as a heterodimer with αIIb, together comprising the platelet-specific integrin αIIbβ3. Its primary ligand is fibrinogen, which acts as a bridge between adjacent platelets, supporting aggregation. Of particular interest, integrin β3 is a highly cysteine-rich protein and disulfide bond rearrangement is known to be crucially important for its activation and it was recently shown to be S-nitrosylated. The cysteines found to be modified in both glycoprotein 1ba and β3 were extracellular, suggesting that the modification may interfere with ligand binding and/or subsequent outside-in signaling functions of these receptors. Indeed, treatment of platelets with AS has previously been shown to inhibit PAC-1 binding, a marker of integrin αIIbβ3 activation, and our results suggest that this inhibition may in fact result from a direct interaction between HNO and the receptor.
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HNO is a small, easily diffusible molecule that can cross biological membranes\(^6\) and thus it is not surprising that several groups of intracellular proteins were also found to be modified by AS. These could primarily be divided into platelet cytoskeletal and cytoskeletal-associated proteins and metabolic proteins. Both categories are instrumental in platelet activation; activation of platelet surface receptors such as integrin \(\alpha_{IIb}\beta_3\) leads to transduction of signals to the platelet cytoskeleton, its subsequent polymerization and re-organization, leading to shape change, while carbohydrate metabolism and glycolysis are thought to be important energy sources for platelet activation. Due to their various roles in platelet activation, interference with the function of these proteins through AS-induced modification could have ramifications for the ability of platelets to aggregate.

The monomeric actin binding protein adenylyl cyclase associated protein 1\(^7\) and the enzymes pyruvate kinase isozyme M1/M2 (and fructose bisphosphate aldolase A (data not shown)) were found to have similar concentration-response curves upon treatment with AS (Figure 3.6(c)). Both show slight responses to doses of AS of 1mM and below (see inset), within the range in which platelet inhibition has been shown to occur. However, there is a dramatic effect at an AS concentration of 10mM. This dramatic increase in modification may be related to the cytotoxic effects of AS, known to occur with high mM concentrations, and therefore may not be physiologically relevant. In a similar manner, the enzyme GAPDH and focal adhesion protein vinculin\(^8\) (Figure 3.6(d)) were grouped as they were found to have similar dose-responses to AS. Both GAPDH and vinculin show little response to AS at doses of 1mM and below, before climbing steeply at the 10mM AS dose, possibly also relating to a cytotoxic dose of AS.
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The final three proteins found to give similar concentration-response curves to AS were actin, gelsolin and α-enolase (Figure 3.6(e)). Gelsolin is an actin-binding protein involved in actin severing and capping\textsuperscript{49}, while α-enolase is an enzymatic protein. Modifications of these proteins were identified; however, while there was a trend of increasing modification with increasing dose of AS, there was no steep increase in the slope of the curve. There was greater variation in the data for these three proteins, largely due to low intensity peak areas of the fragment ions of the modified precursor. In summary, the ten modified proteins showed five different general trends in response to the reaction with AS (Figure 3.6(a-e)), indicating that HNO may target different proteins with different selectivities and specificities, an area which warrants more in depth investigation.

Redox regulation of cysteine residues has emerged over recent years as a key modulator of protein function. Indeed, in the case of six of the ten proteins identified as nitroxylated in our study, redox-based modifications of their cysteine residues, often in pathological or stressed situations and leading to alterations in protein function, have previously been reported. Recently, evidence has begun to come to light regarding the processes involved in regulating these redox-based post-translational modifications, showing that many of these are specific and reversible modifications that play a key role in protein function. Of particular relevance to our findings, an enzyme which catalyses the conversion of cysteine sulfinic acid to cysteine, known as sulfiredoxin, has recently been identified, leading to the description of a “sulfinic acid switch” in proteins whose role in the regulation of protein function is currently under investigation\textsuperscript{50}. This enzyme may be able to remove the sulfinamide modification, or alternatively, it is plausible that the sulfinamide modification may be converted \textit{in vivo} to sulfinic acid, and then be removed by the enzyme.
3.4 Conclusion

In the rapidly evolving field of protein regulation by redox-based direct post-translational modification\textsuperscript{51}, HNO-induced nitroxylation is one of the more recent to emerge. Despite recent progress, this area is still lacking readily available tools to unambiguously identify modified proteins, and to pinpoint the exact site of modification within the protein. The widely applicable mass spectrometry-based method we have developed can identify nitroxylated proteins, either on a single protein or a proteome-wide level, and can be applied to discover the modification of specific cysteine residues within proteins. Indeed, this approach has allowed for the first time the identification of HNO-modified proteins in a biologically-relevant setting. Platelets are known to be inhibited by HNO and this study reveals \textit{ex vivo} targets of HNO in platelets, and also gives insights into the mechanism of the inhibition, as many of the target proteins are involved in platelet activation pathways. Given the current excitement regarding HNO's potential as a therapeutic agent, for example for the treatment of heart failure\textsuperscript{31-13}, and its recent success in inhibiting angiogenesis and breast cancer growth \textit{in vitro}\textsuperscript{52}, the approach outlined in this paper should be of use to all in the biomedical community interested in further elucidating the targets and mechanism of action of nitroxylation.
3.5 References

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4 MULTIPLE NEUTRAL LOSS MONITORING (MNM): A MULTIPLEXED METHOD FOR POST-TRANSLATIONAL MODIFICATION SCREENING
(MNM DEVELOPMENT ON THE QTRAP)

4.1 Introduction

Many researchers in proteomics are concerned with the analysis of low-abundance proteins as they tend to be regulatory proteins that are involved in environmental changes and cell signaling. Post-translational modifications are responsible for determining the activity of these proteins and they are a very common mechanism employed by cells for regulating biological processes such as gene transcription and translation, apoptosis, anti-apoptosis, protein degradation, etc. There are over 300 different types of post-translational modifications with each type of modification having one or more unique functions within the cell. Identification of post-translational modifications is typically performed on triple quadrupole instruments using precursor ion scans on the charged marker ion or neutral loss scans on the characteristic uncharged fragments. A neutral loss scan is performed on a triple quadrupole instrument by scanning the ionized peptides or proteins through the first quadrupole (Q1). The ions are then fragmented in the second quadrupole (Q2) through low-energy collisions with a collision gas in the collision cell. The third quadrupole is then scanned with a fixed offset to Q1 specific to the modification and the charge state of the precursor peptide, transmitting the precursor ion minus the modification, i.e. the product ion of the neutral loss, to the detector. This neutral loss analysis identifies the presence of a modification as well as the associated precursor ion; however, as this scan function requires

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that a quadrupole be scanned, the duty cycle is in the range of 0.1%, i.e. if a mass range of 1000 Th is scanned in 1 Th increments, only 1/1000 of the time is spent transmitting the ion to the collision cell. The duty cycle drops even lower if a quadrupole time-of-flight mass spectrometer is used for the identification of neutral losses. This time-consuming process requires at least one analysis per post-translational modification as one must specifically search for a particular modification from a peptide of some particular charge state. This limits the total number of modifications screened in LC-MS/MS experiments, especially considering their inverse relationship with sensitivity in a fixed timeframe. As well, due to the poor duty cycle, the acquisition rate must be rather long and thus the neutral loss scan (as well as the precursor ion scan) is not routinely used for the analysis of sub-picomole quantities of peptides by LC-MS\(^5\). For these reasons, a novel scan technique has been developed to overcome the limitations of the conventional methods used to monitor for post-translational modifications.

4.2 Experimental

4.2.1 Reagents

The following peptides (bold amino acid residues indicate the site of modification) were obtained from the following sources: sulphated tyrosine (DYMGMDF) (Novabiochem, Darmstadt, Germany), phosphotyrosine (NRVYIHPF), phosphoserine (SAPPNLWAAQR), glycosylated serine (HLLVSNGGGEIEER) (gifts from BRC, Vancouver, BC, Canada), oxidized methionine (HDMNKVLDL) (Sigma-Aldrich, St. Louis, MO, USA). Bovine Serum Albumin (BSA) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Sequencing grade trypsin was obtained from Roche Diagnostics (Laval, Que.,
Formic Acid, acetonitrile, ethanol, and trifluoroacetic acid were purchased from Fisher Scientific (Whitby, Ont., Canada).

4.2.2 Mass Spectrometry

Method development was performed with peptides containing post-translational modifications at 5 µM in methanol/water/formic acid (50:45:5). Neutral loss collision energy profiling for the method development of the Multiple Neutral Loss Monitoring (MNM) scan was performed on a 2000 Q-TRAP instrument (Applied Biosystems/MDS Sciex Framingham, MA, USA) in nanospray mode using the Enhanced Product Ion (EN) scan which essentially traps all product ions utilizing Q3 as a linear ion trap. Neutral Loss Collision energy profiles of all neutral losses were collected by varying the collision energy from 5 to 150 eV in increments of 2 V for the collision energy acceleration potential. For each neutral loss, three neutral loss collision energy profiles were collected and then averaged.

A new scan was developed to be able to perform Multiple Neutral Loss Monitoring which allowed Q1 to be used as a high mass filter (that was set with a low mass cut off of m/z 350). This cut off was selected to eliminate the low mass background of the solvent. Acceleration voltages of 10, 15, 20, 25, and 30 V was applied from Q0 to Q2 to fragment the transmitted peptides in Q2. The peptides were trapped in Q3, then sequentially scanned out. Analysis of complex samples was performed at sub-micromolar concentrations of the modified peptide alone or spiked into a Bovine Serum Albumin (BSA) digest producing a standard mixture of approximately 250 femtomoles of modified peptides in a 250 femtomole BSA digest in 1% formic acid. The Bovine Serum Albumin digest was prepared by reducing, alkylating, and digesting the BSA overnight using sequencing grade trypsin. Both
analyses were performed on the 2000 Q-TRAP with an Ultimate nano-HPLC (LC Packings, Sunnyvale, CA, USA) using the Multiple Neutral Loss Monitoring (MNM) scan. HPLC separations used water:acetonitrile:formic acid with gradient elution. All scans were collected with a scan speed of 1000 amu/s.

4.2.3 Computational Analyses

Resulting LC-MS/MS spectra were analyzed using correlation analyses. A threshold was applied to each mass spectrum that was three times the lowest signal in the mass spectrum. The lowest signal is defined as the peak in the mass spectrum produced from a single charged particle striking the first dynode of the electron multiplier. This ensures that only peaks that were at least three times this intensity were analyzed. This routinely produced mass spectra that contained 4-12 peaks. An autocorrelation analysis was then performed on the entire MNM spectrum to identify apparent mass shifts that corresponded to known neutral losses. Equation 4.1 was used for the autocorrelation analysis:

\[
 r = \frac{\sum_{i=a}^{b} [(X_{1i} - X_{1M})(X_{2(i-d)} - X_{2M})]}{\left[\sum_{i=a}^{b} (X_{1i} - X_{1M})^2\right]^{1/2} \left[\sum_{i=a}^{b} (X_{2(i-d)} - X_{2M})^2\right]^{1/2}}
\]

\text{r = correlation coefficient}
\text{X}_{1i} = \text{intensity of spectrum 1 at m/z i}
\text{X}_{1M} = \text{mean intensity of spectrum 1}
\text{X}_{2(i-d)} = \text{intensity of copy of spectrum 1 at m/z i -d}
\text{X}_{2M} = \text{mean intensity of copy of spectrum 1}
\text{d= apparent mass shift}
\text{a = data acquisition start position (Th)}
b= data acquisition stop position (Th)

In the autocorrelation analysis, the position in the mass spectrum (i) is fixed and the apparent mass shift (d) is varied so as to allow the copy of the mass spectrum (2) to be shifted incrementally down the entire mass spectrum (1), providing correlations seen in the apparent mass shift which would correspond to different neutral losses. The correlation coefficient (r) was plotted against the apparent mass shift (d) to identify the neutral loss.

A convolution mapping analysis using this mass shift was applied to the mass spectrum which identified the precursor ion. Equation 4.2 was used for the convolution mapping analysis:

\[ c = (X_{1i} - X_{1M})(X_{2(i-d)} - X_{2M}) \]  (4.2)

\( c \) = convolution mapping coefficient

In the convolution mapping analyses, the apparent mass shift (d) is fixed, while the position in the mass spectrum (i) is varied in fixed increments (e.g. 0.077 Th). This can be pictured as having a window with width d (e.g. d of 49 for the neutral loss of H$_3$PO$_4$ which is lost from a doubly-charged phosphoserine-modified peptide) shifted along the mass spectrum in increments of 0.077 Th. The intensity of the two peaks that lie on the window edges are then multiplied together to produce a convolution mapping coefficient (c) for every value of (i). The convolution mapping coefficient was plotted against the position (i) in the mass spectrum. The precursor ion was identified as the maximum in the convolution mapping analysis.
4.3 Results and Discussion

4.3.1 Method Design

Modified peptides tend to fragment to either produce a characteristic marker ion or neutral fragment from the precursor ion. As both these marker ions and neutral fragments generally have unique mass increments, they are used as to indicate the presence of some modification. The structure of the precursor ion ([M+nH]⁺), neutral loss fragment and its mass increment, as well as the associated product ion ([M+nH-modification]⁺) for a number of different modifications that were used in this study are illustrated in Illustration 4.1. Specifically, these modified residues are sulfotyrosine with a neutral loss of 80 Da (Illustration 4.1(a)), phosphoserine with a neutral loss of 98 Da (Illustration 4.1(b)), N-acetylhexasine modified-serine (Illustration 4.1(c)), oxidized methionine (Illustration 4.1(d)), and phosphoryrosine (Illustration 4.1(e)).

Utilizing the unique trapping capabilities of a hybrid triple quadrupole/linear ion trap, we previously demonstrated the ability to rapidly identify multiple marker ions with the development of Multiple Precursor Ion Monitoring (MPM). MPM is performed by transmitting all peptides through Q1 to the collision cell, then fragmenting them in parallel. The resulting low mass fragment ions are trapped in Q3, then scanned out and detected, identifying the presence of modifications. This scan function increases the duty cycle to 100%; however, due to constraints of the linear quadrupole ion trap, the extraction efficiency is 20%. Current research with linear ion traps has been investigating the possibilities of increasing this extraction efficiency, but the 20% extraction efficiency still equates to a theoretical 200-fold sensitivity increase compared to the neutral loss scan. Multiple Neutral
Illustration 4.1  The structure of the precursor ions, neutral losses and product ions, as well as the corresponding neutral loss mass for various post-translational modifications:  a) sulfotyrosine, b) phosphoserine, c) N-acetylhexosamine modified serine, d) oxidized methionine and e) phosphotyrosine.
Loss Monitoring (MNM) is an analogous method to MPM; however, only the high mass fragments are scanned out of Q3, thus producing a mass spectrum that contains both the modified peptide and the peptide where the modification has been lost as a neutral fragment (Illustration 4.2(a)). In contrast, as the neutral loss scan function scans both the first and third quadrupole (Illustration 4.2(b)), the precursor ion minus the modification is the only ion to be registered at the detector, identifying both the precursor ion and the modification. Unfortunately, the neutral loss scan is limited to one charge state of one type of modification, while the MNM scan can detect multiple neutral losses regardless of the charge state of the precursor ion.

With the mass spectrum obtained from the MNM scan, further downstream computer processing is required to identify the neutral loss and the associated precursor ion; thus, an autocorrelation analysis is conducted to identify the neutral loss from the precursor ions (Illustration 4.3(a-b)). A copy of the mass spectrum (Illustration 4.3(a)) is created and is overlaid on top of the original mass spectrum. The top mass spectrum is then shifted incrementally to lower m/z values. Theoretically, at a very small mass shift, none of the peaks of the top spectra will be directly overlaid on any of the peaks on the bottom spectra. This is not the case in a real analysis as the background will always be overlaid on top of real peaks, and thus to simplify the autocorrelation analysis, a threshold is applied to the mass spectrum to remove the background and low intensity peaks. At some mass shift, one of the peaks from the top spectrum (ie. M+3H) will be overlaid on top of one of the peaks from the bottom mass spectrum (ie. M+3H-A) corresponding to a mass shift of some modification (ie. modification A) divided by the charge state of the peptide (ie. 3). Similarly, a mass shift of B/2 is seen when M+2H is overlaid on M+2H-B and a mass shift of C is seen when M+H is
Illustration 4.2  Mass Analyzer configuration and corresponding characteristic mass spectra for a) the Multiple Neutral Loss Monitoring (MNM) scan and b) the neutral loss scan.
Illustration 4.3  Theoretical steps of the data processing of Multiple Neutral Loss Monitoring: a) an MNM mass spectrum, b) the resulting autocorrelation analysis identifying the neutral loss(es), and c) the corresponding convolution mapping analysis identifying the associated precursor ion(s).
overlaid on M+H-C. These mass shifts of A/3, B/2 and C are then plotted in the autocorrelation analysis (Illustration 4.3(b)), identifying all of the neutral losses corresponding to all of the different types of modifications that are present in the sample from all of the different charge states of the peptide. With the mass shift (and thus the modification) identified, it is possible to identify the precursor ion. A convolution mapping analysis is applied to the mass spectrum (Illustration 4.3(a)) using the identified mass shift (Illustration 4.3(b)). A window with a width equal to the modification- and charge state-specific mass shift (ie. A/3, B/2, C) detected in the autocorrelation is moved incrementally up the mass spectrum. When both peaks fall on the edges of the window, a large convolution mapping coefficient is produced, identifying the precursor ion (Illustration 4.3(c)). Thus, this analysis is capable of identifying both the modification and precursor ion of every modification that fragments as a characteristic neutral loss from all of the charge states of that peptide. This analysis does not use time-correlation of the elution profiles to identify the precursor and product ions as this is done by the convolution mapping analysis; however, with a high resolution chromatographic separation, comparison of the precursor and product ion elution profiles could be used to confirm the identification. Furthermore, a high resolution liquid chromatographic separation would allow for a quantitative proteomic analysis as previously shown by accurate mass retention time pairs\textsuperscript{11} or protein correlation profiling\textsuperscript{12}, although nonlinearity of the signal response and ion suppression effects may be a concern.

4.3.2 Method Development

MNM scans of a serine-glycosylated peptide spiked into a BSA digest were acquired at a collision energy of 30 eV (Figure 4.1(a)), followed by the autocorrelation analysis of the
Figure 4.1  MNM analysis of glycosylated and phosphorylated peptides. MNM scans using an acceleration potential of 15 V (a,b), corresponding autocorrelation (c,d), and convolution mapping analyses (e,f) of 250 femtomoles of N-acetylhexosamine-modified serine peptide spiked into 250 femtomoles of BSA digest (a,c,e) and 250 femtomoles of phosphoserine-modified peptide spiked into 250 femtomoles of BSA digest (b,d,f).
spectra (Figure 4.1(c)) to identify the neutral loss and then the convolution mapping analysis (Figure 4.1(e)) to identify the associated precursor ion. The autocorrelation analysis of the glycosylated serine modification is not overly complicated by random noise as only 4 peaks are observed in the autocorrelation plot. The peak at a mass shift of 101.5 Th corresponds to the loss of the glycosyl moiety (203 Da) from a doubly-charged peptide, but a number of additional, seemingly unrelated correlations were also identified. The convolution mapping analysis reveals the m/z of the precursor ion (964 Th) as the only signal and no false positives were identified. A similar set of experiments for a phosphorylated serine peptide spiked into a BSA digest were conducted with MNM scans at a collision energy of 30 eV (Figure 4.1(b)), an autocorrelation analysis (Figure 4.1(d)), and a convolution mapping analysis (Figure 4.1(f)). The autocorrelation analysis of the phosphoserine-modified peptide contains a fair degree of noise and spurious peaks. The peak at an apparent mass shift of 49 Th corresponds to the loss of phosphoric acid (98 Da) from a doubly-charged precursor ion. The convolution mapping successfully identifies the precursor ion at 646 Th and no other precursor ions were identified as there was only one peak. The noise and the peaks that do not correspond to a neutral loss seen in the autocorrelation are dependent on the threshold used on the mass spectrum prior to the autocorrelation. The greater the number of peaks within the threshold-applied mass spectrum, the greater the noise and the number of peaks observed in the autocorrelation analysis. The peaks that do not correspond to a characteristic neutral loss for post-translational modifications are the result of two or more signals that are separated by this specific mass to charge ratio. There are three possibilities of how these peaks are generated. The first possibility is that the m/z difference corresponds to the removal of individual amino acid residues as is commonly observed for fragment ion series
of the same charge state such as the y- and b- type ions at higher collision energies. The second possibility is for these spurious peaks to be due to the m/z difference corresponding to the removal of several amino acid residues due to the presence of labile bonds, e.g. N-terminal of proline. The direction of the m/z shift is then dependent on the distribution of the charges on the fragments and results in a “neutral loss” of a peptide fragment, or two fragment ions\(^1\). The neutral loss of individual amino acid residues has previously been observed\(^2,3\). These studies have shown that low collision energies can lead to the formation of neutral fragments carrying the first or first two residues from the peptide, and that certain amino acids are much more likely to cleave as a neutral loss, depending on the charge state of the peptide. In both of these cases, as the m/z differences observed between these fragment ions correspond to the masses of amino acid residues and characteristic neutral losses of post-translational modifications have unique mass shifts that do not usually correspond to these amino acid residue masses, this does not tend to be a problem for the analysis. As subsequent analyses would only be performed on characteristic neutral losses, these peaks are of little importance; however, as the number of spurious peaks decreases, the probability of a random peak occurring at a mass shift corresponding to a neutral loss of interest also decreases. In this context, a combination of low resolution of the quadrupole and isotopic peaks could also lead to the false identification of a modification. This is observed for the neutral loss of a farnesyl moiety from a doubly-charged precursor ion (shift of 102.0 Th) that is incorrectly identified as a neutral loss of an N-acetylhexosamine moiety from a doubly charged precursor ion (shift of 101.5 Th) (data not shown). Upon examination of the autocorrelation data, the maximum correlation was identified at 102.0 Th with 101.5 Th being a lower intensity satellite signal, thus this false positive could be identified, explained,
and excluded. False identifications caused by the neutral loss of amino acid residues (e.g., the neutral loss of 97, 98, and 99 Da for proline, phosphoric acid, and valine, respectively) would be dealt with in a similar manner. Ultimately, the ability of the autocorrelation data to distinguish these interferences will increase with the mass resolution of the instrument. Lastly, the third possibility for these peaks is due to the m/z charge difference between two completely independent signals, either due to fragmentation or the co-elution of several peptides. These correlations are completely random and cannot be predicted. In the case of the co-elution of several peptides, the method benefits from a high-quality liquid chromatographic separation.

Since only four of the peaks within the glycosylated serine MNM mass spectrum (Figure 4.1(a)) were above the threshold, the autocorrelation analysis is very simple. Conversely, the autocorrelation analysis of the serine-phosphorylated peptide is complicated by a large number of peaks with low intensity. This is due to the higher number of signals above the noise. Upon examination of the mass spectrum (Figure 4.1(b)), it is evident that if the intensity of the $[\text{M}+2\text{H}-\text{H}_3\text{PO}_4]^2^+$ product ion increases relative to the rest of the peaks in the spectrum, this condition will lead to an increase in the correlation. Thus, in order to obtain the highest possible number for the correlation coefficient, a ratio of 1:1 of the precursor ion to the precursor ion minus the modification is useful. This 1:1 ratio can be obtained by varying the collision energy applied to the peptide in the collision cell and will be referred to as the Optimal Collision Energy (OCE). Therefore, tight control of the collision energy provides a means of excluding false positives generated by the three possibilities mentioned earlier, as well as its use will lead to the highest possible correlation value.
Enhanced product ion spectra of the phosphoserine-modified peptide were collected over a wide range of collision energies. At low collision energies (Figure 4.2(a)), the abundance of the intact peptide is much greater than the peptide minus the modification. At these low energies, the precursor ion has a low kinetic energy and upon collision with the collision gas in Q2, the peptide will not be very likely to fragment as its energy is below the threshold for fragmentation of the bond that connects the modification to the peptide side chain. The corresponding autocorrelation only contains the peak corresponding to the neutral loss of a mass shift of 49 Th; however, the correlation coefficient of 0.14 is not maximal. At some moderate collision energy (Figure 4.2(b)), the two ions have the same abundance as the precursor ion fragments to produce product ions. The autocorrelation analysis describes the presence of two peaks of equal intensity, one from a correlation from the $y_9^{2+}$ fragment ion and the precursor ion minus the modification at 69.7 Th and one corresponding to the neutral loss. The correlation coefficient for the neutral loss has increased to 0.33 and the analysis contains very little noise. At higher collision energies (Figure 4.2(c)), the peptide minus the modification becomes the dominant species out of the two ions. At these higher collision energies, significant backbone fragmentation of the peptide occurs which would complicate the autocorrelation analysis. This complication is seen in the autocorrelation analysis as a number of peaks are now present. As well, the correlation coefficient corresponding to the phosphoric acid neutral loss has significantly decreased to 0.04. It is evident that the largest correlation coefficient is obtained when the ratio of precursor ion to precursor ion minus the modification approaches one. In this 1:1 case, if these were the only two signals in the mass spectrum, the correlation coefficient would have a maximum value of 0.5. However, with increased collision energies, significant backbone fragmentation also occurs, exemplified by
Figure 4.2 Enhanced Product Ion (EPI) Mass Spectra and autocorrelation analysis of a phosphopeptide. EPI Mass Spectra of a phosphorylated serine-modified peptide (SAPPNLWAQOR) at a collision energy of a) 30 eV, b) 46 eV and c) 60 eV, and the corresponding autocorrelation analyses.
the formation of the y$_9^{2+}$, which causes additional signals to appear in the spectra and reduces the correlation coefficient for the peak at an apparent mass shift of 49 Th, which was due to the loss of phosphoric acid. Regardless, this 1:1 ratio is still desirable. Based on these results, it is also evident that a higher collision energy is required for the MNM scan of the phosphoserine-modified peptide compared to the 30 eV previously used (Figure 4.1(a)) while a collision energy below 30 eV is required for the MNM scan of the serine-glycosylated peptide (Figure 4.1(b)).

Consequently, the dependence of collision energy on the production of modification-specific neutral losses was investigated for a number of different modifications as well as for different charge states, when possible. The results are summarized in Figure 4.3 using two different types of plots, with the structures of the corresponding precursor ions and fragments seen in Illustration 4.1. Neutral loss intensity profiles are plots of the intensity of the precursor ion and the product ion at varying collision energies, while the neutral loss ratio profiles are plots of the ratio of the precursor ion : product ion and product ion : precursor ion. These two ratios have been plotted in the cases when the favourable 1:1 ratio can be obtained as it allows one to visualize that only a narrow collision energy range (~12-15 eV) corresponds to a ratio between 0.5-1. Only one of the ratios is plotted in the remainder of the cases, for which this 1:1 ratio is not reached. As the sulfotyrosine modification is very labile, even at very low collision energies, the M+H - SO$_3$ product ion is the dominant species compared to the precursor ion (Figure 4.3(a)). As the total ion transmission is low at very low acceleration potentials, the OCE is 11 eV for the sulfotyrosine. This would also be the optimal collision energy for the neutral loss scan as this is the apex of the product ion intensity profile, but the product ion is the dominant species and 1:1 ratio is not obtained.
Figure 4.3  Neutral loss intensity profiling and neutral loss ratio profiling of peptides containing post-translational modifications: a) sulfotyrosine, b) phosphoserine, c) N-acetylhexosamine (doubly-charged), d) oxidized methionine, e) N-acetylhexosamine (triply-charged) and f) phosphotyrosine. Ions in the neutral loss intensity profiling plots are denoted as precursor ion (♦) and precursor ion - NL (□), while the ratios in the neutral loss profiling plots are denoted as [precursor ion / precursor ion - NL] (♦) and [precursor ion - NL / precursor ion] (□).
For the phosphoserine (Figure 4.3(b)) and the doubly-charged N-acetylhexosamine-modified serine (Figure 4.3(c)) containing peptides, the OCE is 45 and 81 eV respectively, as seen when the ratio of M+2H : M+2H minus the modification approaches 1 in the neutral loss ratio profile as well as by the intersection point in the neutral loss intensity profiles. In both examples, the precursor ion does not tend to fragment at low collision energies. Increasing the collision energy, the precursor ion begins to fragment to give the neutral loss. Further increasing the collision energy produces the 1:1 ratio of the precursor ion to the product ion (precursor ion minus the modification). Increasing the collision energy a few eV results in a maximum in the product ion neutral loss intensity profile, which would be the optimal collision energy for the neutral loss scan. Continuing to increase the collision energy, both the precursor ion and product ion intensity decreases as both ions are fragmented. As expected from Figure 4.1, the OCE for the serine-phosphorylated peptide is above 30 eV; however, the OCE for the glycosylated serine is also above 30 eV when it was expected to be below 30 eV based on Figure 4.1. The reason for this is that in the MNM scan all charge states of the peptide are transmitted through the first quadrupole. As the triply-charged peptide fragments to produce mainly the marker ions, it also produces the doubly-charged peptide minus the modification, in addition to the unfragmented triply-charged peptide and the triply-charged peptide minus the modification. Therefore, the doubly-charged peptide minus the modification in Figure 4.1(a) is the sum of the fragment ions generated from doubly- and triply-charged species. Thus, its intensity is higher than when the doubly charged precursor ion is isolated in Q1 as was done in Figure 4.3(a). The oxidized methionine (Figure 4.3(d)), triply-charged N-acetylhexosamine-modified serine (Figure 4.3(e)) and phosphotyrosine (Figure 4.3(f)) containing peptides do not tend to fragment to
give a large abundance of the peptide minus the modification, and fail to produce a 1:1 ratio, so the OCE has been reached when the signal of the peptide minus the modification is at a maximum at 50, 51, and 60 eV, respectively. It should be noted that the triply-charged glycosylated serine tends to produce very little of the neutral loss compared to the doubly-charged precursor ion. Instead, it fragments to produce known modification-specific marker ions, which was also the case with the doubly-charged phosphotyrosine modification.

As seen here with peptides, there is an optimal collision energy for each modified peptide where the intensity of the modified peptide is equal to the intensity of the peptide where the modification has fallen off. This concept of an optimal collision energy has previously been shown for glycosides using a neutral loss scan and thus they recommended the use of a collision energy gradient. A similar procedure was proposed for PTM marker ion monitoring with a stepped collision energy approach. When the neutral loss collision energy profiles of several modifications are combined into a single reference graph (Figure 4.4), it becomes evident that a collision energy gradient is necessary for the MNM scan function (as would it be for a neutral loss scan) as each modification has a unique optimal collision energy dependent on the type of modification and charge state of the peptide. More importantly, at the optimal collision energy of the phosphoserine modification, neither of the other modifications would be identified since one is completely fragmented (sulfotyrosine) and the other has not yet begun to fragment (N-acetylhexosamine).

Schlosser and others have previously shown for phosphorylated peptides that the optimal collision energy is also (linearly) dependent on the size of the peptide. Therefore, only the development of the MNM scan with a collision energy gradient would allow for the collection of mass spectra at each optimal collision energy, producing the most statistically
Figure 4.4 Optimal Collision Energy reference graph for three labile post-translational modifications: sulfotyrosine, phosphoserine, and N-acetylhexosamine-modified serine.
significant autocorrelation analysis. Implementing the collision energy into the MNM scan further improves the selectivity of the analysis if a false positive occurs due to a random correlation. In any case, false positives will not be analyzed if this correlation is seen at some collision energy that does not correspond to the characteristic collision energy for that neutral loss. Thus, implementing collision energy as a key parameter in the development of the MNM scan will lead to a much more selective analysis given that peptide mass, charge state, and sequence are also taken into account. Therefore, operating this scan with a collision energy gradient would also facilitate the analysis of multiple modifications on a single peptide. Multiple modifications of the same type are easily identified at their optimal collision energy as the autocorrelation analysis would report multiples of a given modification-specific mass shift (eg. $2 \times 49 \text{ Th} = 98 \text{ Th}$ for a doubly-charged, doubly-phosphorylated peptide), and the subsequent convolution mapping analysis would identify the origin of the single and multiple mass shifts. In contrast, multiple modifications of different types can only be identified in a sequential fashion, as the mass shift of the more labile modification would be detected at low, and that of the more stable modification at high, collision energy in the autocorrelation. However, sequential convolution analysis would still identify one as the product of the other as the product ion of the first neutral loss is only transient and not present in the lowest collision energy spectra.

As the collision energy is defined as the collision energy acceleration potential multiplied by the charge state of the peptide, it is impractical to operate the MNM scan with an actual collision energy as we are transmitting precursor ions of different charge states through the mass analyzer. Thus, we will use the optimal collision energy acceleration potential when referring to the optimal collision energy, which is 11, 22, 40, 25, 17 and 30 V
for the six different species, in the order used in Figure 4.3. Furthermore, as seen in the neutral loss ratio profiles for the modifications that fragment to give the 1:1 ratio, as well as the neutral loss intensity profile for the sulfotyrosine and the stable modifications, the scan should be operated with the collision energy increasing in increments of 5 V. Increments of greater than 5 V may not identify the modification as the analysis may occur outside of the optimal range. The use of a collision energy gradient with increments smaller than 5 V will lead to too much time being spent fragmenting the precursor ion outside of the optimal collision energy range. Therefore, it was concluded that testing of the MNM scan should be performed at 5 different acceleration potentials 10, 15, 20, 25 and 30 V. The maximum acceleration potential was set to 30 V because as the collision energy increases, the degree of backbone fragmentation dramatically increases and this would complicate the autocorrelation analysis.

4.3.3 Method Evaluation

The MNM analysis of an oxidized methionine-containing peptide in a BSA digest was carried out to illustrate the importance of the collision energy gradient (Figure 4.5). Investigation of the autocorrelation analysis and MNM mass spectrum at a collision energy acceleration potential of 10 V indicates that very little of the precursor ion minus the modification has been formed, which is why the correlation coefficient for the autocorrelation signal at 32 Th is very low. At a collision energy acceleration potential of 20 V, the intensity of the [M+2H-CH_{3}SOH]^{2+} peak has increased in intensity in the MNM spectrum leading to an increase in the value of the correlation coefficient. At a collision energy acceleration potential of 30 V, the intensity of the [M+2H-CH_{3}SOH]^{2+} peak has
Figure 4.5  MNM analysis of 250 femtomoles of an oxidized methionine-containing peptide in 250 femtomoles of a BSA digest taken at collision energy acceleration potentials of 10, 20 and 30 V.  a) Total Ion Chromatogram with the corresponding MNM mass spectrum of the oxidized methionine containing peptide at a collision energy acceleration potential of b) 10 V, c) 20 V, and d) 30 V, and the corresponding autocorrelation analysis at e) 10 V, f) 20 V, and g) 30 V.
increased again in the MNM spectrum as has the correlation coefficient; however, the number of spurious peaks has also increased dramatically. Therefore, the analysis of this peptide is best performed between 20 and 30 V, which is in agreement with the optimal collision energy acceleration potential that was determined to be 25 V in Figure 4.3.

The selectivity of the MNM scan was tested by mixing four modified peptides (N-acetylhexosamine, oxidized methionine, phosphoserine and sulfotyrosine) creating a 500 femtomole per microlitre mixture in 0.1% formic acid and then loading 500 femtomoles on column. Indeed, all four peptides were detected in a single MNM experiment (data not shown). These four peptides were then spiked into a BSA digest and were successfully identified from a more complex biological matrix that mimicked an enzymatic digest of a modified protein (Figure 4.6). The mixture was analyzed at five different collision energy acceleration potentials, and each of the five corresponding total ion chromatograms showed the presence of a large number of peptides (Figure 4.6(a)). The total ion chromatogram that corresponded to the OCE for each modification was then used to extract the MNM spectrum at every time point, and an autocorrelation of each MNM spectrum was performed. The autocorrelation coefficient corresponding to the apparent mass shift of the neutral loss was then collected from each of the autocorrelation analyses, which allowed for the construction of an extracted ion chromatogram of autocorrelation coefficients. Figure 4.6(b) is a composite of the four extracted ion chromatogram autocorrelation coefficients, which is then followed by a convolution mapping analysis (Figure 4.6(c)) that was collected at the time corresponding to the autocorrelation coefficient maxima. This identified the precursor ions responsible for the apparent mass shifts identified in Figure 4.6(b). The peaks at 36.6 and 77.3 min in Figure 4.6(b) were attributed to oxidized methionine as the mass spectra showed
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Figure 4.6. MNM analysis of a mixture of serine glycosylated, oxidized methionine, sulfotyrosine, and phosphoserine modified peptides in a BSA digest: a) Total Ion Chromatogram at a collision energy acceleration potential of 10 V, b) overlayed reconstructions of the Extracted Ion Chromatograms autocorrelation coefficients of the apparent neutral losses of sulfotyrosine at 10 V, glycosylated serine at 20 V, phosphoserine at 25 V and oxidized methionine at 25 V, and c) convolution mapping analysis identifying the modified precursor ions.
a mass shift of 32 (64 for the loss of CH$_3$OH divided by a charge state of 2). The peaks at 40.7, 42.4, and 45.2 min were attributed to the N-Acetylhexosamine modification as the mass spectra showed a mass shift of 101.5 (203 for the loss of the N-Acetylhexosamine moiety divided by a charge state of 2). The peak at 49.5 min was attributed to phosphoserine due to an apparent mass shift of 49 (98 for the loss of H$_3$PO$_4$ divided by a charge state of 2) and the peaks at 54.9 and 77.0 min were attributed to sulfotyrosine due to an apparent mass shift of 80 (80 for the loss of SO$_3$ divided by a charge state of 1). The subsequent convolution mapping analysis (Figure 4.6(c)) identified the modified precursor ions that were associated with the apparent mass shifts. The peak at 36.6 min was successfully identified as the oxidized methionine peptide as it had a m/z of 550.7 Th. The other peak that was identified as an oxidized methionine containing peptide is a false positive that is due to the correlation of the singly charged sulfotyrosine modified peptide minus the sulfo moiety and some other ion. In the analysis of the glycosylated peptides, the peak at 40.7 min was successfully identified as being serine glycosylated as it had a m/z of 963.8 Th, while the two other peaks at 42.4 and 45.2 min were identified as false positives. The peak at 49.5 min was successfully identified as the phosphoserine containing peptide as it had a m/z of 645.7 Th and the peak at 77.0 min was identified as the singly charged sulfotyrosine containing peptide with a m/z of 1175.0. The peak at 54.9 min was identified as a false positive. All four of the modified peptides were successfully identified by this analysis with very few false positives identified. In any case, as all of the false positives were associated with some peptide within the sample, further analysis of these peptides would still yield valuable information.
4.4 Conclusion

The Multiple Neutral Loss Monitoring (MNM) scan function successfully identified a number of different modifications that may not have been identified in a neutral loss scan unless each modification was specifically monitored. The MNM scan has thus been shown to be a very powerful method for comprehensive post-translational modification monitoring. Further investigation of the dependence of the optimal collision energy on the peptide size, charge state, and sequence are required to better define their relationships, which could be fashioned into an algorithm to facilitate the MNM scan. Applications of the MNM scanning method for the complete analysis of protein and protein complexes from biological matrices will be the next step.
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4.5 References


5 IMPROVED MULTIPLE NEUTRAL LOSS MONITORING (MNM) FOR A MORE EFFECTIVE MULTIPLEXED POST-TRANSLATIONAL MODIFICATION ANALYSIS (IMPROVED MULTIPLE NEUTRAL LOSS MONITORING)\(^a\)

5.1 Introduction

It is becoming increasingly evident that many different post-translational modifications (PTMs) play an essential role in proper protein and cellular function\(^1\)\(^-\)\(^7\). Current methods to analyze for post translational modifications target only one particular type of modification which, although selective, can be insensitive and time-consuming\(^5\). Researchers have begun to develop novel methodologies for the global analysis of post-translational modifications to overcome some of these hurdles. The majority of these approaches utilize mass spectrometry as it is a tool that is very amenable to studying PTMs because every modification imparts a specific mass change on/onto the modified amino acid. Novel computer algorithms for database-reliant approaches have utilized this mass change to mine MS and MS/MS data for multiple types of modifications\(^8\)\(^-\)\(^{16}\).

Alternatively, many different types of post-translational modifications can fragment, upon collision induced dissociation (CID) within the mass spectrometer, to produce a characteristic fragment ion (marker ion) or characteristic neutral loss fragment. Although these fragments would previously have been monitored by the precursor ion or neutral loss scans respectively, more sensitive multiplexed methods are beginning to be developed that utilize unique configurations of tandem mass spectrometers\(^17\)\(^-\)\(^{23}\). These techniques generally operate the first mass analyzer as a high mass filter (or fully open), followed by collision

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\(^a\) A version of this chapter will be submitted for publication. Hoffman, M.D.; Rogalski, J.C.; Sniatynski, M.J.; LeBlanc, J.C.Y.; Duchoslav, E.; Kast, J. Improved Multiple Neutral Loss Monitoring (MNM) for a more effective Multiplexed Post-Translational Modification Analysis.
induced dissociation in a collision cell, and then the second mass analyzer is scanned. One of these techniques, Multiple Neutral Loss Monitoring (MNM)\textsuperscript{21}, was previously performed on a triple quadrupole instrument where Q3 has linear ion trapping capabilities. The method was shown to successfully identify the modified peptides, but did suffer from false positive identifications. Here we investigate the sources of these false positives, and introduce methods to circumvent the issue of false positive identifications, leading to a more effective PTM analysis.

5.2 Experimental

5.2.1 Reagents

Alpha-casein (bovine milk), iodoacetamide, and dithiothreitol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sequencing grade trypsin was obtained from Roche Diagnostics (Laval, Que., Canada). Formic Acid, acetonitrile, methanol, and trifluoroacetic acid were purchased from Fisher Scientific (Whitby, Ont., Canada).

5.2.2 Mass Spectrometry

Protein samples were reduced, alkylated and digested overnight with sequencing grade trypsin\textsuperscript{24}. All experiments were performed by loading 3-4 picomoles of a single protein digest on to a C\textsubscript{18} column for nanoHPLC separation. Experiments were performed using: an electrospray ionization (ESI) QTRAP 2000 (Applied Biosystems/MDS Sciex, Concord, Ont. Canada) which was coupled to an Ultimate nano-HPLC (LC Packings, Sunnyvale, CA, USA), or an ESI quadrupole time-of-flight QSTAR XL (Applied Biosystems/MDS Sciex, Concord, Ont. Canada). All HPLC separations used the following mobile phases: mobile phase A 0.1% formic acid/95% water/5% acetonitrile and mobile
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phase B 0.1% formic acid/20% water/80% acetonitrile. Mobile phase B was ramped linearly from 0% to 30% over 50 minutes, then up to 90% B in 7 minutes, remaining at 90% B for 5 minutes, and then the column was equilibrated at 0% B for 20 minutes.

Protein identifications were performed by an information dependent acquisition (IDA) whereby an MS scan was followed by a product ion analysis, where the most abundant doubly to quadruply charged ions present in the MS scan were selected for fragmentation in the product ion analysis. The mass spectra were extracted to Mascot generic format files by a script within Analyst 1.4.2 or Analyst QS 1.1 (the software that controls the QTRAP and QSTAR, respectively) and then searched against the mammalian SwissProt Database (v.45; 52,097 sequences) using Mascot v. 1.925.

The MNM scan was set up as previously discussed21, using Q1 as a high mass filter. The low mass cut off was set to 400 Th for the ESI experiments. The low mass cut offs were selected in an effort to remove the low mass background of the solvents from the ESI experiments. Acceleration voltages between 10 - 50 V were applied from Q0 to Q2 to fragment the transmitted peptides in the collision cell (collision gas pressure of 3-4x10⁻² torr; collision gas of nitrogen). The peptides were then trapped in Q3 and scanned out. All QTRAP experiments were collected with a scan speed of 1000 amu/s. An MNM Graphic User Interface (GUI) tool was used to analyze the spectra26 which uses mathematical correlation procedures previously discussed21,27.
5.3 Results and Discussion

5.3.1 Verification of the known modifications on alpha-Casein

Following the MNM validation studies in chapter 4, further QTRAP-based MNM studies were performed on tryptic digests of the multiply modified proteins: bovine asialofetuin, chicken egg ovalbumin and bovine alpha casein; however, the analysis succumbed to some false positives. To investigate the source of the false positive identifications, a systematic investigation of the MNM method was completed with the standard protein, alpha casein. As a first step, it was necessary to verify that the batch of alpha casein used for the studies was in fact modified as described in the literature (Table 5.1) or else it would not be an accurate assessment of the MNM method.

To verify that our batch of the standard did in fact contain these known modifications, the protein digest was analyzed by an MS analysis, followed by an Information Dependent Acquisition (IDA) product ion analysis on an ESI Q STAR XL system. A Mascot search was performed that included the variable modification of phosphorylation (S,T). The analysis identified four singly phosphorylated peptides (Table 5.1) – two of which originated from alpha-S2-casein (peptides 40-56, 153-164), while the other two were derived from alpha-S1-casein (peptides 119-134, 121-134). Phospho-peptide 40-56 was only present as the triply charged form and did not show evidence of a \( \text{H}_3\text{PO}_4 \) neutral loss in the product ion spectra. Phospho-peptide 119-134 was largely present as the triply charged precursor with only a small amount of the doubly charged precursor, whereas phospho-peptides 153-164 and 121-134 were largely present as the doubly charged form. All three of the doubly charged phospho-peptides fragmented to produce a neutral loss of \( \text{H}_3\text{PO}_4 \) in their product ion spectra. A doubly charged precursor ion at 964.3 Th was observed in the MS spectrum (S/N...
Table 5.1. Known modifications for the protein α-Casein as given in the literature along with the results of the IDA product ion analysis and MNM analysis.
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of 3); however, due to its low intensity, a product ion spectrum could not be collected and thus it cannot be confirmed that it was the doubly modified peptide 58-73 (no other charge states of this peptide were found). No other precursor ions were observed in the MS spectra that corresponded to the more highly modified peptides or the unmodified version of these peptides. Thus, for the ensuing MNM analyses of the alpha casein digest, only the four identified modified peptides were used to gauge the success of the method.

5.3.2 Troubleshooting the QTRAP MNM Analysis

Investigation of the true positive PTM identifications, the severity of false positive PTM identifications and the cause of the false negative PTM identifications is essential to improve the MNM method. The false negatives, modified peptides that are not identified by the method, would include the multiply-modified peptides that were not identified in the previous QSTAR analysis, which may have been due to substoichiometric modification and/or a lower ionization efficiency. False negatives are a limitation of both the sample and the instrument as the problem is predominantly a sensitivity issue. This problem is not a limitation of the method as the QTRAP is operated with the highest possible duty cycle under the MNM configuration. This sensitivity issue was investigated by comparing analyses of three picomoles of the alpha casein digest, run by the following four methods on the QTRAP: 1) a 1.4 second MNM scan, 2) a 14 second neutral loss scan, 3) a 3 second neutral loss scan, and 4) a 7.3 second information dependent acquisition (IDA) MSMS approach. The MNM scan identified 4 phosphopeptides (Table 5.1), whereas the 3 second neutral loss scan did not identify any phosphopeptides (Fig. 5.1(a)), the 14 second neutral loss scan identified 3
Figure 5.1. Comparison of MS approaches for monitoring the phosphorylation modification in alpha-Casein. Analysis of a 3 picomole alpha-Casein tryptic digest by a) a 3 second Neutral Loss scan (Neutral Loss of 49 Th; collision energy acceleration potential of 40V), b) a 14 second Neutral Loss Scan (Neutral Loss of 49 Th; collision energy acceleration potential of 40V) and c) a information dependent acquisition MS/MS analysis, all performed on a QTRAP.
phosphopeptides with signal to noise ratios ranging from 2 to 6 (Fig.5.1(b)); and the 7.3 second IDA MS/MS approach identified 3 phosphopeptides (Fig.5.1(c)) from the Mascot search. None of the methods identified phospho-peptide 40-56 of α-S2-casein. The earlier QSTAR analysis showed this peptide to be triply charged and to not fragment to produce the characteristic neutral loss. Thus, the approaches that rely on the neutral loss for detection of the modification (MNM and the neutral loss scan) should not be expected to find the modification; however, the IDA MSMS approach does not rely on the neutral loss to detect the modification, and thus should have identified this peptide.

The MNM method identified more modified peptides than any of the other methods, which was expected from the high duty cycle of the approach; however, none of the methods identified the highly modified peptides (Table 5.1). Possible means of circumventing this sensitivity problem for the MNM analysis would be to load more sample into the mass spectrometer, use a more sensitive instrument or enrich for the modified peptides (ie. titanium dioxide enrichment of phosphopeptides)\textsuperscript{28}. However, these strategies would either increase the number of false positives in the MNM analysis (as the abundance/variety of ions will increase) or limit the number of modifications that could be monitored.

Although the false negative identifications are not necessarily a problem with the method, false positive identifications, signals in the convolution maps of the MNM analysis that are not from modified precursor ions, are a limitation of the method. It was thus crucial to determine the causes and frequency of false positives. The MNM analysis of alpha casein was performed at five different collision energy acceleration potentials, with the total ion chromatogram (Fig.5.2(a)) displayed to present the elution of the peptides and their relative abundances, and the associated convolution mapping chromatogram (Fig.5.2(b)) is shown at
Figure 5.2. MNM analysis of a 3 picomole alpha-Casein tryptic digest on a QTRAP. a) Total ion Chromatogram, b) Convolution mapping chromatogram at a m/z shift of 49 and a collision energy acceleration potential of 40V, and the associated Convolution Maps at times c) 28 min, d) 39 min, e) 41 min, f) 47 min, g) 51 min, h) 71 min and i) 75 min.
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a collision energy acceleration potential of 40 V. The MNM convolution mapping chromatogram is a plot of the sum of the peaks in each of the convolution maps (Fig.5.2(c-i)), at each time point in the analysis, at a mass shift of 49 Th. This mass shift corresponds to a loss of $H_3PO_4$ (98 Da) from a doubly charged phosphopeptide. Thus, the convolution mapping chromatogram (Fig.5.2(b)) is used to indicate every occurrence of a phosphopeptide within the LC run, whereas the convolution maps are used to identify the modified precursor ions (Fig.5.2(c-i)). A total of seven peaks were identified within the elution period (Fig.5.2(b)), and the sources of the 49 Th mass shift can be observed in the convolution maps (Fig.5.2(c-i)).

Similarities can be seen between the convolution mapping chromatogram (Fig.5.2(b)) and the total ion chromatogram (Fig.5.2(a)), with respect to both the location and height of the peaks. As well, it is interesting to note that the two largest peaks in the convolution mapping chromatogram (Fig.5.2(b)) have convolution maps with a large number of precursor ions identified (Fig.5.2(f,g)). The more abundant the precursor ion, the more abundant the number of detectable fragment ions in the MNM mass spectra. As the number of detectable fragment ions increases, so does the probability of a correlation of the mass shift of interest between two ions by coincidence, leading to an increased rate of false positives. This relationship was investigated by determining the source of each peak within the convolution maps (Fig. 5.2(c-i)).

There are four theoretical sources of false positives when studying mass shift signals in mass spectrometry: satellite signals of isotopic patterns, the neutral loss of individual amino acid residues, the removal of several amino acid residues due the presence of labile bonds or two completely independent signals, either from the same peptide or from the co-
elution of different peptides, all of which have been previously discussed\textsuperscript{21}. As the m/z differences observed between the neutral loss of amino acid residues, as well as the removal of several amino acid residues, do not correspond to the characteristic neutral loss of H\textsubscript{3}PO\textsubscript{4} from the phospho-peptides of interest, this is not a problem for this MNM analysis. Thus, the impact of satellite signals of isotopic patterns and the correlation of two completely independent signals can be seen when interpreting the MNM analysis of alpha casein, as shown by the origin of the correlation of the signals (Table 5.2). As the software used to analyze the MNM data\textsuperscript{26} centroids and deisotopes the peaks in the MNM spectra prior to the correlation and convolution analysis, there should be very few false positives caused by satellite signals of isotopic patterns. As expected, there are only two such occurrences (precursor ions 798.2 and 831.3 Th) within Table 5.2 as they are the result of correlations between the second isotope of the M+2H and the second isotope of the M+214-H\textsubscript{3}PO\textsubscript{4}. These false positives have resulted from the software’s inability to deisotope the low resolution QTRAP data (Yves LeBlanc, personal communication), which is highlighted by the low resolution signals of the precursor ions (Fig.5.3(a,b)). Higher resolution signals are thus required for the GUI to properly deisotope the MNM mass spectra (Table 5.2).

To determine whether the remaining false positives are caused by two completely independent signals from the same peptide or from the co-elution of different peptides, time course elution profiling of the correlated signals was performed. Correlated signals with elution profiles that completely overlap indicate that the false positive is the result of a correlation between ions originating from the same peptide, whereas elution profiles that do not overlap indicate that the false positive is from the co-elution of different peptides. Time course elution profiling confirmed that the remaining false positives are all produced from 2
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* letters a-e refer to the time course elution profiles in Figure 5.4

Table 5.2. Origin of the precursor ions identified in the MNM convolution mapping analysis of α-Casein

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Figure 5.3. MNM mass spectra of alpha casein highlighting the low resolution of precursor ions a) 830.8 Th and b) 797.8 Th generated by the QTRAP. It is the low resolution of these peaks that resulted in the GUI’s inability to determine that the peaks at a) 831.3 Th and b) 798.2 Th were the second isotopes of 830.8 Th and 797.8 Th, and thus did not successfully deisotope the spectra prior to the convolution mapping analysis.
signals from the same peptide as the elution profiles all match (Figure 5.4). Only a subset of the time course elution profiles (Figure 5.4) is given as all of the profiles displayed the same trend in that the profiles all match. Once it was determined that the correlated signals were from the same peptide, the MNM total ion chromatogram (at a collision energy acceleration potential of 10 V) was used to determine the time at which each peptide eluted and the peaks within the MNM spectra were assigned using an in silico peptide fragmentation. These assignments were confirmed by comparing the MNM mass spectra to the IDA MS/MS analysis on the QSTAR. Based on the fragment ion assignments in Table 5.2, all of the 49 Th correlations have m/z shifts involving the correlated signals between an m/z of 48.96 and 49.10 Th. This is problematic as a resolution in the tens of thousands would be required to distinguish between a neutral loss of H$_3$PO$_4$ from a doubly charged precursor and the other nominal mass interferences in order to determine whether or not the identification is a true positive.

Detailed inspection of the correlated signals revealed that all of the false positive identifications come from peaks within the MNM mass spectra that are singly- rather than doubly-charged peaks, which would be required if the 49 Th m/z shift corresponded to a loss of H$_3$PO$_4$. A correlation-based analysis was performed on the mass spectra to eliminate the peaks that did not correspond to doubly-charged precursor and fragment ions. Unfortunately, the analysis was not capable of determining the charge state, largely due to the low resolution of the mass spectra (although noise and signal intensity were also an issue in certain cases). Computational charge state determination, and thus false positive elimination, would be much better suited for higher quality data (high resolution, low noise), requiring a different mass spectrometric instrument such as a QqTOF. Lastly, all of the false positives caused by
Figure 5.4. Time course elution profiling of the correlated ions extracted from the Total ion Chromatogram at a collision energy acceleration potential of 40V. The correlated ions (high m/z (pink) and low m/z (blue)) are shown for a selection of the precursor ions a) 797.8 Th, b) 964.3 Th, c) 830.8 Th, d) 440.4 Th, e) 441.9 Th, and f) 553.5 Th.
the correlation of two completely independent signals have been attributed to correlations between two fragment ions. Therefore, provided the identified precursor is not produced by in source fragmentation, the precursor will not be observed in the accompanied MS scan. As such, all of the false positives caused by the correlation of two independent signals could be eliminated (Table 5.2) as they were not present in the MS analysis with a significant signal to noise (S/N>5). These approaches, working in unison, have successfully highlighted the false positive identifications and their respective sources, resulting in a more efficient PTM analysis by the MNM approach.

5.4 Conclusion

The Multiple Neutral Loss Monitoring (MNM) scan has been shown to be the most sensitive means of analyzing post-translational modifications on a QTRAP. As well, the source of false positives has been investigated, and methods to circumvent this issue have been discussed, leading to a more effective PTM analysis. Other MS configurations, such as a MALDI source and higher resolution mass analysis, may also decrease the probability of false positives. In the case of the MALDI source, fragmentation of a singly charged precursor ion may lead to an increased production of the neutral loss while decreasing the production of the false positive producing fragment ions, as seen in chapters 2 and 3. As well, a higher resolution mass analysis would allow for the computational determination of the charge state of precursor and fragment ions, and thus the subsequent elimination of peaks that do not match the charge state of the neutral loss.
5.5 References

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(12) Bandeira, N. Biotechniques. 2007, 42, 687, 689, 691 passim.
Chapter 5: Improved Multiple Neutral Loss Monitoring


6 A MULTIPLEXED POST-TRANSLATIONAL MODIFICATION (PTM) MONITORING APPROACH ON A MATRIX ASSISTED LASER DESORPTION / IONIZATION TIME-OF-FLIGHT / TIME-OF-FLIGHT MASS SPECTROMETER (MULTIPLEXED PTM MONITORING ON A MALDI TOF/TOF)*

6.1 Introduction

Post-translational modifications (PTMs) are important for determining the activity of proteins, and are a very common mechanism employed by cells for regulating biological processes. Their tremendous biological importance has led to a recent surge in modification analysis by mass spectrometry. As one needs fragmentation of the modified peptide (derived from such proteins by enzymatic digestion) for mass spectrometric PTM analysis, Matrix Assisted Laser Desorption Ionization (MALDI) Time-of-Flight (TOF) has never been the preferred approach to monitor post-translational modifications. This is because only post source decay (PSD) has been available for fragmentation on MALDI TOF instruments and PSD varies with amino acid sequence in unpredictable ways and does not provide control over the peptide dissociation. This issue has been the motivation behind the development of the first generation of tandem mass spectrometric configurations employing collision induced dissociation (CID) with MALDI ionization.

In order to be able to perform CID, a mass analyzer must be able to select an ion of some particular m/z. Most mass analyzers, such as quadrupoles, can do this inherently, but if a time-of-flight mass analyzer is used as the first mass analyzer, an external ion gate or ion

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selector is mandatory, as TOF's cannot be operated as mass filters. Ion selection has conventionally been performed on TOF instruments by placing a single electrostatic ion-deflector at the end of the field free region of the TOF tube. The deflector is quickly triggered to turn off and then back on again, acting as a gate which transmits only a narrow ion velocity range, isolating a precursor ion. With such a setup, the mass resolution of the ion selection process is very limited (approximately 100-300). Consequently, a number of improvements have occurred in gating technology in order to improve the resolution of the ion selection process. This improved gating technology, combined with a delayed extraction source before each TOF section, has allowed for the development of a high resolution MALDI-TOF/TOF mass spectrometer, suitable for protein/peptide sequence and modification analysis by collision-induced dissociation. This ability to use CID has opened the door for the development of new methodologies for analyzing post-translational modifications.

Modifications have conventionally been monitored with precursor ion scans or neutral loss scans on triple quadrupole mass spectrometers, as the scans selectively screen for fragment ions or neutral loss fragments that are characteristic of the modification. Both scans have a very low duty cycle and are rather inefficient, as each particular marker ion requires its own precursor ion scan, and each neutral loss of interest requires one neutral loss scan (per charge state), limiting the total number of modifications that can be screened in LC-Electrospray Ionization (ESI) MS/MS experiments. Conversely, MALDI-based off-line techniques do not superimpose any limitations on the acquisition time and make possible the re-analysis of specific samples if the targets are stored. Consequently, they provide an attractive alternative to ESI-based approaches, but their full potential has yet to be explored.
As there are over 300 different types of post-translational modifications\textsuperscript{14}, mass spectrometric methods capable of simultaneously monitoring numerous different post-translational modifications could be a very useful resource. This goal is just beginning to be realized, as can be seen by recent developments in MS methodologies, such as the General precursor ion like scan\textsuperscript{15}, Multiple Precursor Ion Monitoring (MPM)\textsuperscript{16} and Multiple Neutral Loss Monitoring (MNM)\textsuperscript{17}. These methods have been shown to be a more efficient approach to monitor for post-translational modifications (PTMs) for several reasons: they can simultaneously monitor for several marker ions related to one particular modification, increasing the selectivity; they can monitor for several marker ions (or neutral losses) related to different modifications, increasing the completeness of the analysis; and they do not require one to scan the first mass analyzer (c.f. the precursor ion or neutral loss scan), thus decreasing the required scan time.

The MNM and MPM scans, which are specific for tandem mass spectrometers, are performed by using the first mass analyzer as a high pass filter, transmitting only peptide ions to the collision cell, and not the low mass chemical noise which would suppress the marker ion signals. The collision cell is then used to fragment all of the peptides simultaneously, producing the characteristic fragment ions and neutral losses of interest, which are then separated by the second mass analyzer and detected. The MNM scan requires further downstream computational processing to identify the neutral loss and the associated precursor ion\textsuperscript{17,18}. Conversely, the MPM scan strategy identifies the presence of marker ions, but does not identify the associated precursor ion, illustrating the need for an MS/MS approach, targeted MPM (tMPM), to identify the precursor ion. Here we show the feasibility of a multiplexed PTM monitoring approach on a MALDI TOF/TOF as well as develop and
evaluate the concept of targeted MPM. Furthermore, the effect of combining the MNM strategy with MALDI ionization has been investigated in order to refine the approach.

6.2 Experimental

6.2.1 Reagents

Bovine Serum Albumin (BSA), Human Serum Albumin (HSA), and α-cyano-4-hydroxy cinnamic acid (CHCA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sequencing grade trypsin was obtained from Roche Diagnostics (Laval, Que., Canada). Formic Acid, acetonitrile, and trifluoroacetic acid were purchased from Fisher Scientific (Whitby, Ont., Canada).

6.2.2 Peptides

The following peptides were used in this study, with the position of their modified residue indicated in bold: phosphoserine-containing \textit{SAPPNLWAAQR} was a gift from the Biomedical Research Centre (BRC) (Vancouver, BC, Canada) and N-acetyl hexosamine modified serine \textit{HLLVSNVGGDGEEIER} was purchased from the BRC. Unmodified \textit{GAPVPYPDPLEPR} was also obtained as a gift from the BRC, and then myristoylated on the N-terminal glycine \textit{GAPVPYPDPLEPR} as described previously\textsuperscript{19}. Phosphotyrosine-containing \textit{NRVYIHPF} and unmodified \textit{p-ELYENKPRPFL} were purchased from Sigma-Aldrich (St. Louis, MO, USA). \textit{p-ELYENKPRPFL} was then either mono-methylated\textsuperscript{20}, tri-methylated\textsuperscript{21}, or acetylated\textsuperscript{22} on the lysine.

6.2.3 Mass Spectrometry

All experiments were performed on a 4700 Proteomics Analyzer MALDI TOF/TOF controlled by the 4700 Explorer V2.0 software (Applied Biosystems, Framingham, MA,
USA). The software was used as is and did not need to be modified to conduct any of the experiments, with all of the settings manually adjusted in the acquisition method. The following conditions were used, unless mentioned otherwise: sub-picomole quantities (generally 250-500 femtomoles) of peptides were spotted onto the MALDI target using the dried droplet method and α-cyano-4-hydroxy cinnamic acid (CHCA) matrix. The attenuator setting used was 4500 (arbitrary units) and all data used was extracted from tandem mass spectra acquired with atmospheric gas (air) as the collision gas at a pressure of 1-2x10^{-6} torr (3-4x10^{-8} torr for MNM experiments) and a collision energy of 1000 eV. Data illustrated in the plots are the result of a total of seven data series being collected, averaged and plotted. Each spectrum was collected with a total of 1000 shots (50 shots/sub-spectrum, 20 sub-spectra). Due to the specific demands of each experiment, the following conditions were used for each individual study:

6.2.3.1 Sample consumption. The intensity of the marker ion was measured after every 10,000 shots (100 shots/sub-spectrum, 100 sub-spectra) up to a cumulative total of 1,280,000 shots.

6.2.3.2 DE source 2 timing and transmission efficiency. The timed ion selector (TIS) transmission window (TW) was set to a width of 400 Th (± 200 Th from the centre of the transmission window, or a given m/z) and the centre of the transmission window was stepped in increments of 20 Th, resulting in an increasing offset between the m/z of the peptide being analyzed and the centre of the transmission window. The signal to noise (S/N) ratio of the marker ion was recorded at increasing TW offsets and normalized to the S/N ratio of the 0 m/z offset (i.e. transmission window centred on the m/z of the peptide).
6.2.3.3 Effect of TIS transmission window width on the S/N ratio. The Marker ion signal to noise ratios were collected with a 0 TW offset for each TIS transmission window width.

6.2.3.4 The ability of the TIS to localize a precursor ion. Four survey MPM scans were performed at the m/z ranges of 400-800, 800-1200, 1200-1600 and 1600-2000 Th. When the survey MPM spectrum contained the marker ion, its TIS transmission window was split in half, and 2 targeted MPM scans were performed with the smaller TIS transmission windows. This process was repeated until the precursor ion was identified to a narrow TIS transmission window of 3.125 Th.

6.2.3.5 MNM / tMPM analysis of a complex sample. Sample solutions containing approximately 1 picomole/μl of the myristoylated, acetylated and phosphotyrosine modified peptides for the tMPM experiment or approximately 1 picomole/μl of the tri-methylated, N-acetyl hexosamine and phosphoserine modified peptides for the MNM experiment were prepared in a 2 picomole/μl BSA protein digest (1 μl / sample spot). MNM and tMPM scans were collected with each spectrum containing a total of 5000 shots (100 shots/sub-spectrum, 50 sub-spectra). Marker ion intensities were obtained from the analysis of 3 sample spots, averaged and then plotted. In the case of the three marker ions of the myristoylation modification, the geometrical mean intensity of the three marker intensities was calculated using equation 5.1 and plotted:

\[ y = \left[ (X_1)(X_2)(X_3) \right]^{\frac{1}{3}} \]  

(5.1)

Autocorrelation mass shifts of 98 (H3PO4) from phosphoserine, 59 [N(CH3)3] from tri-methyl lysine and 203 (HexNAc) from N-Acetyl hexosamine serine were used for neutral loss
identification and convolution mapping was used for precursor ion localization, as previously described\textsuperscript{17,18}.

6.2.3.6 Comparison of tMPM to other MS/MS approaches. Both product ion scans and tMPM scans were collected with each spectrum containing a total of 5000 shots (100 shots/sub-spectrum, 50 sub-spectra). The phosphotyrosine modified peptide was spiked into a 1-2 picomole/ul mixture of an AcHSA/BSA/\textalpha-casein protein digest at varying concentrations. Marker ion intensities for each sample concentration were obtained from the analysis of 3 sample spots, the intensities were averaged and then plotted. All of the data plotted were taken from spectra obtained with a TIS transmission window width of 10 Th.

6.3 Results and Discussion

6.3.1 Method Design

Post-translational modifications (PTMs) have conventionally been identified in mass spectrometry by observing the fragmentation of modified peptides. Upon fragmentation, any of the over 300 different known PTMs\textsuperscript{14} may dissociate from the peptide as a characteristic charged fragment ion (marker ion) or as a neutral fragment. It is unknown how these modifications dissociate during MALDI high-energy CID analysis as the majority of marker ion analysis is performed using electrospray low-energy CID. A selection of these modifications has been analyzed on a MALDI TOF/TOF instrument, and the associated marker ions that are expected and whether or not they could be detected are summarized in Table 6.1.

Signals in the MS/MS spectra were identified at all of the known marker ion m/z values, as summarized in Table 6.1, indicating that all of these different types of
### Table 6.1: Formation of marker ions from the fragmentation of 250-500 femtomole quantities of modified peptide

<table>
<thead>
<tr>
<th>Modification</th>
<th>Known Marker Ions (Th)</th>
<th>Observed in MS/MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>phosphotyrosine</td>
<td>216 (phosphotyrosine immonium ion$^{25}$, -79(PO$_4^-$), -97(H$_2$PO$_4^-$)$^{27}$</td>
<td>216, -79, -97</td>
</tr>
<tr>
<td>acetylated lysine</td>
<td>126 (acetylated lysine immonium ion with a loss of ammonia$^{24}$, 143 (acetylated lysine immonium ion)$^{12}$</td>
<td>126, 143</td>
</tr>
<tr>
<td>myristoylated N-terminal glycine</td>
<td>211 (myristoyl + H$^+$), 240 (a$_i$ ion), 268 (b$_i$ ion)$^{19}$</td>
<td>211, 240, 268</td>
</tr>
<tr>
<td>N-acetyl hexosamine</td>
<td>204 (HexNAc+H$^+$), 186 (HexNAc+H$^+$ - H$_2$O), 168 (HexNAc+H$^+$ - 2H$_2$O)$^{36}$</td>
<td>204, 186</td>
</tr>
<tr>
<td>mono-methylated lysine</td>
<td>98 (immonium ion of monomethylated lysine with a loss of ammonia)$^{25}$</td>
<td>98</td>
</tr>
</tbody>
</table>
modifications can be detected below approximately 500 picomoles of peptide with a MALDI-TOF/TOF instrument. This was unexpected for a number of reasons. Firstly, the precursor ion generated by the MALDI technique is predominantly singly charged, which leads to a lower probability of marker ion formation upon fragmentation compared to the fragmentation of multiply-charged species in electrospray ionization. This was observed in a previous study\textsuperscript{19} where the farnesyl fragment ion could be identified easily in electrospray experiments, while similar concentrations of the same peptide could not produce a detectable marker ion signal using MALDI high energy CID or MALDI post source decay. Secondly, although high energy CID mass spectra on a MALDI-TOF/TOF instrument are typically very rich in low mass ions\textsuperscript{28} (resulting from both charge-induced and charge-remote fragmentation pathways\textsuperscript{29}), the commonly used PTM marker ions are generally produced by low energy pathways as found in low-energy CID. Therefore, the additional low-mass ions produced from high-energy CID provide little to no additional modification information. Thus, it was presumed that the PTM marker ions would be produced and would then be further fragmented through a high-energy pathway under high energy CID conditions; however, in general, we have seen that this is only the case at collision gas pressures greater than 5x10\textsuperscript{-6} torr (data not shown). As well, since the same quantity of each of the modified peptides was spotted on the MALDI target, but the different marker ions were present at different intensities, it becomes apparent that some marker ions may have a higher propensity to form or sequester a charge than others, although peptide ionization efficiency is also a factor. The likelihood of marker ion formation will depend on a number of factors: the fragment's proton affinity as well as its stability, the stability of the remaining bonds in the peptide, the sample complexity, the activation energy of the fragmentation pathway, the stability of the
associated neutral products, steric factors for rearrangement reactions, and the site of the charge. The finding that marker ions are observed for each of the PTMs tested opens the door for the development of a scan strategy that is analogous to “precursor ion scans”. The fact that the time-of-flight (TOF) technique analyzes all marker ions in parallel allows for a multiplexed analysis similar to other multiplexed approaches developed on a hybrid quadrupole/linear ion trap (Multiple Precursor Ion Monitoring) and on quadrupole/time-of-flight instruments.

As PTM marker ion production is prevalent on a MALDI TOF/TOF instrument, a means of directly monitoring for these marker ions and identifying the associated precursor ion(s) is essential to achieve a comprehensive PTM analysis. A schematic of such an approach is described in Illustration 6.1. The analysis would begin with a survey Multiple Precursor Ion Monitoring (MPM) scan which transmits all of the peptides to the collision cell, where they are fragmented simultaneously. The fragment ions are then separated by the second TOF and detected in a single spectrum. This first step in the analysis identifies the presence of any and all marker ion(s) of interest, but provides no information about the associated precursor ion(s). Although this type of scan is achievable with quadrupole mass analyzers, a problem arises when employing TOF mass separation. As mass discrimination in a TOF analyzer is based on ions of different masses having different velocities, mass dispersion of the ions will occur in the first TOF and these ions will then arrive at the timed ion selector (TIS) and then the delayed extraction (DE) source 2 at different times. This is problematic for the survey MPM scan because the DE source 2 is pulsed, which ensures that the ions entering the second TOF are spatially focused. If the timed ion selector was kept
Illustration 6.1 Schematic of the targeted Multiple Precursor Ion Monitoring (tMPM) approach. Survey MPM scans are performed in order to identify the presence of a modification. Once a modification has been identified, the targeted MPM approach is employed to identify the associated modified precursor ion(s).
open over the entire mass range, and the DE source 2 was not pulsed, ions would be continually entering the TOF and nonsensical spectra would be obtained. Conversely, one could keep the timed ion selector open over the entire mass range and pulse DE source 2, but only the ions that are within the volume of the second source when it is pulsed would be accelerated into the second TOF. Therefore, the principles governing TOF mass analysis do not allow one to operate the MPM scan with one survey scan covering the entire mass range. Instead, a number of 'survey' MPM scans must be performed with a medium sized TIS transmission window stepped to increasing mass to charge values (i.e. 1000-1100 Th, 1100-1200 Th, 1200-1300 Th, etc) and then linked. This creates an analogue of MPM on a MALDI TOF/TOF to identify the presence of a modified peptide or protein.

In order to localize the precursor ion which produced the marker ion of interest, a 'targeted MPM' (tMPM) experiment needs to be performed (Illustration 6.1): Once all of the survey MPM scans are completed, the survey MPM scans are searched for the presence of a marker ion. When a peak corresponding to the m/z of some marker ion is observed from one of the survey MPM scans, the TIS transmission window used for that particular survey MPM scan will be divided into 2 smaller windows, and MPM spectra collected for both. As an example, if a marker ion was determined to be present in the 1000-1100 Th survey MPM scan, two targeted MPM scans would be performed with TIS transmission windows of 1000-1050 Th and 1050-1100 Th. The transmission window which still produces the marker ion will again be divided by 2, and two MPM spectra will again be collected (while the other transmission window that did not produce a marker ion will not be further analyzed). This process will continue until the TIS transmission window is small enough to localize the precursor ion. At which time, the targeted MPM cycle will repeat if another modified
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precursor has been identified, or if there are no more modified precursors to be localized, the analysis will continue with the next sample spot.

In order to develop efficient and practical conditions for the targeted MPM and its combination with the MNM scan on the MALDI TOF/TOF, a number of parameters need to be considered. The first parameter is the rate of consumption of sample from the MALDI target, which is required to determine if the sample will be consumed before the survey and targeted MPM scans are completed. The second parameter is the maximum TIS transmission window width for the survey scan and the effect of the delayed extraction pulse from source 2 on this wide TIS transmission window. This is important as the widest practical TIS transmission window is required to minimize the total number of survey MPM and MNM scans. The third parameter, the efficiency and accuracy of the timed ion selector in localizing the precursor ion, is required to determine to what resolution targeted MPM can localize the m/z of the modified precursor.

6.3.2 Method Development

6.3.2.1 Sample consumption. As the targeted MPM and MNM strategy requires a number of survey scans to cover the entire mass range, as well as a number of targeted MPM scans with an exponentially decreasing TIS transmission window to locate the precursor ion, sample consumption must be sufficiently slow to allow for a large number of scans to be performed before the sample is exhausted. That is, the intensity of the marker ion must remain sufficiently intense throughout the relatively large number of shots needed for a tMPM experiment. In order to determine whether the rate of consumption is independent of the type of modification being analyzed, similar concentrations of the different modified peptides were spotted on the MALDI target and analyzed. Figure 6.1a illustrates the effect of
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the total number of cumulative shots on the marker ion intensity. The immonium ion of acetylated lysine with a loss of ammonia (126 Th), the phosphotyrosine immonium ion (216 Th), and the myristoyl fragment ion (211 Th) all show very similar rates of consumption. The normalized plot, shown in the inset, indicates that the rate of consumption is independent of the type of modification. Since the selected marker ions differ significantly in type and chemical structure, it is reasonable to assume that most marker ions will behave in a similar manner and thus are not consumed at different rates. Furthermore, the dependence of the rate of sample consumption on the laser attenuator setting (fluence) and type of matrix (Figures 6.1b,c), as well as dependence on the peptide quantity (Figure 6.1d) were investigated by monitoring the intensity of the phosphotyrosine immonium ion from the peptide NRVYIHPF to determine if any of these variables adversely affected sample consumption. Increasing the laser attenuator setting produced lower initial yields of marker ions, and increased sample consumption rates (Figure 6.1b), while varying the peptide quantity did not affect the rate of sample consumption (Figure 6.1d), as observed from the normalized plot. The effect of the matrices 3-hydroxy picolinic acid (3HPA), 3,5-dimethoxy-4-hydroxy-cinnamic acid (sinapinic acid, SA), α-cyano-4-hydroxy cinnamic acid (CHCA) and 2,5-dihydroxybenzoic acid (DHB) were also examined. SA and 3HPA did not produce marker ions, regardless of the laser fluence, while DHB required a high laser fluence to produce a low yield of the phosphotyrosine marker ion (Figure 6.1c) and CHCA produced a high yield of the marker ion at a low laser attenuator setting (Figure 6.1b). These results confirmed that CHCA is the best choice of matrix for the fragmentation analysis of low mass peptides due to its ability to provide ions at good sensitivities with low sample consumption rates and consequently, CHCA was used as the matrix for the remaining experiments.
Figure 6.1 The effect of sample consumption with increasing total number of shots. The effect of sample consumption with increasing total number of shots on the a) type of modification, b) the laser fluence with CHCA as a matrix, c) the laser fluence with DHB as a matrix and d) the peptide concentration, plotting total signal intensity as well as normalized intensity (inset).
Chapter 6: Multiplexed PTM monitoring on a MALDI TOF/TOF

As tandem mass spectrometric analyses on this instrument are generally conducted with between 1,000 and 5,000 shots in order to provide statistically significant intensities, the sample consumption is sufficiently low to complete many survey MPM/MNM scans and targeted MPM scans, regardless of the type of modification that is being analyzed, depending on the other scan parameters.

6.3.2.2 DE source 2 timing and transmission efficiency. In the aim of minimizing the total number of survey MPM and MNM scans necessary to cover the entire m/z range, and therefore minimizing sample consumption, the m/z range transmitted to the collision cell should be as broad as possible. The major concern with increasing the width of the TIS transmission window is that the settings of the delayed extraction pulse of source 2 are optimized for an ion with an m/z value that would be in the center of the transmission window. The second source is pulsed based on the calculated flight time of this m/z to the centre of the second source. Consequently, one would expect that the larger the offset between the center of the transmission window and the m/z of an actual precursor ion to be transmitted, the lower the transmission efficiency of the actual precursor ion into the second TOF. This is due to the fact that the larger the m/z offset, the larger the velocity difference of the ion, resulting in the ion being farther away from the centre of source 2 when it is pulsed. All ions that are in the volume of source 2 will be accelerated into the second TOF analyzer; however, the amount of energy the ion picks up is dependent on its location when the second source is pulsed. Thus, if the ion is not centred in the second source, the effective voltage experienced by the ion of interest will be lower or higher than if the ion was in the centre of the source. This leads to a decrease in the resolution and signal to noise ratio of the peak
corresponding to the marker ion. If the ion of interest is not in the volume of Source 2 when it is pulsed, it will not be accelerated into the TOF, and therefore not analyzed at all.

Consequently, the influence of m/z offset on the precursor transmission was examined using a low mass, phosphotyrosine-modified peptide ([M+H]$^+$ = 1126.5 Th) and a higher mass, acetyl-lysine containing peptide ([M+H]$^+$ = 1741 Th). The low-mass peptide (Fig. 6.2(a)) displayed high transmission efficiency (0.4-0.5) within a ± 20 Th offset compared to when the peptide was centred in the transmission window. Any larger m/z offset resulted in a low transmission efficiency of the ions to the second TOF (less than 0.2). This indicated that a TIS transmission window width of up to 40 Th could be used for the survey MPM and MNM scan at low m/z values. The higher mass peptide (Fig. 6.2(b)) showed a slower drop off of precursor transmission with increasing m/z offset with a transmission of approximately 0.4 at +/- 60 Th. This trend was confirmed in the analysis of other low and high mass peptides. This difference in TIS transmission window tolerance is because two heavier ions separated by mass difference, Δm, are separated by a smaller distance than two light ions separated by the same mass difference. Thus, for higher m/z values, the volume of source 2 can contain a wider spread of masses when source 2 is pulsed. As well, the effect of the m/z offset of the precursor ion relative to the center of the transmission window was investigated at various TIS transmission window widths, with maximal S/N signal to noise (S/N) ratios obtained when the actual precursor ion was centered on the transmission window (data not shown).

6.3.2.3 Effect of TIS transmission window width on the S/N ratio. Another factor affecting the use of a broad TIS transmission window is a possible decrease in the S/N ratio with increasing TIS transmission window size. Increasing the transmission window
Figure 6.2 The effect of peptide mass on the transmission efficiency of ions to the second TOF with varying m/z offsets. The m/z offset is the difference between the centre of the transmission window and the actual precursor m/z for a) a low m/z peptide (NRVpYIHPF, 1062.5 Da) and b) a high m/z peptide (p-ELYEN(Ac)KPRRFIL, 1740 Da).
size could lead to an increase in chemical noise that is transmitted through, or generated upon fragmentation in, the collision cell without increasing the transmission of the precursor of interest. This effect was investigated by observing the S/N ratio of the phosphotyrosine marker ion, using different TIS transmission window widths (Figure 6.3). While very narrow TIS transmission windows generate a low S/N ratio for the marker ion, the S/N ratio increases as the TIS transmission window width increases, reaching a maximum at a transmission window width of 25-50 Th. Further increasing the TIS transmission window width leads to a decrease in the S/N ratio. The same general trend was observed when different concentrations of the phosphotyrosine-containing peptide were spotted on the MALDI target, as well as when different peptides were analyzed (data not shown). The initial increase in the S/N ratio with increasing TIS transmission window width is due to the wider window, as it ensures that all of the peptide ions are transmitted to the second TOF. Conversely, the decrease in the S/N ratio at very large TIS transmission window widths is the result of increased noise. As the peptide sample was very simple (the modified and unmodified forms of the peptide were the only peaks present in the MS spectrum), peaks originating from the sample were very limited.

When the phosphotyrosine modified peptide was spiked into a mixture of HSA, BSA and α-casein protein digests, the sample was much more complex as it contained a high number of peptide signals within the mass spectrum. Accordingly, more of the sample ions were present in each transmission window, resulting in an overall decrease of the S/N ratio at all of the various TIS transmission window widths, due to the higher noise, as well as ion suppression. This effect became more pronounced as the protein digest concentration increased from 50 femtomoles/spot to 250 femtomoles/spot (Figure 6.3). Additionally, this
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Figure 6.3 The effect of the TIS transmission window width on DE source 2. Determined from the signal to noise ratio of the phosphotyrosine marker ion by fragmenting 250 femtomoles of the precursor, NRVpYIHPF (●), 250 femtomoles of NRVpYIHPF in 50 femtomoles of the protein digest (■), and 250 femtomoles of NRVpYIHPF in 250 femtomoles of the protein digest (▲).
result is also due to the inability of the reflectron mirror to compensate for the energy spread produced from sample ions not being centred in source 2 when it was pulsed. As the focusing is not ideal for these sample ions, the resolution and S/N ratio of these signals is decreased, because these ions experience slightly more (or slightly less) of the acceleration potential of source 2, leading to a decrease in resolution and mass accuracy. In turn, this would decrease the S/N ratio of a marker ion signal which was produced from a precursor ion centred in the transmission window and in source 2 when it was pulsed. Lastly, as the concentration of the protein digest increased, there was a slight increase in the S/N ratios of the phosphotyrosine marker ion with the wide transmission windows (100, 200, 400 Da) relative to that of the maxima (window width of 25-50 Da). With a large transmission window, as the sample complexity increases, more and more ions are transmitted through the TIS to fragment in the collision cell by high energy CID. As high energy CID produces a wide variety of fragment ions, the probability of nominal mass interferences with the marker ion of interest increases, leading to an apparent increase in the S/N ratio of the marker ion. These findings verified that large TIS transmission window widths could not be used with the survey MPM and MNM scans for the analysis of complex samples.

Lastly, if the TIS transmission window is not centered on the m/z of the actual modified precursor ion, the S/N ratio of the marker ion will depend on both the m/z offset and the width of the TIS transmission window. These factors need to be considered when developing the targeted MPM approach in order to ensure that sufficiently high S/N ratios are observed for the marker ion.

6.3.2.4 The ability of the TIS to localize a precursor ion. A third parameter that must be investigated is whether or not the timed ion selector allows sufficient control and
resolution to locate the precursor ion of the modified peptide using an exponentially decreasing TIS transmission window. The TIS used in this instrument is a double sided timed ion selector which essentially works as follows: the first deflector is operated as a low mass filter, which does not transmit any ions through until it is switched open. The second deflector is operated as a high mass filter, maintained in the open state and then switched to the closed state. In a simplified view, the resolution is limited by how fast the two deflectors can be switched off and on, and thus whether or not one precursor can be separated from another precursor of a similar m/z, which would allow for the successful identification of the modified precursor ion.

The phosphotyrosine peptide (NRVYIHPF) was again spotted on the MALDI target and analyzed. Four survey MPM scans were performed on the m/z ranges of 400-800, 800-1200, 1200-1600 and 1600-2000 Th (Figure 6.4a). The TIS transmission window which produced a mass spectrum containing the 216 Th marker ion (800-1200 Th) was split in half, and targeted MPM analyses were performed on the m/z range spanned by those smaller transmission windows. The process of halving the TIS window which produces a mass spectrum containing the marker ion was repeated until the precursor ion was localized to a narrow TIS transmission window of 3.125 Th.

For all of the following TIS transmission window widths: 200 Th (Figure 6.4b), 100 Th (Figure 6.4c), 50 Th (Figure 6.4d), 25 Th (Figure 6.4e), 12.5 Th (Figure 6.4f), 6.25 Th (Figure 6.4g) and 3.125 Th (Figure 6.4h), the maximum marker ion signal to noise ratio (as well as the maximum peak intensity) corresponded to the TIS transmission window that should contain the precursor ion. The timed ion selector can therefore be manipulated to blindly locate a modified precursor of unknown m/z by using marker ion S/N values (or
Figure 6.4  Investigation of the accuracy of the Timed Ion Selector. Unknown precursor ion m/z localization (NRVpY1HPF, 1062.5 Da) using the timed ion selector with an exponentially decreasing TIS transmission window of a) 400 Th, b) 200 Th, c) 100 Th, d) 50 Th, e) 25 Th, f) 12.5 Th, g) 6.25 Th and h) 3.125 Th. The bars in the plots are used to illustrate the TIS transmission window widths, with the location of the centre of the TIS transmission window being the data points and the markers on each side of the bars indicating the lower and upper limits of the TIS transmission window.
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intensities) as an indicator. Targeted Multiple Precursor Ion Monitoring (tMPM) can thus be successfully performed on a MALDI TOF/TOF instrument.

Based on these results (Figures 6.1-6.4), the following parameters were found to be optimal for the targeted MPM approach: TIS transmission window widths of 40 Th for the m/z range of 1000-1160, 60 Th for 1160-1340, 80 Th for 1340-1580, 100 Th for 1580-1880 and 120 Th for 1880-2000 were used, as the allowable offsets between the centre of the TIS window and precursor m/z were shown to increase with increasing precursor ion mass (Figure 6.2a,b). Since the laser has a frequency of 200 Hz, and 5000 shots were collected per spectrum, it would take 25 seconds to complete one scan. Thus, with the aforementioned setup, targeted MPM would require 13 survey MPM scans with approximately 9 targeted MPM scans for the analysis of one spot on the MALDI target.

When the targeted MPM strategy was tested on 500 femtomoles of a phosphotyrosine-containing peptide spiked into a 1-2 picomole mixture of HSA/BSA/α-casein digest, using the aforementioned acquisition parameters, it readily identified the precursor ion of the modified peptide (data not shown), showing that even in a complex sample matrix, tMPM can use marker ion intensities to identify an associated precursor ion. Moreover, MNM could now be combined with targeted MPM for a global PTM discovery method, as the same parameters are applicable to both.

6.3.3 Method Evaluation

Targeted MPM was then combined with MNM to achieve modification monitoring that is as comprehensive as possible; the tMPM approach will localize the precursor ion for all modifications that fragment to produce marker ions, while those that dissociate as neutrals will be found by MNM17. Sample solutions, one containing approximately 1 picomole/ul of
the phosphotyrosine (Figure 6.5a), myristoylated (Figure 6.5c) and acetylated (Figure 6.5e) modified peptides in a 2 picomole/ul BSA protein digest for the tMPM experiment and the other containing approximately 1 picomole/ul of the phosphoserine (Figure 6.5b), trimethylated (Figure 6.5d), and N-acetyl hexosamine (Figure 6.5f) modified peptides in a 2 picomole/ul BSA protein digest for the MNM experiment were prepared. In the tMPM experiment, marker ion intensities for the 3 different modifications (216 Th for the phosphotyrosine marker ion; 126 Th for the acetyl-lysine marker ion; and geometrical mean of 211, 240 and 268 Th for the myristoyl-glycine marker ions) were extracted from each survey MPM scan and plotted. In the MNM experiment, autocorrelation analyses of each of the survey MNM scans were performed to identify the modifications. Convolution mapping was used for precursor ion localization, as previously described\textsuperscript{17,18}. The widths of the bar graphs are used to depict the width of the TIS transmission windows.

All of the modified peptides were identified by MNM or tMPM. As well, the maxima in the marker ion intensity plots (Figure 6.5a,c,e) corresponded to the correct TIS transmission windows containing the modified peptides. Targeted MPM then correctly localized and identified the modified peptides. Similarly, the maxima in the autocorrelation coefficient plots (Figure 6.5b,d,f) always corresponded to the correct TIS transmission windows containing the modified peptides. For the MNM analysis, two false positives were observed: one false positive was obtained for phosphorylation (Figure 6.5b) as there is a correlation between a peptide ion and the fragment ion of a different peptide; and one false positive was obtained for trimethylation (Figure 6.5d) which is due to the correlation of two peptide ions as they were both within the transmission window (1580-1880 Th). The number of false positives identified here is much lower than what has previously been observed with
Figure 6.5  Multiplexed Post-translational modification monitoring. tMPM analysis of a) phosphotyrosine, c) myristoyl-glycine, and e) acetyl-lysine-modified peptides in a BSA digest; MNM analysis of b) phosphoserine, d) tri-methylated-lysine, and f) N-acetyl hexosamine serine modified peptides in a BSA digest. The widths of the bar graphs are used to depict the width of the TIS transmission windows.
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MNM using electrospray ionization, which employed a pre-fractionation step\textsuperscript{17}. This is because the majority of false positives have, in the past, been due to a correlation between two fragment ions as the MNM spectrum is complicated by singly-, doubly-, and triply charged fragment ions. Conversely, MALDI fragment ions are singly charged, with neutral loss fragmentation being heavily favoured\textsuperscript{19}, features which are very favourable for the MNM technique.

With the tMPM analysis, there were also very few false positives, with the error rate being related to the selectivity of the marker ion. When there is more than one marker ion for a particular modification, the selectivity dramatically increases, as seen for the myristoyl modification (Figure 6.5c). Overall, when combined, tMPM and MNM successfully identified a wide variety of different PTM's in a complex sample.

In order to determine the effectiveness of Targeted MPM as a scan function, the tMPM strategy was compared to various MS/MS strategies available on the MALDI-TOF/TOF (Figure 6.6) with samples containing a varying amount of the phosphotyrosine-containing peptide spiked into a fixed amount of HSA/BSA/α-casein protein digest. Product ion analyses were performed on every peak observed in the MS spectrum (S/N ratio greater than 10, m/z range 1000-2000 Th) by fragmenting the peaks in order of ascending and descending peak intensity. In general, it was observed that when fragmenting the ions in ascending intensity order, the phosphotyrosine marker ion intensities were very low. This is because at low sample concentrations, even though the ion is fragmented early in the analysis, the precursor ion concentration is too low to generate a statistically significant quantity of marker ions. At high peptide concentrations, by the time the precursor ion is subjected to fragmentation, a high degree of sample consumption has occurred, resulting in a
Figure 6.6  Comparison of the effectiveness of targeted MPM to available MS/MS approaches on the MALDI-TOF/TOF. Marker ion intensities of a phosphotyrosine-containing peptide spiked at different concentrations into a fixed amount of a complex peptide mixture (of HSA/BSA/α-casein protein digest) are plotted.
low concentration of peptide that cannot generate a statistically significant quantity of marker ions. Conversely, when fragmenting the peaks in descending intensity order, identification of the modified precursor ion was excellent at high peptide concentrations, but the performance decreased as the spike concentration decreased. This is because at high peptide concentrations, the precursor ion will be fragmented very early in the analysis and very little of the peptide will have been consumed; however, at low peptide concentrations, the peptide will be analyzed late in the analysis and a large portion of the peptide will have been consumed.

In comparison to these experiments, targeted MPM readily identified the modified precursor ions at high concentrations; however, as the concentration decreased, so did the marker ion intensity. At spike concentrations of 100 femtomoles and below, none of the three strategies were capable of identifying the phosphotyrosine immonium ion. Closer investigation to determine why tMPM failed at low spike concentrations revealed that the noise was higher in the survey MPM scans than in the MS/MS scans due to the increased TIS transmission window range of the scan, which led to S/N ratios that were too low to trigger a tMPM scan. Applying narrower TIS transmission windows to the survey MPM scans is not practical as this would decrease the probability of identifying the peptide during the exponentially decreasing tMPM scans, if the sample consumption rate is high. As well, since the precursor ion was very rarely centered in any of the survey MPM scans or the targeted MPM scans, this led to slightly lower intensities. We investigated the possibility of overcoming this drawback by using survey MPM scans with mass ranges that overlapped by half of their size. This led to higher intensities and S/N values in the survey tMPM scans as the precursor ion had a greater probability of being centered in the TIS transmission window,
but lower intensities and S/N ratios in the exponentially decreasing tMPM scans as a result of the increase in the number of scans prior to the precursor ion localization. Overall, tMPM with overlapping mass ranges performed slightly better than the tMPM strategy alone and significantly better than the ascending intensity MS/MS strategy, but could not achieve the signal intensities of the descending MS/MS strategy.

6.4 Conclusion

Targeted Multiple Precursor Ion Monitoring (tMPM) is a new scan strategy developed on a MALDI-TOF/TOF that is capable of identifying the presence of a modified peptide by detecting the marker ion in a non-specific MS/MS scan, and then localizing the precursor ion m/z value. The strategy is also capable of monitoring for different PTMs as well as a number of marker ions related to a particular PTM simultaneously. In combination with MNM, PTM monitoring on a MALDI TOF/TOF can be comprehensive, providing a very selective analysis with complete PTM coverage.

The velocity dispersion that occurs in the first time-of-flight mass analyzer has proven to be a setback, specifically in the operation of DE source 2, hindering the efficiency of the tMPM and MNM approaches on a MALDI TOF/TOF instrument; however, the most recent improvements in instrumentation, found in the 4800 MALDI TOF/TOF Proteomics Analyzer, will greatly improve both the tMPM and MNM work flow for a number of reasons. It is more sensitive, allowing one to obtain the same signal in fewer shots, facilitating more defacto experiments. The timed ion selector (TIS) has been improved significantly allowing one to hone in on precursors that are separated by a smaller m/z difference. Lastly, the distance between source 2 and the source 2 grid is at least 3 times
larger, which means that the effective width of the TIS window can be larger as more ions will be in the volume of the second source when it is pulsed. These upgrades to the 4800 would decrease the number of survey MPM scans required, as well as the number of shots necessary per spectrum thus decreasing the analysis time per sample spot, making way for method automation and high throughput analysis. Unfortunately, there is only a slight increase in mass resolution (from 2000-5000 on the 4700 Proteomics Analyzer to 2000-6000 on the 4800 Proteomics Analyzer) and no change in mass accuracy (~50 ppm) on the 4800 MALDI TOF/TOF compared to the 4700, and thus neither instrument can distinguish marker ions from most nominal mass interferences.

Alternatively, applying this strategy to an instrument where the first mass analyzer can be operated as a high mass filter with no mass dispersion (such as a quadrupole), would allow for a single survey MPM and MNM scan, while the mass analyzer could still be operated with an exponentially decreasing transmission window to isolate and identify the precursor ion. Furthermore, the use of electrospray ionization would facilitate the marker ion's propensity to sequester a charge, promoting the formation of marker ions with the tMPM scan, because the precursor ion is multiply charged. Consequently, tMPM would work better on tandem mass spectrometers that are fitted with an electrospray ion source and an initial quadrupole mass filter; however, the lack of software control with current instrumentation prevents this implementation. Conversely, MNM has been shown here to be an improvement with MALDI compared to the earlier setup\textsuperscript{17}. Future studies will need to include testing the MNM method on a MALDI QqTOF, in order to keep the TOF mass accuracy and resolution as well as the MALDI source, ensuring all fragments are singly charged thus decreasing the complexity of the spectra, while increasing the degree of neutral
losses. It is anticipated that these setups will provide the optimal conditions for the two approaches.
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6.5 References


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Chapter 7: Concluding Remarks

7 CONCLUDING REMARKS

7.1 Conclusion and Discussion

7.1.1 PTM Fragmentation Studies

The study of collision-induced reporter fragment ions and neutral loss fragments by mass spectrometry began approximately 15 years ago with the investigation of phosphorylation\(^1\) and glycosylation\(^2,3\). In the years to follow, mass spectrometric analysis continued to focus on these two modifications because of the tremendous importance of phosphorylation events in signal transduction and of glycosylation in cellular responses and cell-cell interactions.

In recent years, studies have begun to show the importance of other post-translational modifications within the cell. This realization has spurred research into the characteristic fragmentation of some of the over 300 known types of post-translational modifications\(^4,5\). More specifically, these biological studies have been the motivation behind our efforts to characterize both lipid modifications and HNO-induced cysteine modifications on proteins. Lipid modifications have been implicated in cellular localization/targeting to cellular membranes and membrane tethering, as well as other functions\(^6,7\). On the other hand, the function of HNO-induced modifications has yet to be fully elucidated; however, a number of pharmacological effects of HNO have already been shown\(^8-15\). As a result, three classes of lipid modifications: myristoylation, farnesylation, and palmitoylation; as well as HNO-induced cysteine nitroxylation, were studied by a comprehensive mass spectrometric analysis in order to provide the biological community with an additional tool to characterize these PTMs.
In the study of the lipid modifications, the fragmentation of the three different modifications was fully characterized and the most efficient methods of monitoring each PTM were determined for a number of different MS instruments. With respect to HNO-induced modifications, the characteristic fragmentation of cysteine nitroxylation (sulfinamide and sulfonic acid) was characterized and then utilized to study one of HNO's known pharmacological effects, the effect of HNO on platelets.

This is a great step forward for the analysis of lipid-modified proteins, but a number of barriers still face the analysis of lipid-modified proteins, including a low solubility of hydrophobic proteins, a low ionization efficiency, low recoveries of peptide extraction from in-gel digestions and most importantly, very difficult chromatographic separations that usually results in low recoveries from reversed phase separations. In any case, the findings from the lipid modification analysis have had an important effect as they have been referenced in a number of reviews\textsuperscript{16-21} as well as successfully being used as a tool for targeting lipid modification analysis by mass spectrometry\textsuperscript{22,23}. With respect to the HNO-induced modification, although the neutral loss produced from the sulfonic acid was not observed in 100% of the modified proteins, in our experience, many of the known characteristic fragmentations of other PTMs are also not observed in all cases. Many times the production of the neutral loss, or alternatively the production of marker ions, is heavily influenced by the precursor ion’s charge state. In any case, it is our belief that the success of the HNO-induced modification study will be the step forward that is necessary to link pharmacological effect to changes in protein function when dealing with HNO. On a bigger scale, we have contributed to the ever-growing library of known PTM fragmentation products\textsuperscript{17}. 

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7.1.2 Multiplexed PTM analysis

It is becoming clear that the comprehensive analysis of post-translational modifications is essential for a complete understanding of a cell’s biology. Traditional approaches are incapable of such a feat as they target only one particular type of modification. This has led to the creation of a new research field that aims to solve this problem. This was the motivation behind the development of Multiple Neutral Loss Monitoring and targeted Multiple Precursor ion Monitoring. As the library of known PTM fragmentation products continues to grow, we recognized that the unique operation of a mass spectrometer could effectively utilize this library for comprehensive modification analysis.

Multiple Neutral Loss Monitoring (MNM) was originally designed, developed and evaluated on a 2000 QTRAP instrument. The method proved to be capable of identifying post-translational modifications from low levels of modified peptides; however, the method was plagued by false positives. MNM analysis of modified protein standards was then conducted on the QTRAP, and false positives remained an issue. A comprehensive investigation of the sources of the false positives was completed with alpha-Casein and methods of circumventing the causes were explored. As the earlier lipid and HNO modification studies indicated that neutral losses were produced in a higher abundance from fragmentation of precursor ions generated by MALDI, a de facto MNM experiment was designed, developed and evaluated on a MALDI TOF/TOF. The MALDI study did lead to an increase in the production of the neutral loss fragment ion relative to other fragment ions as well as a decrease in false positives. As well, the MALDI TOF/TOF also allowed for the demonstration of a new concept, targeted Multiple Precursor Ion Monitoring (tMPM), which
was the continuation of the earlier approach, Multiple Precursor Ion Monitoring (MPM)\textsuperscript{24}. The operation of these two methods allowed us to demonstrate comprehensive PTM monitoring.

### 7.2 Future Directions

Although the physics of a TOF do not allow the first mass analyzer to be operated as a high mass filter in the MNM MALDI TOF/TOF experiments, we determined that MNM did benefit from a MALDI source as fragmentation of precursor ions generated by MALDI led to an increase in the production of the neutral loss, as well as a decrease in the production of other fragment ions that had previously generated false positives. As well, in order to further improve the MNM method, it has become clear that higher resolution spectra could be analyzed computationally to isolate false positives. MNM experiments have been conducted on both ESI and MALDI quadrupole time of flight instruments; however, we are still in the process of analyzing the data, as the graphical user interface supplied by Sciex is in the process of being reconfigured for the new format of the data files. In this author's opinion, the MNM method would most greatly benefit from a vacuum MALDI TOF Q\textsubscript{LIT} TOF setup. MALDI ensures the production of singly-charged precursor ions which are beneficial for the production of neutral fragments, while vacuum MALDI would ensure that the precursor ions maintain their internal energy deposited, both from the matrix and collisions experienced during the delayed extraction pulse. The TOF would then provide a time frame (on the order of tens of microseconds) necessary for metastable fragmentation, while the quadrupole linear ion trap would trap the precursor and resulting PSD-generated fragment ions, as well as remove the mass dispersion generated by the TOF. The ions could
then be transmitted in a concise packet to an orthogonal acceleration reflectron TOF, which would provide the necessary resolution for the MNM analysis.

It is the goal of this research group to combine the two multiplexed PTM monitoring strategies with *in vivo* crosslinking of proteins in an effort to identify protein-protein interaction partners, as well as the post-translational modifications that have either been added or removed to the proteins of interest as a result of these interactions. From there, the next goal will be to quantify these changes in protein-protein interactions and post-translational modifications by Stable Isotope Labeling of Amino acids in Cell culture (SILAC), and use this approach as a tool to study disease. A schematic of this approach is given below in Illustration 7.1.

Many of the approaches discussed here, as well as previously in the introduction, show great promise; however, many, if not all, are at the proof of concept stage of development, and have yet to be implemented in routine analysis. Within the near future, several of these techniques will likely be further developed and validated, becoming essential tools for the analysis of biological samples. As a recent study has quantified global, site-specific phosphorylation dynamics in cellular signaling networks\(^{25}\), an achievement that would be impossible without computational analysis, the combination of such an approach with a comprehensive PTM analysis will provide a true understanding of a cell’s biology. This attests to the importance of the continual development of analytical techniques that can be applied to the Life Sciences.
Illustration 7.1. Workflow for the quantitative analysis of protein-protein interactions and post-translational modifications. a) Normal cells are grown in SILAC medium 1 (light isotope enriched) while b) stimulated cells (or diseased cells) are grown in SILAC medium 2 (heavy isotope enriched). Cells are treated with para-formaldehyde (crosslinking agent) to induce crosslinking of proteins to their interaction partners and then lysed. An antibody for protein 1 (grey) is used to purify protein 1, as well as any cross linked proteins (protein 2, blue) from the cell lysate. From this point, there are two possible cases: If the post-translational modification (red) is important for the protein-protein interaction (Case 1), only the modified form of protein 2 will be purified. Alternatively, if the modification is not involved in the protein-protein interaction, both the modified and unmodified version of protein 2 will be purified. The proteins are then digested and analyzed by mass spectrometry using a multiplexed PTM analysis to quantify all of the various PTMs. The intensity of the precursor ions from the a) normal cells are compared to that of the b) stimulated cells indicating the degree of up or down regulation of the modification (Case 2, green) or of the modification and protein-protein interaction (Case 1, red).
7.3 References


APPENDIX 1

UBC RESEARCH ETHICS BOARD CERTIFICATE OF APPROVAL

The University of British Columbia
Office of Research Services
Clinical Research Ethics Board – Room 210, 828 West 10th Avenue, Vancouver, BC V5Z 1L8

ETHICS CERTIFICATE OF EXPEDITED APPROVAL

PRINCIPAL INVESTIGATOR: INSTITUTION / DEPARTMENT: UBC CREB NUMBER:
Juergen Kast UBC/Medicine, Faculty H07-01943

of Biomedical Research Centre

UBC CREB NUMBER:

INSTITUTION(S) WHERE RESEARCH WILL BE CARRIED OUT:

UBC

INSTITUTION(S) WHERE RESEARCH WILL BE CARRIED OUT:

UBC

Other locations where the research will be conducted:

Vancouver (excludes UBC Hospital)

Not applicable

CO-INVESTIGATOR(S):

Dana V. Devine

SPONSORING AGENCIES:

Canadian Blood Services

PROJECT TITLE:

Separation and characterization of biomarkers in platelets and blood plasma

THE CURRENT UBC CREB APPROVAL FOR THIS STUDY EXPIRES: September 24, 2008

The UBC Clinical Research Ethics Board Chair or Associate Chair, has reviewed the above described research project,

including associated documentation noted below, and finds the research project acceptable on ethical grounds for research

involving human subjects and hereby grants approval.

DOCUMENTS INCLUDED IN THIS APPROVAL:

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CERTIFICATION:

In respect of clinical trials:

1. The membership of this Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Division 5 of the Food and Drug Regulations.
2. The Research Ethics Board carries out its functions in a manner consistent with Good Clinical Practices.
3. This Research Ethics Board has reviewed and approved the clinical trial protocol and informed consent.

APPROVAL DATE:

September 14, 2007

September 24, 2007

August 3, 2007
form for the trial which is to be conducted by the qualified investigator named above at the specified clinical trial site. This approval and the views of this Research Ethics Board have been documented in writing.

The documentation included for the above-named project has been reviewed by the UBC CREB, and the research study, as presented in the documentation, was found to be acceptable on ethical grounds for research involving human subjects and was approved by the UBC CREB.

Approval of the Clinical Research Ethics Board by:

Dr. Caron Strahlendorf, Associate Chair