Mass Spectrometric Analysis of Thiol Proteins/Peptides Following Selenamide Derivatization And Electrolytic Reduction of Disulfide Bonds

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This dissertation titled

Mass Spectrometric Analysis of Thiol Proteins/Peptides Following Selenamide Derivatization And Electrolytic Reduction of Disulfide Bonds

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Abstract

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**Mass Spectrometric Analysis of Thiol Proteins/Peptides Following Selenamide Derivatization And Electrolytic Reduction of Disulfide Bonds**

Director of Dissertation: Hao Chen

Ambient mass spectrometry (MS) methods such as desorption electrospray ionization-mass spectrometry (DESI-MS) provides direct ionization of analytes with little or no sample preparation. The capability for liquid samples analysis allows DESI-MS to be coupled with many devices such as electrochemical cells and liquid chromatography (LC) for novel analytical applications. The online coupling of a thin-layer electrochemical flow cell with liquid sample DESI-MS can be applied for investigating different electrochemical reactions of biological molecules such as electrolytic reduction of disulfide-containing peptides/proteins. In addition, electrochemistry (EC) can assist the top-down characterization of proteins by electrolytic reduction of the disulfide bonds, in which sequence coverage can be significantly improved for electron-capture dissociation (ECD) and collision-induced dissociation (CID) analysis. Furthermore, the online coupling of LC with liquid sample DESI-MS was established, which allows a wide range of elution flow rates, online derivatization via reactive DESI to solve the post-column derivatization problems, also integration with EC. In addition, reactive DESI, which exploits the potential for coupling specific ion/molecule reactions with the ionization event, greatly improves the selectivity and efficiency for saccharide detection.
A systemic mass spectrometric investigation of a novel strategy for labeling biological thiols using selenamide reagents, involving the cleavage of the Se-N bond and form a new Se-S bond was introduced. Among 20 natural amino acids, the reaction is highly selective to thiol-containing peptides and proteins and occurs rapidly in high yield. The derivatization is also reversible upon addition of reducting reagents. The MS/MS dissociation behaviors of resulting peptide ions upon CID and electron-transfer dissociation (ETD) were investigated. In the positive ion mode, derivatized peptide ions exhibit tag-dependent CID dissociation pathways, allowing fast screening of peptides/proteins containing free cysteine residues in a mixture. By contrast, ETD dissociation of the two selenamide-derivatized peptide ions show the facile loss of the tag, providing insight into the mechanism for electron-based ion dissociation. The derivatization reaction and related ion dissociation chemistry would find extensive applications in proteomics, including the derivatization of thiol protein/peptides arising from online electrolytic reduction mentioned above.
Dedication

Dedicated to my parents, husband, and my daughter for their continuous love, care, and support
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XIC of the +10 protein ion) and b) -2.1 V (the superscripts on the charge numbers labeled in the spectrum indicates the number of the added ebselen tags to the reduced protein ions). Reprinted (Adapted) with permission from Zhang, Y.; Dewald, H. D.; Chen, H. J. Proteome Res. 2011, 10, 1293-1304. Copyright 2011 American Chemical Society.

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Figure 5.2. (a) Scheme of an apparatus for the coupled LC/MS using DESI interface, in which the eluent flow was reduced to 4.5 µL/min using a splitter; (b) acquired UV chromatogram (266 nm) using a C18 column and the isocratic mode (baseline corrected); DESI-MS mass spectra showing the separated (c) NE, (d) NM and (e) DA. Reproduced Zhang, Y.; Yuan, Z.; Dewald, H. D.; Chen, H. Chem. Comm. 2011, 47, 4171-4173. by permission of The Royal Society of Chemistry.

Figure 5.3. (a) Scheme of the apparatus for LC/reactive DESI-MS; reactive DESI-MS mass spectra showing the ions of products generated from the reactions between N-methyl-4-pyridineboronic acid iodide and (b) NE, (c) NM or (d) DA, respectively (background was subtracted). Reproduced Zhang, Y.; Yuan, Z.; Dewald, H. D.; Chen, H. Chem. Comm. 2011, 47, 4171-4173. by permission of The Royal Society of Chemistry.

Figure 5.4. Acquired DESI mass spectra showing the products after electrochemical oxidation of (a) NE, (b) NM and (c) DA following chromatographic separation. The structures of ions resulting from the oxidation of NE and DA were marked in (a) and (c), respectively. Reproduced Zhang, Y.; Yuan, Z.; Dewald, H. D.; Chen, H. Chem. Comm. 2011, 47, 4171-4173. by permission of The Royal Society of Chemistry.

Figure 5.5. (a) Scheme of the apparatus for LC/EC/DESI-MS; (b) acquired UV chromatogram (254 nm) showing the separation of the peptide tryptic digest (baseline corrected); DESI-MS mass spectra of AGCK/TFTSC with a cell potential of c) 0.0 V and d) -1.5 V (background was subtracted). Reproduced Zhang, Y.; Yuan, Z.; Dewald, H. D.; Chen, H. Chem. Comm. 2011, 47, 4171-4173. by permission of The Royal Society of Chemistry.
Figure 5.6. CID MS² spectra of a) m/z 933 and b) m/z 741. Reproduced Zhang, Y.; Yuan, Z.; Dewald, H. D.; Chen, H. Chem. Comm. 2011, 47, 4171-4173. by permission of The Royal Society of Chemistry................................. 159

Figure 5.7. CID MS² spectra of a) [AGCK+H]⁺ (m/z 378) and b) [TFTSC+H]⁺ (m/z 558). Reproduced Zhang, Y.; Yuan, Z.; Dewald, H. D.; Chen, H. Chem. Comm. 2011, 47, 4171-4173. by permission of The Royal Society of Chemistry................................................................................................... 160

Figure 6.1 Scheme of the apparatus for online coupling of a thin-layer µ-PrepCell™ electrochemical flow cell with a Bruker 12 T FT-ICR-MS using DESI as the interface. Reprinted (Adapted) with permission from Zhang, Y.; Cui, W.; Zhang, H.; Dewald, H. D.; Chen, H. Anal. Chem. 2012, 84, 3838-3842. Copyright 2012 American Chemical Society. ......................................................... 165

Figure 6.2. DESI-MS spectra acquired when a solution of 15 µM β-lactoglobulin A in methanol/water (1:1 by volume) containing 0.5% formic acid flowed through the thin-layer electrochemical cell with an applied potential of (a) 0.0 V and (f) -1.2 V; ECD MS/MS spectra of (b) +16 ion of the intact β-lactoglobulin A (m/z 1148.0) and (g) +19 ion of the reduced β-lactoglobulin A (m/z 967.1); The marked ECD backbone cleavage sites of (c) β-lactoglobulin A and (h) reduced β-lactoglobulin A; CID MS/MS spectra of (d) +16 ion of the intact β-lactoglobulin A (m/z 1148.0) and (i) +19 ion of the reduced β-lactoglobulin A (m/z 967.1); The marked CID backbone cleavage sites of (e) β-lactoglobulin A and (j) reduced β-lactoglobulin A. Reprinted (Adapted) with permission from Zhang, Y.; Cui, W.; Zhang, H.; Dewald, H. D.; Chen, H. Anal. Chem. 2012, 84, 3838-3842. Copyright 2012 American Chemical Society. ......................................................... 169

Figure 6.3. DESI-MS spectra acquired when a solution of 15 µM lysozyme in methanol/water (1:1 by volume) containing 0.5% formic acid flowed through the thin-layer electrochemical cell with an applied potential of (a) 0.0 V and (f) -1.5 V (the zoomed-in mass range for +14~+16 for the reduced lysozyme is shown in the inset); CID MS/MS spectra of (b) +10 ion of the intact lysozyme (m/z 1430.5) and (g) +15 ion of the reduced lysozyme (m/z 954.7); The marked CID backbone cleavage sites of (c) lysozyme and (h) reduced lysozyme; ECD MS/MS spectra of (d) +10 ion of the intact lysozyme (m/z 1430.5) and (i) +15 ion of the reduced lysozyme (m/z 954.7); The marked ECD backbone cleavage sites of (e) lysozyme and (j) reduced lysozyme. Reprinted (Adapted) with permission from Zhang, Y.; Cui, W.; Zhang, H.; Dewald, H. D.; Chen, H. Anal. Chem. 2012, 84, 3838-3842. Copyright 2012 American Chemical Society. ......................................................... 172
**Figure 7.1.**  
a) Reactive DESI mass spectrum showing the ionic species produced via the interaction of charged microdroplets generated by ESSI of a mixture of phenylboronic acid and 3-nitrophenylboronic acid (0.2 mM each in H$_2$O with pH=9 adjusted by NH$_4$OH) with fructose (10 mM in aqueous solution); 
b) CID MS$^2$ spectrum of the fructose reaction product ion of m/z 328; 
c) reactive DESI mass spectrum showing the ionic species produced via the interaction of charged microdroplets generated by ESSI of 3-nitrophenylboronic acid (0.2 mM in water with pH=9 adjusted by NH$_4$OH) with glucose (5 mM in aqueous solution); 
d) CID MS$^2$ spectrum of the glucose reaction product ion of m/z 328. Reprinted (Adapted) from Zhang, Y.; Chen, H. Int. J. Mass Spectrom. 2010, 289, 98–107, Copyright (2010), with permission from Elsevier. ................................................................. 184

**Figure 7.2.**  
a) Reactive DESI mass spectrum showing the ionic species produced via the interaction of charged microdroplets generated by ESSI of aqueous solution containing N-methyl-4-pyridineboronic acid iodide (0.2 mM) and tetramethylammonium chloride (0.2 mM, served as an internal standard) with glucose (5 mM in water). The inset shows the detection of the ion of the DESI reaction product (m/z 282) in the analysis of 10 µM of glucose in water; 
b) CID MS$^2$ spectrum of the ion of the glucose DESI reaction product (m/z 282); 
c) reactive DESI mass spectrum showing the ionic species produced via the interaction of charged microdroplets generated by ESSI of aqueous solution containing 2-picoline-4-boronic acid (0.2 mM) and tetramethylammonium chloride (0.2 mM, severed as an internal standard) with glucose (5 mM in water). Reprinted (Adapted) from Zhang, Y.; Chen, H. Int. J. Mass Spectrom. 2010, 289, 98–107, Copyright (2010), with permission from Elsevier. ................................................................. 188

**Figure 7.3.**  
Reactive DESI mass spectrum showing the ionic species produced via the interaction of charged microdroplets generated by ESSI of aqueous solution containing both N-methyl-4-pyridineboronic acid iodide (0.2 mM) and Girard’s reagent T (0.2 mM) with glucose (5 mM in water). Reprinted (Adapted) from Zhang, Y.; Chen, H. Int. J. Mass Spectrom. 2010, 289, 98–107, Copyright (2010), with permission from Elsevier. ................................................................. 189

**Figure 7.4.**  
a) ESI mass spectrum of a mixed solution of N-methyl-4-pyridineboronic acid iodide (0.2 mM in water) with glucose (0.1 mM in urine), and the mixing ratio is 3:1 by volume; 
b) reactive DESI mass spectrum is the ionic species produced via the interaction of charged microdroplets generated by ESSI of N-methyl-4-pyridineboronic acid iodide (0.2 mM in water) with glucose (0.1 mM in urine); reactive DESI mass spectra showing the ionic species produced via the interaction of charged microdroplets generated by ESSI of N-methyl-4-pyridineboronic acid iodide (0.2 mM in acetonitrile) with c) glucose (0.1 mM in urine) and d) glucose (0.5 mM in human serum).
The insets in c) and d) are the instrument responses versus the glucose concentrations in the urine and serum samples, respectively. The discrete lines represent the upper and lower boundaries of the 95% confidence intervals; the solid lines represent the calibration curves obtained from the average data points of three replicates of standard solutions. Reprinted (Adapted) from Zhang, Y.; Chen, H. *Int. J. Mass Spectrom.* 2010, 289, 98–107, Copyright (2010), with permission from Elsevier.

**Figure 7.5.** Reactive DESI mass spectra are the ionic species produced via the interaction of charged microdroplets generated by ESSI of N-methyl-4-pyridineboronic acid iodide (0.2 mM in water) with a) galactose (5.2 mM in water) and b) maltoheptaose (5 mM in water); c) CID MS² spectrum of the ion of the DESI reaction product from maltoheptaose (m/z 1254). Reprinted (Adapted) from Zhang, Y.; Chen, H. *Int. J. Mass Spectrom.* 2010, 289, 98–107, Copyright (2010), with permission from Elsevier.

**Figure 7.6.** a) The apparatus for tip-sampling liquid sample DESI; b) Extracted ion current chromatogram of the ion of the DESI reaction product (m/z 282) for five glucose samples (200 nL, 5 mM in water) sequentially analyzed by reactive DESI using N-methyl-4-pyridineboronic acid iodide (0.2 mM in water) as the chemical reagent. Reprinted (Adapted) from Zhang, Y.; Chen, H. *Int. J. Mass Spectrom.* 2010, 289, 98–107, Copyright (2010), with permission from Elsevier.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AE</td>
<td>Auxiliary electrode</td>
</tr>
<tr>
<td>AI-ECD</td>
<td>Activated-ion electron-capture dissociation</td>
</tr>
<tr>
<td>APCI</td>
<td>Atmospheric pressure chemical ionization</td>
</tr>
<tr>
<td>APPI</td>
<td>Atmospheric pressure photoionization</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CAD</td>
<td>Collisional-activation dissociation</td>
</tr>
<tr>
<td>CI</td>
<td>Chemical ionization</td>
</tr>
<tr>
<td>CID</td>
<td>Collision-induced dissociation</td>
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<tr>
<td>CSD</td>
<td>Charge state distribution</td>
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<tr>
<td>DA</td>
<td>Dopamine</td>
</tr>
<tr>
<td>DAPCI</td>
<td>Desorption atmospheric pressure chemical ionization</td>
</tr>
<tr>
<td>DAPPI</td>
<td>Desorption atmospheric pressure photoionization</td>
</tr>
<tr>
<td>DART</td>
<td>Direct analysis in real time</td>
</tr>
<tr>
<td>DBDI</td>
<td>Dielectric barrier discharge ionization</td>
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<tr>
<td>DEMS</td>
<td>Differential electrochemical mass spectrometry</td>
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<tr>
<td>DESI-MS</td>
<td>Desorption electrospray ionization mass spectrometry</td>
</tr>
<tr>
<td>DI</td>
<td>Desorption Ionization</td>
</tr>
<tr>
<td>DPD</td>
<td>$N,N$-dimethyl-$p$-phenylenediamine</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EC</td>
<td>Electrochemistry</td>
</tr>
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<td>ECD</td>
<td>Electron-capture dissociation</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
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<tr>
<td>EC/LC/MS</td>
<td>Electrochemistry/liquid chromatography/mass spectrometry</td>
</tr>
<tr>
<td>EC/MS</td>
<td>Electrochemical mass spectrometry</td>
</tr>
<tr>
<td>ELDI</td>
<td>Electrospray-assisted laser desorption/ionization</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray ionization</td>
</tr>
<tr>
<td>ESSI</td>
<td>Electrosonic spray ionization</td>
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<tr>
<td>ET</td>
<td>Electron-transfer</td>
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<td>ETcaD</td>
<td>Post-ETD collisional activation</td>
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<td>ETD</td>
<td>Electron-transfer dissociation</td>
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<tr>
<td>eV</td>
<td>Electron-volt</td>
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<tr>
<td>FAB</td>
<td>Fast atom bombardment</td>
</tr>
<tr>
<td>FL</td>
<td>Fluorescence</td>
</tr>
<tr>
<td>FT-ICR-MS</td>
<td>Fourier transform ion cyclotron resonance mass spectrometry</td>
</tr>
<tr>
<td>FT-IR</td>
<td>Fourier transform infrared spectroscopy</td>
</tr>
<tr>
<td>GI</td>
<td>Geometry-independent</td>
</tr>
<tr>
<td>GPx</td>
<td>Glutathione peroxidase</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>GSSG</td>
<td>L-Glutathione disulfide</td>
</tr>
<tr>
<td>SORI</td>
<td>Sustained off-resonance irradiation</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulins G</td>
</tr>
<tr>
<td>IR</td>
<td>Infrared</td>
</tr>
<tr>
<td>IRMPD</td>
<td>Infrared multiphoton dissociation</td>
</tr>
<tr>
<td>kD</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>LAESI</td>
<td>Laser ablation electrospray ionization</td>
</tr>
<tr>
<td>LC</td>
<td>Liquid chromatography</td>
</tr>
<tr>
<td>LC/APCI-MS</td>
<td>Liquid chromatography/Atmospheric pressure chemical ionization mass spectrometry</td>
</tr>
<tr>
<td>LC/DESI-MS</td>
<td>Liquid chromatography/desorption electrospray ionization mass spectrometry</td>
</tr>
<tr>
<td>LC/EC/DESI-MS</td>
<td>Liquid chromatography/electrochemistry/desorption electrospray ionization mass spectrometry</td>
</tr>
<tr>
<td>LC/MS</td>
<td>Liquid chromatography/mass spectrometry</td>
</tr>
<tr>
<td>LC/reactive DESI-MS</td>
<td>Liquid chromatography/reactive desorption electrospray ionization mass spectrometry</td>
</tr>
<tr>
<td>LMJ-SSP</td>
<td>Liquid microjunction surface sampling probe</td>
</tr>
<tr>
<td>LOD</td>
<td>Limit of detection</td>
</tr>
<tr>
<td>LTP</td>
<td>Low temperature plasma</td>
</tr>
<tr>
<td>MALDESI</td>
<td>Matrix-assisted laser desorption electrospray ionization</td>
</tr>
<tr>
<td>MALDI</td>
<td>Matrix-assisted laser desorption ionization</td>
</tr>
<tr>
<td>MD</td>
<td>Magic diamond</td>
</tr>
<tr>
<td>MRFA</td>
<td>Met-Arg-Phe-Ala</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>MS/MS</td>
<td>Tandem mass spectrometry</td>
</tr>
<tr>
<td>MS^n</td>
<td>Multiple stages MS</td>
</tr>
<tr>
<td>MW</td>
<td>Molecule weight</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>m/z</td>
<td>mass-to-charge ratios</td>
</tr>
<tr>
<td>N₂</td>
<td>Nitrogen</td>
</tr>
<tr>
<td>nano-ESI</td>
<td>Nano-electrospray ionization</td>
</tr>
<tr>
<td>ND-EESI</td>
<td>Neutral desorption extractive electrospray ionization</td>
</tr>
<tr>
<td>NE</td>
<td>Norepinephrine hydrochloride</td>
</tr>
<tr>
<td>NETD</td>
<td>Negative electron-transfer dissociation</td>
</tr>
<tr>
<td>niECD</td>
<td>Negative-ion electron-capture dissociation</td>
</tr>
<tr>
<td>NLS</td>
<td>Neutral loss scan</td>
</tr>
<tr>
<td>NM</td>
<td>DL-normetanephrine hydrochloride</td>
</tr>
<tr>
<td>NPSP</td>
<td>N-(phenylseleno)phthalimide</td>
</tr>
<tr>
<td>PA</td>
<td>Proton affinity</td>
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<tr>
<td>PIS</td>
<td>Precursor ion scan</td>
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<tr>
<td>PTM</td>
<td>Post-translational modifications</td>
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<tr>
<td>QIT</td>
<td>Quadrupole ion trap</td>
</tr>
<tr>
<td>QQQ</td>
<td>Triple quadrupole</td>
</tr>
<tr>
<td>RE</td>
<td>Reference electrode</td>
</tr>
<tr>
<td>RF</td>
<td>Radio frequency</td>
</tr>
<tr>
<td>S/N</td>
<td>Signal to noise ratio</td>
</tr>
<tr>
<td>SRM</td>
<td>Selected reaction monitoring</td>
</tr>
<tr>
<td>TCEP</td>
<td>Tris(2-carboxyethyl)phos-phine hydrochloride</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>TOF</td>
<td>Time-of-flight</td>
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<tr>
<td>Abbr.</td>
<td>Full Form</td>
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<tr>
<td>-------</td>
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</tr>
<tr>
<td>TS</td>
<td>Thermospray</td>
</tr>
<tr>
<td>TSP</td>
<td>Thermospray ionization</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>UW</td>
<td>Utah-Washington mechanism</td>
</tr>
<tr>
<td>WE</td>
<td>Working electrode</td>
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<tr>
<td>XIC</td>
<td>Extracted ion chromatogram</td>
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Chapter 1: Introduction

1.1. Goals

The main focuses of this Ph. D. dissertation include a systemic MS investigation of a novel strategy for labeling biological thiols using selenamide reagents, and the applications of liquid sample DESI-MS technology in coupling EC with MS. Compared with the traditional ionization methods, such as electrospray ionization (ESI) technique, DESI is capable of ionizing samples in ambient environment with little or no sample preparation.

1.2. Project Overview

Chapter 2 introduces a novel strategy for labeling biological thiols using selenamide reagents for analysis by MS. This reaction involves the cleavage of Se-N bond by thiol to form a new Se-S bond. By testing different samples including amino acids, thiol-containing peptides and proteins, it shows the reaction is highly selective, rapid, reversible, and efficient. Among 20 amino acids, only cysteine is reactive toward Se-N containing reagents and the reaction occurs in seconds. With the addition of dithiothreitol (DTT), peptides derivatized by selenium reagents can be recovered. The high reaction selectivity and reversibility provide potential in both selective identification and isolation of thiols from mixtures. Equally importantly, the thiol derivatization yield is striking, as reflected by 100% conversion of protein \( \beta \)-lactoglobulin A using ebselen within 30 s. In addition, preliminary applications such as rapid screening of thiol peptides from mixtures and identification of the number of protein free and bound thiols have been
demonstrated. The unique selenium chemistry uncovered in this study would be valuable in the MS analysis of thiols and disulfide bonds of proteins/peptides.\(^1\)

The follow-up work shown in chapter 3 is related to the systematic investigation of MS/MS dissociation behaviors of selenamide-derivatized thiol peptide ions upon CID and ETD. In the positive ion mode, derivatized peptide ions exhibit tag-dependent CID dissociation pathways. For instance, ebselen-derivatized peptide ions preferentially undergo Se–S bond cleavage upon CID to produce a characteristic fragment ion, the protonated ebselen (\(m/z\) 276), which allows selective identification of thiol peptides from protein digest as well as selective detection of thiol proteins from a protein mixture using precursor ion scan (PIS). In contrast, \(N\)-(phenylseleno)phthalimide (NPSP)-derivatized peptide ions retain their phenylselenenyl tags during CID, which is useful in sequencing peptides and locating cysteine residues. In the negative ion CID mode, both types of tags are preferentially lost via the Se–S cleavage, analogous to the S–S bond cleavage during CID of disulfide-containing peptide anions. In consideration of the convenience in preparing selenamide-derivatized peptides and the similarity of Se–S of the tag to the S–S bond, we also examined ETD of the derivatized peptide ions to probe the mechanism for electron-based ion dissociation. Interestingly, facile cleavage of Se–S bond occurs to the peptide ions carrying either protons or alkali metal ions, while backbone cleavage to form \(c/z\) ions is severely inhibited. These results are in agreement with the Utah-Washington (UW) mechanism proposed for depicting electron-based ion dissociation processes.\(^2\)

In addition to the selenium chemistry project, I also worked on the applications of EC online combined with liquid sample DESI-MS. In chapter 4, a structural analysis of
biologically active peptides and proteins containing disulfide bonds by EC/DESI-MS was introduced, in which the sample undergoes electrolytic disulfide cleavage in an electrochemical flow cell followed by online MS detection. The formation of a disulfide bond bridge is an important post-translational modification (PTM) for proteins. Using this EC/DESI-MS method, the disulfide-containing peptides can be quickly identified from enzymatic digestion mixtures, based on the abrupt decrease in their relative ion abundances after electrolysis. Peptide mass mapping and tandem MS (MS/MS) analysis of the ions of the resulting free peptide chains can possibly establish the disulfide linkage pattern and sequence the precursor peptides. In this regard, the method provides much more chemical information than previous analogous electrochemical analyses. In addition, derivatization of thiols by selective selenamide reagents, involving the new derivatization chemistry mentioned above, is useful for easy recognition of reduced peptide ions and the number of their free thiols. Furthermore, electrolytic reduction of proteins leads to increased charges on the detected protein ions, revealing the role of disulfide bonds on maintaining protein conformation. This electrochemical mass spectrometric method is fast and does not need chemical reductants, potentially having valuable applications in proteomics research.\(^3\)

Another project demonstrates a liquid chromatography/mass spectrometry (LC/MS) method using DESI as a versatile interface, and three variant apparatus were introduced, including LC/DESI-MS, LC/reactive DESI-MS, and LC/EC/DESI-MS. This coupling allows a wide range of elution flow rates, online derivatization \textit{via} reactive DESI (avoid the post-column derivatization problems including an increased time delay
and peak broadening), and further combination with EC, which are summarized in chapter 5.\textsuperscript{4}

Covalent disulfide bond linkage in a protein is an important challenge for MS-based top-down protein structure analysis\textsuperscript{5-6} as it reduces the backbone cleavage efficiency for MS/MS dissociation. My additional project shown in chapter 6 presents a strategy for solving this critical issue via integrating EC online with a top-down MS approach. In this approach, proteins undergo electrolytic reduction in an electrochemical cell to break disulfide bonds and then undergo online ionization into gaseous ions for tandem MS analysis by ECD and CID. The electrochemical reduction of proteins allows one to remove disulfide bond constraints and also leads to increased charge numbers of the resulting protein ions. As a result, sequence coverage was significantly enhanced, as exemplified by \(\beta\)-lactoglobulin A (24 vs 75 backbone cleavages before and after electrolytic reduction, respectively) and lysozyme (5 vs 66 backbone cleavages before and after electrolytic reduction, respectively). This methodology is fast due to the fast nature of electrochemical conversion and would have an important impact in high-throughput proteomics research.\textsuperscript{7}

Chapter 7 describes an improved method for the detection of sugars via \textit{in-situ} derivatization with modified phenylboronic acid PhB(OH)\textsubscript{2} using reactive DESI. The study described in chapter 7 employed two modified phenylboronic acids including 3-nitrophenylboronic acid and \(N\)-methyl-4-pyridineboronic acid iodide as the reagents for reactive DESI. In contrast with their parent compound PhB(OH)\textsubscript{2}, enhanced sensitivity of using 3-nitrophenylboronic acid was observed due to the stabilization of the resulting
boronate ester anion by the electron-withdrawing nitro group. In the case of \( N \)-methyl-4-pyridineboronic acid iodide, the higher sensitivity obtained is attributed to the efficient ionization of both the reactive DESI reagent and reaction product since the precursor acid with a quaternary ammonium group is pre-charged. In this case, additional important features are found: it is applicable to neutral and acidic saccharide solutions, facilitating the analysis of biological fluids without the need to adjust pH; simply by changing the spray solvent from water to acetonitrile, the method can be used for direct glucose analyses of both urine and serum samples \textit{via} online desalting, due to the low solubility of salts of these biofluids in the sprayed organic solvent; \( N \)-methyl-4-pyridineboronic acid iodide shows higher reactivity compared with phenylboronic acid in the reactive DESI; the ions of saccharide DESI reaction products undergo extensive ring or glycosidic bond cleavage upon CID, which might be useful in the structure elucidation of saccharides. In addition, a variant sample introduction protocol using pipette tips for saccharide solutions was also demonstrated in the reactive DESI experiments, allowing the analysis of multiple samples of a small volume (e.g., 200 nL) in a short period of time (e.g., five samples in 4 min). The method reported in this study with improved sensitivity and high selectivity along with online desalting and high throughput capability could find useful applications in saccharide analysis in complicated biological samples.\(^8\)

1.3. Selenium Chemistry

In 1818, the element selenium was discovered by a Swedish chemist Berzelius, which was named after the Greek goddess of the moon, Selene. In 1973, with the report of two bacterial enzymes containing selenium, formate dehydrogenase\(^9\) and glycine
reductase\textsuperscript{10}, selenium biochemistry started to emerge. Meanwhile, the biochemical role of selenium in mammals was evidently recognized due to selenium as a part of the active site of the antioxidant enzyme glutathione peroxidase (GPx).\textsuperscript{11-12} Many organoselenium compounds have been studied as biological models that are able to simulate catalytic functions established by natural enzymes, such as ebselen ([2-phenyl-1,2-benzisoselenazol-3(2H)-one], see its structure \textbf{1} in Figure 1.1), which is shown to act as a GPx mimic.\textsuperscript{13-15} and an anti-inflammatory drug since being found.\textsuperscript{13, 15} The proposed mechanism for the observed GPx activity of ebselen was in Figure 1.1.\textsuperscript{12, 16} Based on this cycle, ebselen reacts rapidly with GSH to produce the selenenyl sulfide \textbf{2}, and then it will react with excess GSH to yield selenol \textbf{3} and disulfide compound GSSG, and selenol \textbf{3} was proven to be the predominant molecular species responsible for the GPx activity of ebselen.\textsuperscript{17-19} However, Mugesh \textit{et al.} recently reported that ebselen as a GPx-like redox catalyst is indeed inactive and the reaction of ebselen with PhSH only produces the Se-S product, not disulfide PhSSPh, even with excess amount of PhSH.\textsuperscript{16}
1.4. Mass Spectrometry (MS)

MS is an analytic technique which measures the mass-to-charge ratio (m/z) of ions, which has become one of the most popular and powerful modern physical-chemical methods to study the complexities of elemental and molecular processes in nature. And the advent of new methods of ion generation, new mass analyzers and novel tools for data processing has made it possible to analyze almost all chemical from small organic compounds, large biological molecules, to whole living cells/tissues.

A mass spectrometer usually contains ion source, mass analyzer and detector. A typical MS procedure includes that the sample was first loaded and vaporized, and then
ionized by different methods, the generated charged particles are separated depending on their $m/z$ in a mass analyzer, such as time-of-flight (TOF), quadrupole mass analyzer, ion trap, by electromagnetic field, which are further detected. At last, the ion signal will be processed into mass spectra. There are two laws for the dynamics of ions in electric and magnetic fields in vacuum, which are Lorentz force law and Newton’s second law shown below.

1. Lorentz force law: $F = Q (E + v \times B)$

2. Newton’s second law (motion in non-relativistic): $F = m \times a$

Here $F$ is the force to the ion, $Q$ is the charge of the ion, $E$ is the electric field, and $v \times B$ is the vector cross product of the ion velocity and the magnetic field, $m$ is the mass of the ion, and $a$ is the acceleration. For example, quadrupole mass analyzer consists of four parallel circular rods. There are two voltages applied to the rods, including a radio frequency (RF) voltage applied between one pair of rods and the other and a direct current voltage further applied on the RF voltage. Ions travel down the quadrupole among the rods. And only the ions of a certain $m/z$ will be detected by a detector at a ratio of voltages while the others will collide with the rods due to the unstable trajectories. A linear series of three quadrupoles can be used, which is also called triple quadrupole mass spectrometer, in which $Q_1$ and $Q_3$ quadrupoles are used as mass filters, and $q_2$ quadrupole acts as a collision cell that is an RF-only quadrupole and cannot be employed to be a mass filter.

Two ionization methods founded about two decades ago are most outstanding and useful. The first method is ESI, demonstrated by John Fenn, which is applicable to
the analysis of solution samples, and the second one is matrix-assisted laser desorption ionization (MALDI)\textsuperscript{22} which is applicable to the analysis of solids, belonging to the family of desorption ionization (DI). Both of them have had an enormous effect on the analysis of biology and life sciences, which have conquered protein mass spectrometry since their invention in 1980s, and they have triggered the explosion in applications of MS for protein study. In addition, the ion source of ESI operated in the ambient environment was another breakthrough.\textsuperscript{23-24}

1.5. Desorption Electrospray Ionization Mass Spectrometry (DESI-MS)

Generally, moderate to extensive sample preparations are always necessary for most MS applications, and then followed by introduction of the sample into the high vacuum conditions prior to MS analysis, which can increase the possibility of contamination during sample handling and limit for \textit{in-situ} analysis.

With the development of ambient ionization methods including DESI\textsuperscript{25} and direct analysis in real time (DART),\textsuperscript{26} these drawbacks can be overcome. Ambient MS\textsuperscript{24} is a recent advance in the field. In these methods, samples are examined in the open environment and typically little or no sample preparation is required, which allows \textit{in-situ} analysis while preserving all attributes associated with MS analysis. These approaches open a new area in mass spectrometry.

1.5.1. History and Background

DESI was first introduced by Prof. Graham R. Cooks’ research group at Purdue University in 2004 and is now available commercially from Prosolia Inc.\textsuperscript{25} As a
representative ambient ionization method, DESI\textsuperscript{24} is successful in the fast analysis of various analytes, which can be applicable to liquid samples,\textsuperscript{27} solid samples, frozen solutions and also adsorbed gases,\textsuperscript{23} including pharmaceuticals,\textsuperscript{28-30} proteins,\textsuperscript{31} metabolites,\textsuperscript{32-33} drugs of abuse,\textsuperscript{34-35} chemical warfare agents,\textsuperscript{36} explosives,\textsuperscript{37-39} TLC plates,\textsuperscript{40} bacteria,\textsuperscript{41} even intact tissues for certain components,\textsuperscript{42-44} etc. After the advent of DESI,\textsuperscript{25} a whole new family of ambient ionization methods and variants emerged and now there are almost 30 ambient ionization methods for mass spectrometry reported,\textsuperscript{45} such as DART,\textsuperscript{26} desorption atmospheric pressure chemical ionization (DAPCI),\textsuperscript{46} electrospray-assisted laser desorption/ionization (ELDI),\textsuperscript{47} matrix-assisted laser desorption electrospray ionization (MALDESI),\textsuperscript{48} liquid microjunction surface sampling probe method (LMJ-SSP),\textsuperscript{49} desorption atmospheric pressure photoionization (DAPPI),\textsuperscript{50} dielectric barrier discharge ionization (DBDI),\textsuperscript{51} neutral desorption extractive electrospray ionization (ND-EESI),\textsuperscript{52} laser ablation electrospray ionization (LAESI),\textsuperscript{53} and low temperature plasma (LTP),\textsuperscript{54} etc. For DESI method, the variants include reactive-DESI (reactions accompanying desorption),\textsuperscript{55} geometry-independent (GI) DESI,\textsuperscript{56} non-proximate (stand-off) detection DESI (transport of sample ions at long distances),\textsuperscript{46} transmission-mode DESI,\textsuperscript{57} liquid sample DESI,\textsuperscript{27} nanospray DESI\textsuperscript{58} were gradually introduced to facilitate direct analysis of various samples and increase selectivity and sensitivity for trace samples analysis. DESI is characterized by high speed and throughput, soft ionization, high sensitivity (sub-nanogram range), molecular specificity, positive and negative ionization, low substrate/surface requirements, low
matrix and salt sensitivity, universal applicability, and quantitative accuracy and precision.\textsuperscript{59}

1.5.2. DESI Ionization Source

Figure 1.2 is the typical DESI setup, in which ionization occurs \textit{via} the interaction of charged microdroplets generated in a pneumatically assisted electrospray of an appropriate solvent (typically mixtures of methanol, water and acetic acid) by applying a high voltage of 3-6 kV with solid phase samples spotted on the surfaces. The source consists of two main parts, a sprayer assembly and a surface assembly, both of them mounted on a source base. The solvent nebulizer is made of deactivated fused silica capillary, similar to the one used in electrosonic spray ionization (ESSI).\textsuperscript{60} Nitrogen (N\textsubscript{2}) is used as the nebulizing gas at a linear velocity of approximately 350 m/s. The gas jet consists of electrosprayed aqueous microdroplets and free gas-phase ions, which is directed onto the analyte on a surface at various incident angles usually from 25\textdegree{} to 80\textdegree{} depending on the analyte. The resulting droplets, ions, and neutrals are collected at a shallow angle from the surface, and then transferred as a result of electrostatic and pneumatic forces to a mass spectrometer equipped with an atmospheric pressure interface. The sprayer is typically mounted onto a xyz linear moving stage, which allows adjustment of the sprayer-to-MS or sprayer-to-surface distance and to be positioned at any chosen point with respect to the sample. The moving stage also has a tangent arm drive miniature stage that allows precise angular adjustment from 0\textdegree{} to 90\textdegree{}. The surface holder is mounted on a separated xyz moving stage. (Figure 1.2)
Signal intensity in DESI spectra depends on incident angle ($\alpha$), collection angle ($\beta$), tip-to-surface distance ($d_1$), MS inlet-to-surface distance ($d_2$), and other geometric parameters, as defined in Figure 1.3 (b). Additionally, there are geometry effects for the spray methods, especially for DESI. When optimized, the angles and distances in Figure 1.3 (a) are stable for a long period while the shallow take-off angle has proven doubtful by some investigations. Thus, GI DESI, non-proximate (stand-off) detection DESI, and transmission-mode DESI were demonstrated to solve this problems. In addition, the velocity of nebulization gas, the flow rate of spray solvent, and the potential applied to the sprayer also affect the DESI performance. The type of surface analyzed is also one of the factors that affect the ionization process.
1.5.3. DESI Ionization Mechanisms

DESI has two kinds of ionization mechanisms including heterogeneous charge-transfer mechanism and droplet pick-up mechanism of ionization. Droplet pick-up has been suggested as the primary ionization mechanism in DESI although there is evidence for chemical sputtering (reactive ion/surface collisions) and gas-phase ionization.
processes (e.g., charge transfer, ion-molecule reactions, and volatilization/desorption of neutrals followed by ionization).\textsuperscript{23, 55, 61-63} According to the later one, the surface is pre-wetted by initial droplets, forming a solvent layer to dissolve the surface analytes, and then these dissolved analytes are picked up by later arriving droplets \textit{via} impacting the surface, creating the secondary droplets with the dissolved analytes. Gas-phase ions are then formed from these secondary droplets. The resulting gas-phase ions have internal energy values around 2 eV,\textsuperscript{64} similar to those in ESI and ESSI.\textsuperscript{64} Due to the formation of cold ions, DESI is defined as a soft ionization method, which can give ESI-like mass spectra, especially for proteins and polypeptides.

1.5.4. Reactive DESI

DESI involving conditions such as temperature, solvent, pressure, \textit{etc.} is remarkably similar with solution-phase chemical reactions. Thus, it is normal to apply the established solution-phase chemistry in DESI and add some reagents to the spray solvent or use gas-phase reagents to improve performance. Reactive DESI, one of the variants of DESI, was used to increase the specificity of ionization of particular analytes, such as non-polar analytes cholesterol, fat-soluble vitamins, saccharides, and anabolic steroids, for which DESI is normally less sensitive. The examples of reactive DESI reactions are summarized in Figure 1.4. Chapters 5 and 7 show the applications of reactive DESI using modified phenylboronic acids to react and ionize various sugars.
Figure 1.4. Examples of reactions used in reactive DESI experiments to improve sensitivity of detecting cholesterol,\textsuperscript{65} anabolic steroids,\textsuperscript{66} cis-diols,\textsuperscript{67} phosphonate esters,\textsuperscript{68} and cyclic acetals.\textsuperscript{59, 69}

1.5.5. Liquid Sample DESI

In our laboratory, we extended the conventional DESI method\textsuperscript{25} to direct liquid sample analysis,\textsuperscript{27} and this modified liquid sample DESI method is used in my research study covered in chapters 4-7. The prototype apparatus is in Figure 1.5. This new DESI method can be applied for the direct analysis of liquid samples on the surface, which are continuously delivered through a silica capillary by a syringe pump onto a surface, and
then picked up by the charged droplets generated by DESI sprayer. The resulting ions are analyzed by a mass spectrometer. The liquid sample DESI-MS can be useful in analyzing many biological samples such as urine and blood, and it allows sample detected from their native environments. Furthermore, due to the capability for direct analysis of liquid samples, it is possible to couple liquid sample DESI-MS with other devices such as electrochemical cells or separation technologies for novel analytical applications. Such a study including instrumentation and applications is exemplified in chapters 4-6.

Figure 1.5. Scheme of the DESI-MS apparatus for the direct analysis of liquid samples. 1: ESSI source; 2: sample introduction silica capillary (this capillary can be replaced by a syringe needle so that liquid samples can be delivered to the surface directly from a pumped syringe, as demonstrated later in the text); 3: porous Teflon surface; 4: curtain plate of Q-trap instrument. Reproduced in part with permission from Miao, Z.; Chen, H., J. Am. Soc. Mass Spectrom. 2009, 20, 10-19. Copyright 2009, Springer.

In addition, there are some unique features and several interesting applications of liquid sample DESI-MS. For example, it is easier to desorb large proteins directly from
solution such as high mass proteins (e.g., bovine serum albumin (BSA) with MW of 66 kDa), than from dried samples on surface, probably due to less aggregation in solution than in the solid form. Recently, it was reported that liquid sample DESI-MS is capable for direct ionization of large noncovalent protein complexes (>45 kDa) and proteins (up to 150 kDa). Furthermore, the coupling of liquid sample DESI-MS with microfluidics, microextraction probing protein conformation in solution, as well as for kinetic study were reported.

1.6. Electrochemical Mass Spectrometry (EC/MS)

1.6.1. History and Background

EC is a well-established technique, which has broad applications in biomedical and biological fields. However, the complicated electrochemical reactions indicate that a small amount of byproduct may affect the electrochemical behavior. Thus, EC was combined with other ex-situ and later in-situ characterization methods such as electron microscopy, electron spectroscopy, vibrational spectroscopy (Raman, FTIR, UV), and MS, which can provide information about the species either on the electrode surface or in the solution. However, in terms of sensitivity and specificity, MS is more advantageous. Vibrational spectrometry is less quantitative than the others and all other methods suffer from chemical interferences. In 1971, Bruckenstein first demonstrated the coupling of EC with MS. However, the limited applications for the combination of EC and MS were found up to date due to the initial practical problems in interfacing EC with MS and the lack of published applications. A mass spectrometer as a detector can provide complementary information such as product identification and structural characterization
because the redox reactions often cause mass changes. In addition, the chemical structure can be further elucidated by tandem MS analysis via the fragmentation of the analyte ions.\textsuperscript{82}

1.6.2. Coupling of EC with MS

The online combination of EC and MS is a direct and sensitive method in identifying the electrochemical reaction products or intermediates, leading to extensive applications in bioanalysis and mechanistic studies of redox reactions. Compared with offline EC/MS, online coupling has unique advantages of allowing an integrated and automated system, which is useful in high-throughput analysis; direct and time-resolved analysis of reaction products with minimized dead volume. The schematic view of configurations for combination of EC, LC, and MS, and their main applications are in Figure 1.6.\textsuperscript{82}
Previously, the first EC/MS coupling was demonstrated in 1971, which was applied for *in situ* mass spectrometric determination of volatile electrode reaction products by using a porous electrode, and then Wolter reported differential electrochemical mass spectrometry (DEMS) for the online MS detection of gaseous and volatile electrochemical products in real time. Afterwards, EC coupled with MS was realized using different ion sources/interfaces as thermospray (TS), fast atom bombardment (FAB), inductively coupled plasma, particle beam.
atmospheric pressure chemical ionization (APCI)/atmospheric pressure photoionization (APPI), and especially ESI.

1.6.3. EC/ESI-MS

ESI demonstrated by John Fenn has become one of the most popular ionization methods used in MS analysis, which is a soft ionization technique with a lot of possibilities. There have been several groups reported on EC/ESI-MS systems. In 1995, EC was coupled with ESI-MS by Van Berkel, et. al., who combined three types of electrochemical cells with ESI-MS, and Bond, et. al. studied metal-diethyldithiocarbamate complexes by a two-electrode system. The advantages of EC/ESI-MS coupling are that ESI is valuable in ionizing nonvolatile, polar, thermally labile compounds and large biological molecules.

This method has been used for identification of generated species by electrochemical reactions, for example, Van Berkel and Zhou ionized neutral analytes including perylene, anthracene, ferrocene, tetrabutylammonium tetrafluoroborate etc. Detection of unstable reaction products with transfer times of a few seconds between the EC cell and the entrance of the mass spectrometer, such as the unstable radical cation products generated from EC oxidization of polycyclic aromatic hydrocarbons were investigated.

In terms of elucidating reaction mechanisms and kinetics, Lev and co-workers studied the oxidation mechanism of N,N-dimethyl-p-phenylenediamine (DPD) by online EC/ESI-MS; To realize online chemical tagging, Girault et. al. modified free cysteine residues using quinone addition.
For mimicking metabolic reactions, for example, EC was online coupled with MS to simulate metabolic oxidation reactions introduced by Bruins and co-workers; the coupling with chromatographic separation, such as Karst group used electrochemistry/liquid chromatography/mass spectrometry (EC/LC/MS) to simulate the detoxification mechanism of paracetamol in the body; and oxidative cleavage of peptides/proteins.

Given that electrochemical reactions have been found to occur at the interface between the liquid and the spray capillary for electrospray ionization, there have been many publications describing ESI as an electrolytic cell. Compared with traditional EC analysis, some advantages with the use of the inherent electrolysis in an ESI metal capillary emitter were found, including the consumption of less amount of sample. In comparison with a separate EC flow cell on-line with ESI-MS, the instrumental setup is simpler but less flexible. The potential cannot be controlled without affecting the high voltage on the ESI emitter. All of the ESI current, the material of the capillary, and the amount of the electroactive pecies present in the sample solution, will influence the electrochemical potential of the spray capillary.

1.6.4. EC/DESI-MS

Although online EC/MS coupling has been developed in different fields since 1971, there are some inherent problems of the coupling interface that have not been solved, such as the issues of solvent conflict and voltage conflict. For the solvent conflict, electrolysis reactions normally use high concentrations of supporting electrolyte (typically about 0.1 M). In MS, high concentrations of salts would cause peak
suppression and fouling of the interface plates and lenses of MS instrument.\textsuperscript{112} Thus, the traditional electrolytes for EC like nonvolatile inorganic salts are not suitable for MS. More MS compatible and volatile solvents such as formic acid, ammonia hydroxide, and ammonia acetate are used. The second conflict is the voltage. For EC/ESI-MS, high voltage for ESI source is used to produce the charged ions. While, a small voltage for redox reactions is used for electrolysis. For the safety concern, the small voltage applied to the EC cell is either floated or decoupled from the high voltage used for ionization. This increases the instrumentation complexity, and causes many electrode cross-talk, which may also produce undesired reactions.\textsuperscript{75}

By using the direct sampling liquid sample DESI as the interface mentioned above for coupling MS with EC, the two problems can be overcome. In 2009, EC/DESI-MS method was first demonstrated in our group (Figure 1.7),\textsuperscript{141} which has several unique and valuable features. First, there is no conflict between the small potential applied to the EC cell and the high voltage used for spray ionization in the EC/DESI-MS because the two potentials are physically separated. In contrast, the EC system usually needs to be either floated or decoupled from the ESI source in EC/ESI-MS. Thus, the EC/DESI-MS method leads to simpler instrumentation and also has the freedom to choose either positive or negative ionization modes while performing either reduction or oxidation reactions in the EC cells. Second, as DESI can ionize aqueous samples without addition of organic additives, there is more freedom to use aqueous solvent compared with EC/ESI-MS. Third, DESI appears to have high salt tolerance. It was shown that, good MS signal of the analyte could be still obtained even when 10 mM NaCl was used as an
electrolyte.\textsuperscript{141} Fourth, there is no background signal from oxidation of the analyte caused by DESI ionization like dopamine (DA) oxidation, only generating dopamine quinine.\textsuperscript{141} This feature is valuable for the detection of reaction intermediates or products from the EC event in the cell with no interference from the MS ionization step.

\textit{Figure 1.7.} Scheme of the apparatus for online coupling of a thin-layer electrochemical flow cell with DESI-MS. WE, working electrode; AE, auxiliary electrode; and RE, reference electrode.\textsuperscript{141} Reproduced in part with permission from Li, J.; Dewald, H. D.; Chen, H., \textit{Anal. Chem.} 2009, 81, 9716-9722. Copyright 2009, ACS.

Bases on this online EC/DESI-MS method, several electrochemical reactions of analytical importance were demonstrated, especially for the electrolytic reduction of disulfide-containing peptides and proteins, which are introduced in chapters 4-6.
1.7. Tandem Mass Spectrometry

Tandem MS also known as MS/MS involves multiple steps of mass analysis, usually involving some forms of fragmentation. It has been proven useful for the identification and characterization of ions and for complex mixture analysis. In addition, multiple stage MS ($MS^n$) separation can be achieved by implementing tandem MS in either space or time. For instance, one mass analyzer can isolate a peptide ion generated from a mixture entering into a second mass analyzer where it has collisions with inert gas, causing fragmentation. This dissociation process is called CID. A third mass analyzer then analyzes the fragments generated from the peptide ion dissociation. Furthermore, tandem MS can also be done in a single mass analyzer such as in a quadrupole ion trap (QIT). There are different methods to dissociate ions for tandem MS, including CID, surface-induced dissociation (SID), infrared multiphoton dissociation (IRMPD), laser-induced ionization/dissociation (LID), ECD, electron induced dissociation (EID), EDD (electron detachment dissociation), ETD, metastable ion dissociation (MAD), etc. In 1984, a common nomenclature for sequence ions in mass spectra of peptides was first proposed by Roepstorff and Fohlman. (Figure 1.8) The cleavage of the C-N bonds generates $y$- and $b$- ions, which are the fragments where the positive charges are on the C- and N-terminus of the original peptide ion, respectively. And the C$_\alpha$-C and N-C$_\alpha$ bonds cleavages produce $a$-/x-ions and $c$-/z-ions.¹⁴²
In addition, there are four major scan modes using tandem MS, including PIS, product ion scan, neutral loss scan (NLS), and selected reaction monitoring (SRM). For PIS, only done with space based mass spectrometers like triple quadrupole (QqQ), the precursor masses are scanned in the first mass analyzer and undergo dissociation in the second mass analyzer; then the product ions are selected in the third mass analyzer. The PIS was used in the work shown in chapter 3.

1.7.1. Collision-Induced Dissociation (CID)

CID is one of the most popular activation methods in the tandem MS to obtain structural information from small to large molecules such as peptides and proteins, which refers to energetic collisions with a neutral target gas. The process of collisional activation followed by fragmentation of the ion is commonly called CID. The structural information obtained from CID of a peptide or protein ion is highly dependent on the experimental conditions used. There are three commonly used regimens for effecting CID of gas-phase biological ions, including “high-energy” CID (fast activation), “low-energy”
CID (slow activation), and ion-trapping CID (very slow activation), based on the three time regimens for activation methods used in tandem MS distinguished by McLuckey and Goeringer in 1997. Figure 1.9 is the typical ranges of activation times for a number of activation methods, with the collision-based methods of interest here highlighted.

**Figure 1.9.** Activation time scale (time between activating events, e.g., gas-phase collision or photon absorption) for a wide variety of methods used to dissociate biological ions, with the gas-phase collision-based methods highlighted in bold. Reproduced in part with permission from McLuckey, S. A.; Goeringer, D. E., J. Mass Spectrom. 1997, 32, 461-474. Copyright 1997, John Wiley and Sons.

The “high-energy” CID is typically used for CID affected at kilo-eV precursor ion kinetic energies, with a low target gas pressure, and only single or at most five collisions
can take place. Due to the high kinetic energy of the ions, the time scale for dissociation is usually on the order of a few microseconds. Sector-based instrumentations have been used for many CID experiments under this regimen. And the use of kilo-eV collisions are increased in tandem TOF instruments. The advantages of CID conducted at kilo-eV energy and low collision numbers include reproducibility and accessibility to side chain cleavages for isomeric and isobaric ion distinction.145

The “low-energy” CID generally refers to CID conducted in quadrupole or other multipole collisions cells and is characterized by collision energy less than 100 eV with target gas pressures chosen to allow a long (tens to hundreds collisions) intervals between individual collisions. The CID process time scale is on the order of a few hundred microseconds to a few milliseconds. The eV energy range is applied in tandem quadrupole instruments and hybrid instruments which combine quadrupole collision cells with other types of mass analyzers for precursor selection and/or product mass analysis.145

For ion-trapping CID, there are two forms of ion traps, including electrodynamic QITs and electrostatic/magnetic ICR ion traps, and the difference between them is the mechanism how they store ions and perform mass analysis. But their behaviors with respect to CID are very similar. CID in both instruments is characterized by hundreds of collisions at about tens of eV collision energies and is qualitatively similar to the “low-energy” quadrupole CID mentioned before. An important difference between “low-energy” CID and ion-trapping CID is that the trapping nature of these instruments
requires that very long time scales can be used to achieve very high dissociation
efficiency (75–100%) and to access very slow dissociation channels.\textsuperscript{145}

For the CID study of peptide and protein ions, $b$- and $y$- ions are the most useful
ion types for sequencing, there are other ion types (e.g., $c$, $x$, $z$, $a$, $d$, $w$, $v$, immonium
ions) used for spectral identification and database searches, but these are typically
observed at higher collision energies (e.g., “high-energy” CID).\textsuperscript{146-148} A marked
difference between “low-“ and “high-energy” CID is the abundant dissociation of amino
acid side chains forming $d$, $w$, and $v$ type ions (Scheme 1.1). Side-chain cleavages are
useful for distinction of isomeric and isobaric amino acids in peptide sequencing.\textsuperscript{146}
Another characteristic of the “high-energy” CID process is the abundance of immonium
ions in the low mass range region.\textsuperscript{147, 149} Immonium ions are used as sequence qualifiers,
i.e., good indicators of the presence or absence of a particular amino acid in the peptide
sequence.

\begin{center}
\textit{Scheme 1.1.} Structures of fragments arising from side-chain cleavage ($d$-, $v$-, and $w$-
ions), with Val as residue.\textsuperscript{146} Reproduced in part with permission from Wysocki, V. H.;
\end{center}
CID has been widely used in bottom-up, top-down, and shot gun proteomics, but it still has some limitations, such as the analysis of PTMs. When the ions with PTMs are collisionally activated, the PTMs are easy to lose, leading to the difficulty in localization of the PTMs.\textsuperscript{150} In addition, other side effects such as sequence scrambling causing misleading ions have been observed.\textsuperscript{151-152} In the field of structural biology, CID has shown boundaries in determining subunit topology for some quaternary noncovalent protein assemblies, due to significant protein subunit unfolding during activation (multicollision CID), yielding highly charge-asymmetric monomers, thus limiting the amount of relevant substructure information for such noncovalent complexes.\textsuperscript{153-155}

1.7.2. Electron-Capture Dissociation (ECD)

Gas-phase ion-electron and ion-ion reactions are gaining popularity for the activation of peptide ions in tandem MS. ECD,\textsuperscript{156} a gas phase ion fragmentation technique for structural elucidation in \textit{Fourier transform ion cyclotron resonance} mass spectrometry (FT-ICR-MS),\textsuperscript{156-157} which has been established for top-down sequencing. The first ECD-type mass spectra were observed by a UV photodissociation experiment. In the experiments, when the protein ions were trapped in an FT-ICR-MS cell and irradiated by 193 nm laser pulses, the charge reduction effect and $c$, $z$ fragmentation were observed.\textsuperscript{158} Later, the UV laser was replaced by a standard EI source (filament-based electron gun) and the ECD technique was born. ECD was first demonstrated by Roman Zubarev and Neil Kelleher when in Fred McLafferty's lab in 1998,\textsuperscript{156} and since then more and more groups are adopting this approach.
ECD spectra of the multiply charged protein ions usually produce the c- and z-type ions caused by the cleavage of the N-Cα bond,\textsuperscript{156} which has some unique features including the preferential cleavage of disulfide bonds in gaseous multiply-protonated proteins by low-energy electrons due to the high S-S affinity for H• atoms,\textsuperscript{6} location of PTMs,\textsuperscript{159-162} preservation of the non-covalent bonds,\textsuperscript{163} and extensive backbone cleavage.\textsuperscript{164} However, the mechanism of ECD is still under debate and both the Cornell mechanism and the UW mechanism have been proposed.\textsuperscript{165} The first one was originally formulated by the Cornell group,\textsuperscript{6,166} the electron was captured at a charge state, followed by hydrogen transfer to a proximate amide carbonyl forming an aminoketyl intermediate, and the resulting fragments are the residues of the peptide N-terminus and C-terminus, denoted as c and z ions, respectively (Scheme 1.2).\textsuperscript{165}

\begin{center}
\end{center}
The second mechanism was proposed independently by the groups of Simons and Tureček. The UW mechanism considers that the electron was first captured at the backbone carbonyl, and generated an anion-radical superbase, which can exothermically abstract a proton from a proximate proton donor group to generate fragile aminoketyl radicals, thus triggering backbone dissociation and undergoing facile N-C\textsubscript{\alpha} bond cleavage, generating the \( c \) and \( z \) ions (Scheme 1.3).\textsuperscript{165}

\begin{center}
\includegraphics[width=0.7\textwidth]{scheme1_3.png}
\end{center}

\textit{Scheme 1.3.} UW mechanism.\textsuperscript{165} Reproduced in part with permission from Chen, X. H.; Tureček, F., J. Am. Chem. Soc. 2006, 128, 12520-12530. Copyright 2006, ACS.

However, there are still some challenges for this ECD method. For example, sometimes it is difficult to separate the fragments cleaved by ECD due to the intramolecular interactions of the residues in large proteins. The non-covalent intramolecular bonding surviving ECD fragment is an obstacle to obtaining abundant
backbone fragmentation in the sequencing of proteins larger than 20 kDa. To solve this problem, activated-ion electron-capture dissociation (AI-ECD) was introduced. Protein ions are first preheated by collisions with intert gas molecules/atoms or by irradiation with IR laser light, and then ECD is applied. This preheating of protein ions can cause unfolding of the protein, and break the intramolecular interactions. This approach extended top-down mass spectrometry to proteins with masses greater than 200 kDa.

FT-ICR-MS is the most effective device for the proteins sequence analysis by ECD, although it is costly and requires more expertise than other mass spectrometers. The high mass resolving power of FT-ICR-MS instruments resolves overlapping fragment ions well to obtain confident peak assignment. Furthermore, a significant advantage obtained with ECD is that it can fragment protein ions, which can be utilized for analysis of large biomolecules. Bruker, IonSpec and Thermo produce the commercial FT-ICR-MS instruments used for ECD experiments without hardware modification. Also, a more efficient low-energy source, based on an indirectly-heated dispenser cathode can reduce the ECD experiment time from seconds to milliseconds, which improves the ECD efficiency. Therefore most top-down analyses were done with FT-ICR-MS instruments.

With the recent technological development, ECD has found a lot of applications in biomacromolecule research, including the characterization of intact proteins, nucleic acids, the characterization of protein conformation, the location of protein-ligand binding-site, and the protein-protein assembly characterization, ect.
In addition, negative-ion electron-capture dissociation (niECD)\textsuperscript{185} was recently developed to allow the localization of PTMs and \textit{de novo} sequencing for acidic peptides which have the improved ionization efficiency in the negative-ion mode compared to the positive-ion mode. The peptide anions can capture 3.5-6.5 eV electrons, generating the radical species with increased charge and yielding peptide backbone bond fragmentation analogous to that observed in cation ECD/ETD, including the preservation of PTMs and higher sequence coverage compared to CID.\textsuperscript{185}

1.7.3. Electron-Transfer Dissociation (ETD)

In addition to the prevalent ECD approach, ETD is also one of electron-induced fragmentation methods for fragmenting peptides. ETD was first invented by the Donald Hunt group,\textsuperscript{186-187} which shares many similarities with ECD, such as the preferential and extensive N-C\textsubscript{α} bond cleavage, the preservation of the labile modifications, and the ability to differentiate certain isomeric amino acid residue by secondary and radical-induced rearrangement.\textsuperscript{188-190} Furthermore, the mechanism of ETD is still debated like ECD. ETD is a method to fragment peptides utilizing ion/ion chemistry,\textsuperscript{187, 191-193} which dissociates peptides by transferring an electron from a radical anion to a protonated peptide, inducing the fragmentation of the peptide backbone, and then causing the cleavage of the N-C\textsubscript{α} bond (Scheme 1.4), the same as ECD, which also produces the \textit{c} and \textit{z}-type ions.\textsuperscript{188}
The differences between ETD and ECD are that ETD uses a RF quadrupole ion trapping device instead of an FT-ICR-MS for ion trapping and detection. The advantage is that the RF ion trap mass spectrometers are low-cost, low-maintenance, and widely accessible compared with the FT-ICR-MS. In addition, for ETD, some energy is consumed to overcome the electron affinity of the anion reagent, and the collisional cooling is afforded in a higher pressure ion trap. Thus, ETD is considered to be a “colder” fragmentation method than ECD, which can preserve PTMs, even sulfations, the most labile of PTMs, giving the sequence information.

Although ETD has advanced performance, the efficiency of top-down ETD fragmentation is reduced by noncovalent interactions. It is therefore necessary to disrupt the noncovalent interactions before the products ions are detected individually from the ETD event, which can be achieved by post-ETD collisional activation (ETcaD).
ETcaD is a supplemental collisional-activation dissociation (CAD) method targeting the nondissociated (intact) electron-transfer (ET) product species ([M +2H]^{+}) to the improvement of the ETD efficiency for peptide ions. In addition, the ETD efficiency can be improved by modifying the peptides with fixed charge tags.

ETD-based top-down MS has been used in-depth for characterization of PTMs in large peptides, small- and medium-sized proteins, and noncovalent protein complexes. To date, it has been applied for the structural analysis of intact 150 kDa monoclonal antibodies, immunoglobulins G (IgGs). In addition, the negative electron-transfer dissociation (NETD) is compatible with fragmenting peptide and protein ions with the backbone cleavage at the C-C_α bond, resulting in a- and x-type product ions.
Chapter 2: The Study of Highly Selective, Rapid and Efficient Thiol Derivatization Using Selenium Reagents by Mass Spectrometry


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2.1. Introduction

Biological thiols, such as glutathione (GSH) and thiol proteins, are critical physiological components found in animal tissues and fluids and involved in an extremely important cellular functions.\(^{204-205}\) In the past decades, chemical characterization of biological thiols has attracted significant attention. Various measurement methods have been developed including UV,\(^{206}\) fluorescence (FL) spectroscopy,\(^{207-209}\) and MS,\(^{210-215}\) in which the derivatization of thiol groups with a suitable chemical reagent is necessary for increasing thiol stability and improving detection selectivity.

While spectroscopic methods such as FL are very useful for detection and imaging of thiol compounds, MS can provide molecular weight and structural information. The inherent sensitivity and chemical specificity offered by MS\(^{216-219}\) is essential in further identifying biological thiols and investigating their physiological functions on a molecular level. Also, a number of excellent MS studies of thiols and disulfides of proteins/peptides based on the novel ion chemistry have been reported.\(^{213-214,220-223}\)
At present, MS labeling strategies are mainly based on three types of chemical reactions. Nucleophilic substitution such as using heptafluorobutyl chloroformate\textsuperscript{224} and iodoacetamide is commonly used for the analysis of protein tryptic digest;\textsuperscript{225} however, these reagents are not specific for thiol compounds, that is, they can also couple with amino/hydroxyl groups. The second approach involves Michael-addition of thiols onto unsaturated C=C bonds such as using acrylate\textsuperscript{226} or maleimide derivatives as common labeling agents.\textsuperscript{227} These reagents have selectivity towards thiols and have been often used in practical thiol analysis. However, the Michael-addition product is irreversible, which cannot allow enriching and purifying analyte compounds from complex matrices.\textsuperscript{228} The third approach involves thiol exchange reaction such as using 5,5'-dithiobis(2-nitrobenzoic acid) known as Ellman’s reagent;\textsuperscript{229-230} a large excess amount of the disulfide reagent is necessary to ensure complete derivatization of all thiols in the sample. As a result, the newly formed disulfides in the reaction can possibly further react with residual thiols of the target molecule to form undesirable disulfides. For example, Udgaonkar \textit{et al.} used Ellman’s reagent in a 100-fold molar excess to label the thiol protein based on the exchange reaction in the study of the cooperativity of a fast protein folding reaction by MS.\textsuperscript{230}

Besides the problems mentioned above, these derivatization reactions have other limitations, including a long reaction time, low conversion yield or more than one possible site for tagging. Therefore, new derivatization chemistry suitable for MS detection of thiol, particularly with high thiol selectivity, fast reaction speed, good
reaction reversibility and high conversion yield, is still needed for the structural analysis of peptides and proteins in biological samples.

Because selenium is an essential element \textit{in vivo}^{231} and a key component of selenoproteins known as antioxidant enzymes, selenium chemistry has recently attracted increasing attention. Ebselen, 2-phenyl-1,2-benzisoselenazol-3(2H)-one (Reagent 1, Scheme 2.1), has been considered as a mimetic of GPx^{232-235} and an anti-inflammatory drug since being found.\textsuperscript{13, 15}

The mechanism for ebselen-catalyzed thiol oxidation has been proposed:\textsuperscript{12, 16} the Se-N bond of ebselen is cleaved by thiol RSH to produce the corresponding selenenyl sulfides Se-S, which further react with excess thiol RSH to produce selenol and disulfide compound RSSR. However, Mugesh \textit{et al.} recently reported that ebselen as a GPx-like redox catalyst is indeed inactive and the reaction of ebselen with PhSH only produces the Se-S product, not disulfide PhSSPh, even with excess amount of PhSH.\textsuperscript{16}

Very recently, based on this novel selenium chemistry, Tang’s group synthesized fluorescent probes carrying Se-N bonds and used them for detecting and imaging thiols in living cells\textsuperscript{209, 236} by FL. It was shown that the FL probes capture cellular thiols selectively in the presence of diverse species such as inorganic metal ions, DA, histamine, \textit{L}-adrenaline, \textit{etc}. Following that, Zhang \textit{et al.} used piazselenole containing Se-N bond to probe physiological thiols based on electrochemical reactions.\textsuperscript{237} Highly specific derivatization of thiols using compounds containing Se-N bonds makes them promising candidates to derivatize peptides and proteins for subsequent MS characterization. Although the analysis of protein thiols and disulfide bonds is important
in proteomics applications, the selenium chemistry received surprisingly limited attention. Here we present a systematic study of derivatization of amino acids, peptides and proteins using Se-N containing reagents and examine the utility of this chemistry for analytical applications involving mass spectrometry.

In this study, a series of reactions of two Se-N containing reagents including ebselen and NPSP (Reagent 2, Scheme 2.1), with various biological thiol compounds such as cysteine, reduced peptide GSH and β-lactoglobulin A protein were carried out. In Scheme 2.1, the Se-N bonds in reagents 1 and 2 are cleaved by thiols to form new Se-S bonds as shown in products 3 and 4, respectively (Scheme 2.1). Our experimental results showed that this thiol derivatization reaction is highly selective, rapid, reversible and efficient (quantitative in some cases) at room temperature. Based on these revealed features of the reaction, associated analytical applications were explored, such as the selective identification of thiol-containing peptides from mixtures, and measurement of the number of cysteine residues of proteins. In addition, the dissociation behaviors of the derivatized protein/peptide ions were also examined.

2.2. Experimental Section

2.2.1. Chemicals and Materials

Twenty amino acids, angiotensin II human (FW: 1046 Da), bradykinin acetate (FW: 1060 Da) and MRFA (Met-Arg-Phe-Ala) acetate salt (FW: 523 Da), β-lactoglobulin A from bovine milk (MW: 18369 Da), tris(2-carboxyethyl)phosphine hydrochloride (TCEP), DTT, TPCK-treated trypsin from bovine pancreas (MW: ~23.8 KDa), ammonium bicarbonate, 1,4-benzoquinone and NPSP were purchased from Sigma-Aldrich (St. Louis, MO). GSH (reduced form, MW: 307 Da) and ebselen were obtained from TCI America (Tokyo, Japan) and Calbiochem (Cincinnati, OH), respectively. HPLC-grade methanol and acetonitrile from GFS Chemicals (Columbus, OH) and Sigma-Aldrich (St. Louis, MO) were used and acetic acid was purchased from Fisher Scientific (Pittsburgh, PA). The de-ionized water used for sample preparation was
obtained using a Nanopure Diamond Barnstead purification system (Barnstead International, Dubuque, IA).

2.2.2. Instrumentation

ESI of samples was performed using a Thermo Finnigan LCQ DECA Mass Spectrometer (San Jose, CA) or a hybrid triple-quadrupole-linear ion trap mass spectrometer (Q-trap 2000; Applied Biosystems/MDS SCIEX, Concord, Canada). The sample injection flow rate was 10 µL/min. A high voltage of +5 kV was applied to spray probe for the positive ion mode. For Thermo Finnigan LCQ DECA Mass Spectrometer, the optimized heated transfer capillary tube temperature was 150 °C. CID was used for further structural confirmation of the product ions. Data acquisition was performed using Xcalibur™ (Version 2.0.7, Thermo Scientific, CA, USA). For Q-trap 2000 Mass Spectrometer, the mass spectrometer curtain gas (N₂) was kept as 20 (manufacturer’s units) and declustering potential was set at 10 V. CID was carried out to provide ion structural information using enhanced product ion scan mode. PIS was used to select reaction products by monitoring the characteristic fragment ions and N₂ was used as collision gas. Data acquisition was performed using the Analyst software (Version 1.4.2, Applied Biosystems/MDS SCIEX, Concord, Canada). Deconvolution of mass spectra was carried out using Mag-Tran 1.03b2 software (Amgen Inc., Thousand Oaks, CA) written based on the ZScore algorithm.²³⁹

High resolution MS

High-resolution LTQ-Orbitrap mass spectrometer (Thermo Electron, Bremen, Germany) with a modified nano-electrospray ionization (nano-ESI) source was used for
collecting high resolution data. The system was operated in the positive ion mode with a resolving power of 60,000 at m/z 400. *Molecular Weight Calculator* (http://ncrr.pnl.gov/software/) was used to simulate isotope distribution for thiol-derivatized products.

2.2.3. Method

*Protein reduction*

175 µL of 0.1 mM β-lactoglobulin A in methanol/water (1:1 by volume) containing 2% acetic acid and 17.5 µL of 50 mM TCEP in 20 mM ammonium bicarbonate aqueous solution were mixed resulting in the molar ratio of 1:50 (protein:TCEP). The protein was reduced by TCEP for 3.5 h at room temperature. Then Millipore-ZipTip® Pipette Tips were used to remove TCEP and ammonium bicarbonate via desalting.

2.3. Results and Discussion

2.3.1. Reaction Selectivity

In this experiment, the selectivity of the derivatization reaction was examined first using amino acids as reaction substrates and using ebselen as a reagent. It is found that ebselen reacts exclusively with side chains of cysteine residues that contain a free thiol. In Figure 2.1, the ESI-MS spectrum displays the reaction of ebselen (10 µM) with cysteine (5 µM) in the presence of methionine, tryptophan and valine (5 µM for each) in methanol/water (1:1 by volume) containing 0.5 % acetic acid. A dominant peak at m/z 397 corresponds to the reaction product between ebselen and cysteine. The inset is the
zoom-in area for the reaction product ion C_{16}H_{17}O_{3}N_{2}SSe (m/z 397) (bottom) in comparison with the calculated product isotope distribution (top). Exact match is observed, confirming the peak assignment. The characteristic isotope distribution helps to identify products of the derivatization reaction. Upon CID, the ion at m/z 397 fragments into m/z 379, 353 and 276, by losses of one water molecule, one carbon dioxide, and one cysteine, respectively, confirming the product ion structure and the covalent nature of the newly formed bond. In addition, peaks at m/z 118, 122, 150, 205 and 276 are seen in the Figure 2.1, corresponding to the protonated valine, cysteine, methionine, tryptophan, and ebselen, respectively. We also tested the reaction of ebselen with cysteine in the presence of 19 other natural amino acids. Again, only the reaction product of ebselen and cysteine was observed, demonstrating that ebselen has exclusive specificity towards cysteine derivatization. Similarly, it was also found that another selenium reagent 2, has similar selectivity for cysteine in the presence of other amino acids. Furthermore, in observation, the derivatization reaction takes place rapidly and is completed in seconds at room temperature.\textsuperscript{19,235} This is an advantage for shortening analysis time in real-world applications, particularly for high throughput proteomics analysis.
Figure 2.1. ESI-MS spectrum of the selective reaction of ebselen with cysteine in the presence of methionine, tryptophan and valine. The insets show the agreement of the simulated product peak isotopic distribution (top) with the recorded reaction product ion of m/z 397 (bottom). Reprinted (Adapted) with permission from Xu, K.; Zhang, Y.; Tang, B.; Laskin, J.; Roach, P. J.; Chen, H. Anal. Chem. 2010, 82, 6926–6932. Copyright 2010 American Chemical Society.

2.3.2. Reactions with Peptide Thiols

In addition to reacting with amino acid cysteine, both ebselen and the selenium reagent 2 were also tested with thiol peptides. Figure 2.2a is ESI-MS spectrum showing the reaction of GSH (0.1 mM, the structure of this tripeptide is in the Figure inset) with ebselen (0.2 mM) in acetonitrile/water (1:1 by volume) containing 1% acetic acid. As expected, the reaction product resulting from the reaction of ebselen with GSH (m/z 583) was observed. As shown in the CID spectrum of m/z 583 (Figure 2.2b), water loss (the
formation of m/z 565), backbone cleavages (the formation of m/z 508 and m/z 454), and the protonated GSH (m/z 308) and ebselen (m/z 276) were seen, confirming the product ion structure and the addition of peptide thiol onto ebselen (step a of the eq.1, Scheme 2.1). It can be seen that, besides backbone cleavages, Se-S bond cleavage was also observed leading to the formation of the fragment ion of m/z 276 and 308, which may be driven by the re-formation of the five-membered ring of ebselen as assisted via the nucleophilic attack of selenium by the adjacent amide nitrogen of the ebselen tag (the inset of Figure 2.2b).

Based on this hypothesis, the CID behavior of the derivatized peptide ions could be tuned, simply by removing the adjacent amide group of the derivatizing selenium reagent. The reagent 2 was also tested. Figure 2.2c is ESI-MS spectrum showing the reaction of GSH (0.1 mM) with reagent 2 (0.5 mM) in acetonitrile/water (1:1 by volume) containing 1% acetic acid, in which the reaction product (m/z 464) is seen. As shown in eq. 2 (Scheme 2.1), the derivatized thiol tag does not contain amide group as the reagent 2 is split during the reaction. Indeed, the CID of the product ion at m/z 464 (Figure 2.2d) does not lead to the cleavage of Se-S bond. Instead, it shows the loss of one water molecule (the formation of m/z 446), the backbone cleavage (the formation of m/z 389, 335 and 318) and the cleavage of S-C bond (the formation of m/z 189). In this case, the selenium tag is stable, surviving the CID process, which would be valuable in top-down proteomics studies (e.g., for pinpointing thiol location). In Figure 2.2c, one peak at m/z 191 corresponding to the protonated PhSe(=O)OH probably arose from the impurity of the reagent 2 used, which was in technical grade.
Figure 2.2. a) ESI-MS spectrum of the reaction of GSH with ebselen; b) CID MS² spectrum of ebselen derivatized GSH product ion (m/z 583); c) ESI-MS spectrum showing the reaction of GSH with reagent 2; d) CID MS² spectrum of the product ion of GSH derivatized by reagent 2 (m/z 464); e) ESI-MS spectrum of the selective reaction of ebselen with GSH in the presence of angiotensin II, bradykinin and MRFA; f) PIS based on the loss of the characteristic fragment ion of m/z 276 for the selective detection of GSH. Reprinted (Adapted) with permission from Xu, K.; Zhang, Y.; Tang, B.; Laskin, J.; Roach, P. J.; Chen, H. Anal. Chem. 2010, 82, 6926–6932. Copyright 2010 American Chemical Society.
In the case of labeling by ebselen, the facile cleavage of Se-S could be useful in selective detection of cysteine containing peptides from mixtures, based on the resulting characteristic fragment ion of $m/z$ 276. In this experiment, selective reaction of ebselen (0.1 mM) with GSH (50 µM) in the presence of other peptides of angiotensin II, bradykinin and MRFA (50 µM for each) in methanol/water (1:1 by volume) containing 1% acetic acid was carried out. In Figure 2.2e, the ESI-MS spectrum displays that the reaction of ebselen with GSH is highly selective, only a product ($m/z$ 583) resulting from the reaction of ebselen with GSH was detected. However, the spectrum has multiple peaks and appears complicated. PIS was applied to ions generated by ESI of the same reaction mixture and a much clearer spectrum containing only $m/z$ 276 (the protonated ebselen) and 583 (the protonated ion of ebselen-derivatized GSH) are seen (Figure 2.2f), in which cysteine-containing peptide GSH can be rapidly identified.

Reversibility of a derivatization reaction is very important as it allows enrichment and purification of analyte compounds from complex matrices. However, as already mentioned above, commonly used thiol derivatization reactions such as nucleophilic substitution and Michael-addition are irreversible, limiting their analytical utilities. In this study, the reversibility of the thiol derivatization reaction was investigated by using DTT as reductant. Figure 2.3 is the ESI-MS spectrum revealing the recovery of GSH ($m/z$ 308) 30 min after adding DTT (0.5 mM) to the reaction mixture containing GSH (5 µM) and ebselen (10 µM). In the spectrum, one can see that $m/z$ 583, the protonated molecule of the ebselen-derivatized GSH, disappears upon reaction with DTT. In addition, $m/z$ 553 arose, probably indicating that the resulting reduction product, ebselen selenol
(Compound 5, Scheme 2.1), was air sensitive and readily oxidized by O₂ in the ambient environment into a more stable diselenide Se-Se products.¹⁹,²⁴¹ The CID spectrum of \( m/z \) 553 shows the loss of PhNH₂ and the formation of the protonated ebselen (\( m/z \) 276), in agreement with the peak assignment. The \( m/z \) 575 corresponds to the sodiated molecule of the diselenide. These results clearly demonstrate the excellent reversibility of the thiol derivatization. In combination with the extraordinary selectivity of the reaction, this reactivity can be used in a variety of applications focused on enrichment and purification of biological thiols from cells and tissues.

Figure 2.3. ESI-MS spectrum showing the recovery of unmodified GSH 30 min after adding DTT to the reaction mixture of GSH and ebselen. Reprinted (Adapted) with permission from Xu, K.; Zhang, Y.; Tang, B.; Laskin, J.; Roach, P. J.; Chen, H. Anal. Chem. 2010, 82, 6926–6932. Copyright 2010 American Chemical Society.
2.3.3. Reaction with Protein Thiols

Selenium derivatizing reagents were used for identification of free cysteine residues in proteins due to the high selectivity for thiol groups. β-Lactoglobulin A used as a model system in this study (162 amino acid residues) contains two disulfide bridges (Cys\textsuperscript{66}-Cys\textsuperscript{160} and Cys\textsuperscript{106}-Cys\textsuperscript{119}) and one free Cys\textsuperscript{121}.\textsuperscript{242}

Figure 2.4a is the ESI-MS spectrum of β-lactoglobulin A (5 µM) in methanol/water (1:1 by volume) containing 1% acetic acid. Multiply charged ions of β-lactoglobulin A are detected and deconvolution of the mass spectrum provides the protein mass of 18,364 Da (the inset of Figure 2.4a). In Figure 2.4b, ESI-MS spectrum shows the reaction of β-lactoglobulin A (5 µM) and ebselen (10 µM) in methanol/water (1:1 by volume) containing 1% acetic acid. Deconvolution of the mass spectrum of ebselen-derivatized β-lactoglobulin A provides a mass of 18,639 Da (the inset of Figure 2.4b). After derivatization of β-lactoglobulin A by ebselen, a mass gain of 275 Da was observed, indicating addition of one ebselen (MW 275 Da) to the sole free cysteine residue of the protein. Furthermore, only the ions of the ebselen-derivatized β-lactoglobulin A were seen in the spectrum, (Figure 2.4b) suggesting that all of the protein was reacted with ebselen and quantitative conversion yield was obtained.

High-resolution mass analysis using Orbitrap MS was used for unambiguous identification of the derivatization product. Figure 2.4c is the Orbitrap MS spectrum showing the exact match between the simulated (shown in blue discrete line) and experimentally observed (shown in black solid line) isotope distribution of +16 charge state of the derivatized β-lactoglobulin A ions. Furthermore, we performed tandem MS
analysis using LTQ-Orbitrap. High resolution Orbitrap CID MS² spectrum of +16 derivatized β-lactoglobulin A ions (m/z 1166) contains fragment ions y'_{139}^{+12} and y'_{130}^{+11} that carry the selenium tags. This suggests that the derivatization site is located on the last 130 amino acid residues of the protein, in agreement with the position of the free cysteine residue in β-lactoglobulin A (Cys^{121}). These results indicate the potential use of the selenium derivatization strategy in top-down proteomics studies.

In the case of β-lactoglobulin A derivatization, ebselen was compared with one commonly used thiol tagging reagent, 1,4-benzoquinone. It was found by ESI-MS that the protein can be fully reacted with ebselen 30 s after mixing (The spectrum is the same as the one in Figure 2.4b). By contrast, under the same conditions (e.g., concentrations and solvents used for the reaction were kept the same), only ca. 30% protein was derivatized with 1,4-benzoquinone 30 s after mixing the protein and the reagent. This indicates that the labeling using selenium reagents is much faster and more efficient than using the Michael-addition reagents such as 1,4-benzoquinone. The result suggests that the selenium chemistry would be quite useful in high throughput analysis of thiol containing proteins.
High selectivity and efficiency of the selenium chemistry investigated in this study makes it useful in identification of the number of free and bound thiol groups in proteins, which is of importance in the protein structural analysis. The reaction of intact protein β-lactoglobulin A with ebselen as described above shows that the protein has only one free cysteine residue. We further examined the derivatization reaction with reduced...
protein. In the experiment, the $\beta$-lactoglobulin A was first reduced by TCEP which is known to be more stable and effective to reduce disulfide bonds than DTT.$^{243}$ After reduction and removal of the excess amount of TECP, reagent ebselen was added to the protein solution for thiol derivatization. In Figure 2.5, the reduced $\beta$-lactoglobulin A containing three selenium tags has the high relative abundance. It is likely that the reduction of disulfide bond of Cys$^{66}$-Cys$^{160}$ is easier than the other Cys$^{106}$-Cys$^{119}$ bond leading to reduced protein mainly with three free thiols.$^{244}$ Another possible reason is that the reduced protein is partially folded so that it is not easy for the relatively large ebselen reagent to access all of the free thiols. Nevertheless, in Figure 2.5, the multiply charged ions of fully modified proteins with five selenium tags were clearly observed, suggesting that the reduced protein has a maximum of five free cysteine residues. This indicates that the four additional free cysteine residues result from reduction and the protein has two disulfide bonds prior to reduction, which is exactly in agreement with the known structure of $\beta$-lactoglobulin A as mentioned before. Thus, it is possible to identify the number of free cysteine, total cysteine residues and disulfide linkages in proteins, using a simple approach based on the selenium chemistry.
Figure 2.5. ESI spectrum showing the derivatized protein resulting from reacting the reduced β-lactoglobulin A with ebselen. The superscript in the charge number shows the number of selenium tags added to the protein after derivatization. Reprinted (Adapted) with permission from Xu, K.; Zhang, Y.; Tang, B.; Laskin, J.; Roach, P. J.; Chen, H. Anal. Chem. 2010, 82, 6926–6932. Copyright 2010 American Chemical Society.
2.4. Conclusions

In summary, ebselen and NPSP, as Se-N bond containing compounds, are excellent labeling reagents for characterization of thiol-containing compounds by mass spectrometry. In this study we examined a series of reactions of these two selenium reagents with amino acids, peptides and proteins. Our study reveals that the thiol derivatization reaction is highly selective, rapid, reversible and efficient (quantitative in the case of β-lactoglobulin A derivatization by ebselen), which is of high value in proteomics research. In comparison to the well-known Michael-addition reactions used for thiol tagging, selenium reagents appear to be more efficient and faster. Analytical applications stemming from this investigation include: i) fast screening of peptides/proteins containing free cysteine residues from complex mixtures; ii) identification of the number of free and bound thiols of proteins and their locations using MS/MS experiments. Given the significance of thiols in life and the important reaction features uncovered, it is expected that there will be many novel MS applications based on the powerful selenium chemistry reported in this study.
Chapter 3: Tandem MS Analysis of Selenamide-Derivatized Peptide Ions

3.1. Introduction

Biological thiols such as GSH and thiol proteins are critical physiological components found in animal tissues and involve in a plethora of important cellular functions.\textsuperscript{204} Due to the inherent sensitivity and chemical specificity (e.g., molecular weight and structural information), MS\textsuperscript{210-211} has attracted significant attention in characterization of biological thiols. A number of elegant MS studies of thiols and disulfides of proteins/peptides based on the novel ion chemistry have been reported.\textsuperscript{213, 221, 245-248} For MS analysis of thiol proteins/peptides, the derivatization of thiol groups with a suitable chemical reagent is often necessary for increasing thiol stability and improving detection selectivity or for enrichment and purification purposes. Furthermore, MS/MS can benefit from the modification of thiols. For instance, \textit{N,N}-dimethyl-2-chloroethylamine\textsuperscript{249} was used to increase the charge state of cysteine-containing toxin ions to meet the requirement for performing ETD.\textsuperscript{197} In addition, it has been shown that thiol derivatization using (3-acrylamidopropyl)-trimethyl ammonium chloride significantly improves the percent fragmentation and sequence coverage for peptide ions upon ETD.\textsuperscript{250} Remarkably, quinone-modified cysteine was shown to facilitate site-selective cleavage by photodissociation.\textsuperscript{251}
Traditional thiol labeling strategies are mainly based on nucleophilic substitution (e.g., using heptafluorobutyl chloroformate\textsuperscript{224} and iodoacetamide\textsuperscript{225}), Michael-addition with unsaturated C=C bonds (e.g., using acrylate\textsuperscript{252} or maleimide derivatives\textsuperscript{227}), or thiol exchange reaction (e.g., using the Ellman’s reagent\textsuperscript{229-230}). However, these protocols have some limitations such as low reaction selectivity, long reaction time, or low conversion yield. In chapter 2, I introduced a new methodology to label biological thiols using selenamide reagents for MS analysis, which involves the cleavage of Se-N bond by thiol to form a new Se-S bond.\textsuperscript{1} The data show that the reaction is highly selective, rapid, reversible and efficient. In order to better implement this new thiol derivatization reaction for protein/peptide analysis by mass spectrometry, it is therefore necessary to well understand the tandem MS/MS dissociation behavior of derivatized protein/peptide ions, which is the motivation and focus of this study. On the other hand, due to the significance of ECD\textsuperscript{156} and ETD\textsuperscript{197} in top-down proteomics research, their mechanisms arouse lots of attention and are still under debate.\textsuperscript{156, 165, 167} One of the approaches for mechanism elucidation is to introduce various tags with different electron or proton affinities to model peptides and then to test the effects of the tags on the dissociation.\textsuperscript{253-256} In consideration that the preparation of selenamide-derivatized proteins/peptides is handy as mentioned above and that the formed Se-S bond is structurally similar to the S-S bond, ETD of the derivatized peptide ions was also investigated with aim to provide some new insight to the electron-based ion dissociation mechanism.

In this study, several free cysteine-containing peptides were chosen as examples and systemically investigated their ion dissociation pathways after derivatization by
selenamide reagents such as ebselen and NPSP, under CID (in both positive and negative ion modes) and ETD conditions. For the ebselen tag, it appears to be easily lost upon CID, probably due to the presence of an adjacent amide group in the tag, consistent with previous observation. The phenylselenenyl tag from NPSP derivatization can survive in the CID process, leading to the formation of a series of $b/y$ ions from backbone cleavage of peptides in the positive ion mode. Interestingly, both tags can be removed upon CID in the negative ion mode, which is similar to the known CID behavior of disulfide bonds. During ETD of derivatized peptide ions, selenium tag loss is dominant while the backbone cleavage is suppressed. Similar phenomenon was also observed for alkali metal-containing peptide ions and these results can be explained by the UW mechanism proposed for ECD dissociation.

3.2. Experimental Section

3.2.1. Chemicals and Materials

Peptides HCKFWW (MW 906.1 Da), NRCSQGSCWN (MW 1154.3 Da), bovine pancreas insulin (MW 5733.5 Da), $\alpha$-lactalbumin from bovine milk (type III, calcium depleted, $\geq 85\%$, MW 14178 Da), $\beta$-lactoglobulin A from bovine milk (MW 18369 Da), lysozyme from chicken egg white (MW 14300 Da), pepsin from porcine gastric mucosa (MW $\sim 35$ KDa), NPSP, TCEP and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (St. Louis, MO). Ebselen was purchased from Calbiochem (Cincinnati, OH). HPLC-grade methanol from GFS Chemicals (Columbus, OH) and acetonitrile from Sigma-Aldrich (St. Louis, MO) were used and acetic acid was purchased from Fisher Scientific (Pittsburgh, PA). The de-ionized water used for sample preparation was
obtained using a Nanopure Diamond Barnstead purification system (Barnstead International, Dubuque, IA).

3.2.2. Instrumentation

High-resolution LTQ-Orbitrap mass spectrometer (Thermo Electron, Bremen, Germany) with a modified nano-ESI source was used for collecting CID and ETD data of derivatized peptide ions. All ETD experiments were conducted with fluoranthene as the radical anion electron-transfer reagent. The PIS was performed using a hybrid triple-quadrupole-linear ion trap mass spectrometer (Q-trap 2000; Applied Biosystems/MDS SCIEX, Concord, Canada). PIS was used to screen the ebselen tag-containing precursor ions based on the formation of the characteristic fragment ion of m/z 276 (i.e., the protonated ebselen) and N₂ was used as collision gas.

3.2.3. Method

**Preparation of selenamide-derivatized proteins/peptides**

Peptide thiols were derivatized by reaction with 5~10 time excess amount of ebselen or NPSP in either methanol/water (1:1 by volume) containing 1% acetic acid or acetonitrile/water (1:1 by volume) containing 1% acetic acid at room temperature.

The derivatized insulin chain B was obtained via reacting reduced insulin with the selenamide reagents. First TCEP in 20 mM ammonium bicarbonate aqueous solution was added to insulin in acetonitrile/water (1:1 by volume) containing 0.1% TFA in the molar ratio of 1:50 (protein/TCEP) for 3.5 h at room temperature. Then Millipore-ZipTip® Pipette Tips were used to remove TCEP and ammonium bicarbonate via desalting. After
that, excess amount of ebselen (ebselen: protein= 6:1) or NPSP (NPSP: protein=11:1) were added to derivatize the reduced insulin.

**Derivatization of insulin peptic digest and protein mixture**

Digestion of insulin was performed by incubating insulin and pepsin at a molar ratio of 50: 1 in water containing 1% acetic acid at 37 °C for 11 h. Then TCEP was added to the peptic digested insulin in the molar ratio of 1:20 (protein:TCEP) for several days at room temperature. After that, ebselen (ebselen: protein=20:1) was added to derivatize the insulin digest.

Ebselen (50 µM) was reacted with β-lactoglobulin A (10 µM) in the presence of α-lactalbumin, lysozyme, and insulin (10 µM for each) in methanol/water (1:1 by volume) containing 1% acetic acid for 30 s.

3.3. Results and Discussion

Various biological thiols, such as peptide HCKFWW containing one free cysteine, NRCSQGSCWN having two free cysteines, and insulin chain B carrying two cysteines, were chosen for examination in this study. Following derivatization by ebselen or NPSP, the sample was directly ionized by ESI and it was found that the number of tags in the main derivatization products agrees with the number of the free cysteines of the peptide substrates reacted. Then, the derivatized peptide ions were mass-selected for tandem MS analysis.
3.3.1. CID MS/MS of the Positive Ions of Selenamide-Derivatized Peptides

Positive ion CID MS/MS of ebselen-derivatized peptide ions was first carried out. Figures 3.1a-c show the CID MS/MS mass spectra of doubly charged $[\text{HC}^{*}\text{KF}^{+}]^{2+}$ ($m/z$ 591.0; see its structure in Figure 3.1a; the asterisk “*” denotes one ebselen tag on the peptide cysteine group and such a denotation is applicable throughout the text), doubly charged $[\text{NRC}^{*}\text{SQGSC}^{*}\text{WN}^{+}]^{2+}$ ($m/z$ 852.8; structure is in Figure 3.1b), and triply charged $[\text{FVNQHLC}^{*}\text{GS}^{*}\text{HLVEALYLVC}^{*}\text{GERGFFYPKA}^{+}]^{3+}$ ($m/z$ 1317.0; structure is in Figure 3.1c), respectively. Upon CID, facile Se-S bond cleavage occurs to $m/z$ 591.0 (Figure 3.1a), leading to the formation of singly and doubly charged ions of HCKFWW ($m/z$ 906.5 and 453.8) via the loss of one protonated ebselen and one neutral ebselen, respectively. Indeed, the protonated ebselen ($m/z$ 276.1) was also observed in Figure 3.1a. This process might be driven by the re-formation of the five-membered ring of ebselen as assisted via the nucleophilic attack of selenium by the adjacent amide nitrogen in the ebselen tag.

The result is consistent with our previous observation in the case of ebselen-derivatized GSH, a tripeptide with one cysteine residue. In Scheme 3.1, the cleavage of Se-S bond during CID takes place in two pathways: one is to lose neutral ebselen (pathway a, eq. 1) and the other is to lose the protonated ebselen (pathway b, eq. 1). Also, in comparison to the Se-S bond dissociation, the backbone cleavage to $b/y$ ions is negligible in this case and only a tiny peak of $y_4$ ion was seen. The facile Se-S bond cleavage appears to be a general trend and was also observed during CID of the other two
peptides containing two ebselen tags (Figures 3.1b and c). In Figure 3.1b, two major fragment ions of \( m/z \) 1429.4 and 715.2 were observed, corresponding to the singly and doubly charged ions of NRCSQGSCWN with one ebselen tag, respectively, arising from the losses of one protonated ebselen and one neutral ebselen from \([\text{NRC*SQGSC*WN+2H}]^{2+}\) (\( m/z \) 852.8). The \( m/z \) 1429.4 was also further examined by MS\(^3\), which shows that the ebselen tag is either on its Cys\(^3\) or on the Cys\(^8\) residue. It indicates that there is no regioselectivity for the dissociation of its precursor ion of \( m/z \) 852.8 with regard to ebselen tag loss. Another fragment ion of \( m/z \) 1154.4 (Figure 3.1b), the protonated NRCSQGSCWN originating from \( m/z \) 1429.4 dissociation by further loss of the ebselen tag, was detected, along with the protonated ebselen and \( b/y \) fragment ions such as \( b_4^* \), \( b_5^* \), \( b_6^* \), \( b_7^* \) and \( y_9^{**} \) with low abundances resulting from backbone cleavage (again, the asterisk indicates one ebselen tag and the double asterisks indicate two ebselen tags).

In the case of insulin chain B, \( m/z \) 1837.9 and 1225.7 representing the doubly and triply charged ions of chain B with one ebselen tag were produced upon CID, again, via the loss of one protonated ebselen and one neutral ebselen from the precursor ion of \( m/z \) 1317.0 upon CID. Also, the doubly and triply charged ions of the free chain B were seen at \( m/z \) 1700.5 and 1134.3, respectively, as a result of secondary dissociation of \( m/z \) 1837.9 and 1225.7 by further loss of the remaining ebselen tag. These results above clearly suggest that ebselen-derivatized peptide cations preferentially undergo Se-S bond cleavage during CID via losses of both neutral and protonated ebselen (the exception of no protonated ebselen observed in Figure 3.1c is probably due to the low mass cut-off in
the linear ion trap of the LTQ-Orbitrap used). Both the formation of the characteristic fragment ion of \( m/z \) 276 and the facile loss of neutral ebselen from the CID of derivatized thiol peptide ions would be useful to identify thiol-containing peptides from mixtures such as protein digests, using either PIS or NLS, and the former application is demonstrated below.

Figure 3.1. Positive ion CID MS/MS mass spectra of (a) \([\text{HC}^+\text{KFWW}+2\text{H}]^{2+}\) (\( m/z \) 591.0); (b) \([\text{NRC}^+\text{SQGSC}^+\text{WN}+2\text{H}]^{2+}\) (\( m/z \) 852.8); (c) \([\text{FVNQHLC}^+\text{GSHLVEALYLVC}^+\text{GERGFFYTPKA}+3\text{H}]^{3+}\) (\( m/z \) 1317.0); (d) \([\text{HC}^+\text{KFWW}+2\text{H}]^{2+}\) (\( m/z \) 531.8); (e) \([\text{NRC}^+\text{SQGSC}^+\text{WN}+2\text{H}]^{2+}\) (\( m/z \) 733.8); (f) \([\text{FVNQHLC}^+\text{GSHLVEALYLVC}^+\text{GERGFFYTPKA}+3\text{H}]^{3+}\) (\( m/z \) 1238.0). Reprinted (Adapted) from Zhang, Y.; Zhang, H.; Cui, W.; Chen, H. J. Am. Soc. Mass Spectrom., 2011, 22, 1610-1621. with kind permission from Springer Science and Business Media.
Scheme 3.1. CID MS/MS processes for \([\text{HC*KFWW+2H}^2]^+\) (eq. 1) and \([\text{HC*KFWW-H}^-]\) (eq. 2). Reprinted (Adapted) from Zhang, Y.; Zhang, H.; Cui, W.; Chen, H. *J. Am. Soc. Mass Spectrom.*, 2011, 22, 1610-1621. with kind permission from Springer Science and Business Media.

As a demonstration, insulin (its sequence is in the inset of Figure 3.2b) was digested by pepsin and then reduced by TCEP. The reduced protein digest mixture was derivatized via the selective reaction with ebselen and then subject to ESI-MS analysis. In Figure 3.2a, the ESI-MS spectrum shows that a series of ebselen-derivatized thiol peptide ions \([\text{FVNQHLC*GSHL+3H}^3]^+\) \((m/z\) 510.8), \([\text{HLC*GSHL+2H}^2]^+\) \((m/z\) 521.4), \([\text{LVC*GERGF+2H}^2]^+\) \((m/z\) 579.2), \([\text{QCCASVC*SL+2H}^2]^+\) \((m/z\) 596.0) (the exact position of the ebselen tag in this case is uncertain), \([\text{LVC*GERGFF+2H}^2]^+\) \((m/z\) 652.0), \([\text{YLVC*GERGF+2H}^2]^+\) \((m/z\) 659.8), \([\text{YC*N+H}^+]\) \((m/z\) 674.3), \([\text{VC*SL+H}^+]\) \((m/z\) 696.1), \([\text{QCC*ASVC*SL+2H}^2]^+\) \((m/z\) 733.5) (the exact positions of the two ebselen tags are not certain in this case), \([\text{FVNQHLC*GSHL+2H}^2]^+\) \((m/z\) 765.6), \([\text{LVC*GERGFFYT+2H}^2]^+\) \((m/z\) 774.6).
(m/z 783.1), [NYC*N+H]^+ (m/z 788.2), [ASVC*SL+H]^+ (m/z 854.0), and [LVC*GERGFF+H]^+ (m/z 1303.2) were observed (listed in the Figure 3.2a inset).

In addition, [FVNQHLCGSHL+2H]^2+, [LVCGERGF+H]^+, [QCCASVCSL+H]^+, [LVCGERGFF+H]^+ were seen at m/z 627.8, 880.5, 914.3, and 1027.1, probably resulted from the incomplete derivatization reaction by ebselen owing to the presence of the excess amount of reductant TCEP (indeed, we found that, like DTT, TCEP can reduce the ebselen-derivatized peptides such as GSH in a separate experiment). Furthermore, [ALY+H]^+, [GIVE+H]^+, [VEAL+H]^+, [FVNQ+H]^+, [YQLE+H]^+, [YTPKA+H]^+, and [QLENY+H]^+ were detected at m/z 366.1, 417.2, 431.3, 507.4, 552.2, 579.2, and 666.4, respectively (the inset of Figure 3.2a). These ions correspond to seven additional peptides which do not have free cysteine residues. Because of no cysteine group, no corresponding ebselen-derivatization ions were detected for these peptides. Thus, in this insulin digest, totally eighteen peptides were observed (listed in the Figure 3.2b inset), covering 100% sequence of insulin. Meanwhile, [2ebselen+H]^+ was also detected at m/z 550.9.

Due to the multi-component nature of the sample, the ESI-MS spectrum in Figure 3.2a has multiple peaks and appears complicated. Using the Q-trap instrument, PIS was employed to screen those peptide containing cysteine residues. By monitoring the product ion of m/z 276, all of the ebselen-derivatized peptides ions mentioned above were selectively detected corresponding to eleven thiol peptides, (Figure 3.2b, the list of detected thiol peptides is in the inset). This experiment provides a simple and rapid method to selectively identify thiol peptides in complex protein digest mixtures. This method also complements our previous method for selective identification of disulfide
bond-containing peptides in protein digests based on their response to electrochemical reduction.\textsuperscript{3}

Furthermore, this selective strategy is also applicable to thiol proteins. In another experiment, a protein mixture containing $\beta$-lactoglobulin A, $\alpha$-lactalbumin, lysozyme, and insulin was derivatized \textit{via} the selective reaction with ebselen and then analyzed by ESI-MS. In Figure 3.3a, the ESI-MS spectrum shows that the multiply charged ions of $\alpha$-lactalbumin, lysozyme, and insulin, together with one ebselen-derivatized $\beta$-lactoglobulin A ions. PIS was then applied to screen the thiol protein ions. In Figure 3.3b, the ebselen-derivatized $\beta$-lactoglobulin A ions were selectively detected by monitoring the product ion of $m/z$ 276. Thus, it can be seen that the ebselen derivatization in conjunction with PIS is powerful in analysis of both thiol peptides and proteins in mixtures.
1. m/z 366.1 [ALY+H]^+  
2. m/z 417.2 [GIVE+H]^+  
3. m/z 431.3 [VEAL+H]^+  
4. m/z 507.4 [FVNQHLC+H]^+  
5. m/z 510.8 [FVNQHLC+3H]^+  
6. m/z 518.8 [FVNQHLC+3H]^+  
7. m/z 550.9 [2ebselen+H]^+  
8. m/z 552.2 [YQLE+H]^+  
9. m/z 579.2 [2ebselen+H]^+  
10. m/z 596.0 [QCCASVCSL+2H]^+  
11. m/z 627.8 [FVNQHLCGSHL+2H]^+  
12. m/z 652.0 [LVCGERGF+3H]^+  
13. m/z 659.8 [YLVCGERGF+2H]^+  
14. m/z 666.4 [QLENY+H]^+  
15. m/z 674.3 [YCN+H]^+  
16. m/z 696.1 [QLENY+H]^+  
17. m/z 733.5 [QCCASVCSL+2H]^+  
18. m/z 765.6 [FVNQHLCGSHL+2H]^+  
19. m/z 783.3 [LVCGERGFYT+2H]^+  
20. m/z 788.2 [NYCN+H]^+  
21. m/z 854.0 [ASVC*SL+H]^+  
22. m/z 880.5 [LVCGERGF]  
23. m/z 914.3 [QCCASVCSL]^+  
24. m/z 1027.1 [LVCGERGF+H]^+  
25. m/z 1303.2 [LVC*GERGFF+H]^+  

* = ebselen tag

**Figure 3.2.** (a) ESI-MS mass spectrum of the reaction of reduced pepsin digested insulin with ebselen. The inset shows the list of all the peptide peaks; (b) PIS based on the loss of the characteristic fragment ion of m/z 276 upon CID shows the selective detection of thiol peptides in reduced pepsin digested insulin. The insulin sequence and a list of detected cysteine-containing and non-cysteine containing peptides are shown in the inset. Reprinted (Adapted) from Zhang, Y.; Zhang, H.; Cui, W.; Chen, H. *J. Am. Soc. Mass Spectrom.*, 2011, 22, 1610-1621. with kind permission from Springer Science and Business Media.
Figure 3.3. (a) ESI-MS mass spectrum showing a mixture of β-lactoglobulin A, α-lactalbumin, lysozyme, insulin after reaction with ebselen; (b) PIS based on the monitoring of the characteristic fragment ion of m/z 276 upon CID shows the selective detection of containing protein β-lactoglobulin A in a protein mixture. Reprinted (Adapted) from Zhang, Y.; Zhang, H.; Cui, W.; Chen, H. J. Am. Soc. Mass Spectrom., 2011, 22, 1610-1621. with kind permission from Springer Science and Business Media.
In stark contrast to ebselen tagging, peptide ions with phenylselenenyl tags arising from NPSP derivatization display drastically different CID dissociation behaviors, in which the tags are preserved and consecutive backbone cleavages occur (eq. 1, Scheme 3.2). This is probably due to the lack of intramolecular nucleophilic attack of selenenyl sulfide bond by adjacent amide nitrogen of the selenium tag as in the case of ebselen. In Figure 3.1d, extensive b and y ions \((b_2', b_4'\cdot NH_3, b_5', b_5'\cdot NH_3, y_1, y_1\cdot NH_3, y_2, y_2\cdot NH_3, y_3, y_4, y_5')\) were seen (the single prime indicates one phenylselenenyl tag).

Again, similar phenomena were observed for CID of the NRCSQGSCWN ion with two phenylselenenyl tags, \([\text{NRCSQ'GSC'WN+2H}^2]^+\) \((m/z\ 733.8, \text{Figure 3.1e})\), and of insulin chain B ion with two phenylselenenyl tags, 

\[\text{[FVNQHL'C'GSHLVEALYLVC'GERGFYTPKA+3H}^3]^+\ \ (m/z\ 1238.0, \text{Figure 3.1f}).\]

For the former peptide ion, mainly b and y ions such as \(b_2, b_3', b_4', b_5', b_6', b_7', b_8'', b_9''\), \(b_9''\cdot 2^+, y_2, y_3', y_4', y_5', y_6', y_7', \) and \(y_8''\) were observed (Figure 3.1e; the double prime indicates two phenylselenenyl tags in the fragment ions). In the latter, \(b_3, b_4\cdot NH_3, b_5, b_6, b_7, b_8'\cdot 2^+, b_9''\cdot 2^+, b_9''\cdot 2^+, y_4, y_8, y_9, y_{11}, y_{14}', y_{15'}, y_{15'}\cdot NH_3, y_{22'}\cdot NH_3^2, y_{23'}, y_{24''}\cdot 2^+, y_{25''}\cdot 2^+\) were detected (Figure 3.1f).

It turns out that the phenylselenenyl tag could facilitate peptide sequencing and be valuable in pinpointing the location of cysteine residues of peptides. For example, based on the distinct selenium isotope distribution of \(y_5'\) which is absent for \(y_4\) (inset Figure 3.1d), it is clear that \(y_5'\) contains the tag and thus a cysteine residue while \(y_4\) does not. This result is in agreement with the peptide structure (the cysteine is located in the 2\(^{\text{nd}}\) residue of the peptide HCKFWW) and also emphasizes the high selectivity of the NPSP
derivatization toward cysteine. The derivatization selectivity was further confirmed by the appearance of fragment ion pairs of $b_2/b_3'$ and $b_7'/b_8''$ in the case of peptide NRCSQGSCWN (having Cys$^3$ and Cys$^8$) and the generation of the fragment ion pairs of $y_{11}/y_{14}'$ and $y_{23}/y_{24}''$ in the case of insulin chain B (having Cys$^{12}$ and Cys$^{24}$). In particular, the pair $y_{11}/y_{14}'$ reveals that one of the two cysteines is located between the 12$^{\text{th}}$ and 14$^{\text{th}}$ residues of the chain B.

3.3.2. CID MS/MS of the Negative Ions of Selenamide-Derivatized Peptides

Selenium and sulfur, two elements in the Group VIB, exhibit many expected similarities in chemical properties.$^{258-259}$ Indeed, they have similar electronegativities of 2.55 and 2.58 (Pauling scale), respectively. The Se-S bond is also close to the S-S bond, both of which can be electrolytically reduced.$^{260}$ It is known that S-S undergoes marginal cleavage under positive ion CID except with high energy or with metal ions.$^{220}$ But S-S undergoes facile cleavage under negative ion CID.$^{211}$ Proton abstraction from the C$_\alpha$ and C$_\beta$ of the cysteine residue causes diverse fragmentation reactions including the cleavage of disulfide bonds, and persulfides, thioaldehydes, and dehydroalanine residues at the cysteine position are generated upon negative ion CID.$^{261}$

Thereby we also investigated the dissociation behavior of the selenamide-derivatized peptide ions containing Se-S bonds under negative ion CID conditions. The basic cleavages of Se-S bond in ebselen- and NPSP-derivatized peptide ions are outlined in eq. 2 (Schemes 3.1 and 3.2). For the fragmentation of ebselen-derivatized peptide anions, it is similar to that of their cationic counterparts and the loss of neutral ebselen is the major dissociation pathway (eq. 2, Scheme 3.1). As shown in the CID MS/MS mass
spectrum of \([\text{HC}^*\text{KFWW-H}^-]\) (\(m/z\) 1179.0, Figure 3.4a), \(m/z\) 904.5 corresponding to \([\text{HCKFWW-H}^-]\), was generated by loss of one ebselen. In the case of CID of the NPSP-derivatized peptide anion, \(\beta\)-elimination and \(\alpha\)-elimination processes give rise to the thiaoaldehyde and dehydroalanine residues at the cysteine side chain, respectively (eq. 2, Scheme 3.2). These processes can be seen in the CID MS/MS mass spectrum of \([\text{HC}^*\text{KFWW-H}^-]\) (\(m/z\) 1060.0, Figure 3.4b), which results in intense fragment ions of \(m/z\) 902.3 and 870.4 due to \(\alpha\)- and \(\beta\)-eliminations. It does confirm that Se-S cleavage occurs in negative ion CID as expected. The negative ion CID might be useful in the analysis of acidic peptides as those peptides prefer to form anions.
Figure 3.4. CID MS/MS mass spectra of the negative ion of (a) [HC*KFWW-H]⁻ (m/z 1179.0); (b) [HC'KFWW-H]⁻ (m/z 1060.0). Reprinted (Adapted) from Zhang, Y.; Zhang, H.; Cui, W.; Chen, H. J. Am. Soc. Mass Spectrom., 2011, 22, 1610-1621. with kind permission from Springer Science and Business Media.
Scheme 3.2. CID MS/MS processes for [HC'KFWW+2H]²⁺ (eq. 1) and [HC'KFWW-H]⁻ (eq. 2). Reprinted (Adapted) from Zhang, Y.; Zhang, H.; Cui, W.; Chen, H. J. Am. Soc. Mass Spectrom., 2011, 22, 1610-1621. with kind permission from Springer Science and Business Media.

3.3.3. ETD MS/MS of Selenamide-Derivatized Peptide Ions

Both ECD¹⁵⁶ and ETD¹⁹⁷ are powerful dissociation methods which often give rise to extensive backbone cleavages. In ECD experiments, low-energy electrons were reacted with peptide cations in the ICR cell located in the center of the magnetic of a Fourier-Transfer Ion Cyclotron mass spectrometer (FT-ICR-MS).¹⁵⁶ ETD utilizes ion/ion reactions¹⁹³, ¹⁹⁷, ²⁶² to transfer electron to peptide ions for dissociation but is regarded to have similar mechanism as ECD.

An early proposed mechanism for ECD (also known as the Cornell mechanism¹⁵⁶) starts with electron localization at positively charged ammonium or guanidinium groups of a peptide that generates a hypervalent center,⁶ which transfers a hydrogen atom H⁺ to a carbonyl oxygen of an amide group to form an aminoketal radical intermediate. The aminoketal radical intermediate subsequently undergoes the cleavage
of the N-C\(_{\alpha}\) bond to form c/z ions.\(^{263}\) It has been previously shown that disulfide bonds can be preferentially broken by ECD for the reason that S-S has a higher affinity for H\(^{+}\) atom compared with the amide carbonyl.\(^6\) However, the mechanism of ECD and ETD fragmentations are still not thoroughly understood.\(^{156, 165, 167, 264}\) Even though the Cornell mechanism provided a reasonable picture for ECD, some backbone fragmentations were not easily explained.\(^{253}\)

An alternative mechanism, now known as the UW mechanism,\(^{165, 167}\) has been proposed to explain the origin of c and z fragment ions. In this theory, direct electron attachment to Coulomb-stabilized amide \(\pi^*\) orbitals makes the amide group an exceptionally strong base with a proton affinity (PA) in the range of 1100-1400 kJ/mol.\(^{265}\) The amide anion radical is able to abstract a proton to result in an intermediate identical to the aminoketyl cation radical proposed in the Cornell mechanism and can undergo the same N-C\(_{\alpha}\) bond cleavage.

Recently, one of the powerful approaches for the ECD/ETD mechanistic elucidation is to introduce various tags with different electron or proton affinities to model peptides and to test the effect of the tags on the dissociation upon electron capture/transfer.\(^{253-256}\) In consideration of convenient preparation of selenamide-derivatized proteins/peptides and the structural similarity of the resulting Se-S bond to S-S bond, electron-based dissociation behaviors of the derivatized peptide ions were also examined, with purpose to provide some insightful information about the mechanism of the electron-based dissociation.
Figures 3.5a and d show the ETD MS/MS mass spectra of \([\text{HC}^*\text{KFWW}+2\text{H}]^{2+}\) and \([\text{HC'}\text{KFWW}+2\text{H}]^{2+}\), respectively. In both cases, preferential cleavage of selenium tag takes place predominantly, giving rise to the main fragment ion of \(m/z\) 906.5, the protonated peptide ion without tag. Also, \(m/z\) 1063.3 corresponding to the radical cation \([\text{HC'}\text{KFWW}+2\text{H}]^{2+}\) as a result of one electron capture is in Figure 3.5d. Few \(c\) ions with very low abundances from backbone cleavage were observed. The ETD process for the doubly charged ion of HCKFWW with one selenium tag is in eq. 1, Scheme 3.3. The cleavage of the Se-S bond can be expedited by direct electron-capture as stabilized by Coulomb effect (by the positively charged arginine or terminal amino of the peptide) followed with bond cleavage, leading to loss of radical \(\text{RSe}^*\) and thiolate anion. Subsequent proton transfer occurring to the thiolate anion forms singly charged HCKFWW.

This proposed mechanism follows the UW pattern and was further testified by ETD of the corresponding alkali metal-containing peptide ion. Figure 3.6 is ETD MS/MS mass spectrum of \([\text{HC'}\text{KFWW}+\text{Na}+\text{K}]^{2+}\) (\(m/z\) 562.2) and the similar phenomena was observed with preferential cleavage of phenylselenenyl tag taking place to form the fragment ion of \(m/z\) 967.3 (\([\text{HCKFWW}+\text{Na}+\text{K}-\text{H}]^+\)) (see the proposed mechanism in eq. 2, Scheme 3.3). Also, \(m/z\) 1124.3 corresponding to \([\text{HC'}\text{KFWW}+\text{Na}+\text{K}]^{3+}\) is in Figure 3.6 as charge reduced species.

Again, in Figures 3.5b and e, the NRCSQGSCWN peptide ions with two ebselen and phenylselenenyl tags also display similar ETD mass spectra. No \(c\) or \(z\) ions were observed, instead only few \(b/y\) ions with low abundances were detected. In both cases
(Figures 3.5b and e), preferential cleavage of one selenium tag occurs predominantly, generating the main fragment ions of \( m/z \) 1429.4 and 1310.4, respectively. The ETD mass spectra of \([\text{FVNQHLC}^*\text{GSHLVEALYLVC}^*\text{GERGFFYPKA}+3\text{H}]^{3+}\) and \([\text{FVNQHLC}'\text{GSHLVEALYLVC}'\text{GERGFFYPKA}+3\text{H}]^{3+}\) were in Figures 3.5c and f, respectively. Likewise, there are no \( c \) or \( z \) ions observed, either. The cleavage of one ebselen tag occurs to either Cys\textsuperscript{7} or Cys\textsuperscript{19}, giving rise to the fragment ion of \( m/z \) 1838.0 (Figure 3.5c).

Because of the presence of two selenenyl sulfide bonds in selenamide-derivatized NRCSQGSCWN and insulin chain B, the electron transfer to the tag completely suppresses the backbone cleavage, resulting in no formation of \( c/z \) ions. These results indicate that the selenium tags can efficiently quench the formation of \( c/z \) ions in the ETD of peptide ions and the mechanism can be accounted for by the UW mechanism.
Figure 3.5. ETD MS/MS mass spectra of (a) [HC*KFWW+2H]^2+ (m/z 591.0); (b) [NRC*SQGSC*WN+2H]^2+ (m/z 852.8); (c) FVNQHLC*GSHLVEALYLVC*GERGFFYTPKA+3H]^3+ (m/z 1317.0) (d) [HC*KFWW+2H]^2+ (m/z 531.8); (e) [NRC*SQGSC*WN+2H]^2+ (m/z 733.8); (f) [FVNQHLC*GSHLVEALYLVC*GERGFFYTPKA+3H]^3+ (m/z 1238.0). Reprinted (Adapted) from Zhang, Y.; Zhang, H.; Cui, W.; Chen, H. J. Am. Soc. Mass Spectrom., 2011, 22, 1610-1621. with kind permission from Springer Science and Business Media.
**Figure 3.6.** ETD MS/MS mass spectrum of [HC'KFWW+Na+K]^{2+}(m/z 562.2). Reprinted (Adapted) from Zhang, Y.; Zhang, H.; Cui, W.; Chen, H. *J. Am. Soc. Mass Spectrom.*, 2011, 22, 1610-1621. with kind permission from Springer Science and Business Media.

**Scheme 3.3.** Dissociation processes for the doubly charged HCKFWW carrying two protons and one selenium tag (eq. 1) and for the doubly charged HCKFWW carrying one sodium, one potassium and one selenium tag (eq. 2) upon ETD. Reprinted (Adapted) from Zhang, Y.; Zhang, H.; Cui, W.; Chen, H. *J. Am. Soc. Mass Spectrom.*, 2011, 22, 1610-1621. with kind permission from Springer Science and Business Media.
3.4. Conclusions

In conclusion, unimolecular ion dissociation behaviors of selenamide-labeled thiol peptide ions upon CID and ETD were investigated. It is evident that derivatized peptide cations undergo tag-dependent CID dissociation pathways. Ebselen-derivatized peptide cations display the unique fragment ion of $m/z$ 276 upon dissociation, which is useful for selective identification of thiol peptides and proteins in mixture. The robust phenylselenenyl tag is useful in peptide sequencing and locating of cysteine residues in peptides. In the negative ion mode CID, both types of tags are preferentially lost via the Se-S cleavage, similar to S-S bond. In addition, the preferential cleavage of Se-S bond occurs over the formation of $c/z$ ions during ETD activation to both protonated and alkaliated peptides, following the UW mechanism. Given the significance of thiol residues in proteins/peptides, the selective derivatization by selenamides and the rich ion dissociation chemistry revealed in this study would be useful in the proteomics research.
Chapter 4: Online Mass Spectrometric Analysis of Proteins/Peptides Following Electrolytic Cleavage of Disulfide Bonds

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4.1. Introduction

Redox-active disulfide bonds are one of the most common protein PTM and provide reversible covalent cross-linkages in native proteins for maintaining the three-dimensional structures of proteins and their biological activities.\textsuperscript{210-211} Such a linkage plays a critical role in the activity of enzymes and is also a key structural feature of biologically active peptide hormones such as somatostatin, oxytocin, and [Arg\textsuperscript{8}]-vasopressin.\textsuperscript{266} The presence of the disulfide linkages increases the complexity for the protein structure elucidation by MS. The cleavage of disulfide bond is often essential for the protein/peptide analysis as dissociation of a reduced protein/peptide ion can give rise to more structurally informative fragment ions than that of the intact counterpart.\textsuperscript{210-211} The traditional protocol to break a disulfide bond is chemical reduction using an excess amount of reagents like DTT or TCEP. However, the reduction usually takes one-half to several hours and the removal of the excess amount of reductant is time-consuming and troublesome. Besides chemical reduction, other novel approaches include the cleavage of disulfide bonds via laser-based ionization,\textsuperscript{267-268} ultraviolet photodissociation,\textsuperscript{269} negative ion dissociation,\textsuperscript{211, 261, 270} ECD,\textsuperscript{6} ETD,\textsuperscript{199} plasma-induced oxidation,\textsuperscript{271} reactive ELDI,\textsuperscript{272} or using new ion chemistry.\textsuperscript{214, 220}
An alternative way for reducing disulfide bonds without involving chemical reductants is electrolytic reduction. It is well known that disulfide bonds can undergo reductive cleavage on an amalgam electrode surface. Previous investigations showed that such a reduction followed with electrolytic oxidation of the resulting thiols back to disulfides in an electrochemical flow cell can be coupled with either HPLC or electrophoresis separation, which are useful for the simultaneous detection of thiol- and disulfide-containing peptides in mixtures. Although these techniques are elegant and quite sensitive, they do not provide any structural information for the detected peptides. Also, the separation processes involved take a long time and the disulfide-containing peptides cannot be detected if they do not elute from the HPLC column.

DESI has recently been introduced for direct ionization of analytes with little or no sample preparation. This technique is successful in the fast analysis of a variety of different analytes ranging from pharmaceuticals to tissue imaging. In addition to being used regularly for solid sample analysis of surfaces, DESI has been extended to the analysis of liquid samples in several laboratories.

In liquid sample DESI experiments, ionization occurs via interactions of the sample with charged microdroplets generated in a pneumatically assisted DESI spray and subsequent desolvation of the resulting secondary microdroplets containing the sample analyte. It has been shown that liquid sample DESI allows convenient on-line coupling of MS with EC, microfluidics, and single droplet microextraction. It can also be used for monitoring protein conformational changes in solution.
In the combined EC/DESI-MS experiments, previous data showed that the products generated from redox reactions such as DA oxidation and disulfide bond reduction can be directly desorbed and ionized from an electrochemical cell by DESI for online MS analysis.\textsuperscript{141} The method is a new development in the field of EC/MS which has a history of 40 years.\textsuperscript{81} Different ionization methods such as TS and ESI\textsuperscript{111-113, 138, 281} were employed in previous EC/MS studies, which has found numerous applications such as the identification of electrochemically generated species, mimicking biologically relevant electrochemical reactions, oxidative cleavage of peptide backbones, and online chemical tagging. In our EC/DESI-MS method,\textsuperscript{27,141} some unique and valuable features originating from using DESI as the coupling interface have been revealed, including a simplified coupling apparatus owing to no requirement to separate the small potential applied to the electrochemical cell from the high voltage used for spray ionization, tolerance to inorganic salt electrolytes, and the freedom to choose traditional solvents for electrolysis, as well as, to use either positive or negative ionization modes.

In this study, the EC/DESI-MS method has been explored for analysis of protein/peptide digest mixtures, various biologically active peptides (both intra- and inter-disulfide bond containing peptides) and intact proteins. It is one part of our effort toward the utilization of EC/DESI-MS in proteomics research, as motivated by the fact that many proteins (e.g., disulfide or metal ion containing proteins) are electroactive species and the investigation of protein electrochemistry is still very limited.\textsuperscript{282}

In this experiment, we first optimized the experimental conditions to improve the performance of an amalgam electrode for reduction by polishing its surface with a silk
handkerchief and using low concentration of protein and peptide samples to reduce possible chemical adsorption on the electrode. Through the analysis of various types of samples by the EC/DESI-MS, several interesting findings are uncovered, including: i) disulfide-containing peptides can be quickly identified from enzymatic digest mixtures, simply based on their large relative ion abundance changes before and after electrolysis; ii) in conjunction with tandem MS analysis and peptide mass mapping, the disulfide linkage pattern and sequence information for the examined peptides could be determined; iii) in comparison to traditional chemical reduction, the reported online EC/DESI-MS method is much faster (completed in few minutes) and has no requirement to remove chemical reductants for obtaining good ionization efficiency; iv) in the case of peptides having symmetric chains or intra-peptide disulfide bonds, selenamide reagents can be used to label the reduced peptides, a rapid and highly selective thiol derivatization strategy recently developed in our laboratory, which aids in the recognition of the reduced peptide ions, the number of free thiols in the reduced peptides, and the type of disulfide bond in the precursor peptides examined; v) the role of disulfide bonds in maintaining protein folding can be revealed, based on the charge state distribution (CSD) changes after electrolytic reduction.

4.2. Experimental Section

4.2.1. Chemicals and Materials

Peptides [Arg\textsuperscript{8}]-conopressin G and somatostatin 1-14 were purchased from Bachem (King of Prussia, PA). Ebselen was purchased from Calbiochem (Cincinnati, OH). TPCK-treated trypsin from bovine pancreas (MW: ~23.8 KDa), pepsin from
porcine gastric mucosa (MW: ~35KDa), NPSP, ammonium bicarbonate, TCEP hydrochloride solution, mercury (triply distilled, 99.9999% purity), L-glutathione disulfide (GSSG), [Arg⁸]-vasopressin acetate salt, oxytocin, bovine pancreas insulin, α-lactalbumin from bovine milk (type III, calcium depleted, ≥85%), and HPLC-grade acetonitrile were all purchased from Sigma-Aldrich (St. Louis, MO). Acetic acid and HPLC-grade methanol were obtained from Fisher Scientific (Fair Lawn, NJ) and GFS Chemicals (Columbus, OH), respectively. The silk handkerchief was purchased from Royal Silk Direct, Inc. (Princeton, NJ). The de-ionized water used for sample preparation was obtained using a Nanopure Diamond Barnstead purification system (Barnstead International, Dubuque, IA).

4.2.2. Instrumentation

A home-built apparatus for coupling a thin-layer electrochemical flow cell with either a Thermo Finnigan LCQ DECA ion trap mass spectrometer (San Jose, CA) or a hybrid triple quadrupole-linear ion trap mass spectrometer (Q-trap 2000, Applied Biosystems/MDS SCIEX, Concord, Canada) by liquid sample DESI was used and described previously in detail.¹⁴¹ The thin-layer electrochemical flow cell consisted of a WE embedded in PEEK and separated from a stainless steel AE by two Teflon gaskets (0.01 inch thick each) and a Ag/AgCl (3 M NaCl) RE contacting the sample solution through a small hole in the AE. The WE used was a dual amalgam electrode (3 mm diameter) or an amalgam electrode (6 mm diameter) for reduction. The procedure used to polish the electrodes is as follow; the residual mercury was removed by 6 M nitric acid from the gold surface, and then the electrode was polished sequentially with water, 15, 3,
and 1 µm diamond and 0.5 µm alumina powders on a grit pad or a nylon pad. Also the electrode block was rinsed using methanol and water and then air-dried. After that, the electrode was coated with a drop of mercury for 10 min, and the excess amount of mercury was removed by one edge of an index card. The mercury surface was further polished with a piece of silk handkerchief until the smooth surface was obtained. After kept overnight (at least 6 hours), the amalgam electrode was used for reduction. A ROXY potentiostat (Antec Leyden, Netherlands) was used to apply potentials to the electrochemical cell for reduction of analytes that flowed through the cell. Sample solutions were degassed by argon purging for 20 min to remove dissolved oxygen prior to the injection to the cell for electrolysis. The reduced species flowed out of the cell via a short piece of fused silica connection capillary (i.d. 0.1 mm, length 4.2 cm) and underwent interactions with the charged microdroplets from DESI spray for ionization. The capillary outlet was placed about 2~3 mm downstream from the DESI spray probe tip and kept in line with the sprayer tip and the mass spectrometer’s inlet. The spray solvent for DESI was methanol/water (1:1 by volume) containing 1% acetic acid and injected at a rate of 5 µL/min and a high voltage of + 5 kV was applied to the spray probe. The flow rate for sample solutions passing through the electrochemical cell for electrolysis was 3 µL/min.
4.2.3. Method

*Tryptic Digestion*

Digestion of peptides were carried out using TPCK-treated trypsin with a ratio of 1:100 (enzyme/peptide) in 25 mM ammonium bicarbonate aqueous solution for 3 h at 38 °C incubation to finish digestion.\(^{284}\)

*Peptic Digestion*

Digestion of insulin was performed by incubating insulin and pepsin at a molar ratio of 50:1 in water containing 1% acetic acid at 37 °C for 6.5 h.\(^{220}\) In the comparison experiment using chemical reduction, TCEP was added to the peptic digested insulin in the molar ratio of 1:20 (protein/TCEP) for 2 h at room temperature.

*Safety precautions*

As mercury is toxic, the preparation for the amalgam electrode should be carried out in a hood. The mercury waste should be disposed in a special mercury waste container containing sublimed sulfur.
4.3. Results and Discussion

4.3.1. Reduction of Peptides Containing Disulfide Bonds in Enzymatic Digests


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<th>Sequences of peptides obtained from digestion</th>
<th>Denotation</th>
<th>MW (Da)</th>
<th>Sequences of reduced peptides and their MW (Da)</th>
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<td>1062.3</td>
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<td>AGCK</td>
<td>P2 932.4 (932.5*)</td>
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Note: MW labeled with “*” in the parenthesis refers to the measured MW from the m/z values; The small differences between the measured MW and the theoretic MW of peptides P3-P7 were caused by the low resolution of the ion trap instrument in the analysis of multiply charged ions of these peptides.

“**” refers to “not detected”.
4.3.1.1. Trypsin Digested [Arg^8]-Conopressin G

Electrolytic reduction of disulfide bonds of trypsin digested [Arg^8]-conopressin G (MW: 1062.3 Da) was first carried out using the EC/DESI-MS apparatus. The tryptic digestion breaks [Arg^8]-conopressin G in its middle backbone to generate a peptide (denoted as P1) containing asymmetric chains linked by one disulfide bond (chain A: CFIR; chain B: NCPR, Table 1).

Figure 4.1a is the DESI-MS spectrum acquired when a solution of trypsin digested [Arg^8]-conopressin G (0.1 mM) in methanol/water (1:1 by volume) containing 1% acetic acid flowed through the thin-layer electrochemical cell with no potential applied to the cell. Peaks corresponding to the doubly and triply charged P1 were detected at m/z 512.9 and 342.3, respectively. When a -2.0 V potential was applied to the amalgam WE, two new peaks at m/z 538.0 and 489.0 appeared, corresponding to the protonated free chain A, [CFIR+H]^+, and the protonated free chain B, [NCPR+H]^+ (Figure 4.1b), respectively.

These assignments were consistent with their MS^2 spectra showing that m/z 538 dissociates into fragment ions of y_1, y_1-NH_3, y_2-NH_3, and y_4-NH_3 ions (Figure 4.1c) and m/z 489 produces fragment ions of y_1, y_1-H_2O, y_1-H_2O-NH_3, y_2-NH_3, and y_4-NH_3 ions upon CID (Figure 4.1d; other peaks seen in the spectrum are from the background contribution due to the low abundance of the precursor peptide ion). This result demonstrates that EC/DESI-MS is applicable for reducing the disulfide bonds of peptides in enzymatic digest and the resulting peptide chain products can be directly detected online by MS without separation, suggesting its utility in mixture analysis.
Figure 4.1. DESI-MS spectra acquired when a solution of 0.1 mM trypsin digested [Arg⁸]-conopressin G in methanol/water (1:1 by volume) containing 1% acetic acid flowed through the thin-layer electrochemical cell with an applied potential of a) 0.0 V and b) -2.0 V; CID MS² spectra of c) the protonated chain A (m/z 538) and d) the protonated chain B (m/z 489) resulted from the electrolytic reduction. The downward arrows in a) indicate that the ion abundance will drop during electrolysis, the upward arrows in b) indicates the appearance of new reduced peptide product ions. Such a labeling is applicable in Figures 4.2. and 4.3. as well. Reprinted (Adapted) with permission from Zhang, Y.; Dewald, H. D.; Chen, H. J. Proteome Res. 2011, 10, 1293-1304. Copyright 2011 American Chemical Society.

4.3.1.2. Trypsin Digested Somatostatin 1-14

In the experiment described above, the conversion yield was low and the working amalgam electrode had a short life time. By polishing the electrode surface with silk handkerchief and also lowering the concentration of the samples injected into the electrochemical cell, an enhanced reduction yield and elongated life-time of the amalgam
were achieved. Presumably, the polishing made the amalgam surface more uniform and using diluted sample solution reduced the possible chemical adsorption onto the amalgam surface.

Under these optimized conditions, electrolytic reduction of disulfide bonds of trypsin digested somatostatin 1-14 (MW: 1637.9 Da) was examined. The sequence of somatostatin 1-14, a peptide containing an intra-peptide disulfide bond, is shown in Table 1. After tryptic digestion, peptide (denoted as P2) containing asymmetric chains linked by one disulfide bond (chain A: AGCK; chain B: TFTSC) was obtained, together with another peptide NFFWK.

Figure 4.2a is the DESI-MS spectrum acquired when a solution of trypsin digested somatostatin 1-14 (35 µM) in methanol/water (1:1 by volume) containing 1% acetic acid flowed through the thin-layer electrochemical cell with no potential applied to the cell. The singly, doubly and triply charged P2 were detected at \( m/z \) 933.3, 467.3, and 312.3, respectively (these peaks are listed in Figure 4.2a inset for clarity). In addition, one sodium adduct peak \([\text{P2+ Na}]^+ (m/z 955.3)\) was seen, probably due to ubiquitous sodium ions (e.g., from the water used for preparing the sample solution). Furthermore, ions of the peptide NFFWK, \([\text{NFFWK}+\text{H}]^+ (m/z 741.3)\), \([\text{NFFWK}+\text{Na}]^+ (m/z 763.3)\), and \([\text{NFFWK}+2\text{H}]^{2+ (m/z 371.4)}\), were also observed.

When a -1.8 V potential was applied to the cell for reduction, two new ions of \( m/z \) 378.1 and 558.3 were generated, corresponding to the protonated ions of free chain A and chain B of P2, respectively (Figure 4.2b). Strikingly, abrupt changes in relative abundances of disulfide containing peptide ions before and after electrolysis were
observed (the data are summarized in Table 2). For instance, the relative ion abundances of ions associated with the peptide P2, \([P2+H]^+\), \([P2+Na]^+\), \([P2+2H]^{2+}\) and \([P2+3H]^{3+}\), decreased to a considerable extent (by 59-100 % reduction) when the cell was turned on (Table 4.2). In particular, the triply charged P2 \([P2+3H]^{3+}\) (m/z 312.3) completely disappeared after electrolysis (Figure 4.2b). By contrast, the relative abundances for ions associated with the peptide NFFWK without disulfide bonds, \([\text{NFFWK}+H]^+\), \([\text{NFFWK}+\text{Na}]^+\), and \([\text{NFFWK}+2\text{H}]^{2+}\), remained nearly unchanged (instead, a small increase of 2-5% were observed for the latter two ions probably caused by signal fluctuation during ionization, Table 4.2). This result is exactly in line with the fact that NFFWK is not electroactive while the disulfide-containing peptide P2 is. The pronounced changes in relative ion abundance due to electrolysis are useful to distinguish disulfide containing peptides from those without disulfide bonds in the enzymatic digest mixtures. As the electrolysis is simply controlled by turning on and off the cell potential, such a differentiation is simple and fast.

In addition, in Figure 4.2c, the electro-generated chain A ion (m/z 378) gives rise to the fragment ions of \(b_2\), \(b_3\), \(b_4\), \(b_4\)-H\(_2\)O, \(y_1\), \(y_2\), and \(y_3\) ions upon CID, from which its sequence can be determined as AGCK (the cysteine residue is in the 3\(^{rd}\) position in chain A). Likewise, the protonated chain B (m/z 558) dissociated into \(a_2\), \(b_2\), \(b_3\), \(b_4\)-H\(_2\)O, \(b_5\), \(b_5\)-H\(_2\)O and \(y_3\) ions (Figure 4.2d), which reveals its sequence to be either TFTSC or FTTSC (in other words, the cysteine is located in the 5\(^{th}\) position in chain B). In comparison to the fact that the CID of the protonated intact P2 (m/z 933) only produces \(A1-A4/B(y_1)\), \(A1-A4/B(y_2)\), \(A1-A4/B(y_3)\), \(B1-B5/A(b_3)\) and \(B1-B5/A(y_2)\) ions (spectrum not
shown; A1-A4/B(y1) refers to a fragment with y1 ion of B chain linked with an intact A chain; the notation is applicable to other fragment ions), the electrolytic reduction of disulfide bond allows one to obtain more sequence information of the examined peptide.

More importantly, in this experiment, the connectivity of the disulfide bond in P2 can be inferred, based on the measured molecule weight (MW) information of the precursor peptide and its reduced peptide chains (both theoretic and measured MWs of the peptides and their reduced peptide chains are listed in the Table 4.1 with the measured MWs labeled with “*”). As discussed above, we know that electroactive P2 in the mixture contains disulfide bonds and the two new peptides AGCK and TFTSC are generated after the sample electrolysis. It is found that the sum of the measured MWs of AGCK and TFTSC (934.4 Da) is 1.9 Da higher than that of the precursor peptide P2 (932.5 Da). This indicates that AGCK and TFTSC are the two chains of P2, and P2 has one disulfide bond bridging these two chains. According to the sequence information of each chain revealed by CID as mentioned above, it is therefore clear that the disulfide bond linkage in P2 is the one bridging Cys3 of chain A and Cys5 of chain B (Table 4.1).

These results confirmed that the EC/DESI-MS can be used for reduction of disulfide bonds of peptides in enzymatic digest mixtures. Such an experiment is versatile. One utility is the fast recognition of disulfide-containing peptides from the digest, based on the abrupt changes of the relative ion abundances before and after electrolysis. The MS and MS/MS analysis following electrolytic reduction further provide the sequence information of the peptides and the connectivity of the disulfide linkage.
Figure 4.2. DESI-MS spectra acquired when a solution of 35 µM trypsin digested somatostatin 1-14 in methanol/water (1:1 by volume) containing 1% acetic acid flowed through the thin-layer electrochemical cell with an applied potential of a) 0.0 V and b) -1.8 V; CID MS² spectra of c) the protonated chain A (m/z 378) and d) the protonated chain B (m/z 558). Reprinted (Adapted) with permission from Zhang, Y.; Dewald, H. D.; Chen, H. J. Proteome Res. 2011, 10, 1293-1304. Copyright 2011 American Chemical Society.
Table 4.2. Relative abundance changes after the electrolysis. Reprinted (Adapted) with permission from Zhang, Y.; Dewald, H. D.; Chen, H. J. Proteome Res. 2011, 10, 1293-1304. Copyright 2011 American Chemical Society.

<table>
<thead>
<tr>
<th>Proteins/Peptides</th>
<th>Peptide ions</th>
<th>m/z</th>
<th>Relative Abundances when cell was turned off (RA_{off})</th>
<th>Relative Abundances when cell was turned on (RA_{on})</th>
<th>(RA_{on}/RA_{off}) \times 100%</th>
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4.3.1.3. Pepsin Digested Insulin

The success in the peptide digest analysis encouraged us to test the method for protein digests. Bovine pancreas insulin (MW: 5733.5 Da, sequence shown in Table 4.1) is known to have A and B chains linked by two disulfide bonds, and the chain A of insulin has an additional intra-peptide disulfide bond. After pepsin digestion, five disulfide-containing peptides denoted as P3, P4, P5, P6, and P7 along with seven additional peptides ALY, GIVE, VEAL, FVNQ, YQLE, YTPKA, and QLENY were obtained (Table 4.1). The reason to use pepsin in this case is because that pepsin digestion occurs in acidic environment, in which the possible disulfide bond rearrangement can be precluded.²¹⁰

Figure 4.3a shows the DESI-MS spectrum acquired when a solution of pepsin digested insulin (10 µM) in methanol/water (1:1 by volume) containing 1% acetic acid flowed through the thin-layer flow cell without any potential applied to the cell. The triply and doubly charged ions of P3 (m/z 692.2, 1037.5), P4 (m/z 855.1, 1281.5), and P5 (m/z 722.1 and 1082.5) were seen in the spectrum. Also, the doubly charged P6 and triply charged P7 were detected at m/z 770.0 and 641.3, respectively. In addition, ions of other peptides in the peptic digest, including [YTPKA+2H]²⁺ (m/z 290.6), [ALY+H]⁺ (m/z 366.4), [GIVE+H]⁺ (m/z 417.4), [VEAL+H]⁺ (m/z 431.4), [FVNQ+H]⁺ (m/z 507.5), [YQLE+H]⁺ (m/z 552.5), [YTPKA+H]⁺ (m/z 579.6), and [QLENY+H]⁺ (m/z 666.5) were observed and are listed in Figure 4.3a inset.

As expected, when a -1.6 V potential was applied to the cell (Figure 4.3b), the relative abundances for the ions of disulfide-containing peptides P3-P7 decreased.
significantly by 34~60% (detailed abundance change information is given in Table 4.2).
Alternatively, the other seven peptides without disulfide bonds are not sensitive to
electrochemical reduction and their relative ion abundances have marginal changes (the
maximum decrease is only by 3.5%, Table 4.2). This result emphasizes that the disulfide-
containing peptides can be distinguished from others based on their responses to
electrolytic reduction, even in a complex mixture of protein digest.

In addition, when the electrochemical cell was turned on, new peaks of \( m/z \) 384.3,
514.8, 628.5, 656.5, 766.6, 880.6, 913.4, 1027.5, 1254.6, and 1311.5 were observed,
corresponding to \([\text{HLCGSHL+2H}]^{2+}\), \([\text{LVCGERGFF+2H}]^{2+}\), \([\text{FVNQHLCGSHL+2H}]^{2+}\),
\([\text{GIVEQCCASVCSL+2H}]^{2+}\), \([\text{HLCGSHL+H}]^{+}\), \([\text{LVCGERGF+H}]^{+}\), \([\text{QCCASVCSL+H}]^{+}\),
\([\text{LVCGERGF+H}]^{+}\), \([\text{FVNQHLCGSHL+H}]^{+}\), and \([\text{GIVEQCCASVCSL+H}]^{+}\),
respectively (Figure 4.3b). The appearance of these new peaks shows that six free peptide
chains, HLCGSHL, LVCGERGFF, FVNQHLCGSHL, GIVEQCCASVCSL,
LVCGERGF and QCCASVCSL generated from the electrolytic reduction of the pepsin
digested insulin, were detected.

Based on the measured MWs (Table 4.1), we could assign these peptide chains to
their precursor peptides. As shown in the Table 4.3, among 15 possible combinations of
the six detected peptide chains, only the sum of the MWs of two chains
GIVEQCCASVCSL and HLCGSHL (2076.9 Da) is fairly close to the MW of P3 (2073.3
Da), which differ by 3.6 Da in mass, which suggests that P3 consists of two chains,
GIVEQCCASVCSL and HLCGSHL, and the mass difference of 3.6 Da indicates that P3
contains two disulfide bonds.
In a similar way, we also can relate the chain pairs of GIVEQCCASVCSL/FVNQHLCGSHL and QCCASVCSL/FVNQHLCGSHL to P4 and P5, respectively, which are both bridged via two disulfide bonds as well. Among these three precursor peptides, P4 is the largest one in the insulin digest. However, as the A chain ions of P6 and P7 are missing in the DESI-MS spectrum (Figure 4.3b, only B chains LVCGERGFF and LVCGERGF were detected), it is not possible to identify the chain compositions of P6 and P7. The reason that both A chain ions of P6 and P7 can not be observed after reduction is probably because these chains lack the basic amino acid residues and thereby have low ionization efficiencies. Similar phenomena of missing chain A in the spectra of reduced insulin samples was noticed before.281, 285
**Figure 4.3.** DESI-MS spectra acquired when a solution of pepsin digested insulin (10 μM) in methanol/water (1:1 by volume) containing 1% acetic acid flowed through the thin-layer electrochemical cell with an applied potential of a) 0.0 V and b) -1.6 V (the extracted ion chromatogram (XIC) of [HLCGSHL+2H]^2+ (m/z 384) is shown in the inset); c) ESI-MS spectrum of peptic digested insulin (10 μM) reduced by TCEP in methanol/water (1:1 by volume) containing 1% acetic acid. The m/z 251.4 is the protonated TECP and the ion of its oxidized form appears at m/z 267.3. Reprinted (Adapted) with permission from Zhang, Y.; Dewald, H. D.; Chen, H. J. Proteome Res. 2011, 10, 1293-1304. Copyright 2011 American Chemical Society.
Table 4.3. Assignment of the two chains for precursor peptides P3-P5 based on the measured MWs. Reprinted (Adapted) with permission from Zhang, Y.; Dewald, H. D.; Chen, H. J. Proteome Res. 2011, 10, 1293-1304. Copyright 2011 American Chemical Society.

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<th>Combination of two chains (MW in Da)</th>
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<th>Assigned precursor peptides (MW in Da)</th>
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In addition, the connectivities of disulfide bonds in this case can be possibly assigned based on information acquired by tandem MS analysis. For instance, the doubly charged ion (m/z 628) of the chain B of P4 generated from electrolysis gives rise to fragment ions of $b_4$, $b_4$-$NH_3$, $b_5$, $b_5$-$NH_3$, $b_6$, $b_7$, $b_7$-$NH_3$, $b_8$, $b_9$, $b_{10}^{2+}$, $y_4$, $y_5$, $y_7$, $y_8$-$NH_3$, $(y_9$-$NH_3)^{2+}$, $y_9$, $y_9^{2+}$ and $y_{10}^{2+}$ upon CID (Figure 4.4e). This set of fragment ions originates from the cleavage of all of the backbone amide bonds of the chain B ion and determines that the chain sequence is FVNQHLCGSHL with one cysteine residue located at the 7th
amino acid site. Since the P4 contains two pairs of disulfide bonds, it is therefore clear that its chain A has three cysteines, of which two of them are paired up and the other one is linked with the Cys\textsuperscript{7} of the B chain FVNQHLCGSHL.

CID spectrum of the singly charged A of P4 (\(m/z\) 913) was further examined (Figure 4.4d), and its fragment ions of \(b_7\), \(b_8\), \(b_9\), \(b_{10}\), \(b_{11}\), \(b_{12}\), \(y_{11}\)-NH\textsubscript{3}, \(y_{12}\), and \(y_{13}\)-NH\textsubscript{3} suggests that the chain A sequence is GIXXXXXASVCSL (X represents an unknown residue). As the CID of triply charged P4 ion (\(m/z\) 856, Figure 4.4b) produces B1-11/A(\(y_8\)^{2+}), B1-11/A(\(y_9\)^{2+}), B1-11/A(\(y_{10}\)^{2+}), and B1-11/A(\(y_{11}\)^{2+}) via the loss of GIVEQ, GIVE, GIV and GI fragments from the chain A, it can be further inferred that the chain A sequence is GIVEQXXASVCSL. Because chain A is known to have three cysteines, it sequence should be GIVEQCCASVCSL, which agrees with its measured MW (Table 4.1). Also, it can be further inferred that the Cys\textsuperscript{6} in the chain A is paired up with Cys\textsuperscript{11} of the chain to form an intra-peptide bond, since the backbone cleavage between these two sites is missing in the CID of triply charged P4 ion (Figure 4.4b). The conservation of the same amino acid residue region during the CID of both P3 and P5 ions (Figures 4.4a and c) was also noticed, confirming such a disulfide linkage assignment. Thus, in P4, the Cys\textsuperscript{7} of chain A forms one inter-peptide bond with Cys\textsuperscript{7} of chain B.

It is therefore evident that both the connectivities of disulfide bonds and the sequence of P4 (Table 4.1) can be determined using the information acquired from this EC/DESI-MS method. In comparison to the previous electrochemical detection of thiol- and disulfide-containing peptides,\textsuperscript{283,278} our method provides much more chemical
information as a result of the online mass spectrometric detection such as the connectivities of disulfide bonds and the sequence information.
Figure 4.4. CID MS$^2$ spectra of a) [P3+3H]$^{3+}$ (m/z 693), b) [P4+3H]$^{3+}$ (m/z 856) and c) [P5+3H]$^{3+}$ (m/z 722), d) [GIVEQCCASVCSL+H]$^{+}$ (m/z 913) and e) [FVNQHLCGSHL+2H]$^{2+}$ (m/z 628). Reprinted (Adapted) with permission from Zhang, Y.; Dewald, H. D.; Chen, H. J. Proteome Res. 2011, 10, 1293-1304. Copyright 2011 American Chemical Society.
We also tested the analysis of the pepsin digested insulin using chemical reduction with TCEP reagent, a traditional protocol to break a disulfide bond. In Figure 4.3c, in addition to some non-cysteine containing peptide ions, only +1 and +2 ions of free chain HLCGSHL was observed at $m/z$ 766.5 and 384.6, respectively. The other five cysteine-containing peptides that were detected in the DESI-MS spectrum (Figure 4.3b) were not seen in Figure 4.3c. It is likely that these ion signals were suppressed by the excess amount of TCEP used in the chemical reduction.

In our EC/DESI-MS analysis, this issue is avoided because the method does not involve chemical reductants. Also, electrolytic reduction of disulfide is much faster than chemical reduction which usually requires 30 min to 2 h for completion. For example, in the case of insulin digest, the electrochemical cell was turned on only for about 1 min (Figure 4.3b inset is the time when the cell was turned on and off) and a good DESI-MS spectrum was acquired. In most cases of the EC/DESI-MS experiments, it is noticed that reduced products could be detected even when the reduction potential was applied for only 30 s (although the electrolytic reduction is instantaneous when the potential is turned on, there is a time delay for transferring the product from EC cell to MS instrument). The short reduction time required and online DESI-MS detection thereby expedite the analysis process.
4.3.2. Reduction of Peptides Containing Intra-Peptide or Symmetric Inter-Peptide Disulfide Bonds

4.3.2.1. [Arg$^8$]-Conopressin G

In the analysis described above, all disulfide-containing peptides resulting from enzymatic digestion contain asymmetric chains with different MWs. However, biologically active peptides containing an intra-peptide disulfide bond (e.g., oxytocin) or symmetric chains linked with an inter-peptide disulfide bond (e.g., GSSG) also occur. For these peptides, the peak of reduced peptide ion might overlap with the $^{34}$S isotopic peak of the intact peptide ion in the acquired MS spectrum. To overcome this problem, our strategy is to adopt fast and selective derivatization of the reduced peptides, using selenamide reagents such as ebselen and NPSP, a new protein/peptide thiol labeling method recently developed in our laboratory. Compared to other reactions to derivatize thiols such as thiol exchange and Michael-addition reactions, selenamide reagents introduced in chapter 2 can selectively and rapidly derivatize protein/peptide thiols in seconds, satisfying the requirement for the recognition of reduced peptides with free thiol groups via online derivatization.

[Arg$^8$]-vasopressin (MW: 1084.2 Da), a peptide containing one intra-peptide disulfide bond was chosen for investigation first. When a solution of 0.1 mM [Arg$^8$]-vasopressin doped with NPSP flowed through the thin-layer electrochemical cell with no potential applied, the singly and doubly charged ions of [Arg$^8$]-vasopressin were detected at $m/z$ 1083.7 and 543.1, respectively. Also, $m/z$ 637.0 and 723.9 from the background were observed (Figure 4.5b).
When a -2.0 V potential was applied, the intensities of \( m/z \) 1083.7 and 543.1 decreased. On the other hand, one new peak at \( m/z \) 699.5 corresponding to the doubly charged ion of the reduced peptide CYFQNCPRG labeled with two selenium tags was clearly observed (Figure 4.5c), which is well separated from the doubly charged intact peptide ion \( m/z \) 543.1 (without derivatization, the resulting doubly charged ion of the reduced peptide would appear at \( m/z \) 544.1 and overlap with the \( S^{34} \) isotopic peak of \( m/z \) 543.1). The double addition of the selenium tags with the reduced peptide (the reaction equation is in Figure 4.5a) indicates that the precursor peptide \([\text{Arg}^8]\)-vasopressin has one intra-peptide disulfide bond. After reduction, the reduced peptide carries two free cysteines that allow double addition of selenium tags.

As expected, upon CID, the derivatized reduced peptide ion of \( m/z \) 699 (Figure 4.5d) shows a series of backbone cleavages, giving rise to a series of \( b \) and \( y \) ions (\( b_2, b_3, b_5, y_2, y_3, y_4, y_5, y_6, \) and \( y_7 \)) plus \( a_2, a_3-NH_3, \) and \( a_4-NH_3 \). In contrast, the doubly charged intact \([\text{Arg}^8]\)-vasopressin ion (\( m/z \) 543.1) (Figure 4.5e) dissociates into fewer fragment ions upon CID owing to the presence of the disulfide bond linkage.

Again, this data demonstrates that increased structural information can be obtained via electrolytic reduction of disulfide bonds. Another ion arising from the reduction was observed at \( m/z \) 643.1, corresponding to the reduced peptides labeled with one selenium tag and one mercury atom. The CID spectrum of \( m/z \) 643 (Figure 4.6) shows that the mercury is covalently attached to the peptide at the first cysteine residue. The origin for this mercury-thiolate product remains unclear and we did not see the similar products for other peptides examined in this study. It is likely that the mercury-
thiolate product was formed *via* anodic oxidation of thiol product in the presence of mercury\(^{286}\) or *via* disulfide bond reduction on mercury surface as an intermediate.\(^{273}\)

In addition, similar EC/DESI-MS results were obtained for oxytocin (MW: 1007.2 Da), another biologically active peptide containing one intra-peptide disulfide bond. The electrolytic reduction cleaved its disulfide bond and reduced oxytocin with two free cysteines underwent the double addition with ebselen, another selenamide reagent, which was detected by DESI-MS. The spectra and reaction equation are in the Figure 4.7.
Figure 4.5. a) Scheme of the derivatization of reduced [Arg$^8$]-vasopressin (sequence CYFQNCPRG) by NPSP; DESI-MS spectra acquired when a solution of [Arg$^8$]-vasopressin acetate salt (0.1 mM) and NPSP (0.5 mM) in acetonitrile/water (1:1 by volume) containing 1% acetic acid flowed through the thin-layer electrochemical cell with an applied potential of b) 0.0 V and c) -2.0 V; CID MS$^2$ spectra of d) the doubly charged ion of intact [Arg$^8$]-vasopressin (m/z 543) and e) the doubly charged ion of the reduced [Arg$^8$]-vasopressin with two phenylselenenyl tags (m/z 699). Reprinted (Adapted) with permission from Zhang, Y.; Dewald, H. D.; Chen, H. J. Proteome Res. 2011, 10, 1293-1304. Copyright 2011 American Chemical Society.
Figure 4.6. CID MS² spectrum of the ion (m/z 643). Reprinted (Adapted) with permission from Zhang, Y.; Dewald, H. D.; Chen, H. J. Proteome Res. 2011, 10, 1293-1304. Copyright 2011 American Chemical Society.
Figure 4.7. a) Derivatization of reduced oxytocin using ebselen; DESI-MS spectra acquired when a solution of 1 mM oxytocin and 0.5 mM ebselen in acetonitrile/water (1:1 by volume) containing 1% acetic acid flowed through the thin-layer electrochemical cell with an applied potential of b) 0.0 V and c) -1.8 V. Reprinted (Adapted) with permission from Zhang, Y.; Dewald, H. D.; Chen, H. J. Proteome Res. 2011, 10, 1293-1304. Copyright 2011 American Chemical Society.
4.3.2.2. Glutathione Disulfide (GSSG)

GSSG (MW: 612.6 Da), the oxidized form of the cysteine-containing tripeptide GSH, contains two symmetric chains linked with one inter-peptide disulfide bond. It is known from the previous study\textsuperscript{141} that the peak of singly charged GSH ($m/z$ 308) generated by electrolytic reduction of GSSG overlaps with the $^{34}$S isotopic peak of the doubly charged GSSG ion ($m/z$ 307). In this study, this issue can be overcome using on-line rapid derivatization of the product GSH by ebselen.

In the experiment, the solution of GSSG was doped with ebselen and injected through the electrochemical flow cell. The singly and doubly charged GSSG ions at $m/z$ 613.0 and 307.0, respectively, were detected (Figure 4.8a). When -2.0 V potential was applied to the WE of the cell, a new peak $m/z$ 582.9 appears (Figure 4.8b), which corresponds to the protonated ion of the derivatized GSH with one ebselen tag and clearly separated from the GSSG peaks. This assignment was verified by its CID MS\textsuperscript{2} spectrum which shows major fragment ions of the protonated ebselen and GSSG. Also, the intensities of [GSSG+H]\textsuperscript{+} and [GSSG+2H]\textsuperscript{2+} decreased, indicating the consumption of GSSG during electrolysis. Unlike [Arg\textsuperscript{8}]-vasopressin and oxytocin which contain an intra-disulfide bond, the reduced GSSG product contains one free cysteine residue so that only the single addition of ebselen to GSH is possible. This feature is useful in determining whether or not the disulfide bond of the examined peptide is in inter- or intra-peptide form. Further, the number of selenium tags on the reduced peptides points out the number of their free cysteine residues.
Figure 4.8. DESI-MS spectra acquired when a solution of GSSG (0.25 mM) and ebselen (0.5 mM) in methanol/water (1:1 by volume) containing 1% acetic acid flowed through the thin-layer electrochemical cell with an applied potential of a) 0.0 V and b) -2.0 V. Reprinted (Adapted) with permission from Zhang, Y.; Dewald, H. D.; Chen, H. J. Proteome Res. 2011, 10, 1293-1304. Copyright 2011 American Chemical Society.
4.3.3. Reduction of Intact Proteins Containing Disulfide Bonds

We further examined the protein α-lactalbumin (MW: 14178.0 Da) from bovine milk for the EC/DESI-MS. This protein (sequence is in Figure 4.9a inset) has 123 amino acid residues and contains four disulfide cross-links (Cys\textsuperscript{6}-Cys\textsuperscript{120}, Cys\textsuperscript{28}-Cys\textsuperscript{111}, Cys\textsuperscript{61}-Cys\textsuperscript{77}, and Cys\textsuperscript{73}-Cys\textsuperscript{91}) to maintain and stabilize its three-dimensional structure in solution.\textsuperscript{287} To investigate the electrolytic reduction of the disulfide bonds in bovine α-lactalbumin, we also adopted the selenamide derivatization strategy as it is helpful to recognize the multiply charged reduced protein ions from those intact protein ions using our ion trap mass spectrometer with unit mass resolution.

Figure 4.9a is the DESI-MS spectrum acquired when a solution of α-lactalbumin (20 µM) and ebselen (0.2 mM) in acetonitrile/water (1:1 by volume) containing 1% acetic acid flowed through the thin-layer electrochemical cell, the multiply charged ions of intact α-lactalbumin with a charge distribution of +8 ~ +12 centered at +10 were detected. When a -2.1 V potential was applied to trigger protein reduction, the abundance of α-lactalbumin ions decreased as exemplified by the XIC of the +10 protein ion (m/z 1418.9, Figure 4.9a inset). Besides, the multiply charged ions of α-lactalbumin carrying one, two, three and four ebselen tags arose (Figure 4.9b), suggesting that the protein underwent electrolytic reduction and the subsequent chemical reactions with ebselen (an EC mechanism). Also, based on the maximum number of the ebselen tags, it is indicative that the protein underwent two disulfide bond cleavages, leading to totally four free cysteine residues for derivatization. The partial reduction of disulfide bonds in α-lactalbumin suggests that the remaining two disulfide bonds are sterically hindered in the
interior area of the protein while the other two are accessible for the electrolytic cleavage on the amalgam surface.

Indeed, according to the literature report, $^{288}$Cys$^6$-Cys$^{120}$ is the easiest one to be reduced and Cys$^{28}$-Cys$^{111}$ is the second easiest one to be cleaved. Our experimental results show that $\alpha$-lactalbumin protein can be electrochemically reduced and the resulting reduced proteins were detected online by MS. It is very likely that these partially reduced $\alpha$-lactalbumin ions can be interrogated by MS/MS to provide more structurally informative fragment ions than the intact one, which was not further explored in our experiment as a result of the low resolving power of the ion trap used.

In addition, examination of Figure 4.9b also reveals the role of disulfide bonds on maintenance of the protein conformational structures. As mentioned above, liquid sample DESI offers a convenient way to probe protein conformational changes in solution since the sample can be ionized directly without sample pre-treatment.$^{73}$ A simple but effective way to detect the conformational change of proteins is to record the shift of CSD of protein ions.$^{289-291}$ Typically, when a protein is in the folded structure, a narrow CSD in low charge states is observed while CSD is broadened and shifted to high charge states after unfolding. This is probably because that, in comparison to a folded protein, the unfolded one has a greater capacity to accommodate a significantly large number of charges on its surface.

In this experiment, electrochemical reduction can serve as a good stimulant to trigger protein conformational changes simply via electrolytic reduction processes, which can be online monitored with DESI-MS. Prior to electrolysis, the intact $\alpha$-lactalbumin
exhibits the CSD of +8~+12 with the most abundant peak located at +10 ion. These charge numbers are relatively low, even under the denaturing solvent environment (containing acetonitrile and 1% acetic acid) in this experiment, probably owing to the presence of four disulfide bonds which limits the extent of the protein unfolding.

After electrolysis, the CSD of the reduced α-lactalbumin ions with ebselen tags has shifted to higher charge states (+9 ~ +16) and the most abundant charge state centered at +11 (indicated with unlined charge number). This CSD change could stem from the addition of selenium tags or the conformational changes of the protein as a result of the disulfide bond cleavages.

The former speculation is less unlikely because the addition of selenium tags in the peptide cases discussed above (e.g., oxytocin and GSH) does not help to increase the charges of derivatized peptide ions. No higher charge state was observed in the case of protein β-lactoglobulin A after derivatization by ebselen in my previous experiment. It is more likely that the observed CSD shift is a result of the cleavage of disulfide bonds in the protein.

In this experiment with α-lactalbumin, the electrolytic cleavage of two disulfide bonds results in protein unfolding so that the protein can carry more charges. This phenomenon is in agreement with the early ESI report that increased charge occurred to the detected protein ions after the cleavage of their disulfide bonds. It is also consistent with the previous report that the two-disulfide form of α-lactalbumin retains only half the secondary and tertiary structures of the intact α-lactalbumin. Indeed, once the α-lactalbumin was completely reduced by TECP and then ionized by ESSI, a variant form
of ESI, a further CSD shift to +19 was noticed. These results suggest that, like the first two disulfide bonds reduced in the electrolysis, the two remaining disulfide bonds are also responsible in keeping protein folded. Thus, it can be seen that the EC/DESI-MS can provide unique insights into the role of disulfide bonds of a protein in maintaining its conformation.

**Figure 4.9.** DESI-MS spectra acquired when a solution of α-lactalbumin (20 µM) and ebselen (0.2 mM) in acetonitrile/water (1:1 by volume) containing 1% acetic acid flowed through the thin-layer electrochemical cell with an applied potential of a) 0.0 V (the insets show the intact protein sequence and the XIC of the +10 protein ion) and b) -2.1 V (the superscripts on the charge numbers labeled in the spectrum indicates the number of the added ebselen tags to the reduced protein ions). Reprinted (Adapted) with permission from Zhang, Y.; Dewald, H. D.; Chen, H. J. Proteome Res. 2011, 10, 1293-1304. Copyright 2011 American Chemical Society.
4.4. Conclusions

EC/DESI-MS is a powerful tool for the structural elucidation of different types of disulfide bond containing peptides and proteins and for mixture analysis of protein/peptide digests. Based on the relative ion abundance change during electrolysis, one can identify the disulfide-containing peptides from others in enzymatic digest mixtures. In conjunction with mass mapping and tandem mass analysis, peptide sequencing and location of the disulfide linkages is possible. Adoption of selective selenamide derivatization reactions facilitates the analysis of intra- and inter-peptide disulfide containing peptides by the EC/DESI-MS. The effect of disulfide bonds on maintaining protein conformational structures can also be investigated based on the CSD shifts of protein ions after reduction. The reported electrochemical mass spectrometry for disulfide analysis is fast, simple and controllable by switching on/off the electrolysis potential. No chemical reductant is involved so that direct ionization after reduction is feasible. In addition, unlike the gas-phase ion activation methods used for the cleavage of disulfide bond which could lead to backbone dissociation, our electrochemical reduction takes place in solution and is very selective to disulfide bonds.
5.1. Introduction

LC/MS is an analytical technique that combines the physical separation capability of LC with the mass analysis power of MS. Because of its unparalleled capability in mixture analysis, the significance of LC/MS is hard to overstate and the coupling of LC with MS was realized by using various ionization methods such as chemical ionization (CI), thermospray ionization (TSP), APCI and predominantly ESI.

A recent breakthrough in the field of MS is the advent of ambient mass spectrometry such as DESI and DART which provide direct ionization of analytes with little or no sample preparation. Very recently, the coupling of LC to DART-MS has been reported, which showed that DART is compatible with eluents containing phosphate buffers at high elution flow rates. However, the combination of LC with MS using DESI has not been realized so far, because traditional DESI deals with the analysis of solid samples on surfaces. In our laboratory, DESI has been extended to direct analysis of continuous flow liquid samples. Liquid sample DESI can be used to ionize a wide range of molecules, from small organic molecules to high mass proteins. Different from ESI, liquid sample DESI can be used to directly analyze salt-containing sample solutions and does not require “make-up” solvents/acids to be doped in a sample prior to ionization. For instance, urine samples or an electrolyzed solution (from an
electrochemical cell) can be directly ionized by DESI.\textsuperscript{27,141} Thus it is intuitive to explore the LC/MS interfaced by DESI.

In this study, we have successfully combined LC with MS using liquid sample DESI for the first time. DESI is shown to tolerate a wide range of elution flow rates (e.g., up to 1.8 mL/min, even when the eluent does not contain any organic solvents). Reactive DESI,\textsuperscript{8,67} in which a chosen chemical reagent is doped with the DESI spray solvent, is applicable in this LC/DESI-MS method, for selective derivatization of separated analytes following the chromatographic separation. Furthermore, we have introduced an electrochemical cell between the LC and DESI-MS to construct a combined LC/EC/DESI-MS system, which allows the separated analytes to further undergo redox reactions prior to MS detection. The conjunction with electrochemistry broadens LC/DESI-MS applications such as fast structural elucidation of disulfide-containing peptides from enzymatic digests as demonstrated in this work.

5.2. Experimental Section

5.2.1. Chemicals and Materials

Norepinephrine hydrochloride (NE), DL-normetanephrine hydrochloride (NM), DA, ammonium formate (\(\geq 99.995\%\) metals basis), and HPLC-grade acetonitrile were obtained from Sigma-Aldrich (St. Louis, MO). Acetic acid, formic acid, \(N\)-methyl-4-pyridineboronic acid and HPLC-grade methanol were purchased from Fisher Scientific (Fair Lawn, New Jersey), Spectrum (Gardena, CA), Wako Pure Chemical Industries, Ltd. (Japan) and GFS Chemicals (Columbus, OH), respectively. The de-ionized water used for sample preparation was obtained using a Nanopure Diamond Barnstead purification
system (Barnstead International, Dubuque, IA). A mixture solution of NE, NM and DA (3 mg/mL) were prepared in water.

5.2.2. Instrumentation

All experiments were carried out using a Thermo Finnigan LCQ DECA or DECA MAX ion trap mass spectrometer (San Jose, CA) and a Perkin Elmer HPLC system (Perkin Elmer, Shelton, CT) with an Agilent C18 column (250 mm×4.6 mm i.d.). The DESI spray voltage was set at +5 kV and the nebulizing gas (N₂) pressure used was 170 psi. Unless specified otherwise, the DESI spray solvent was 1% acetic acid in acetonitrile or in methanol/water (1:1 by volume) and sprayed at 10 µL/min. In this study, three different apparatus configurations, including the prototype LC/DESI-MS, LC/reactive DESI-MS and LC/EC/DESI-MS, were introduced and described below, showing the versatility of the DESI interface.

5.2.3. Method

Digestion of somatostatin 1-14

4 mg/mL digested somatostatin 1-14 (MW: 1637.9 Da) carried out using TPCK-treated trypsin with a ratio of 1:100 (enzyme/peptide) in 25 mM ammonium bicarbonate aqueous solution for 3 h at 38 °C incubation.

Separation of the tryptic digest of somatostatin 1-14

A binary solvent (solvent A: water containing 0.09% TFA; solution B: acetonitrile/water (3:2 by volume) containing 0.09% TFA) was employed. A 2-min elution with 100% A solution, 10-min linear gradient from 0 to 100 % solution B, 5-min
elution with 100% solution B, 3-min linear gradient from 0 to 100 % solution A and 3-min elution with 100% solution A was used for HPLC separation. The volume of the digest loaded for separation was 5 µL. 4 mg/mL tryptic digested somatostatin 1-14 first underwent LC separation, an ASI adjustable splitter was used to reduce the flow rate to 5 µL/min and then electrochemical reduction prior to DESI ionization. The rest eluents were flowed through the UV detector (detection wavelength was set at 254 nm). A thin-layer µ-PrepCell™ electrochemical flow cell equipped with a MD electrode (diameters: 12 × 30 mm, Antec Leyden, Netherlands) as the WE was employed and a Roxy™ potentiostat (Antec Leyden, Netherlands) was used to apply a potential of −1.5 V to the cell for triggering electrolytic reduction. The reduced species flowed out of the thin-layer cell via a short piece of fused silica capillary (i.d. 0.1 mm, 4.7 cm long) and then underwent DESI ionization.

5.3. Results and Discussion

5.3.1. LC/DESI-MS

The prototype LC/DESI-MS apparatus (Figure 5.1a) allows the direct DESI-MS analysis of the eluent with a high flow rate without splitting.

As a demonstration, a 10 µL mixture consisting of three neurotransmitter compounds (3 mg/mL each), NE, NM, and DA, first underwent LC separation using an isocratic elution with the mobile phase being 50 mM aqueous ammonium formate (pH 3.0 adjusted with formic acid), flowed through the UV detector (detection wavelength was set at 266 nm) and then was subject to ionization by DESI. As shown in the chromatogram (Figure 5.1b), three peaks were observed, corresponding to NE, NM and
DA, respectively. In the experiment, the connection between the UV detector and DESI ion source was a piece of fused silica capillary (i.d. 0.1 mm). As a result of the high eluent flow rate of 1.8 mL/min used, a stable liquid jet emerging from the outlet of capillary was produced. Interestingly, it is convenient to use DESI to ionize the jet sample via directing the charged droplet beam from DESI spray to interact with the jet. A similar phenomenon was noted in fast kinetics study by our group.74

In Figures 5.1c-e, the acquired DESI-MS mass spectra of the three separated NE, NM, and DA, clearly show the corresponding protonated ions at m/z 170, 184 and 154, respectively. In addition, the XICs of m/z 170, 184 and 154 are in the Figure insets. These XICs agree well with the UV chromatogram and there is less than 3s delay between the UV and DESI-MS detection, owing to the high elution flow rate used. These results suggest that DESI is a feasible interface for LC/MS, even at a high flow rate which would suit the usage of an ordinary analytical separation column without splitting or fits the need of preparative chromatography. Noted that, in this experiment, the aqueous eluent containing no organic solvent was ionized directly by DESI. This is in contrast to the previous LC/APCI-MS study in which acetonitrile needed to be added into the eluent via a post-column addition tee for efficient ionization.294 In Figures 5.1c-e, m/z 152 came from background. In Figure 5.1d, m/z 166 arose from the protonated NM (m/z 184) by loss of one water molecule.

We further tested the low elution flow rate for the DESI interface. When an ASI adjustable splitter was used to reduce the flow rate to 4.5 µL/min, DESI-MS spectra with high signal intensities were also obtained for the three separated transmitter compounds
(Figure 5.2). In this case, the total amount of each analyte compound introduced into the DESI ion source was 75 ng, suggesting good sensitivity with DESI ionization. Thus one can see that the DESI interface allows a wide range of flow rates for LC/MS coupling. We also compared DESI with traditional ESI as an interface in this LC/MS experiment. At high elution flow rate (>1 mL/min), ESI failed to produce ion signals due to the formation of a “flood” in the ion source. At low flow rates (4.5 μL/min) ESI is more sensitive than DESI but by less than 10 fold.

![Diagram of a prototype LC/DESI-MS apparatus]

Figure 5.1. (a) Scheme of a prototype LC/DESI-MS apparatus; (b) acquired UV chromatogram showing the separation of NE, NM and DA (baseline corrected); DESI-MS mass spectra showing the detection of (c) NE, (d) NM and (e) DA following the chromatographic separation. Reproduced Zhang, Y.; Yuan, Z.; Dewald, H. D.; Chen, H. Chem. Comm. 2011, 47, 4171-4173. by permission of The Royal Society of Chemistry.
Figure 5.2. (a) Scheme of an apparatus for the coupled LC/MS using DESI interface, in which the eluent flow was reduced to 4.5 μL/min using a splitter; (b) acquired UV chromatogram (266 nm) using a C18 column and the isocratic mode (baseline corrected); DESI-MS mass spectra showing the separated (c) NE, (d) NM and (e) DA. Reproduced Zhang, Y.; Yuan, Z.; Dewald, H. D.; Chen, H. Chem. Comm. 2011, 47, 4171-4173, by permission of The Royal Society of Chemistry.

5.3.2. LC/Reactive DESI-MS

Post-column derivatization in LC/MS is often necessary, especially when the analyte is difficult to ionize. Typically, the protocol for derivatization is to introduce a chemical reagent solution that merges with the chromatographic eluent via a Tee mixer. Such a mixing causes an increased time delay for MS ionization, leading to peak broadening resulting from diffusing effects.
In our LC/DESI-MS experiment, reactive DESI can be performed, in which a selective chemical reagent can be doped with the DESI spray solvent so that the analyte can be derivatized and ionized simultaneously via ion/molecule reactions. For demonstration, we still used the neurotransmitter mixture as the sample and adopted selective boronic acid chemistry for derivatization. It is known that phenylboronic acid can selectively bind cis-diol to form a stable cyclic boronate via complexation reactions (the reaction equations are in Figure 5.3a), which will be introduced in details in chapter 7. In this study, 0.1 mM N-methyl-4-pyridineboronic acid iodide in acetonitrile was used as the DESI spray solution to provide a reagent ion of the positively charged N-methyl-4-pyridineboronic acid. The permanent charge carried by the reagent ion is helpful in enhancing the sensitivity of DESI-MS analysis. In Figure 5.3a, the mixture was subject to LC separation (under the same elution conditions as mentioned above), flow splitting and then DESI ionization.

In Figures 5.3b and d, the product ions of $m/z$ 271 and 255, arising from the complexation reactions of the reagent ions with NE and DA, respectively, via losses of two molecules of water, were clearly detected. In contrast, no such product ion was seen for the compound NM in Figure 5.3c as one of its cis-diol was methylated. Only $[\text{NM+H}]^+$ ($m/z$ 184), $[\text{NM-H}_2\text{O+H}]^+$ ($m/z$ 166) and $[\text{NM+CH}_3\text{CN+H}]^+$ ($m/z$ 225) were observed. It is evident that both NE and DA containing the cis-diol functionality react with the reagent ion selectively while NM with one protonated hydroxyl group does not, consistent with their structures. It can be seen that LC/reactive DESI-MS is applicable to
in-situ derivatization after chromatographic separation, recognizing the structural functionalities of the analytes and increasing detection selectivity.

**Figure 5.3.** (a) Scheme of the apparatus for LC/reactive DESI-MS; reactive DESI-MS mass spectra showing the ions of products generated from the reactions between N-methyl-4-pyridineboronic acid iodide and (b) NE, (c) NM or (d) DA, respectively (background was subtracted). Reproduced Zhang, Y.; Yuan, Z.; Dewald, H. D.; Chen, H. *Chem. Comm.* 2011, 47, 4171-4173. by permission of The Royal Society of Chemistry.

### 5.3.3. LC/EC/DESI-MS

The addition of an EC component with LC/MS has proven useful in many applications such as mimicking biologically relevant electrochemical reactions, increasing analyte ionization efficiency via electrochemical conversion, and on-line chemical tagging. In this regard, ESI has been the dominant technique. However, it has
been revealed that liquid sample DESI has valuable characteristics that facilitate the combination of EC with MS. These include a simplified coupling apparatus with no need to separate the small potential applied to the electrochemical cell from the high voltage used for spray ionization, tolerance to inorganic salt electrolytes, and the freedom to choose traditional solvents for electrolysis, as well as, to use either positive or negative ionization modes. Therefore it is advantageous to integrate an EC component into the LC/MS by DESI.

In Figure 5.5a, our LC/EC/DESI-MS assembly employs a thin-layer ReactorCell™ or µ-PrepCell™ electrochemical flow cell equipped with a MD electrode (Antec Leyden, Netherlands) as the WE. The cell is placed between splitter and the DESI source. The splitter is used to control the flow rate, in consideration with the fact that the lower the flow rate, the higher the electrochemical conversion yield can be obtained. After chromatographic separation, compounds in the eluent undergo redox reactions in the cell and the electrolyzed species flowing out of the thin-layer cell via a short piece of fused silica capillary are then subject to DESI ionization. A Roxy™ potentiostat (Antec Leyden, Netherlands) was used to apply a potential to the cell for triggering electrolytic oxidation or reduction.

As a proof-of-principle experiment, the neurotransmitter mixture was first tested with the LC/EC/DESI-MS. As expected, when the electrochemical cell was on, the ions corresponding to the oxidized products of NE and DA were seen at m/z 168 and 152, respectively (Figure 5.4). By contrast, no product ion resulting from oxidation of the
electrochemically inactive NM was observed. These results showed the viability of the combination of LC, EC and MS by DESI.

Figure 5.4. Acquired DESI mass spectra showing the products after electrochemical oxidation of (a) NE, (b) NM and (c) DA following chromatographic separation. The structures of ions resulting from the oxidation of NE and DA were marked in (a) and (c), respectively. Reproduced Zhang, Y.; Yuan, Z.; Dewald, H. D.; Chen, H. Chem. Comm. 2011, 47, 4171-4173. by permission of The Royal Society of Chemistry.
The LC/EC/DESI-MS coupling has unique applications in proteomics, for instance, in fast structural elucidation of disulfide-containing peptides via online electrochemical reduction. Traditionally, the presence of the disulfide linkages increases the complexity for the protein/peptide structure elucidation by MS. Thus the cleavage of disulfide bonds is often essential as dissociation of a reduced protein/peptide ion can give rise to more structurally informative fragment ions than that of the intact counterpart.^{211} The traditional protocol to break a disulfide bond involves the use of chemical reductants like DTT which is time consuming. It is also troublesome to get rid of excess amount of reductants afterwards.

In this study, a disulfide-containing peptide somatostatin 1-14 (MW 1637.9 Da) was chosen as an example for demonstration, which was digested by trypsin to produce a peptide mixture, AGCK/TFTSC (this peptide has two chains of AGCK and TFTSC linked by a disulfide bond), and NFFWK (their sequences are in the inset of Figure 5.5b).

This digest mixture was examined using the LC/EC/DESI-MS. The separation of the mixture was carried out using a gradient elution. In Figure 5.5b, the two peptides eluted at 15.51, and 17.46 min, respectively (these assignments were confirmed by MS/MS spectra acquired, Figure 5.6). Other peaks in Figure 5.5b were also observed, probably originating from the trypsin used.

Figure 5.5c is the DESI-MS of the peptide AGCK/TFTSC eluted at 15.51 min, when no potential applied to the cell. The singly, doubly and triply charged peptide ions were detected at \( m/z \) 933.2, 467.1, and 311.7, respectively. When a \(-1.5\) V potential was applied to the cell for reduction, these peptide ion peaks totally disappeared (Figure 5.5d),
indicating a 100% reduction yield (probably due to a large MD electrode of 12×30 mm² used). Instead, two new ions of $m/z$ 378.1 and 558.0 were observed. As the electrochemical reduction occurs after chromatographic separation, it is reliable to conclude that the two ions are from the reduction products of the AGCK/TFTSC. Indeed, they correspond to the protonated ions of two chains AGCK and TFTSC, respectively. Further, the sum of the MWs of the two products (378.1+558.0−2=934.1 Da) is higher than that of precursor peptide (932.2 Da) by 1.9 Da, suggesting that the precursor peptide has one disulfide bond.

In addition, tandem MS analysis was performed to elucidate the electro-generated ion structures, for establishing the disulfide bond connectivity as well as the sequence of the peptide AGCK/TFTSC. Upon CID, the $m/z$ 378.1 gives rise to $b_2$, $b_3$, $b_4$, $y_1$, and $y_2$ fragment ions (Figure 5.7a), from which its sequence can be determined as either AGCK or GACK (cysteine is in 3rd position). Likewise, the $m/z$ 558.0 dissociates into $a_2$, $b_2$, $b_3$, $b_4$, $b_5$, and $y_3$ ions (Figure 5.7b), which reveals its sequence to be either TFTSC or FTTSC and the cysteine residue to be the 5th position in chain. Thus it can be seen that the sole disulfide bond of AGCK/TFTSC bridges the 3rd residue of one chain with the 5th residue of the other chain.

These results show that, by using the LC/EC/DESI-MS, one can obtain useful sequence information of the examined peptide and explicitly establish the connectivity of the disulfide bond. Also, the protonated peptide NFFWK containing no disulfide bond remained unchanged once the cell potential was applied. This suggests that LC/EC/DESI
can also be used to differentiate disulfide-bond containing peptides from others in an enzymatic digest.

Figure 5.5. (a) Scheme of the apparatus for LC/EC/DESI-MS; (b) acquired UV chromatogram (254 nm) showing the separation of the peptide tryptic digest (baseline corrected); DESI-MS mass spectra of AGCK/TFTSC with a cell potential of c) 0.0 V and d) -1.5 V (background was subtracted). Reproduced Zhang, Y.; Yuan, Z.; Dewald, H. D.; Chen, H. Chem. Comm. 2011, 47, 4171-4173. by permission of The Royal Society of Chemistry.
Figure 5.6. CID MS$^2$ spectra of a) m/z 933 and b) m/z 741. Reproduced Zhang, Y.; Yuan, Z.; Dewald, H. D.; Chen, H. Chem. Comm. 2011, 47, 4171-4173. by permission of The Royal Society of Chemistry.
Figure 5.7. CID MS² spectra of a) [AGCK+H]⁺ (m/z 378) and b) [TFTSC+H]⁺ (m/z 558). Reproduced Zhang, Y.; Yuan, Z.; Dewald, H. D.; Chen, H. Chem. Comm. 2011, 47, 4171-4173, by permission of The Royal Society of Chemistry.
5.4. Conclusions

In summary, the study shows that DESI can be a versatile interface for combing LC with MS, which allows a wide range of elution rates, online derivatization using reactive DESI and integration of EC, which should find many valuable applications in mixture analysis, EC and experiments related to proteomics.
Chapter 6: Electrochemistry-Assisted Top-Down Characterization of Disulfide-Containing Proteins

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6.1. Introduction

Top-down MS\textsuperscript{179, 184, 298-303} has unparalleled strength in protein analysis, which can be used to rapidly identify protein identity\textsuperscript{5, 184, 300} and to provide extensive molecular connectivity information including labile PTMs\textsuperscript{304}.

Despite these striking advantages, it has some challenging issues. For instance, in the case of large proteins, the fragments resulting from ECD\textsuperscript{305} may not be separated from each other due to non-covalent intramolecular interactions of the residues\textsuperscript{304}. Demonstrated solutions for this issue include prefolding-dissociation (PFD)\textsuperscript{169} and AI-ECD\textsuperscript{170} which unfold proteins and break the intramolecular interactions prior to ECD.

Likewise, covalent disulfide bond linkage in a protein represents another challenge for top-down protein analysis. Disulfide bond is one of the most common PTMs (~19% proteins contain multiple disulfide bonds\textsuperscript{306}), which is vital for maintaining the protein structure stability\textsuperscript{307}. However, the dissociation efficiency of the protein backbone by electron-based ion dissociation is greatly reduced in the presence of disulfide bonds as the latter would be preferentially broken\textsuperscript{5-6}, which is exemplified by no fragment ions\textsuperscript{6} observed for ECD of ribonuclease A ions and a poor sequence coverage for the disulfide-bond protected regions of antibody IgGs\textsuperscript{308}. Thus top-down protein
analysis with high sequence coverage is limited to those without disulfide bonds or those with relatively simple disulfide linkages like insulin.\textsuperscript{6}

For proteins containing complicated disulfide bonds such as lysozyme,\textsuperscript{309} chemical reduction of disulfide bonds using reducing reagents like DTT are often performed prior to top-down analysis. Such a chemical reduction is usually not carried out online with MS analysis of proteins/peptides. Offline reduction therefore could limit the applications of top-down analysis for shotgun proteomics or high throughput analysis. Furthermore, the chemical reduction takes minutes to hours, and the removal of the excess amount of reductants is also time-consuming and troublesome.\textsuperscript{211}

To tackle this critical problem, our strategy in this study is to couple EC with top-down MS, in which proteins are rapidly reduced electrochemically followed with online MS and tandem MS analysis. Remarkably, detectable protein backbone cleavages was significantly enhanced by 3-13 folds with the assistance of electrochemical reduction that removes the protein disulfide bond constraints, as revealed by the ECD and CID data of two chosen proteins, $\beta$-lactoglobulin A and lysozyme.

6.2. Experimental Section

6.2.1. Chemicals and Materials

$\beta$-Lactoglobulin A from bovine milk (MW: 18363 Da), lysozyme from chicken egg white (MW: 14300 Da), and formic acid (~98\%) were all purchased from Sigma-Aldrich (St. Louis, MO). HPLC-grade methanol was obtained from GFS Chemicals (Columbus, OH). The de-ionized water used for sample preparation was obtained using a
Nanopure Diamond Barnstead purification system (Barnstead International, Dubuque, IA).

6.2.2. Instrumentation

The configuration of the EC/DESI-MS apparatus is in Figure 6.1. The electrochemical flow cell consists of a MD WE (12×30 mm² surface area, Antec BV, Netherlands), a titanium AE and a HyREF™ RE. The WE and AE were separated by two spacers (50 µm thickness). A ROXY potentiostat (Antec BV, Netherlands) was used to apply potentials to the electrochemical cell for the reduction of proteins that flowed through the cell. The protein samples were prepared in methanol/water (1:1 by volume) containing 0.5% formic acid. The reduced proteins flowed out of the cell via a piece of fused silica connection capillary (i.d. 0.1 mm, 7.5 cm long) and underwent interactions with the charged microdroplets from the DESI spray for ionization. The capillary outlet was placed about 1 mm downstream from the DESI spray probe tip and kept in line with the DESI sprayer tip and the MS inlet orifice. The flow rate for sample solutions passing through the electrochemical cell for electrolysis was 4 µL/min. The spray solvent for DESI was methanol/water (1:1 by volume) containing 1% acetic acid and injected at a flow rate of 5 µL/min. A high voltage of +5 kV was applied to the spray solvent for generating charged droplets with nebulization gas N₂ pressure being 170 psi.

To acquire ECD spectra, the cathode filament of the FT-ICR instrument was conditioned at 1.6 A. To record the spectra the ECD lens was set to 10 V, and a pulse length of 50 ms and ECD bias of 0.7 V was employed. For CID, the collision voltage was set between 11 and 18 V for ions of different m/z values. Each acquired spectrum was the
average of 100~120 broadband 1 M time-domain transient. The Bruker SNAP 2.0
algorithm was used to pick the peaks and Bruker Data Analysis 4.0 was used to generate
fragment mass lists which were searched using Bruker BioTools and ProsightPTM, and manually checked, to assign the cleavage sites in the protein sequence.

Figure 6.1. Scheme of the apparatus for online coupling of a thin-layer µ-PrepCell™
electrochemical flow cell with a Bruker 12 T FT-ICR-MS using DESI as the interface.
Reprinted (Adapted) with permission from Zhang, Y.; Cui, W.; Zhang, H.; Dewald, H. D.;

6.2.3. method

The experimental apparatus of this methodology consists of a thin-layer µ-
PrepCell™ electrochemical flow cell (Antec BV, Zoeterwoude, Netherlands) online
coupled with a Bruker 12 Tesla SolariX FT-ICR-MS (Bruker Daltonics, Bremen,
Germany) using DESI as an interface. In our laboratory, DESI has been shown to be
suitable for transfer/ionize electrolyzed compounds from solution to the gas phase for MS
detection.3-4, 27, 141

6.3. Results and Discussion

6.3.1. CID and ECD for Electrolytic Reduced $\beta$-Lactoglobulin A

$\beta$-lactoglobulin A containing two disulfide bridges (Cys$^{66}$-Cys$^{160}$ and Cys$^{106}$-
Cys$^{119}$) and one free Cys$^{121}$ (refer to Figure 6.2c for its sequence) was first chosen as an
elementary for this study.

Figure 6.2a is the DESI-MS spectrum acquired when a solution of 15 µM $\beta$-
lactoglobulin A in methanol/water (1:1 by volume) containing 0.5% formic acid flowed
through the electrochemical cell with no potential applied to the cell, the multiply
charged ions of intact $\beta$-lactoglobulin A with a CSD of +10~+18 with the most intense
peak at +15 were detected. When the $+16$ protein ion ($m/z$ 1148.0) was selected for ECD,
only 17 c ions were observed in the acquired spectrum, arising from the cleavage of the
free protein N-terminal where there is no disulfide bond (the major c ions are labeled in
the ECD MS/MS spectrum of Figure 6.2b and all detected fragment ions are marked in
Figure 6.2c). There was no detected fragment ion resulting from broken backbone bonds
that are protected by either or both of the disulfide bonds Cys$^{66}$-Cys$^{160}$ and Cys$^{106}$-Cys$^{119}$.
This result can be accounted for by the aforementioned reason that the cleavage of
protein backbone bonds by ECD in the presence of disulfide bonds has low efficiency,5-6,
and the observation of fragment ions from those disulfide bond-protected regions
requires the cleavage of both backbone and the disulfide bonds.
Likewise, in the case of CID of the +16 ion, it is difficult to produce protein fragment ions via backbone cleavages of the residues enclosed by two disulfide bonds because CID in the positive ion mode is known to be unable to effectively cleave disulfide bonds. As a result, few fragment ions \( b_{16}, b_{17}, b_{18}, b_{19}, b_{20}, b_{21}, b_{22}, b_{23}, b_{24}, b_{25}, \) and \( b_{26} \) were generated from N-terminal cleavages.\(^6,169,312\) (Figures 6.2d and e)

By contrast, when a -1.2 V potential was applied to the electrochemical cell to trigger online disulfide bond reduction, the CSD of protein ions was shifted to higher charges with the appearance of a new population of +19~+22 (Figure 6.2f), indicating a conformational change occurring to the protein due to the reduction.\(^313-315\) This phenomenon is in agreement with the early ESI report that increased charges of the protein ions occurred after the cleavage of disulfide bonds\(^314\) and my previous work in chapter 4.\(^3\) Indeed, the reduction of disulfide bonds in a protein causes the unfolding of the protein, thus allowing the protein to have a greater capacity to accommodate a larger number of charges on its surface.\(^313-315\)

Thus the newly produced +19 ion was chosen for ECD analysis, under the same experimental conditions used for ECD of +16 unreduced protein ion as described above. As shown in the ECD MS/MS spectrum (Figure 6.2g) and the labeled sequence map (Figure 6.2h), 40 \( c \) ions and 26 \( z \) ions were generated. In addition, 23 \( b \) and 12 \( y \) ions were observed in the CID of +19 ion.\(^6,169,312\) (Figures 6.2i and j) These results represent a significant increase the backbone cleavage after electrolytic reduction (totally 73 backbone cleavages from the combined ECD and CID data) in comparison to that before reduction (totally 24 backbone cleavages from the combined ECD and CID data). This
change, the approximately 3 fold enhancement in observable backbone cleavages, is ascribed to electrochemical assistance in removing S-S constraints. It might be also contributed to the resulting increased charge states as a result of disulfide bond reduction.\textsuperscript{179,316} Although the number of ECD cleavages could be further improved by pre-activation of the precursor ions,\textsuperscript{169} this was not performed because the purpose in this study is to investigate the effect of electrochemical reduction.
Figure 6.2. DESI-MS spectra acquired when a solution of 15 µM β-lactoglobulin A in methanol/water (1:1 by volume) containing 0.5% formic acid flowed through the thin-layer electrochemical cell with an applied potential of (a) 0.0 V and (f)–1.2 V; ECD MS/MS spectra of (b) +16 ion of the intact β-lactoglobulin A (m/z 1148.0) and (g) +19 ion of the reduced β-lactoglobulin A (m/z 967.1); The marked ECD backbone cleavage sites of (c) β-lactoglobulin A and (h) reduced β-lactoglobulin A; CID MS/MS spectra of (d) +16 ion of the intact β-lactoglobulin A (m/z 1148.0) and (i) +19 ion of the reduced β-lactoglobulin A (m/z 967.1); The marked CID backbone cleavage sites of (e) β-lactoglobulin A and (j) reduced β-lactoglobulin A. Reprinted (Adapted) with permission from Zhang, Y.; Cui, W.; Zhang, H.; Dewald, H. D.; Chen, H. Anal. Chem. 2012, 84, 3838–3842. Copyright 2012 American Chemical Society.
6.3.2. CID and ECD for Electrolytic Reduced Chicken Lysozyme

We further examined chicken lysozyme, a 14 kDa protein having 129 amino acid residues and containing four disulfide bonds (Cys\textsubscript{6}-Cys\textsuperscript{127}, Cys\textsubscript{30}-Cys\textsuperscript{115}, Cys\textsubscript{64}-Cys\textsuperscript{80}, and Cys\textsubscript{76}-Cys\textsuperscript{94}). The latter two disulfide bonds are intertwined and further enclosed by the former two bonds (Figures 6.3c).

Figure 6.3a is the DESI-MS spectrum acquired when a solution of 15 µM lysozyme in methanol/water (1:1 by volume) containing 0.5% formic acid flowed through the electrochemical cell, the intact lysozyme exhibits the CSD of +7~+10 with the most abundant peak located at +9. Interestingly, no fragments were observed upon CID of +10 ion of \(m/z\) 1430.5 (Figures 6.3b and c). This result is not surprising as the protein has 4 cross-linked disulfide bonds that protect most of the protein sequence.

Again, ECD of +10 ion (\(m/z\) 1430.5) gives rise to few fragment ions of \(c_2\), \(c_3\), \(c_4\), \(c_{12}\), and \(z_8\) with low abundances. (Figures 6.3d and e) This is in agreement with the previous report about the ECD of intact lysozyme ions.\textsuperscript{309} In this case, although the backbone dissociation efficiency is very low, the cleavage of one disulfide bond (Cys\textsubscript{6}-Cys\textsuperscript{127}) by ECD occurred, evidenced by the formation of \(c_{12}\), and \(z_8\).

When a -1.5 V potential was applied to the cell to reduce the protein, the CSD of the protein ions was broadened and shifted to the higher charge states (+7~+16, Figure 6.3f). It appears that the electrochemical reduction can serve as a stimulant to trigger protein conformational change by reducing S-S linkages to eliminate the protein tertiary structure, which can be monitored online with DESI-MS detection. As shown in the CID mass spectrum of +15 ion (\(m/z\) 954.7) of the reduced lysozyme (Figure 6.3g) and the
labeled backbone cleavage sites (Figure 6.3h), $b_7$, $b_9$, $b_{18}$, $b_{27}$, $b_{28}$, $b_{29}$, $b_{30}$, $b_{31}$, $b_{39}$, $y_6$, $y_{10}$, $y_{24}$, $y_{25}$, $y_{26}$, $y_{28}$, $y_{30}$, $y_{31}$, $y_{32}$, $y_{33}$, $y_{37}$, $y_{40}$ and $y_{41}$ were generated after electrolytic reduction of lysozyme, which is in stark contrast with the CID of intact protein ion (Figure 6.3b).

In addition, much more backbone cleavages took place with ECD. As shown in Figures 6.3i and j, 36 $c$ ions and 14 $z$ ions were observed upon ECD of the newly produced +15 ion (this ECD result is similar to ECD of chemically reduced mononitrated lysozyme which shows 59 fragment ions$^{309}$).

Thus, it can be seen that, with electrochemical assistant to remove disulfide bond linkages, the sequence information for lysozyme was remarkably enhanced. Based on the combined CID and ECD data, totally 5 and 66 unique backbone cleavages were resulted before and after electrolysis, respectively). Other methodologies have been reported for enhancing the top-down dissociation efficiency, including CID of low charged lysozyme ions,$^5$ which showed a maximum of 13 backbone cleavages. In contrast, our method is more effective.
Figure 6.3. DESI-MS spectra acquired when a solution of 15 µM lysozyme in methanol/water (1:1 by volume) containing 0.5% formic acid flowed through the thin-layer electrochemical cell with an applied potential of (a) 0.0 V and (f) -1.5 V (the zoomed-in mass range for +14~+16 for the reduced lysozyme is shown in the inset); CID MS/MS spectra of (b) +10 ion of the intact lysozyme (m/z 1430.5) and (g) +15 ion of the reduced lysozyme (m/z 954.7); The marked CID backbone cleavage sites of (c) lysozyme and (h) reduced lysozyme; ECD MS/MS spectra of (d) +10 ion of the intact lysozyme (m/z 1430.5) and (i) +15 ion of the reduced lysozyme (m/z 954.7); The marked ECD backbone cleavage sites of (e) lysozyme and (j) reduced lysozyme. Reprinted (Adapted) with permission from Zhang, Y.; Cui, W.; Zhang, H.; Dewald, H. D.; Chen, H. Anal. Chem. 2012, 84, 3838-3842. Copyright 2012 American Chemical Society.
6.4. Conclusions

It is evident that substantially increased backbone cleavage by ECD and CID (3-13 fold increments as shown with the two chosen proteins in this study) occurs to the electrolytic reduced protein in comparison to intact protein, producing many more structurally informative fragment ions. The electrolytic reduction for the disulfide bonds is fast and takes place instantaneously when the reduction potential is applied. Equally importantly, it is a clean and “green” approach as it does not involve the use of chemical reductants. Overall, the online EC employed is well suited for the top-down protein analysis, which would be particularly valuable in high-throughput proteomics applications. It is also an addition to the study of proteins by combined electrochemistry and mass spectrometry.\textsuperscript{138-139}
Chapter 7: Detection of Saccharides by Reactive Desorption Electrospray Ionization (DESI) Using Modified Phenylboronic Acids


7.1. Introduction

Sugars are essential biological molecules which play fundamental roles in various fields. First, saccharides, either alone or as constituents of glycoproteins, proteoglycans and glycolipids, are critical to cellular events such as recognition, proliferation and signal transduction. Second, saccharides are also key intermediates in bio-fuel processing in which bio-mass is converted into sugars and then into ethanol or methanol. In addition, many body tissues depend on glucose as a primary source of energy. Due to their significant properties, the detection of saccharides is of great importance.

Also, because they usually occur in complex biological matrices, the detection method must have high selectivity and sensitivity. A number of different methods have been developed for detecting saccharides, including electrochemical enzyme-based approaches, FL detection, potentiometric detection. MS is also commonly used for carbohydrate analysis due to its inherent sensitivity and capability for providing molecular structure information. However, intact saccharides are poor analytes for MS and derivatization is often employed prior to the analysis. For instance, the detection sensitivity was shown to be enhanced considerably via the derivatization using various chemical reagents such as the Girard’s reagent T, 1-phenyl-3-methyl-5-pyrazolone, and trimethyl-(p-aminophenyl) ammonium derivatives.
Ambient mass spectrometry\textsuperscript{24, 279} has recently been introduced to provide direct ionization of analytes with little or no sample preparation. DESI developed by Cooks and co-workers\textsuperscript{25} and DART developed by Cody and et al.\textsuperscript{26} were the first two of this new family of technologies. It has been shown that DESI is of great value in the fast analysis of a variety of different analytes ranging from pharmaceuticals to tissue imaging.\textsuperscript{31, 40-41, 43, 50, 332-335} In DESI, ionization occurs \textit{via} the interaction of charged microdroplets generated in a pneumatically assisted electrospray of an appropriate solvent with solid phase samples on surfaces. In addition to being used regularly for solid sample analysis from surfaces, DESI has been recently extended to allow the direct analysis of liquid samples.\textsuperscript{27, 57, 71, 336-338} Reactive DESI\textsuperscript{28, 38-39, 66-67, 339} is a further development in DESI that exploits the potential for coupling specific ion/molecule reactions\textsuperscript{219, 340-347} with the ionization event and so greatly improves the selectivity and efficiency with which compounds with specific functionalities are detected. It involves the use of a spray solution that contains specific reagents intended to allow particular ionic reactions during the sampling process. Compared with traditional methods\textsuperscript{329} employing solution phase derivatization followed by ESI, the online derivation in reactive DESI is much faster (typically taking seconds for one sample analysis), thereby speeding up the analytical process.

It is well known that boronic acids react with \textit{cis}-dial containing compounds in basic aqueous medium through reversible ester formation (Scheme 7.1 is the equilibria between phenylboronic acids and diols).\textsuperscript{348-350} Such strong binding allows boronic acids to be used as the recognition moiety in the construction of sensors for saccharides,\textsuperscript{322, 351}
as nucleotide and carbohydrate transporters,\textsuperscript{352} and as affinity ligands for the separation of carbohydrates and glycoproteins.\textsuperscript{353} In a previous communication,\textsuperscript{67} we reported a reactive DESI method for rapid and selective detection of \textit{cis}-diols employing the boronic acid chemistry. It was shown that monosaccharides like glucose and fructose on surfaces can be desorbed and ionized \textit{via} a heterogeneous ion/molecule reaction with phenylboronate anions \( \text{PhB(OH)}_3^- \) to form esters under ambient conditions. The experiment is unusual in that the product ions resulting from the selective reaction between phenylboronic acid anions and diol compounds were directly detected by mass spectrometry following the reactive interaction event. However, no quantitative analysis was performed in the previous preliminary study.

In this study, the method was improved and extended, focusing on reactions between two modified phenylboronic acids, 3-nitrophenylboronic acid and \(N\)-methyl-4-pyridineboronic acid iodide (structures are in Scheme 7.2), and saccharides (e.g. fructose and glucose) using reactive DESI, in an attempt to develop a highly selective and sensitive method for \textit{in-situ} saccharide detection in complicated biological matrices. The purpose of using modified phenylboronic acids instead of using the parent phenylboronic acid\textsuperscript{67} is to improve the boronic acid reactivity, to enhance the abundance of the reactant ions (thus the reaction yield), or to enable the reaction to occur under neutral or acidic physiological environments.\textsuperscript{354} This study was also driven by our interest in the fundamental ion chemistry at atmospheric pressure and in mechanistic studies of DESI experiments.

Scheme 7.2. The structures of 3-nitrophenylboronic acid, \(N\)-methyl-4-pyridineboronic acid iodide and 2-picoline-4-boronic acid. Reprinted (Adapted) from Zhang, Y.; Chen, H. \textit{Int. J. Mass Spectrom.} 2010, 289, 98–107, Copyright (2010), with permission from Elsevier.
7.2. Experimental Section

7.2.1. Chemicals and Materials

Phenylboronic acid was purchased from Fluka (Ronkonkoma, NY), 3-nitrophenylboronic acid, glucose, fructose, galactose, N-acetyl-d-glucosamine, maltose, cellobiose, maltoheptaose and human serum (type AB male, from clotted male whole blood) were purchased from Sigma–Aldrich (St. Louis, MO). N-methyl-4-pyridineboronic acid was purchased from Wako Pure Chemical Industries, Ltd. (Japan). All solvents used were of HPLC grade purity.

7.2.2. Method and Instrumentation

The apparatus (Scheme 7.3a) designed for liquid sample DESI\(^2\) was first employed for the reactive DESI, in which an ESSI\(^6\) source was used to generate charged microdroplets containing the modified phenylboronic acid reagent and analyte samples of saccharide solutions were introduced via a fused-silica capillary by flow injection onto the DESI surface for desorption and ionization (i.e., the saccharides in solution underwent homogenous phase reactions with the modified phenylboronic acid introduced in charged micro-droplets generated by ESSI).

The spray voltage was set at +5 kV for the positive ion mode and −5 kV for the negative ion mode. The optimized heated transfer capillary tube temperature was 250 °C. The nebulizing gas (N\(_2\)) pressure for ESSI was 170 psi. The ESSI spray solvent used for 3-nitrophenylboronic acid was water (pH was adjusted by adding NH\(_4\)OH) and the solvent for N-methyl-4-pyridineboronic acid iodide was either water or acetonitrile. ESSI spray solutions were injected at a flow rate of 3–5 µL/min by a syringe pump.
Saccharide samples were dissolved in water, urine or human serum and then introduced onto a Teflon surface at a flow rate of 1–5 µL/min by a syringe pump for DESI analysis. In the case of serum samples, in order to prevent the mass spectrometer from being blocked by the desorbed serum sample, a piece of filter paper (not in Scheme 7.3a) was used to cover the inlet of the sample transfer silica capillary so that the serum can be filtered prior to be desorbed and ionized by DESI.

The derivatized sugar product ions were collected and detected using either a Thermo Finnigan LCQ DECA Mass Spectrometer (San Jose, CA) or a Thermo Finnigan LCQ DECA MAX Mass Spectrometer (San Jose, CA). The limit of detections (LODs), defined as three times of standard deviations of the blanks, were calculated using the calibration curve slopes and the standard deviations of the blanks. The 95% confidence intervals for the calibration curve, the slope, and the intercept were calculated from the averages of three replicates for each standard concentration. CID was also used for further structural confirmation of the product ions.

In an alternative apparatus, a small volume of saccharide solutions (200 nL) was introduced as a thin layer of liquid film contained in a pipette tip (VWR pipette tips, 1.5 cm long), which was analyzed directly without air drying. The spray-generated microdroplets containing the chemical reagent of N-methyl-4-pyridineboronic acid iodide were allowed to pass through the saccharide liquid film for ionization. The angle between the electrospray tip and saccharide sample and the angle between the sample and capillary inlet to the mass spectrometer were both set to 0°. This DESI geometry is similar to the transmission mode of DESI reported by Brodbelt’s group.57,338
Furthermore, the distances between the ESSI source and the pipette tip and the distance between the pipette tip and the heated capillary inlet were approximately 2 mm, and the DESI spray solution was injected at a flow rate of 5 µL/min.


7.3. Results and Discussion

7.3.1. Phenylboronic Acids with Electron-withdrawing Groups

In this experiment, 3-nitrophenylboronic acid was the first modified phenylboronic acid chosen for the reactive DESI study. First, we compared the reactivity of parent phenylboronic acid and 3-nitrophenylboronic acid in reactive DESI. Figure 7.1a is the reactive DESI mass spectrum showing interaction of the sprayed charged droplets containing both phenylboronic acid and 3-nitrophenylboronic acid (0.2 mM each in H₂O
with pH=9 adjusted using NH₄OH) with fructose (10 mM in aqueous solution) continuously introduced onto a Teflon surface by a syringe pump through a silica capillary.

The product ion of $m/z$ 328 resulting from the reaction of 3-nitrophenylboronic acid boronate anion ($m/z$ 184) and fructose by loss of two molecules of water (eq. 1, Scheme 7.4) is observed as the base peak in the spectrum. As shown in its CID spectrum (Figure 7.1b), water loss (the formation of $m/z$ 310), sugar ring cleavage (the formation of $m/z$ 284 and 250) and loss of nitrobenzene (the formation of $m/z$ 205) were seen, confirming the product ion structure. Similarly, in Figure 7.1a, the resulting product ions of $m/z$ 283 from the unmodified phenylboronic acid is generated via loss of two water molecules from the complexation of phenylboronate PhB(OH)$_3^-$ ($m/z$ 139) and fructose. Thus, by comparing the abundance of two major of products ions ($m/z$ 328 vs. $m/z$ 283 is about 16: 1), it can be clearly seen that 3-nitrophenylboronic acid is much more reactive than phenylboronic acid, in the competitive reaction with fructose.

This comparison is further confirmed by running the reactive DESI experiments using the two DESI reagents separately under the same experimental conditions and the intensity of the product ion from 3-nitrophenylboronic acid is about 15 times higher than that from phenylboronic acid (see spectra in Figure 7.1). This higher reactivity of 3-nitrophenylboronic acid than phenylboronic acid is because the introduction of electron-withdrawing groups into the ring of a phenylboronic acid stabilizes the boronate form of the acid, favoring ester formation$^{355}$ (e.g., the binding constants of phenylboronic acid and 3-nitrophenylboronic acid with catechol dye, an aromatic diol compound at a pH=7.5
buffer are 1500 and 6110 M<sup>-1</sup>, respectively<sup>356</sup>). In Figure 7.1a, m/z 310 and 265 are produced from water loss of the product ions of m/z 328 and 283, respectively. The deprotonated 3-nitrophenylboronic acid (m/z 166) and deprotonated phenylboronic acid (m/z 121) are also observed. The ion of m/z 346 corresponds to the adduct of deprotonated 3-nitrophenylboronic acid (m/z 166) and fructose. One major peak at m/z 315, corresponds to deprotonated 3-nitrophenylboronic anhydride (commercially available 3-nitrophenylboronic acid contains various amounts of anhydride) and m/z 333, a deprotonated dimer ion of 3-nitrophenylboronic acid. Furthermore, the peaks at m/z 154 ([phenylboronic acid +O<sub>2</sub>F<sup>-</sup>]) and m/z 199 ([3-nitrophenylboronic acid +O<sub>2</sub>F<sup>-</sup>]) come from the dissolved oxygen in the aqueous solution, as evidenced by the observation that these peaks disappeared if degassed spray solutions were used.

![Diagram](image)

**Scheme 7.4.** The equations of modified phenylboronic acids reacted with sugars. Reprinted (Adapted) from Zhang, Y.; Chen, H. *Int. J. Mass Spectrom.* 2010, 289, 98–107, Copyright (2010), with permission from Elsevier.
Reactive DESI using 3-nitrophenylboronic acid as the chemical reagent can be also used to ionize glucose directly from aqueous solutions. Figure 7.1c is the reactive DESI mass spectrum showing interaction of the sprayed charged droplets containing 3-nitrophenylboronic acid anions (0.2 mM in water with pH=9 adjusted by NH₄OH) with glucose (5 mM in aqueous solution).

In Figure 7.1c, the resulting product ion, shown at m/z 328, was generated via loss of two molecules of water from the reaction of 3-nitrophenylboronate (m/z 184) and glucose. Also, the deprotonated anhydride peak is observed (m/z 315) and m/z 346 is the simple adduct of deprotonated 3-nitrophenylboronic acid (m/z 166) and glucose. The CID spectrum of m/z 328 resulting from glucose (Figure 7.1d) shows the consecutive loss of water (the formation of m/z 310, 292 and 274) and the further loss of C₂H₄O₂ and C₃H₆O₂ via ring cleavage from the fragment ion m/z 310 gives rise to m/z 220 and 250.

In this study, the LOD for glucose in water using 3-nitrophenylboronic acid was determined to be 0.11 mM (S/N=3, SIM scan mode was used to monitor the ion of m/z 328), lower than the LOD for glucose in water using phenylboronic acid (0.4 mM, S/N=3, SIM scan mode was used to monitor the ion of m/z 283). The linear dynamic ranges observed for 3-nitrophenylboronic acid and phenylboronic acid are 0−2.0 mM (linear regression coefficient R²=0.96) and 0−20 mM (R²=0.99), respectively.
Figure 7.1. a) Reactive DESI mass spectrum showing the ionic species produced via the interaction of charged microdroplets generated by ESSI of a mixture of phenylboronic acid and 3-nitrophosphylboronic acid (0.2 mM each in H$_2$O with pH=9 adjusted by NH$_4$OH) with fructose (10 mM in aqueous solution); b) CID MS$^2$ spectrum of the fructose reaction product ion of m/z 328; c) reactive DESI mass spectrum showing the ionic species produced via the interaction of charged microdroplets generated by ESSI of 3-nitrophosphylboronic acid (0.2 mM in water with pH=9 adjusted by NH$_4$OH) with glucose (5 mM in aqueous solution); d) CID MS$^2$ spectrum of the glucose reaction product ion of m/z 328. Reprinted (Adapted) from Zhang, Y.; Chen, H. Int. J. Mass Spectrom. 2010, 289, 98–107, Copyright (2010), with permission from Elsevier.
7.3.2. Phenylboronic Acids with Permanent Positive Charge Tags

In addition to using negative reactant ions such as the 3-nitrophenylboronate (m/z 184) mentioned above, we also employed permanent positively charge-labeled phenylboronic acid ions generated from the ESSI spray of N-methyl-4-pyridineboronic acid iodide. The purpose of using permanent charge-labeled compounds is to improve the sensitivity of the method by increasing the ionization efficiency of both the reactive DESI reagents and products. This charge-labeling strategy has been widely used in previous mass spectrometry studies; for example, the cationic derivatization of oligosaccharides with Girard's T reagent via hydrazone formation gave significant increase in detection sensitivity as compared with the underivatized oligosaccharides when analyzed by MALDI or ESI mass spectrometry; also, betaine aldehyde was recently used as a reactive DESI reagent for rapid and selective analysis of cholesterol in serum and tissues.

In Figure 7.2a, the product ion of m/z 282 arose from the interaction of charged microdroplets generated by ESSI of N-methyl-4-pyridineboronic acid iodide (0.2 mM in water) with glucose (5 mM in H2O). The reaction equation is in eq. 2, Scheme 7.4. Upon CID (Figure 7.2b), water losses give rise to the fragment ions of m/z 264 and 246. In addition, the fragment ion of m/z 252 and m/z 222 arises from the loss of CH2O and C2H4O2 and the formation of m/z 192 and 162 is a result of ring cleavage from m/z 282 by loss of C3H6O3 and C4H8O4, respectively, consistent with the proposed ion structure in eq. 2, Scheme 7.4. In Figure 7.2a, the base peak of m/z 120 is produced from the dehydration of the reactant ions of m/z 138, due to the relatively high temperature of the
heated ion transfer capillary of the mass spectrometer used for ion desolvation in the experiment.

The LOD for glucose using N-methyl-4-pyridineboronic acid iodide was determined to be 6.9 µM (S/N=3, SIM scan mode was used to monitor the product ion of \(m/z\) 282), which is much lower than that of using phenylboronic acid, probably due to the favorable effect of charge-labeling on the ionization efficiency, as mentioned above. The LOD for glucose using N-methyl-4-pyridineboronic acid iodide was determined to be 6.9 µM (S/N=3, SIM scan mode was used to monitor the ion of \(m/z\) 282), which is much lower than that of using phenylboronic acid, probably due to the favorable effect of charge-labeling on the ionization efficiency, as mentioned above.

The inset of Figure 7.2a is the obtained spectrum for the analysis of 10 µM of glucose in water using the reactive DESI and the linear dynamic range from 0 µM to 500 µM was observed for this method (\(R^2=1.00\)). To further confirm this charge-labeling effect, another reactive DESI experiment using 2-picoline-4-boronic acid (Scheme 7.2), a neutral compound structurally similar to the N-methyl-4-pyridineboronic acid iodide, was also carried out. In Figure 7.2c, the ionic species are produced via the interaction of charged microdroplets generated by ESSI of containing 2-picoline-4-boronic acid (0.2 mM) and tetramethylammonium chloride (0.2 mM, severed as an internal standard) with glucose (5.0 mM in water). It has been shown that the ratio of the intensity of the ion of the DESI reaction product (\(m/z\) 282) in the case of N-methyl-4-pyridineboronic acid iodide (Figure 7.2a) to the intensity of the internal standard ion, Me₄N⁺ (\(m/z\) 74), is 0.65, which is much higher than the corresponding ratio of 0.1 in the case of 2-picoline-4-
boronic acid (Figure 7.2c), in agreement with the hypothesis that the sensitivity enhancement for $N$-methyl-4-pyridineboronic acid iodide does stem from the charge-labeling effect.

We further compared the reactivity of the $N$-methyl-4-pyridineboronic acid iodide with other cationic sugar derivatizing reagents like the Girard’s reagent T in this reactive DESI experiment. When an aqueous solution containing both $N$-methyl-4-pyridineboronic acid iodide (0.2 mM) and Girard’s reagent T (0.2 mM) was sprayed and the glucose (5.0 mM in water) was chosen as sample, it was found that the intensity of the ion of $m/z$ 282 from $N$-methyl-4-pyridineboronic acid iodide is ca. 12 times higher than that of the ion of the DESI reaction product ($m/z$ 294) from Girard’s reagent T (Figure 7.3), suggesting that the former reagent has higher reactivity in the reactive DESI experiments. This result also indicates that the formation of boronic acid ester is easier than the formation of hydrozone for saccharides in the DESI conditions.

Unlike phenylboronic acid or 3-nitrophenylboronic acid, $N$-methyl-4-pyridineboronic acid iodide can be used to detect glucose in acidic conditions. It has been found that the ion of the DESI reaction product ($m/z$ 282) was readily observed even when the glucose solution was acidified by acetic acid to pH 2. This feature of reactive DESI will facilitate the direct sugar detection in both neutral and acidic physiological conditions.
Figure 7.2. a) Reactive DESI mass spectrum showing the ionic species produced via the interaction of charged microdroplets generated by ESSI of aqueous solution containing N-methyl-4-pyridineboronic acid iodide (0.2 mM) and tetramethylammonium chloride (0.2 mM, served as an internal standard) with glucose (5 mM in water). The inset shows the detection of the ion of the DESI reaction product (m/z 282) in the analysis of 10 µM of glucose in water; b) CID MS² spectrum of the ion of the glucose DESI reaction product (m/z 282); c) reactive DESI mass spectrum showing the ionic species produced via the interaction of charged microdroplets generated by ESSI of aqueous solution containing 2-picoline-4-boronic acid (0.2 mM) and tetramethylammonium chloride (0.2 mM, served as an internal standard) with glucose (5 mM in water). Reprinted (Adapted) from Zhang, Y.; Chen, H. Int. J. Mass Spectrom. 2010, 289, 98–107, Copyright (2010), with permission from Elsevier.
To test the analytical utility of the reactive DESI using N-methyl-4-pyridineboronic acid iodide as reagent, a urine sample (pH=6) was chosen to be examined. Urine glucose monitoring is a potentially valuable noninvasive way of monitoring diabetes control as an alternative to blood glucose monitoring. 361-362

First, we simply tested direct ESI of glucose-containing urine (0.1 mM glucose) mixed with N-methyl-4-pyridineboronic acid iodide (0.2 mM) aqueous solution (the mixing volume ratio of 1: 3). As shown in the ESI mass spectrum (Figure 7.4a), we failed to detect the product signal at \( m/z \) 282 and there is a high noise background in the spectrum. This is probably because of the well-known suppression of ion signals in ESI in the presence of abundant salts from urine.
When reactive DESI was performed to examine the urine sample containing 0.1 mM of glucose by spraying \( N \)-methyl-4-pyridineboronic acid iodide in water (the injection flow rates of urine sample and spray solution are 1 \( \mu \)L/min and 3 \( \mu \)L/min, analogous to the mixing of two individual solutions the ESI experiment mentioned above; the concentration of 0.1 mM was selected for testing because it is lower than the LOD of 1.3 mM for glucose oxidase-based sensors\(^{363} \) and the LOD of 2 mM for dipsticks\(^{364} \) used for urine glucose monitoring), the peak of \( m/z \) 282 is detected and the detection was confirmed with CID of the mass selected \( m/z \) 282 that shows the correct fragmentation pattern as described above (c.f. Figure 7.2b), emphasizing the known salt tolerance of DESI.\(^{27,365} \) This is probably because DESI ionization does not involve in the direct spray of salt-containing urine, thus decreasing the complexity caused by salts. However, the signal of \( m/z \) 282 is weak (2.5x10\(^4 \) accounts, Figure 7.4b) and background noise level is still high, probably because the merged secondary charged droplet still contains salts as the spray solution and urine are completely miscible.

Interestingly, when the spray solvent of water was replaced by acetonitrile, the intensity of \( m/z \) 282 was considerably enhanced (with intensity of 7.8x10\(^5 \) accounts, Figure 7.4c) and much lower level of noise was observed. Again, upon CID, the product ion of \( m/z \) 282 undergoes dehydration and ring cleavage (c.f. Figure 7.2b), confirming its assignment. It is likely that the pronounced solvent effect is due to the low solubility of urine salts in sprayed organic solvent of acetonitrile. Thus, as the charged acetonitrile microdroplets containing \( N \)-methyl-4-pyridineboronic acid iodide pick up the sugar in urine on the surface, salts in urine were being excluded so that the resulting secondary
charged droplets contain low amounts of salts. Indeed, when 900 uL of acetonitrile was added to 300 uL of the saturated sodium chloride solution (in the ratio of 3:1 by volume), we found that there was salt precipitate formed in the mixed solution, in agreement with our hypothesis.

The phenomenon of salt exclusion in this reactive DESI experiment (i.e. the in-situ desalting using organic spray solvent for DESI ionization to gain enhanced sensitivity) is analogous to that previously observed in the fused-droplet ESI (FD-ESI) experiments\textsuperscript{366} in which ultrasonically nebulized sample aerosols (e.g. peptides and proteins) are ionized via fusion with charged methanol droplets.

In our study, samples were injected directly without the nebulization process and the urine analysis did not involve any sample pre-treatment processes such as extraction or drying, enabling in-situ desalting and derivatization and thus suggesting a novel and fast protocol for applying DESI to the trace analysis of complicated biological fluids with high salt content. It is also found that a linear dynamic range of 0-1 mM of glucose in urine can be achieved ($R^2 = 0.95$, see the calibration curve in the inset of Figure 7.4c). The calibration curve is displayed with the upper and lower confidence intervals (discrete lines) at the 95% confidence level. The slope and intercept are $(5.2 \pm 0.6) \times 10^6$ L/mmol and $(5.5 \pm 0.9) \times 10^5$, respectively.
Figure 7.4. a) ESI mass spectrum of a mixed solution of N-methyl-4-pyridineboronic acid iodide (0.2 mM in water) with glucose (0.1 mM in urine), and the mixing ratio is 3:1 by volume; b) reactive DESI mass spectrum is the ionic species produced via the interaction of charged microdroplets generated by ESSI of N-methyl-4-pyridineboronic acid iodide (0.2 mM in water) with glucose (0.1 mM in urine); reactive DESI mass spectra showing
the ionic species produced via the interaction of charged microdroplets generated by ESSI of N-methyl-4-pyridineboronic acid iodide (0.2 mM in acetonitrile) with c) glucose (0.1 mM in urine) and d) glucose (0.5 mM in human serum). The insets in c) and d) are the instrument responses versus the glucose concentrations in the urine and serum samples, respectively. The discrete lines represent the upper and lower boundaries of the 95% confidence intervals; the solid lines represent the calibration curves obtained from the average data points of three replicates of standard solutions. Reprinted (Adapted) from Zhang, Y.; Chen, H. Int. J. Mass Spectrom. 2010, 289, 98–107, Copyright (2010), with permission from Elsevier.

In addition to the urine glucose test, reactive DESI was also performed to examine glucose-containing human serum samples using N-methyl-4-pyridineboronic acid iodide as the chemical reagent and acetonitrile as the spray solvent. Figure 7.4d is the mass spectrum obtained from the direct detection of serum containing 0.5 mM of glucose in serum using the reactive DESI and the ion of the DESI reaction product (m/z 282) can be seen clearly. The inset of Figure 7.4d is the linear dynamic range of 0–0.5 mM ($R^2=0.98$) for the analysis. The calibration curve is also displayed with the upper and lower confidence intervals (discrete lines) at the 95% confidence level. The slope and intercept are $(1.3\pm0.1)\times10^5$ L/mmol and $(4.5\pm1.6)\times10^3$, respectively.

7.3.3. Reactions with Other Saccharides

Different mono-, di- and oligosaccharides, including galactose, mannose, N-acetyl-d-glucosamine, maltose, cellobiose and maltoheptaose, were also tested by the reactive DESI using N-methyl-4-pyridineboronic acid iodide as chemical reagent. In all cases, the ions of the DESI reaction products were observed and their MS/MS data are summarized in Table 7.1. Figures 7.5a and b are the reactive DESI mass spectra of the ionic species produced via the interaction of charged microdroplets generated by ESSI of
N-methyl-4-pyridineboronic acid iodide (0.2 mM in water) with galactose (5.2 mM in water) and with maltoheptaose (5 mM in water), respectively. In the case of galactose, the ion of the DESI reaction product (m/z 282) appears as the base peak and its high intensity is probably because one favored conformer of galactose in water, galactopyranose, contains cis-diol functionality in the ring carbons 3 and 4. Besides the ion of m/z 282, sodium adduct ion of galactose (m/z 203) is also observed as sodium ion is ubiquitous and has high binding affinity toward carbohydrate compounds. In the case of oligosaccharides of maltoheptaose, CID MS² spectrum of the ion of the DESI reaction product (m/z 1254) is the consecutive glycosidic cleavages to produce a series of y and z ions (Figure 7.5c), probably involving a charge-remote cleavage mechanism (see the proposed structure and cleavages of the product ion in the inset of Figure 7.5c). The formation of a series of y and z ions could be useful in the structure elucidation of oligosaccharides.
Table 7.1. Results of saccharides examined by reactive DESI results using N-methyl-4-pyridineboronic acid iodide as the chemical reagent (0.2 mM in water). Reprinted (Adapted) from Zhang, Y.; Chen, H. *Int. J. Mass Spectrom.* 2010, 289, 98–107, Copyright (2010), with permission from Elsevier.

<table>
<thead>
<tr>
<th>Saccharides</th>
<th>Formula Weight (Da)</th>
<th>Ions of DESI Reaction Product (m/z)</th>
<th>Fragment Ions Observed in MS/MS (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>glucose</td>
<td>180.16</td>
<td>282</td>
<td>264, 252, 246, 222, 204, 192, 180, 176, 162, 138, 120</td>
</tr>
<tr>
<td>mannose</td>
<td>180.16</td>
<td>282</td>
<td>264, 252, 222, 204, 192, 162, 120</td>
</tr>
<tr>
<td>galactose</td>
<td>180.16</td>
<td>282</td>
<td>264, 252, 246, 222, 206, 204, 192, 162, 180, 176, 138, 120</td>
</tr>
<tr>
<td>N-acetyl-D-glucosamine</td>
<td>221.21</td>
<td>323, 222</td>
<td>305, 287, 257, 245, 222, 204, 192, 162, 138, 120</td>
</tr>
<tr>
<td>maltose</td>
<td>360.32</td>
<td>444</td>
<td>426, 384, 354, 324, 282</td>
</tr>
<tr>
<td>cellobiose</td>
<td>342.30</td>
<td>444</td>
<td>426, 384, 354, 324, 284, 282</td>
</tr>
<tr>
<td>maltoheptaose</td>
<td>1153.02</td>
<td>1254</td>
<td>1236, 1188, 1092, 1074, 930, 912, 768, 750, 606, 444</td>
</tr>
</tbody>
</table>
Figure 7.5. Reactive DESI mass spectra are the ionic species produced via the interaction of charged microdroplets generated by ESSI of N-methyl-4-pyridineboronic acid iodide (0.2 mM in water) with a) galactose (5.2 mM in water) and b) maltoheptaose (5 mM in water); c) CID MS² spectrum of the ion of the DESI reaction product from maltoheptaose (m/z 1254). Reprinted (Adapted) from Zhang, Y.; Chen, H. Int. J. Mass Spectrom. 2010, 289, 98–107, Copyright (2010), with permission from Elsevier.
7.4. High Throughput Analysis using Tip-Sampling

One intrinsic advantage of DESI is the capability for high throughput analysis. In this study of reactive DESI experiments, a variant sample introduction protocol using pipette tips was also demonstrated, allowing analyzing multiple samples of a small volume in a short period of time.

In Figure 7.6a, a small volume (200 nL) of saccharide solutions was introduced as a thin layer of liquid film contained in a pipette tip. The spray-generated microdroplets containing the chemical reagent of N-methyl-4-pyridineboronic acid iodide were allowed to pass through the saccharide liquid film for ionization. The angle between the electrospray tip and saccharide sample and the angle between the sample and capillary inlet to the mass spectrometer were both set to 0° (such a linear arrangement is similar to that reported previously in the transmission mode DESI experiments, which is helpful to overcome the geometry problem for enhanced DESI sensitivity). Figure 7.6b is the extracted ion current chromatogram of the glucose DESI reaction product (m/z 282) for five glucose samples (200 nL, 5 mM in water) sequentially analyzed by reactive DESI (in the experiment, another pipette with a new sample was manually placed in between the ESSI source and MS inlet for sample replacement once the analysis of last sample was done). As each sample was ionized, the ion signal of m/z 282 arose and it took 4 min to complete the analysis of the five glucose samples.

This demonstration on the ionization of small volume of samples in high throughput manner might be of value in dealing with the analysis of precious biological samples in small volumes.
**Figure 7.6.** a) The apparatus for tip-sampling liquid sample DESI; b) Extracted ion current chromatogram of the ion of the DESI reaction product (m/z 282) for five glucose samples (200 nL, 5 mM in water) sequentially analyzed by reactive DESI using N-methyl-4-pyridineboronic acid iodide (0.2 mM in water) as the chemical reagent. Reprinted (Adapted) from Zhang, Y.; Chen, H. *Int. J. Mass Spectrom.* 2010, 289, 98–107, Copyright (2010), with permission from Elsevier.
7.5. Conclusions

In summary, in comparison to the prototype reactive DESI of using phenylboronic acid, the employment of its derivatives with electron-withdrawing and especially permanently charged groups significantly enhances the sensitivity of the method and expands the application scope to acidic solution samples. The secondary ionization nature of DESI allows one to have freedom to select favorable spray solvents and chemical reagents, which allows both online desalting and online derivatization for analysis of high salt-containing biofluid samples. Given the high selectivity, fast analysis speed, salt tolerance and high throughput capability of the reactive DESI as well as the significance of saccharides, this method reported would be valuable in saccharide analysis in many complex biological samples.
Conclusions and Future Work

A novel strategy for labeling biological thiols using selenamide reagents for analysis by MS was introduced in chapter 2, and the systematic investigation of MS/MS dissociation behaviors of selenamide-derivatized thiol peptide ions upon CID and ETD was shown in chapter 3. These two projects can be classified as MS study for selenium chemistry. In addition, four projects related to the applications for liquid sample DESI-MS were introduced in chapters 4-7, including a structural analysis of biologically active peptides and proteins containing disulfide bonds by EC/DESI-MS, the online coupling of LC and DESI-MS, EC-assisted top-down characterization of disulfide-containing proteins by EC/DESI-MS, and detection of saccharides by reactive DESI using modified phenylboronic acids. Thus, six projects were mentioned.

In summary, for MS investigation of selenium chemistry, the two selenamide compounds are excellent labeling reagents for characterization of thiol-containing compounds by mass spectrometry. This thiol derivatization reaction is highly selective, rapid, reversible and efficient, which can have some analytical applications including fast screening of thiol-containing peptides/proteins from complex mixtures, and identifying the number of free and bound thiols of proteins and their locations by tandem MS. In addition, unimolecular ion dissociation behaviors of selenamide-labeled thiol peptide ions upon CID and ETD were further investigated. The derivatized peptide cations undergo tag-dependent CID dissociation pathways, which is useful not only for selective identification of thiol peptides and proteins in mixture but also for peptide sequencing and locating of cysteine residues in peptides. Further, both protonated and alkaliated
selenamide-labeled thiol peptide give the preferential cleavage of Se-S bond over the formation of c/z ions during ETD, which following the UW mechanism. Given the significance of thiols in life, it is expected that there will be many novel MS applications based on the powerful selenium chemistry. For the follow-up work, the synthetic work is very necessary since we are limited by two selenamide reagents. If we can have more Se-N compounds, which can increase the sensitivity, and even possibly survive in ETD process, it will be useful in the study of selective electrolytic reduction of proteins.

Additionally, online EC coupled with DESI-MS is a powerful tool for both bottom-up and top-down approaches in protein analysis, which can be applied for the structural elucidation of different types of disulfide-containing peptides and proteins and for mixture analysis of protein/peptide digests. Based on the relative ion abundance change during electrolysis, the disulfide-containing peptides from others in enzymatic digest mixtures can be identified very quickly. In conjunction with mass mapping and tandem mass analysis, peptide sequencing and location of the disulfide linkages is possible. Adoption of selective selenamide derivatization reactions facilitates the analysis of intra- and inter-peptide disulfide containing peptides by the EC/DESI-MS with low resolution. The effect of disulfide bonds on maintaining protein conformational structures can also be investigated based on the CSD shifts of protein ions after reduction. In addition, for top-down in protein analysis, the substantially increased backbone cleavage by ECD and CID occurs to the electrolytic reduced protein in comparison to intact protein, producing many more structurally informative fragment ions. This electrochemical mass spectrometry for disulfide analysis is fast, simple and controllable
by switching on/off the electrolysis potential. No chemical reductant is involved so that
direct ionization after reduction is feasible. Besides, unlike the gas-phase ion activation
methods used for the cleavage of disulfide bond which could lead to backbone
dissociation, our electrochemical reduction takes place in solution and is very selective to
disulfide bonds. As both peptides from protein digests and intact proteins can be reduced,
this method should find important applications. Also, DESI can be a versatile interface
for combing LC with MS, which allows a wide range of elution rates, online
derivatization using reactive DESI and integration of electrochemistry, which should find
many valuable applications in mixture analysis, electrochemistry and experiments related
to proteomics. In the future, our group will focus on the electrolytic reduction for some
large biomolecules (MW is larger than 20 kD), like antibodies. Also for proteins
containing multiple disulfide bonds, there is a possibility to achieve selective cleavage of
certain disulfide bonds by using controlled reduction potentials; such an investigation
is under way. We will use different potentials to reduce different disulfide-containing
proteins/peptides in order to obtain the optimum reduction yield, which suggests the
feasibility of such an investigation. In addition, the top-down analysis can be carried out
for the selectively electrolytic reduced proteins. As disulfide bonds play an important role
in protein conformations and functions, this electrochemical mass spectrometric method
would find its valuable utilities in proteomics research.

For the last project, reactive DESI work, compared with the prototype reactive
DESI of using phenylboronic acid, the employment of its derivatives with electron-
withdrawing and permanently charged groups significantly enhances the sensitivity of the
method and expands the application scope to acidic solution samples. It is possible to
select favorable spray solvents and chemical reagents due to the secondary ionization
nature of DESI, which allows both online desalting and online derivatization for analysis
of high salt-containing biofluid samples. Given the high selectivity, fast analysis speed,
salt tolerance and high throughput capability of the reactive DESI as well as the
significance of saccharides, this method reported would be valuable in saccharide
analysis in many complex biological samples. In addition, this method has been applied
in LC/DESI-MS work instead of post-column derivatization which successfully avoids an
increased time delay and peak broadening.
References


99. Pretty, J. R.; Evans, E. H.; Blubaugh, E. A.; Shen, W.; Caruso, J. A.; Davidson, T. M., Minimisation of sample matrix effects and signal enhancement for trace analytes


277. Sun, Y.; Smith, D. L.; Shoup, R. E., Simultaneous detection of thiol- and disulfide-containing peptides by electrochemical high-performance liquid


312. Ryan, C. M.; Souda, P.; Halgand, F.; Wong, D. T.; Loo, J. A.; Faull, K. F.; Whitelegg, J. P., Confident assignment of intact mass tags to human salivary cystatins


Appendix A

Publications


Books


Patents

