Summer 2014

# Gas-Phase Ion/Ion Reactions Of Biomolecules: An Examination Of Carboxylate Reactivity And Arginine Based Non-Covalent Complexes

Nathan Zachary Barefoot
*Purdue University*

Graduate School ETD Form 9
(Revised 12/07)

# PURDUE UNIVERSITY
## GRADUATE SCHOOL
## Thesis/Dissertation Acceptance

This is to certify that the thesis/dissertation prepared

By Nathan Z. Barefoot

Entitled GAS-PHASE ION/ION REACTIONS OF BIOMOLECULES: AN EXAMINATION OF CARBOXYLATE REACTIVITY AND ARGININE BASED NON-COVALENT COMPLEXES

For the degree of Master of Science

Is approved by the final examining committee:

Scott A. McLuckey
Chair

Mary J. Wirth

Marcy H. Towns

To the best of my knowledge and as understood by the student in the *Research Integrity and Copyright Disclaimer (Graduate School Form 20)*, this thesis/dissertation adheres to the provisions of Purdue University's "Policy on Integrity in Research" and the use of copyrighted material.

Approved by Major Professor(s): Scott A. McLuckey

Approved by: R. E. Wild 7-22-2014
Head of the Graduate Program Date

# GAS-PHASE ION/ION REACTIONS OF BIOMOLECULES: AN EXAMINATION OF CARBOXYLATE REACTIVITY AND ARGININE BASED NON-COVALENT COMPLEXES

A Thesis

Submitted to the Faculty

of

Purdue University

by

Nathan Z. Barefoot

In Partial Fulfillment of the

Requirements of the Degree

of

Master of Science

August 2014

Purdue University

West Lafayette, Indiana

To my family: Mom, Dad, and Pap

thank you so much for all your love and support

# ACKNOWLEDGMENTS

First of all I would like to thank all of my family for all of the support they have given me over the years. I want to especially thank my mom who has always encouraged me to try new things and to persevere through tough situations. I know that this is one of the reasons that I have been able to make it this far through the many challenges and difficulties of graduate school. I also want to thank my grandfather who has been a constant inspiration to me throughout my life.

I want to thank my high school chemistry Mike Long who was one of the first people to get me interested in chemistry and went out of his way to try and get students to become fascinated with the field of science. I feel lucky to have had so many excellent teachers throughout my life, both in and out of school, but I know that I would not have made it to Purdue without his support and guidance. I also want to thank Dr. Scott A. McLuckey who has served as my advisor and mentor for these past few years. His guidance and support has allowed me to learn so much more about the field of science. He is an excellent role model and genially cares about every student in his research lab. I feel privileged that I have had the opportunity to work with him.

I want to thank all of the members of the McLuckey research group, especially Will and Marija for training me during my first few years within the group and for helping my with the many questions that asised about my research. I want to thank my

friends Brian, Justine, Stacey, Tanya, and Taylor for always providing a new perspective on my work whenever it seemed that I ran out of ideas.

Lastly, I want to thank our collaborators at AB Sciex for the assistance and help with the instrumentation and willingness to take the time to teach us the inner workings of the mass spectrometers. I also want to thank my committee members for sharing their time and providing feedback.

TABLE OF CONTENTS

Page

Page

# LIST OF FIGURES

Figure Page

Figure Page

# LIST OF SCHEMES

# LIST OF ABBREVIATIONS

| | |
|---|---|
| AC | Alternating Current |
| BT-CID | Beam Type Collision Induced Dissociation |
| CID | Collision Induced Dissociation |
| CRM | Charge Residue Model |
| Da | Mass in Daltons |
| DC | Dipolar Current |
| DDC | Dipolar Direct Current |
| ESI | Electrospray Ionization |
| HPLC | High-Performance Liquid Chromatography |
| ICR-MS | Ion-Cyclotron Resonance Mass Spectrometer |
| IEM | Ion Evaporation Model |
| LIT | Linear Ion Trap |
| MALDI | Matrix Assisted Laser Desorption/Ionization |
| MCP | Multi-Channel Place detector |
| *m/z* | Mass-to-Charge Ratio |
| MS | Mass Spectrometry |

| | |
|---|---|
| MS/MS | Tandem Mass Spectrometry |
| MSAE | Mass-Selective Axial Ejection |
| $MS^n$ | Tandem Mass Spectrometry |
| MTP-NHS | S-methyl 5,5′-thiodipentanoylhydroxysuccinimide |
| nESI | Nanoelectrospray Ionization |
| NHS | N-Hydroxysuccinimide |
| PTM | Post-Translational Modification |
| QIT | Quadrupole Ion Trap |
| QMF | Quadrupole Mass Filter |
| $Q_n$ | $n^{th}$ Quadrupole Mass Analyzer |
| QqQ | Triple Quadrupole |
| RF | Radio-Frequency |
| TOF-MS | Time-of-Flight Mass Spectrometer |

# ABSTRACT

Barefoot, Nathan Z. M.S., Purdue University, August 2014. Gas-Phase Ion/Ion Reactions of Biomolecules: An Examination of Carboxylate Reactivity and Arginine Based Non-Covalent Complexes. Major Professor: Scott A. McLuckey

The advent of Electrospray Ionization with the ability to generate multiply charges ions has contributed significantly to the study of gas-phase ion/ion reactions. With the tools available in mass spectrometry it has been shown that these reactions are effective at transforming one type of gaseous ion into another through a series of reactions. This work examines some of these reactions and their application to field of proteomics specifically focusing on the amino acids of arginine and lysine. NHS reagents have been shown to react to both of these molecules with in the gas-phase under different conditions but have relies on negatively charged reagent ions. The effect of a positively fixed-charge reagent in investigated through a series of reactions and compared to the effects observed in the positive mode.

Additionally arginine has been shown to form non-covalent interactions with the sites of phosphorylation of phosphopeptides, a type of biologically important post translational modification. Though the use of this interaction it was observed if ion/ion reactions could aide in the simplification of a digest analysis. Through these non-covalent interactions it may be possible to selectively alter the charge of the analyte to allow for easier detection.

# CHAPTER 1: AN INTRODUCTION TO MASS SPECTROMETRY AND IONIZATION TECHNIQUES

At its most basic level mass spectrometry can be thought of as an analytical technique used to determine the mass of exceedingly small charged particles and has advanced significantly over the course of its one hundred year history. Although the roots of mass spectrometry can be traced all the way back to research conducted in the 19$^{th}$ century on discharge tubes, the first true example of a mass spectrum emerged in 1912 from the work of Sir J. J. Thomson[1, 2]. In his parabola mass spectrograph, ions were sent through parallel magnetic and electric fields such that they were deflected and separated by both their velocity and mass-to charge ration or *m/z* [3]. This effect was typically observed on photoplates until a set of experiments were run where the magnetic field was scanned and the deflected ions were detected with electronics to produce one of the first mass spectrums as shown in Figure 1.1. Since that time the field of mass spectrometry has grown and has emerged to become an almost indispensable analytical tool for a variety of fields in chemistry and biochemistry. However, its utility is most apparent in the realm of biological sciences where its unique abilities are used in the analysis of biological molecules and have led to the development of the fields of proteomics, lipidomics, genomics, and much more. The technologies available today, in regards to a biological molecule, allow for the investigation of both primary and three-dimension structures as well as an inquiry of the molecules reactivity.

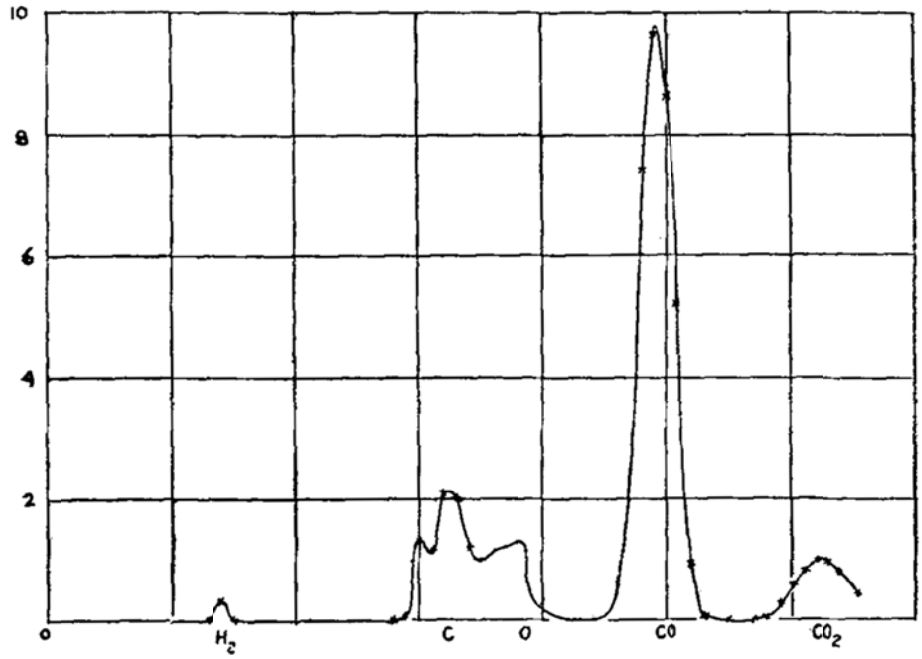



Figure 1.1 Mass spectrum produced by work from J. J. Thomson using a parabola mass spectrograph coupled to an electroscope for detection (Adopted from ref. 1).

When thinking about the function of a mass spectrometer or understanding a mass spectrometry experiment, it is useful to divide the system into three different segments. The first segment consists of a method for generating gas phase ions from the sample of interest (*i.e.,* ionization). Secondly, after the sample has been ionized it can be subjected to a probe method used to activate the ions and in most cases induce some form of fragmentation. The final step in the process is mass analysis or detection where all of the remaining ions are separated by their *m/z*.

## 1.1 Quadrupole Mass Spectrometers

With the advancement of technology a number of different mass analyzers have been developed that far exceed the ability of Thomson's original parabola mass

spectrograph. However, each technique still relies on the implementation of electric fields, magnetic fields, or a combination of the two in order to selectively separate ions by their mass-to-charge rations. One such device is a quadrupole mass filter, sometimes referred to as a quadrupole mass filter or QMF, which in appearance is constructed of four metal rods arranged in a parallel array and positioned in a square configuration. Separation of ions in the quadrupole is accomplished through the use of both RF and DC potentials that are superimposed on one another[4]. Opposite sets of road pairs are coupled together and the electric field is applied such that one set of rod pairs is in phase with the RF and the other is out of phase, as illustrated in Figure 1.2. This allows for the creation of a quadrupolar field.Under ideal circumstances the rods would have a hyperbolic structure. Most quadrupoles are actually constructed of cylindrical rods which

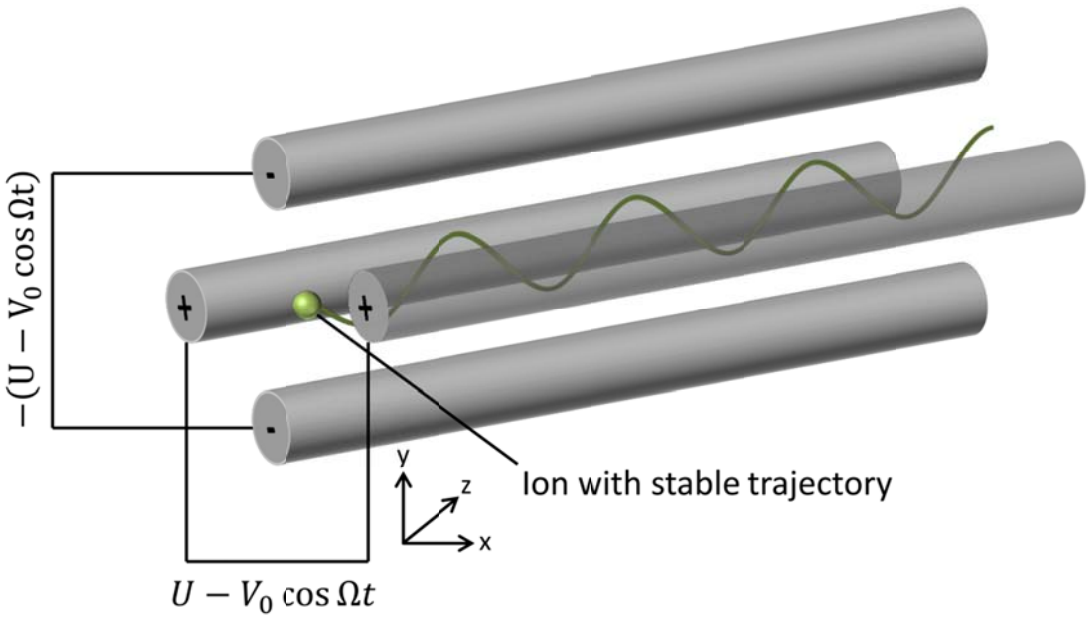



Figure 1.2 Illustration of a quadrupole mass filter consisting of two rod pairs with the applied electric potentials used to create the quadrupolar field and a visual of an ion with stable trajectory.

can only approximate an ideal electric field[5]. For this device the term quadrupolar refers to the nature of the electric field and not the structure of the quadrupole mass filter itself. Inside a quadrupolar field the forces on an ion are dependent upon the square of the distance from the origin, in this case the center of the trap[6].

The combination of RF and DC potentials applied to the rods is shown in equation 1.1 as well as illustrated in Figure 1.2 above.

$$\Phi_0 = \pm U - V\cos\Omega t \qquad (1.1)$$

Here $\Phi_0$ is the total potential applied to the rods, U is the DC potential, V is the RF potential, and Ω is the angular frequency of the RF. The combination of these two fields traps ions in the x- and y-directions (sometimes referred to as radially) while they are still free to move through the quadrupole in the z-direction (sometimes referred to as axially). The quadrupolar nature of the trapping electric field operates such that the force on the ion increases the further it moves from the center of the quadrupole. In other words the more distant an ion is from the center of the trap the more force is exerted upon it by the electric field. Depending on the values of the RF and DC potentials applied to the quadrupole the complex motions of the ions can either be described as stable or unstable. As the names would suggest, stable ions traverse the length of the rods and remain trapped within the device whereas unstable ions are displaced from the center of the trap and are either removed from the quadrupole or are impacted against the rods. Through these motions ions can be either selectively isolated based on their *m/z* or scanned through the quadrupole in a mass selective manner to generate a mass spectrum. The

stability or instability of a given ion is determined by the Mathieu equation, a linear second-order differential equation discovered in 1868[7].

Much of the following information reguarding the treatment of the Mathieu equation is based on the information presented in several resources unless otherwise noted.[4, 6, 8] The commonly accepted form of the Mathieu equation is given in equation 1.2 below:

$$\frac{d^2u}{d\xi^2} + (a_u - 2q_u \cos 2\xi)u = 0 \qquad (1.2)$$

where u represents displacement from the axis, $\xi$ is a dimensionless parameter equal to $\Omega t/2$, and $a_u$ and $q_u$ are dimensionless trapping parameters related to DC and RF potentials respectively. This equation can be solved for the two trapping parameters which relate the given properties of an ion and the inherent conditions of the mass spectrometer. These relationships are given below in equations 1.3 and 1.4.

$$a_u = \frac{8zeU}{mr_0^{\,2}\Omega^2} \qquad (1.3)$$

$$q_u = \frac{-4zeV}{mr_0^{\,2}\Omega^2} \qquad (1.4)$$

Here m is the mass of the ion, $r_0$ is the distance between the center of the trap and the rod pairs, e is the elementary charge or $1.602 \times 10^{-19}$ coulombs, and z is the charge of the ion. With these two equations it is possible to determine which ions will have a stable trajectory and which will have an unstable trajectory while passing through the quadrupole. In other words these equations indicate whether or not an ion will be trapped within the electric field or ejected under a given set of conditions. Since $r_0$ is a constant

that does not change unless the dimensions of the quadrupole are physically altered and most instruments operate with a fixed Ω, the only variables are the *m/z* of the ion and both the RF and DC potentials.

In order to better illustrate how these equations determine an ion's stability it can be helpful to generate a graph in dimensionless $a_u$, $q_u$ space. Here $q_u$ and $a_u$ values are graphed onto the x- and y-axis respectively of a graph as shown in figure 1.3. Since the electric fields of the quadrupole trap the ions in two dimensions the ion's trajectory along both axes must remain stable otherwise the ion will be ejected. To illustrate these Mathieu Stability regions it then becomes necessary to show the solutions to equations 1.3 and 1.4 for both the x and y planes of the quadrupole. The blue trace in the figure shows that stability regions where an ion's trajectory is stable along the x-axis for given $a_u$ and $q_u$ values and the red trace show shows the same for the y-axis. The areas where the two traces overlap, indicated and labeled as A, B, C, and D, are regions where an ion's trajectory along both axes is stable. If conditions were adjusted such that a given ion was located in one of the traces but outside these four regions it would only be trapped by the electric field in one direction while ejected from the quadrupole in the other.

Of the four areas of overlapping stability in Figure 1.3, commonly referred to as a Mathieu Stability Diagram, most quadrupole mass filters operate only within region A. The other three areas, while allowing for the effective trapping of ions inside the device, are typically only used for research and not implemented in common practices[2]. As mentioned previously, the only variables that can be adjusted to affect an ion's stability are the instrumental parameters of U and V. Through either adjusting these parameters or scanning them it becomes possible to isolate ions of a particular *m/z* or to generate a mass

spectrum. Both of these functions will be described using Figure 1.4, which shows a close-up view of area A from Figure 1.3 above, and the trapping parameters $a_u$ and $q_u$.

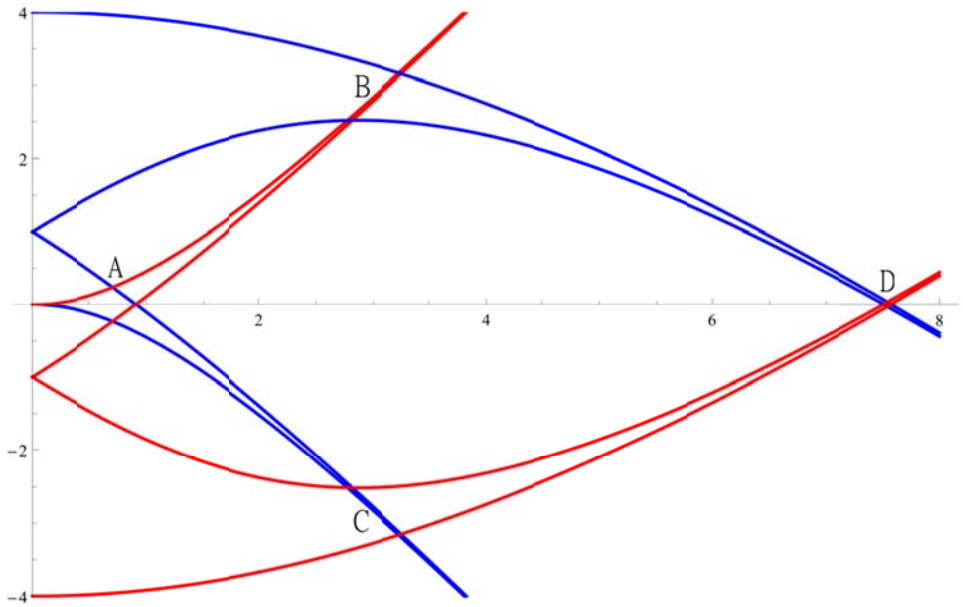



Figure 1.3 Graphical representation of the Mathieu stability regions for a quadrupole mass filter with the red trace showing ions stable along the y-axis and the blue trace showing the same for the x-axis.

The DC potential applied to the quadrupole affects only parameter $a_u$ and when zero potential is applied all of the ions lie along the $a_u = 0$ line. Under these conditions all ions entering the quadrupole are transmitted provided that their *m/z* has them within the bound region and at a $q_u$ value between 0 and 0.908. The RF potential can be adjusted to allow different *m/z* ranges through and in effect can allow the quadrupole mass filter to function as either a high-pass or low-pass filter under these conditions. In this mode of operation the quadrupole in simply functioning to transport ions from one area to another while keeping them focused at the center of the trap. It is possible to construct a

quadrupole without a DC power supply such that it would only be operating in this manner. . These devices are referred to as either transport quadrupoles or RF only quadrupoles.

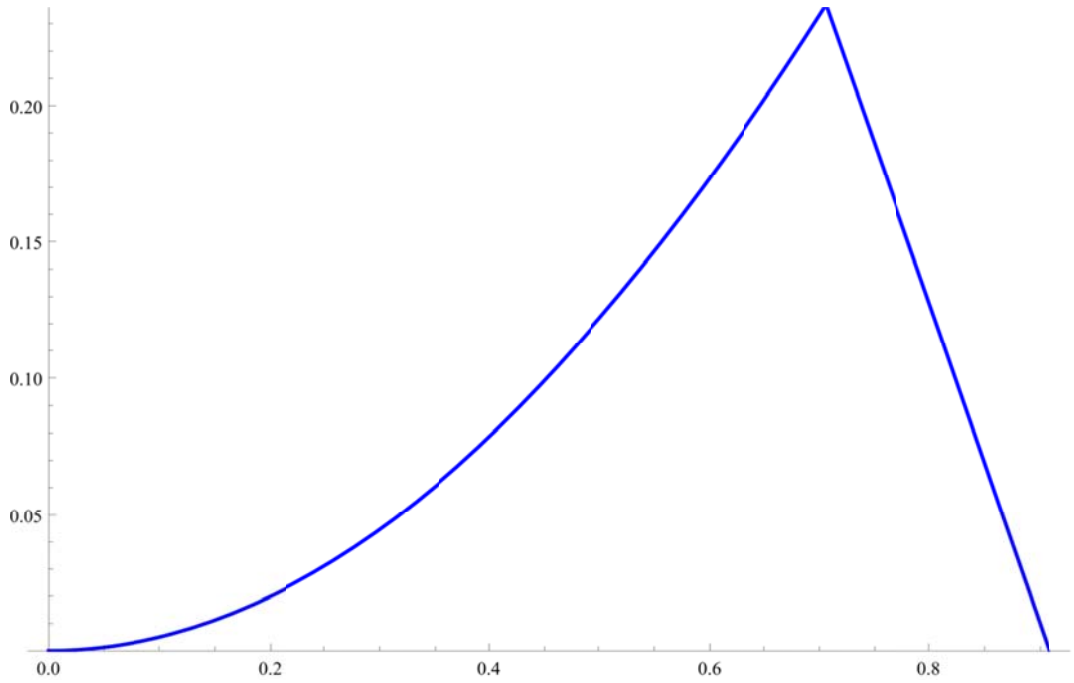



Figure 1.4 Representation of a portion of the Mathieu Stability Diagram illustrating the typical operating area for most quadrupole mass filters.

In order to isolate a ion of a particular *m/z* inside a quadrupole mass filter it is necessary to use a combination of RF and DF potentials and will be most easily explained again by using Figure 1.4 for illustration purposes. The first step involves adjusting the RF potential such that the *m/z* value of interest is exactly below the apex of the stability diagram, or at a $q_u$ value of 0.706. Once that is accomplished the DC potential is applied such that the same ion is moves up on the stability diagram to around an $a_u$ value of

0.236. As the DC potential is applied, all ions other than the particular *m/z* of interest are moved into a region on the stability diagram where their trajectories become unstable and are ejected from the trap. The closer the ion is brought to the apex of the stability diagram the tighter and cleaner the isolation will become (i.e. less ions with differing *m/z* will be present within the quadrupole). If the ion is either brought to close to the apex or is brought above the apex then some of the desired species will also be ejected from the quadrupole. Once these conditions are tuned the quadrupole mass filter will only transmit ions of the narrow *m/z* window selected and all other ions will be in regions of instability and ultimately ejected.

Similar principles are used to selectively scan ions through the device in order to produce a mass spectrum. From a practical standpoint it should be noted that this approach assumes that the same sample is passing through the quadrupole for the entire duration of the procedure. A fixed ratio between $a_u$ and $q_u$ is selected to define a mass scan line and the instrument is programmed to adjust the potentials of the RF and DC currents such that it essentially scans this line in the stability diagram. The only requirements for the ratio are that $a_u/q_u$ is greater than zero. Using Figure 1.4 as a reference the line would essentially start at the origin and increase linearly with a slope dependent on the ratio. Each *m/z* value would be essentially isolated and passed through the trap selectively in a process referred to as a Mass-Selective Stability scan. The slope of this line, and hence the ratio between $a_u$ and $q_u$, determines the resolution of the scan. If the slope is low then a broad range of ions will able to mass through at different *m/z* and adversely affect the resolution. Alternatively if the slope is high but still remains within the stable regions of the diagram then only a narrow window can pass through the

quadrupole. In this way there is a trade-off between the resolution of the instrument and the signal of the scan as the tighter the isolation, the more likely the conditions will begin to remove the ions of interest. The resolution of the QMF can also be affected by the number of cycles the ions spend within the quadrupolar field.

## 1.2 Linear Ion Traps

While extremely versatile, the quadrupole mass filter is somewhat limited in its use given that ions can only flow continuously through the device. One alternative design developed in the 1953 is to take the rods of the quadrupole and essentially bend them in a circle to form a donut shaped 3-dimensional trap that differs from the linear QMF[9]. This mass analyzer is composed of a central ring electrode sandwiched between two end cap electrodes and commonly referred to as a 3-D trap or a quadrupole ion trap (QIT). The ions are still trapped with a quadrupolar field and overall the QIT operates in a similar manner to the quadrupole mass filter albeit with different $a_u$ and $q_u$ trapping parameters. The most significant difference is that the QIT can trap ions both radially and axially within the quadrupolar field while a QMF is only capable of radial confinement. As a result ions can be pulsed into the device rather than flowing through in a continuous manner. Once trapped the ion population can be stored within the electric field for some time and potentially subjected to further reactions or analysis.

There are a number of different advantages to being able to trap ions and subject themto further scrutiny that are not available with a traditional QMF[10, 11]. This has led to the creation of a linear ion trap which functions in a manner identical to a QMF in terms of the trapping parameters with the notable exception that is can also store ions in the

axial or z-direction. To accomplish this two electrostatic lenses are placed on opposite ends of the quadrupolar rod set and are connected to a DC power supply. When the ions enter the quadrupole they are trapped within the quadrupolar field by the RF radially and by the repulsive DC potential applied to the lenses axially. The combination of these two fields traps the ions in the linear device which is referred to as either a linear ion trap (LIT) or a 2-dimensional quadrupole ion trap. Essentially the only difference between a QMF and a LIT is the addition of the two lenses for confinement of ions in the z-direction.

### 1.2.1 Mass Selective Axial Ejection

Once the ions have entered the linear ion trap and are successfully contained with both the RF and DC the issue now becomes how to selectively eject the ions and generate a mass spectrum. Given the nature of the electric field is it possible to eject the ions either axially or radially[11]. If one were to attempt to use the same procedure as for the QMF and scan U and V with a fixed potential the results would prove to be ineffective at the ions are no longer continuously passing through the quadrupole. This would simply serve to isolate a single species of ion, the *m/z* where the scan stopped, and remove everything else. To generate a mass spectrum in this manner one would need to sequentially inject, isolate, and scan out each different *m/z* which would be prohibitively time consuming. Therefore another method is required.

The theory behind the operation of the QMF, and thus the LIT, as discussed above is based on several assumptions that do not necessarily hold true in practice. First the mathematical treatment assumes an ideal quadrupolar field which can only be created

using infinitely long hyperbolic shaped rods. In practice most rod sets are somewhere between 10 and 30 cm in length and machined to be cylindrical rather than hyperbolic. Under these conditions the quadrupolar field most ideally exists at the center of the mass analyzer in regards to its length and decreases in strength at either end of the rod set. The electric fields present at either end of the quadrupole mass filter are referred to as fringe fields and are formed in part by higher-order fields present due to the non-ideal shape of the rods and the effects of the potential allied to the nearby electrostatic lens[12]. Under normal circumstances these fringe fields can result in a reduction in performance of quadrupole mass filters but for a LIT they can be utilized for mass-selective axial ejection[13, 14].

As a result of the fringing fields at the end of the quadrupole there are two different forces affecting ions in the region. The first is due to the reduction of the quadrupolar potential which results in an axial electric field that directs ions out of the linear ion trap. The second force is due to the trapping potential created by the electric field applied to the nearby electrostatic lens that pushes the ions in the opposite direction and back into the trap. It is possible to overly these two forces to show the electric field experiences in the axial direction by trapped ions which is illustrated in figure 1.5. The dashed line in the figure represents the so called cone of reflection created by the electric fields present at the end of the quadrupole rod set[10, 13, 14]. For ions on the left side of the cone the net force experienced is repulsive away from the exit while ions on the right are ejected axially from the trap and experience an attractive electric field. Through these forces it is possible to eject ions in a mass-selective manner by adjusting the potential applied to the exit lens and controlling the radial amplitude of the ions.[14]

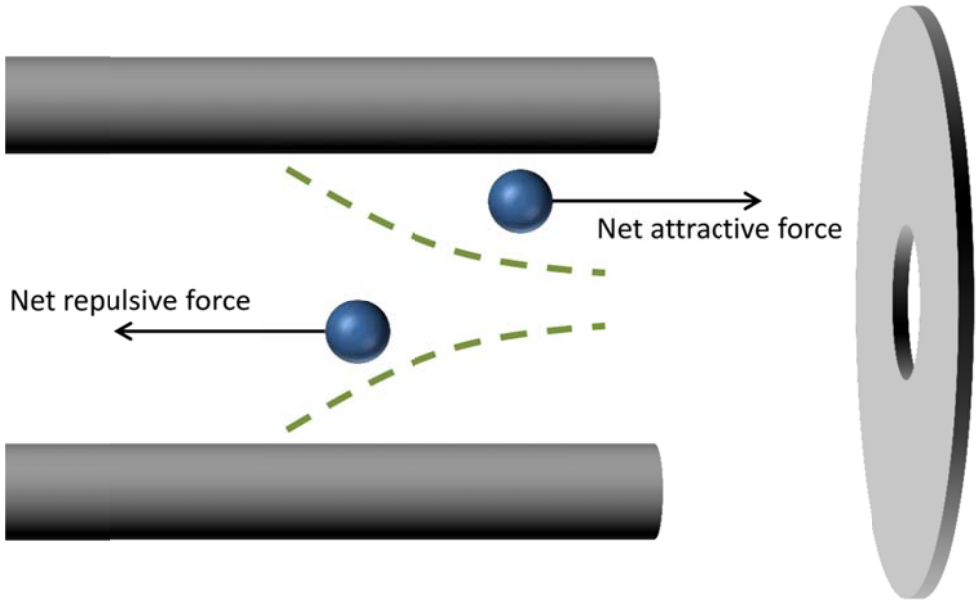



Figure 1.5 An example of the cone of reflection experienced by an ion due to the fringing fields in a quadrupole mass analyzer and utilized for mass-selective axial ejection.

When trapped within a LIT, ions are thermalized by the presence of nitrogen gas and coalesce at the center of the trap with minimal radial amplitude. Through mass-selective axial ejection (MSAE) it is possible to apply a supplemental AC potential to a rod pair in the quadrupole mass analyzer that can increase the radial amplitude of ion of select *m/z*. In this manner each individual *m/z* can be given enough radial amplitude to pass through the cone of reflection and be ejected from the LIT. The applied potential is small enough such that the ions are ejected axially before they impact the rod pairs and are lost. This mass-selective process allows for the generation of a mass spectrum as the supplemental AC potential and DC potential on the electrostatic lens are scanned.

## 1.3 Electrospray Ionization

Since mass spectrometry involves the separation of ions by their *m/z* through the use of either electric or magnetic fields it is necessary to generate ions from any sample of interest. There are a variety of techniques for generating ions that have evolved significantly since Thomson's work with the parabola mass spectrograph. One of the most common is electrospray ionization or ESI which first came to popularity through the work of Fenn in the late 1980s[15] [16]. Essentially ESI involves flowing a solution containing a dilute concentration of the analyte through a capillary tube and applying a voltage. The polarity of the potential applied determines the polarity of the ions produced. Unlike some other ionization techniques, ESI, is capable of producing multiply-charged ions and was first used to obtain the molecular weight of proteins and biomolecules[17].

An overall illustration of the electrospray process is illustrated in figure 1.6 below and shows the sample from the capillary tube to the inlet of the mass spectrometer. For the process to work a certain onset voltage is required that varies depending on the nature of the sample and solvent. When the voltage isreached the buildup of electric charge within the solution causes the formation of a cone at the end of the capillary referred to as a Taylor cone. From this cone a series of small droplets are formed about 1.5 μm in diameter that contain the analyte and any other molecules present within the solution. As these droplets flow from the capillary and travel to the mass spectrometer the solvent begins to evaporate increasing the charge per unit volume within the droplet. Eventually the droplet begins to deform and form a Taylor Cone, similar to the one formed in the capillary, as a result of the concentration of charge. This cone then emits a series of

smaller droplets that continue this pattern of evaporation and division until reaching the mass spectrometer[2, 18]. At this point either most of the solvent has been evaporated or will be removed in the inlet of the instrument leaving only the charged ions.

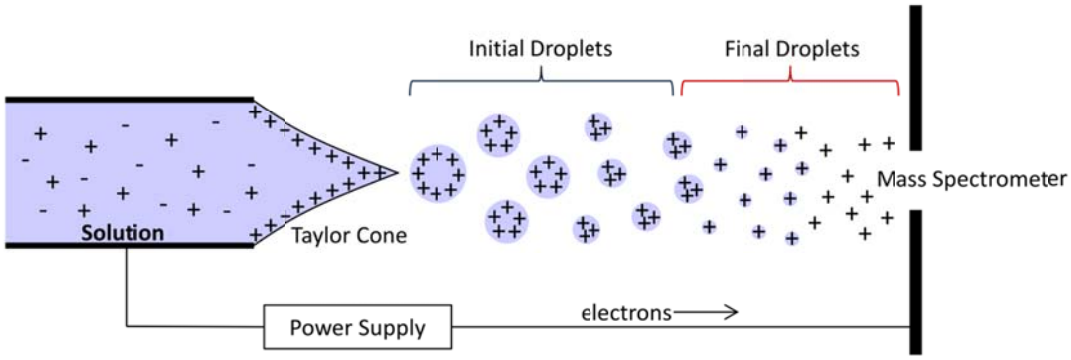



Figure 1.6 Schematic representation of the electrospray ionization process form the formation of the Taylor Cone to the inlet of the mass spectrometer

During the droplet migration from the capillary to the instrument the charges contained within a droplet are determined by the Rayleigh limit shown in equation 1.4.

$$q = 8\pi\sqrt{\varepsilon_0 \gamma R^3} \qquad (1.4)$$

Here q is the charge of the droplet, $\varepsilon_0$ is the permittivity of free space, γ is the surface tension, and R is the diameter of the droplet. This equation calculates the theoretical maximum amount of charge that a droplet can hold before becoming unstable. Most of the charge droplets begin to fragment somewhere with between 90 and 100% of this limit. The fragment droplets are then about 0.1μm in diameter and can continue the process of evaporation and division.

### 1.3.1 Ion Evaporation Model

Currently there are several prevailing theories for the mechanism behind electrospray ionization. One of these theories is known as the Ion Evaporation Model or IEM and is typically understood to be the most likely process for how ions of small molecules are formed[19]. As the charges build up on the surface of the droplet the molecule inside the droplet is also charged. The interactions of these forces, both of which are the same polarity, result in the ion being ejected from the droplet. This process is illustrated in figure 1.7and of the two process illustrated can be thought of as ion desertion. A recent study suggested a method whereby a large molecule could be formed through this method. If a protein were to become denatured in solution then one of the ends of the sequence could become charged and emerge from solution. Due to the repulsion of charges this would effectively pull the remainder of the protein out of the droplet and place charges on some of the other residues. Although the paper referred to this as the chain ejection model it is still quite similar to the ion evaporation model[20].

### 1.3.2 Charge Residue Model

The charge residue model is different from the ion evaporation model in that it is believed to be one of the processes for how large ions, such as proteins, are formed during ESI[21]. Compared to the IEM this process can be thought of as desorption and is also illustrated in Figure 1.2. As the solvent evaporates from the droplet the molecule remains inside. This theory assumes that there is only one analyte molecule present. As the solvent continues to evaporate the charges are places on the molecule to form an ion

and the process continues until all the solvent is evaporated. What remains is only a charged ion containing any non-volatile components of the droplet such as salts[17, 20].

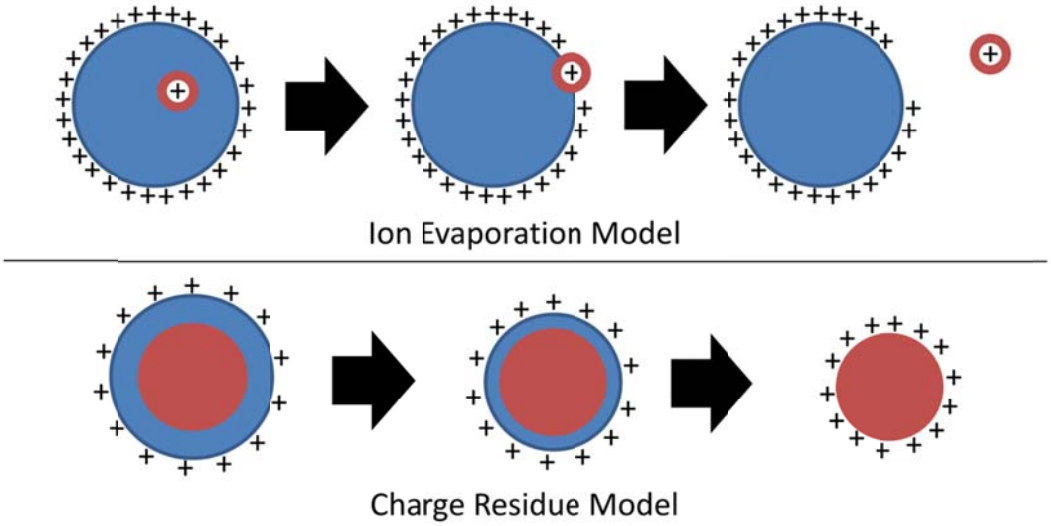



Figure 1.7 Diagram showing two proposed mechanisms for electrospray ionization; the ion evaporation model and the charge residue model.

## 1.4 References


1. Thomson, J. J., XIX. Further experiments on positive rays. Philosophical Magazine Series 6 1912, 24 (140), 209-253.

2. de Hoffmann, E.; Stroobant, V., Mass Spectrometry: Principles and Applications. Wiley: 2007.

3. Thomson, J. J., XXVI. Rays of positive electricity. Philosophical Magazine Series 6 1911, 21 (122), 225-249.

4. March, R. E.; Todd, J. F., Quadrupole Ion Trap Mass Spectrometry. Wiley: 2005.

5. Denison, D. R., Operating Parameters of a Quadrupole in a Grounded Cylindrical Housing. Journal of Vacuum Science & Technology 1971, 8 (1), 266-269.

6. March, R. E., Quadrupole ion traps. Mass Spectrometry Reviews 2009, 28 (6), 961-989.

7. Mathieu, E., Mémoire sur le mouvement vibratoire d'une membrane de forme elliptique. J. Math. Pure Appl. (J Liouville) 1868, 13, 137.

8. March, R. E.; Todd, F. J.; (Eds.), Practical Aspects of Trapped Ion Mass Spectrometry Series, Vol I-V. CRC Press: Boca Raton, FL.

9. Paul, W.; Steinwedel, H., A NEW MASS SPECTROMETER WITHOUT A MAGNETIC FIELD. Journal Name: Zeitschrift fuer Naturforschung 1953, a8, Medium: X; Size: Pages: 448-50.

10. Hager, J. W., A new linear ion trap mass spectrometer. Rapid Communications in Mass Spectrometry 2002, 16 (6), 512-526.

11. Schwartz, J. C.; Senko, M. W.; Syka, J. E. P., A two-dimensional quadrupole ion trap mass spectrometer. Journal of the American Society for Mass Spectrometry 2002, 13 (6), 659-669.

12. Hager, J. W., Performance optimization and fringing field modifications of a 24-mm long RF-only quadrupole mass spectrometer. Rapid Communications in Mass Spectrometry 1999, 13 (8), 740-748.

13. Londry, F. A.; Hager, J., Mass selective axial ion ejection from a linear quadrupole ion trap. Journal of the American Society for Mass Spectrometry 2003, 14 (10), 1130-1147.

14. James, W. H., Linear Ion Trap Mass Spectrometry with Mass-Selective Axial Ejection. In Practical Aspects of Trapped Ion Mass Spectrometry, Volume IV, CRC Press: 2010; pp 545-571.

15. Fenn, J. B.; Mann, M.; Meng, C. K.; Wong, S. F.; Whitehouse, C. M., Electrospray ionization for mass spectrometry of large biomolecules. Science 1989, 246 (4926), 64-71.

16. Mann, M.; Meng, C. K.; Fenn, J. B., Interpreting mass spectra of multiply charged ions. Analytical Chemistry 1989, 61 (15), 1702-1708.

17. Kebarle, P.; Verkerk, U. H., Electrospray: From ions in solution to ions in the gas phase, what we know now. Mass Spectrometry Reviews 2009, 28 (6), 898-917.

18. Gomez, A.; Tang, K., Charge and fission of droplets in electrostatic sprays. Physics of Fluids (1994-present) 1994, 6 (1), 404-414.

19. Iribarne, J. V.; Thomson, B. A., On the evaporation of small ions from charged droplets. The Journal of Chemical Physics 1976, 64 (6), 2287-2294.

20. Konermann, L.; Ahadi, E.; Rodriguez, A. D.; Vahidi, S., Unraveling the Mechanism of Electrospray Ionization. Analytical Chemistry 2012, 85 (1), 2-9.

21. Dole, M.; Mack, L. L.; Hines, R. L.; Mobley, R. C.; Ferguson, L. D.; Alice, M. B., Molecular Beams of Macroions. The Journal of Chemical Physics 1968, 49 (5), 2240-2249.

# CHAPTER 2: GAS-PHASE ION/ION REACTIONS AND CHARACTERIZATION OF BIOMOLECULES VIA MASS SPECTROMETRY

Mass Spectrometry is a powerful tool for the analysis of bio-molecules. Its versatility, sensitivity, and analysis speed have led to the development of the field of proteomics. Probably one of the most significant factors contributing to this development was the advent of soft ionization techniques such as ESI and matrix assisted laser desorption/ionization or MALDI during the 1980s.[1,2] This allowed researchers to ionize these large molecules without subjecting them to fragmentation leaving the intact structure for further analysis. One of the most common mass analyzers for this additional analysis are ion traps, specifically the linear ion trap. Given its ability to trap ions for a prolonged period it allows for these ionized biomolecules to be subjected to additional reactions or structural inquiry.

## 2.1 Introduction to Proteomics: Top-Down vs. Bottom-Up

In regards to the study of proteins and peptides there are two competing approaches through which structural and sequence information can be obtained. The first approach is commonly referred to as bottom-up[3] while the second is top-down[4-6]. In the bottom-up approach to protein analysis a protein is first digested and broken into much small peptide fragments, typically through the use of an enzymatic protease reagent such

as trypsin or chymotrypsin. Once the sample has been completely digested the resulting solution is analyzed by a mass spectrometer where the peptide sequences are identified and used to determine the sequence of the intact starting protein. Most of the time this identification is accomplished by comparing the mass spectrum produced from the digest to a database containing similar information for a number of proteins.

There are several advantages and also several disadvantages to the bottom-up approach for protein analysis. One of the biggest benefits is the small molecular weight typically seen with peptides in comparison to proteins that allows for its implementation on a variety of instruments and a variety of mass analyzers. Linear ion traps, for example, typically have an upper mass range of several thousand *m/*z which limits their ability to analyze intact proteins and protein complexes while time-of-flight, TOF, and ion-cyclotron resonance, ICR, mass spectrometers have good resolution up to a hundred thousand *m/z* or farther. However, the approach has significant drawbacks such as the complexity of the solution once the protein has been digested which when not subjected to appropriate separation and cleanup could result in inaccurate mass information being used for the database search. Additionally not all of the fragments of the protein are successfully ionized which typically results in a loss of relevant information for protein identification or study, especially if it should contain a post-translational modification (PTM) such as a phosphorylation site. The bottom-up approach is typically not considered to be effective for analyzing these PTMs because of this loss of information.

Alternatively the top-down approach is quite different and as one might suspect relies on beginning with the whole and intact protein or protein complex as opposed to the smaller fragments. The overall concept behind the top-down method is that all of the

sample preparation performed in various solutions from digestion to purification, and cleanup be performed within the mass spectrometer itself through a variety of methods. This allows for the complete protein to be sequenced and studied without any loss of information, additionally most if not all PTMs can be preserved for further analysis using fragmentation methods and are only lost if the method itself is responsible. However, there are several drawbacks to this approach which mostly stem from its immaturity compared to the bottom-up methodology. Due to the limitations in instrumentation and technology, bottom-up methods have been studied for years whereas the top-down approach is just beginning to become widely implemented. Given that the entire protein is being analyzed it requires an instrument that can operate as extremely high mass ranges with excellent mass accuracy and resolution. This requires a TOF, IRC, or orbitrap mass analyzer: although through the use reactions inside the mass spectrometer and ESI it is possible to use a LIT for top-down studies. Both the overall principles of top-down and bottom-up analysis are depicted in Figure 2.1 below.

### 2.1.1 Structural Interrogation and Fragmentation Methods

With ESI it is possible to generate a protein ion in an exceedingly high charge state compared to other soft ionization methods such as MALDI which primarily produces singlely charged or low charge stated ions[2, 7]. This means that the protein ion can contain enough charges that its *m/z* is within the operational range of an LIT and be subjected to additional structural interrogation. For this type of experiment a useful instrument design is referred to as a triple quad mass spectrometer, sometimes abbreviated QqQ. Here three quadrupole mass filters are placed in a sequential

arrangement with additional ion optics and a detector. Typically the first and third rod sets, identified by the labels Q1 and Q3, are fully functional RF/DC quadrupoles while the second rod set, or q2, is an RF only quadrupole and identified with the labeling scheme of QqQ by the lower case lettering.

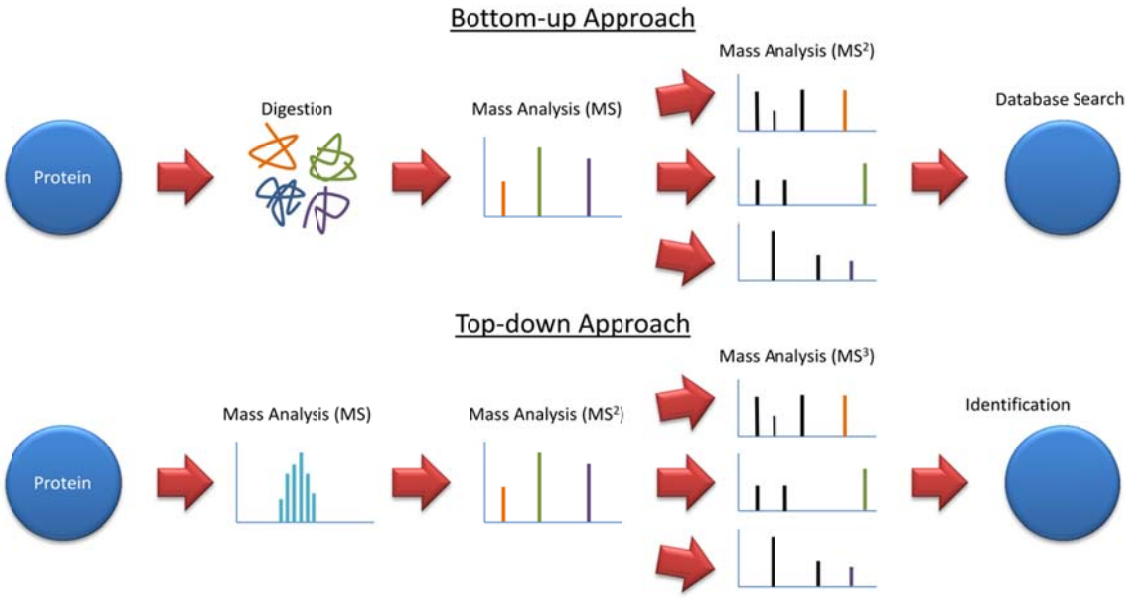



Figure 2.1 Illustration of top-down and bottom-up approaches for protein analysis using mass spectrometry

The triple quad can be operated such that a desired protein charge state is isolated from all other species present within the electrospray in Q1 and subjected to some form of activation in q2 that causes fragmentation. Once the parent ion has been fragmented the resulting species can be mass selectively scanned out of Q3 to produce a mass spectrum. This is one possible technique that can be used to obtain sequence information for a protein or peptide although mass spectrometry as a whole and not just the triple

quad platform excels in providing this information. There are a variety of methods available to activate and fragment the ion of interest that have a very broad range for the amount of energy that can be applied and the timescale of the event. Surface induced dissociation or SID, for example is an extremely fast process whereby the ion is fragmented by colliding with a surface. At the other end of the spectrum is BIRD or Blackbody infrared radiative dissociation which is a slow heating process that slowly adds energy to the ion until the bonds begin to break.[1]

In the spectrum between these two processes are some techniques that are far more commonly applied. Ultra violet photo-dissociation (UVPD) and infrared multi-photon dissociation (IRMPD) are two processes that involve the use of a laser to induce fragmentation. For UVPD the ion of interest must contain a structure that is UV active and will absorb the necessary wavelength of light while IRMPD is a slower multi-photon process. Electron transfer dissociation (ETD) and electron capture dissociation (ECD) are another two processes that cause fragmentation by the transfer of an electron to the ion of interest. ECD requires the electrons to be generated within the instrument and ETD requires that the electron be transferred from a reagent molecule. Both processes are similar and produce similar fragmentation. Probably the most common and universal fragmentation method is collision induced dissociation or CID. It essentially involves applying energy to the ion of interest such that it collides with the background gases present within the mass spectrometer and eventually fragments. Given the fact that the method work for any ion species at any *m/z* it is considered to be universal.

## 2.2 Overview of Collision Induced Dissociation

Collision induced dissociation is a technique that is used to fragment ions of interest through the application of kinetic energy and can be accomplished in both a high energy and a low energy fashion[8, 9]. For simplicity this discussion will be focused on the application of collision induced dissociation within a QMF or a LIT although the technique can be applied to several other mass analyzers. Essentially the concept relies on applying additional energy in some fashion to the analyte ion that causes it to have collisions with background gasses inside the mass spectrometer. In most situations this gas is nitrogen or helium. If the energy of the collision is sufficiently high then the energy transferred to the analyte ion through the collision will result in bond fragmentation. Alternatively if the energy of the collision does not transfer enough energy to the ion for fragmentation then the ion will still become collisionally activated but remain intact. When unimolecular dissociation does occur it is governed by the principle of quasi-equilibrium theory or RRKM theory.

### 2.2.1 High Energy CID

The amount of energy imparted to an ion as a result of CID can vary depending on the situation and the experiment. Mass analyzers based on QMF, particularly triple quadrupole mass spectrometers, and linear ion traps are capable of both high energy and low energy CID. High energy processes rely on passing the beam of ions or an ion cloud through a collision cell that is operated at a slightly higher pressure compared to the rest of the instrument. The collision cell still under a partial vacuum is fed either nitrogen or helium gas to allow for more gas molecules/targets to be present. When the ions are

passed through they are accelerated with great deal of kinetic energy such that their collisions with the background gas will result in the transfer of the energy to internal energy and cause fragmentation. At these energies the excitation of the ion is electronic rather than vibrational and occurs as such a speed that no chemistry is possible during the activation.

### 2.2.2 Low energy CID

An alternative to high energy CID is to use an approach that relies on lower energies and slower speeds to cause fragmentation of the ions. Conceptually the idea is still the same in that the fragmentation occurs due to the transfer of energy from a collision with a background gas molecule. The difference is the amount of energy involved and with this method, sometimes referred to as a slow heating method, the ability to have chemistry occur during the process. In an LIT or QMF each ion oscillates through the instrument with a specific secular frequency that is specific to the *m/z* of the ion population. It is possible to apply a supplemental AC potential at the frequency and apply additional energy to the ion. This causes its oscillation to increase in amplitude and cause more collisions with the background gas eventually causing fragmentation. Since the heating is a slow process it is possible for chemistry to occur during the heating such as a complex being given just enough energy to react before fragmenting. Additionally since the energy applied to the ion is controlled it is possible to adjust how that energy is applied; high energy over a short time or low energy over a long time.

## 2.3 Ion Chemistry

Given the variety of control present within a mass spectrometer the degree to which an ion can be manipulated is quite impressive. Within a simple LIT alone it is possible to isolate an ion, mass-selectively detect a large population of ions, fragment an ion, etc. However, there are also a series of reactions that can be performed in the gas phase that allow for the transformation of the ion from one type to another.[10] One type of reaction is known as charge inversion where the charge of an ion is transformed from one polarity to another either by adding or removing protons of through the formation of a complex with another ion. The various types of reactions include proton transfer, metal transfer, metal cation attachment, electron transfer, cation transfer, etc.

Although some of these phenomena have been observed since the early experiments were performed on J.J. Thomson's parabola mass spectrograph, the development of ion/ion reactions is somewhat more recent. In order for a reaction to occur in the gas phase between two oppositely charged ions it is necessary for them two species to form a long lived complex of simple pass close enough for a transfer of charge to occur. Given the ability to manipulate these ions in the gas-phase, ion/ion reactions represent a powerful analytical tool for the study of biomolecules via mass spectrometry.

## 2.4 Protein and Peptide Fragmentation

In the study of proteomics mass spectrometry is a widely used tool for sequence and structural analysis. During fragmentation of these ions when they are protonated most of the bond cleavage, whether induced with CID or some other method, occurs

along the peptide backbone. A systematic methodology for naming this fragmentation was originally proposed in 1984 and is illustrated within figure 2.2 below[11]. Here the bonds identified as a, b, and c are related to the N-terminus with the subscript representing the number of that type of bond away from the end of the protein or peptide. The labels x, y, and z represent the same information but correspond to fragments from the C-terminus.

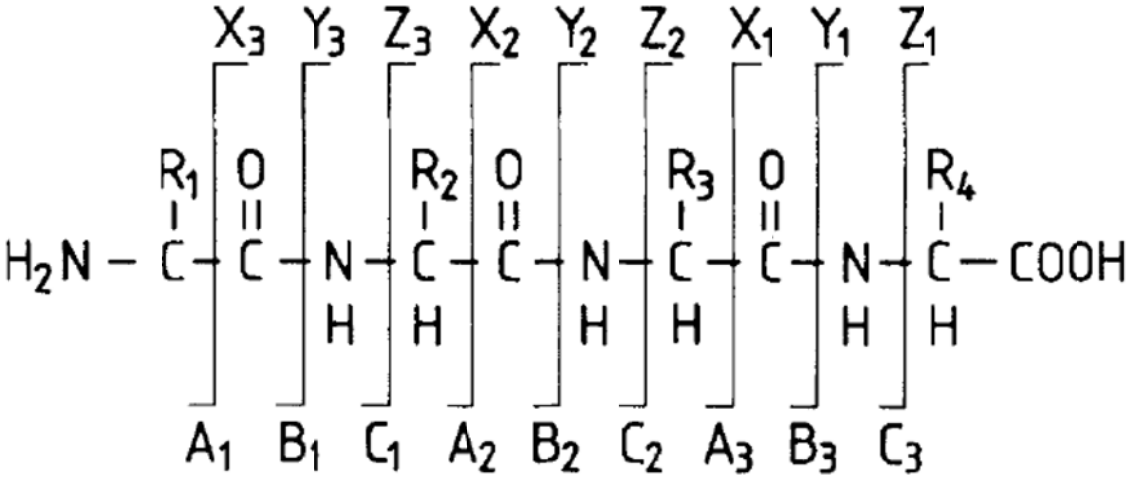



Figure 2.2 Diagram illustrating a nomenclature proposed for the fragmentation of peptides and proteins (adopted from ref. 11)

## 2.5 References

1. de Hoffmann, E.; Stroobant, V., Mass Spectrometry: Principles and Applications. Wiley: 2007.

2. Fenn, J. B., Mann, M., Meng, C. K., Wong, S. F., Whitehous, C. M., Science 1989, 246, 64-71

3. Aebersold, R., Goodlett, D. R., Chemical Reviews 2001, 101, 269–295

4. McLafferty, F. W., Kelleher, N. L., Begley, T. P., Fridriksson, E. K., Zubarev, R. A.; Horn, D. M., Current Opinions in Chemical Biology 1998, 2, 571-578

5. Kelleher, N. L., Analytical Chemistry 2004, 76, 196A-203A

6. Reid, G. E., McLuckey, S. A., Journal of Mass Spectrometry 2002, 37, 663-675

7. Karas, M., Hillenkamp, F., Analytical Chemistry 1988, 60, 2299-2301

8. McLuckey, S. A., Goeringer, D. E., Journal of Mass Spectrometry 1997, 32, 461-474

9. Wells, M. J., McLuckey, S. A., in Methods in Enzymology, ed. A. L. Burlingame. Academic Press, 2005, vol. Volume 402, pp 148-185.

10. Prentice, B. M.; McLuckey, S. A., Gas-phase ion/ion reactions of peptides and proteins: acid/base, redox, and covalent chemistries. Chemical Communications 2013, 49 (10), 947-965.

11. Roepstorff, P.; Fohlman, J., Letter to the editors. Biological Mass Spectrometry 1984, 11 (11), 601-601.

# CHAPTER 3: EXPERIMENTAL CONDITIONS

## 3.1 Instrumental Specifics: QTRAP 2000

Experiments listed within the following chapters were primarily performed on a modified prototype AB Sciex QTRAP 2000 hybrid triple quadrupole/linear ion trap mass spectrometer (AB Sciex, Concord, ON, Canada) shown in figure 3.1 below[1]. The instrument was modified to apply alternating current (AC) to the electrostatic trapping lenses designated IQ2 and IQ3 allowing for the simultaneous trapping of ions of opposite polarities in the q2 collision cell. This quadrupole rod set is partially sealed within a container and incorporates an additional nitrogen gas line so that it can operate at a higher pressure than the rest of the mass spectrometer. This nitrogen flow is controllable to allow for the adjustment of the pressure within the collision cell. Normally this higher pressure is utilized to increase the efficiency of ion/ion reactions.

Two custom built pulsed nano-ESI emitters situated at the orifice function to alternately inject anions and cations when indicated by the Datalyst control software.[2] Nano-ESI requires significantly smaller sample volumes compared to normal ESI as it relies on flow rates that are on the order of nanoliters per minute as opposed to microliters per minute (REF). The emitters are powered by external Ortec Model 556 high-voltage power supplies connected to a custom build pulse generator. When triggered by the software this pulse generator sends the power to the nano-ESI emitters for sample

injection into the mass spectrometer; during the remainder of the analysis the voltage at the emitter is zero.

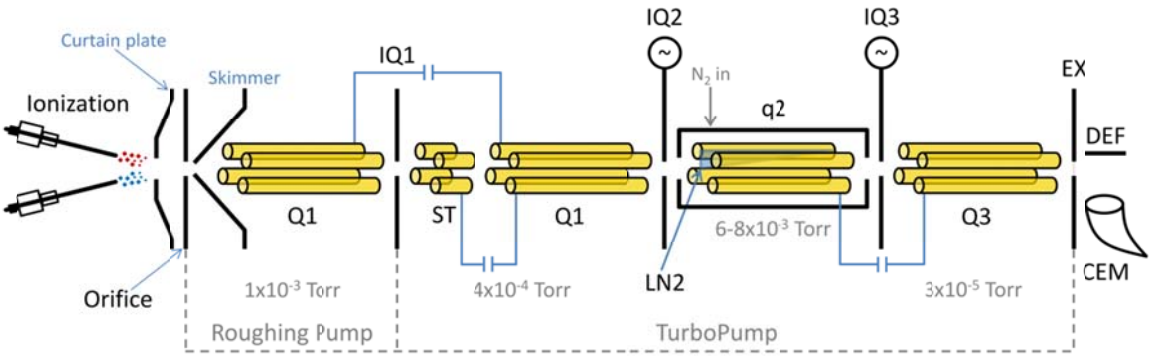



Figure 3.1 Schematic diagram of a modified/prototype AB Sciex QTRAP 2000 hybrid triple quadrupole/linear ion trap mass spectrometer

Once generated, each polarity of ions passes through Q1 where mass selection is possible through use of a RF/DC isolation before exiting into the collision cell. Here ion/ion reactions are performed due to the increased efficiency at higher pressure although the instrument can be easily modified to perform the reactions in Q3 as opposed to q2. The change simply involves altering which pair of electrostatic trapping lenses receives that additional AC trapping potential. Both ion polarities are mutually stored in q2 for times ranging from 50 – 500 ms until the desired product is formed. After the reaction, further mass selection and collisional activation is possible in the Q3 quadrupole with the linear ion trap design allowing for $MS^n$ experiments.

The IQ2 and IQ3 lenses on the instrument have been modified to apply a supplemental AC current in addition to the normal DC potential. This allows for both

positive and negative polarities of ions to be trapped within the q2 collision cell for a mutual storage reaction.[3,4] The first polarity is injected and trapped with DC potential while the second polarity is being sprayed and isolated in Q1. Once both reagents are in the trap the AC is applied for a desired period of time and then the DC potential is reapplied eject any ions of the undesired polarity. From here the sample is transferred to Q3 for further analysis.

With this instrument platform two different types of activation are possible. The first is ion trap CID which involves electrostatically trapping the ions in Q3 and applying a supplementary AC potential at the secular frequency of the ions of interest to cause fragmentation. This is accomplished through the use of an external waveform generator that is tuned to the desired frequency and triggered by the computer software. Alternatively is it possible to accelerate the ions between q2 and Q3 to cause dissociation in a process that involved a rapid intake of energy compared to the slow heating of conventional CID. This process is commonly referred to as beam type CID (BT-CID). Mass analysis is accomplished with mass selective axial ejection (MSAE) from Q3 into a discrete electron multiplier.[5]

## 3.2 Instrumental Specifics: API QSTAR

Several of the experiments performed in the following chapters were accomplished using a prototype modifier AB Sciex API QSTAR *Pulsar I* mass spectrometer (AB Sciex, Concorde, ON, Canada)[6]. The overall instrument schematic is shown in figure 3.2 but is operated in a similar capacity to the QTRAP 2000 described in detail above. Unlike the QTRAP design which features a triple quadrupole QqQ

configuration the QSTAR relies instead on a Qq-TOF arrangement commonly referred to as a QTOF design. Here two linear ion traps are placed at the front end of the instruments for isolation, ion manipulation, and storage before the ions are passed to a time of flight (TOF) mass analyzer. The TOF separates ions by a difference in velocity as then travel through a regions of space within a region of the device called the flight tube.

At the beginning of the analysis sequence ions are accumulated inside the quadrupole before being pulsed into the TOF and in front of an accelerator plate. This electrode applies an acceleration voltage to each ion in the ion cloud that causes them to travel down the flight tube and towards the detector. At this potential energy of the electrode is converted into kinetic energy ions of different *m/z* are given different velocities. This is illustrated below in equation 3.1.

$$`t^2 = \frac{m}{z}\left(\frac{L^2}{2eV_s}\right) \tag{3.1}$$

Here L represents the length of the flight tube regions, $V_s$ is the accelerating potential applied to the ion, e is the elementary charge or $1.602 \times 10^{-19}$ coulombs, and t is the time required to traverse distance. L.

The TOF mass analyzer on the QSTAR instrument is a reflectron TOF which means that as the ions travel away from the accelerating plate they eventually approach a series of lenses called an ion mirror that have a repulsive potential. This mirror then slowly stops the ions before reflecting them back towards the detector located at the top of the flight tube adjacent to the accelerator place. This results in a longer flight length which increases separation of the ion populations and corrects for the kinetic energy

distribution of the ions from the accelerating potential. Given the distribution present within an ion cloud it is not possible to apply an identical accelerating potential to all the ions which can result in ion of the same *m/z* traveling at different speeds. Since separation of ions within the TOF is based on the flight time this effect results in decreased resolution and performance difficulties. The reflector corrects for this problem because ions of different kinetic energies will penetrate the repulsive field of the ion mirror to varying degrees. Ions with a high velocity will pass farther than those with low velocities such that the additional time spent within the reflectron will allow for all ions with the same *m/z* to impact the detector at the same time

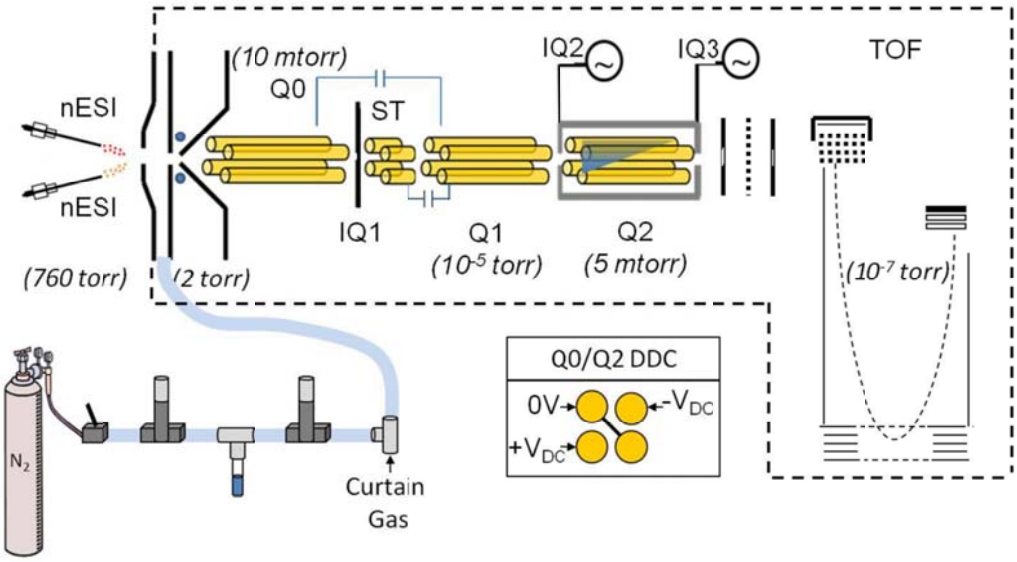



Figure 3.2 Schematic diagram of a prototype modified API QSTAR *Pulsar I* mass spectrometer

For the QSTAR instrument ions are first passed through a series of ion guides or focusing mechanisms before entering Q1 where mass selection and isolation are possible.

From here the ions pass through to the collision cell which much like the QTRAP 2000 operates at a higher pressure and has been modified to allow for mutual storage reactions. Afterwards the ions are injected into the TOF for orthogonal acceleration and detected with a multi-channel place (MCP) detector. The instrument is equipped with custom build nano-EST emitters that are capable of generating ions in both positive and negative modes. Each emitter is controlled by the Datalyst control software and an external power supply with a pulse generator. Typically one of the emitters is connected to an internal instrument power supply that operates in the same polarity that the instrument is configured for.

In order to perform ion activation and fragmentation the QSTAR is equipped with traditional ion trap CID in Q1 and beam type CID is also possible between Q1 and q2. However since the ion/ion reactions take place in q2 during a mutual storage setup it is necessary to transfer any reactions products back to Q1 since q2 is not equipped to apply the supplemental AC necessary for ion trap CID. Additionally the instrument is also equipped with another slow heating process can also be used to fragment ion called dipolar direct current or DDC[7]. This operated by the application of a broadband waveform to a pair of opposite electrodes in the rod set of a linear ion trap. When activated the DDC applies energy to ions across a large range is not a mass selective process as is ion trap CID. This allows for uniform fragmentation of a range of species.

## 3.3 Materials

` In electrospray ionization the solvent composition affects the voltage required for formation of the Taylor Cone and ionization of the sample. In order to generate positively

charged ions protein, peptide, and reagent samples were dissolved in a mixture of 49.5% water, 49.5% methanol, and 1% glacial acetic acid. When necessary the pH of the solution could be adjusted to decrease with pH and produce higher charge states. Solutions intended for the production of negative ions were composed of 49.5% water, 49.5% methanol, and 49.5% ammonium hydroxide. Both the ammonium hydroxide and glacial acetic acid are volatile compounds which function to adjust the pH of the solution without clogging the emitter when the solvent begins to evaporate. The emitters were prepared with borosilicate glass capillaries that had and outer diameter of 1.5mm and an inner diameter of 0.86 mm. They were by a P-87 Flaming/Brown micropipette puller (Sutter Instruments, Novato, CA).

## 3.4 References

1. Hager, J. W., A new linear ion trap mass spectrometer. Rapid Communications in Mass Spectrometry 2002, 16 (6), 512-526.

2. Liang, X.; Xia, Y.; McLuckey, S. A., Alternately Pulsed Nanoelectrospray Ionization/Atmospheric Pressure Chemical Ionization for Ion/Ion Reactions in an Electrodynamic Ion Trap. Analytical Chemistry 2006, 78 (9), 3208-3212.

3. Xia, Y.; Wu, J.; McLuckey, S.; Londry, F.; Hager, J., Mutual storage mode ion/ion reactions in a hybrid linear ion trap. Journal of the American Society for Mass Spectrometry 2005, 16 (1), 71-81.

4. Xia, Y.; Liang, X.; McLuckey, S., Pulsed dual electrospray ionization for Ion/Ion reactions. Journal of the American Society for Mass Spectrometry 2005, 16 (11), 1750-1756.

5. Londry, F. A.; Hager, J., Mass selective axial ion ejection from a linear quadrupole ion trap. Journal of the American Society for Mass Spectrometry 2003, 14 (10), 1130-1147.

6. Xia, Y.; Chrisman, P. A.; Erickson, D. E.; Liu, J.; Liang, X.; Londry, F. A.; Yang, M. J.; McLuckey, S. A., Implementation of Ion/Ion Reactions in a Quadrupole/Time-of-Flight Tandem Mass Spectrometer. Analytical Chemistry 2006, 78 (12), 4146-4154.

7. Webb, I. K.; Londry, F. A.; McLuckey, S. A., Implementation of dipolar direct current (DDC) collision-induced dissociation in storage and transmission modes on a quadrupole/time-of-flight tandem mass spectrometer. Rapid Communications in Mass Spectrometry 2011, 25 (17), 2500-2510.

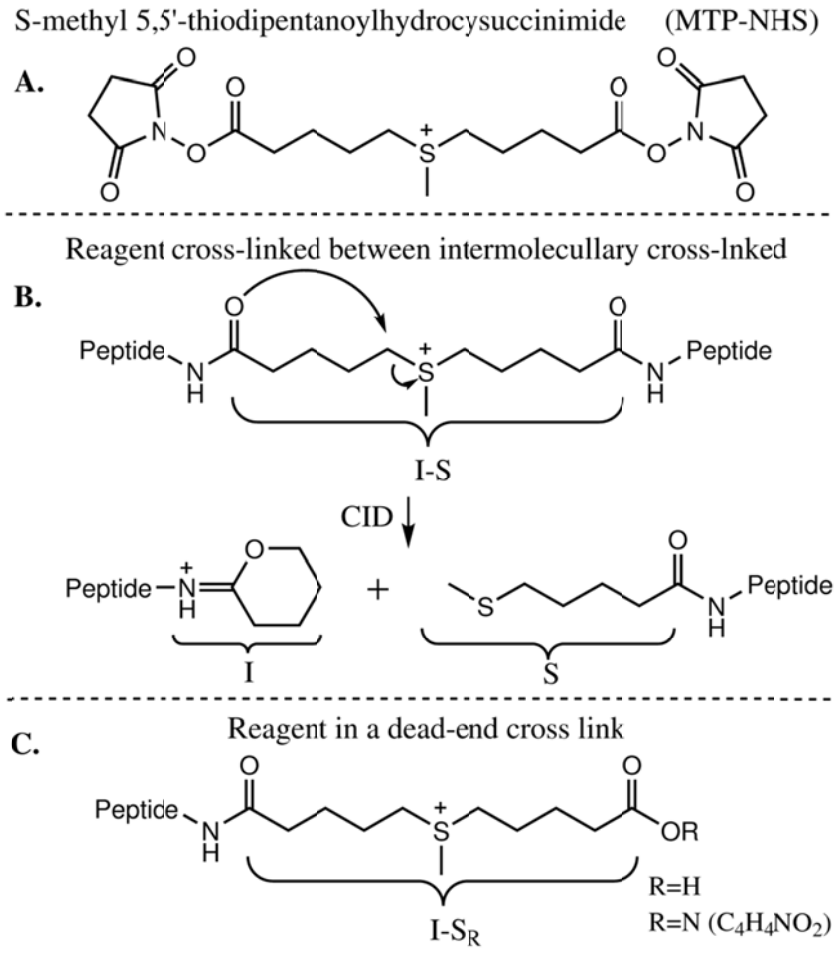
# CHAPTER 4: EXAMINING A FIXED CHARGE, GAS PHASE CLEAVABLE CROSS-LINKING REAGENT FOR USE IN PROTEIN STRUCTURAL IDENTIFICATION

## 4.1 Introduction

The use of chemical crosslinking reagents is a common practice in proteomics for studying protein structure[1, 2]. These reagents attach to specific amino acids in the protein and based on their spacer length can give information about the distances between different residues leading to three-dimensional information about the protein[3, 4]. A large number of crosslinking reagents based off the ability of NHS esters to react with primary amines have been developed. However these experiments can produce very complex data due to the large number of reactions possible[3]. Crosslinking reagents can form intramolecular, intermolecular, and dead-end links representing a crosslink between two residues in the same protein, a crosslink between two residues in different proteins, and a crosslink attached only to one residue with the other end unreacted. On possible solution to allow for the differentiation of these three distinct reactions is to modify the crosslinking reagent to incorporate a gas phase cleavable bond, a practice which lends itself well to tandem mass spectrometry[5-7]. Upon activation the cross linking reagent fragments at a specific bond while each end still remains attached to an amino acid residue in the protein/peptide structure. This then allows for addition interrogation of the overall structure[8].

Previous work has shown that ion/ion reactions can be used to form covalent bonds in the gas phase between crosslinking reagents and un-protonated primary amines. The reagents bis[sulfosuccinimidyl] suberate (BS3) and 3,3'-dithiobis [sulfosuccinimidylpropionate] (DTSSP) were used to form intramolecular and intermolecular links between the peptides KAGK, YGGFLK, KGAILKGAILR, and RKRARKE[5]. Sulfo-NHS based reagents were necessary to both hold the negative charge on the reagent and to allow for the formation of a long lived gas-phase complex between the peptide and reagent, thus permitting the modification to take place. Recently a new cross-linking reagent shown in figure 3A was developed that shows promise for use in ion/ion reaction without the use of sulfo-NHS[8].

The reagent S-methyl 5,5′thiodipentanoylhydroxysuccinimide (MTP-NHS) incorporates a sulfonium ion into the spacer arm which functions as not only a fixed charge site but also a gas-phase cleavable bond. Upon collisional activation in a tandem mass spectrometry experiment the reagent fragments as shown in figure 3B form either the I or S modification both of which are different masses. Another alternative is for the reagent to under only one reaction such that it does not for a cross-link between two points in the peptide and contains one unreacted-NHS functionality. This is commonly referred to as a dead-end link and does not provide as much information and when both sites are covalently bound. For a dead-end link as shown in figure 3C it is possible for the NHS group to remain attached or to fall off resulting in either the I-SH or the I-SN structure as shown. This allows for the identification of the cross linked sites and the distinction between the three cross-linked products[3].




Scheme 4.1 Possible fragmentation options and pathways for the MTP-NHS reagent. (A) The complete structure of the MTP-NHS reagent. (B) Structure of the reagent when cross linked-between two peptides and the gas phase cleavable products. (C) Structure of the reagent in a dead-end link, still gas phase cleavable.

## 4.2 Experimental Section

### 4.2.1 Materials

The MTP-NHS reagent was synthesized by members of Dr. Gavin E. Reid's research group at Michigan State University following previously published procedures[8],

LIAGDGAILR was purchased from SynPep, DGAILDGAILD, and LIAGDGAILK was purchased from NeoBioSci (Cambridge, MA), the N-hydroxysuccinimide ester of 4-trimethylammonium butyrate (TMAB-NHS) was obtain from Dr. Fred Regnier. Methanol, glacial acetic acid, acetic anhydride, and ammonium hydroxide were purchased from Mallinckrodt (Phillipsburg, NJ).

Acetylation of the N-terminus and any un-protonated primary amines was accomplished by dissolving 1 nmol of peptide in 20 μL of 50 mM ammonium bicarbonate solution and 50 μL of acetylation reagent solution. This reagent was prepared by combining 20 μL of acetic anhydride and 50 μL of methanol. The reaction was allowed to proceed for two hours.[9]

Acetylation of only the N-terminus of a peptide with a lysine residue present was accomplished by first dissolving 0.3 mg of sulfo-NHS acetate in 400 μL of carbonate buffer at pH=5. The 100 μL of peptide solution at a concentration of 1 mg/mg of peptide was added to the sulfo-NHS solution and the reaction was allowed to proceed for two hours. The desired produce was separated via an Agilent HPLC using previously reported methods[7].

### 4.2.2 Mass Spectrometer

Experiments were performed using a modifier prototype AB Sciex QTRAP 2000 triple quadrupole/linear ion trap mass spectrometer (AB Sciex, Concorde, ON, Canada).[10] The instrument was equipped with a custom build dual nano-electrospray ionization source capable of generating ions at different polarities and described in more detail in

chapter 3. Primarily the experimental procedure consisted of first generating and isolating in Q1 the desired charge state of the peptide to be reacted with the MTP-NHS reagent. In most all cases the ion was either in a $[M-2H]^{2-}$ or a $[M-3H]^{3-}$ charge state such that the resulting product would be negatively charged. After isolation the peptide was stores in q2 while the singly positively charges MTP-NHS was generated and injected into the instrument and also isolated while passing through q2.

Both ion populations were subjected to mutual storage within the collision cell for a reaction time varying between 100 and 500 ms before the remaining positively charges species were ejected from the trap. Any remaining negatively charges ions were transferred to Q3 where the resulting complex between the peptide and the MTP-NHS was subjected to fragmentation through CID to obtain further structural information. Occasionally additional $MS^3$ spectra were obtained by performing a RF/DC isolation on the CID produce in question and fragmenting the ion further.

## 4.3 Results and Discussion

Preliminary experiments with the MTP-NHS cross-linking reagent seemed to indicate that it was modifying arginine present in several model peptides. Previous work has only observed reactions between NHS esters and un-protonated primary amines in the gas phase with any arginine residues present viewed as only charge bearing sites. Additionally the loss of either the NHS of sulfo-NHS from any complex formed was viewed as a signature of covalent modification. In an experiment shown below in figure 4.1, the model peptide Ac-LIAGDGAILR was reacted with MTP-NHS via an ion/ion reaction which produced a complex between the peptide and reagent after a single NHS

loss. This loss is believed to be a signature of covalent modification (REF). However, with the N-terminus acetylated no reactive residue remains unless the arginine is reacting with the reagent.

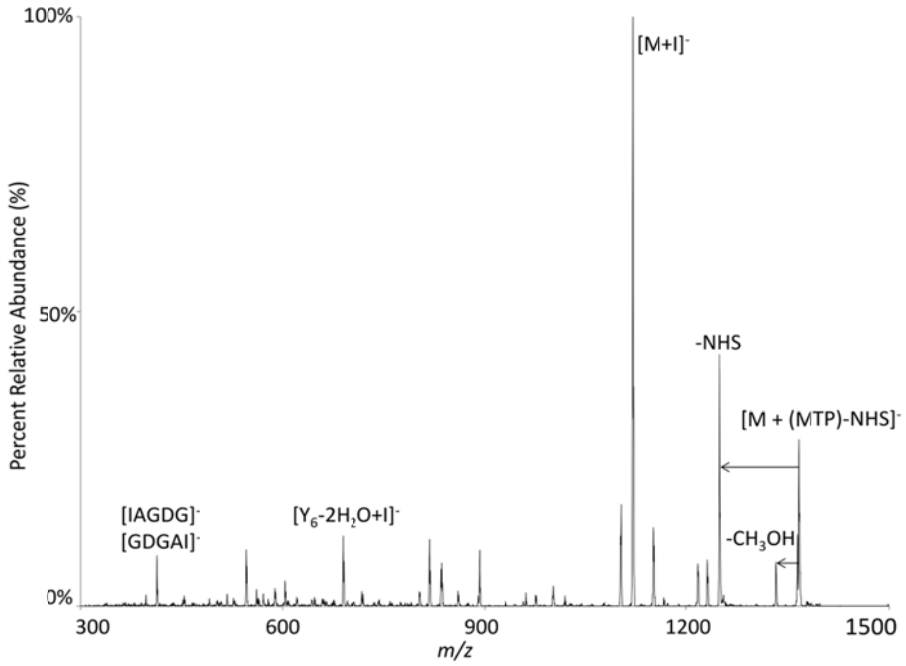



Figure 4.1 Mass Spectrum produced from ion trap CID of the complex [AC-LIAGDGAILR + (MTP-NHS) –NHS]$^-$ formed from the reaction between Ac-LIAGDGAILR and MTP-NHS.

Isolation and subsequent fragmentation of the complex with ion trap CID produced the mass spectrum shown in figure 4.1. The apparent loss of an additional NHS is observed along with the addition of the I portion of the mass spec cleavable cross-linker to the peptide. Several water losses are observed and the loss of methanol from the original complex, believed to be the result of a transfer of the methyl group on the sulfur atom to a carbonate followed by loss of the newly formed methanol. Further isolation

and fragmentation of the second NHS loss and the [M+I]- complex failed to produce any additional information. A possible explanation for this being that CID of negative ions has been shown to primarily result in side chain losses with little fragmentation of the peptide backbone. Consequently, structural assignment becomes difficult as the majority of structural information for interpretation is obtained from backbone fragmentation.

Seeing a single loss of NHS with the peptide Ac-LIAGDGAILR could be explained by the MTP-NHS reacting with arginine residue; however, this does not explain the additional second loss of NHS from the complex upon ion trap CID. Continuing with the assumption that previous work is correct and this loss is indeed a signature of covalent modification the only other possible sites for modification in this peptide are a carboxylate group on the C-terminus or the aspartic acid or the possibility that the arginine is undergoing a second reaction. The information obtained from figure 4.1 does not contain enough structural information to answer this question so another experiment was performed. The peptide DGAILDGAILD was selected as it contained a large number of carboxylate groups and could adequately hold the negative charge necessary for the ion/ion reaction.

Unlike the experiment with Ac-LIAGDGAILR, where no intact electrostatic complex between the peptide and reagent was observed, DGAILDGAILD and MTP-NHS did appear to form a complex. Upon isolation and ion trap CID the mass spectrum shown in figure 4.2 was produced. Unfortunately due to the large number of carboxylates in the peptide a significant number of water losses are apparent that dominate the spectrum. This was not unexpected as carboxylate groups tend to readily lose water upon dissociation in negative mode[11]. Of interest in this spectrum is not only the loss of NHS,

which could be attributed to a dead-end link attached to the N-terminus of the peptide, but also the second loss of NHS that is apparent but at much lower abundance. This seems to provide support to the theory that the carboxylates are reacting with the reagent. Additionally a complex corresponding to a loss of 245 Da from the starting complex was observed which does correspond to the mass of the I substituent of the reagent with the NHS functionality still attached. This is supported in that this complex was only formed from fragmentation of the starting [DGAILDGAILD+(MTP-NHS)]- and not from subsequent fragmentation of the resulting complex after a single NHS loss. Additionally fragmentation of the 245 Da loss peak produced the [M+I]- complex providing further justification for this assignment.

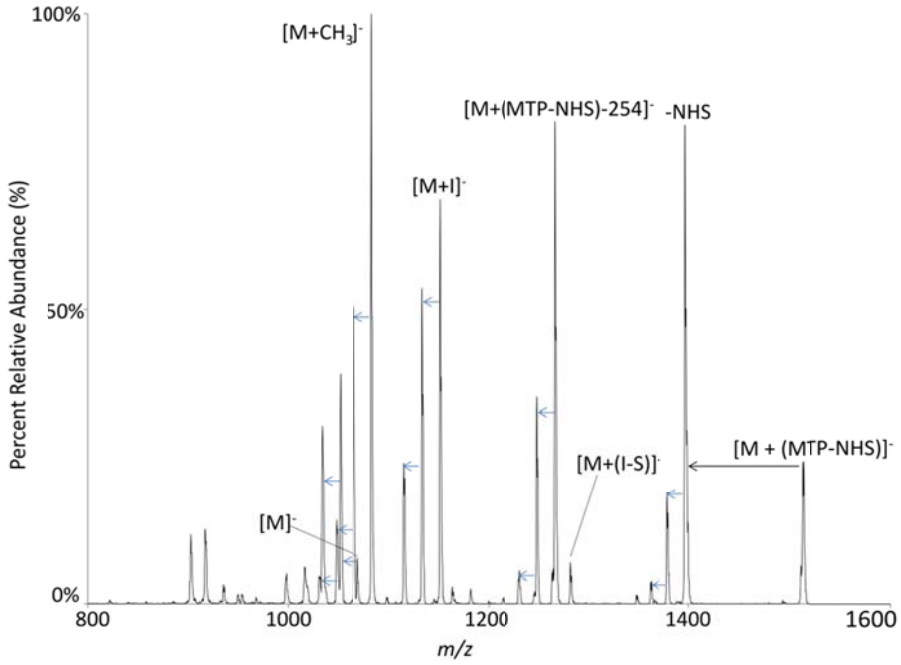



Figure 4.2 Mass spectrum produced from ion trap CID of the complex [DGAILDGAILD + (MTP-NHS)]$^-$ formed from the reaction between [DGAIlDGAILD – 2H]$^{2-}$ and MTP-NHS$^+$. The symbol ← represents a water loss.

Assuming this assignment is correct, it would mean that the reagent broke in half with a portion of it still electrostatically bound to the peptide. This is not surprising given that MTP-NHS is designed to be easily cleavable in the gas phase. What is surprising is that only one half of the fragmented MTP-NHS was observed and that only the I substituent was seen and not the S substituent. Between the two portions of the reagent conventional wisdom would indicate that the S substituent would be more likely as contains the sulfur atom which acts as a "sticky group" electrostatically binding the reagent fragment to the peptide. In order to determine whether

In order to make a more definitive determination of whether the reagent was indeed modifying carboxylate groups within the peptide the experiment was repeated with the N-terminus of DGAILDGAILD acetylated. The spectrum produced from ion trap CID of the complex is shown below in Figure 4.3. Not surprisingly the complex of the peptide and the enact reagent with a single NHS loss was observed as a produce of the ion/ion reaction, unlike the experiments with Ac-LIAGDGAILR. The spectrum in figure 4.3 is interesting in that the enact peptide is observed indicating that a significant portion of the Ac-DGAILDGAILD was only bound electrostatically to the reagent and no covalent modification took place. In addition to a series of water losses there is also evidence of the transfer of a methyl cation from the sulfonium ion o the reagent to the peptide. This is indicated by the addition of 15 Da to Ac-DGAILDGAILD labeled in figure 4.3 as [M+CH3]- which also exhibits a series of water losses and possibly a methanol loss that is not labeled due to the complexity of the spectrum in that region.

Also of interest from this experiment is the formation of another complex whose mass corresponds to the MTP-NHS reagent fragmenting and a portion of it remaining

electrostatically bound to the reagent. The presence of this complex complex labeled as [M+(MTP-NHS)-254]- along with the large quantity of unmodified peptide present would seem to indicate that the reagent does not react with carboxylates. However, there is a small peak in the spectrum that aligns with a single loss of NHS from the entire starting electrostatic complex. The presence of this complex along with the lack of any real sequence information from fragmentation of the peptide backbone in this spectrum or any other spectrum obtained from additional fragmentation of the complexes shown in figure 4.3 unfortunately still prevents any conclusions on carboxylate reactivity from being drawn.

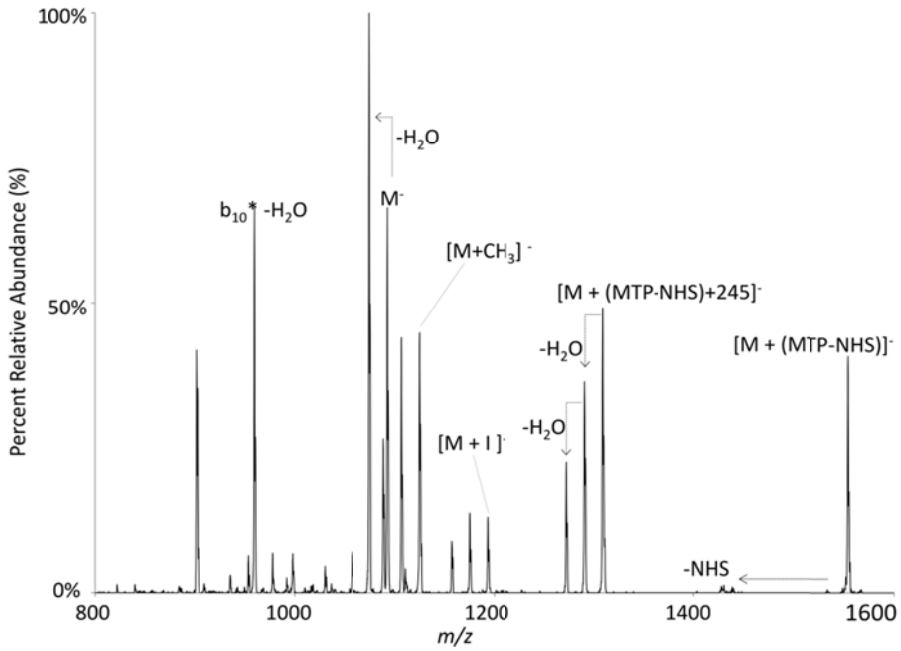



Figure 4.3 Mass spectrum produced from ion trap CID of the complex [AC-DGAILDGAILD + (MTP-NHS)]$^{-}$ formed from the reaction between [Ac-DGAILDGAILD – 2H]$^{2-}$ with MTP-NHS$^{+}$

The other possibility to explain the loss of more NHS residues that reactive sites present in the peptide was that arginine was somehow undergoing more than one covalent modification. This was examined first by repeating the experiments performed with Ac-LIAGDGAILR but using Ac-LIAGDGAILK containing only an n-terminal acetylation. This would compare the reactivity of MTP-NHS between arginine and lysine as the remainder of the peptide is the same. The results of this experiment are shown below in figure 4.4A. As was observed with the Ac-LIAGDGAILR the intact electrostatic complex was not observed and the complex formed from the ion/ion reaction already lost one NHS functionality indicating that this loss is extremely fast. Additionally the loss of the second NHS group is observed although in much more significant abundance that for the reaction with Ac-LIAGDGAILR. The additional of the I substituent of MTP-NHS to Ac-LIAGDGAILK can be observed seemingly indicating that at least one site on the pepeide was covalently modified. However, little structural information from the peptide backbone was obtained with the exception of the presence of a modified y6 ion after two water losses. Observing something after two water losses without seeing any of the compound present in the spectrum before the losses of water is odd, but the mass of the peak with the label does correspond to the compound indicated and the assignment is supported by additional fragmentation of the [M+I]- complex shown in figure 4.4B. However it cannot be unambiguously stated that the modification occurred without additional backbone fragments present. The unlabeled peaks in the spectrum can correspond to multiple complexes and it was not possible with the data available to unambiguously identify them.

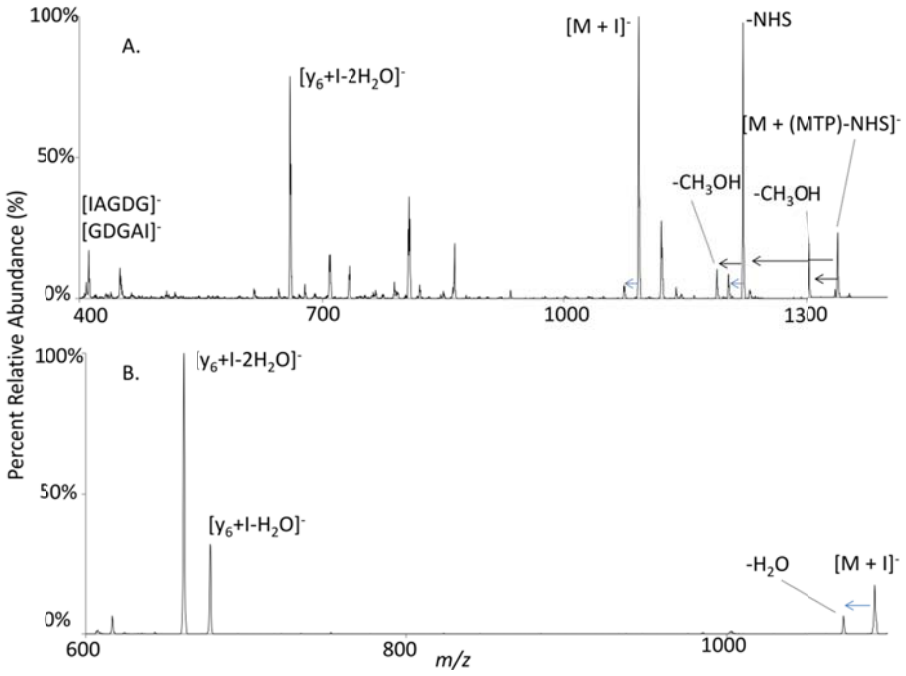



Figure 4.4 (A) Mass Spectrum produced from ion trap CID of the complex [N-terminal-Ac-LIAGDGAILK + (MTP-NHS)]$^{-}$ formed from the reaction between [N-terminal-Ac-LIAGDGAILK -2H]$^{2-}$ and MTP-NHS$^{+}$. (B) MS$^{3}$ experiment showing ion trap CID of [N-terminally-AC-LIAGDGAILK + (MTP-NHS)]$^{-}$. The symbol ← represents a water loss.

Given that the range of experiments performed were unable to determine exactly what was happening with MTP-NHS reagent when reacted with a peptide it was decided to attempt a reaction with a smaller reagent of similar structure. For this to be effective the new reagent needed to contain a fixed positive charge and incorporate a NHS functionality as opposed to sulfo-NHS. The selected reagent was the N-hydroxysuccinimide ester of 4-trimethylammonium butyrate (TMAB-NHS) which featured a fixed charge quaternary amine and whose structure is shown in figure 4.5. Upon ion/ion reaction with Ac-DGAILDGAILD and TMAB-NHS an intact complex was formed without the loss of the NHS substituent. Upon ion trap CID the complex fragmented to shown a single NHS loss and the additional loss of 3-carboxypropyl-

trimethylammonium (CP-TMA) which is the reagent TMAB-NHS without the NHS functionality. This pattern had been observed with previous experiments and indicates a situation where non covalent modification occurred and the two species were only bound electrostatically. This would seem to add further evidence to support the conclusion that the MTP-NHS was not reacting with carboxylate resides assuming that the previous interpretation of the data was correct. Unfortunately although there is some evidence throughout all of the experiment conducted that would seem to indicate the reactivity of the carboxylates now of the experiments performed offer conclusive proof for or against their reactivity.

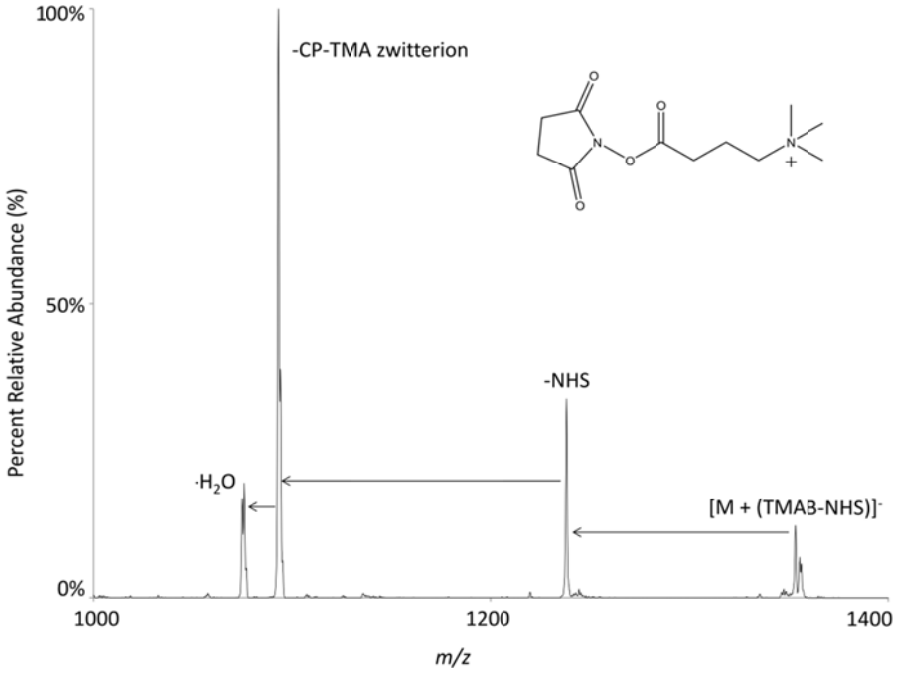



Figure 4.5 Mass Spectrum produced from ion trap CID of the complex $[Ac\text{-}DGAILDGAILD + (TMAB\text{-}NHS)]^-$ formed from the reaction between $[Ac\text{-}DGAILDGAILD - 2H]^{2-}$ and $MTP\text{-}NHS^+$

## 4.4 Conclusions

While approaching the investigation of a different phenomenon, the reactivity of histidine residues in the gas-phase, another member of the research group made a discovery offering evidence to support the reactivity of the carboxylate residues. Using reagents based on sulfo-NHS an isotope labeling experiment was performed in which a sample was prepared in $O^{18}$ labeled water. This caused any $O^{16}$ present within the sample of interest to be replaces with the $O^{18}$ such that it could be tracked during the course of the ion/ion reaction based on the mass.

## 4.5 References

1. Sinz, A., Chemical cross-linking and mass spectrometry to map three-dimensional protein structures and protein–protein interactions. Mass Spectrometry Reviews 2006, 25 (4), 663-682.

2. Chakravarti, B., Lewis, S.J., Chakravarti, D.N., Raval, A., Three-dimensional structures of proteins and protein complexes from chemical cross-linking and mass spectrometry: a biochemical and computational overview. Curr. Proteom 2006, 3, 1-21.

3. Tubb, M. R.; Silva, R. A. G. D.; Fang, J.; Tso, P.; Davidson, W. S., A Three-dimensional Homology Model of Lipid-free Apolipoprotein A-IV Using Cross-linking and Mass Spectrometry. 2008.

4. Singh, P.; Panchaud, A.; Goodlett, D. R., Chemical Cross-Linking and Mass Spectrometry As a Low-Resolution Protein Structure Determination Technique. 2010.

5. Mentinova, M.; McLuckey, S. A., Covalent Modification of Gaseous Peptide Ions with N-Hydroxysuccinimide Ester Reagent Ions. Journal of the American Chemical Society 2010, 132 (51), 18248-18257.

6. Mentinova, M.; McLuckey, S., Intra- and Inter-Molecular Cross-Linking of Peptide Ions in the Gas Phase: Reagents and Conditions. Journal of The American Society for Mass Spectrometry 2011, 22 (5), 912-921.

7. Mentinova, M.; Barefoot, N.; McLuckey, S., Solution Versus Gas-Phase Modification of Peptide Cations with NHS-Ester Reagents. Journal of The American Society for Mass Spectrometry 2012, 23 (2), 282-289.

8. Lu, Y.; Tanasova, M.; Borhan, B.; Reid, G. E., Ionic Reagent for Controlling the Gas-Phase Fragmentation Reactions of Cross-Linked Peptides. Analytical Chemistry 2008, 80 (23), 9279-9287.

9. Reid, G.; Simpson, R.; O'Hair, R. J., A mass spectrometric and ab initio study of the pathways for dehydration of simple glycine and cysteine-containing peptide [M+H]+ ions. Journal of the American Society for Mass Spectrometry 1998, 9 (9), 945-956.

10. Hager, J. W., A new linear ion trap mass spectrometer. Rapid Communications in Mass Spectrometry 2002, 16 (6), 512-526.

11. Bowie, J. H.; Brinkworth, C. S.; Dua, S., Collision-induced fragmentations of the (M- H)− parent anions of underivatized peptides: An aid to structure determination and some unusual negative ion cleavages. Mass Spectrometry Reviews 2002, 21 (2), 87-107.

# CHAPTER 5: SELECTIVE CHARGE INVERSION IN THE GAS PHASE OF PHORPEPTIDES FROM A TRYPTIC DIGEST USING AN ARGINING BASED REAGENT

## 5.1 Introduction

Posttranslational modifications (PTMs) are alterations made to the structure of proteins after they are created or translated by mRNA. A number of different covalent modifications are possible such as nitrosylation, methylation, glycosylation, etc. and each can have an influence on the function of the protein[1]. Consequently understanding PTMs and being able to easily identify them is extremely important and relevant to the study of proteins in the field of proteomics. One such modification that is the focus of study is phosphorylation which involved the addition of a phosphate group to a protein typically on serine, threonine, and tyrosine residues. Phosphorylation plays a large role in various cellular processes functioning in a number of situations as an on/off switch and understanding it is relevant to understanding how these processes work.

Recently researchers at the national institute of health have studied non-covalent complexes formed between two peptides as these interactions can play a role in understanding protein interactions. The focus in particular of a series of studies has been on the ability of sulfation and phosphorylation sites to interact with guanidinium groups on the amino acid arginine to form a strong interaction. A series of peptides containing serine, threonine, and tyrosine sites of phosphorylation were studies for their ability to

interact with peptides containing sequential arginine residues. Non covalent complexes of varying strength were formed for all of the amino acids and the potential exists to use these interactions and ion chemistry as a selection tool for selection for phosphopeptides from a mixture.[2-4]

Ion/ion reactions are a powerful for studying protein and peptide interactions and in particular charge inversion reactions are relevant to the study of these interactions.[5] A charge inversion reaction occurs when an ion of one polarity forms a complex with a multiple charged ion of another polarity. The resulting electrostatic complex has the same polarity of charge as the multiply charged ion thus inverting the charge of the original species. If the interaction is long lived it is also possible for multiple charges to transfer to the starting compound allowing for the charge inversion of the starting species and not just the formation of a charge inverted complex. This reaction can be applied to the study of non-covalent interactions between phosphorylation sites and arginine residues. A solution containing several phosphopeptides can be sprayed in the negative mode forming negative ions. That mixture can then undergo an ion/ion reaction with a positively charged arginine based reagent resulting in the formation of a non-covalent interaction between any phosphopeptides in the mixture. The resulting complex would be positively charged thus charge inverting the phosphopeptides. Furthermore if the reagent reactivity could be tuned so that it formed the strongest interactions with only phosphorylation sites is would be possible to selectively charge invert only phosphopeptides in a mixture allowing for easier identification and analysis.

## 5.2 Experimental Section

### 5.2.1 Materials

Methanol, glacial acetic acid, acetic anhydride, and ammonium hydroxide were purchased from Mallinckrodt (Phillipsburg, NJ). The peptides pYGGFL, sYGGIG, RR, and RRR were purchased from NeoBioSci (Cambridge, MA), leucine encephalin (YGGFL), ubiquitin, a-casein, and b-casein were purchased from Sigma-Aldrich (St. Louis, MO). Protein digestions were performed by members of Dr. Andy Tao's research group at Purdue University.

Acetylation of the N-terminus and any un-protonated primary amines was accomplished by dissolving 1 nmol of peptide in 20 μL of 50 mM ammonium bicarbonate solution and 50 μL of acetylation reagent solution. This reagent was prepared by combining 20 μL of acetic anhydride and 50 μL of methanol. The reaction was allowed to proceed for two hours.[6]

Methyl esterification of the c-terminus of a peptide was accomplished by first allowing 250 μL of dry methanol to cool in a -20oC freezer for twenty minutes. Then 40 μL of acetyl chloride were added dropwise to the methanol and the resulting mixture was allowed to sit for five minutes. 50 μL of a stock peptide solution containing the peptide as a concentration of 1 mg/mL in water was combined with 100 μL of the acidified methanol and the reaction was allowed to proceed for two hours.[7] Protein digestions were performed by members of Dr. Andy Tao's research group at Purdue University

### 5.2.2 Mass Spectrometer

Experiments were performed using a modifier prototype AB Sciex QTRAP 2000 triple quadrupole/linear ion trap mass spectrometer (AB Sciex, Concorde, ON, Canada).[8] The instrument was equipped with a custom build dual nano-electrospray ionization source capable of generating ions at different polarities and described in more detail in chapter 3. The basic experiment consisted of several portions that began with the control steps to ensure that only peptides which underwent the charge inversion reaction were detected. The digest sample was sprayed in negative mode to confirm its composition and determine the mass which should be observed assuming that the charge inversion experiment functioned as intended. Additionally the conditions in the instrument were adjusted to maximize the amount of singly charged species present within the sample unless otherwise specified. Finally the conditions within the collision cell were optimized such that only a minimal loss of signal for the digest sample occurred when mutual storage was activated.

After the signal from the digest sample was optimized the next step consisted of optimizing the reagent. Since a specific charge state was chosen for each experiment the desired *m/z* was isolated in Q1 with an RF/DC isolation. From here the sample was injected into the collision cell and the mutual storage settings were activated. This insured that both the positively and negatively charged species were stable within the quadrupole under the specified conditions. For the reaction, both samples were injected into the instrument sequentially and trapped in q2 for a period of time ranging from 100-500 ms. From here the negatively charges ions were ejected from the trap while an rf ramp was performed to remove any unreacted reagent. Since any charge inverted peptides or

complexes would have a higher *m/z* than the reagent the rf was ramped up such that is ejected the small reagent ion from the trap while continuing to contain the larger product ions.

From here everything was transferred to Q3 for initial analysis. Any complexes formed were isolated using an RF/DC isolation and subjected to fragmentation via CID. The transfer process for the ions into Q3 was kept at a low enough energy to prevent the occurrence of beam-type CID. Essentially only MS and $MS^2$ scans were obtained for this series of experiments unless otherwise indicated. In addition several experiments were performed on a prototype modifier AB Sciex API QSTAR *Pulsar I* mass spectrometer (AB Sciex, Concorde, ON, Canada) that was described in detail in chapter 3. The procedure for these experiments was quite similar to those performed on the QTRAP 2000 with the exception that no CID was performed and after mutual storage the ions were simply injected into the TOF for mass analysis.

## 5.3 Results and Discussion

For an initial investigation into the ability of an arginine reagent to charge invert phosphopeptides, the tripeptide RRR was selected as a reagent. This was chosen as it contained several arginine residues adjacent to each other which is necessary for the formation of a non-covalent complex and it is relatively small to decrease the overall mass of the complex. The first experiment performed was to charge invert the model peptide pYGGFL which proved to be successful although a significant amount of proton transfer from the reagent to the peptide was observed in addition to complex formation show in figure 5.1. This is not surprising and is to be expected although it is necessary to

minimize the amount of proton transfer product produced so as to maximize the amount of non-covalent complex obtained.

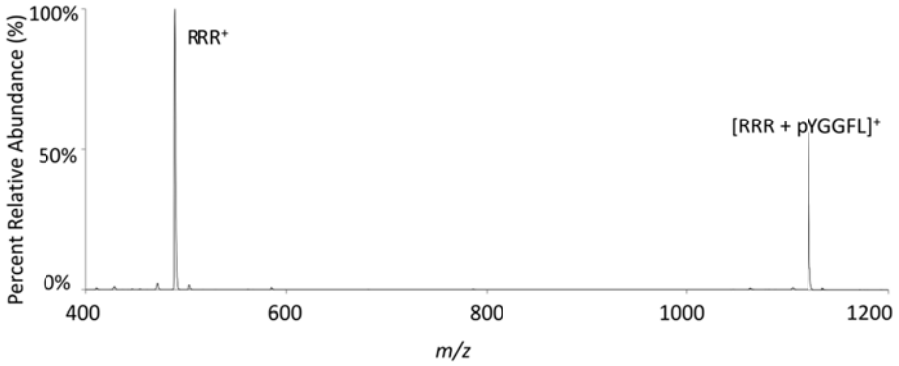



Figure 5.1 Charge inversion reaction between $[RRR+2H]^{2+}$ and $[pYGGFL-H]^{-}$

As RRR has successfully been show to form a non-covalent complex and charge invert a phosphopeptide the next step was to attempt selective charge inversion. In this case the protein α-casein, which contains several phosphorylation sites, was digested with trypsin and the resulting mixture was sprayed in negative mode. The goal of the experiment was to only charge invert the phosphopeptides so that all other peptides in the mixture would not react causing them not to be detected or alternatively steal a proton from the reagent resulting in neutralization. The results of this ion/ion reaction, which was performed on the QSTAR, are shown in figure 5.2 A. A significant amount of proton transfer occurred as expected considering that the amount of peptides in the mixture far outweighs those containing a phosphorylation site. However, almost not peptides in the digest where charge inverted or formed a complex with RRR and of the peaks present in the spectrum only one could be identified. Additionally as the label indicates this peptide

does not contain any phosphorylation leading to speculation about the effectiveness of RRR as a reagent. A similar experiment was performed with β-casein and comparable results were obtained and displayed in figure 5.2B.

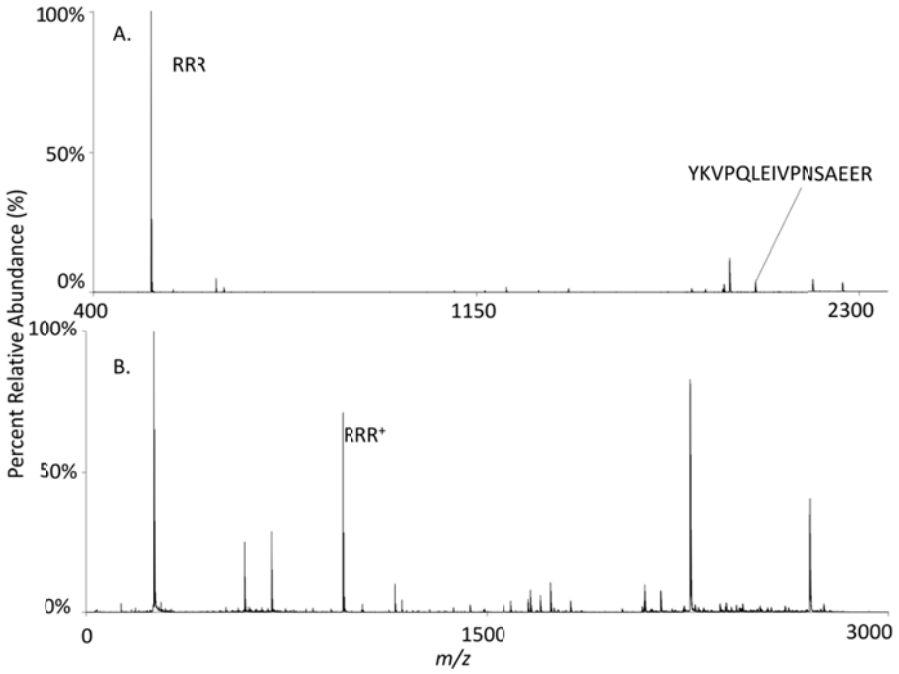



Figure 5.2 (A) Mass Spectrum produced from the charge inversion reaction between $[RRR+2H]^{2+}$ and a tryptic digest of the phosphopeptide α-casein. (B) Charge Inversion reaction between a digest of b-casein and $[RRR+2H]^{2+}$. The peaks to the left of the $RRR^{+}$ are fragments of the tripeptide and the remaining peaks could not be identified.

These results would seem to indicate that RRR is too good of a reagent in that it can charge invert compounds in the digest beyond the phosphopeptides present. In order to confirm this idea another ion/ion reaction was performed where RRR was reacted with a tryptic digest of ubiquitin. Ubiquitin does not contain any phosphorylation sites so the theory was that that it would be a good way to demonstrate the selectivity of the reagent.

It was already known RRR could for a non-covalent complex with phosopeptides but this experiment would give insight into the tripeptide's ability for form complexes with other compounds that might be in the digests. The results are shown in figure 5.3 and go a long was to show that RRR by itself is not selective enough to accomplish the ultimate goal of this project. A large number of peptides formed a non-covalent complex with RRR and several actually underwent a double proton transfer in that they went from a single negative charge to a single positive charge. This experiment seemed to demonstrate the necessity to select a new charge inversion reagent that could be more selective to only forming interactions with phosphorylation sites

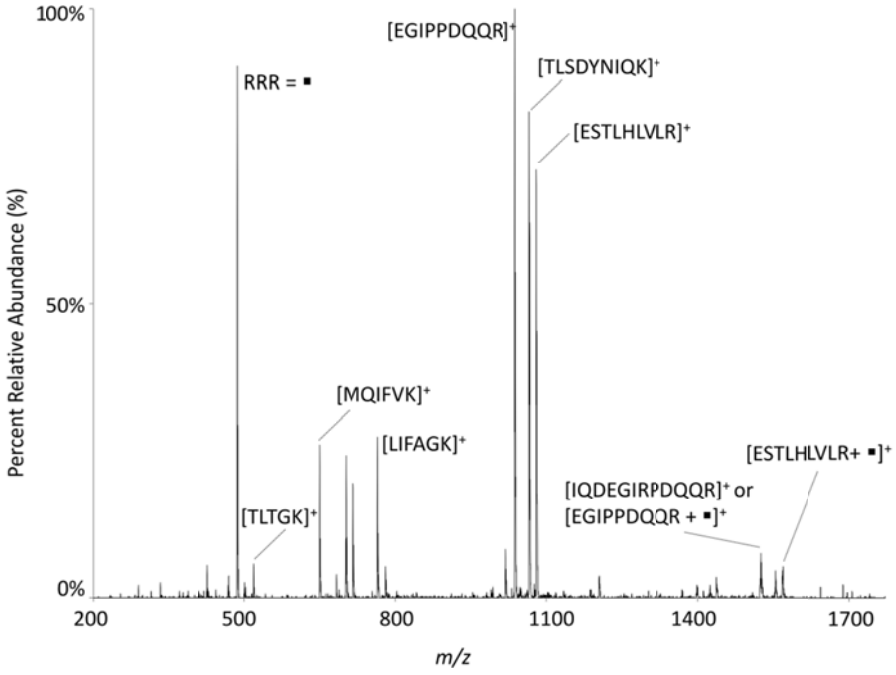



Figure 5.3 Mass spectrum produced from the charge inversion reaction between $[RRR+2H]^{2+}$ and the tryptic digest of ubiquitin.

Rather than creating a new peptide and attempting to change to overall structure to something of the form RXR, the existing tripeptide was acetylated at the N-terminus and methyl esterified at the C-terminus to form Ac-RRR-OMe. To show that this would still form a strong non-covalent interaction with phosphorylation sites it was reacted with a mixture containing three model peptides. These peptides were YGGFL, pYGGFL, and sYGGIG. The YGGFL was present to initially observe if Ac-RRR-OMe was selective at least for the phospho- and sulfopeptides in this mixture. sYGGIG was used instead of sYGGFL in order to distinguish the peptide from pYGGFL as both peptides have similar weights and could not be distinguished with the resolution present on the QTRAP 2000. Figure 5.4 contains the results of this experiment and show that Ac-RRR-OMe does not charge inverts YGGFL and as expected the interaction with the sulfation is stronger than the interaction with phosphate groups. This is consistent with observations made by previous experiments 7*. The addition of 98 Da to Ac-RRR-OMe was observed however this could come from either peptide as sYGGIG can lose $H_2SO_4$ and pYGGFL can lose $H_3PO_4$. Both species can form an interaction with Ac-RRR-OMe and CID of the peak in question was uninformative. Of interest to note is that this experiment was repeated several times and the 98 Da addition to Ac-RRR-OMe was not always present indicating that it might be dependent upon the energy used to transfer to ions between q2 and Q3. However, the presence of the complex was not consistent enough to make this determination.

To further examine the selectivity of Ac-RRR-OMe a charge inversion reaction was performed with the ubiquitin digest use previously with RRR. The results were similar with the key difference that the charge inverted products formed in significantly

lower quantities that observed with RRR. This experiment alone was not sufficient to demonstrate selectivity so the experiment was performed again only now the model peptide pYGGFL was spiked into ubiquitin digest. This allowed for a direct comparison between the abundances of the charge inverted phosphopeptide with the other charge inverted species and the results are displayed in figure 5.5. The top portion of the spectrum labeled A is the ubiquitin digest spiked with pYGGFL which is at a concentration similar to the most abundant peptides in the mixture but is not itself the most abundant compound. There are several unlabeled peaks in the spectrum that were not identified as they do not correspond to any peptides that would be produced in the digest.

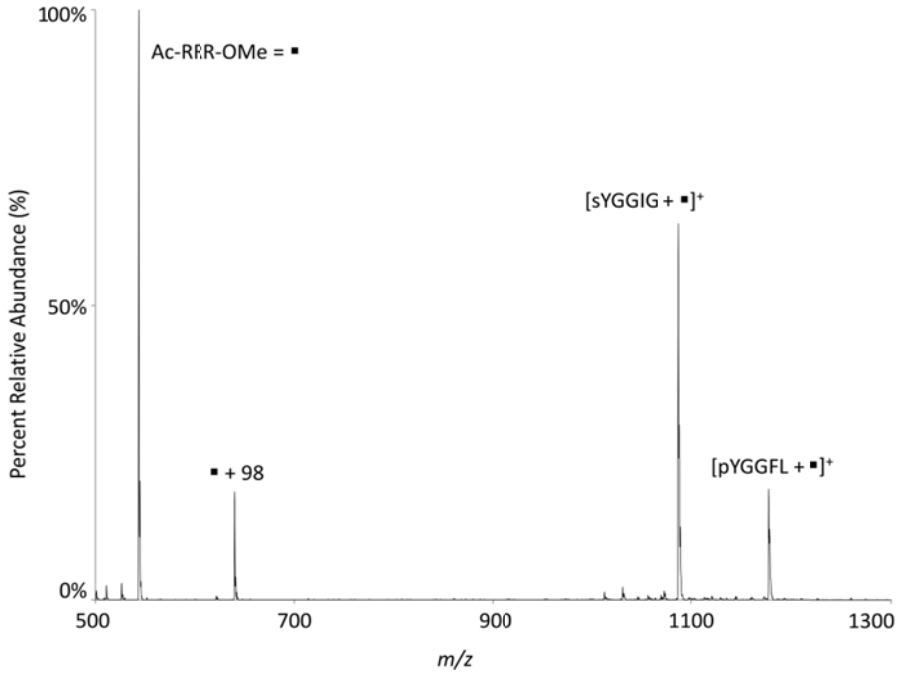



Figure 5.4 Mass spectrum produced from the charge inversion reaction between [Ac-RRR-OMe +2H]$^{2+}$ and a mixture of three peptides [YGGFL-H]$^-$, [pYGGFL-H]$^-$, and [sYGGFL-H]$^-$.

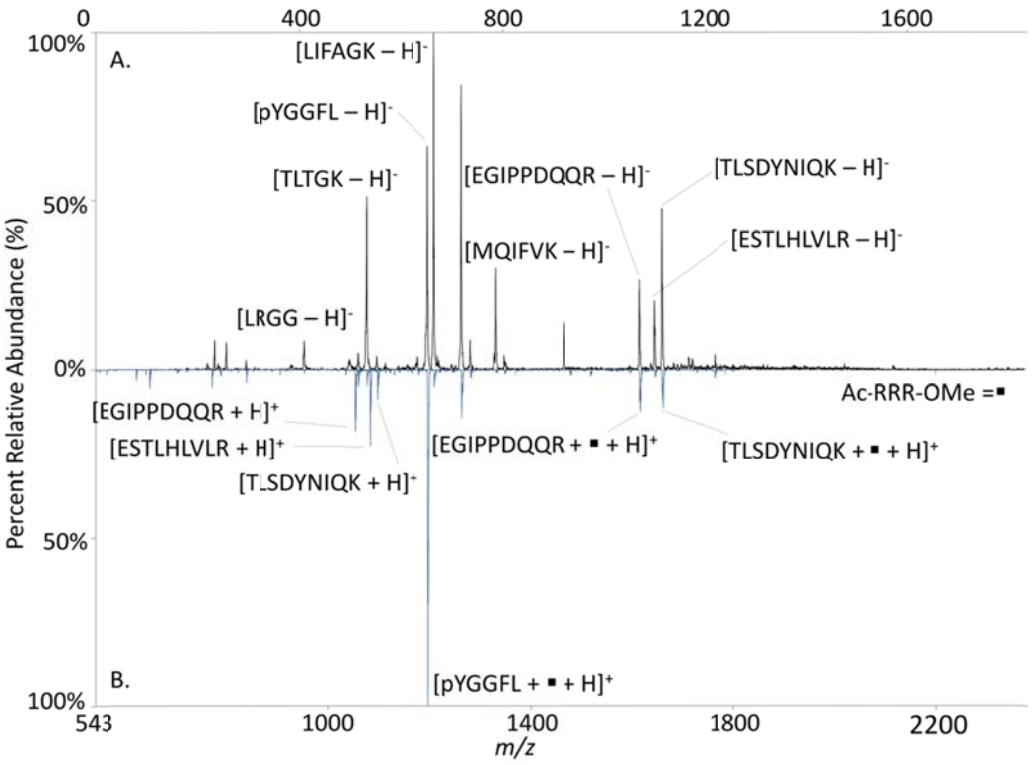



Figure 5.5(A) Mass spectrum from negative nano-ESI of a digest of ubiquitin spiked with $[pYGGFL\text{-}H]^-$ compared to (B) the mass spectrum produced from the charge inversion reaction between $[Ac\text{-}RRR\text{-}OMe+2H]^{2+}$ and the tryptic digest of ubiquitin spiked with $[pYGGFL\text{-}H]^-$.

The bottom portion of Figure 5.5 labeled as spectrum B represents the products of the charge inversion reaction and has been adjusted so that the peaks are shifted from the peaks in spectrum A by the mass of Ac-RRR-OMe. Therefore any peaks corresponding to peptides that were charge inverted line up on both spectrums for easier interpretation of the data. It should also be noted that when performing this experiment a large amount of proton transfer occurred producing singly charged Ac-RRR-OMe which is not present of spectrum B due to the shifting of the masses. The most abundance compound formed from the charge inversion reaction of [Ac-RRR-OMe + pYGGFL]+ are desired for the

selective charge inversion project but several other peptides contained within the digest were also charge inverted. The two peptides EGIPPIDQQR and TLSDYNIQK not only formed non-covalent complexes but also underwent double proton transfer. On the other hand the peptide ESTLHLVLR underwent double proton transfer and also most likely formed a non-covalent complex but that is unlabeled due to its abundance being in the noise of the spectrum. This result is interesting as the peptides that formed larger amounts of the complex contain more basic sites indicating that they might have some involvement in the reaction.

Although the results displayed in figure 5.5 are not ideal in that peptides other than phosphopeptides were charge inverted the Ac-RRR-OMe could still be a useful reagent. If it's possible to use higher transfer energies between q2 and Q3 when performing the reaction to dissociate the complexes with peptides not containing a phosphorylation site the charge inversion is still selective to a degree. Even though other peptide might charge invert and undergo a double proton transfer, the phosphopeptide can still be distinguished by a mass difference as long as the interaction between Ac-RRR-OMe and the phosphate is the strongest.

In all proceeding experiments the only peptides shown to for non-covalent complexes with the arginine based reagents in an ion/ion reaction have had phosphorylation sites on only tyrosine residues. It has been shown that the interaction between phosphorylated tyrosine and arginine is stronger than with phosphorylated serine or threonine. In order to determine whether these experiments would be successful it was necessary to examine a peptide containing a phorphorylation site on something other than arginine so the peptide LPISASHpSpSKTR was selected. However the mass of a singly

charged complex between this peptide and RRR is above the upper mass limit of the QTRAP 2000 which is 1770 m/z. Consequently it was necessary to use triply charged RRR for this experiment to react with singly charged peptide and form a double charged complex of $[LPISASHpSpSKTR+RRR]^{2+.}$ The results of this experiment are shown in figure 5.6 below with the small unlabeled peaks below RRR correspond to fragments of the tripeptide. The spectrum was scaled to the abundance of the complex peak as a significant amount of proton transfer occurred. However, this experiment does show that the charge inversion experiment is successful when the phosphorylation is on a serine as opposed to a tyrosine.

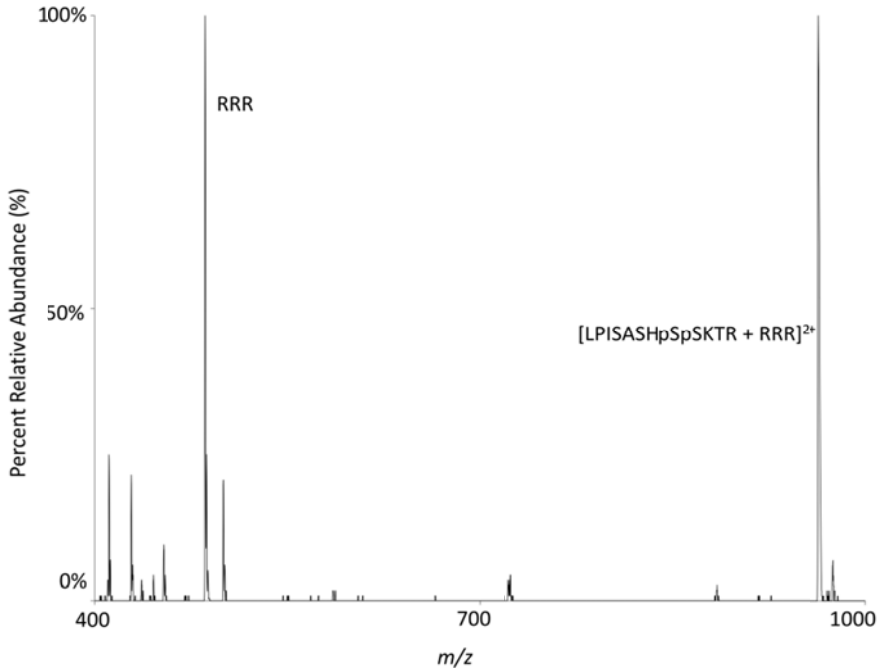



Figure 5.6 Mass spectrum produced from the charge inversion reaction between $[RRR+3H]^{3+}$ and $[LPISASHpSpSKTR-H]^{-}$. Abundance scaled to $[LPISASHpSpSKTR+RRR]^{2+}$.

## 5.4 Conclusions

Based on the results obtained with the experiments above it would appear that the reagents selected for the charge inversion process are too strong. Essentially the peptides based on the amino acid arginine such as RRR and Ac-RRR-OMe seem to form interactions with not only the phosphopeptides but also the other peptides present within the solution. Further investigation may show that it could be possible to separate these two different complexes if the strength of the interaction is different. This could be accomplished using a form of beam-type CID within the instrument such that the energy imparted would be sufficient to fragment the undesirable complexes but leave the phosphopeptide complexes intact. Alternatively it may be possible to use a reagent based on a different amino acid with a weaker interactions but preliminary experiments indicate that this is unlikely.

## 5.5 References

1. Nelson, D. L. D., Lehninger principles of biochemistry. 2008.

2. Jackson, S. N.; Moyer, S. C.; Woods, A. S., The Role of Phosphorylated Residues in Peptide–Peptide Noncovalent Complexes Formation. Journal of the American Society for Mass Spectrometry 2008, 19 (10), 1535-1541.

3. Jackson, S. N.; Wang, H.-Y. J.; Yergey, A.; Woods, A. S., Phosphate Stabilization of Intermolecular Interactions. Journal of Proteome Research 2005, 5 (1), 122-126.

4. Woods, A. S.; Wang, H.-Y. J.; Jackson, S. N., Sulfation, the Up-and-Coming Post-Translational Modification: Its Role and Mechanism in Protein−Protein Interaction. Journal of Proteome Research 2007, 6 (3), 1176-1182.

5. McLuckey, S. A., The emerging role of ion/ion reactions in biological mass spectrometry: considerations for reagent ion selection. Eur J Mass Spectrom (Chichester, Eng) 2010, 16 (3), 429-36.

6. Reid, G.; Simpson, R.; O'Hair, R. J., A mass spectrometric and ab initio study of the pathways for dehydration of simple glycine and cysteine-containing peptide [M+H]+ ions. Journal of the American Society for Mass Spectrometry 1998, 9 (9), 945-956.

7. Nudelman, A.; Bechor, Y.; Falb, E.; Fischer, B.; Wexler, B. A.; Nudelman, A., Acetyl Chloride-Methanol as a Convenient Reagent for: A) Quantitative Formation of Amine Hydrochlorides B) Carboxylate Ester Formation C) Mild Removal of N-t-Boc-Protective Group. Synthetic Communications 1998, 28 (3), 471-474.

8. Hager, J. W., A new linear ion trap mass spectrometer. Rapid Communications in Mass Spectrometry 2002, 16 (6), 512-526.