Development of Cleavable Peptide Probes for Mass Spectrometry Based Immunoassays

THESIS

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Abstract

Molecular diagnostics based on antibodies and complementary DNAs is the method of choice for high-throughput protein biomarker detection. Most of these methods rely on colorimetric detection via enzymatic reactions or fluorescent probes. However, there are some restrictions regarding probe stability and multiplexed capabilities. Herein, we propose a novel mass spectrometry-based diagnostic platform for rapid and multiplexed detection of protein disease biomarkers. The core of this approach is the use of available cleavable isobaric peptides as mass reporter in immunoassays. All peptides probes are rationally designed to have the same molecular weight but dissociate to give specific fragment ions when subjected to collision-induced dissociation (CID), providing well-defined spectra resolution in a single experiment for multiple biomarkers.

The project has three main aspects: (i) optimization of conditions for peptide ion generation and fragmentation to obtain highly sensitive mass reporters with characteristic fragment ions, (ii) design and incorporation of the selected mass reporter peptides into a cleavable probe unit, and (iii) subsequently use the cleavable probe for immunoreaction, on wax-printed paper substrates. The cleavable probe unit will feature three functional characteristics: (a) sulfur-containing (-NCS) groups for coupling to antibodies specific to the protein biomarker of interest, (b) easily ionizable peptide molecules for sensitive MS detection, and (c) a cleavable linkage allowing release of the peptide. To assess the
peptide suitability as mass reporters, nano-electrospray ionization (nESI) and paper-spray ionization were used to generate ions for mass spectrometry (MS) characterization.

Three peptides were designed and synthesized: AKRRG, RRGKA, and GARKK, all having molecular weight of 586 Da. nESI-MS analysis of these peptides showed a predominant peak at \( m/z \) 294 for doubly protonated \([M+2H]^{2+}\) ions and minor signals at \( m/z \) 196 and 587 as triply \([M+H]^{3+}\) and singly charged \([M+H]^{+}\) species, respectively. All peptides can be sensitively detected at 3 nM concentration levels, in tandem MS (MS/MS) mode. Most importantly, fragment ions from the three isobaric peptides produced in MS/MS are different, providing a simple way to differentiate them as mass tags for different diseases. For example, CID of \([M+H]^{+}\) species from GARRK produced a signature fragment ion at \( m/z \) 441 via elimination of lysine (K) residue. Similarly, CID of \([M+2H]^{2+}\) ions from GARRK yielded signature fragment ion at \( m/z \) 230. CID data for all peptides tested are discussed in details. Linear calibrations curves are easily generated for \([M+2H]^{2+}\) ions in 3–100 nM concentration range using the characteristic fragment ions.

Moreover, we observed that fragmentation efficiencies of the \([M+2H]^{2+}\) ions from the selected peptides were different. Ion mobility experiments, however, revealed that the three \([M+2H]^{2+}\) peptide ions have comparable cross sections. This result suggests that ability to derive unique fragment ions from isobaric peptides with different efficiencies may be related to the mobile proton mechanism where restrictions in the movement of charge affect ease of fragmentation. Bio-conjugation of the GARRK peptide-probe is achieved. A peptide-probe was successfully obtained and future applications on paper-
based microfluidic device will allow quantification through paper-spray mass spectrometry.
Dedication

This dissertation is dedicated to all the people who have supported me through this journey; my family, friends and mentors.
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First of all, I want to thank my advisor, Dr. Badu-Tawiah who challenged me to reach my full potential as a scientist, as well for his guidance and support. I also want to thank all my colleagues in my research group who have all contributed to my learning. I specifically want to thank Jay Kim and Yosef Maher for experimental contributions. Other people in my group who helped in training who I want to thank include Deidre Damon, Suming Chen, Qiong Qiong Wan and Savithra Jayaraj. I want to also thank Alyssa Stiving in the Wysocki group for providing the use, collection and data handling for Ion Mobility, as well the Jaroniec group for providing the Lyophilizer. I also want to thank Kedwin Rosa in the Forsyth group for his availability in addressing specific synthetic concerns.
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Chapter 1: Introduction

1.1 Direct-to-Consumer Diagnostics and Point–of-Care Testing

Clinical diagnosis of diseases typically involves a patient-to-doctor encounter in which the patient is ordered to conduct an off-site laboratory analysis in which patient’s biological fluids such as urine, blood, serum, saliva, etc. are sampled and analyzed. The diagnosis is done in a laboratory, equipped with large and complex instrumentation that require highly trained personnel. Traditional clinical diagnosis using bio-fluids involves tedious, multi-step protocols that can become time-consuming. Moreover, early diagnosis and health monitoring can become essential for rapid decision-making, which is expected to aid in disease treatment and management. For countries of low-resource settings, a simple patient-to-doctor encounter can be highly limited, and early, prompt diagnosis can become essential for the detection of infectious diseases. Thus, traditional methods for disease monitoring and biomarker detection can render clinical diagnostics tedious and unreliable.

There is an area of opportunity to develop technologies that enable portable, reliable, rapid, simple and low-cost methods for disease diagnostics and health monitoring. In the last couple of decades with advances in medical technologies, point-of-care (POC) and direct-to-consumer (DTC) technologies have become possible that enable patient diagnosis to be done in-site (physician’s office, hospital, at-home) and
thus allowing efficient and rapid healthcare delivery. With the success of certain home-based technologies such as pregnancy tests, there is a growing emphasis toward prevention and early disease diagnoses for multiple conditions in a non-laboratory setting that would support and transform “personalized” patient care.3,6

The traditional detection methods of choice for commercial DTC and POC technologies for disease diagnostics are the polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA).7–9 PCR is a target amplification technique that reliably detects the presence of just a few copies of a nucleic acid sequence.7 Even though PCR is a highly sensitive technique, it requires extensive sample preparation, which makes it complex and expensive, limiting POC applications.10

On the other hand, ELISA is a more common technique employed in POC applications. ELISA is a colorimetric-based signal amplification technique in which a specific enzyme is used as the catalytic entity to increase the signal that results in the presence of a substrate, achieved in an immunoassay format.8 These traditional immunoassay methods however, rely on enzymatic, fluorescent or particle labels to detect the presence of protein biomarkers through colorimetric methods. Even though current POC methods offer functionality advantages such as portable and color read-outs for fast and easy-to-read results, they often compromise sensitivity and stability, affecting quantitative performance.11–12,13 The flaws of existing techniques for POC devices often lead to concerns regarding reliability for positive and negative read-outs if not read in a specific time frame, which also decreases accuracy.13–15 Thus, current methods employed
in POC limit the use of such techniques for clinical setting and traditional in-lab techniques are often preferred.

1.2 Types of Immunoassays

Current POC assay kit technologies centralized their formats as lateral flow assays and come in different configurations.\(^1\) Two major types of lateral flow immunoassays are either through a competitive or “sandwich” (non-competitive) format.

The non-competitive “sandwich” immunoassay is used for the identification of specific and large analytes, and it requires two antibodies that bind to non-overlapping epitopes on the analyte molecules. The sample analyte binds to an immobilized antibody (on a solid support), the analyte is later trapped (hence “sandwiched”) by a second antibody which binds to a different epitope in the analyte molecule. \(^1\) The second antibody acts as a report, the presence of which is detected by attaching an enzyme (ELISA) or a fluorophore to yield a colorimetric signal (in Figure 1). Sandwiched ELISA is by far the most common immunoassay method used in clinical diagnosis. \(^1\)

![Figure 1](image)

**Figure 1.** Non-competitive “sandwich” ELISA in which an antibody which has been immobilized attaches to a specific antigen, then a second enzyme-linked antibody is added in which a colorimetric signal is read by adding a colored substrate
In a competitive ELISA, binding is based upon a competition reaction between a labeled and unlabeled antigen to a limited amount of antibody sites. First the primary antibody with the “labeled” antigen to be measured is incubated, forming an Antigen-Antibody complex. Then the complex is added into a solid support in which the same “unlabeled” antigen has been immobilized. Unbound antibodies are washed away. Both antigens go through a competitive reaction trying to bind to the antibody sites. Then, an enzyme–linked secondary antibody specific to the primary antibody is introduced, and a substrate specific to the enzyme is subsequently added. The enzyme-induced activity is measured through a colorimetric signal as illustrated in Figure 2. This technique is usually applied to smaller analytes in more complicated matrices.

![Figure 2.](image)

In this thesis, we will focus on a non-competitive based immunoassay technique for protein biomarker identification in a MS-based method.
1.3 Electrospray Ionization Mass Spectrometry and Protein/Peptide Fragmentation

Detection of protein biomarker for clinical diagnosis often depends on two analytical characteristics to enable the effective evaluation of a specific medical condition: sensitivity and specificity. Mass spectrometry (MS) has proven to be a fast, highly sensitive and specific technique for routine analysis of biomarkers in clinical samples such as tissues and biofluids. Therefore, MS has become the leading technique of choice for biomarker studies. Typical proteomics experiments involve either “top-down” or “bottom-up” approach by either fragmenting an intact protein or digesting the protein and fragmenting the peptides, respectively.

Mass spectrometry involves (a) generation of ions, (b) separation of ions by their mass-to-charge \((m/z)\) ratio and (c) detection of the ions by which both the \(m/z\) value and ion abundance of a specific chemical species are determined. Electrospray ionization (ESI) and matrix-assistant laser/desorption/ionization (MALDI) techniques have facilitated protein and peptide structural characterization transforming the area of proteomics. In MALDI, single protonate species are observed, as opposed to ESI in which single protonated species are observed with corresponding generation of multiply charged ionic species whose identity can be described by

\[
[M + nH]^n+ 
\]  

ESI is a “soft ionization” technique that involves the injection of a dilute sample solution in a polar volatile sample through a needle or capillary. A high voltage (\(~5\) kV)
of direct current (DC) potential is applied to the tip of the needle or capillary, which generates a plume of electrically charged droplets at ambient pressures from the solution in a Taylor cone. A sheath gas (N\textsubscript{2}) is applied that improves nebulization and directs the spray of charged droplets into the inlet of the instrument. The droplets decrease in size by evaporation as they reach the inlet, in which the charged analyte is finally released from the droplet and enters the mass spectrometer, a depicted diagram can be observed in Figure 3. Charge density on the decreasing droplets increases until Coulombic repulsion balances surface tension and minimizes potential energy. This stage of the process is regarded as the “Rayleigh limit” in which the number of elementary charges $e$ is described by

$$z_r = \frac{8\pi}{\epsilon} \sqrt{\frac{\epsilon_0 \gamma R^3}{e}}$$

$R$ is the droplet radius, $\epsilon_0$ is vacuum permittivity and $\gamma$ is surface tension. The gas-phase ions that enter the mass spectrometer are produced by the highly charged nanodroplets produced at the Rayleigh limit through a process known as jet fission. There are two principal mechanisms that have been proposed which describe formation of gas-phase ions from the highly charged nanodroplet: ion evaporation model (IEM) and charge residue model (CRM). IEM is based on the fact that electric field from the highly charged nanodroplet is high enough and causes ejection of small ions. Small molecular weight species are thought to be formed through this process. CRM suggests that solvents evaporate from the highly charged nanodroplet until the charge is left on the gas-phase analyte; high molecular weight analytes (e.g. proteins) are thought to be
favored by this model. Gaseous analyte ions are produced with low internal energy, $E_{\text{int}}$, thus regarded as a “soft” ionization, and allows the transfer of intact protein/peptide ions from solution to the gas phase.\textsuperscript{24}

![Figure 3. Schematic illustrating the operational configuration and mechanism of electrospray ionization (ESI). Figure adapted from reference 31](image)

Some advantages exist for ESI over MALDI; ESI allows the detection of large analytes by MS with limited $m/z$ range by providing multiply charged ions, which in turn facilitates ion dissociation in some tandem MS experiments.\textsuperscript{28} Fragmentation of protein and peptides are subsequently observed in an experiment called tandem MS (MS/MS). In this experiment, fragment ions are formed through collision-induced dissociation (CID) where a precursor ion is activated by collision with a neutral background gas.\textsuperscript{36} MS reveals information regarding molecular mass, while MS/MS via CID provides information on peptide/protein amino acid sequence by inducing energy to the ions, in which structural characterization can be determined.\textsuperscript{37} The cleavage of the peptide bonds results in fragment ions, which the mechanism can be explained with the mobile proton model. The model claims that the protonated peptides activated under low energy
conditions fragment by charge directed reactions.\textsuperscript{38} It states that a proton in a protonated peptide, with added energy, can migrate to amide nitrogen atoms and produce backbone cleavages, such as \(b_n/y_x\) ions.\textsuperscript{39, 40}

A special nomenclature for peptide fragmentation was first proposed by Roespstorff \textit{et al.}\textsuperscript{41} and modified by Biemann \textit{et al.}\textsuperscript{42} which is now used to identify fragment ions when different bonds are cleaved in the peptide backbone. Two fragment ions are typically observed in CID, the \(b_n\) and \(y_x\) ions.\textsuperscript{43} When the amino terminal retains the charge, the \(b_n\) ions are formed and observed in the spectra. However, if the c-terminus retains the charge the \(y_x\) ion is produced and observed. These peptide fragment ion types are considered to be most useful for sequence structure determination.\textsuperscript{43, 44} Mueller \textit{et al.}\textsuperscript{45} and Cordero \textit{et al.}\textsuperscript{46} showed that a \(y\)-ion structure is produced by a protonated amino acid or a protonated truncated peptide. Yalcin \textit{et al.}\textsuperscript{47} showed that \(b\)-ions structure are produced when the peptide backbone ends in a stable cyclic oxazolone ring. Examples for common structures for a \(b_4\) and \(y_3\) ion for a penta-peptide are demonstrated in Figure 5. However, some other ion types are also produced and may be observed in the tandem mass spectrum as shown in Figure 4.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{peptide_fragmentation.png}
\caption{Nomenclature of ion types produced in collision-induced dissociation}
\end{figure}
Understanding peptide fragmentation nomenclature will help in interpreting the MS/MS spectra. In ESI, peptides may exhibit multiple charged state (1+, 2+ or even 3+) and this will change the appearance of the MS/MS spectra. In this thesis, three penta-peptides are characterized and considered for diagnostic applications.

1.4 Applications of Ambient Ionization, MS Miniaturization and Microfluidics for Clinical Diagnosis

One of the disadvantages of using current mass spectrometric methods for protein biomarker detection in clinical samples is that they usually require tedious and time consuming pretreatment steps and they are done using expensive, big, high-resolution mass spectrometers, which require trained personnel to interpret such data. Thus, the high costs, space and resources required to maintain current MS approaches for diseases diagnosis would sometimes make MS technology fall short in resource-limited clinical settings. To fully take on the advantages and justify the use of MS for clinical diagnosis in POC settings, alternative sampling and analysis methods must be considered that would make routine MS-based diagnosis possible through the simplification of analytical procedure and instrumentation.
Fortunately, potential applications of routine MS diagnosis in a POC setting have already been demonstrated with the development of ambient ionization techniques through the introduction of Desorption/Ionization Electrospray (DESI)\textsuperscript{48} and direct analysis in real time (DART)\textsuperscript{49}, just to mention a few. These methods have been named “ambient ionization” because they offer the characteristics of (a) desorbing analytes directly from samples in condensed phase, which (b) ionizes the samples in open air and results in no sample preparation.\textsuperscript{50} Tedious sample pre-treatment steps such as separation, extraction and purification of complex biological matrices can be avoided. Moreover, ambient MS allows different sample forms (solid, liquid and gases) to be analyzed on most common mass spectrometers with no instrument modification.\textsuperscript{51}

There are three main or “original” types of ambient ionization techniques; ESI based, plasma based, or laser assisted techniques. Multiple subtypes and variations have been developed over the years. DESI was the first ESI based ambient ionization type for solid-liquid extraction analysis. DESI involves an electrospray source that creates charged droplets when directed to a solid sample, in which the droplets pick-up analyte ions that are analyzed in the MS.\textsuperscript{48} Atmospheric pressure chemical ionization (APCI) is an ionization technique that generates gaseous ions at atmospheric pressures through corona discharge or plasma formation.\textsuperscript{52} An example of a conventional plasma based ionization technique is DART, which generates ions by penning and APCI mechanisms in which ions; electrons and metastable atoms are produced.\textsuperscript{49} APCI and ESI paved the road for more sophisticated ambient ionization techniques.\textsuperscript{53} “Soft” laser based techniques have also been developed such as electro-spray assisted laser desorption
ionization (ELDI) in which analytes are produces by a pulsed laser and positioned in an ESI plume. Diagram of these different ionization techniques set-up are shown in Figures 6a-d.

![Diagram of ionization techniques](image)

**Figure 6.** Examples of ambient ionization techniques illustrated using (a) desorption electrospray ionization (DESI) (b) direct analysis in real time (DART) (c) paper-spray ionization (PS) and (d) electrospray-assisted laser desorption ionization (ELDI).

Ambient ionization has paved the road for the development of alternative ionization techniques for MS chemical analyses that can be highly applicable for POC settings. The attractive features (e.g., ease of use, simplicity and rapid nature) of ambient ionization make it optimal for coupling to portable MS devices that can be used for in-field analysis of complex biological mixtures. Portable MS devices would provide the benefits of MS sensitivity and specificity in a clinical setting, which would
avoid the high cost involved in big instrumentation.

One type of ambient ionization that has become a potential technique for POC devices due to its unique characteristics is paper-spray ionization. Paper-spray ionization involves the spotting of sample into the paper substrate cut into a sharp tip; charged droplets are emitted from the wet paper tip after the application of a DC high voltage. Typical spray solvent applied is methanol/water (1:1, vol/vol) solution. Paper spray (PS) ionization was introduced by the Cooks group in 2010 and has been proved to be useful for fast, qualitative and quantitative analysis of complex mixtures, such as whole blood. PS has been proven to be a quick, simple and robust ionization technique for clinical samples such as bio fluids, in which analysis is achieved in less than 1 minute, and has minimal sample consumption.

With the increased interest of developing simple, portable and low-cost POC technologies, paper-based microfluidic analytical devices have been developed. Paper applications in microfluidic devices are optimal due to low-cost, simplicity and minimal storage requirements, making them optimal for use in low-resource environments and POC settings. Moreover, microfluidic devices made of paper have become well established and developed for commercial applications in clinical diagnosis, such as the glucose meter and blood analyzer cartridges (i-STAT system).

Wax printed-paper has been shown to provide easy manipulation in microfluidic devices by creations of patterns, which produces independent channels that allow ease of multi-step processes in one single device due to capillary action. Thus, paper-based
microfluidic devices capable of multiplex biomarker detection have been explored.\textsuperscript{66-68} Using paper for simple POC microfluidic devices capable of multiple disease diagnosis would minimize time, waste and complexity of current commercial methods. If merged with the advantages of sensitive and robust PS ambient ionization, can transform current POC technologies. Damon, \textit{et. al.} demonstrated that a 2D-wax printed-paper substrate creates microfluidic channels and high sensitivity, and quantification of drugs were observed for raw urine.\textsuperscript{69} Thus, PS ionization has become an attractive and valuable technique that could be coupled to microfluidic paper-based analytical devices (µPADs) to enable multiplexed protein biomarker detection on handheld MS instruments.\textsuperscript{56} In this thesis, the initial efforts to couple µPADs to portable mass spectrometers is described through the development of low-molecular isobaric peptides for applications in paper-based immunoassays.

1.5 Mass Spectrometry Based Immunoassays

Even though traditional µPADs have demonstrated to be a simple and straightforward as a POC diagnosis tool using colorimetric detection,\textsuperscript{70} there are quantitative challenges that current methods face, in which stability and sensitivity is often compromised.\textsuperscript{10} Thus, there are needs to develop an alternate diagnostic technique that would enable robust and reliable results for portable, on-demand clinical diagnosis. MS analysis provides the versatility of analyzing big and small biomolecules depending on the instrument and ionization technique of choice. Direct MS detection, however, has
limitations regarding sensitivity for protein biomarker analysis, thus developing techniques that allow a MS signal amplification have been of particular interest.\textsuperscript{71} That is, instead of analyzing big protein biomarkers directly for clinical diagnosis, “tagging” using small molecules such as gold nanoparticles,\textsuperscript{72-74} photolabile tags\textsuperscript{75, 76} and ionic probes\textsuperscript{77} have been developed, just to name a few. Tagging protein biomarkers offers advantages such as providing higher sensitivity, avoids pretreatment steps (e.g. protein digestion) and reduces instrumentation requirements (e.g. LC/MS/MS, MALDI) as those used in current mass spectrometric immunoassays.\textsuperscript{78-80}

Cleavable probes for MS-based bioassays have been developed\textsuperscript{9, 81, 77} in which mass tag entity is conjugated into a unit that binds to an antibody, and generates antibody-antigen complexes. This “bio-conjugation” approach to a mass tag in an MS experiment provides various advantages such as overcoming lack of stability associated with enzymatic or fluorescent tags, as well the opportunity to include readily available elements as reporter tags. Combining the use of a cleavable probe into an MS-based immunoassay with a mass reporter allows the realization of the multiplexing capabilities of mass spectrometers as indicated by their large peak capacity and dynamic range. Multiplexed capabilities developed in this thesis is unique in that all peptide mass tags are designed to have the same molecular weight (isobaric) so that they can be selected in a single MS/MS experiment for multi-biomarker quantification. Although isobaric, each peptide tag dissociates in CID to give a unique fragment ion, which is then used to determine the presence of a specific disease biomarker.
In this thesis, the development of a novel probe containing the isobaric peptides is proposed for application in MS paper-based immunoassay. The MS paper-based immunoassay method was introduced by our group in 2016, in which the detection of malaria *Pf*HRP2 antigen and two cancer antigens Ca-125 and CEA was successfully detected through a sandwich immunoassay on paper. The experimental steps consists of (a) fabrication of an aldehyde functionalized wax printed paper substrate that allows the capture antibody to be immobilized into the paper, (b) excess aldehyde functional groups are blocked with tris-buffer saline, (c) a selective sandwich immunoreaction on the paper substrate by the use of a conjugated detection antibody on a mass reporter probe and (d) MS analysis of the mass reporter. This experimental workflow is described in Figures 7 and 8. This technique enables comparable sensitivity to a typical ELISA method, and allows convenience in storage and reliable detection over time with increased stability and low sample consumption.

![Diagram of MS paper-based immunoassay workflow](image)

**Figure 7.** MS paper-based immunoassay summarized workflow as developed by Chen, et. al.⁷⁷
Figure 8. Paper substrate aldehyde functionalization and subsequent immobilization of capture antibody for MS paper-based immunoassays by Chen, et. al. 

When the capture antibody has been immobilized into the paper, it can sandwich the antigen by the addition of a second antibody, which is conjugated to an “ionic probe.” Chen, et. al. developed for the first time an ionic probe, which consists of a pH sensitive triple, charged amine conjugated to a unit with an isothionate group (-NCS) that binds to the amine group in the detection antibody. The triple charged amine is later released by hydrolysis and identified using tandem-MS analysis. Chen, et. al. proved that the tag is directly related to the amount of antigen added. The ionic probes are shown below.
Figure 9. Ionic Probes for MS-based immunoassay, charged amine mass reporter (in red) group is cleaved through a pH sensitive bond for (a) 4-(4-isothiocyanatophenethoxy) N,N,N-trimethyl-4-oxobutan-1-aminium chloride (ITBA), MW=307 Da and (b) 2-(4-isothiocyanatophenethoxy)- N,N,N-trimethyl-2-oxoethanaminium chloride (ITEA), MW=279 Da developed by Chen, et. al.\textsuperscript{77}

However, the two quaternary amines used as mass reporters in paper MS based immunoassays have a few limitations such as limited starting reagent availability, multi-step synthetic reaction steps, and multiplexing capabilities. Multiple tandem-MS analyses for the quantification of more than one analyte has to be done. Ideally, we would like the MS paper-based immunoassay to allow the simultaneous detection of analytes in one tandem-MS experiment.

1.6 Cleavable Isobaric Peptides as Mass Reporters for MS-paper Based Immunoassay Probe

Multiplexed protein biomarker quantification in MS and proteomics studies have been routinely done using commercially available approaches such as isobaric tag for relative and absolute quantitation (iTRAQ)\textsuperscript{82} and tandem mass tag (TMT)\textsuperscript{83}. A typical
experiment involves extracting the protein from clinical samples and digesting the protein, in which different peptides are labeled and mixed, and one single precursor ion is monitored. Reporter ions identify multiple peptides and useful relative intensity information can be gathered. However, the limitation these methods includes extent on multiplexing capabilities (up to 8 samples). Plus, these are not often used in an immunoassay format.

In this thesis, isobaric peptides tags are explored as potential mass reporters in MS paper-based immunoassays (Figure 7). The peptides were rationally designed and synthesized based on three characteristics (a) include amino acids with available protonation sites such as the amino and guanine groups in lysine and arginine, respectively, (b) form a small peptide (penta-peptide) in a mass window of <1000 m/z and (c) contain very similar or identical protonation sites but differ in sequence. In this case, the isobaric peptides share a common parent mass. Exposure to collisional activation in tandem MS experiments, however, yields characteristic fragment ions belonging to a specific peptide. This would provide, in theory, a novel detection method of protein biomarkers in a single MS/MS experiment. The use of isobaric peptide mass tagging would also offer unlimited possibilities in creating a library of isobaric mass reporters that could be used to tag multiple analytes of interest. A similar approach was adopted by Kwong et al., who demonstrated the use of isobaric mass tags to track the response of ten biomarker peptides which compared favorably with commercial isobaric tags (e.g., 8-plex iTRAQ).
Figure 10. Cleavable peptide probe design as proposed in this thesis, isobaric peptides used as mass reporters, which are released by a pH sensitive cleavable bond, which is bind to a unit that allows bio conjugation through the (-NCS) group

Three isobaric peptide tags are synthesized and characterized in this thesis: GARRK, AKRRG and RRGKA, all having the molecular weight of 586.7 Da. The nature and possibility of coupling them into a pH cleavable probe is studied. The peptide will be incorporated into a pH sensitive ester cleavable bond, which links the peptide to the conjugation unit (Figure 10). The -NCS group will react with amines in the lysine residues of the detection antibody, allowing a bio-conjugation.
Chapter 2: Methods

2.1 Ionization Sources

2.1.1 Nano Electrospray Ionization

Nano electrospray ionization (nESI) is an attractive technique for the analysis and characterization of biomolecules due to the fact that it requires small samples volumes, and the multiple protonation allows the analysis on a low mass window. In order to characterize the isobaric peptides, nESI was used; here, where a borosilicate glass capillary (O.D. 1.5mm, I.D. 0.86 mm, 10 cm length, Sutter Instruments, Novato. CA, USA) was pulled to a sharp tip using a micropipette puller (Model P-97, Sutter Instrument Co., NovatoCA, USA) generating the nESI emitter tip The nESI glass capillary was filled with 10 μL of sample solution, and a silver electrode was inserted into the open part of the capillary. Spray voltage of 1.5-2kV was applied to the analyte solution via the silver electrode, which subsequently emitted charged micro-droplets for electrospray ionization (Figure 11); a distance of ~8 mm was maintained between nESI emitter tip and the mass spectrometer inlet. Full MS mass range 50 -1000 m/z.
Figure 11. Nano electrospray ionization (nESI) experimental set-up in which a high voltage is applied to a silver electrode, which is placed inside a pulled capillary containing sample solution.

2.1.2 Paper-spray Ionization (PS)

Paper-spray ionization was also used for characterization and reproducibility studies of the selected isobaric peptides as well for peptide-probe conjugation. The wax printing for the paper substrates was done in a Xerox ColorQube8870 wax printer (Norwalk, CT) which was used to print triangular patterns on Whatman (Maidstone, UK) cellulose paper. The paper was heated to 130°C for 30 seconds to allow wax to penetrate through. The wax printed-paper was cut into approximate 70mm² triangles (9mm base and 16mm height). The wax printed-paper substrate is used (Figure 12), which is fixed in place by an alligator metal clip, 5 µL of analyte is placed on the paper substrate followed by 10 µL of spray solvent. A high DC voltage of 3 kV is applied to the wet paper through the metal clip. Distance to the MS is kept at ~5 mm.
Figure 12. Wax-printed paper-spray ionization experimental set-up; high voltage is applied to a wetted channel of a wax printed triangular paper-substrate as described by Damon, et. al. 69

2.2 Mass Spectrometer

For all mass spectrometry studies, experiments were carried out with a Thermo Fisher Scientific Velos Pro LTQ (San José, Ca, USA), which is an ion trap mass spectrometer. The instrument was operated in full mass spectrum mode or MS/MS mode, which reveals product ions produced by CID, which were monitored. The commercially attached ESI source was removed from the instrument and replaced with either nESI or paper-spray set-up as previously described. Transfer capillary temperature is kept 150-250 °C, with 3 micro scans and 100 ms injection time. nESI and paper-spray parameters were kept at a 50-1000 Da mass range. The normalized collision energy for CID was kept at 20-45%. Thermo Fisher Scientific XCalibur 2.2 SPI was the software used for MS system control and data acquisition, in which spectra are averaged for 10-40 seconds. For
characterization purposes, full MS and MS/MS of peptides was recorded by selection/activation through CID of the singly and doubly charged ion peptide.

Figure 13. Schematic showing the various components of Thermo Scientific Velos Pro LTQ, adapted from reference 109

2.3 Reagents

Custom peptides: GARRK, AKRRG and RRGKA (MW = 586.70 Da, pI=12) were purchased from Thermo Scientific (Waltham, MA, USA). Deuterated GARRK peptide was also obtained from Thermo Scientific (MW=589.70 Da, pI=12). All peptide samples were prepared in 100% water from a Milli-Q water purification system (Millipore, Billerica, MA) in (1:1, vol/vol) Methanol/Water with 1% formic acid for characterization purposes. 2-(4-isothiocyanatophenyl)ethan-1-ol was purchased from Organix, Inc. (Woburn, MA). Di-tert-butyl dicarbonate, N,N- dicyclohexylcarbodiimide (DCC), methanol, dimethyl amino pyridine (DMAP) and anhydrous dimethylformamide
(DMF), potassium periodate, 10x phosphate buffered saline (PBS), 2,4-dinitrophenylhydrazine (2,4-DNP) were all purchased from Sigma Aldrich (St. Louis, MO, USA). Sodium bicarbonate and formic acid was purchased from Fischer Scientific (San José, CA, USA). GE PD-Midi Trap G-10 was purchased from GE Healthcare (Chicago, IL, USA). Whatman No. 1 chromatography paper and glycerol were purchased from VWR (Radnor, PA, USA). Anti-Malaria PfHRP2 IgG monoclonal antibodies (ABMAL-0444 (Clone 44), ABMAL 0445 (Clone 45)) were purchased from Arista Biologicals Inc (Allentown, PA). ABMAL 0444 (Clone 44) was used as the capture antibody and ABMAL 0445 (clone 45) was used as the detection antibody. UltraCruz TM Micro G-25 Spin Columns were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Borosilicate theta capillaries purchased from Sutter Industries (Novato, CA, USA).

2.4 Ion Mobility and Collisional Cross Sections (CCS) Determination

Ion Mobility experiments were conducted using a Waters Synapt G2S instrument (Manchester, UK) which allows for the extra dimension of traveling wave-ion mobility separation in addition to standard mass spectra collection. A nESI source was used in which pulled borosilicate glass emitters contained the sample solution as explained in the previous section. The ion source temperature was set at 20°C and 1.2 kV potential was applied to the nanospray emitter using a platinum wire. The instrument parameters were as follows: IMS wave height 23.0V, transfer wave height 20V. The helium cell gas flow was 120 mL/min, the IMS gas flow was 60 mL/min. About 10 μM of Poly-DL-Alanine
was used as a calibrant for collisional cross section (CCS) calibration curves. MS and drift time data was gathered for all peptides of the singly and doubly charged ion peaks. Drift times of all isobaric peptides and the calibrants were determined using MassLynx and CCS was calculated using Origin and Excel.

CCS of the peptides was obtained by the following protocols reported previously.\textsuperscript{85} The calibration curves for Poly-DL-alanine were constructed from singly charged ions in which n=6-14 and z=1 (427-995 m/z), as well for doubly charged ions n=13 and z=2 (462-924 m/z). The drift times (ms) for each of these ion calibrant masses were obtained from the TWIM-MS raw data. The drift times (t\textsubscript{D}) are corrected for m/z dependent flight times

\[ t'_D = t_D - c \sqrt{m/z} \] (3)

The constant c is determined empirically and obtained from the instrument. The literature CCS (\(\Omega_{He}\)) obtained for Poly-DL-alanine are corrected for the charge and reduced mass

\[ \Omega' = \frac{\Omega_{He}}{z} \sqrt{\mu} \] (4)

Calibrated CCS values are determined by plotting the calibrant literature CCS (\(\Omega_{He}\)) as a function of the final corrected drift times (t\textsubscript{D}”), where x is an exponential factor determined from the plot of ln (\(\Omega’\)) as a function of ln (t\textsubscript{D}”)

25
\[ t_\text{D}'' = t_\text{D}' \frac{z}{\sqrt{\mu}} \]  

(5)

CCS of an ion is obtained from the relationship of the ion mobility (K) and the ion neutral CCS (Ω) which is related under the Mason-Schamp equation

\[ K = \frac{3ze}{16N\Omega k_b T\mu} \]  

(6)

In which z is the integer charge, e is elementary charge, N is drift gas number density and \( k_b \) is Boltzmann constant, T is the absolute temperature and \( \mu \) is the ion neutral reduced mass.

2.5 Synthesis of Cleavable Peptide Probe

The method of synthesis for creating GARRK-based cleavable probe unit is described below. The primary amine of GARRK peptide was first protected in a catalyst-free chemo-selective N-tert-butyloxycarbonylation (Boc) of the primary amine,\(^{86, 87}\) since it may be reactive towards the isothianate group of the conjugation unit in the final cleavable probe (Figure 10). For the Boc protection reaction, 0.011mmol of GARRK peptide and at least 1.2 mol equivalent of di-tert-butyl dicarbonate was added to 3 mol equivalents of sodium bicarbonate in water. The reaction was left to stand overnight at room temperature. The protected peptide product was purified and lyophilized. The Boc protection was confirmed by paper-spray MS by selecting \( m/z \) 344 \( \rightarrow \) 294 for doubly
charged peak, and $m/z$ 687 $\rightarrow$ 587 for singly charged peak, in which the characteristic fragment of GARRK is observed.

The protected peptide then acts as the carboxylic acid, which reacts with the conjugation unit (alcohol) through a Steglich-esterification reaction. To a stirred solution of 0.00628 mmol protected GARRK peptide in DMF (1 mL), 3-10% mol DMAP and 2-4 mol equivalents of 2-(4-isothiocyanatophenyl)ethan-1-ol is added in room temperature, 1.1 mol equivalents of DCC is then added at 0°C for 5 minutes. The reaction was then kept overnight, at room temperature to react. The product is then filtered off and the solvent removed by pressurized evaporation in vacuum. The probe product is confirmed by selecting $m/z$ 851 and observing the characteristic, singly charged fragment ion of GARRK peptide. The product was diluted in PBS solvent to form a probe solution of 0.2 mM, decanted and stored in -80 °C until use.

2.6 Preparation of Aldehyde-Functionalized Paper

Whatman No.1 chromatography paper was functionalized with aldehyde groups by soaking the Whatman No.1 chromatography paper in 0.03M KIO4 at 65 for 2 hours. After the reaction, the sheets were washed three times with deionized water for one minute each and pouring it off. The sheets are blotted with paper towels and left to dry for about 6hrs and stored in a desiccator immediately. A wax pattern is printed into the paper, the protocol is adopted from the paper-spray ionization studies. The sheets are placed in an oven for about 30 seconds so the wax melts through the paper. This creates hydrophilic zones (areas without wax) and hydrophobic wax barriers in the paper. The
presence of aldehyde groups in the paper was verified by the 2,4- DNP test. A 2,4-DNP solution is used by preparing 300mg in concentrated sulfuric acid (1.5mL), the solution is then added to a mixture of water (2mL) and ethanol (7mL). The solution is mixed, and 3µL is added to the test zone of functionalized paper and incubated for 1 minute. The remaining solution is blotted and the test zone is washed with 3 washes of 5µL of water. The color of the test zone should turn bright orange. The wax printed paper is stored in a desiccator until used.

2.7 Bio-conjugation

In this section, the conjugation of the synthesized peptide probe unit to proteins (i.e., anti-malaria antibodies) is described. The peptide probe was dissolved into PBS buffer solution to form 0.2 mM probe solution. The reporter anti-malaria antibody (dAb) was selected for conjugation. For this, a reaction mixture consisting of 1:10 ratio of dAb:probe is needed. For example, for a 0.2 mM solution of probe, 100 µL of the concentration solution was mixed with 25 µL of the detection antibody (10.5 mg/mL) in 10 mM PBS buffer (pH 7.4), yielding a total reaction volume of 125 µL. The reaction was left overnight at 4°C. The isothiocyante functional group of the peptide probe reacts with the amine group of the lysine residues of the antibody and forms the thiourea bond. Size-exclusion columns with a Sephadex matrix was used to purify the excess probe (PD-10 Desalting column and Micro G-25 Spin column). In order to verify the bio-conjugation, 0.1 M NH₄OH solution was added to the purified dAb-peptide probe
conjugate to hydrolyze the labeled ester bond, releasing the GARRK peptide which can be picked up and analyzed by paper spray mass spectrometry.
Chapter 3: Results and Discussion

3.1 Isobaric peptide design and mass reporters

Three readily available and short chain isobaric peptides were rationally designed based on their capability to produce positive ions, thus basic amino acids (His, Lys, Arg) were considered. The structures of isobaric peptides considered are shown in the figure below (Figure 14)

Figure 14. Structural schematic of three different isobaric peptides (a) GARRK (b) RRGKA and (c) AKRRG
The charge on peptide ions, derived from electrospray, are highly influenced by various factors such as the instrument and ionization method of choice, voltage, concentration and properties of the peptide. Thus, instrument and method parameters of these peptides using nESI and PS were maintained constant. The main difference between the peptide is the overall peptide sequence. The final charge generation observed for a peptide not only depend on the specific mechanism of ion generation (i.e., CRM versus IEM), but also on the number of basic sites in a peptide, which aids in the determination of charges that can accommodate during the process of ESI.

GARRK, RRGKA and AKRRG share the same amount of 3 basic side chain residues (2 Arg and 1 Lys) and a basic site in the primary amine (N-terminus). Since these peptides are isobaric, they contain the same molecular weight and same number of basic residues, thus their full MS is nearly identical. The addition of formic acid in aqueous solution facilitates protonation, and the resultant full MS spectra reveal the presence of singly, doubly and triply charged ions at m/z 587, 294 and 196, respectively (Figure 15). The doubly protonated ion is the most abundant in the full mass spectra for all the isobaric peptides. The presence of adjacent Arg residues in all the peptides produces high charge retention, depending on their proximities with respect to the N or C-terminus, which subsequently affects the ease of fragmentation. This observation has previously been shown experimentally, where peptides containing more than one Arginine residue tend to show more intense doubly charged fragment ion peaks than those containing less basic residues, dominantly retaining the charge.
3.1.1 Mass Spectrometric Characterization of Characteristic Fragment Ions

MS/MS product ion spectra for the singly charged ion, $m/z$ 587, for all the peptides is shown below in Figure 16
Figure 16. nESI-MS product ion MS/MS spectra of singly charged precursor ions, \( m/z \) 587, providing characteristic fragment peaks for each isobaric peptide (a) GARRK (b) RRGKA and (c) AKRRG. All peptide solutions were prepared in (1:1, vol/vol) MeOH/Water with 1% formic acid.

The spectra in figure 16 shows that GARRK, RRGKA and AKRRG reveal low abundance of singly charged \( b_4^+ \) fragment. However, due to the high abundance of the
doubly charged ions, it was selected used for further studies. Each peptide yielded unique fragment ions as shown in the MS/MS mass spectra below when the \([\text{M}+2\text{H}]^{2+}\) ions were subjected to CID fragmentation (Figure 17).

![Mass Spectra](image)

**Figure 17.** nESI-MS product ion MS/MS spectra of doubly charged precursor ions, \(m/z\) 294, providing characteristic fragment peaks for each isobaric peptide (a) GARRK (b) RRGKA and (c) AKRRG. All peptide solutions were prepared in (1:1, vol/vol) MeOH/Water with 1% formic acid

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GARRK has an abundant characteristic \( y_3^{2+} \) fragment ion at \( m/z \) 230, while RRGKA fragments to give a characteristic \( b_4^{2+} \) ion at \( m/z \) 249, and AKRRG yielded a characteristic fragment of \( m/z \) 256 also a \( b_4^{2+} \) ion. It can be noted that GARRK and RRGKA offer the most abundant characteristic fragment ions at the same ionization conditions, whereas AKRRG produces limited fragment ion upon activation. It can be noted that, for AKRRG and RRGKA, the cleavages yielding the predominant fragment ions occurred when the amino terminal fragment retained the charge to produce b ions. On the other hand, GARRK fragments to retain the charge at the carboxyl-terminal to yield an abundant y ion. Both of the preferred dissociation pathways are illustrated in Figure 18 for each isobaric peptide.

![Figure 18](image)

**Figure 18.** Schematics showing the fragmentation and characteristic fragment ions observed in the product ion MS/MS experiments for AKRRG, RRGKA and GARRK

A study done by Tabb *et. al.* demonstrated that the position of basic residues within peptides influences the peak intensities of b and y series ions. \(^90\) Arg shows the
most dominant effect out of all the basic residues, in affecting charge retention in ESI. This can be explained by considering that the pKa=12.5 for Arginine; it is the most basic amino acid and is always found protonated in pH’s lower than it’s pKa. This is consistent with the results shown above in Figures 16-17. Moreover, it has also been studied that the position of Arg residue in a peptide sequence also influences intensity of the ion series observed, more than any other basic residue in a peptide, known as “competitive enhancement”. The addition of other basic residues can also affect the series intensity. The residues for each peptide at the characteristic fragment ion are comparable, however AKRRG at b₄²⁺ is the only peptide that does not include a basic residue in the terminal where charge is retained (N-terminus). The “y” series dominates for GARRK, producing y₃²⁺ ions, because the charge is highly concentrated in the C-terminus, where the basic residues are clustered.

The basic residues in each of the peptides highly affect the fragmentation pathway as shown in these penta-peptides. This can be attributed to the highly basic amino acid (Arg), which dominates the fragmentation pathway and has the highest proton affinity, a proton will prefer to bind to Arg, followed secondly by Lys, and lastly, the N-terminus. Moreover, in order to further investigate the characteristic fragment ion capabilities in a multiplex analysis, a (1:1:1, vol/vol/vol) mixture of the peptides was studied using nESI. MS/MS of doubly charged ion reveal the characteristic fragment ions of each peptide in the solution mixture (Figure 19), thus demonstrating the potential for multiplex reporter ions for protein biomarkers.
Figure 19. nESI MS/MS spectra with shared doubly charged precursor ion (294 m/z) is subjected to CID, demonstrates the characteristic fragment ions of the isobaric peptides in a (1:1:1, vol/vol/vol) mixture using 1% formic acid in (1:1, vol/vol) MeOH/Water

3.1.2 Fragmentation Efficiency of Isobaric Peptides

As previously observed in the MS/MS data for the doubly charged ion fragmentations, AKRRG yields the smallest abundant fragment ion in comparison to the other peptides. We evaluated this existing trend in fragmentation efficiency by increasing the concentration for all the peptides while holding CID energy constant at 20.

Figure 20. Fragmentation efficiency of isobaric precursor peptide ions prepared in MeOH/Water (1:1, vol/vol) with 1% formic acid for (a) increasing concentration, n=5 maintaining CID constant at 20 and (b) increasing CID, fragment ions m/z 256, 230 and 249 were monitored for AKRRG, GARRK and RRGKA, respectively
The trend is clear from Figure 20, where GARRK easily dissociates producing the highest abundance of $y_3^{2+}$ fragments, while the $b_4^{2+}$ fragment ions from RRGKA and AKRRG follow. GARRK and RRGKA have around the same magnitude of fragment ion intensity signal, whereas AKRRG differs slightly (one order of magnitude less) for each increasing concentration. It is evident from the peptide design that both GARRK and RRGKA have basic residues closest to the N-terminus and C-terminus where charge is mostly retained by the basic residues. However, AKRRG, has internal clustering of charge in the middle of the peptide sequence. Tabb$^{90}$ stated that randomly displaced basic residues in the middle of the sequence may produce a mix of b/y ions that could potentially be competing in the observed spectra, thus explaining the lower fragmentation efficiency observed for AKRRG.

Moreover, GARRK has a slight higher efficiency than RRGKA; the mobile proton model could possibly explain this. In which for GARRK, the charge is highly clustered in the C-terminus and a mobile proton is right next to the amide bond, favoring highly abundant and efficient $y_3^{2+}$ ion generation when little energy is applied. On the other hand, arginine at the N-terminus of RRGKA holds the most basic site capable of protonation, the mobile proton transfers to the amide bond therefore favoring a $b_4^{2+}$ ion pathway, but this would require additional energy to occur. Additional mobile proton model discussion is shown further to support results of ion conformation for collisional cross section data.
3.1.3 Fragmentation Ion

Chen, et. al. evaluated the capabilities of the developed ITEA and ITBA for MS paper-based immunoassay at nM concentrations.\textsuperscript{77} Thus comparable linearity, reproducibility and ease of fragmentation from the penta-peptide fragment ions in PS analysis was desirable for probe development. Calibration curves were obtained for all three peptides, in which good reproducibility and linearity was obtained as seen in 21a-c. However, it is evident that GARRK exhibited excellent linearity, probably due to the ease with which it fragments. Comparable sensitivity (i.e., the slope of the calibration curves) was observed for all isobaric peptides as illustrated in Figure 21. Although all the isobaric peptides qualified to be used as cleavable probe for immunoreactions, we decided to optimize the methodology using the GARRK.
3.1.4 Collisional Cross Section Study

The penta-peptides considered in this study clearly show differences in the ion fragmentation efficiencies as previously discussed, based on charge-directed mechanism.
as explained by the mobile proton model. Thus, the preferred fragmentation pathway is thought to be dependent on (a) the amino acid sequence order in the peptides, and (b) the shape and size of the respective isobaric peptide in gas-phase conformations, which is dependent on the way the charge is distributed. The existence of the later effect was examined by measuring the collisional cross sections (CCS) of each peptide. IM-MS has become a valuable tool for ion chemistry because it is able to differentiate the structure and conformation of gas phase ions. IM-MS rapidly separate ions based on their mobilities in a background gas, which depend on the structural shape and charge, as determined by their experimental drift times. The CCS can be determined by the drift times of ions and it provides valuable information into ion structure.

A traveling wave (TW) IM-MS was used for this study, in which spatially non-uniform radio frequency (rf) and direct current (dc) electric fields are used. CCS determined from TWIM-MS instruments are directly related to a corrected parameter, ion drift time, to determine CCS values of analytes. Thus, the analysis of unknown CCS is done using known CCS values from calibrant libraries. A calibrant is needed to correct for experimental parameters because TWIM-MS experiments are performed using a range of parameters like temperature, gas compositions, pressures and modes of separation. These differences can make direct correlation between drift times and CCS challenging for peptide ion assignments.

In this research, poly-DL-alanine was chosen as the calibrant, in which theoretical values for CCS ($\Omega_{He}$) have been determined. The calibration would correct for differences in parameters. Poly-DL-alanine was used as a calibrant due to similar
characteristics with the peptide ions considered in this study, which is required to ensure CCS accuracy. Poly-DL-alanine yields singly, doubly and triply protonated peptides using ESI and has low molecular mass, which makes it optimal for comparison with the short-chained peptides in this study. The literature $\Omega_{\text{He}}$ value for poly-DL-alanine was plotted vs. the corrected drift times, and the resulting calibration curves for 3 replicates as is shown below in Figure 22.

![Graphs](image)

**Figure 22.** IM-MS calibration curves for Poly-DL-alanine calibrant, CCS ($\Omega_{\text{He}}$) as a function of the corrected drift time, $t_{D''}$ for three different trials
From the calibration curves obtained of Poly-DL-alanine, the value for corrected drift time, $t_0^{\prime\prime}$ as a function of literature CCS ($\Omega_{He}$) a linear regression was obtained. With the linear regression, the following relationship is obtained

$$t_0^{\prime\prime} = m(\Omega) + b \quad (7)$$

This relationship allowed the calculation of unknown CCS ($\Omega$) from the experimental $t_0^{\prime\prime}$ of the isobaric peptides. The results obtained for the CCS are shown in Table 1.

<table>
<thead>
<tr>
<th>Peptides</th>
<th>[M+H] (Ω)</th>
<th>RSD</th>
<th>[M+2H]$^2+$ (Ω)</th>
<th>RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>AKRGG</td>
<td>243.5400</td>
<td>0.62</td>
<td>342.9854</td>
<td>0.53</td>
</tr>
<tr>
<td>GARRK</td>
<td>248.3591</td>
<td>0.29</td>
<td>342.4677</td>
<td>0.60</td>
</tr>
<tr>
<td>RRGKA</td>
<td>243.2440</td>
<td>0.17</td>
<td>342.4251</td>
<td>0.82</td>
</tr>
</tbody>
</table>

For AKRGG, GARRK and RRGKA, the CCS were determined for three trials and averaged for the singly (m/z 587) and doubly (m/z 294) charged ions with their corresponding errors (i.e., relative standard deviation, RSD). Table 1 reveals that GARRK has the highest CCS for a singly charged ion, while AKRGG and RRGKA have slightly lower but comparable CCS values. The 3% CCS difference for a singly charged ion between GARRK and the other two isobaric peptides is evaluated.
GARRK peptide sequesters the protonation site for a singly charged ion at the Arg residue, thus it makes sense that $b_4^+$ would be the most abundant fragment ion as observed in the MS/MS spectrum in Figure 16. Similarly, AKRRG clusters the protonation site in the Arg residue, thus the fragmentation ion $b_4^+$ is observed. Moreover, RRGKA peptide sequesters the charge at the Arg residue in the N-terminus, producing a $b_4^+$ fragmentation as observed. Since there is no competing charge sites in a singly charge ion, there is an absence of Coulombic repulsion, and the CCS observed for all the peptides show no significant difference.

The hypothesized protonation sites for the singly charged ions in the penta-peptides are

$$\text{Gly-Ala-Arg-ArgH}^+-\text{Lys}$$

$$\text{Ala-Lys-Arg-ArgH}^+-\text{Gly}$$

$$\text{ArgH}^+-\text{Arg-Gly-Lys-Ala}$$

A clear trend in the obtained CCS data shows that as the ion charge increases, the collisional cross sections for the peptides increases. The singly charged ions exhibit more folding than the doubly charged ion, and causes the more compact CCS to be observed. Similar studies which have involved the characterization of CCS of isomeric peptides have been explored by Wu et al.\textsuperscript{99} and Pepin et al.\textsuperscript{100}. In their studies, they have reported that a similar trend of increasing CCS is observed for highly charged states. The singly charged precursor ions stabilize the charge (located in the highly basic Arg group) by backbone carbonyl groups forming a charge center; thus a tightly folded precursor ion conformation is observed, or salt bridges could also be possible.\textsuperscript{101, 102}
For the doubly charged peptides, no significant differences in CCS are observed between the isobaric peptides as seen in Table 1. However, the way in which the charge is arranged in the sequence for a doubly protonated peptide depends on the mobile proton, while one of the protons is sequestered by the most basic site. For GARRK, it was previously discussed that one of the arginines sequesters the charge, and it is thought in this work that the arginine in the C-terminus is also charged. Due to the presence of highly basic groups, closest to the C-terminus, the fragmentation pathway for C-terminus charge retention is preferred, and thus the $y_3^{2+}$ ion is observed. For AKRRG, it is hypothesized based on the mobile proton model that the charge is first retained by one of the arginine’s and the mobile proton on the second arginine is free and transfers its charge to the amides on the backbone with sufficient energy. Since charge is more randomly distributed between the N and C-terminus, the $b_4^{2+}$ are not as abundant, consistent with the statements done by Tabb, et. al.90 Moreover, the peptide RRGKA, is first thought to sequester its charge on the N-terminal arginine residue, and the mobile proton transfers to the peptide amide bond, favoring the observed $b_4^{2+}$ pathway. Thus, with this discussion, taking into consideration coulombic repulsion and the mobile proton model, the protonation sites that are thought to be most favorable for the doubly charged isobaric peptides are

\[
\text{Gly-Ala-Arg}^+\text{-Arg}^\text{H}^+\text{-Lys}
\]
\[
\text{Ala-Lys-Arg}^+\text{-Arg}^\text{H}^+\text{-Gly}
\]
\[
\text{Arg}^+\text{-Arg}^+\text{-Gly-Lys-Ala}
\]
GARRK, AKRRG RRGKA have a total of 4 basic sites (three basic residues and N-terminal). Schnier, et. al. proposed a model that predict the number of charge states for arginine containing peptides formed by the process of ESI which is determined by their gas-phase proton transfer reactivity.\textsuperscript{103} Schnier, et. al. concluded from their results that for arginine containing peptides, there was a strong correlation between the coulumbic repulsion and the maximum number of charges that an ion could retain in the ESI process. With the model that Schnier, et. al. developed, the authors concluded that by simply counting the number of total basic sites in a peptide and dividing it by 1.75, an accurate number of charge states can be obtained. By using his methodology, it can be determined that the average maximum charged states for the three isobaric peptides is closest to 2, and they’re successfully observed for all the peptides as supported by the Full MS spectrum obtained in Figure 15.

Dongre, et. al., however, stated that protonation of adjacent arginine residues for doubly charged peptides is highly likely due to proton sequestering.\textsuperscript{104} Due to the highly adjacent arginine sites, it would make sense that coulombic repulsion on the peptide backbone are decreased by resulting in an elongated precursor ion conformation.

The doubly charged ions demonstrate comparable CCS between the isobaric peptides and no significant difference is observed between the peptide doubly charged ions. Therefore, it is concluded that the differences in fragmentation efficiencies observed for the isobaric peptides (GARRK > RRGKA > AKRRG) is not due to differences in the cross section of the peptides. Instead, the locations and distribution of the basic (protonation) sites in a peptide is the most important factor in influencing fragmentation
behavior of the isobaric peptides, which affect the conformation of the precursor ions. The mobile proton effect may also play a role as previously described and helps in the understanding of the fragmentation efficiencies observed.

For example, according to mobile proton model for doubly charged peptides, RRGKA has an immobilized proton at the arginine in the N-terminus, and a mobile proton at the adjacent arginine, which transfers to the amide bond with added energy yielding a $b_4^{2+}$. However, the mobile proton in RRGKA is closer to the N-terminus, thus, it would make sense that it doesn’t fragment as efficiently as GARRK. The peptide GARRK may require less energy for proton mobilization due to the charge being close to the peptide amide bond that results in a $y_3^{2+}$, with charge being retained in the C-terminus. Although the N-terminus of AKRRG is less basic than RRGKA, the sequestration of the protons occurs at the center of the peptide further reducing AKRRG fragmentation efficiency below that of RRGKA. Overall, the IM-MS experiments were highly reproducible, however there was always some small amount of error involved from measurement uncertainties in the calibrant ions.  

3.2 Cleavable Probe Unit Design and Synthesis

The overall cleavable probe unit design that will be explored in this thesis was previously discussed in the introductory chapter, in Figure 10. The final probe unit is showed in the diagram below (Figure 25): there are a few challenges that should be address about the probe design (a) first, the selection of a very stable and sensitive peptide that yields high abundance of characteristic fragment ions, (b) protection of the
highly reactive basic sites in the peptide (i.e., primary amine) through an amino-protection reaction – this was necessary to prevent self-cross coupling among peptides and (c) the coupling of the peptide to the report antibody through reaction between the isothiocyante functionality in the probe and amines in lysine residues of the antibody. From the three isobaric peptides studied, GARRK peptide was selected for coupling into a cleavable probe unit for mass spectrometry based immunoassays.

GARRK peptide was considered for coupling into a cleavable peptide probe unit, for mass spectrometry based immunoassay applications due to the ease with which it fragments to give abundant fragment $y_3^{2+}$ and $b_4^+$ ions. GARRK N-terminus has a pKa = 9, while the basic residues Lys pKa = 10.5 and Arg pKa = 12.5. Thus, GARRK contains a highly reactive primary amine that could potentially allow self-cross coupling through reaction with isothiocyanate group in the probe for the purposes of the reaction conditions studied. (The isothiocynate group is intended to react with primary amine groups in the Lys residue from the detector antibody) Thus, a Boc protected GARRK was first prepared using solution-phase chemistry; the protected peptide was then coupled to the probe through a Steglich-esterification. The following discussions focus on the description of the procedures used to achieve the final peptide probe.

3.2.1 Amino-Protection Reaction

In order to incorporate the peptide into the conjugate probe unit the reactivity of the final probe design must be considered. The most reactive amino group in the isobaric
peptide must be protected due to possible interactions with the NCS group within the final probe. Thus, the reactive amino group was protected through a di-tert-butyl dicarbonate (Boc) protection. Boc groups have been described to be a very efficient and cheap method for primary amine protection group for alpha amino acids and amino acid residues in solution and solid phase peptide chemistry. For the purpose of this thesis, our goal is to protect the primary amino group in the peptide. The arginine residues in the peptides always maintain protonation due to the high pKa of the guanidine group (pKa=12.5), and are therefore non-nucleophilic. Moreover, the lysine side chain has a side chain that is always maintained protonated in the solution phase synthesis as well, since the pKa = 10.5. Thus, the primary amine at the N-terminus is considered to be the possible reaction site for the isothiocyanate when pH = 9. The Boc protection reaction occurs as described in Figure 23.

![Figure 23](image_url)

**Figure 23.** Boc-protection of reactive primary amine in GARRK peptide (i.e., amine in lysine residue) using established protocol under aqueous basic conditions. The Boc-GARRK peptide was obtained, MW= 686.7 Da
Figure 24. MS³ of Boc-GARRK protected peptide for (a) doubly (MS³) and b) singly (MS²) charged precursor peptide ions

Mass spectra of the Boc protected GARRK peptide reveals that there is one reactive amino group, which is expected to be the primary amine at the N-terminus. The protected peptide will have a mass shift of 100 Da compared with the unprotected peptide. In this case, ions at m/z 687 indicate the singly charged species whose product ion MS/MS spectrum is shown in Figure 24b. The doubly charged peak of the protected peptide, however, is the most abundant ion, yielding a peak at m/z 344. MS³ spectra for the doubly charged ion show the characteristic fragment ion expected for GARRK peptide m/z 230 (Figure 24a).
3.2.2 Synthesis of Cleavable Boc-GARRK Probe Unit through Esterification Reaction

The purified Boc protected GARRK peptide was further reacted in a subsequent step through a Steglich esterification reaction in solution phase (Figure 25). In the Steglich-Esterification reaction, the carboxylic group of the protected peptide formed an ester bond with the alcohol group in the NCS-containing cyclic probe. The esterification product for the peptide-conjugated probe has a mass of 850 Da.

![Diagram showing the synthesis of the cleavable Boc-GARRK probe unit through esterification reaction](image)

**Figure 25.** Esterification reaction steps for peptide probe product; the peptide contains the carboxylic acid and the probe has the alcohol end, in the presence of DCC and DMAP catalyst, the product is obtained, MW = 850 Da

![MS/MS spectrum of the conjugated cleavable peptide probe product from esterification reaction](image)

**Figure 26.** MS/MS of 851 peak, conjugated cleavable peptide probe product from esterification reaction
The final synthesized cleavable Boc-GARRK probe unit was characterized using tandem MS experiments. The MS/MS spectra for the singly charged ion of the peptide probe (m/z 851, Figure 26) shows that the protected peptide dissociates to give the characteristic GARRK $b_4^+$ fragment ion at m/z 441, and shows a loss of the conjugate unit by revealing the mass of the protected peptide at m/z 687. The signal obtained for this experiment was low, and the singly charged ion for the peptide probe was only accessible by tandem MS. There could be various possibilities for such a low signal, the most probable due to potential side products.
Chapter 4: Conclusions and Future Work

4.1 Conclusions

Three isobaric penta-peptides (GARRK, AKRRG and RRGKA) were designed, synthesized, characterized and evaluated as possible mass reporters for MS-based immunoassay. Selection was based on reproducibility and ease with which the peptide produced abundant characteristic fragment ions when subjected to collisional activation. The peptides GARRK, AKRRG and RRGKA share singly, doubly and triply charged precursor ions in the full MS analysis, however, MS/MS reveals different fragment ions. The protonation sites of the singly and doubly charged ions demonstrated to be dependent on the localization of the basic residues in the peptide sequence, and this affects the fragmentation pathway observed for each peptide, rendering unique fragment ions in tandem MS. The requirements to synthesize and utilize the isobaric peptide probes in multiplexed immunoassay capabilities are explored. A cleavable peptide probe was created using a solution-phase synthetic strategy, which involved Boc protection and subsequent condensation of the C-terminus to the conjugation unit. This thesis proves a successful method to obtain a peptide-probe for future bio-conjugation and MS paper-based experiments.
This thesis gathers novel possibilities of developing isobaric peptide-probes for protein biomarker detection. In order to develop highly efficient and stable isobaric peptide-probes, the peptide sequence considered should yield abundant fragment ions through specific fragmentation pathways. This information gathered is especially useful in improving peptide-probe stability needed for on-demand direct-to-customer MS-based immunoassay applications.

4.2 Future Work

Even though the focus of this work was achieved, that is to rationally develop characterize, and study the capabilities of isobaric peptides to generate a novel peptide probe with multiplexing capabilities, a high yield of the peptide probe was not achieved. This could be due to various experimental limitations such as the adapted coupling reaction method, which could generate challenges regarding stability of the final peptide probe. The current probe involves the use of an isothiocyanate (NCS) group, which is amine-reactive and binds to lysine residues in antibody proteins through acylation in mild conditions. The peptide sequence could be re-considered to remove any lysine residues that could potentially interfere with stability of final probe for future studies. Moreover, alternative peptide-probe coupling techniques could be considered by using solid-phase peptide synthesis (SPPS) that could alleviate concerns for side products by site-directed
protections. Alternative cleavage linkers could also be considered, since the current pH sensitivity linker is released in basic conditions.
References


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