The Development of an Antibody Affinity Enrichment and Mass Spectrometry-based Assay (iMALDI) for the Characterization of EGFR and EGFR Isoforms from Human Brain Cancer Tissue

by

Brinda Shah
BSc, University of British Columbia, 2007

A Thesis Submitted in Partial Fulfillment
of the Requirements for the Degree of

MASTER OF SCIENCE

in the Department of Biochemistry and Microbiology

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Abstract

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EGFR (Epidermal Growth Factor Receptor) is a protein that is ubiquitous in the human body. Aberrant activity of EGFR or its isoforms is implicated in a number of cancers, notably brain cancer. An isoform of EGFR, EGFRvIII (EGFR variant III), is particularly relevant to brain cancer since it is only naturally found in brain tumour tissue. However, the presence and activity of EGFRvIII is not well characterized. I hypothesize that the different levels of EGFRvIII expression and its expression relative to wild type EGFR in human brain tumour tissue can be used to diagnose the different stages and progression of disease in the glioblastoma multiforme (GM) type of brain cancer.

The work presented in this thesis is an attempt to develop a method for the accurate and absolute quantitation of EGFRvIII from brain tumour tissue. Using iMALDI (immunoMALDI), which combines the high-specificity of MALDI mass spectrometry with antibody immunoaffinity enrichment, I have optimized and developed a high-throughput technique for sensitive, specific and quantitative detection and differentiation of EGFR and EGFRvIII. I have also demonstrated a proof-of-concept by applying this assay to the isolation and detection of these proteins from brain tumour tissue.

Table of Contents

Supervisory Committee ii
Abstract iii
Table of Contents iv
List of Tables vii
List of Figures viii
Acknowledgments x
Chapter 1 Introduction 1
Glioblastoma Multiforme and its Expression of EGFR and EGFRvIII 1
Detection of EGFR and EGFRvIII in Biological Samples 4
Mass Spectrometry-based Methods for Detection of EGFR and EGFRvIII 6
iMALDI for the Quantitative Detection and Differentiation of EGFR and its Isoforms 13
Chapter 2 Synthesis and Characterization of Peptides and Antibodies 15
Introduction 15
Materials and Methods 21
Synthesis of Peptides and Stable Isotope Standards 21
Derivation of Polyclonal Antibody 22
Enzyme-linked Immunosorbent Assay 23
Sensitivity of Detection of Peptides 24
iMALDI Assay for Testing Antibody Affinity 24
Results 25
Discussion 30
Conclusion 32
Chapter 3 Optimization of Experimental Parameters of iMALDI Assay 33
Introduction 33
Materials and Methods 37
Washing of Protein G Beads 38
iMALDI Assay for Testing Limit of Detection 38

Antibody Concentration Optimization 39
Antibody Incubation Optimization 39
Peptide Incubation Time Optimization 40
One-step vs. Two-step Method 41
Results 42
Discussion 52
Conclusion 55
Chapter 4 Analysis of Biological Sample: U87MGΔEGFR Cell Line 57
Introduction 57
Materials and Methods 60
Cell Culture of U87MG and U87MGΔEGFR Cell Lines 60
Cell Lysis 61
BCA Assay to Measure Total Protein Concentration of Cell Lysate 62
Tryptic Digestion of Cell Lysate 62
iMALDI Analysis 63
Results 63
Discussion 67
Conclusion 69
Chapter 5 Analysis of Biological Samples: Glioblastoma Tumour Tissues 71
Introduction 71
Materials and Methods 73
Sonication of Tissue and Extraction of Proteins 73
BCA Quantitation 73
Tryptic Digestion of Tissue Lysate 74
iMALDI Analysis of Digested Tissue Lysate 74
RT-PCR Assay 75
Results 75
Discussion 83
Conclusion 87
Chapter 6 Characterization of Antibody Binding to Alkylated Peptide 88
Introduction 88

Materials and Methods 89
Preparation of Alkylated Peptides 89
iMALDI Capture of Modified and Unmodified Peptide 89
Results 90
Discussion 94
Conclusion 95
Chapter 7 Summary and Conclusions 96
Detergent Removal 96
Optimization of Incubatioin Time and Antibody Concentration 97
Selection of the Alkylating Agent 98
Selection of the Antibody 99
Brain Tissue Extraction 101
Conclusion 102
Bibliography 104
Appendix A List of Abbreviations 114
Appendix B Theoretical Peptide Information 117
Appendix C Ethics Committee Approval 122

List of Tables

Table 2.1 Synthesized Peptide Sequences and Masses. 25
Table 2.2 Limit of Detection of Peptides Directly on Target Plate Without Enrichment. 27
Table 2.3 Preliminary iMALDI data with Protein G magnetic beads containing Tween-20 28
Table 3.1 Preliminary iMALDI data with Protein G magnetic beads after detergent exchange with CHAPS. 44
Table 5.1 83
Table 5.2 Results from the RT-PCR Analysis of the Five Glioblastoma Tumour Samples and Comparison to the iMALDI results. 83

List of Figures

Figure 1.1 General Schematic of Ionization of Analytes Using the MALDI (Matrix Assisted Laser Desorption Ionization) Technique. 7
Figure 1.2 A Schematic Representation of the Ionization of Particles by MALDI and Their Separation Based on Time-of-Flight. 8
Figure 1.3 The Nomenclature of Fragmentation Patterns of a Peptide. 10
Figure 1.4 Analytical Scheme of the iMALDI Assay. 13
Figure 2.1 The Sequence Difference Between the Tryptic Peptides EGFR and EGFRvIII. 16
Figure 2.2 Schematic of Interaction between Magnetic Bead, Protein G, Antibody and Target Peptide. 20
Figure 2.3 ELISA Titration of Rabbit Polyclonal Antibodies Raised Against EGFRvIII Peptide. 26
Figure 2.4 Mass Spectrum from Preliminary iMALDI Analysis 29
Figure 3.1 Structures of Detergents used in the iMALDI Assay 34
Figure 3.2 Resulting MALDI TOF/TOF Mass Spectrum after Buffer Exchange from Tween 20 to CHAPS-containing Solution. 43
Figure 3.3 Optimization of Antibody Concentration in Experimental Samples. 46
Figure 3.4 Optimization of Antibody Binding Time 48
Figure 3.5 Optimization of Peptide Binding Time. 49
Figure 3.6 Comparison of One-step and Two-step iMALDI Method 51
Figure 3.7 An Updated and Optimized Analytical Scheme for iMALDI Analysis of EGFR and EGFRvIII 56
Figure 4.1 iMALDI Analysis of U87MGΔEGFR Cell Line. 65
Figure 4.2 Quantitation Curve for Endogenous EGFRvIII in the U87MGΔEGFR Cell Line. 66
Figure 4.3 MALDI-MS Spectrum Showing Capture of Spiked-in EGFR Wild-type Peptide from U87MG Control Cell Line. 67
Figure 5.1 iMALDI analysis of Glioblastoma tumour sample 1 (GM1). 76

Figure 5.2 iMALDI Analysis of Glioblastoma Tumour Sample 2 (GM2) 77
Figure 5.3 iMALDI Analysis of Glioblastoma Tumour Sample 3 (GM3). 78
Figure 5.4 iMALDI Analysis of Glioblastoma Tumour Sample 4 (GM4). 79
Figure 5.5 iMALDI Analysis of Glioblastoma Tumour Sample 5(GM5) 80
Figure 5.6 iMALDI Analysis of Normal Brain Sample 1 (NM1) 81
Figure 5.7 iMALDI Analysis of Normal Brain Sample 2 (NM2) 82
Figure 6.1 MALDI-MS Spectrum of Completely Alkylated EGFRvIII Peptide............. 91
Figure 6.2 MALDI TOF/TOF Spectrum to Confirm a 1:1 mixture of Unmodified and Modified EGFRvIII Peptide 92
Figure 6.3 MALDI-MS Spectrum of Both Alkylated and Non-alkylated EGFRvIII Peptide after Antibody Capture. 93

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Chapter 1
Introduction

Glioblastoma Multiforme and its Expression of EGFR and EGFRvIII

Glioblastoma multiforme (GM) is the most common and malignant type of cancer of the central nervous system (Clarke et al., 2010). The survival rate of affected patients is very low (<5%) over a period of less than 5 years (Quick et al., 2010). The symptoms that are presented depend on the location of the tumour, but frontal lobe and temporal lobe involvement can present the most common symptoms of rapid memory loss and noticeable personality changes (Clarke et al., 2010). A glioblastoma is particularly dangerous because it can be asymptomatic until the tumour reaches a large size, so early detection is imperative for better prognosis (Bruzzone et al., 2009). If a tumour is visualized by magnetic resonance imaging (MRI), a stereotactic biopsy or craniotomy is performed with tumour resection and the tumour is screened for cancer-indicating markers. The genetic abnormalities that are screened to indicate the presence of a cancerous tumour include the mutation of the *p53* gene, *p16/cdkn2* gene inactivation, monosomy of chromosome 10, and overexpression or mutation of the epidermal growth factor receptor (*egfr*) gene (Bruzzone et al., 2009; Lang et al., 1994).

Epidermal growth factor receptor (EGFR) is a 170-kD cell surface receptor comprised of 1210 amino acids that is ubiquitous in the human body. It is part of the four-membered EGF-family of receptors (also known as the ErbB family). The receptor is activated upon binding of a ligand, most often EGF (epidermal growth factor), and subsequent

dimerization with another receptor from the ErbB family. This dimerization process is part of the regulation of this protein in that the receptor cannot be activated or cannot perform its downstream tasks until it has dimerized. The dimerization stimulates its intrinsic intracellular tyrosine kinase activity such that several tyrosine residues on the intracellular tail of the protein become phosphorylated. This action provides binding sites for other proteins in the EGFR pathways and initiates a signal transduction cascade *via* the MAPK, Akt, and JNK pathways, finally leading to the activation of DNA synthesis and cell proliferation mechanisms (Mitsudomi et al., 2010). As a result of these primary functions, EGFR is considered an oncogene. Any anomaly that causes the *EGFR* gene to stay in a constitutively active state results in uncontrolled cell division (Gan et al., 2009). Mutations that allow upregulation or overactivity of EGFR have been associated with a number of cancers, especially lung cancer and breast cancer.

In cancers of the brain, a particular isoform of EGFR, termed EGFRvIII (EGFR variant 3), is often implicated in the development of cancerous growth (Gan et al., 2009). EGFRvIII is a splicing variant of EGFR that is caused by the deletion of exons 2-7 out of a total of 26 exons, resulting in the creation of a novel glycine residue at the site of deletion. This deletion also causes the loss of amino acids 6 through 273, and the new protein product has a mass of approximately 145 kD (Wikstrand et al., 1998). The deleted exons are part of the extracellular binding domain, so EGFRvIII does not bind to a ligand (Ekstrand et al., 1994; Nishikawa et al., 1994; Moscatello et al., 1996). Instead, the protein spontaneously dimerizes and activates the phosphorylation of its internal tyrosine residues (Han et al., 1996). As a result, EGFRvIII remains in a constitutively

active state which leads to uncontrolled cell growth (Ayuso-Sacido et al, 2009). Some evidence suggests that EGFRvIII acts *via* different signalling cascades which cause more aggressive cell overgrowth (Zeineldin et al., 2010). EGFRvIII also confers enhanced tumorigenicity, possibly by reducing apoptotic events. Therefore, an increase in cell growth and decrease in cell death amounts to a net tumour growth (Zeineldin et al., 2010).

Despite the recognition that EGFRvIII is an integral part of tumour development in the brain, the activity of EGFRvIII is not well characterized. The signalling network of EGFRvIII is incomplete and it is not known how the different levels of EGFRvIII protein affect cell overgrowth, partly due to a lack of a quantitative method for detection (Yoshimoto et al., 2008). Furthermore, the relationship between EGFR and EGFRvIII is not well understood, as many tumours show the simultaneous presence of both proteins (Yu et al., 2008). If different levels of EGFRvIII or EGFRvIII + EGFR presence indicate how aggressive the tumour growth will be or what the progression of the disease will be, it becomes imperative that any assay be able not only to distinguish between the two proteins but also to quantitate the relative levels of both proteins in tissue. The ability to perform absolute quantitation is also an asset because the absolute levels of both proteins may have an impact on tumorigenicity of the tissue. Absolute quantitation is also important for future development of a clinical assay.

Since EGFRvIII is exclusively found in tumours, particularly brain tumours, a proper understanding of the activity of this protein is important for the development of targeted

therapeutics for this disease (Lo et al., 2001; Kuan et al., 2009; Mischel et al., 2003). **I hypothesize that the presence of EGFR and EGFRvIII in brain tumour tissue will correlate to disease state and progression**. To accomplish this task, I have taken a unique mass spectrometry-based approach rather than the traditional methods such as immunohistochemistry or polymerase chain reaction (PCR) which are of limited use for accurate protein quantitation.

Detection of EGFR and EGFRvIII in Biological Samples

Immunohistochemistry (IHC) and PCR methods are among the most common methods for detection of EGFR and EGFRvIII. IHC is used extensively for grading and classification of tumour tissue based on the presence of the receptor. IHC is also considered semi-quantitative in that the signal intensity increases with increasing number of receptors present (von Wasielewski et al., 2008). In contrast, PCR tests are based on the presence of mRNA copies of EGFR and the over-amplification of the *EGFR* gene is detected. PCR tests are also very common for EGFRvIII detection (Yoshimoto et al., 2008). IHC for the detection of EGFRvIII has been developed recently, but the unavailability of specific anti-EGFRvIII antibodies has hindered the development of this method (Nishikawa et al., 2004).

iMALDI is a more suitable method to determine the quantity of EGFR and EGFRvIII because both traditional methods have some disadvantages in the accurate detection of EGFR and EGFRvIII. IHC is particularly prone to false positives, especially because a signal cannot be *verified* as true or false (Barrett et al., 2007). Background staining can

result from several aspects of this methodology, ranging from diffusion of the antigen to contamination from other antibodies in a polyclonal antibody mixture (Tawfik et al., 2006). False positives and background signal affect all laboratory methods, although controls can be implemented to counter these effects. However, even after the controls are used, IHC cannot give a definitive answer as to whether a particular antibody is indeed binding the target antigen in that a positive signal cannot be verified by a protein sequence (Di Leo et al., 2002). Additionally, IHC cannot perform quantitation to the level of accuracy that is required to understand the details of EGFRvIII activity.

PCR, on the other hand, is considerably more accurate than IHC and the false positive rate is low (Yoshimoto et al., 2008). PCR can also be semi-quantitative in that the detection of the presence of the mRNA is more quantifiable than IHC. The sequence of the amplified portion of cDNA can be confirmed in order to validate the presence of the target gene. However, the rapid degradation and fragile nature of mRNA within cells poses a disadvantage to a PCR assay. PCR tests are highly sensitive so great care and precautions need to be taken in order to reduce the possibility of contamination (Klein, 2002). Most importantly, mRNA-based data can be unreliable when the mRNA expression does not correlate well with protein expression or activity (Greenbaum et al., 2003). Often times, not all mRNA copies in the cell are translated into protein copies (Sjogren et al., 1996). Moreover, since the receptor in the cell is the entity that is effective in signalling downstream proteins, intuitively, it makes more sense that the quantity of the protein will be a better indicator of disease state and progression than the quantity of mRNA (Yates III, 2000).

Mass Spectrometry-based Methods for Detection of EGFR and EGFRvIII

Mass spectrometry is an essential tool in contemporary research on cancer diagnostics (Diamandis, 2004; Rodland, 2004). Mass spectrometers follow a general layout that consists of: an ionization source, which converts the analytes or molecules into charged particles; a mass analyzer, which separates the ions based on its mass (or mass-to-charge ratio, m/z) by applying an electric and/or magnetic field; and a detector, which detects these ions. The ionization source determines the types of molecules that are ionized and this affects which ions are ultimately detected. The source can create charged particles, either negative ions (negative ion mode) or positive ions (positive ion mode). Two of the most common types of ionization sources for MS of proteins and peptides are MALDI (matrix assisted laser desorption ionization) and ESI (electrospray ionization) (Sparkman et al., 2007).

In MALDI, the analyte is co-crystallized with a light absorbing organic acid (matrix) on a solid metal surface. The acidic matrix facilitates the ionization of the analyte. A laser is applied to the crystallized complex, and the resulting energy is absorbed by the matrix and transferred to the analyte. The charged analyte molecules are subsequently separated by the mass analyzer (Figure 1.1) (Karas et al., 1987). A common mass analyzer used in conjunction with a MALDI ionization source is a time-of-flight (TOF) analyzer (Figure 1.2). In a TOF, ions that are charged in the ionization source are accelerated into a field-free tube until they reach the detector. The amount of time that the ion takes to reach the detector is proportional to the mass-to-charge ratio(m/z) of the ion (Guilhaus M., 1995).

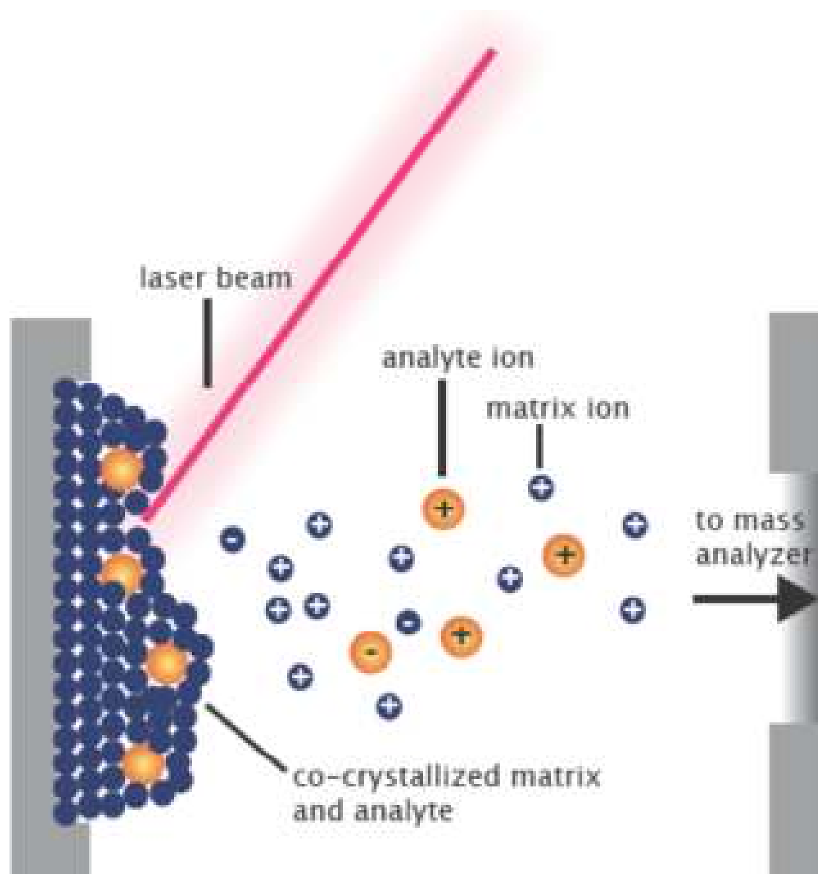

Figure 1.1 General Schematic of Ionization of Analytes Using the MALDI (Matrix Assisted Laser Desorption Ionization) Technique. *A laser is applied to a co-crystallized matrix and analyte sample on a target plate. The analytes in the sample are ionized and are directed into the mass analyzer.*

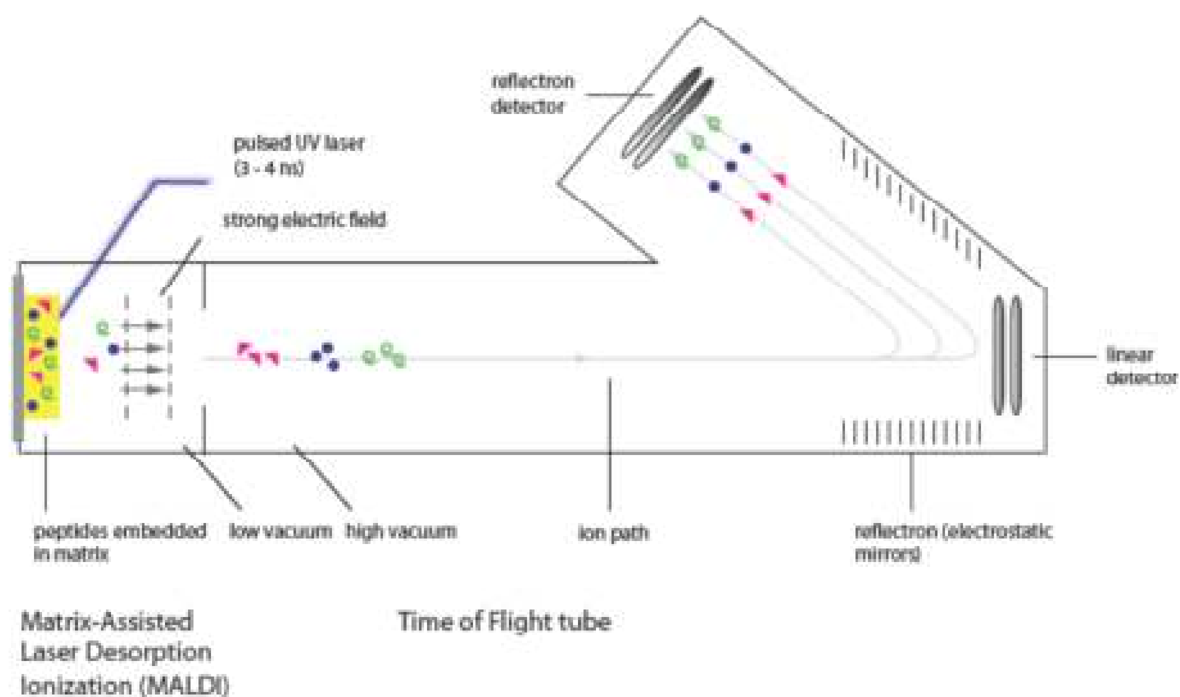

Figure 1.2 A Schematic Representation of the Ionization of Particles by MALDI and Their Separation Based on Time-of-Flight. *Ions (coloured icons) travel through a field-free region of the time-of-flight tube to strike the linear detector where their flight time is measured.. Alternatively, ions with the same mass-to-charge ratio but slightly different velocities travel past a set of electrostatic mirrors (the reflectron) where their velocities are equalized before hitting the detector. Operation in the reflectron mode provides higher resolution, whereas the linear mode provides greater sensitivity.*

Although MS is able to detect the mass of the analytes in a sample, because many analytes may have the same molecular weight (and thus the same parent or precursor ion m/z value), to know the specific identity of the analyte requires a more specific approach. Successive fragmentation of protein or peptide precursor ions in conjunction with MS (termed 'tandem MS' or MS/MS, or MS^n depending on the number of fragmentations and mass analysis steps) is a popular technique to increase the specificity. The two MS runs can be accomplished by placing two mass analyzers in tandem. For instance, in a MALDI TOF/TOF instrument, a MALDI ionization source is coupled to two TOF mass analyzers.

The most common type of fragmentation method used in a MALDI TOF/TOF instrument is Collision Induced Dissociation/Decay (CID) (Wells et al., 2005). In this fragmentation method, the molecules collide with inert gas in a specific chamber within the MS instrument (Yost et al., 1978; Morgan et al., 1978). A particular molecule is detected in the MS spectrum, , and that molecule will subsequently be selected for fragmentation. The MS/MS analysis will yield a spectrum consisting of fragments from the selected ion. The fragmentation of peptides is predictable, and these fragments are named according to the Roepstorff nomenclature (Figure 1.3) (Roepstorff et al., 1984). For example, b and y ions originate from cleavage of the amide bonds and contain the N and C termini, respectively. As a result, the sequence of an analyte can be determined from the mass-to-charge ratios of these ions. This can be used to confirm the identity of a peptide (and therefore the identity of a protein) that is present in a sample.

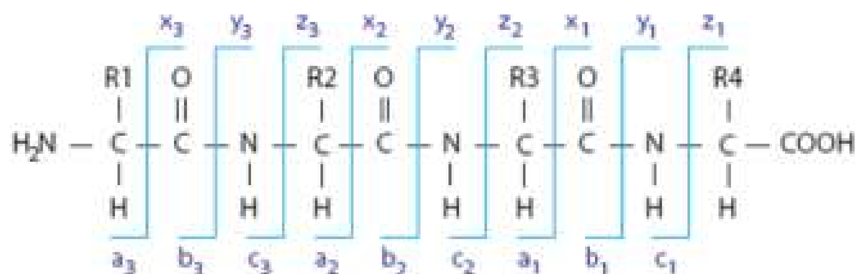

Figure 1.3 The Nomenclature of Fragmentation Patterns of a Peptide.

Surface-enhanced laser desorption ionization (SELDI) (Hutchens, T and T Yip, 1993), developed by Ciphergen Biosystems (Fremont, CA), was proposed as a method for the detection of biomarkers from several cancers and from various biological materials (Espejel et al., 2008; Petricoin et al., 2004). SELDI is the combination of MALDI mass spectrometry and a surface modified target. In this method, the biological sample is first affixed on the target plate *via* an affinity group and then overlaid with a matrix. The downstream steps are identical to conventional MALDI mass spectrometry. Several studies have investigated the role of EGFRvIII in the tumour cell using this SELDI-TOF MS method (Whelan et al., 2008; Kumar et al., 2008). However, the major disadvantage of SELDI is that special surface-modified targets are required.

However, other enrichment methods coupled to mass spectrometry have emerged as powerful methods for cancer diagnostics (Aebersold et al., 2005; Faca et al., 2007). The added enrichment step is imperative for minimizing background noise and detecting low-abundance molecules (Huttenhain et al., 2009). A recent study utilized a

phosphoproteomics approach for elucidating the EGFRvIII signalling network (Huang et al., 2007). Since EGFR is a tyrosine kinase and all downstream signalling occurs *via* phosphorylation events, this approach was ideal. In this method, phosphorylation levels of various hypothesized downstream proteins were analyzed using iTRAQ labelling and liquid chromatography coupled to a QqTOF (quadrupole-quadrupole time-of-flight) mass spectrometer. Prior to MS analysis, several steps of immunoaffinity enrichment and IMAC (immobilized metal affinity chromatography) were performed to minimize background binding and to enrich for phosphopeptides. The results were then compared to EGFRvIII activity in the same sample (Huang et al., 2007). Although this method was very helpful in characterizing relationships within the data set, it was only capable of relative and not absolute quantitation.

Absolute quantitation of protein expression may aid in the understanding of the tumour development process, particularly if the levels of protein present indicate the stage or progression of any given tumour sample (Ong et al., 2005). Determining the absolute amounts of EGFR and EGFRvIII expression in a cancer cell is therefore important for the development of an assay to be used for clinical diagnostics. Two absolute quantitation methods, SISCAPA (stable isotope standards and capture by anti-peptide antibodies (Anderson et al., 2004)) and iMALDI (immunoMALDI (Warren et al., 2004; Jiang et al., 2007)) utilize immunoaffinity enrichment coupled to mass spectrometry. In both techniques, stable isotope-labelled internal standards are used to provide absolute quantitation (Figure 1.4). The major difference between the two methods is whether or

not the analyte is eluted before analysis, and the downstream mass spectrometric instrumentation used.

iMALDI utilizes MALDI-TOF mass spectrometry. In iMALDI, a peptide of interest is first selected from a target protein and an anti-peptide antibody is generated against the peptide. Stable isotope-labelled standards are synthesized according to FMOC chemistry which are essentially identical to the target peptide but are slightly heavier because of the incorporation of heavy isotopes. Affinity beads with immobilized anti-peptide antibodies are then used to capture the peptide from the solution and thereby to enrich the peptides from a proteolytically-digested complex mixture (Shah et al., 2010). The standard is spiked into the mixture and is co-captured along with the endogenous peptide. After the enrichment step, the beads are placed directly onto the MALDI target plate. The sample spot is then overlaid with matrix and the captured peptides are eluted off the beads by the matrix solvent and are ionized by the laser. The ions are detected at specific mass-to-charge ratios. In the positive ion mode, these m/z values correspond to the protonated molecular ions $(M+H)^+$ of the corresponding peptides. Following the identification of the signal corresponding to the peptide of interest, this ion can be selected for further fragmentation and sequencing by MS/MS. Since the sequence of the target peptide is previously known (it was used for the generation of antibodies and synthesis of standard peptides), this sequencing step confirms the identity of the captured and detected peptide (Jiang et al., 2007).

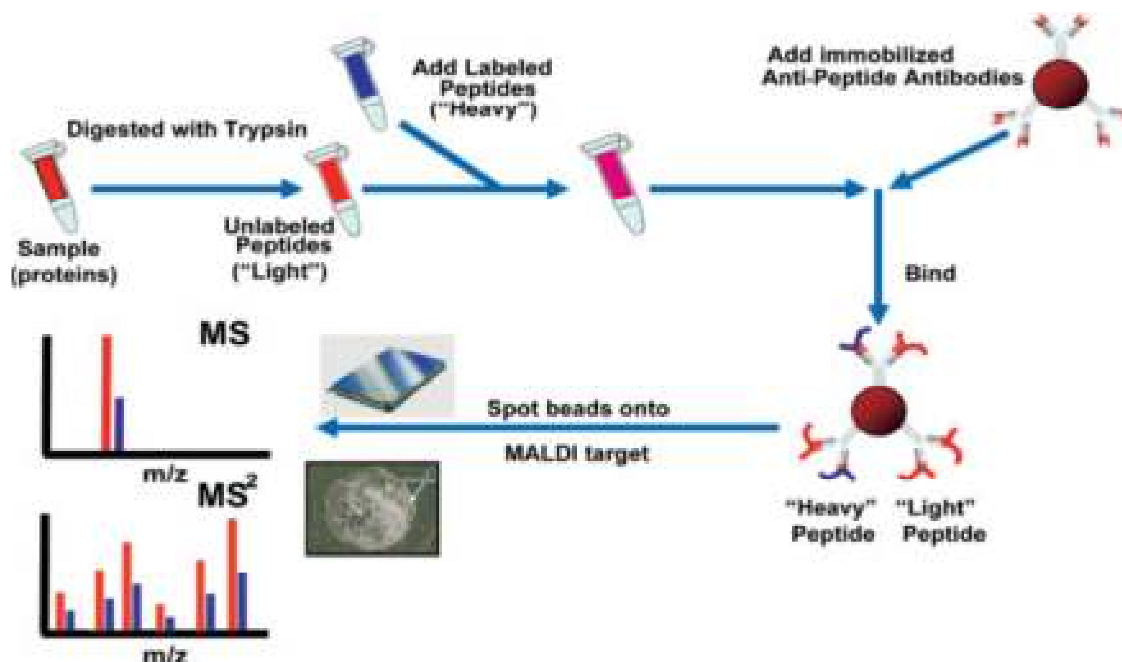

Figure 1.4 Analytical Scheme of the iMALDI Assay. *Sample proteins are digested with trypsin, mixed with a stable isotope labelled version of the target peptide, incubated with anti-peptide antibody beads, spotted onto a MALDI target plate and subsequently analyzed by MS and MS/MS using MALDI TOF/TOF instrumentation.* (Reprinted with Permission by Wiley and Sons 2007)

iMALDI for the Quantitative Detection and Differentiation of EGFR and its Isoforms

Although EGFR and EGFRvIII have already been detected in glioblastoma tissue, lack of absolute quantitative protein data has hindered the development of a reliable clinical method. The main objectives of this study, therefore, were to apply the iMALDI technique to the detection, differentiation, and quantitation of EGFR and EGFRvIII in human brain tissue.

To achieve this objective, a target peptide was selected from the EGFRvIII protein which had a similar -- but not identical -- sequence to wild-type EGFR. An anti-peptide antibody was then generated by EzBiolab Inc. which captured both the EGFR (wild type) and EGFRvIII (isoform) peptides due to the similarity in sequence. MALDI MS was used to differentiate between these peptides as distinct peaks (because of their different molecular weights) and MS/MS sequencing was utilized to confirm their identity. Isotopically-labelled standard peptides were spiked in and co-captured, thus providing absolute quantitation of the endogenous peptides. After establishing the experimental parameters of this workflow, the iMALDI assay was applied to cell lines and human brain tumour tissue.

Chapter 2
Synthesis and Characterization of Peptides and Antibodies

Introduction

The EGFRvIII protein differs from the EGFR protein by a deletion of the extracellular binding domain and addition of an added glycine residue (Yamakazi et al., 1988). A unique peptide is created in this process whereby the EGFR protein contains the tryptic peptide sequence R'NYVVTDHGSCVR and EGFRvIII contains K'**G**NYVVTDHGSCVR. If both proteins are digested with trypsin, these peptides differ by only one amino acid (Figure 2.1). Since the wild-type peptide sequence is contained *within* the sequence of the variant, polyclonal antibodies against the peptide sequence NYVVTDHGSCVR should capture *both* peptides. I have also made synthetic versions of these two peptides to test their sensitivity to detection on the mass spectrometer and their interaction with the polyclonal antibodies. In addition, stable isotope-labelled standard peptides for absolute quantitation were synthesized.

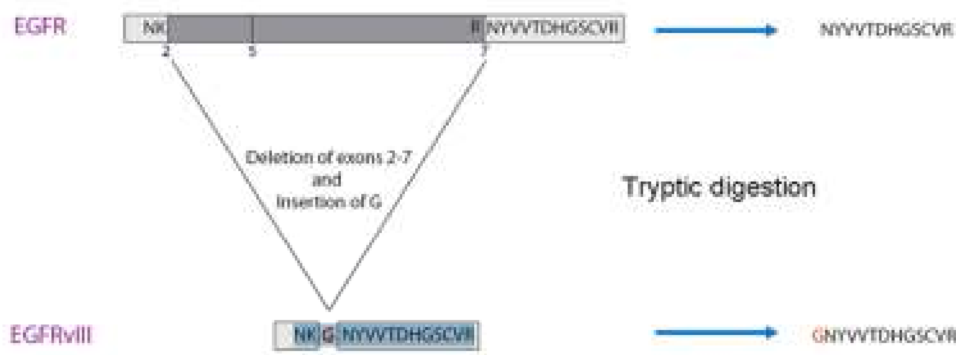

Figure 2.1 The Sequence Difference Between the Tryptic Peptides EGFR and EGFRvIII. *The EGFR protein sees a deletion of exons 2-7 and an insertion of a glycine residue to form the EGFRvIII protein. Upon trypsin digestion, EGFRvIII yields a 13-amino acid peptide with a glycine at the C terminus, whereas the EGFR protein yields the same peptide but without the glycine.*

The creation of synthetic peptides for laboratory use was pioneered by the solid-phase peptide synthesis (SPPS) work of Robert Bruce Merrifield (Merrifield, R.B. 1964; Stewart J., 1976). Unlike protein expression, peptides are difficult to express using bacteria. SPPS circumvents this problem and also allows the synthesis of peptides with specific modifications or unconventional amino acids. In general, peptide chains are built on linkers that are covalently bound to small porous beads. The free N terminus of the growing peptide chain is coupled to a single amino acid unit. The added amino acid has a protected N terminus which needs to be unprotected prior to the addition of the next amino acid unit. After successive deprotection and coupling cycles, the completed peptide is cleaved off the bead under acidic conditions. Fmoc, an acronym for *9H-*(f)luoren-9-yl(m)eth(o)xy(c)arbonyl, is one of the protective groups used in this method (Merrifield B., 1997). For the stable isotope-labelled standards, amino acids with

incorporated heavy isotopes of nitrogen (^{15}N), carbon (^{13}C), or oxygen (^{18}O) are used in lieu of the normal amino acids. The result is the creation of a heavier peptide with the same sequence as its light counterpart. The heavy peptide is indistinguishable with respect to antibody binding; however, the difference in mass can be detected by the MS.

Polyclonal antibodies are commonly used for specific precipitation (immunoprecipitation) of a particular antigen (Hanly et al., 1995). Antibodies are large molecules that are an integral part of the humoral immune system and are responsible for recognizing specific sites on an antigen, the epitope. The B lymphocytes of the humoral immune system are responsible for the production of immunoglobulins that are specific to the antigen (Janeway C., 2001). Polyclonal antibodies for research purposes are generated by eliciting an immune response in an animal that is exposed to the antigen of interest. Peptides tend to be too small to generate a large enough immune response, so successful generation of polyclonal antibodies against a peptide are done by coupling the peptide to a carrier molecule which is much more immunostimulatory than the peptide. This response stimulates multiple B cell (lymphocytes that are responsible for the production of antibodies) clones, thus making the response polyclonal (Hanly et al., 1995).

The most common type of animal chosen for polyclonal antibody production is the rabbit. Other mammals such as mice have also been used, but mice generally provide insufficient amounts of antibody due their small size. In addition to providing a large amount of serum for antibody purification, young adult rabbits also provide a very

vigorous immune response, thereby making them the desirable means of antibody production (Hanly et al., 1995). Furthermore, rabbit antibodies, both monoclonal and polyclonal, tend to show more specificity and less cross-reactivity than mouse antibodies (Rossi et al., 2005).

After peptide-based affinity purification of the polyclonal antibodies, they are screened for activity by ELISA. ELISA (enzyme-linked immunosorbent assay) is a means by which the positive binding of the antigen to the antibody is tested. In peptide ELISA, the peptides are immobilized on a microtiter plate and the antibody is washed over the peptides and allowed to bind. Between each step, the plate is washed with a detergent-containing solution to remove any non-specifically bound molecules. Typically, a chromogenic reporter molecule, such as a labelled secondary antibody, is allowed to bind and is used to detect the presence of the primary antibody. An enzymatic substrate is added in the final step, prior to measuring the absorbance or fluorescence or luminescence of the plate wells, to determine the presence of binding and the quantity which was bound (Lequin R., 2005).

Prior to optimizing the iMALDI assay, the sensitivity of detection of the peptides with mass spectrometry *without* affinity enrichment was determined. The limit of detection (LOD) of a peptide by mass spectrometry depends on attributes such as the amino acid make up of the peptide, the solvent, the matrix, and the type of mass spectrometer (Trauger et al., 2002). All of these characteristics affect the ability of the peptide to ionize and therefore its ability to be detected. Since the iMALDI assay uses MALDI

mass spectrometry, MALDI MS was used to determine the sensitivity of peptide detection.

This LOD experiment was performed by creating a serial dilution of the peptides to include concentrations both above and below the limitations of the instrument. The lowest concentration (the blank) was a control sample to eliminate the possibility of noise peaks with the same m/z as the target peptide. The highest concentration was selected to ensure saturation of the detector.

In this study, a signal-to-noise ratio of 10:1 was used to ensure a confident detection of the target peptide. This threshold is more stringent than most studies, where a S/N of 3:1 is used. Since sensitivity is of utmost importance in this study, a strict S/N ratio cut-off is essential. In this particular LOD experiment, where no other peptides are included in the solution, a signal-to-noise ratio of lower than 10:1 would probably still mean that the target peptide was detected. However, for future biological experiments where the target peptide must be detected in a complex sample, this 10:1 cut-off ensures that background noise is a not a major contributor to the desired signal. Also, most importantly, when the signal-to-noise ratio is too low, it is very difficult to obtain a complete MS/MS spectrum with which to verify the sequence of the peptide.

A preliminary iMALDI assay prior to optimization was also performed in order to confirm and supplement the ELISA results and to anticipate which aspects of the assay required further attention. For this preliminary assay, the method chosen to capture the antibody in the iMALDI assay was Protein G immunoprecipitation. Protein G is a 65-

kDa protein that is native to group C and G Streptococcal bacteria, and binds to the Fc region of most IgG isotypes (Sjobring et al., 1991). It is often used for the specific purification of IgG antibodies. Protein G can be immobilized on several different types of substrates such as agarose or Sepharose for efficient pulldown of IgG molecules (Sjobring et al., 1991). Recently, the use of magnetic metal beads with immobilized protein G has become a popular alternative to agarose or Sepharose beads. These magnetic beads are more uniform in size and have a greater binding capacity and better reproducibility. Assays similar to iMALDI, such as SISCAPA, regularly use these Protein G magnetic beads for the immunoaffinity capture of the primary antibody (Whiteaker et al., 2007).

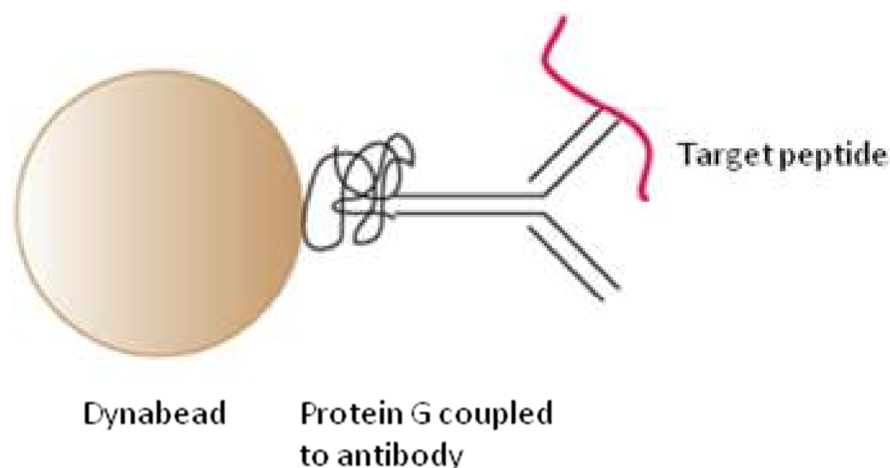

Figure 2.2 Schematic of Interaction between Magnetic Bead, Protein G, Antibody and Target Peptide. *The antibody is positioned on the protein G such that the Fab regions are pointing towards the solution and are free to capture the antigen. (Image not to scale.)*

The use of protein G magnetic beads in this assay has several advantages. The binding of IgG and protein G occurs such that the IgG is oriented with the Fab regions facing the

solution. This ensures that the antibodies are in the proper orientation for binding of the antigen (Figure 2.2). Additionally, crosslinking the protein G and IgG is not necessary since the two proteins have high affinity for each other. Avoiding this crosslinking step is preferable, since the crosslinking procedure may cause excessive loss and is not economical for expensive antibodies (Sjobring et al., 1991).

This non-covalent binding of the primary anti-EGFR antibody to Protein G instead of directly to CNBr beads is a new approach, which I developed and first used in this project. It is an improvement to the standard iMALDI protocol (Shah et al., 2010). Because of the tight binding of protein G and the anti-EGFR antibody, this new procedure simplified the overall iMALDI protocol because the binding of protein G and IgG requires minimal experimental manipulation. It also reduces the likelihood of experimental errors.

Materials and Methods

Synthesis of Peptides and Stable Isotope Standards

The peptides were created using Fmoc chemistry at the UVic-Genome BC Proteomics Centre. The peptides were synthesized on a Prelude peptide synthesizer (Protein Technologies, Tucson, AZ) at a scale of 5 µmol. The C-terminal amino acids were conjugated to TentaGel R resin (Rapp Polymere). Subsequent residues, at a concentration of 100 mM, were double coupled using 20% piperidine as the deprotector and 1H-Benzotriazolium 1-[bis(dimethylamino)methylene]-5chloro-, hexafluorophosphate (1),3-oxide (HCTU) as the activator. Cleavage was performed online with 95:2.5:2.5 trifluoroacetic acid (TFA):water:triisopropylsilane (Sigma-

Aldrich, St. Louis, MO). The cleaved peptides were removed from the synthesizer and their TFA volumes were reduced under a stream of nitrogen. Ice cold diethyl ether (Sigma-Aldrich, St. Louis, MO) was added to precipitate the peptides and, after centrifugation at 13000 rpm for 5 minutes, the ether layer was poured off. The pellets were resolubilized in 0.1% TFA and lyophilized (Modulyod, Thermo Savant).

Purification was carried out by reversed-phase HPLC on an Ultimate 3000 (Dionex, Sunnyvale, CA), monitoring peptide elution at 230nm. Approximately 5 mg of the crude peptides were separated using a Vydac C_{18} (218TP) column (10 x 250mm, 10μm resin) with a linear gradient of 0.1% TFA in water (v/v) and 0.85% TFA at a flow rate of 4mL/minute over 60 minutes.

The fractions of interest were spotted onto a stainless steel MALDI plate and analyzed by MALDI-TOF (Applied Biosystems/MDS SCIEX, Foster City, CA). Fractions with greater than 80% purity were pooled and lyophilized. A small sample of the peptide was sent for Amino Acid Analysis (AAA) at the Advanced Protein Technology Centre at Hospital for Sick Children, Toronto, ON to determine the concentration of the peptides.

Derivation of Polyclonal Antibody

Polyclonal antibodies were generated against the peptide NYVVTDHGSCVR in order to capture both the wild type and variant peptide isoforms (Figure 2.2). The antibodies (antisera) were generated by EZBiolab Inc. (Carmel, IN). Two rabbits were immunized in this process. The carrier used was KLH (keyhole limpet hemocyanin), which was

coupled using a cysteine residue added to the carboxy terminal of the peptide. The rabbits were boosted once prior to serum collection. A total of 1 mL of serum was collected and the proteins were precipitated with ammonium sulphate prior to Protein A chromatography and peptide affinity chromatography. The antibodies were subsequently tested for binding to peptides, and the titer was determined by ELISA.

Enzyme-linked Immunosorbent Assay

One Falcon 3915 Pro-bind 96-well assay plate (Becton-Dickinson) was coated with peptide that had been diluted in distilled water to give approximately 0.1 µg to 2.0 µg per well. The diluted peptides were applied in 100 µL volumes per well and uncoated wells were left as no-peptide controls (only 100 µL of water was added to these wells). Plates were dried at 37 °C overnight. A 200 µL-aliquot of 3% (w/v) powdered milk in 1X Phosphate-Buffered Saline (PBS, Sigma-Aldrich, St. Louis, MO) was added to all wells, and wells were covered with Parafilm in order to prevent evaporation. The plate was then incubated for 1 hour at 37 °C. The wells were then washed 3 times with 1XPBS-0.05% Tween-20 pH 7.4 solution. The anti-EGFR-peptide antibodies were diluted in 1XPBS and 1% (w/v) skim milk powder, and 100 µL were added to each well. The plate was incubated again at 37 °C for 1 hour. After washing the plate 3 times, the secondary antibody was added. The secondary antibody (Caltag goat-anti rabbit IgG/IgM Alk-Phos) was diluted 1:2000 in PBS-Tween 20/1% skim milk, and 100 µL were added to each well. The plates were incubated once more at 37 °C for 1 hour. A100 µL aliquot of substrate (1 pill dissolved in 5 ml diethanolamine buffer and warmed to room temperature) was added to each well. After incubation for 30 minutes at room

temperature in the dark, the absorbance was read at 405 nm on an ELISA plate reader. This procedure was performed at by Immunoprecise Antibodies Ltd., Victoria, B.C.

Sensitivity of Detection of Peptides

A serial dilution of the EGFRvIII peptide (GNYVVTDHGSCVR), the wild-type EGFR peptide (NYVVTDHGSCVR), and the EGFRvIII stable isotope-labelled standard (GNYVVTDHGSCVR*) was prepared to yield concentration of 500 femtomoles/µL, 250 femtomoles/µL, 100 femtomoles/µL, 50 femtomoles/µL, 10 femtomoles/µL, 1 femtomoles/µL, 500 attomoles/µL, 100 attomoles/µL, and 50 attomoles/µL. A 1 µL aliquot of each of the dilutions of all the peptides was spotted onto a stainless steel 386-well MALDI target plate (Applied Biosystems, Foster City, CA) and overlaid with 1µL of α-cyano-4-hydroxycinnamic acid matrix (CHCA) (3 mg/mL, 50% acetonitrile, 0.1% TFA, 1.8 mg/ml ammonium citrate, Sigma-Aldrich, St. Louis, MO) to test the limit of detection of peptide without enrichment. The spots were analyzed using the 4800 MALDI TOF/TOF mass spectrometer (Applied Biosystems, Foster City, CA). For this study, an S/N ratio of >10 was considered a significant signal; any peak with an S/N ratio below 10 was considered as noise.

iMALDI Assay for Testing Antibody Affinity

The iMALDI method was used to test binding of the antibody to the peptides. A 100 µL aliquot of 1XPBS and 5 µL of Protein G Dynabeads® (Invitrogen Inc, Carlsbad, CA) bead slurry were added to several sample tubes. Each sample tube also contained a different concentration of synthetic peptide NYVVTDHGSCVR, ranging from 10

attomoles/µL to 10 picomoles/µL. Each control tube lacked one of the components (no peptide, no antibody, or no beads). Other control tubes contained just beads and just antibody. After an overnight incubation at 4 °C on rotation, each sample was washed three times with 1XPBS and then three times with 25 mM ammonium bicarbonate (AmBic, Sigma-Aldrich, St. Louis, MO). The beads were resuspended in 5 µL of 25 mM AmBic. A 1 µL-aliquot of the beads was spotted onto a MALDI target plate and overlaid with 1 µL of CHCA matrix. The spots were analyzed using the 4800 MALDI TOF/TOF mass spectrometer. This set of experiments was repeated for the EGFRvIII (GNYVVTDHGSCVR) peptide.

Results

The wild type and vIII isoform peptides, as well as their heavy isotopically-labelled counterparts, were synthesized (Table 2.1). An ELISA assay to confirm antigen-antibody binding and the titers were determined (Figure 2.3).

Synthesized Peptide	Name of peptide	m/z value
GNYVVTDHGSCVR	EGFRvIII	1406.6
GNYVVTDHGSCVR*	EGFRvIII SIS	1416.6
NYVVTDHGSCVR	EGFR wild type	1349.6
NYVVTDHGSCVR*	EGFR wild type SIS	1359.6

Table 2.1 Synthesized Peptide Sequences and Masses. *The asterisk (*) denotes the heavy isotope used. For instance, GNYVVTDHGSCVR* indicates that a heavy R residue was used in the synthesis of the peptide. The heavy R residue adds approximately 10 Daltons to the mass of the original peptide.*

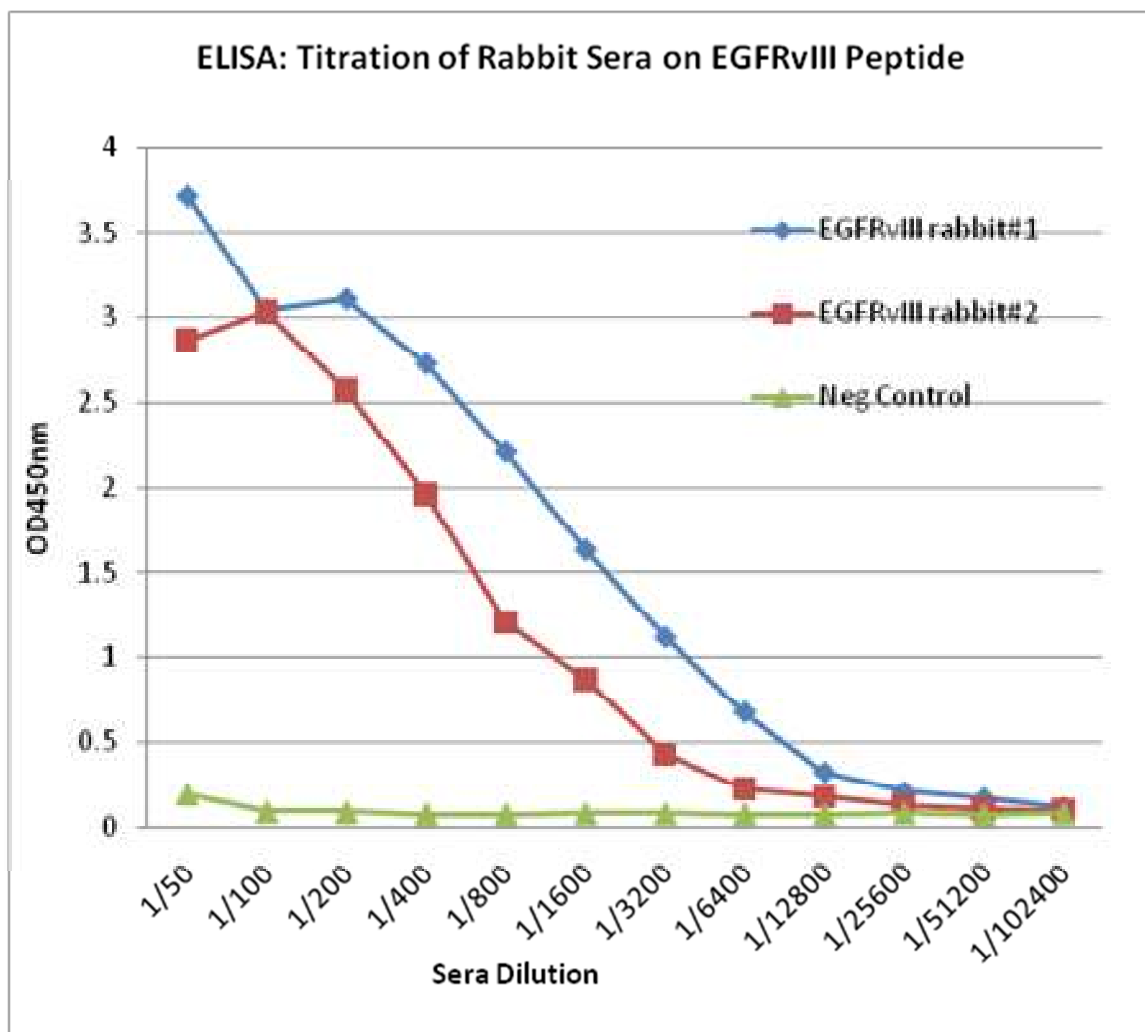

Figure 2.3 ELISA Titration of Rabbit Polyclonal Antibodies Raised Against EGFRvIII Peptide. *Two rabbit polyclonal antisera (EZBiolab Inc.) were titrated against the EGFRvIII peptide (GNYVVTDHGSCVR) (blue line and red line) with polyclonal antisera against a salmon protein used as a negative control (green line).*

The two rabbits showed a slight difference in titer against the EGFRvIII peptide, with the Rabbit #1 serum showing overall slightly higher titer than the Rabbit #2 serum. However, both rabbit sera exhibit a much higher signal than the negative control (a salmon protein), which stayed at near 0% absorbance at all dilutions (Figure 2.3).

The EGFR and EGFRvIII peptides were also spotted directly onto the plate without an enrichment step to determine the limitations of the instrumentation to detect the peptides (Table 2.2). Additionally, a preliminary iMALDI assay was performed to assess the sensitivity of detection with enrichment (Table 2.3 and Figure 2.4).

Concentration on target plate	**GNYVVTDHGSCVR**	**NYVVTDHGSCVR**
500 femtomoles/µL	✓	✓
200 femtomoles/µL	✓	✓
100 femtomoles/µL	✓	✓
50 femtomoles/µL	✓	✓
10 femtomoles/µL	✓	✓
1 femtomole/µL	Barely visible	Barely visible
500 attomoles/µL	Not detected	Not detected
50 attomoles/µL	Not detected	Not detected

Table 2.2 Limit of Detection of Peptides Directly on Target Plate Without Enrichment. *On-plate concentrations of 500 femtomoles/µL to 50 attomoles/µL were analyzed by MALDI TOF/TOF. The checkmark (✓) indicates a strong ion signal in the mass spectrum.*

Both the EGFRvIII and EGFR wild-type peptide were only detected well at concentrations above 10 femtomoles/µL where they gave a S/N ratio of greater than 10:1. Although both peptides were observed at 1 femtomole/µL, the S/N ratio was less than 10:1 (Table 2.2).

Presence of beads in solution (5 µL slurry)	**Presence of antibody beads in solution (1 µg)**	**Amount of EGFRvIII peptide in solution**	**Estimated concentration of EGFRvIII peptide on target plate**	**Ion Signal**
Yes	No	0	0	Not detected
Yes	Yes	0	0	Not detected
Yes	**No**	**5 picomoles**	**0**	**Barely visible**
Yes	Yes	100 attomoles	20 attomoles	Not detected
Yes	Yes	1 femtomole	200 attomoles	Not detected
Yes	Yes	5 femtomoles	1 femtomole	Not detected
Yes	Yes	10 femtomoles	2 femtomoles	Not detected
Yes	Yes	50 femtomoles	10 femtomoles	Not detected
Yes	Yes	100 femtomoles	20 femtomoles	Not detected
Yes	Yes	1 picomole	200 femtomoles	Not detected
Yes	**Yes**	**5 picomoles**	**1 picomole**	**Barely visible**
Yes	Yes	10 picomoles	2 picomoles	✓
Yes	Yes	50 picomoles	10 picomoles	✓
Yes	Yes	100 picomoles	20 picomoles	✓
Yes	Yes	1 nanomole	200 picomoles	✓
Yes	Yes	5 nanomoles	1 nanomole	✓

Table 2.3 Preliminary iMALDI data with Protein G magnetic beads containing Tween-20. *Different concentrations of EGFRvIII peptide between 100 attomoles and 5 nanomoles were incubated with beads and antibody to test the limit of detection after enrichment. Control samples either contained no antibody, no peptide, or just beads. Since one fifth of the beads were plated, theoretically one-fifth of the total peptide in solution was on the plate. Barely visible signal indicates a peptide signal below the 10:1 S/N ratio threshold. (* ✓*) indicates an ion signal above the 10:1 S/N ratio threshold. Detection limits with and without the antibody beads are shown in* ***bold.***

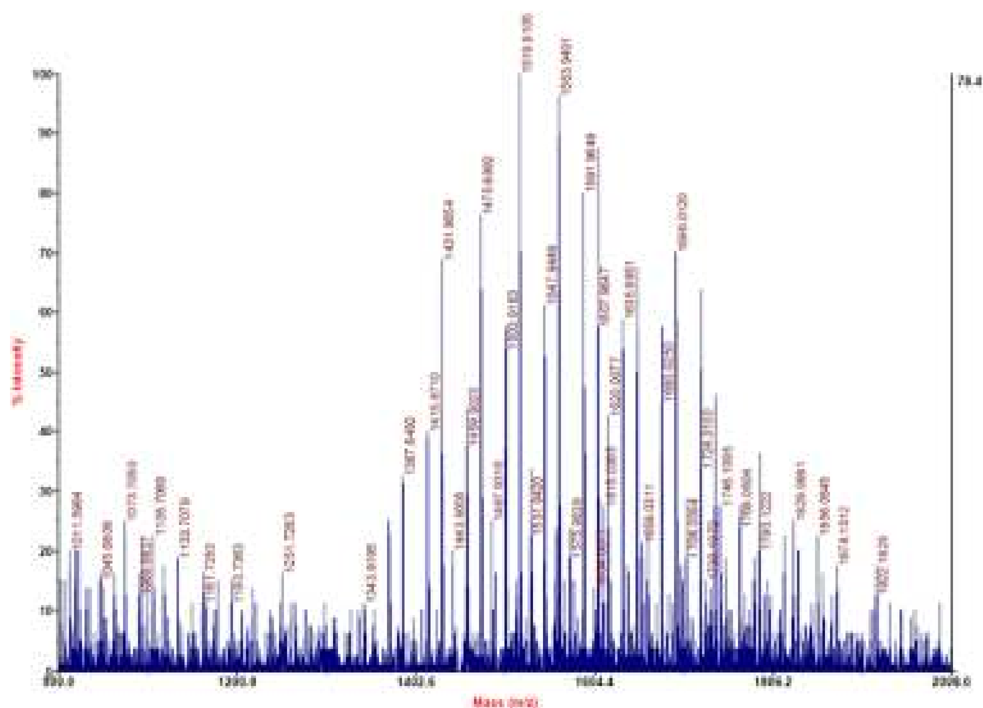

Figure 2.4 Mass Spectrum from Preliminary iMALDI Analysis. *The spectrum resulting from the incubation of Protein-G Dynabeads (Invitrogen, Inc.) with 1 µg of anti-EGFRvIII antibody and 50 femtomoles/µL of peptide is shown. The target peptide peak (m/z 1406.6) is not seen, but many repeating detergent peaks are visible.*

The preliminary iMALDI experiment revealed that the sensitivity of the assay is adversely affected by the presence of Tween-20 detergent in the sample. Because MALDI spectra are normalized to the base peak in the spectra, the target peptide was visible at high concentrations. However, at low concentrations of peptide, the signal was masked by the abundant signals of the detergent (Figure 2.4). Unfortunately, Tween-20 detergent is a component of the storage buffer for the magnetic beads.

Discussion

After performing the preliminary iMALDI assay. it was observed that the sensitivity of the preliminary iMALDI assay was not as high as expected. Not only was non-specific binding observed, but the same concentration of peptide in solution with and without antibody beads gave the same LOD (peptide peak at less than the 10:1 S/N ratio cut-off) (Table 2.3). The sensitivity of the assay was much lower than simply on-plate spotting of the peptides.

The non-specific binding observed was most likely a result of the target peptides non-specifically binding to the surface of the beads or sticking to the surface of the pipette tips, even after the wash steps. Although this is not desirable, it is not unexpected. The hydrophobic surface of the beads is an ideal binding sites for the peptides and it is also common for peptides to adhere to the surface of tubes or pipette tips (Bark et al., 2007; Kraut et al., 2009). When there are no other entities in solution, the effects of this phenomenon are more pronounced. To prevent this, the number of washes could have been increased or different eluting reagents could have been used. Studies also suggest that the use of polystyrene tubes may help to keep the peptides from sticking to the walls (Bark et al., 2007). By performing several longer washes, the signal from these non-specifically-bound peptides decreased to a S/N value of < 5:1. To completely eliminate the detection of this peptide, the number of washes could have been increased even further, or a stronger wash buffer could have been used. However, in this experiment, there was no primary antibody present and no competition for the peptide in the solution. In later experiments where the antibody will be used, it was expected that the peptide

would have higher affinity to the primary antibody than to the protein G or the surface of the magnetic bead, and that it would preferentially bind to the antibody. Moreover, performing more washes reduces the high-throughput capability of this assay and results in bead loss. Also, the use of harsher reagents to strip away peptides from the walls might denature the primary antibodies and might not be compatible with subsequent mass spectrometry. Although these alternative methods might have helped this particular experiment, these methods have little relevance to improving the signal when complex samples are analyzed, where the target peptide is more likely to bind to the antibody than to the walls of the pipette tip or the walls of the tube.

The non-specific binding was not the only problem with this assay. The sensitivity of the assay was severely diminished by the presence of Tween-20 detergent (in the storage buffer for the beads). This poses a serious problem for downstream studies. If the Tween-20 peaks are strong and abundant, they will compete with and suppress the signals from the target peptides, as well as masking these signals, especially since the target peptide is expected to be of low abundance in biological samples. Similar issues were addressed in a SISCAPA study in which the conclusion drawn was that Tween-20 should be replaced by a zwitterionic detergent such as CHAPS (Anderson et al., 2009; Whiteaker et al., 2010). In contrast to a polymer detergent like Tween-20, CHAPS is detected as a single m/z peak in a mass spectrum but still performs the necessary detergent tasks such as improving protein solubilities, bead handling, and reducing non-specific binding.

Conclusion

Since the non-specific binding of the target peptide to other entities in the solution instead of the antibody was not anticipated to be a major issue, this aspect of the procedure was not changed. From these experiments, however, it was determined that the Tween-20 containing buffer would have to be exchanged prior to any iMALDI type analysis. The detergent chosen to replace Tween-20 was CHAPS, based on the findings of other studies that encountered a similar problem.

Chapter 3
Optimization of Experimental Parameters of iMALDI Assay

Introduction

As purchased, the Protein G magnetic beads to be used in the iMALDI assay (Protein G Dynabeads, Invitrogen Inc.) were stored in a Tween 20 containing buffer. Tween 20 (Polysorbate 20) is a widely used detergent for immunoprecipitation and immunoaffinity capture studies due to its ability to reduce non-specific binding (Batteiger et al., 1982). This detergent also reduces the tendency for the magnetic beads to stick to each other or to the walls of the tube. Tween 20 has a mass of 1227 Da but shows several repeating peaks in the mass spectrum do to its polymeric nature (Figure 3.1). This poses a problem for the iMALDI assay because Tween 20 stays in solution and on the beads throughout the procedure, even after successive washings with PBS. Therefore, the repeating peaks on the mass spectrum hide the signal from the desired peptide.

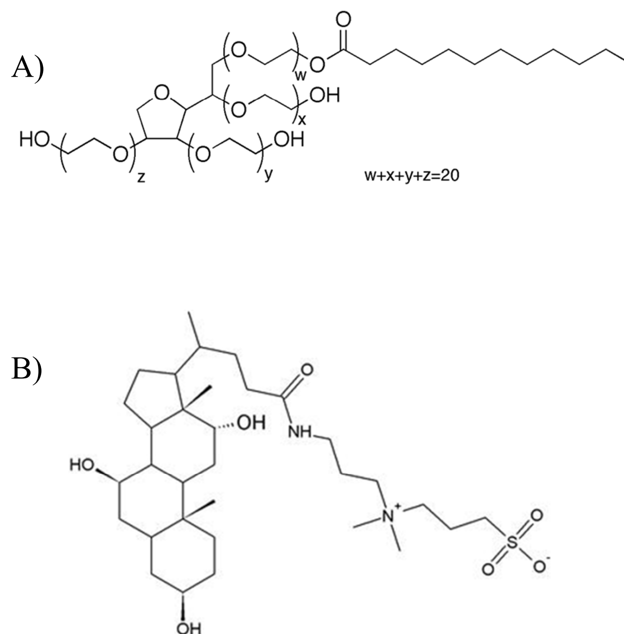

Figure 3.1 Structures of Detergents used in the iMALDI Assay. *A) Tween 20; B) CHAPS.*

As a result, a buffer exchange is necessary in order to proceed with iMALDI-based analysis of samples. The Tween-20 containing buffer is exchanged with a CHAPS containing buffer. CHAPS is a zwitterionic detergent that shows a single peak on the mass spectrum (at m/z 1229.8) but has similar properties to Tween 20 (Figure 3.1). It prevents the beads from sticking to the sides of the sample tube but does not interfere with the signal of the target peptide (Whiteaker et al., 2007). The bead washing procedure was optimized to remove as much Tween 20 from the sample as possible, and the preliminary iMALDI analysis from Section 2.2 was repeated in order to determine the limit of detection of the peptides in a CHAPS-based buffer.

In addition to the buffer optimization, there were two main interactions in the iMALDI workflow that also needed optimization in order to establish a procedure by which biological samples could be analysed. The first is the interaction between the Protein G beads and the primary antibody, and the second is the interaction between the primary antibody and the target peptide. For both these interactions, finding the optimal concentrations and incubation times is important for several reasons. First, it avoids the use of excess sample, antibody, or beads. It also facilitates high-throughput analysis by preventing unnecessarily long binding times. Specifically, the reactions that were optimized were: the concentration of antibody to obtain maximum binding of peptide, the length of time the primary antibody was incubated with the Protein G beads, the length of time the primary antibody and the peptide should be incubated. The high throughput capability of the assay also had to be determined.

The concentration of the antibody was studied to find the optimal concentration for maximum binding to the peptide. This was done by creating a serial dilution of antibody and comparing the amount of peptide bound for each concentration. The time-for-incubation studies were approached by first isolating the interaction being studied. For instance, in order to optimize the antibody and beads interaction, the incubation was done first without the presence of peptide. Also, in order to optimize the antibody and peptide interaction, this incubation was first done before adding the beads. Samples were incubated for several time points (one sample for each time point) and then the missing component (beads or peptides) was added. At this point, all sample tubes were incubated for the same length of time to control this stage of the process.

Another major objective that was addressed here was the high-throughput capability of the assay. The general method of enrichment of peptides using beads and antibodies is that first the beads are incubated with the antibody, a wash is performed to remove any unbound entities, and then the peptide is added to the antibody beads. However, instead of two separate incubations, a new method for simultaneous incubation of the beads, antibody, and peptide together was investigated. Similar one-step methods which eliminate the in-between wash steps have been successfully utilized in various ELISA assays (Houwers et al., 1987; Sorell et al., 2002). This type of one-step incubation method has the potential to reduce the total time of the experiment and reduce sample handling, but it may not be a usable option if it reduces the amount of captured peptide as compared to the traditional two-step process.

All of these experiments were done in a background of BSA (Bovine Serum Albumin) digest to add to the complexity of the reaction mixture and to mimic the conditions that would be encountered in real biological samples, as well as to determine the extent of non-specific binding. BSA digest is used often to simulate background noise or to act as a standard for proteomics studies, since the protein and its peptides are well characterized. As a result, a standard tryptic BSA digest is readily available through several scientific products companies, especially those targeting proteomics researchers. Although the protein digest is not nearly as complex as an authentic biological sample, it adds sufficient amounts of non-specific peptides in the solution to provide a more accurate measure of the analytical parameters of the iMALDI assay.

Additionally, all of the spectra acquired in these experiments were normalized. In MALDI-TOF mass spectrometry, the relative intensities of all detected signals are reported in the mass spectrum. From day-to-day, inter-spectrum variation due to slight differences in overall intensity and performance of the instrument can occur. For this reason, normalization of spectra is imperative for proper analysis and processing of the data (Borgaonkar et al., 2010). Several normalization procedures have been proposed for biomarker analysis using MALDI-TOF TOF data. The most relevant to this study are the use of internal standards (in this case, the stable isotope standards), or mean normalization (Norris et al., 2007). In the first method -- using internal standards -- if all peaks of interest are compared to the intensity of a known standard in a spectrum, then the variability between overall intensities of different spectra is reduced. In mean normalization, the mean signal from each spectrum is divided by the mean signal from all spectra to yield a 'normalization factor, NF'. The NF is then multiplied by the intensity of the peak of interest to yield a more uniform and realistic data set (Borgaonkar et al., 2010; Norris et al., 2007). For this portion of the study, the mean normalization method was selected. Furthermore, each set of experiments was done in a short window of time so as to minimize the inter-spectra variability due to instrument variability.

Materials and Methods

Washing of Protein G Beads

A 1-mL aliquot of Dynabeads ® Protein G magnetic beads (Invitrogen Inc., Carlsbad, CA) was put into a 15 mL conical tube. A 5 mL aliquot of CHAPS buffer (1X PBS pH 7.4, 0.3% CHAPS (Sigma-Aldrich, St. Louis, MO)) was added. After vortexing, the bead suspension was placed on a rotator for 15 minutes to wash at room temperature. A 1-µL aliquot of the beads was spotted onto a 386-well stainless steel MALDI target plate (Applied Biosystems, Foster City, CA) and overlaid with α-cyano-4-hydroxycinnamic acid matrix (CHCA) (3 mg/ml, in 50% acetonitrile, 0.1% TFA, 1.8 mg/mL ammonium citrate, Sigma-Aldrich, St. Louis, MO). The tube was placed near a magnet to collect the beads and hold them in place. The supernatant was subsequently drawn and discarded. Another 5 mL of CHAPS buffer were added and the washing and spotting steps were repeated 4 more times. The beads were resuspended in 1 mL of CHAPS buffer. Each spot was analyzed using the 4800 MALDI TOF/TOF (Applied Biosystems, Foster City, CA), operated in the positive ion reflectron mode.

iMALDI Assay for Testing Limit of Detection

The iMALDI method described in Chapter 2 was performed again, this time using washed beads in CHAPS-containing buffer. One hundred µL of 1XPBS and 5 µL of Protein G Dynabeads® (Invitrogen Inc, Carlsbad, CA) bead slurry were added to several sample tubes. Each sample tube also contained a different concentration of synthetic peptide NYVVTDHGSCVR, ranging in concentration from 10 attomoles/µL to 10 picomoles/µL. Each control tube lacked one of the components (*i.e.,* no peptide, no antibody, or no beads). Other control tubes contained just beads or just antibody. After an overnight incubation at 4 °C with rotation, each sample was washed three times with

1XPBS and then three times with 25 mM ammonium bicarbonate (AmBic, Sigma-Aldrich, St. Louis, MO). The beads were resuspended in 5 μL of 25 mM AmBic. A 1-μL aliquot of the beads was spotted onto a MALDI target plate and overlaid with 1 μL of CHCA matrix. The spots were analyzed using the 4800 MALDI TOF/TOF instrument. This set of experiments was repeated for the EGFRvIII (GNYVVTDHGSCVR) peptide.

Antibody Concentration Optimization

In 6 separate microcentrifuge tubes, 5 μL of washed bead slurry (from 3.1) were added to 100 μL of 100 femtomoles/μL Tryptic BSA digest (Michrom Bioresources, Auburn, CA) diluted in 1XPBS. Each tube contained either 0.2 μg, 0.5 μg, 1 μg, 2 μg, 5 μg, or 7 μg of the polyclonal anti-EGFRvIII antibody. The EGFR peptide (GNYVVTDHGSCVR) was added to the solution at a final concentration of 500 femtomoles/μL (50 picomoles total of peptide in solution). The sample was incubated for 2 hours at room temperature on a rotator. After incubation, the suspensions were washed three times with 100 μL of 25 mM ammonium bicarbonate (AmBic) (Sigma-Aldrich, St. Louis, MO) and resuspended in a final volume of 5 μL. A 1-μL aliquot of beads from each tube was placed directly onto the MALDI target plate. One μL of CHCA matrix was applied to each spot, and the sample was analyzed with the Applied Biosystems 4800 MALDI TOF/TOF. Three replicates of this experiment were performed.

Antibody Incubation Optimization

In 8 microcentrifuge tubes, 5 μL of washed bead slurry were added to 100 μL of 100 femtomoles/μL tryptic BSA digest dissolved in 1XPBS. One μg of antibody was added

to each tube and the tubes were incubated for 15 minutes, 30 minutes, 60 minutes, 120 minutes, 240 minutes, 300 minutes, 360 minutes, and 480 minutes. After these incubation periods, the EGFRvIII peptide (GNYVVTDHGSCVR) was added to produce a final concentration of 50 femtomoles/µL (5 picomoles total of peptide in solution) in each tube, and the tubes were incubated for 2 hours at room temperature. Each sample was washed three times with 25 mM AmBic and resuspended in a final volume of 5 µL. One µL of the beads from each tube was placed directly onto the MALDI target plate. One µL of CHCA matrix was applied to each spot, and the spots were analyzed with the Applied Biosystems 4800 MALDI TOF/TOF. Three replicates of this set of experiments were performed.

Peptide Incubation Time Optimization

In 8 microcentrifuge tubes, 1 µg of antibody was added to 100 µL of 100 femtomoles/µL Tryptic BSA digest diluted in 1XPBS. EGFRvIII peptide (GNYVVTDHGSCVR) was added to each tube to produce a final concentration of 50 femtomoles/µL (5 picomoles total of peptide in solution). The tubes were incubated for either 15 minutes, 30 minutes, 60 minutes, 120 minutes, 240 minutes, 300 minutes, 360 minutes, or 480 minutes. After their respective incubation times, 5 µL of washed bead slurry was added to each tube and incubated for an additional 2 hours at room temperature. Each sample was then washed three times with 25 mM AmBic and resuspended to a final volume of 5 µL. 1 µL of the beads from each tube was spotted directly onto the MALDI target plate. 1 µL of CHCA matrix was applied to each spot and analyzed with the Applied Biosystems 4800 MALDI TOF/TOF. Three replicates of this experiment were performed.

One-step vs. Two-step Method

In 5 microcentrifuge tubes, 5 μL of washed bead slurry was added to 100 μL of 100 femtomoles/μL tryptic BSA digest diluted in 1XPBS. One μg of antibody was added to each tube. The EGFRvIII peptide (GNYVVTDHGSCVR) was added to produce final concentrations of 10 femtomoles/μL (1 picomole total of peptide in solution), 25 femtomoles/μL (2.5 picomoles), 50 femtomoles/μL (5 picomoles), 100 femtomoles/ μL (10 picmoles), or 200 femtomoles/μL (20 picomoles). The samples were then incubated for 2 hours at room temperature. After incubation, the supernatant was removed and the beads were washed three times with 100 μL of 25 mM AmBic, and resuspended to a final volume of 5 μL. One μL of beads from each tube was placed directly onto the MALDI target plated and overlaid with 1 μL of CHCA matrix.

In 5 other microcentrifuge tubes, 5 μL of washed bead slurry were added to 100 μL of 100 femtomoles/μL tryptic BSA digest diluted in 1XPBS. One μg of antibody was added to each tube and incubated for 2 hours at room temperature. The supernatant was removed and the beads were washed 3 times with 100 μL of 1XPBS. Then, the peptides were added (at the same concentrations as above) and incubated for another 2 hours at room temperature. The samples were then washed three times with 100 μL of 25 mM AmBic and resuspended in 5 μL. One μL of the beads from each tube was placed directly onto the MALDI target plate and overlaid with 1 μL of CHCA matrix. The spots from both the one-step and two-step experiment were analyzed with the 4800 MALDI TOF/TOF. Three replicates of this experiment were performed.

Results

The effects of CHAPS substitution for Tween-20 were evaluated by performing several successive washes to dilute the residual concentration of Tween-20 on the beads. After these washes, the abundance of the Tween 20 polymer peaks began to decrease. After 5 washes, the CHAPS peak at m/z 1229.7 was the most prominent peak in the spectrum. However, as seen in the representative spectrum, some Tween 20 peaks did remain, but were greatly reduced in intensity (Figure 3.2).

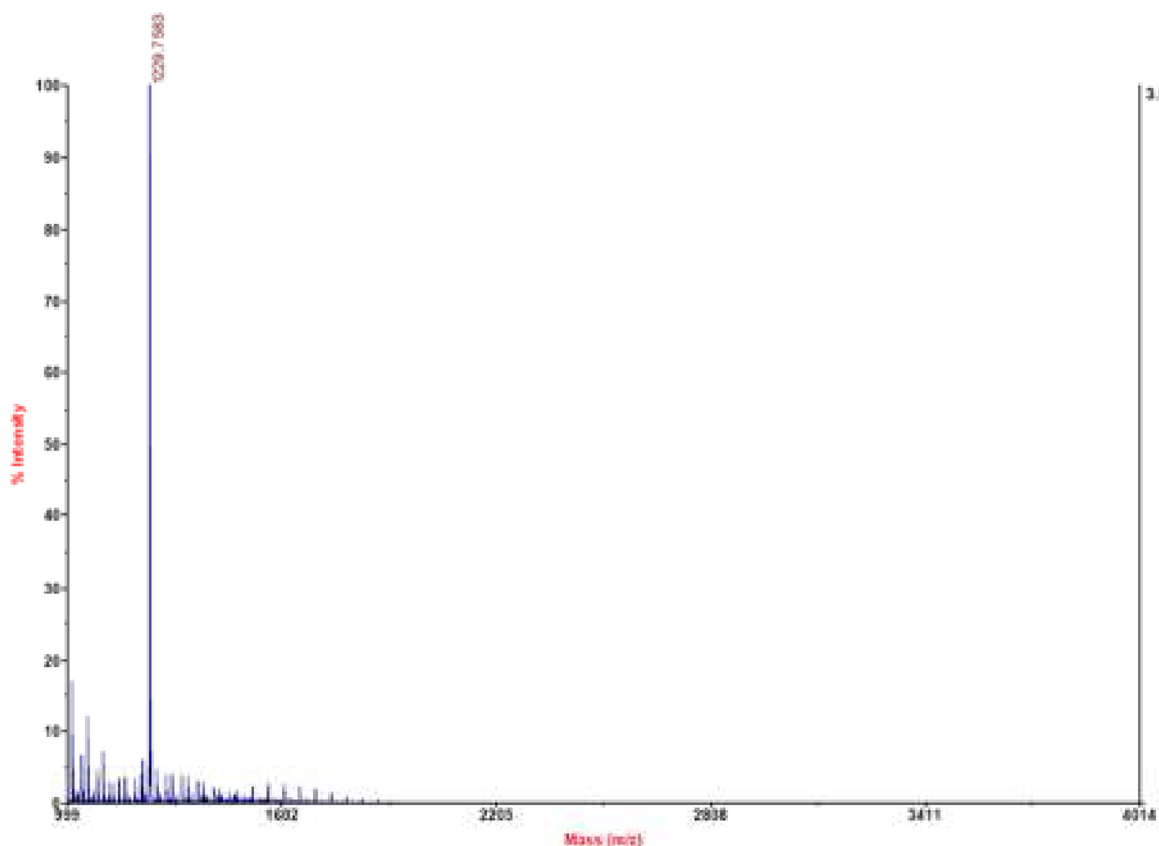

Figure 3.2 Resulting MALDI TOF/TOF Mass Spectrum after Buffer Exchange from Tween 20 to CHAPS-containing Solution. *The Tween 20 storage buffer for the beads was exchanged for CHAPS buffer (0.3% CHAPS, 1XPBS) several times to obtain this spectrum showing a major CHAPS peak at m/z 1229.7 and diminished Tween 20 polymer peaks.*

An iMALDI analysis in buffer was performed to test the limit of detection of the peptides after buffer exchange and enrichment (Table 3.1). A strong peptide signal was seen when at least 10 femtomoles/uL of peptide were present in the solution. EGFRvIII peptide was also detected in the control sample that contained only beads and peptide and no antibody.

Presence of beads in solution (5 µl slurry)	**Presence of antibody beads in solution (1 µg)**	**Amount of EGFRvIII peptide in solution**	**Estimated concentration of EGFRvIII peptide on target plate**	**Ion Signal**
Yes	No	0	0	Not detected
Yes	Yes	0	0	Not detected
Yes	**No**	**5 picomoles**	**0**	**Barely visible**
Yes	Yes	100 attomoles	20 attomoles	Not detected
Yes	Yes	1 femtomole	200 attomoles	Not detected
Yes	Yes	5 femtomoles	1 femtomole	Not detected
Yes	Yes	10 femtomoles	2 femtomoles	Not detected
Yes	Yes	50 femtomoles	10 femtomoles	Not detected
Yes	**Yes**	**100 femtomoles**	**20 femtomoles**	**Barely visible**
Yes	Yes	1 picomole	200 femtomoles	✓
Yes	Yes	5 picomoles	1 picomole	✓
Yes	Yes	10 picomoles	2 picomoles	✓
Yes	Yes	50 picomoles	10 picomoles	✓
Yes	Yes	100 picomoles	20 picomoles	✓
Yes	Yes	1 nanomole	200 picomoles	✓
Yes	Yes	5 nanomoles	1 nanomole	✓

Table 3.1 Preliminary iMALDI data with Protein G magnetic beads after detergent exchange with CHAPS. *Following the exchange of storage buffer in the bead suspension, different concentrations of EGFRvIII peptide between 100 attomoles and 5 nanomoles were incubated with beads and antibody to test the limit of detection after enrichment. Control samples either contained no antibody, no peptide, or just beads. Since one fifth of the beads was plated, theoretically one-fifth of the total peptide in solution was on the plate. Barely visible signal indicates a peptide signal below the 10:1 S/N ratio threshold. (* ✓*) indicates an ion signal above the 10:1 S/N ratio threshold. Detection limits with and without the antibody beads are shown in* ***bold.***

In the experiment to determine the amount of antibody to use in the incubation solution, an increase in the amount of antibody produced an increase in peptide capture and signal. However, the gain in peptide signal was highest after the addition of 1 µg of antibody.

The difference in the signal obtained after the addition of more than 1 μg of antibody was not as substantial as the difference between 0.5 μg and 1 μg (Figure 3.3).

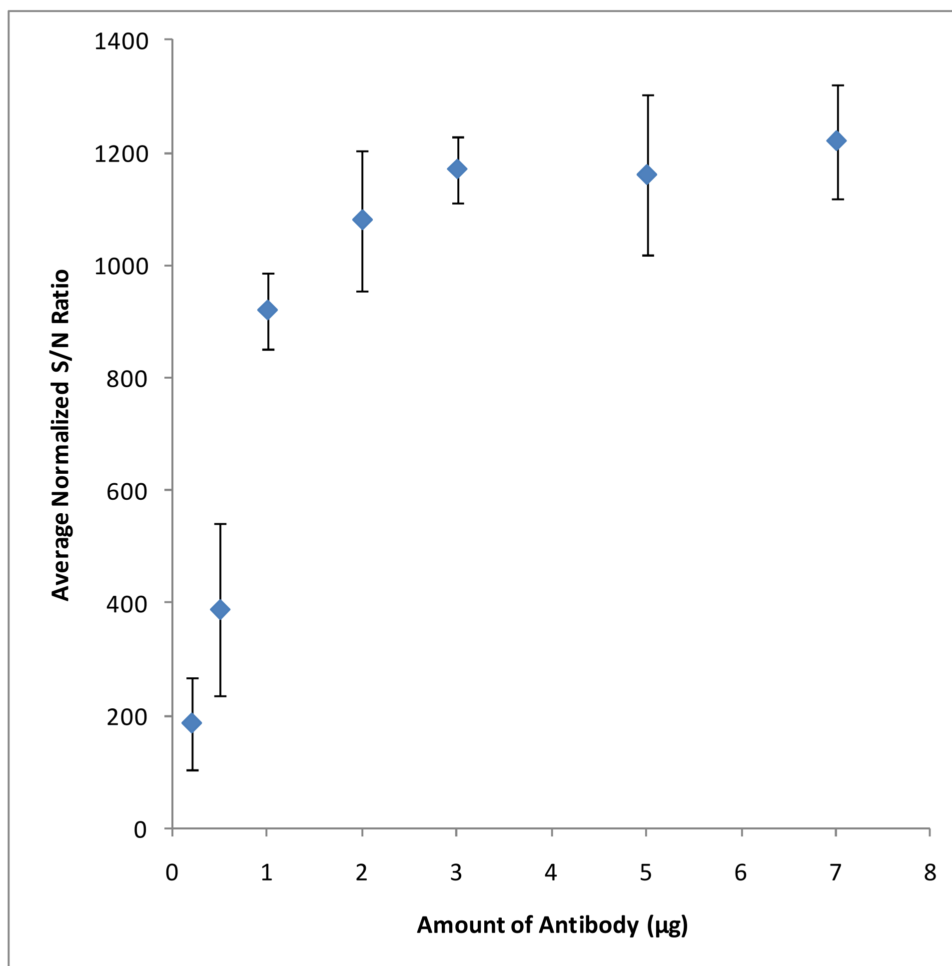

Figure 3.3 Optimization of Antibody Concentration in Experimental Samples. *500 femtomoles/µL (50 picomoles total) of EGFRvIII peptide with varying concentrations of antibody were incubated with BSA digest prior to iMALDI analysis. Mass spectra were acquired on the AB MALDI TOF/TOF (three replicates for each concentration of antibody) and normalized against the mean S/N ratio of all spectra in this series. (◆) represents the average normalized S/N ratio of the EGFRvIII peptide at each antibody concentration.*

When the beads were incubated with the antibody for varying lengths of time, the highest signal-to-noise ratio was obtained at 120 minutes, after which the signal-to-noise ratio started to decrease (Figure 3.4). Nonetheless, the signal at the longer incubation times (200 minutes and above) was still higher than for shorter incubation times (less than 120 minutes). A similar trend was seen when varying the length of incubation with the peptide. A four-hour peptide incubation time gave the highest signal-to-noise ratio, but the signal-to-noise decreased at incubations longer than four hours (Figure 3.5).

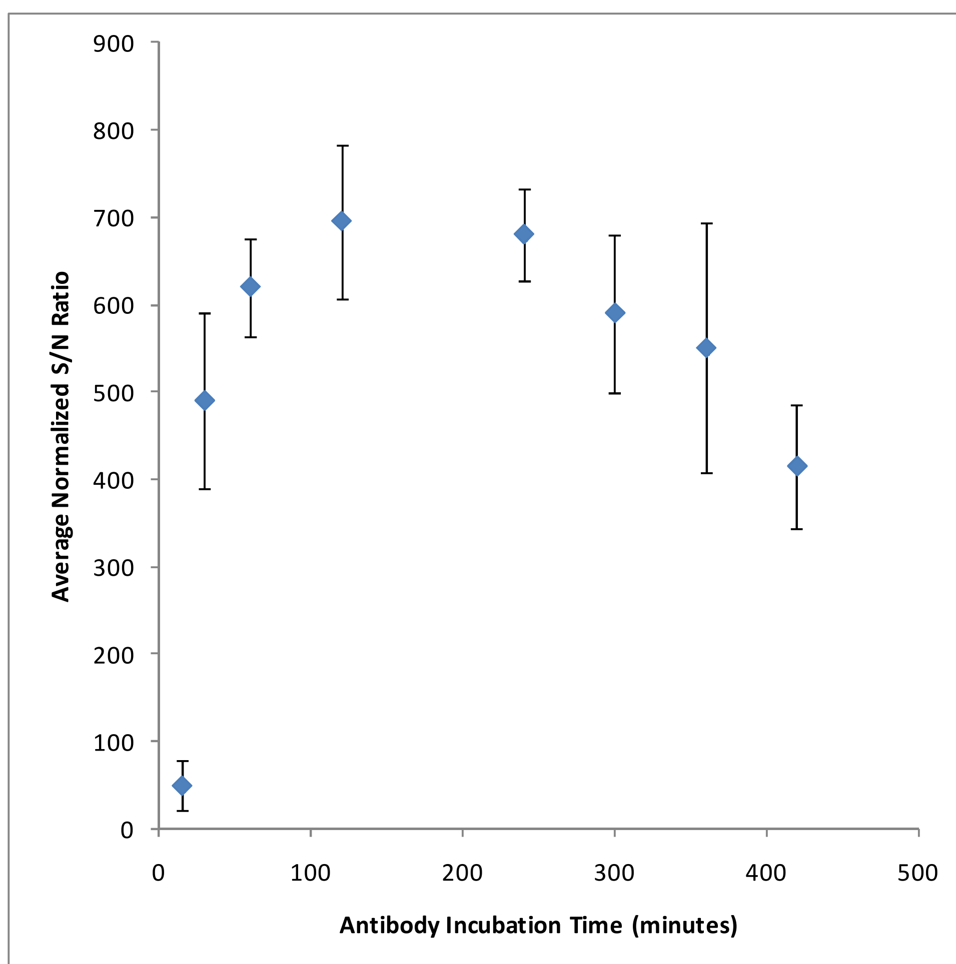

Figure 3.4 Optimization of Antibody Binding Time. *Fifty femtomoles/µL of EGFRvIII peptide were added to 1 µg of antibody and sample was incubated for varying lengths of time prior to iMALDI analysis. Mass spectra were acquired on the AB MALDI TOF/TOF (three replicates for each time point) and normalized against the mean S/N ratio of all spectra in this series. (◆) represents the average normalized S/N ratio of the EGFRvIII peptide at that antibody incubation time.*

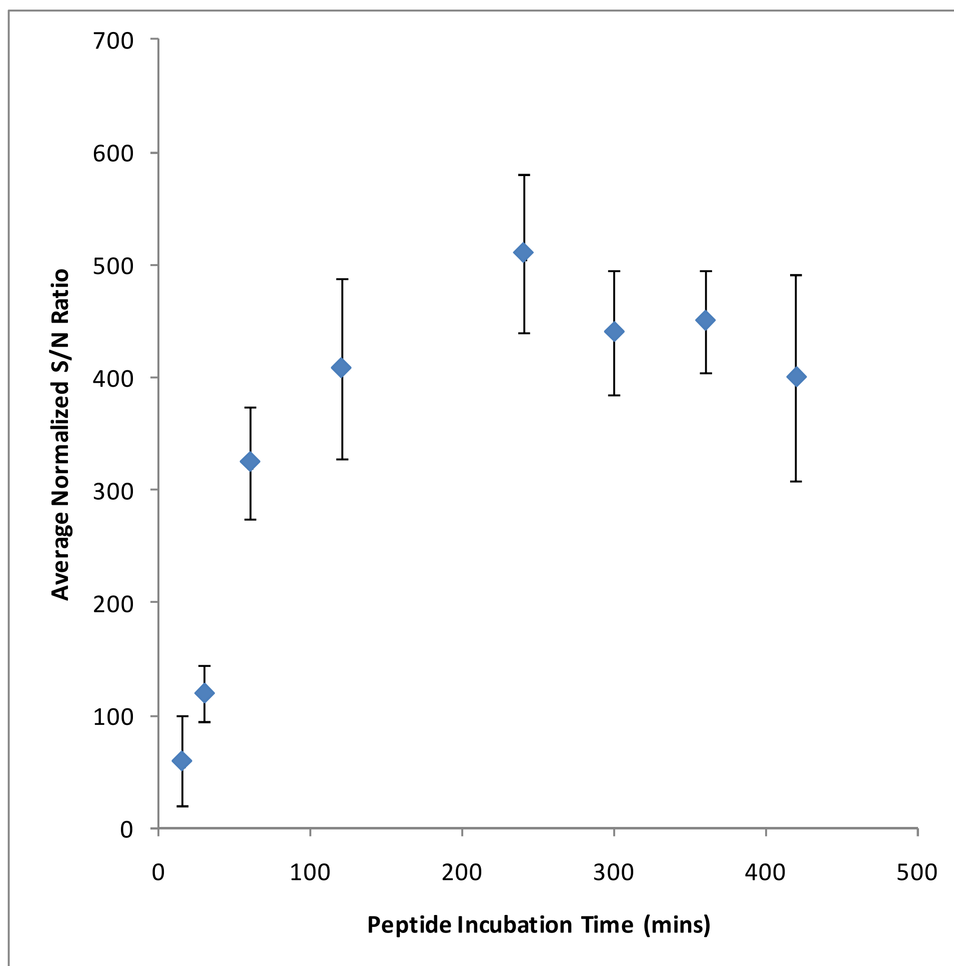

Figure 3.5 Optimization of Peptide Binding Time. *50 femtomoles/μL of EGFRvIII peptide was incubated at varying lengths of time with 1 μg of antibody prior to iMALDI analysis. Mass spectra were acquired on the ABMALDI TOF/TOF (three replicates for each time point) and normalized against the mean S/N ratio of all spectra in this series. (◆) represents the average normalized S/N ratio of the EGFRvIII peptide at that peptide incubation time.*

Finally, the one-step and two-step incubation methods were evaluated and compared. Both types of incubation yielded a similar trend in signal obtained (Figure 3.7). For both experiments, the signal-to-noise ratio increased steadily as the concentration of peptide

was increased. Neither method significantly increased nor improved signal-to-noise ratio compared to the other. Thus, the sensitivity of the new, shorter, one-step protocol was as good as that from the original, longer, two-step protocol.

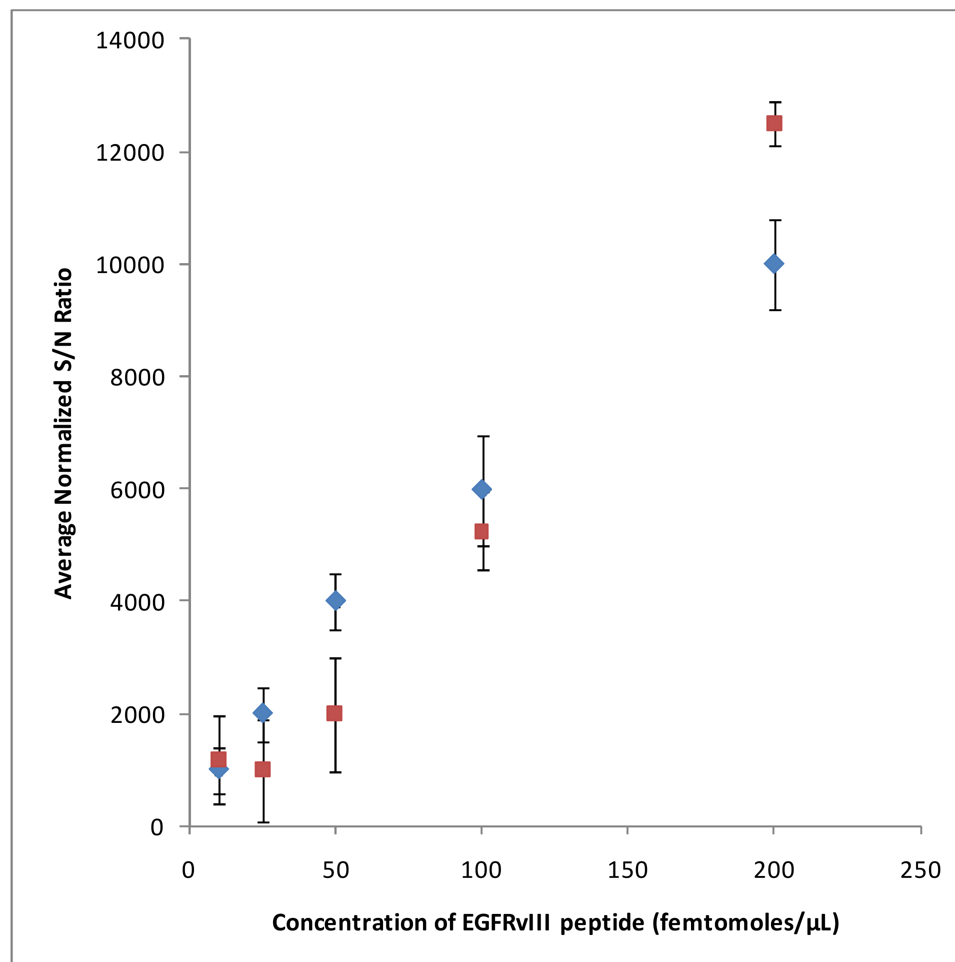

Figure 3.6 Comparison of One-step and Two-step iMALDI Method. *Varying concentrations of EGFRvIII peptide were incubated with antibody and beads together in the one-step method, and 1 µg of antibody was incubated with beads and washed prior to incubation with varying concentrations of EGFRvIII peptide in the two-step method. Mass spectra were acquired on the AB MALDI TOF/TOF (three replicates for each concentration of peptide) and normalized against the mean S/N ratio of all spectra in this series. (■) represents the average normalized S/N ratio of the EGFRvIII peptide at that given concentration of peptide in the one step method. (◆) represents the average normalized S/N ratio of the EGFRvIII peptide at that concentration of peptide in the two step method.*

Discussion

The first stage of the development of this method was the removal of Tween 20 buffer in exchange for CHAPS buffer. After successive washes, it was determined that washing the beads more than 5 times with CHAPS buffer reduces the amount of Tween 20 detergent left in the sample, thereby decreasing noise in the mass spectrum. Prior to capture of the antibody in solution, the beads were thoroughly washed to remove as much Tween 20 detergent as possible. This wash procedure includes more extensive washing with CHAPS than was recommended in the SISCAPA protocol (Whiteaker et al., 2007). Even after the CHAPS wash, the sensitivity was still limited by the residual detergent, since the instrument was capable of detecting even lower amounts of peptide from solution (1 femtomole on target) (Table 2.2 and Table 3.1). However, compared with the preliminary iMALDI assay with the Tween-20 containing beads, the CHAPS-containing beads showed improved sensitivity, with a detection limit of 20 femtomoles with CHAPS compared to 1 picomole with Tween-20. (Table 2.2, Table 2.3, and Table 3.1). Therefore, until beads can be obtained without any detergent, all experiments for this project will have to contain more than 20 femtomoles of peptide as the final concentration on the MALDI target in order to be above the detection limit.

In order to determine the amount of antibody needed to provide sufficient binding sites for the peptides in the sample, several concentrations of antibody were tested against a fixed concentration of peptide. The concentration of peptide used was higher than the expected concentration of the target peptide in a biological sample. Although the highest signal of peptide was obtained with more than 2 µg of antibody, the gain in signal

compared to that of 1 μg of antibody was not large enough to justify the cost of using several micrograms of antibody per sample. Similar SISCAPA studies use 1 μg of antibody for most experiments because the binding capacity of that amount of antibody still exceeds the expected amount of the target biomarker.

In general, biomarkers for disease tend to be present in low abundance in biological samples (Anderson, L 2005). Although EGFR is a common receptor, its abundance in the cell is still low in comparison to known high-abundance proteins and even highly-concentrated cell or tissue lysates would not contain large amounts of EGFR simply due to their abundance in the cell in relation to other proteins (Zhang et al., 2006; Wang et al., 2003). The abundance of EGFR in normal non-cancerous cells is reported to be approximately 100 attomoles/μg of protein (Santini et al., 2006). Although this threshold is used as a cut-off between normal and cancerous tissue, many cancerous tissues express EGFR at more than ten times that amount (Santini et al., 2006). In this case, if 1 μg of antibody (6.6 pmoles) can capture up to approximately 13 picomoles of peptide and if my limit of detection while using the CHAPS-containing beads is 20 femtomoles on plate, I should be able to detect the EGFR and EGFRvIII protein as long as I use sufficient amounts of digested tissue lysate per analysis. For instance, 1 mg of digested tissue lysate protein should contain 100 femtomoles of EGFR peptide, which is more than sufficient to detect based on my limit of detection (20 femtomoles) and the 13-picomole binding capacity of 1 μg of the antibody.

Following those experiments, the optimal time of incubation of the antibody and of the peptide was also determined. In the case of the antibody incubation with the beads, the general trend was that better signal was achieved from a longer incubation period. However, it was observed that very long incubation times resulted in a lower peptide signal (Figure 3.4), indicating that either the antibody-peptide or the antibody-protein G interaction was becoming weaker. Although antibody-antigen interactions are routinely allowed to incubate overnight, I found no evidence to suggest that a longer protein G-IgG incubation time would result in stronger binding. In fact, many protocols suggest an incubation time of 2 hours at room temperature as sufficient for immunoprecipitation reactions (Brocklehurt et al., 2006, Harlow et al., 2006; Ong, S, 2010). A similar trend was seen with incubation times for peptide binding (Figure 3.5). However, for the antibody-peptide interaction, a decrease in signal with increasing time of incubation seemed to result from increasing noise. As the resulting peptide peak was measured as a signal to noise ratio, as the background noise increased, the overall S/N ratio would decrease.

Furthermore, as was demonstrated with other types of studies, both incubations could be carried out at the same time (Houwers et al., 1987; Sorell et al., 2002). That is, the antibody, beads, and peptide sample could be added at the same time for affinity capture. No significant difference was found between the signals obtained by the one-step versus the two-step method. Although the theoretical risk with the one-step method is that not enough time would be allotted for binding all of the target peptides in the sample, the one-step method results in a simpler protocol, a shorter analysis time, and a reduced

chance of bead loss and human error.

Conclusion

Since MALDI mass spectrometry can be very sensitive to background noise from detergents and the fact that MALDI TOF MS spectra display relative intensity, several wash steps were added in order to remove as much Tween-20 detergent as possible. Although this may result in some initial bead loss, the beads that will remain are nearly detergent free.

In determining the amount of antibody to use in each sample, it was shown that the experimental capacity of 1 μg of the antibody was most likely much greater than the concentration of EGFR or EGFRvIII in any biological sample. In other words, although more than 3 μg of antibody would have been ideal for capturing 500 femtomoles/μL of peptide, EGFR or EGFRvIII are not likely to be present in high abundance in a biological sample. It was therefore concluded that, at this stage, 1 μg of antibody provided more than enough binding sites to theoretically capture all of the target peptides in the sample.

Finally, in determining whether to perform iMALDI analyses *via* the one-step or two-step method, it was evident that there was no clear difference in peptide signal between these two methods for this particular application. Since the ideal protein G-antibody incubation time and the ideal antibody-peptide incubation time could both be done in 120 minutes and, both incubations could be carried out at the same time as has been reported

in other types of studies. After considering the advantages and risks of both methods, the one-step method was selected because of its higher-throughput potential, and all subsequent experiments were carried out using the new one-step method. In conclusion, a procedure for washing the Protein G Dynabeads, immunoprecipitating the antibody and the antigens, and releasing antigens for MALDI TOF/TOF analysis was developed (Figure 3.8).

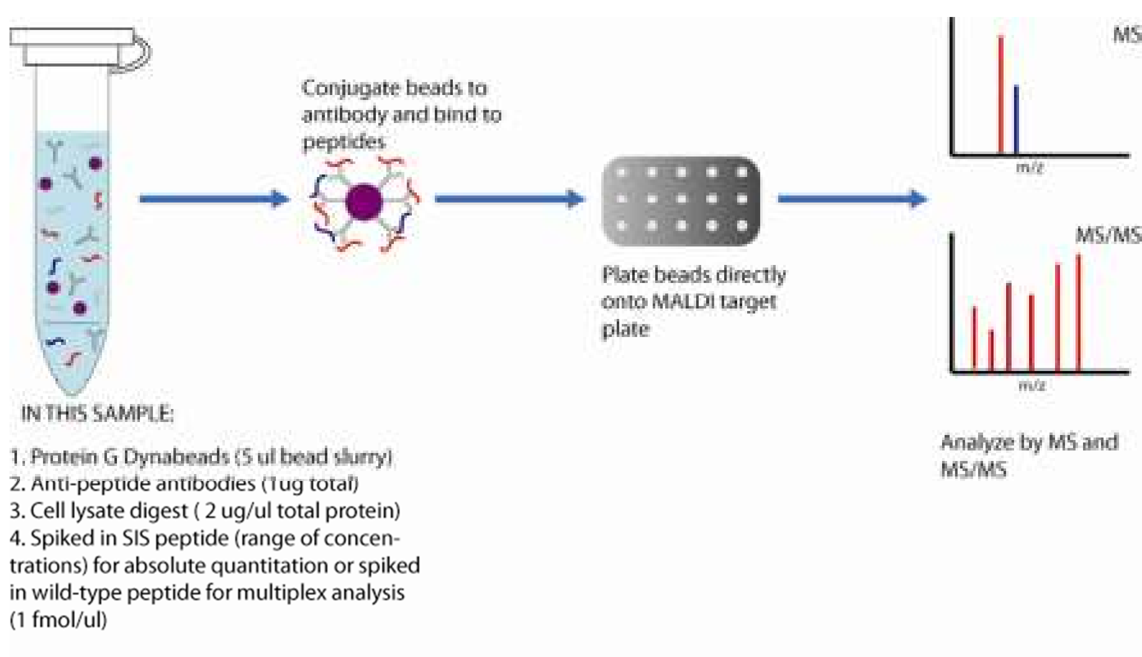

Figure 3.7 An Updated and Optimized Analytical Scheme for iMALDI Analysis of EGFR and EGFRvIII. *In a one-step method, Protein-G Dynabeads, anti-peptide antibodies, biological sample, and heavy peptides are incubated at once to allow for binding of antibodies and antigen, the beads are directly placed on the MALDI target plate, overlaid with CHCA matrix, and analyzed by MALDI TOF/TOF instrumentation.*

Chapter 4
Analysis of Biological Sample: U87MGΔEGFR Cell Line

Introduction

In order to test the assay on the ultimate target sample, the tumour tissue, the assay must first be further developed for cell lines that mimic the complexity and biological makeup of tumour tissue. There are very few cell lines that have been engineered to express the EGFRvIII protein isoform (Zhang et al., 2009). The U87MGΔEGFR cell lines obtained from the Webster Cavenee lab at the Ludwig Institute for Cancer Research, La Jolla, CA was used for this study. The parent cell line, U87MG, was derived from the malignant glioma of a 44 year-old Caucasian female who exhibited stage III (terminal) cancer. It is now one of the most widely-used in-vitro models of human glioblastoma cells for investigating the effect of drugs, therapies, and diagnostic technologies for the detection, treatment, and prevention of brain cancers (Clark et al., 2010).

To understand the activity of EGFRvIII in vivo, the Cavenee group was the first to use a retroviral transfer method to transfer the mutant EGFR into the U87MG gliobastoma cell lines. Their initial analysis showed that the expression of EGFRvIII in these cell lines led to an increase in tumorigenicity and malignancy. The parent cell line, U87MG, expresses neither EGFR nor EGFRvIII (Prigent et al., 1996), and these new EGFRvIII-expressing cells (termed U87MGΔEGFR) also show a significant reduction or loss of expression of wild-type EGFR. Following this study, the cell line was cultivated for research and collaborative use. They have made this cell line available to the scientific community to

support research that will help to better understand the EGFRvIII signalling pathways that lead to tumour development.

Upon receiving the cell line, the cells were grown and cultured at the tissue culture facility in the Watson laboratory at the Deeley Research Centre in Victoria, B.C. The cell lysate was extracted using sonication. Sonication was done very quickly, on ice, and in small batches so the sample would not degrade. Sonication is a way of disrupting the cell membrane and releasing lysate through the application of sound energy. For this project, sonication was preferred to other chemical-based methods of cell lysis because the issue of buffer incompatibility was avoided. This is important because the lysate goes through an immunoprecipitation after the sonication step, where buffer type and buffer pH will be important for preserving antibody stability and activity. Sonication was an attractive method for this assay development project because only simple buffers were required.

After extraction of the cell lysate, tryptic digestion of the sample was performed prior to the iMALDI analysis. Trypsin is a protease that cleaves other proteins by cutting at the carboxyl side of all lysine and arginine amino acid residues. The specificity of the enzyme is due in part to an aspartate residue in the catalytic pocket that attracts and stabilizes the positive charges of lysine or arginine. Normally, an impurity present in trypsin is the enzyme chymotrypsin. For mass spectrometry studies, where enzyme specificity is important, the trypsin used contains an inhibitor -- tosyl phenylalanyl chloromethyl ketone (TPCK) -- that blocks the activity of chymotrypsin but not trypsin.

To prepare a sample for tryptic digestion -- especially if the target peptides contain cysteines -- the proteins within the sample should be reduced first to cleave the disulfide bonds present in the protein. This is normally done by using a strong reducing agent such as dithiothreitol (DTT). After the reduction of the cysteine residues, the re-formation of disulfide bridges need to be prevented. This can be done by alkylating the sulfhydryl groups with a chemical such as iodoacetamide. Following the alkylation step, reducing agents such as DTT are added again to react with any excess alkylating agent in order to quench the alkylation reaction (Herbert et al., 2001). These steps are important for digestion of proteins that contain many disulfide bonds because the complete digestion of a folded protein is difficult if lysine or arginine residues are buried within the folded region (Herbert et al., 2001; Hale et al, 2004). In fact, the target EGFR peptide is known to be disulfide-bridged in the native protein (Hoskins et al., 2009; Dawson et al., 2005).

The presence of low levels of DTT has little effect on the activity of trypsin, but my downstream application of this sample involves the use of antibodies. The construction and activity of antibodies is heavily dependent on disulfide bridges, and the presence of DTT in a sample may be detrimental to the proper functioning of these antibodies, because DTT separates the light and heavy chains of the antibody and denatures the antigen-binding site. As a result, all other extraneous agents including the trypsin need to be removed prior to analysing the mixture of peptides. In these experiments, the cleanup was done by using specialized cartridges with hydrophobic columns that specifically bind to peptides. The peptides were extracted by acetonitrile (ACN), an organic solvent, to obtain a pure solution of peptide. The ACN solvent, whoever, was itself too harsh for the

antibodies which require delicate saline conditions, but it was necessary for the sample preparation portion of the protocol. Therefore, to remove the ACN, samples were lyophilized overnight and resuspended in an appropriate buffer prior to iMALDI analysis.

The assay was then performed to quantitate the absolute amount of free unmodified EGFRvIII in the cell lysate and the copy number was determined. By comparing a sufficient number of samples, the density (i.e., the copy number) of the EGFRvIII protein can help to predict different characteristics of glioblastoma tumours. In the literature, the density of EGFRvIII in U87MGΔEGFR cells is quoted as 2.7-6.8 x 105 copies per cell (Wickstrand et al., 1997). These numbers were generated using an immunoradioassay which measures the number of receptors with radioactive antibodies. The absolute number of receptors per cell measured by iMALDI was compared to the information available in the literature to assess the accuracy of the assay for the detection of endogenous EGFRvIII.

Materials and Methods

Cell Culture of U87MG and U87MGΔEGFR Cell Lines

The cell culture was performed by the Deeley Cancer Research Centre, Victoria BC. A 1 mL aliquot of the cells was thawed quickly in a water bath at 37 °C. Cells were transferred to 9 mL of warm medium (containing MEM (Invitrogen Inc., Carlsbad, CA), 10 % fetal bovine serum (Thermo Scientific, Waltham, MA), 100 units/mL penicillin + 100 µg/mL streptomycin + 5% CO_2) in a 15 mL conical tube. After centrifugation for 5

minutes at 1200 rpm, the supernatant was removed and the pellet was resuspended in 10 mL of warm medium. The cells were divided between two 150-mm plates containing 5 mL of warm medium and placed in an incubator. After two days, the medium was removed and fresh media was added. When cells were 70-90% confluent, they were split in 1:4. The cell layer was briefly rinsed with equal volumes of PBS pH 7.4 (Invitrogen Inc., Carlsbad, CA) and discarded. 3 mL of 0.25% (w/v) trypsin + 0.53 mM EDTA solution (Invitrogen Inc., Carlsbad, CA) was added to the flask and cells were observed under an inverted microscope until the cell layer was dispersed. Ten mL of complete growth medium were added and cells were collected by pipetting, and aliquots of cell suspensions were added to new vessels. The cells were collected by centrifugation and washed with ice cold 1XPBS and the supernatant was aspirated to prepare for cell lysis.

Cell Lysis

The probe of the sonicator was cleaned with successive washes of deionized water, 50% methanol, and 2mM sodium dodecyl sulphate (SDS) (Sigma-Aldrich, St. Louis, MO). Cells were resuspended in 500 µL of 1XPBS containing 1% sodium deoxycholate (w/v) (Sigma-Aldrich, St. Louis, MO). After sonication, the proteins were precipitated with 5 mL of ice-cold acetone (Sigma-Aldrich, St. Louis, MO) overnight at -20 °C. The supernatant was then removed and the proteins were resuspended in 200 uL of 25 mM AmBic.

BCA Assay to Measure Total Protein Concentration of Cell Lysate

The Bicinchoninic Assay Kit (Sigma-Aldrich) was used according to the manufacturer's directions. A dilution series of a 1 mg/mL BSA standard was prepared and dispensed into the first row of a 96-well flat bottomed plate. The two samples were diluted in the same manner and each of the series was dispensed into the second and third row of the plate. Two hundred µL of bicinchoninic acid reagent was dispensed into each well. The plate was briefly vortexed, covered with clear film to prevent evaporation, and incubated for 15 minutes at 60 °C to allow the acid to chelate ions in the solution and turn from a green solution to a purple solution. The optical density was measured with the use of a Multiskan Ex Plate Reader (Thermo Scientific). Based on the absorption spectra of theknown BSA concentrations, the amount of protein present was quantified.

Tryptic Digestion of Cell Lysate

One mg of cell lysate protein was suspended in 100 µL of 25 mM AmBic. Five µL of 200 mM Dithiothreitol (DTT) (Sigma-Aldrich, St. Louis, MO) were added to the sample and incubated for 1 hour at 37 °C. Twenty µL of 200 mM Iodoacetamide were added to the sample and incubated for another hour at 37 °C. To quench, 25 µL of 200 mM DTT were added to the sample and incubated again for 1 hour at 37 °C. Twenty µg of trypsin-TPCK were added and incubated overnight at 37 °C. The sample was then boiled for 30 minutes to deactivate the trypsin.

Solid phase extraction cleanup was performed using Waters® Corp. Oasis HLB Cartridges. The cartridges were conditioned with 1 mL of methanol (VWR, West Chester, PA). The columns were then equilibrated with 1 mL of HPLC-Grade water

(VWR, West Chester, PA), loaded with sample, and eluted with 70% HPLC-Grade Acetonitrile (VWR, West Chester, PA) and 0.1% trifluoroacetic acid (Sigma-Aldrich, St. Louis, MO). The eluted samples were then lyophilized overnight and resuspended in 100 μL of 1XPBS.

iMALDI Analysis

Five μg of protein (approximately 20,000 cells) were used for each analysis. The samples were transferred to 6 microcentrifuge tubes containing 100 μL of 1XPBS and 5 μL of washed bead slurry. In one sample tube, the unlabelled synthetic version of the EGFR peptide (NYVVTDHGSCVR) was spiked in because the cell line should not contain any wild type EGFR. For quantitation, either 5 femtomoles/μL, 10 femtomoles/μL, 15 femtomoles/μL, 20 femtomoles/μL, 25 femtomoles/ μL or 30 femtomoles/μL of the stable isotope standard EGFRvIII peptide (GNYVVTDHGSCVR*) were added to the remaining 5 sample tubes. After incubation for 2 hours at room temperature on a rotator, the samples were washed 3 times with 25 mM AmBic and resuspended in 5 μL of 25 mM AmBic. A 1 μL aliquot of the bead slurry was spotted onto a MALDI target plate and analyzed with the 4800 MALDI TOF/TOF. This set of experiments was performed on both U87MG and U87MGΔEGFR cell lines, and was repeated three times.

Results

The endogenous non-alkylated EGFRvIII peptide (m/z 1406.67) was successfully captured from the digested U87MGdeltaEGFR cell lysate, along with the spiked-in non-alkylated EGFRvIII SIS peptide (m/z 1416.68) (Figure 4.1). A serial dilution of the cell lysate was performed and spiked with a fixed concentration of the EGFR SIS peptide in order to quantitate the amount of endogenous EGFRvIII in the sample. The response was linear over the range of concentrations of the SIS peptide used. The errors were largest for the lowest concentration of SIS peptide (5 femtomoles/μL) and highest concentration of SIS peptide (30 femtomoles/μL) (Figure 4.2).

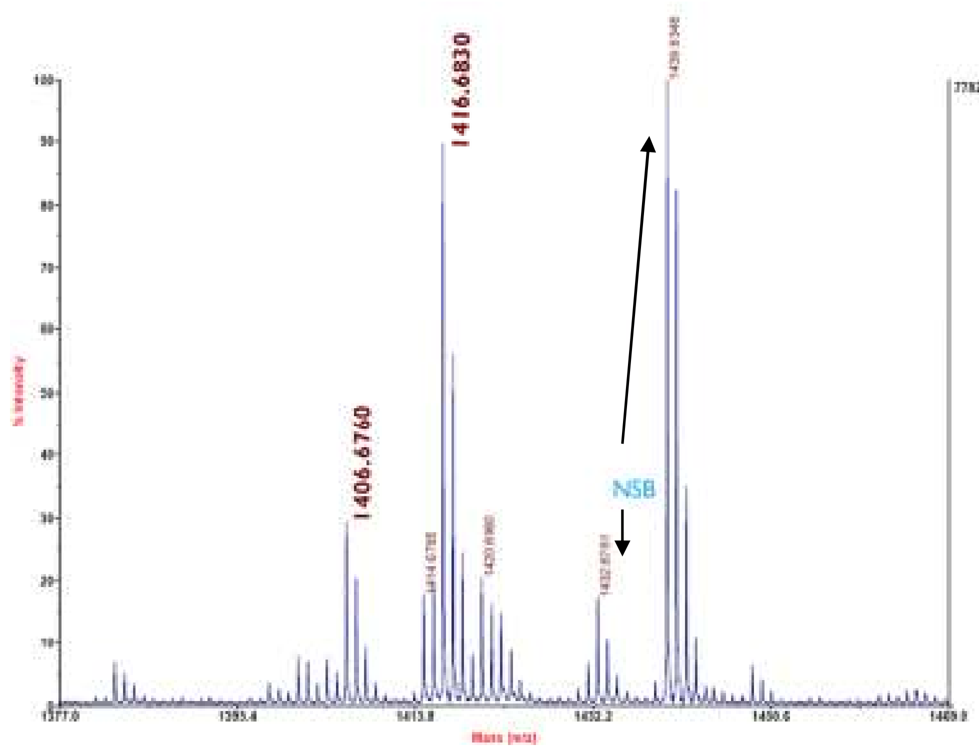

Figure 4.1 iMALDI Analysis of U87MGΔEGFR Cell Line. ***The MALDI*-MS** *spectrum shows the capture and detection of the non-alkylated EGFRvIII peptide at m/z 1406.67 (GNYVVTDHGSCVR) from the U87MGΔEGFR cell line, as well as the spiked-in EGFRvIII stable isotope standard peptide at m/z 1416.68. Background peaks result from non-specific binding (NSB) of other compounds in the cell lysate.*

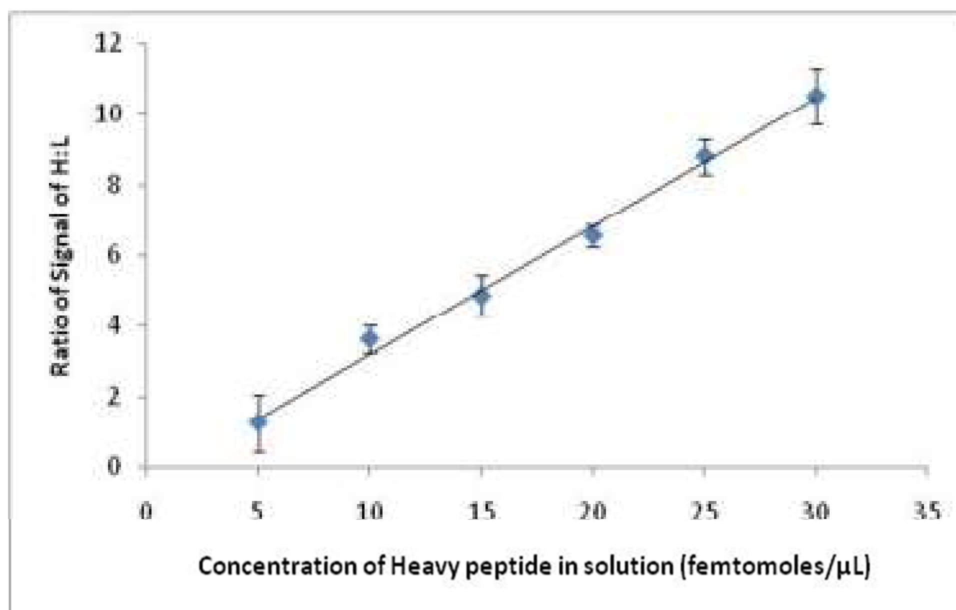

Figure 4.2 Quantitation Curve for Endogenous EGFRvIII in the U87MGΔEGFR Cell Line. *The standard curve was generated using the SIS peptide (heavy, H) spiked in increasing concentrations into trypsin digested cell lysate to measure the unknown amount of endogenous EGFRvIII peptide. The ratio of MS signals of 'Heavy' and 'Light' peptide was plotted against the known concentration of Heavy peptide added to solution. The concentration of total protein in digested cell lysate was approximately 1 mg/mL (n=3).*

The U87MG cell line which does not contain EGFRvIII or wild-type EGFR was used as a control. Endogenous EGFRvIII was not detected, but the spiked-in EGFR wild type peptide was captured and detected (Figure 4.3). In all of the cell lysates, the non-alkylated versions of the peptides were found, not the alkylated versions. Therefore the non-alkylated versions of the SIS peptides were used to generate the standard curves.

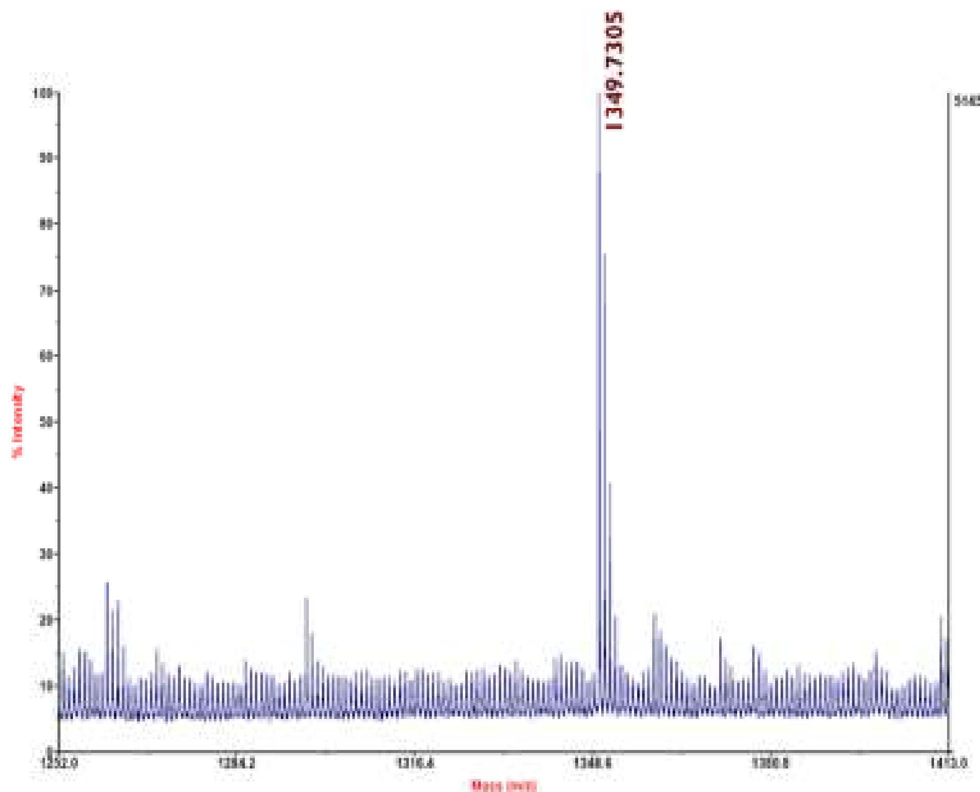

Figure 4.3 MALDI-MS Spectrum Showing Capture of Spiked-in EGFR Wild-type Peptide from U87MG Control Cell Line. ***The*** *EGFR peptide (NYVVTDHGSCVR) was spiked into U87MG control cell line, captured using the iMALDI method with the anti-EGFRvIII antibody, and detected by MALDI-MS.*

Discussion

The stable isotope labelled standard peptide for EGFRvIII was added at known concentrations to create a standard curve which was used to measure the concentration of the endogenous EGFRvIII peptide. Based on the curve, the endogenous peptide was present in my sample at approximately 3 femtomoles/µL. Given that I used about 20,000 cells for each analysis, the number of receptors per cell equals 150,000. Literature

searches reveal that the number of receptors of EGFRvIII per cell range from 270,000-680,000 in the U87MGΔEGFR cell line (Wickstrand et al., 1997; Fernandes et al., 2001). The iMALDI method gives lower than expected values, which may be due to several reasons. The complexity of the sample could be one reason for seeing lower-than-expected values, as it could lead to incomplete reduction and/or alkylation. One solution for this would have been to reduce the amount of total protein in the sample while keeping the amount of reducing and alkylating agents the same in order to reduce their overall 'workload'. Although this approach may result in a greater proportion of my sample being alkylated, it would inevitably decrease the amount of my target peptide in the sample. Other similar methods use chromatographic techniques to reduce the complexity of samples to narrow down the range in which the target peptide is found (Anderson et al., 2010; Whiteaker et al., 2010). However, chromatographic methods such as HPLC would not only add several hours to the iMALDI process, it would add further buffer exchange and chemical steps to the entire process. This would ultimately increase the chances of error in the experiments and provide more opportunities for peptide loss.

Another plausible reason for the discrepancy is incomplete tryptic digestion. Trypsin digestion efficiency is quite variable. Some techniques to improve tryptic digestion efficiency include using different buffers, certain additives, longer digestions, or different temperatures (Proc et al., 2010). For my assay, it was imperative to keep buffers and additives to a minimum because it would involve more clean-up steps and therefore more chance for error and loss. And, any residual buffer would hinder the antibody reaction

downstream and result in both sample and antibody denaturation. Given the fragile nature of the interactions in this assay, any additives or extraneous reagents can render the resultant spectra difficult to interpret (Wierenga et al., 2002).

Interestingly, only the non-alkylated forms of the peptides were detected. This could be because of incomplete reduction and/or alkylation, or because of selective capture of the non-alkylated form by the antibody. These possibilities will be discussed in Chapters 5 and 6, respectively.

Conclusion

The most significant finding of these experiments is that only the non-alkylated versions of the peptides were found in the tissue samples. Although several improvements could have been to the reduction/alkylation/digestion steps which might have improved the signal, a major disadvantage is that high-throughput ability would have been compromised. Since this ability is a priority in designing this iMALDI assay, some of these approaches would not be suitable. Furthermore, my calculated number of receptors was not drastically low compared to other literature. And, none of the other methods could verify their signal as definitively from the EGFRvIII protein. On the contrary, my results can be confirmed by utilizing MS/MS. In this case, while the copy number is lower than expected, the results obtained by this experiment are similar each time and are definitively EGFRvIII peptide. Therefore, since my current assay is both consistent and precise, it can be used to elucidate the EGFRvIII activity in tissue. After doing multiple

experiments and obtaining a copy number in the same range each time, I concluded that although my copy number analysis does not match the literature values, my results are consistent enough to apply this assay to tumour tissues.

Chapter 5
Analysis of Biological Samples: Glioblastoma Tumour Tissues

Introduction

After showing the proof-of-concept with the analysis of cell lines, the assay was evaluated against real tumour tissues from the tumour tissue bank of the London Health Sciences Centre. These brain tumour tissues belonged to end stage glioblastoma multiforme patients of various ages, and were extracted using the stereotactic brain needle biopsy method. In this method, a small hole is drilled near the side of the skull that the tumour is detected. With the help of imaging techniques such as CT or MRI scans, a needle is inserted into the brain tissue and guided to the site of the tumour to remove a portion of it.

After extraction from the patient, these samples were flash frozen in liquid nitrogen and stored at -80 °C at their facilities. Five glioblastoma samples (GM1, GM2, GM3, GM4, and GM5) and two normal brain samples to serve as a control (NM1 and NM2) were received. These 'normal brain' samples did not contain any glioblastoma markers and did not exhibit cancerous tumour growth. Although a small portion of all of the glioblastoma and normal brain tissues was analyzed for other brain tumour markers, the presence of EGFR or EGFRvIII was not tested. Therefore, half of each tissue sample was sent to the Hospital for Sick Children in Toronto, ON for RT-PCR analysis. There, the presence of EGFRvIII is routinely measured using reverse transcriptase PCR for research purposes.

These tissues were treated in an almost identical way to the cell lines. The major difference was that the brain tissue contained a much higher percentage of lipids and a small amount of blood. Sodium dodecyl sulphate (SDS) is by far the most common substance used in solubilizing high-fat tissues for protein extraction. However, SDS is a detergent that can dominate mass spectra and can hinder proteolytic digestion of tissue lysate (Wisniewski et al., 2009). As a result, the majority of fat and blood was removed by doing several rounds of acetone precipitations. Studies report that sonication followed by acetone precipitation is one of several optimal ways for extracting proteins from brain tissue (Ericsson et al., 2007).

Following the extraction and acetone precipitations, the tumour lysate was digested and incubated with antibodies and beads in the same manner as the cell lysates. However, for quantitation, it was not feasible to use the tissues themselves as a means of creating a standard curve because of sample size limitations. Because this was the first time the iMALDI samples were used on brain tissue, there was no estimate of the expected amount of EGFRvIII in the tissue. Using too much heavy standard could suppress the signal of the endogenous peptide. A trial-and-error process to find the range in which the EGFRvIII protein exists in each tissue sample could have used up most of the precious lysate. The approach selected was to start with a set of samples containing a serial dilution of heavy peptide over a broad range of concentrations. A spectrum was then selected where the heavy and natural peptides were detected as strong signals and replicate analyses were performed with only that particular concentration of spiked

standard. Then, the quantitation curve from the previous cell line study was used to roughly quantitate the density (copy number) of the EGFRvIII protein in these tissues. Thus, these experiments also provided the first approximation of the amount of SIS peptide that should be added to each sample. This is useful information and will facilitate further studies of EGFRvIII density in brain tumour samples. This knowledge will minimize the need for a future trial- and- error processes so that future brain tissue samples can be quantitated more accurately.

Materials and Methods

Sonication of Tissue and Extraction of Proteins

The probe of the sonicator was cleaned with successive washes of deionized water, 50% methanol, and 2mM sodium dodecyl sulphate (SDS) (Sigma-Aldrich, St. Louis, MO). The tissue resuspended in 500 µL of 1XPBS containing 1% sodium deoxycholate (w/v) (Sigma-Aldrich, St. Louis, MO). After sonication, the proteins were precipitated out with 5 mL of ice cold acetone (Sigma-Aldrich, St. Louis, MO) overnight at -20 °C. The acetone precipitation was repeated to remove residual blood pigment and to further isolate the proteins. The supernatant was then removed and the proteins were resuspended in 200 µL of 25 mM AmBic.

BCA Quantitation

The BCA assay was performed in the same manner as described in section 4.3. The first row contained a dilution series of the BSA standard. The subsequent rows of the plate contained a dilution series of each tissue lysate. The optical density was measured and

the amount of total protein in each sample was quantified. After quantitation, each sample was diluted in 25 mM Ammonium Bicarbonate to give a 1 mg/mL total protein concentration.

Tryptic Digestion of Tissue Lysate

One mg of protein was diluted in 1 mL of 25 mM AmBic. The proteins were reduced with 5 µL of 200 mM DTT at 37 °C for 1 hour. The alkylation was done with 20 µL of 200 mM iodoacetamide at 37 °C for 1 hour. After quenching with 25 µL of 200 mM DTT at 37 °C for 1 hour, 20 µg of trypsin were added and the sample was further incubated overnight at 37 °C. The samples were then boiled for 30 minutes to deactivate the trypsin. Solid-phase extraction cleanup was performed using the same method described in section 4.4. The samples were then lyophilized overnight and resuspended in 100 µL of 1XPBS.

iMALDI Analysis of Digested Tissue Lysate

A 100 µL aliquot of each sample was placed in a microcentrifuge tube containing 1XPBS, 5 µL bead slurry, 1 µg of antibody and 50 femtomoles/µL of the stable isotope standard peptide (GNYVVTDHGSCVR*). The samples were incubated for 2 hours at room temperature on a rotator. After washing 3 times with 100 µL of 25 mM AmBic, the beads were resuspended in 5 µL of 25 mM AmBic. One µL of the settled beads was spotted on the MALDI target plate and overlaid with 1 µL of CHCA matrix. The spots were then analyzed with the 4800 MALDI TOF/TOF instrument in both MS and MS/MS mode. Each set of experiments was replicated three times on all of the tissues.

RT-PCR Assay

Reverse transcriptase-PCR analysis was performed by Aaron Gajadhar in the laboratory of Dr. Abhijit Guha, University of Toronto. In brief, the total RNA was extracted from tissues and PCR products were produced using the primers 5′-ATGCGACCCTCCGGGACG-3′ and 5′-ATTCCGTTACACACTTTGCGGC-3′ which flank the deletion of exons 2-7. The products were analyzed on a 1% agarose gel and the band representing EGFRvIII cDNA was excised and submitted for sequencing for confirmation of correct product. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as a positive control in this experiment.

Results

The iMALDI assay was applied to five glioblastoma tissues and two normal brain tissues to detect and quantify EGFRvIII in the samples. The endogenous non-alkylated EGFRvIII peptide was only detected in three out of five of the glioblastoma tissues, and the endogenous EGFR wild type peptide was detected in one glioblastoma and both normal tissues (Figures 5.1-5.7). With the exception of GM1, the results of the RT-PCR confirmed the presence of EGFRvIII in the same samples as the iMALDI results (Table 5.2). The RT-PCR results show that EGFRvIII is present in GM1 but the iMALDI did not detect EGFRvIII in GM1. The SIS peptide was detected in all cases.

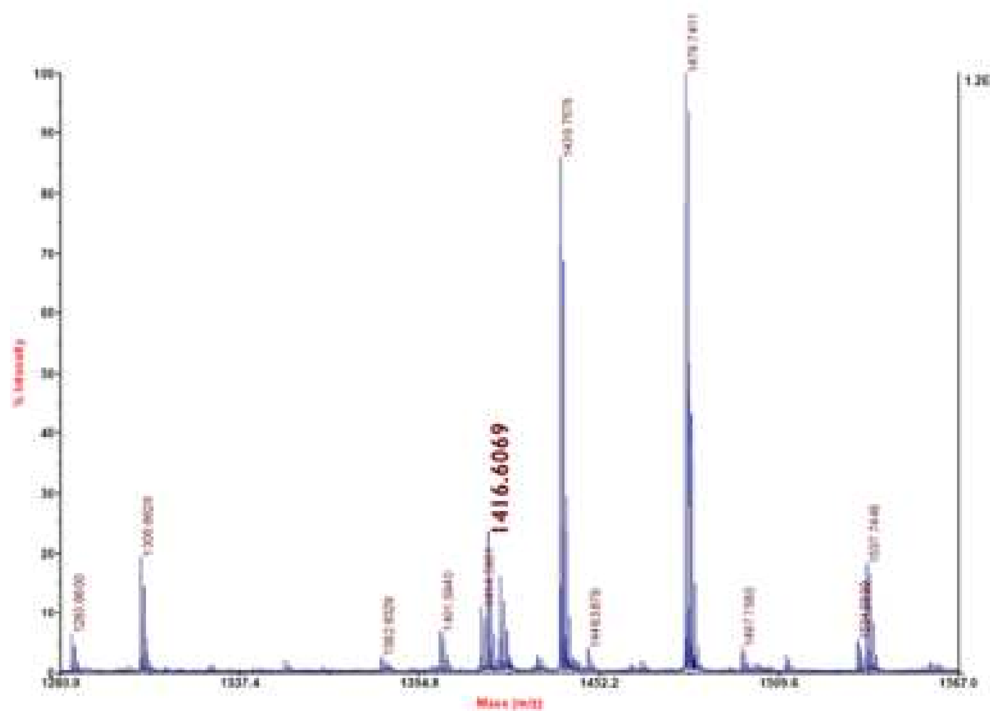

Figure 5.1 iMALDI analysis of Glioblastoma tumour sample 1 (GM1). *A MALDI-MS spectrum showing the capture and detection of spiked-in SIS EGFRvIII peptide (GNYVTDHGSCVR*) at m/z 1416.6069 from GM1. Fifty femtomoles/µL of SIS peptide were spiked into digested tissue lysate.*

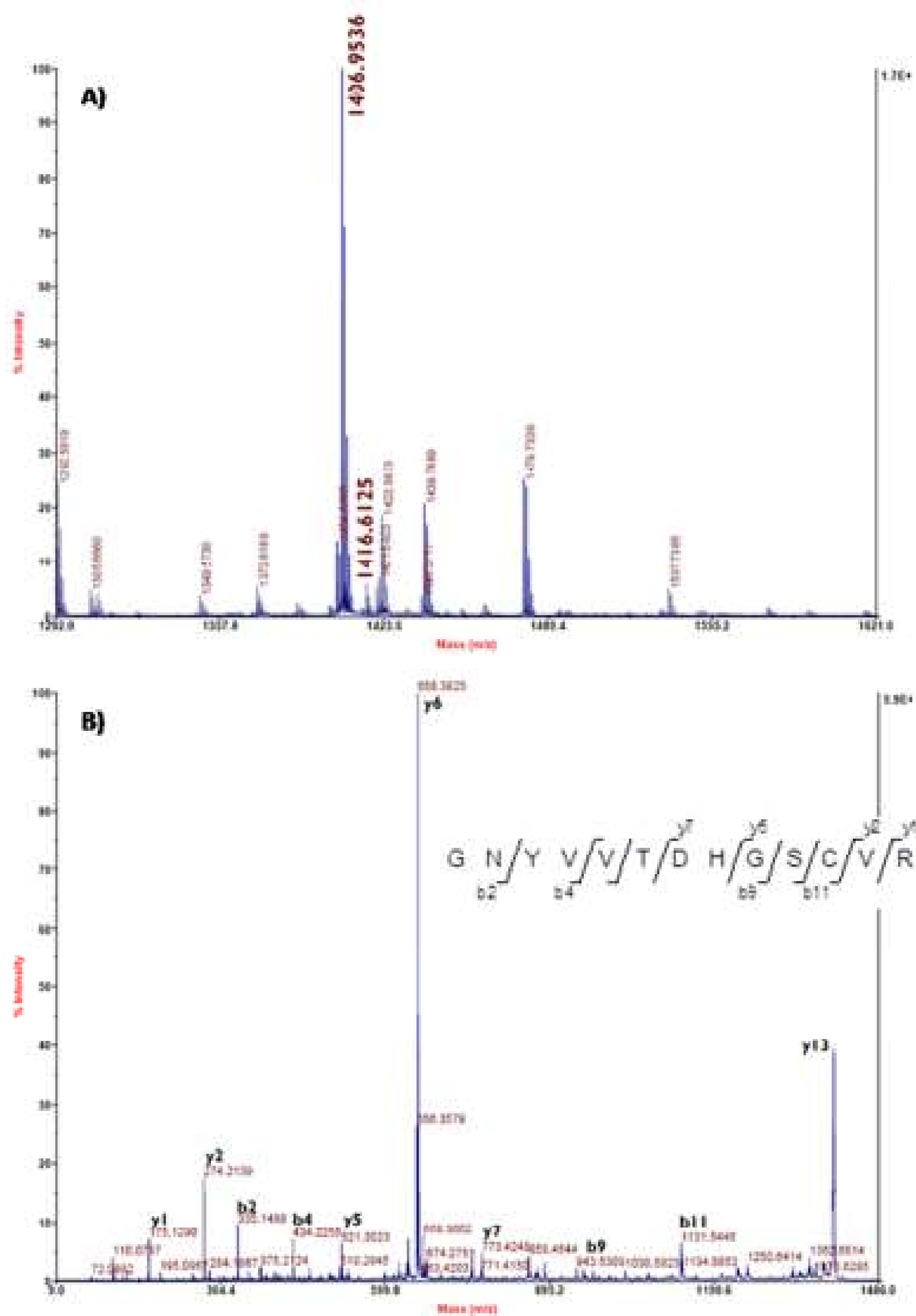

Figure 5.2 iMALDI Analysis of Glioblastoma Tumour Sample 2 (GM2). *A) MALDI-MS spectrum showing the capture of endogenous non-alkylated EGFRvIII peptide (GNYVVTDHGSCVR) at m/z 1406.9536 and spiked-in SIS EGFRvIII peptide (GNYVVTDHGSCVR*) at m/z 1416.6125. B) MS/MS spectrum of MS peak at m/z 1406.9536 to confirm the sequence and identity of peptide.*

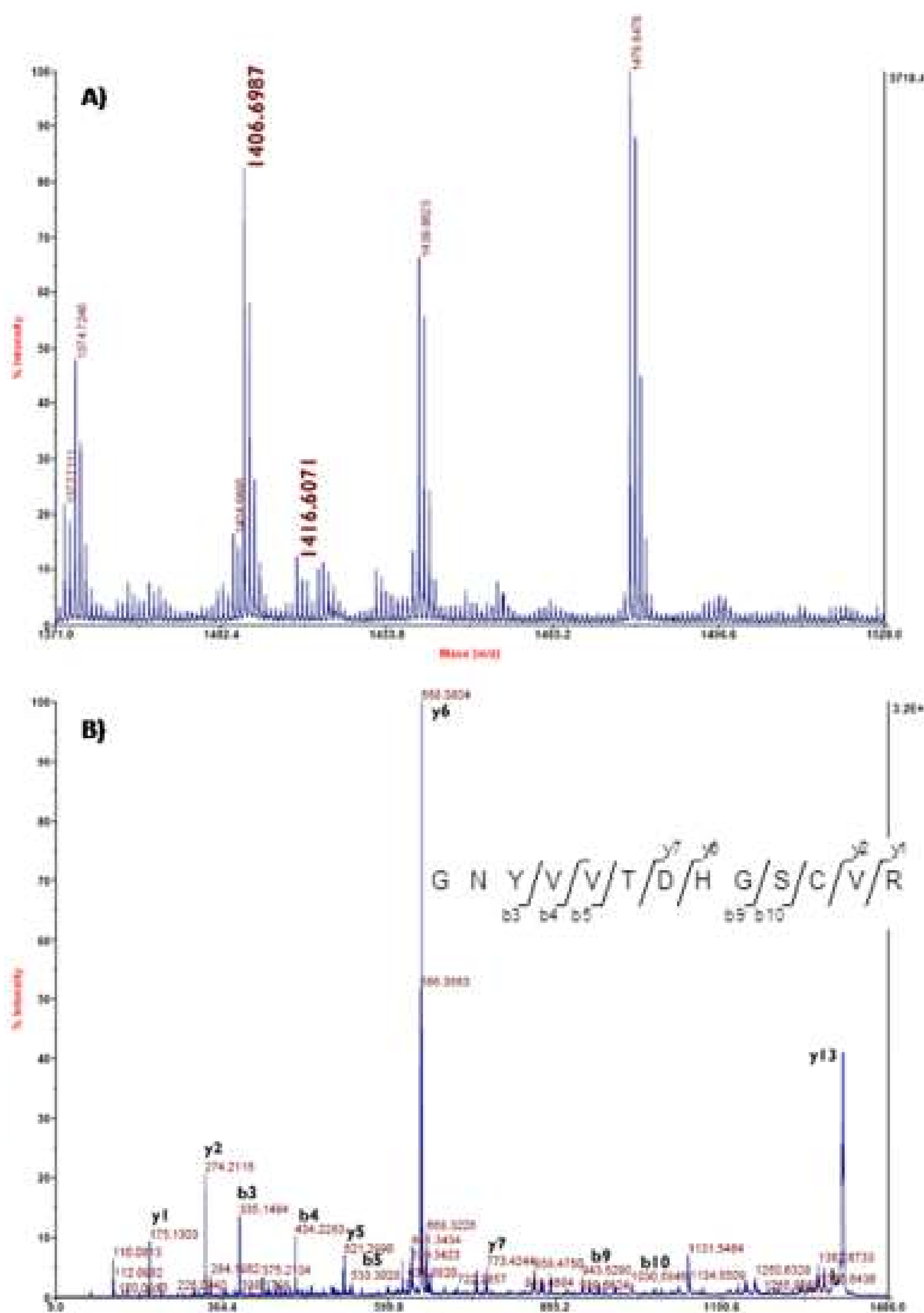

Figure 5.3 iMALDI Analysis of Glioblastoma Tumour Sample 3 (GM3). *A) MALDI-MS spectrum showing the capture of endogenous non-alkylated EGFRvIII peptide (GNYVVTDHGSCVR) at m/z 1406.9536 and spiked-in SIS EGFRvIII peptide (GNYVVTDHGSCVR*) at m/z 1416.6125. B) MS/MS spectrum of MS peak at m/z 1406.9536 to confirm the sequence and identity of peptide.*

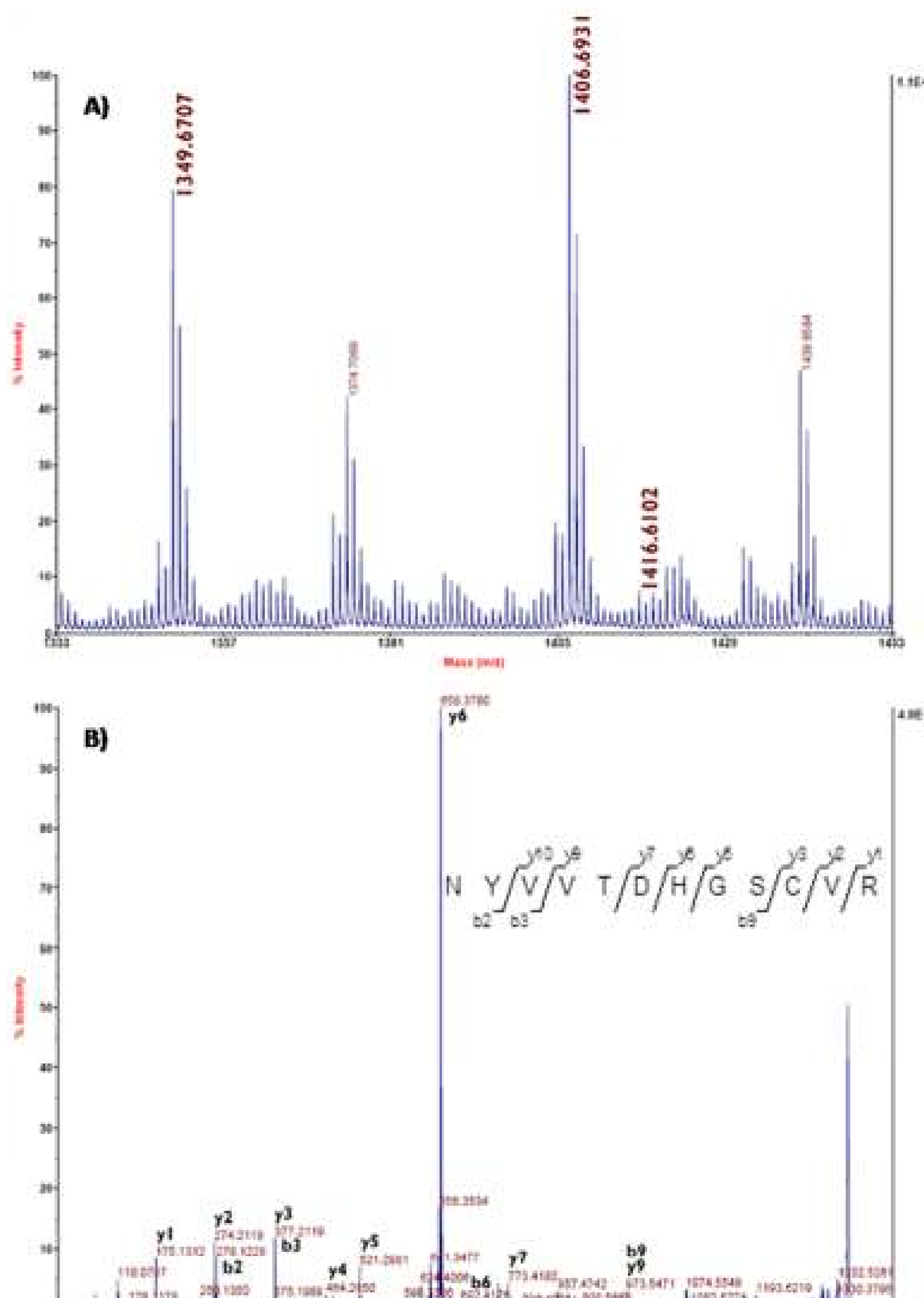

Figure 5.4 iMALDI Analysis of Glioblastoma Tumour Sample 4 (GM4). *A) The capture of endogenous non-alkylated EGFRvIII peptide at m/z 1406.6931, spiked in SIS EGFRvIII peptide at m/z 1416.6102 and endogenous EGFR wild type peptide (NYVVTDHGSCVR) at m/z 1349.6707. Fifty femtomoles/µL of SIS peptide were spiked into the digested tissue lysate. B) MS/MS spectrum of MS peak at m/z 1349.6707 to confirm the sequence and identity of the peptide.*

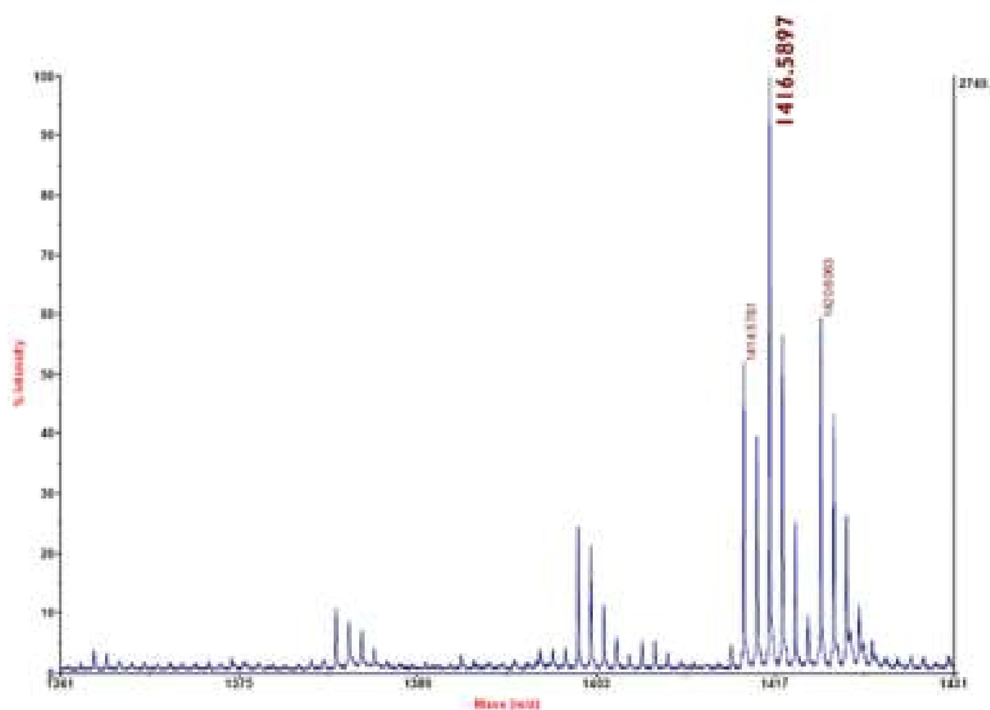

Figure 5.5 iMALDI Analysis of Glioblastoma Tumour Sample 5 (GM5). *A MALDI-MS spectrum showing the capture and detection of spiked-in non-alkylated SIS EGFRvIII peptide (GNYVVTDHGSCVR*) at m/z 1416.5897. 50 femtomoles/µL of SIS peptide was spiked into the digested tissue lysate.*

Figure 5.6 iMALDI Analysis of Normal Brain Sample 1 (NM1). *A) MALDI-MS spectrum showing the capture of endogenous EGFR wild type peptide (NYVVTDHGSCVR) at m/z 1349.5581. B) MS/MS spectrum of the MS peak at m/z 1349.5581 to confirm the sequence and identity of peptide.*

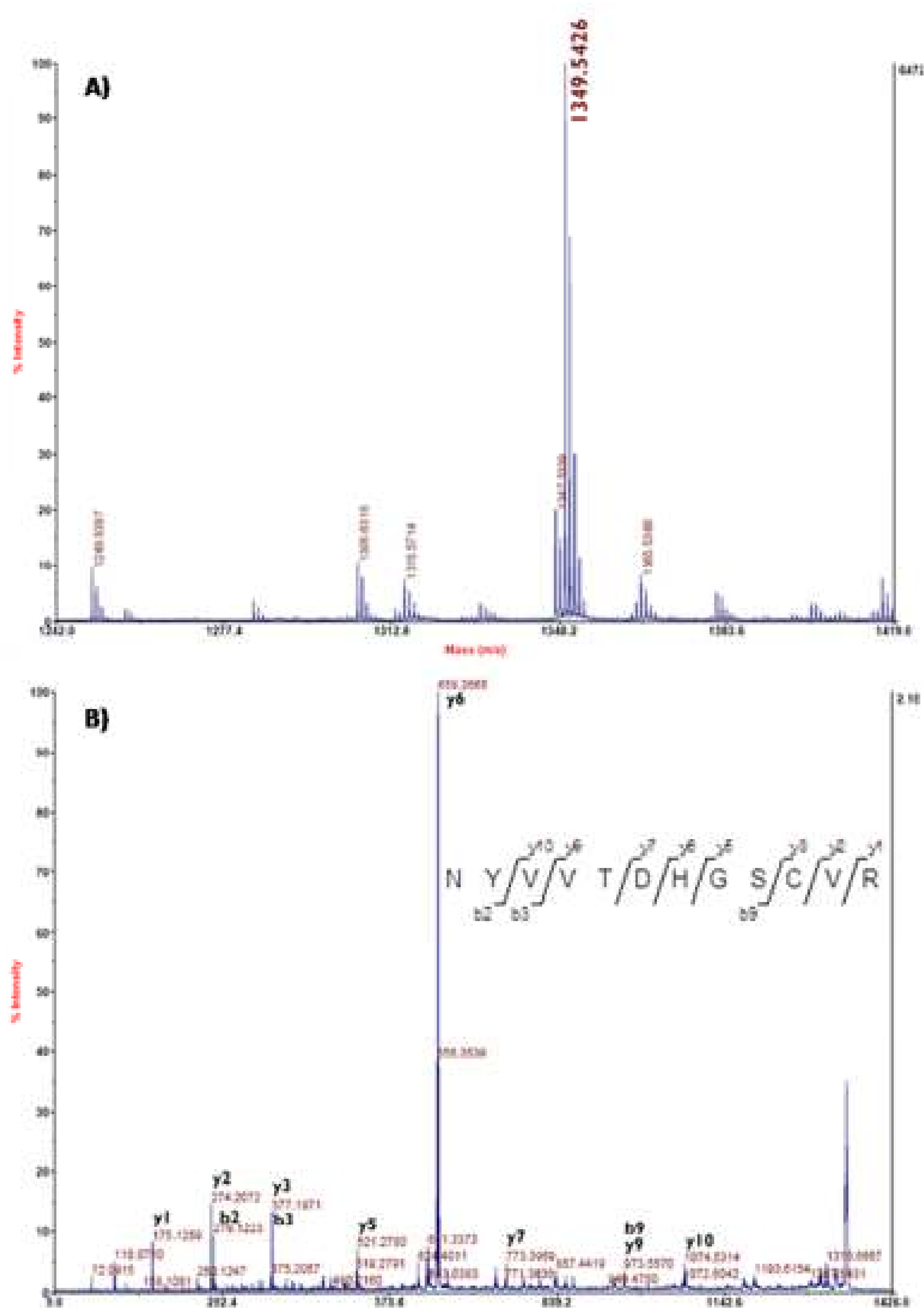

Figure 5.7 iMALDI Analysis of Normal Brain Sample 2 (NM2). *A) MALDI-MS spectrum showing the capture of endogenous EGFR wild type peptide at m/z 1349.5426. B) MS/MS spectrum of the MS peak at m/z 1349.5426 to confirm the sequence and identity of peptide.*

Tumour Sample	EGFR wild type (NYVVTDHGSCVR) m/z = 1349.7	EGFRvIII isoform (GNYVVTDHGSCVR) m/z = 1406.6	Heavy Standard (GNYVVTDHGSCVR) m/z = 1416.6
GM1	Undetected	Undetected	Detected
GM2	Undetected	**Detected**	Detected
GM3	Undetected	**Detected**	Detected
GM4	**Detected**	**Detected**	Detected
GM5	Undetected	Undetected	Detected

Table 5.1 Summary Table of Results from the iMALDI Analysis of the Five Glioblastoma Tumour Samples. *EGFRvIII peptide was detected in GM2-GM4 with a S/N ratio of higher than 10. The wild type EGFR peptide was detected in GM4.*

Tumour Sample	EGFRvIII isoform	Positive control (GAPDH gene)	Correlation with iMALDI results
GM1	+	+++	**No**
GM2	+	+++	Yes
GM3	+++	+++	Yes
GM4	+++	+++	Yes
GM5	-	+++	Yes

Table 5.2 Results from the RT-PCR Analysis of the Five Glioblastoma Tumour Samples and Comparison to the iMALDI results. *The two methods correlated well in all of the samples except for GM1. The RT-PCR gave a positive result for the presence of EGFRvIII mRNA in the tissue. The iMALDI analysis gave a negative result for presence of EGFRvIII peptide in the tissue, at least in concentrations above the iMALDI detection limit of 100 femtomoles/µL in solution.*

Discussion

After evaluating the results from the analysis of brain tissue, it was noticed that there was a discrepancy between the quantitation obtained by the RT-PCR results and the quantitation results from this experiment. The RT-PCR results showed higher levels of endogenous vIII protein in the tissue sample. Additionally, after several attempts, the iMALDI method was not able to detect any vIII protein in GM sample 1. In contrast, the

RT-PCR experiments did detect the mRNA of EGFRvIII in the same sample. An explanation for these results is that the distribution of mRNA copies or protein copies of vIII protein may not have been uniform, which has been known to be problematic in brain tumour tissue (Wickstrand et al., 1998). Alternatively, there could be a lack of correlation between the number of mRNA copies and expressed protein copies of EGFRvIII in this particular sample, as is often the case in biological systems (Tian et al., 2004; Greenbaum et al., 2003). Not enough literature is available which refers to the relationship between mRNA and protein expression of EGFRvIII since it is a growing field of research. However, a lack of correlation actually strengthens the claim that a protein-based assay is necessary for EGFRvIII detection and quantitation because it is possible to have discrepancies in mRNA and protein expression for EGFRvIII.

In contrast, the iMALDI approach includes its own validation in every sample. The detection of the SIS version of the EGFRvIII peptide indicates that the capture and detection portions of the assay were, in fact, working. This means that the native EGFRvIII peptide must have been present *at below the detection limit* in the digests but the mRNA was detected by the incredibly sensitive PCR assay. Since the SIS peptide is added *after* these steps, it will not compensate for problems in the digestion step, if, in fact, they did occur.

It is important to note that although the SIS peptide was detected in all tissues, it was detected with a stronger signal in some samples than others, even though the same amount of SIS peptide per µg of total protein (250 femtomoles of heavy peptide per 200

μg of total protein) was added. One reason for this is that the proteins in brain tissue are known to be distributed unevenly and extracted brain tissue sample composition can be very non-uniform (Krasteva et al., 2003; Ehlert et al., 2000). Although the total protein concentration was the same in all samples after BCA quantitation, the starting composition and distribution of proteins in the original frozen brain sample may have been different from sample to sample. As a result, the relative abundance of certain proteins was undeniably different from tissue to tissue as was seen in the relative intensities of non-specific peaks in the spectra. The intensity of the non-specific peptides affects the intensity of the SIS peptide because of the nature of MALDI TOF MS, but the relative intensity ratios of the SIS to the native peptide should remain the same. This clearly emphasizes the need for an internal standard. The lack of homogeneity of the brain tissue may be an issue to address in future studies. Nevertheless, it must be emphasized that the SIS peptide was detected in all cases. If the SIS peptide is consistently detected, the lack of homogeneity in the sample will have a smaller effect on the detection of the target peptide.

Although endogenous EGFR and EGFRvIII peptides were detected in some of the samples, an insufficient number of samples were tested in order to elucidate a pattern of expression. The samples were obtained from a wide range of demographic qualities such as age or gender, and even though the patients were deceased at the time the samples were received, the tissues were collected at different stages of disease. Also, not all tumours were presented in the same area of the brain. Furthermore, although the 'normal brain' samples were not cancerous, the brain biopsy would have been performed

to test for other brain diseases such as Alzheimer's or repetitive seizures. In that sense, these tissues may not have been disease-free. This information was not made available by the LHSC tumour bank.

After reduction by DTT and alkylation by iodoacetamide, it was expected that all the endogenous vIII peptide (GNYVVTDHGSCVR) would have been alkylated at the internal C residue. However, the peptide detected in these samples did not contain the alkylation and was the reduced form of the peptide. Upon searching further in the spectrum, a very small amount of alkylated peptide was seen, but the signal was too low to generate a strong MS/MS spectrum. As discussed in Section 2.2, the antibody used was raised against the non-alkylated version of the peptide, and might not have captured the alkylated form very efficiently. This possibility will be explored in Chapter 6. Regardless, the detection of unmodified peptide instead of the modified peptide indicates that alkylation using iodoacetamide did not go to completion. A recent study found that alkylation can be a very fragile process in that pH plays a large role in whether the reaction goes to completion (Galvani et al., 2001). Furthermore, the alkylation time is a big factor in that the reaction reaches completion only after 6 hours and the presence of other agents such as reducing agents can aid in the resisting of alkylation (Galvani et al., 2001). First, all these alkylation reactions were done in less than two hours. And second, it was calculated that there was excess of reducing agent in these samples. In addition, a very large excess of DTT was used to quench the iodoacetamide alkylation reaction.

An alternative explanation for these results is that the alkylation *did* go to completion, but that the first reduction with DTT was incomplete, leaving the target peptide as a bridged peptide. The second reduction, with a larger amount of DTT, then reduced this disulfide bond, leaving the non-alkylated peptides.

Conclusion

Further experiments would need to be performed in order to understand the problems in digestion and lack of alkylation in the sample. However, for the purposes of this study, it was more important to examine the possibility of preferential capture of the non-alkylated peptide. In other words, the two main reasons why alkylated peptide would not be detected are 1) the alkylation process was faulty or 2) the antibody did not capture the alkylated products. If one of those possibilities can be eliminated, future iMALDI studies on EGFRvIII detection could optimize that part of the assay. Therefore, the antibody was tested against a 1:1 mixture of alkylated and non-alkylated peptide to determine whether the antibody preferentially captured one or the other. This is to be the subject of the next chapter, Chapter 6.

Chapter 6
Characterization of Antibody Binding to Alkylated Peptide

Introduction

It was originally decided to use non-alkylated peptides for the generating the antibody because of the nature of target peptide. The target peptide is actually involved in a disulfide bond in the native EGFR protein (Hoskins et al., 2009; Dawson et al., 2005). We therefore assumed that a real sample would have to be reduced and alkylated to keep the disulfide bonds from re-forming. This resulted in a challenge for the antibody generation step. Basically, one would have to derivatize only the internal cysteine, while leaving the N-terminal cysteine free to bind to the carrier protein. However, the tumour-tissue experiments showed capture of only the non-alkylated form of the peptides. In light of these results, the antibodies were characterized further in order to determine whether anti-alkylated peptide antibodies will have to be generated.

To determine the relative binding affinities of the, MALDI MS was used to examine differences in binding between the alkylated and non-alkylated forms of the wild-type and vIII variant peptides. The typical method used to find binding “on and off rates” is Surface Plasmon Resonance (SPR). This method provides a means of much more accurate analysis than using MALDI MS. However, some studies have shown that mass spectrometry based analysis of binding kinetics can be a powerful tool prior to performing SPR (Morrissey et al., 2008).

Materials and Methods

Preparation of Alkylated Peptides

In two microcentrifuge tubes, 100 picomoles/µL of the EGFRvIII peptide (GNYVVTDHGSCVR) were brought up in 100 µL of 25 mM AmBic. One tube was kept as control while the other one was alkylated. In the alkylation tube, 5 µL of 200 mM DTT was added and the sample tube was incubated for 1 hour at 37 °C. 20 µL of 200 mM Iodoacetamide was then added and incubated for another 1 hour at 37 °C. In the control tube, 25 mM AmBic buffer was added in the same volumes and times as the alkylation tube. The incubations for the two tubes were done identically as well. Three technical replicates were performed.

iMALDI Capture of Modified and Unmodified Peptide

A 5 µg aliquot of antibody was incubated with 5 µL of bead slurry for 2 hours at room temperature on a rotator. The sample was washed 3 times with 1XPBS and resuspended in 100 µL of 1XPBS. In a separate tube, an estimated 1:1 ratio of alkylated and native peptide were mixed together and cleaned up using µC18 ZipTip® column tips (Millipore, Billerica, MA). One µL of the cleaned-up mixture was spotted onto a MALDI target plate and analyzed to observe a 1:1 ratio of both peptides. The mixture and clean up steps were repeated several times by adjusting the starting amounts of the two peptides in order to obtain the 1:1 signal. Once this signal ratio was obtained, the corresponding peptide mixture was added to the sample tube containing the beads. The sample was then incubated for 2 hours at room temperature on a rotator. After washing 3 times with 100

μL of 25 mM AmBic, the beads were resuspended in 5 μL. One μL of the settled beads were spotted on two different positions and overlaid with 1 μL of CHCA matrix.

Results

The native peptide was first completely alkylated (Figure 6.1). After obtaining a 1:1 mixture of alkylated and non-alkylated peptide (Figure 6.2) and iMALDI capture with washing, the signal of the alkylated version was only slightly less than the non-alkylated peptide (Figure 6.3).

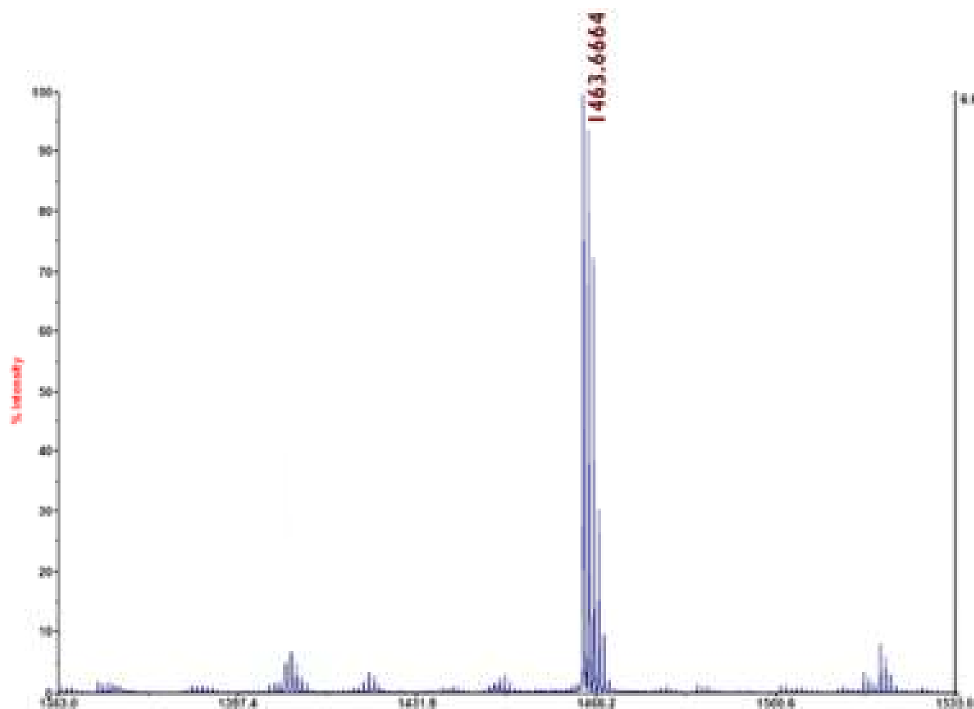

Figure 6.1 MALDI-MS Spectrum of Completely Alkylated EGFRvIII Peptide. *The EGFRvIII synthetic peptide (GNYVVTDHGSCVR) was reduced and alkylated using iodoacetamide (Sigma) and spotted onto a MALDI target plate without enrichment. The completely alkylated peptide was detected at m/z 1463.6664.*

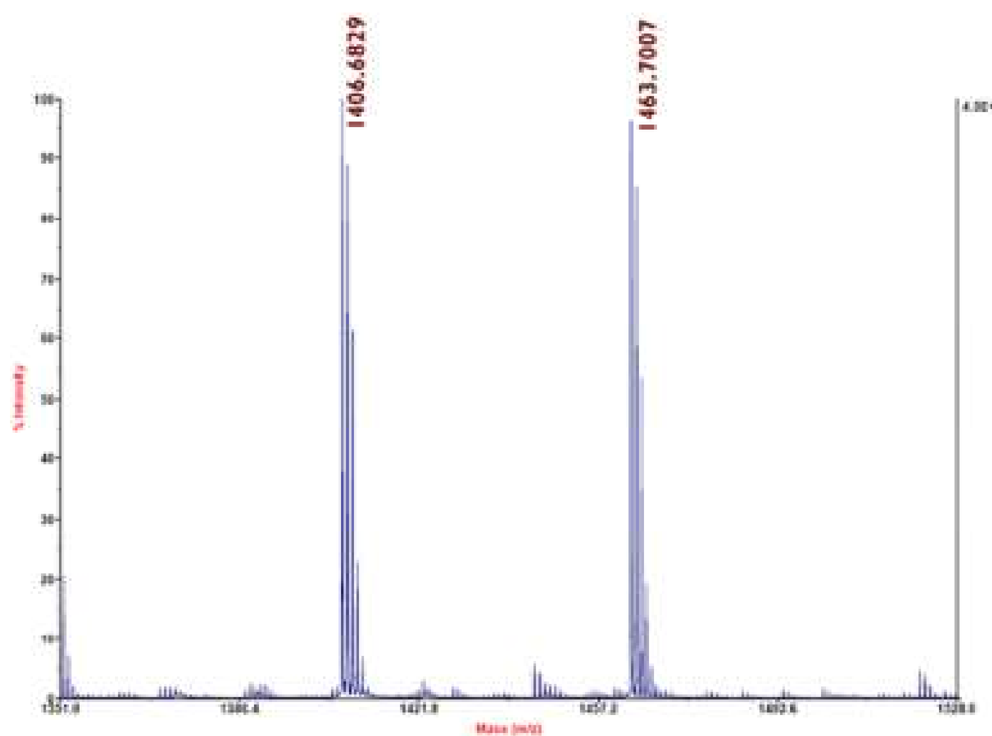

Figure 6.2 MALDI TOF/TOF Spectrum to Confirm a 1:1 mixture of Unmodified and Modified EGFRvIII Peptide. *The alkylated and non-alkylated peptides were mixed in several different ratios and spotted on the MALDI target plate in order to obtain a 1:1 mixture of peptides.*

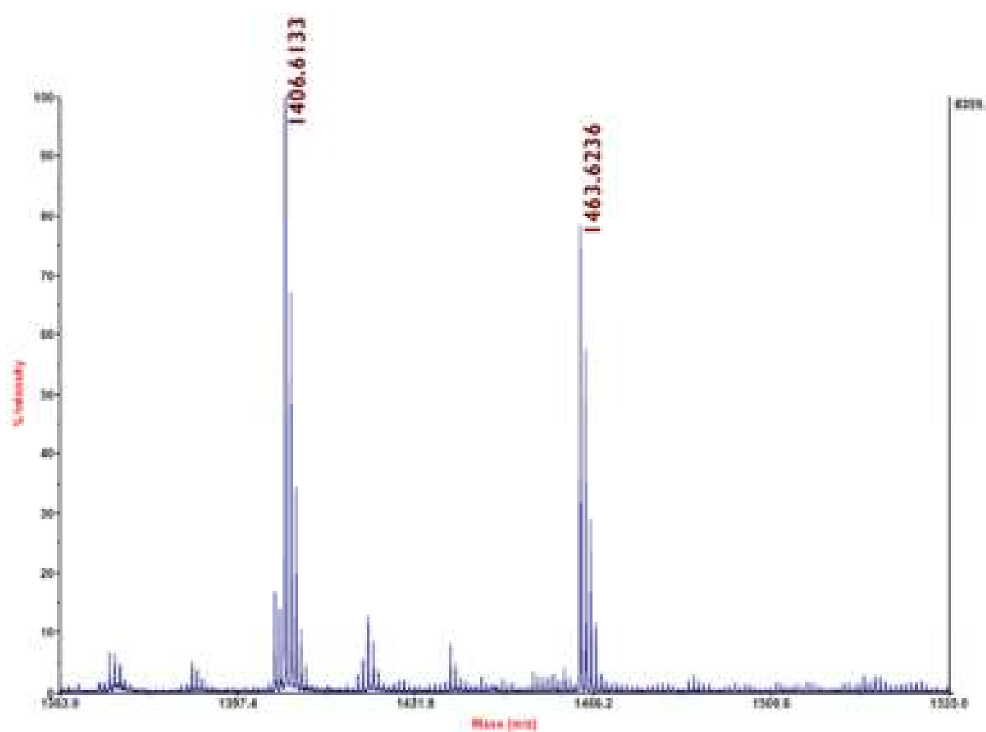

Figure 6.3 MALDI-MS Spectrum of Both Alkylated and Non-alkylated EGFRvIII Peptide after Antibody Capture.*A 1:1 mixture of the two peptides was incubated with anti-EGFRvIII antibody and beads, and washed three times with 25 mM AmBic (Sigma) prior to spotting the beads directly on the MALDI target plate.*

Discussion

The successful completion of these experiments rested on getting the concentration of the unmodified and modified peptide to be in a 1:1 ratio prior to capture with the antibody. This was particularly difficult because there was no direct way to measure the concentration of the modified peptide once it had undergone the reduction and alkylation procedure and clean up with the ZipTip, a step which is necessary to remove the DTT and iodoacetamide before any antibody analysis. The ZipTip cleanup essentially concentrates the peptide in addition to cleaning it up, and it is inconsistent from sample to sample because the capacity of the ZipTip is small in comparison to the large volume of the sample. So, applying the ZipTip clean up to both peptides does not guarantee that they will end up with the same concentration. The only quick way to ensure equal amounts of peptide was by adjusting the volume of the peptide, spotting 1 μL onto the plate, looking for a 1:1 signal, and adjusting the initial volume and repeating these steps to obtain the desired ratio. Eventually, I was successful in mixing several different amounts of the two peptides until I was able to obtain a 1:1 signal in the mass spectrum (Figure 6.2).

Antibody activity can be difficult to predict until it is experimentally validated. In this case, the sequences of both the alkylated and non-alkylated peptide were the same. The only difference was an alkyl group on the cysteine residue that was added during the alkylation process to prevent the formation of dimers. Normally, an epitope of an antibody spans nine residues. Since the antibody used in this study was polyclonal, a

variety of epitopes could, in theory, be recognized. The only way the alkylated and non-alkylated peptides would show a difference in the antibody affinity was if a significant population of the polyclonal antibodies recognized an epitope containing the internal cysteine peptide and/or if the antibodies bound in such a way that the alkyl group would affect binding. Although it is not unusual because post translationally-modified peptides can themselves be used to generate antibodies which can specifically select for this modified peptide (Hebbes et al., 1989), this experiment revealed that there was only a slight decrease in signal of the modified peptide. This slight difference was not enough to conclusively claim a difference in binding affinities of the antibody for the alkylated and non-alkylated forms of the target peptides.

Conclusion

This experiment showed that the antibody was indeed able to capture the modified peptide if there was any alkylated peptide in the digests. Therefore, it was likely the reduction/alkylation reaction that was faulty and will need to be optimized for further iMALDI studies. Additional SPR studies will also have to be performed in order to understand the relationship between the antibodies and the two target peptides. Antibodies specifically against the alkylated peptide may also have to be created in order to perform full quantitation of the target peptides in the tissue if the alkylation process poses additional problems in the future. Alternatively, strategies could be developed to produce the non-alkylated form from tissue samples.

Chapter 7
Summary and Conclusions

The objective of this study was to characterize the molecular relationship between EGFR and EGFRvIII proteins in brain tumour tissue, as well as to provide a comprehensive diagnostic method that was capable of absolute quantitation. The truncation of the EGFR protein to create EGFRvIII and the mechanism of cell signalling and proliferation has not been extensively studied and not well understood. However, it has been shown many times that EGFRvIII is seen only in cancerous tissue, particularly in brain tumours (Gan et al., 2009; Ayuso-Sacido et al, 2009; Zeineldin et al., 2010). Therefore, EGFRvIII offers a unique and specific target for cancer therapeutics. The recent development of an EGFRvIII antibody-based vaccine is an indication that there is growing interest in this protein for therapeutics and the continuation of this trend warrants a much greater understanding of the activity of this protein (Wickstrand et al., 1998; Heimberger et al., 2009).

This goal of this research project was to optimize the previously described iMALDI assay and apply it to the study of EGFRvIII and EGFR in brain tumour tissue. After synthesizing the peptides and obtaining polyclonal antibodies, the various antibodies, beads, and peptide interactions of the assay were optimized to create a high throughput and streamlined method with which to analyze biological samples.

Detergent Removal

The first issue to consider is that of sensitivity. Biomarkers are often of low abundance in biological tissue so the ability to detect these molecules even when they are present in low quantities is of utmost importance (Keshishian et al., 2007). In this case, the sensitivity of the assay was compromised by the Tween 20 detergent buffer used for the storage of beads. The presence of Tween is normally not an issue for immunoassays, because they do not utilize mass spectrometry as a downstream tool. However, for studies that do, similar issues have been reported and buffer exchange has been performed (Anderson et al., 2010; Whiteaker et al., 2007). In the course of this research, a method was developed for removing most of the detergent by exchanging it with CHAPS buffer. However, residual Tween 20 that remained in the sample did prove to be a hindrance when target peptide signal was low. Unfortunately, the residual Tween 20 is extremely difficult to remove. The application of the beads in this assay is quite unique in that the beads are placed directly onto the plate. Any Tween 20 that remains will inevitably be seen in the spectrum, especially since MALDI TOF mass spectrometry shows the entire complement of signals in an m/z range. As a possible solution to this conundrum, Invitrogen® Inc. has provided us with some prototype Protein G Dynabeads that do not contain any detergent. The issue with these beads is that they will stick to the walls of the tubes and pipette tips more than the detergent beads, which may result in bead loss and ultimately sample loss. However, further investigation and optimization in the use of these beads can prove beneficial to this assay.

Optimization of Incubation Time and Antibody Concentration

The other parameters of the assay such as incubation times and antibody concentrations were also optimized. A robust and successful one-step method was developed to capture endogenous EGFRvIII and EGFR wild type peptide from cell lysate and tumour tissues. The development of the one-step incubation method was a new manifestation of the previously described iMALDI method. This improved method streamlined the iMALDI process and made it more high throughput, which is an important consideration when analyzing multiple samples.

However, since a polyclonal antibody was used, these parameters will have to be optimized again when a new batch of antibodies is obtained. Therefore, for future studies, it would be beneficial to create a monoclonal antibody for this project. Although it is more expensive, a monoclonal antibody can be developed to definitively capture the EGFRvIII and EGFR wild type peptides out of tumour tissue. A monoclonal antibody will give rise to an unlimited supply of exactly the same antibody and will eliminate much of the ambiguity and variability that comes with the use of polyclonal antibodies. Monoclonals can also be well characterized with the use of Surface Plasmon Resonance in order to accurately measure on and off rates.

Selection of the Alkylating Agent

It was also noticed during the course of this project that there was an overlap in the masses of the target peptides. For instance, the mass of the alkylated version of the EGFR wild type peptide is exactly the same as the non-alkylated version of the EGFRvIII

peptide. This is due to the fact that an addition of an alkyl group has the same mass as a glycine residue. Although MS/MS was performed whenever a potential target peptide is seen on the mass spectrum, at very low levels the signal can be too weak to produce a clear MS/MS spectrum. Given that the sequences are almost similar, a clear MS/MS spectrum is needed to avoid confusing one peptide with the other. This is why a S/N of 10:1 was used for the LOD.

Three options present themselves for avoiding this problem: 1) an LC separation step could be introduced and these two peptides could be separated chromatographically. This however would turn the iMALDI assay into more of a SISCAPA-type assay (Anderson et al., 2004), with LC-MALDI detection. This would increase the sample handling and would probably reduce the throughput; 2) a different alkylating agent (other than iodoacetamide) could be used which would produce a different mass increment, and (if needed) an antibody could be raised against a peptide modified with this new alkylating agent; or 3) a method could be developed to avoid alkylation entirely. This would mean that the current antibody could be used. This means that all of the method optimization done in the course of this project could be used as well.

Selection of the Antibody

It was found that the polyclonal antibody used for these experiments was created against the native peptide only, whereas the experimental methods of this assay create modified peptides. Since the target peptide contains a cysteine residue that gets alkylated, the next

version of this assay should be done either with an antibody raised against the alkylated peptides, although this may pose somewhat of a challenge because of the need for a free N-terminal cysteine to conjugate to the carrier protein to enhance its antigenicity during antibody production. In discussions with the company generating the antibodies, I have learned, however, that it *would* be possible to synthesize the target peptides with pyridylethylcysteine instead of the "internal" cysteines for the purposes of antibody generation. The free cysteines in the sample would then be modified by pyridylethylation before capture, and the capture would be done using an antibody raised against a target peptide which likewise contained pyridylethylcysteine.

Alternatively, it might be preferable to use the current antibody (raised against the reduced but non-alkylated versions of the target native EGFR peptide) because of the sensitivity demonstrated in the iMALDI method using these non-alkylated peptides. This would require modification of the digestion/reduction/alkylation protocol. It might be possible to design a method either for a "reversible" alkylation, perhaps with MMTS which converts –SH groups to -$SSCH_3$ (Kirley et al., 1989; Smith et al., 1975) or possibly reagents that are chemically-cleavable, similar to chemically-cleavable crosslinkers (Bennett et al., 2000). It might also be possible to keep the cysteines in the sample as free cysteines, possibly by using a small amount of DTT and an acid solution. This would have to be done in such a way as to not denature the capture antibody or interfere with the capture process.

Brain Tissue Extraction

The analysis of brain tissue proved to be difficult. The extraction of proteins from the tissue was an arduous task since the proteins had to be separated from the lipids and the blood. Normally, the extraction of proteins from brain tissue is not difficult. SDS is used to solubilise the proteins in the tissue to obtain a high yield. However, my downstream applications of the protein are unique in that a proteolytic digestion had to be performed, and SDS denatures proteases. SDS could have been used and then removed the SDS from the sample, but even slight amounts of it left behind in the solution could negatively affect the mass spectrometric experiments. Furthermore, the added chemical steps could have increased the chance of error and sample loss. As it is, the selected sonication method used, tends to decrease protein yield by 7.5% on average. Unfortunately, even after several acetone precipitations, a small quantity of lipid may have remained in the solution. Although lipids in solution might have been better than adding SDS and subsequently trying to remove it (it may be as difficult to remove as Tween) before performing a proteolytic digestion, the lipids might have been a hindrance to the BCA quantitation assay, and might also have altered the subsequent alkylation reaction which is performed prior to tryptic digestion.

For future investigations, the protein extraction procedure should be optimized *before* experimenting on human brain tissue. Rat brains offer a similar composition to human brain tissue. Tumour models of rat brains expressing EGFRvIII have been recently created and could be of great use in this project (Yang et al., 2005). Not only would the rat brains offer a similar type of tissue for the development of a proper protein extraction

procedure, it would also provide an appropriate medium for the complete detection and analysis of EGFRvIII prior to analysing human brain tissue. Moreover, a recent study in the Borchers group has demonstrated the wide variability of "optimum" digestion conditions for different proteins (Proc et al., 2010). A digestion protocol optimized specifically for the EGFR protein in brain tissue might also increase the sensitivity of this assay.

Conclusion

Overall, the quantitative aspect of the iMALDI assay was probably affected by incomplete reduction, alkylation, and digestion of tissue. In addition, in order to find trends and patterns in the activity of EGFRvIII, more tumour tissues of different types need to be analyzed. Obtaining tumour tissues has proven to be difficult, but it is necessary in order to further our understanding of this topic. If previous studies are correct in identifying a possible alternate signalling mechanism for EGFRvIII, a multiplexed iMALDI assay could be created whereby EGFRvIII and several of its downstream signalling proteins could be analyzed simultaneously from the same sample. In particular, anti-peptide antibodies can be created to specific proteins in the EGFRvIII signal cascade. They can then be analysed using the iMALDI assay and the samples can be multiplexed incrementally without compromising sensitivity and accuracy. In this manner, the mass spectrometry and antibody based method developed through this project may lead to future insight into the molecular characteristics of EGFRvIII through the creation of a multiplexed assay for use in the clinic.

Despite various setbacks encountered during the course of this project, several proof-of-principle aspects were clearly demonstrated. First, a method to characterize the efficiency and ideal incubation time for antibodies was developed. Also, this was the first iMALDI project to successfully use Protein-G magnetic beads, and this allowed for the development of a new one-step iMALDI protocol. With the use of these beads in conjunction with iMALDI, antibodies can be easily screened for potential downstream uses. Additionally, this method was successful at capturing endogenous peptides from a complex biological sample and detecting them with high sensitivity using mass spectrometry. A “benchmark” concentration for the addition of SIS peptides to brain tissue samples was also determined. The inability to provide absolute quantitation for this project was limited because of the nature of the peptides, antibodies, and upstream digestion/alkylation issues. Studying more tumour tissues and providing absolute quantitative data is likely to provide further insight into the EGFRvIII activity within tumour tissues.

At this point, a general method has been developed to study EGFRvIII and EGFR in biological samples. Future improvements on this method, guided by the results shown here, should aid in the further study of the EGFRvIII protein in brain tissue, specifically their activity pathway in the cell and the influence of the concentration of EGFRvIII on tumour development in the brain.

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Appendix A
List of Abbreviations

μ	micro
μg	microgram
μL	microlitre
A	adenine
AmBic	ammonium bicarbonate
BCA	bicinchoninic acid
BSA	bovine serum albumin
C	cysteine (amino acid) or cytosine (nucleic acid)
cDNA	complementary DNA
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate
CHCA	α-cyano-4-hydroxycinnamic acid
CID	collision induced decay
D	aspartic acid
DTT	dithiothreitol
ECD	electron capture dissociation
EDTA	ethylenediaminetetraacetic acid
EGFR	epidermal growth factor receptor
EGFRvIII	epidermal growth factor receptor variant III
ELISA	enzyme-linked immunosorbent assay
ESI	electrospray ionization
Fab	fragment antigen-binding

FMOC	9H-fluoren-9-ylmethoxycarbonyl
G	glycine (amino acid) or guanine (nucleic acid)
GM	glioblastoma multiforme or glioblastoma multiform tissue
H	histidine
HPLC	high pressure or high performance liquid chromatography
IHC	immunohistochemistry
IMAC	immobilized metal affinity chromatography
iMALDI	immunoMALDI
KLH	keyhole limpet hemocyanin
LOD	limit of detection
MALDI	matrix-assisted laser desorption/ionization
MRI	magnetic resonance imaging
MMTS	Methyl methanethiosulfonate
MS	mass spectrometry
MS/MS	tandem mass spectrometry
N	asparagine
NM	normal brain tissue
PBS	phosphate buffered saline
PCR	polymerase chain reaction
R	arginine
RT-PCR	real time PCR; reverse transcriptase PCR
S	serine
SDS	sodium dodecyl sulphate

SELDI	surface-enhanced laser desorption/ionization
SIS	stable isotope standard
SISCAPA	stable isotope standards and capture by anti-peptide antibodies
SPPS	solid phase peptide synthesis
T	threonine (amino acid) or thymine (nucleic acid)
TFA	trifluoroacetic acid
TOF	time-of-flight
TPCK	tosyl pheynylalanyl chloromethyl ketone
U	uracil
V	valine
Y	tyrosine

Appendix B
Theoretical Peptide Information

EGFRvIII peptide - GNYVVTDHGSCVR

ISOTOPE DISTRIBUTION
Elemental Compositon: C58 H92 N19 O20 S1
Monoisotopic M/Z: 1406.64812
Total Abundance: 100.00%

Isotope Number	m/z	Percent Total	Percent Maximum
0	1406.64812	43.67	100.00
1	1407.65091	32.68	74.83
2	1408.65234	15.78	36.13
3	1409.65371	5.69	13.04
4	1410.65513	1.66	3.81
5	1411.65666	0.41	0.94
6	1412.65831	0.09	0.20
7	1413.66002	0.02	0.04
8	1414.66193	0.00	0.01
9	1415.66407	0.00	0.00
10	1416.66583	0.00	0.00

MSMS SEQUENCE IONS

MH^{+1}(av)	MH^{+1}(mono)
1407.5571	1406.6481

b				Y
---	1	**G**	13	---
172.0717	2	**N**	12	1349.6267
335.1350	3	**Y**	11	1235.5837
434.2034	4	**V**	10	1072.5204
533.2718	5	**V**	9	973.4520
634.3195	6	**T**	8	874.3836
749.3464	7	**D**	7	773.3359
886.4054	8	**H**	6	658.3090
943.4268	9	**G**	5	521.2500
1030.4588	10	**S**	4	464.2286
1133.4680	11	**C**	3	377.1966
1232.5364	12	**V**	2	274.1874
---	13	**R**	1	175.1190

EGFR wild type peptide – NYVVTDHGSCVR

ISOTOPE DISTRIBUTION
Elemental Compositon: C56 H89 N18 O19 S1
Monoisotopic M/Z: 1349.62666
Total Abundance: 100.00%

Isotope Number	m/z	Percent Total	Percent Maximum
0	1349.62666	44.95	100.00
1	1350.62945	32.42	72.14
2	1351.63081	15.27	33.96
3	1352.63213	5.37	11.95
4	1353.63353	1.53	3.41
5	1354.63506	0.37	0.82
6	1355.63669	0.08	0.17
7	1356.63841	0.01	0.03
8	1357.64015	0.00	0.00
9	1358.64213	0.00	0.00

MSMS SEQUENCE IONS

MH^{+1}(av)	MH^{+1}(mono)
1350.5048	1349.6267

b				Y
---	1	N	12	---
278.1135	2	Y	11	1235.5837
377.1819	3	V	10	1072.5204
476.2504	4	V	9	973.4520
577.2980	5	T	8	874.3836
692.3250	6	D	7	773.3359
829.3839	7	H	6	658.3090
886.4054	8	G	5	521.2500
973.4374	9	S	4	464.2286
1076.4466	10	C	3	377.1966
1175.5150	11	V	2	274.1874
---	12	R	1	175.1190

Alkylated EGFRvIII peptide - GNYVVTDHGSC(Carbamidomethyl)VR

ISOTOPE DISTRIBUTION
Elemental Compositon: C59 H94 N19 O20 S2
Monoisotopic M/Z: 1463.66959
Total Abundance: 100.00%

Isotope Number	m/z	Percent Total	Percent Maximum
0	1463.66959	42.43	100.00
1	1464.67236	32.89	77.52
2	1465.67386	16.28	38.37
3	1466.67527	6.02	14.18
4	1467.67672	1.80	4.24
5	1468.67827	0.46	1.07
6	1469.67992	0.10	0.24
7	1470.68164	0.02	0.05
8	1471.68354	0.00	0.01
9	1472.68584	0.00	0.00
10	1473.68729	0.00	0.00

MSMS SEQUENCE IONS

MH^{+1}(av)	MH^{+1}(mono)
1464.4440	1463.6696

b				y
---	1	**G**	13	---
172.0717	2	**N**	12	1406.6481
335.1350	3	**Y**	11	1292.6052
434.2034	4	**V**	10	1129.5419
533.2718	5	**V**	9	1030.4735
634.3195	6	**T**	8	931.4050
749.3464	7	**D**	7	830.3574
886.4054	8	**H**	6	715.3304
943.4268	9	**G**	5	578.2715
1030.4588	10	**S**	4	521.2500
1190.4895	11	**C(Carbamidomethyl)**	3	434.2180

1289.5579	12	**V**	2	274.1874
---	13	**R**	1	175.1190

Alkylated EGFR wild type peptide - NYVVTDHGSC(Carbamidomethyl)VR

ISOTOPE DISTRIBUTION
Elemental Compositon: C58 H92 N19 O20 S1
Monoisotopic M/Z: 1406.64812
Total Abundance: 100.00%

Isotope Number	m/z	Percent Total	Percent Maximum
0	1406.64812	43.67	100.00
1	1407.65091	32.68	74.83
2	1408.65234	15.78	36.13
3	1409.65371	5.69	13.04
4	1410.65513	1.66	3.81
5	1411.65666	0.41	0.94
6	1412.65831	0.09	0.20
7	1413.66002	0.02	0.04
8	1414.66193	0.00	0.01
9	1415.66407	0.00	0.00
10	1416.66583	0.00	0.00

MSMS SEQUENCE IONS

MH^{+1}(av)	MH^{+1}(mono)
1407.3918	1406.6481

b				y
---	1	**N**	12	---
278.1135	2	**Y**	11	1292.6052
377.1819	3	**V**	10	1129.5419
476.2504	4	**V**	9	1030.4735
577.2980	5	**T**	8	931.4050
692.3250	6	**D**	7	830.3574
829.3839	7	**H**	6	715.3304
886.4054	8	**G**	5	578.2715
973.4374	9	**S**	4	521.2500
1133.4680	10	**C(Carbamidomethyl)**	3	434.2180

1232.5364	11	**V**	2	274.1874
---	12	**R**	1	175.1190

Appendix C
Ethics Committee Approval

Human Research Ethics Board
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Tel (250) 472-4545 Fax (250) 721-8960
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Human Research Ethics Board
Certificate of Approval of Waiver

Principal Investigator	Department/School	Supervisor
Brinda Shah **Master's Student**	**BIOC**	**Dr. Christoph Borchers**

Co-Investigator(s):

Project Title: **The development of an Antibody Affinity Enrichment and Mass Spectrometry-based Assay (iMALDI) for the Characterization of EGFR and EGFR isoforms from brain cancer tissue**

Protocol No.	Approval Date	Start Date	Expiry Date
10-217	**27-May-10**	**27-May-10**	**26-May-11**

Certification

This certifies that the UVic Human Research Ethics Board has examined this research protocol and concluded that, in all respects, the proposed research meets the appropriate standards of ethics as outlined by the University of Victoria Research Regulations Involving Human Participants.

This Certificate of Approval is valid for the above term provided there is no change in the protocol. Extensions and/or amendments may be approved with the submission of a "Request for Annual Renewal or Modification" form.

10-217 Sh