Immuno Tandem Mass Spectrometry (iMALDI) Assay for Clinical Diagnostics

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ABSTRACT

JIAN JIANG: Immuno Tandem Mass Spectrometry (iMALDI) Assay for Clinical Diagnostics
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For infectious diseases and cancers, currently there is no ideal diagnostic method. I present here an iMALDI assay that holds the promise as the future platform of clinical diagnostics. It uses immobilized anti-peptide antibodies and MALDI-MS to detect and quantify protein expression and modification levels. By determining peptide MW with MS and sequence with MS/MS, the iMALDI assay is highly sensitive and nearly absolutely specific. Furthermore, this technology is safe and capable of absolute quantitation, multipexing and high-throughput analysis. The iMALDI-based diagnostics has been developed for Francisella tularensis (*F. tularensis*), an infectious bacterium, and the epidermal growth factor receptor (EGFR), a marker for several cancers.

F. tularensis has been designated as one of the ten organisms most likely to be engineered for bioterrorism. Methods for early and specific diagnosis are of critical importance. The F. tularensis iMALDI assay provides unambiguous detection of F. tularensis peptides at attomole levels from peptide solutions, and at low CFU levels from bacteria. It allows absolute quantitation of the *F. tularensis* target peptide and therefore the parent protein. It is able to provide absolute specificity, avoiding "false positives" from the non-specific binding. The assay is also

applied to samples that are more useful for screening large populations, such as nasal swabs and urine. It is also safe.

EGFR is highly expressed in a variety of tumors, and is therefore an important biomarker for cancer diagnosis and a target for cancer therapy. The EGFR iMALDI assay can detect EGFR in the low attomole range in buffer and in one mammalian breast cancer cells. It is highly specific. It also allows absolute quantitation of the target peptide and therefore the parent protein. This technique is capable of detecting EGFR in tumors.

The iMALDI assay can be easily adapted to other target peptides and therefore has broad applications in clinical diagnosis and therapy selection of other pathogens and diseases.

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ABBREVIATIONS

ABC, ammonium bicarbonate;

BSA, bovine serum albumin;

CFU, colony forming units;

CNBr, cyanogens bromide.

CRP, C-reactive protein;

EGFR, epidermal growth factor receptor;

ELISA, enzyme-linked immunosorbent assay;

F. tularensis, Francisella tularensis;

HCCA, α-cyano-4-hydroxycinnamic acid;

HHA, hand-held assay;

HPLC, High Performance Liquid Chromatography;

ID-MS, Isotope dilution-mass spectrometry;

IHC, immunohistochemistry;

iMALDI, immuno Tandem Mass Spectrometry;

LDH, Lactate Dehydrogenase, Lactic Acid Dehydrogenase;

LPS, lipopolysacchride;

LPS, lipopolysacchride;

LVS, Live Vaccine Strain;

MALDI, Matrix-Assisted Laser Desorption/Ionization;

MS, mass spectrometry;

MS/MS (or MS^2), tandem MS;

PBS, phosphate-buffered saline;

PCR, polymerase chain reaction;

PSA, prostate-specific antigen;

RT-PCR, real time-polymerase chain reaction;

SELDI, surface-enhanced laser desorption/ionization MS;

SISCAPA, Stable isotope standards with capture by anti-peptide antibodies;

TOF, Time-of-flight

CITATIONS TO PAST WORKS

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CHAPTER

1 OVERVIEW

1.1 Infectious diseases

In the USA, each year the number of cases of illnesses recognized related to infectious diseases is over 5 million (1). The number of cases unrecognized is significantly greater. Infectious diseases result in substantial morbidity and mortality (2). To win the battle over infectious diseases requires critical and timely intervention that relies on rapid and accurate detection of the pathogen in the acute-care setting and beyond.

Since the anthrax outbreak after the September 11, 2001 terrorist attacks, attention toward the threat of bioterrorism has been considerably increasing. For front-line acute care physicians to initiate appropriate response measures, rapid recognition and accurate diagnosis of real or suspected bioterrorism events is critical (3).

1.2 Cancers

In industrialized countries, cancer is one of the leading causes of death. Each year the number of diagnosed new cases of cancer worldwide is about 10 million. Despite recent substantial progress made in cancer therapy due to advances in molecular medicine, genomics, proteomics, and translational research,

our current efforts to combat cancer are not very successful. Mortality rates for the most prevalent cancers have not been significantly reduced. Some of the best available options to decrease overall mortality from cancer include primary prevention and earlier diagnosis (4). If the disease can be detected early enough, there may be an opportunity to drastically reduce the burden of cancer. The earlier a cancer is detected, the better the chances of treating it successfully. For example, the earliest diagnosis of breast cancer could bring 5-year survival rates up to 98% (5).

1.3 Clinical diagnosis

1.3.1 Time

Not only in cases of infectious diseases where acute care is usually needed, and cancers of which mortality is high, but also in any other cases of diseases, early diagnostics is necessary. The earlier a disease is detected, the better the chances of treating it successfully, and the earlier the patients and their families' suffering ends. Otherwise, some diseases may develop to a more severe condition. The patients would need more care and some may have to stay in hospital. It leaves the patients and their families more suffering and greater economic burden. This also adds more burdens on health care workers and the hospitals, health insurance companies and the whole health care system in terms of workload and expenses. In the worst cases, some diseases that are curable in the beginning or not lethal themselves, just because it was discovered too late, can develop so serious as to become incurable or convert to some other untreatable lethal diseases and even cause death. These are unnecessary at all and should be avoided with best effort.

However, a person usually cannot tell whether he or she is sick or need go to hospital at the very beginning of a disease's development. When we go to see a doctor, the disease probably has already progressed to a certain stage. Since the populace has limited medical knowledge that is apparently not enough for self-diagnosis, this delay is unavoidable. The majority cases of cancer are diagnosed only after tumors have metastasized, which leaves the patient with a grim prognosis and delays treatment. Thus, a rapid reliable diagnostic assay, which allows for accurate identification of pathogens and informed early therapeutic intervention would be invaluable (3).

1.3.2 Costs

As mentioned previously, no matter to the patients or the health care system, slow and delayed diagnosis is expensive in terms of time and effort. The later the treatment is delayed, the more it costs. A diagnostic tool itself should not be too costly to limit its accessibility to the populace either. Otherwise, its application in clinics would be very limited.

1.3.3 Sensitivity

A diagnosis should be sensitive and avoid false negatives. False negatives can cause some consequences as same as slow and delayed diagnosis. Because the disease is not detected, treatment is delayed, and the best timing for treatment may have been missed. Some diseases can progress to severe stage, or even cause death that can be avoided if detected and treated in time. Patients and their families suffer more and longer, with greater economic burdens.

1.3.4 Specificity

When referring to a medical test, clinical specificity refers to the percentage of people who test negative for a specific disease among a group of people who do not have the disease. Here, “specificity” means that there are no “false positives” – i.e., the test result is specific for the target organism/disease.

A diagnostic method, if gives too many false positive results, even if it is rapid, cost-effective and sensitive, it is still not a useful clinical tool. Speed and relatively low cost do not mean poor quality. When a diagnostic method is being evaluated, specificity is another important factor.

For people who do not have the disease, a “false positive” result causes them unnecessary pains and troubles. All efforts to treat an illness that actually does not exist would be wasting our already limited resources. For people who have another disease different from the diagnosed one, it results in a wrong treatment, which can pose great dangers to the patients.

1.3.5 Quatitation

Some diseases, such as cancers, are detected by the detection and quantitaion of some biomarkers that are characteristic of certain types of cancer. The staging of the diseases may also be based on the level of the biomarkers. Not only in clinical but also in research laboratories, quantitation of some specific target reagents, such as proteins, peptides and organisms, can provide a lot of useful information. Therefore, quantitation capability of a diagnostic method is also important and useful.

1.3.6 High throughput analysis

The capability of a diagnostic method for high throughput analysis is important for screening large populations.

1.3.7 Safety

An ideal diagnostic method should be safe. Some methods, such as traditional culture-based methods, because they have to keep the organisms alive, pose hazards not only to the laboratory personnel, but also potentially to the populace if the disease-inducing reagents leak out of the laboratories by any chance. Although strict laboratory practice measures can be taken, no absolute safety can be guaranteed.

1.4 Current techniques

1.4.1 Overview

For infectious diseases and cancers, there are enormous diagnostic methods. Each has its pros and cons. For example, although culture-bases techniques are most widely used in conventional hospital laboratories, these techniques are slow, insensitive, unspecific and hazardous. Immunohistochemistry (IHC) is a sensitive method for cancer diagnosis, but due to its poor staining and high noise level, its specificity is low. Molecular diagnostics have been herald as “the diagnostics of the new millionaire”. Because they detect and analyze on molecular levels, they have a lot of incomparable advantages that conventional methods lack, such as the molecular specificity. They can be specific to DNA or protein level to different extent.

The enzyme-linked immuno sorbent assay (ELISA) and polymerase chain reaction (PCR) are both sensitive and widely used, while PCR is more rapid. Some diagnostics, such as ELISA and PCR, have more or less quantitation capabilities, some very limited though.

Some current diagnosis techniques are reviewed in the following sections, in two categories, cell and molecular based respectively. Some of them are already widely used in clinics for years, some are also widely used in research laboratories, and some are still under development but have potential for future clinical applications.

1.4.2 Cell-based diagnostics

1.4.2.1 Culturing

In a traditional culturing method, the organism is cultured in a medium containing some kind of chemical reagent that will indicate the physical property change of the medium because of the organism growth.

Culturing is widely used in conventional hospital laboratories. However, culturing is cumbersome, requiring intensive labors and additional special settings. It is time consuming. Assays for fastidious pathogens can be extremely prolonged, up to several weeks. Its low sensitivity can result in high false negatives. Characterization of detected pathogens, e.g. discernment of species, strain, virulence factors, requires additional testing and more time. For patients who have received antibiotics, test sensitivity is diminished. Sometimes they are unable to culture certain pathogens in disease states associated with microbial infection.

Because the organisms must be kept alive, it is also hazardous to laboratory personnel.

Limitations of conventional culturing methods make such tests unreliable and inadequate for clinical diagnosis of infectious diseases, not to mention timely and accurate detection of bioterrorism agents in suspected outbreaks.

1.4.3 Molecular-based diagnostics

Molecular diagnostics, heralded as the “diagnostic tool for the new millennium”, is revolutionizing the clinical practices, especially in acute care settings where timely and accurate diagnostic tools are critical for patient treatment decisions and outcomes (3).

Molecular methods diagnose on molecular level through the detection, characterization and/or quantification of signature molecules, such as nucleic acids and proteins. Unlike in conventional culture-based tests, the presence of viable organisms is not required for the identification of infectious agents, such as bacteria, viruses, and fungi, and therefore the detection of difficult to culture microorganisms is possible (6).

Although the costs of molecular testing may be relatively high compared to most traditional clinical laboratory tests, the average cost per test may actually not, and the overall cost to the health system may be ultimately decreased due to improved diagnosis efficiency.

Molecular-based diagnostics can be categorized into two groups, DNA/RNA-based and protein-based.

1.4.3.1 DNA/RNA-based diagnostics

1.4.3.1.1 Fluorescent in situ hybridization (FISH)

Fluorescent in situ hybridization, or FISH, a cytogenetic technique which studies the structure of chromosome material, has emerged as a powerful clinical and research tool. Compared to routine cytology, it is relatively simple, very robust, more sensitive, and thus applicable in material of lesser quality (7).

In FISH, a fluorescent probe binds to those parts of the chromosome with which it shows a high degree of sequence similarity. Then fluorescence microscopy is used to find out where the fluorescent probe binds to the chromosome.

FISH is used to detect and localize specific DNA sequences on chromosomes. It can identify different chromosomal abnormalities including changes in chromosome number, translocations, deletions, rearrangements, and duplications.

1.4.3.1.2 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) is a technique for enzymatically replicating DNA.

The PCR reaction takes place in a thermocycler. Each PCR cycle consists of three major steps. (1) “Denaturation”: heated to a certain temperature, double-stranded template DNA is separated into single-stranded DNA. (2) “Annealing”: temperature lowered, the primers attach to their complementary target single DNA strands. (3) “Elongation”: the DNA polymerase extends the annealed primer along the DNA strand to generate new copy of the target DNA. At the end of each cycle, the newly synthesized DNA strands act as new targets for the next cycle. Subsequently, the cycle repeated multiple times, the target DNA is amplified

logarithmically. The PCR product is then identified by its size using agarose gel electrophoresis.

PCR can be performed quantitatively, using fluorescent dyes and probes to measure the amount of amplified product in real time, which is known as real-time PCR (RT-PCR). It can quantify starting amounts of DNA, cDNA or RNA indirectly. It is oftern used to determine whether a sequence is present or not.

Compared to conventional culture-based techniques, PCR is more sensitive, specific, and rapid; it has been utilized for identifying organisms that are difficult to grow in vitro by existing culture techniques (3). With all required reagents ready and reaction conditions optimized, a PCR reaction usually takes only a few hours. The detection of the reaction results does not take long either.

Clinically, PCR has been used for detection of specific or broad-spectrum pathogen, evaluation of emerging novel infections, surveillance, early detection of bioterrorism agents, and antimicrobial resistance profiling. In medical and biological research labs, PCR is commonly used for a variety of tasks, such as detection of hereditary diseases, identification of genetic fingerprints, diagnosis of infectious diseases, cloning of genes, paternity testing, and DNA computing.

The greatest problem that hampered the widespread use of PCR in clinical settings comes from false-positive results. The predominant reason is the background DNA contamination from the products of earlier PCR reactions. These “carry-over” products may be transmitted through PCR reagents, tubes, pipettes, and laboratory surfaces. Even very minor amounts of carry-over contamination may be amplified, which lead to false-positive results. Although contamination risks can be reduced by meticulous control measures, such as good laboratory practices and

physical separation of pre-amplification and post-amplification areas, these measures are not foolproof. PCR materials can be decontaminated by destroying DNA using, for example, ultraviolet irradiation, chemical treatment, and enzymatic digestion (8, 9). However, these methods at the same time will diminish the assay sensitivity (3).

Also, PCR assays may give false-negative results due to its failure. There may be problems associated with PCR processing. For instance, the primers may not work, the temperatures are possibly not optimized, and the annealing can fail. Because the sample volume that PCR assay accommodates is very small, when the concentration of infectious organisms in the sample is too low, the assay may not be sensitive enough and would yield false-negative results. Efforts to improve an assay's detection sensitivity may need to be individually adjusted based on the assay's clinical application and the microbial pathogen of interest.

Detected pathogens need to be further characterized so that pathogens' species or strains, virulence factors, and antimicrobial susceptibilities can be ascertained. Genetic sequences contain rich sources of information that can be analyzed for this purpose. To acquire these sources of information, several target genes need to be amplified simultaneously in a single reaction, which is called multiplexing. Multiplexing-PCR can be performed with different primer pairs used in a single reaction, but in this case annealing temperatures for each primer pair must be optimized to work correctly within a single reaction. Oftentimes one or more of the target sequences do not amplify. Unless a single primer set can be found suitable for amplification of all these fragments, performing multiplex-PCR is very difficult. In any multiplex-PCR, amplicon sizes should be separated by enough

difference in final base pair length to form distinct bands via gel electrophoresis. Although the real-time PCR with potential use of differentially labeled fluorescent probes to identify multiple amplified products simultaneously in a single assay holds promise (10). Unfortunately, current ability to spectrally differentiate multiple fluorescent signals is quite limited (3).

Typically, PCR products are detected and analyzed by gel-electrophoresis and sequencing techniques. These approaches are laborious, time consuming, and not suitable for clinical applicability. The best way to analyze the resultant PCR products remains unclear.

1.4.3.2 Protein-based diagnostics

1.4.3.2.1 Agglutination

Agglutination test is a blood test used to identify unknown antigens; a known antibody is added to blood with the unknown antigen, and whether or not agglutination occurs helps to indicate whether or not the antigen exists in the blood. It can be used for diagnosis of infection and to identify pathogens and blood types.

Agglutination tests can be used to quantitate the level of antibodies in a sample. Serial dilutions of the sample are made and then a fixed number of red blood cells or bacteria or other such particulate antigen are added. The maximum dilution that gives visible agglutination, called the “titer”, is determined. The results are reported as the reciprocal of the maximal dilution that gives visible agglutination. It is only semi-quantitative.

The agglutination pattern is usually determined by visual examination, which is time consuming, cumbersome, and highly subjective.

1.4.3.2.2 Immunohistochemistry (IHC)

Immunohistochemistry or IHC is widely used in the diagnosis of cancer through detection of specific molecular markers that are characteristic of particular cancer types. In common instances, biopsies are processed into sections with a microtome and the sections are incubated with an appropriate antibody. The antibody is tagged to a visible lable of which the most popular is an enzyme that can catalyse a colour-producing reaction or a fluorophore. The site of antibody binding is then visualized under an ordinary or fluorescent microscope to localize the antigen in cells of a tissue section.

IHC is also widely used in basic research to understand the distribution and localization of biomarkers in different parts of a tissue. It can also be used to visualise the interactions between multiple proteins.

In IHC techniques, there are many potential problems affecting the outcome of the procedure. The two major problems are a poor signal/noise ratio and poor staining. To solve these problems requires time-consuming optimization steps.

1.4.3.2.3 Enzyme-linked immuno sorbent assay (ELISA)

The enzyme-linked immuno sorbent assay, or ELISA, is an immunology technique used to detect the presence of an antibody or an antigen in a sample. It is currently one of the most common applications of protein microarrays (11).

Usually the ELISA test is performed in a 96-well microtiter plate. In a typical “sandwich” ELISA to detect a sample antigen, the well of the plate is coated with a capture antibody which serves to bind and concentrate the antigen onto the surface.

The sample is added for incubation and any antigen present binds to the capture antibody. A detection antibody is added and binds to the same antigen as the capture antibody, but does so at a different site. Then enzyme-linked secondary antibody is added and binds to detecting antibody. A substrate is added later and converted by the enzyme to a detectable form which causes a chromogenic or fluorogenic signal, indicating a positive reaction.

The ELISA can be used for detecting the presence of an antigen or antibody in a qualitative format, or for determining its concentration in a quantitative format. A qualitative ELISA simplely provide a positive or negative reading between which the cutoff is determined by the analyst and may be statistical. In a quantitative ELISA, the target standard is diluted in series, and spot intensities are measured via imagery of the treated arrays and plotted against the protein concentrations. By fitting an appropriate function to the set of (intensity, concentration) measurement pairs, a standard curve is estimated (12). The concentration of the sample is then estimated by fitting the spot intensity into the standard curve.

There are several factors that determine the sensitivity and precision of an ELISA. These include but are not limited to: the affinity of antibodies; the amount of immobilized antigens or antibodies on a solid phase; the incubation time; the eddiciency of enzyme conjugates; methods for signal detection; and signal amplification. Manipulating any of these or other factors could potentially enhance the detection signal and thereby improve the detection sensitivity of an assay. The use of the matched pair of high-affinity reagents for a single analyte also helps to improve both the assay sentitivity and specificity. However, not compromising the ability of a system to determine the true value of the analyte requires extreme care

(13). Moreover, ELISAs can only be applied to proteins for which paired antibodies and an antigen standard are available for the assay, which limits the applications of ELISAs (11).

1.4.3.2.4 Surface-enhanced laser desorption/ionization-mass spectrometry (SELDI-MS)

Surface-enhanced laser desorption/ionization-mass spectrometry, or SELDI-MS is an array based mass spectrometric method that can identify reproducible patterns of protein profiles. It has been applied as a serum/plasma biomarker discovery platform that can be used for diagnosis, prognosis and monitoring of disease progression (14).

SELDI-MS couples protein separation directly to presentation to the mass spectrometer. Proteins of interest are selectively absorbed onto a chemically modified surface and then subjected to mass spectrometric analysis. Based on targets' biochemical properties the surface can be modified in various ways in order to achieve varying affinity to different subsets of proteins for better protein separation. The surface can be modified for anion exchange, cation exchange, normal phase, reversed phase, and immobilized metal affinity chromatography (IMAC). The resulting mass spectra are analyzed by statistical tools to yield protein profiles (15).

This method could work quantitatively if the surfaces used for molecule immobilization are specific for certain proteins (e.g. antibodies or other binders), or they have enough capacity to bind all the proteins applied to the sample (4).

1.4.4 Summary

Each method has its own strengthes and weaknesses. No single method will be optimal in all applications. Our immuno tandem mass spectrometry (iMALDI) technique is developed to address some of the problems associated with these current methods such as culturing, PCR, SELDI and ELISA.

CHAPTER 2

2 IMMUNO TANDEM MASS SPECTROMETRY (iMALDI) ASSAY

2.1 Immuno tandem mass spectrometry (iMALDI) assay

Immuno tandem mass spectrometry (iMALDI) assay is based on immunopurification of a representative peptide followed by identification and quantification of this peptide using MS. It is the future platform of clinical diagnostics, which can be applied to quantify specific diagnostically relevant proteins. This assay is inexpensive, highly sensitive, highly specific, quantitative and potential for high throughput analysis and safe.

2.1.1 Scheme

Basically, anti-peptide antibodies are produced and immobilized on affinity beads. The proteome of interest is proteolytically digested. Isotopically labeled epitope-containing peptides, called “heavy” peptides, are added into the digest as the internal standard for quantitation, and then incubated with the antibody-beads to immuno-adsorb the epitope-containing target peptides. After immuno-adsorption, antibody-beads are arranged in a microarray/spot format on the MALDI-target plate. MALDI matrix solution is then added, which enables the elution of the affinity-bound peptides from the immobilized antibodies permitting MALDI-analysis of the peptides. The relative abundances of the molecular ion signals corresponding to the original or light and heavy peptides are used to quantify the amount of this protein in the

original sample. Absolute specificity can be achieved by mass spectrometric sequencing of the epitope containing peptide, using MALDI-MS/MS (**Figure 1**).

2.1.2 Peptide vs. protein

The iMALDI is based on detection and quantitation of peptides instead of proteins. Because peptides are more stable and less susceptible to denaturation, preserving native structure is not necessary and there's no protein class restriction. The detected peptides can be sequenced for identification.

2.1.3 Bead vs. plate

For affinity capture, anti-peptide antibodies are immobilized on inexpensive affinity beads rather than on the surface of a plate as is done in the conventional protein chip technology, using standard immobilization techniques, therefore no specific protein surface chemistry required. Moreover, compared to limited plate surface on chips, since the surface area of beads is larger, the binding capacity is increased, and therefore using beads surfaces could be more sensitive (16). A planar array sometimes suffers from non-uniformity and slow diffusion of targets to the binding surface. Using beads can circumvent these problems since the reactions proceed in a solution-like environment with solution kinetics. Moreover, the reactions proceed with an active mixing, which helps diffusion and therefore binding of the targets to the immobilized antibodies. This can result in a gain of sensitivity and improve accuracy and reproducibility (17). The beads with different antibodies conjugated to the surface can be mixed together for multiplexing in which different

targets can be detected and analyzed simultaneously in one reaction. Therefore iMALDI assay has the potential for high throughput analysis.

2.1.4 Highly specific capture reagents

Because iMALDI is based on immunoaffinity capture, highly specific antibodies remain a prerequisite. However, the current techniques for generation of antibodies can not guarantee generated antibodies are always highly specific. In cases highly specific antibodies cannot be obtained, antibody mimics, such as aptermers, are alternative candidates.

2.1.5 Costs

A clinical diagnosis should be affordable and accessible to the populace. So its cost is one of the important factors that are critical to its development. For iMALDI, the costs of antibodies and MS instruments seem to be the major expenses.

Generation and production of antibodies need to be cost-effective. It currently costs about $2,000~$2,500 to raise an antibody by a commercial company such as SIGMA-GENYSIS. A package in this price range includes synthesis of the epitope-containing peptide, ELISA analysis of the serum containing the developed antibodies, affinity purification, and host animal maintaining. Although the price is not cheap, the amount of produced antibody is usually enough for analyzing quite an amount of samples. The antibody cost per sample is actually not as high as it appears. Moreover, the technology is still developing. In the future the antibody production will be more cost-effective.

Compared to other types of chemical analysis instruments, mass spectrometers are expensive. But to use iMALDI, one does not have to have a mass spectrometer themselves. They can send the samples to a facility that has the appropriate mass spectrometer for analysis. Therefore the cost of MS instrument is actually not a big concern for iMALDI users.

2.2 Mass spectrometry as the detection and analysis method

MS analysis of peptides is rapid (a few seconds per sample by MALDI-MS), accurate, sensitive, and specific (because of the accurate determination of the molecular weight by MS and the accurate determination of the amino acid sequence by MS/MS). These features allow the analyst to distinguish non-specifically-bound, and even cross-affinity-bound, peptides from the specific target epitope peptide. Mass spectrometry is also well suited for peptide/protein quantitation. MS can perform relative quantitation using reference peptides from two different samples that have been differentially-labeled with stable isotopes, or absolute quantitation if a known amount of a stable-isotope labeled peptide has been added as an internal standard.

2.2.1 Matrix-Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF)

MALDI-TOF MS is an important and popular analytic tool for identifying the mass identity of biomolecules. Typically, samples are mixed with an organic compound that acts as a matrix (protonated at acidic pH) to facilitate desorption and ionization of compounds in the sample. The analyte ions are then accelerated by an

applied high voltage (15-25 KV), separated in a field-free flight tube and detected as an electrical signal at the end of the flight tube.

2.2.1.1 Matrix-Assisted Laser Desorption/Ionization (MALDI)

Several advantages have been demonstrated with MALDI, including spectral simplicity due to singly charged ions, a high mass range (up to >900 kDa), low noise levels, high sensitivity, little sample consumption, short measurement times, average salt tolerance and minimal fragmentation.

2.2.1.2 Time-of-flight (TOF)

A Time-of-flight (TOF) mass analyzer separates ions and measures their m/z based on the time they spend to “fly” from the ion source to the detector. A TOF mass spectrometry can be operated in the linear mode or the reflectron mode. The latter is utilized for our analysis of peptides. Compared to the linear mode of TOF, the reflectron mode considerably improves mass resolution and thus mass accuracies.

In the linear mode, due to the spatial distribution of ions in the ion source and their proximity to the applied electric field, not all the ions receive the same initial kinetic energy. These factors give rise to relatively poor mass resolutions of the order of 100-500. This leads to components in mixtures being unresolved from one another and large errors (~1%) in molecular weight measurements.

A reflectron is constructed of a stack of donut-shaped lens across which a high voltage is applied. The potential applied across the lenses of the reflectron causes the ions that enter it to be gradually repelled. Ions of different kinetic

energies penetrate the mirror to differing degrees. These ions are then reflected down the same or second flight tube to a second detector. The difference in the flight path and time corrects for the differences in the kinetic energies of the ions so that they reach the detector at the same time. Furthermore the reflectron effectively extends the flight tube to almost twice its length, which can be seen to have a dramatic effect on an ion's flight time. So the reflectron mode considerably improves mass resolution and thus mass accuracies.

2.2.2 Tandem MS (MS/MS)

The advantage of MS as readout platform for immunodetection is the high molecular specificity. The specificity is achieved by mass spectrometric sequencing of the epitope containing peptides, using Tandem MS (MS/MS).

In Tandem MS or MS/MS, the peptide with a specific mass is selected, called the "precursor ion" or "parent ion". The precursor ion is fragmented and the fragment ions are separated and detected. Therefore an MS/MS spectrum can provide the sequence information of the precursor peptide ion. A peptide can be fragmented in several ways, before, at or after the peptide bond, giving rise to "a" and "x" ions, "b" and "y" ions, "c" and "z" ions respectively. Usually the "b" and "y" ions that are produced from breaking the peptide bond are annotated.

2.3 Applications

The iMALDI has a wide range of applications, including but not limited to quantification of known clinical markers (peptides, proteins, carbohydrates, small molecules) with molecular specificity, quantification of biological warfare agents, and

identification of protein isoforms, variants, modifications. It can be applied in clinical environment and in army hospital type acute care settings.

2.4 iMALDI vs. ELISA

Like ELISAs, iMALDI is also an antibody-based technique. Instead of binding to a surface, however, anti-peptide antibodies (not anti-protein antibodies) are bound to CNBr-activated sepharose, and the blocking of unreacted sites is also done on the beads. Unlike ELISAs, however, analyte detection in iMALDI is not based on secondary colorimetry or fluorescence, but on the fundamental properties of the bound analyte – its molecular weight and amino acid sequence. The iMALDI technique is sometimes referred to as a “peptide chip” assay because multiple samples can be analyzed by mass spectrometry as an array of different anti-peptide antibody beads on a MALDI target.

Like ELISA, iMALDI does not require extensive protein separation, and low-abundance proteins can be analyzed in the presence of high-abundance proteins, providing both speed and dynamic range. Because peptides are more stable and less susceptible to denaturation, iMALDI should be more reproducible from sample to sample than ELISAs or chip techniques based on anti-protein antibodies, and sample storage requirement should not be as rigorous. Unlike ELISAs, iMALDI requires only one anti-peptide antibody, which lowers the cost of antibody production. Antibodies are immobilized on inexpensive sepharose beads for affinity capture, as in the conventional affinity capture/elution/MALDI technique (18), rather than on the surface of a plate, thus eliminating the need for special surface chemistry. Additional advantages of using affinity beads are less-stringent sample storage

requirements after antibody immobilization, fewer complications resulting from denaturation of the capture antibody in solution, and the ability to incubate several types of beads with small volumes of tryptic-digested biological fluid instead of “soaking” the entire target.

2.5 Summary

In summary, the iMALDI uses only one antibody, so compared to ELISA that uses at least two antibodies, iMALDI is less expensive in terms of antibody production. In iMALDI, serial dilutions of the sample are not required. By using internal standards, iMALDI can be used for absolute quantitations. MS provides molecular specificity and protein isoforms specificity. The iMALDI is also an extremely flexible format. Multiple antibodies can be used for multiplexing. The iMALDI has a wide range of applications. It is the future platform of clinical diagnostics.

CHAPTER 3

3 iMALDI FOR DETECTION OF FRANCISELLA TULARENSIS

3.1 Francisella tularensis (*F. tularensis*)

Francisella tularensis (*F. tularensis*) is a small, nonmotile, aerobic Gram-negative cocco-bacillus that causes tularemia. The bacteria can penetrate unbroken skin, can survive and multiply within macrophages, and can then spread to various organs through the blood (19). Ten bacteria injected subcutaneously (20), 10-50 on contact with unbroken skin (21), 10-50 given by aerosol (22, 23), or 10^2 to10^8 bacteria by ingestion (21) are sufficient to cause infection. Humans who have direct physical contact with infected animals or insects, or have inhaled aerosolized bacteria, have a good chance of becoming infected. Untreated, the mortality rate can be 30% (24). Although normally tularemia in humans is relatively rare, *F. tularensis* has the potential to be engineered for bioterrorism.

Initial symptoms are flu-like and non-specific. They usually appear 3 days after exposure (25). General laboratory tests (CRP, LDH, alkaline phosphatase, leukocytes, etc) are insufficient for diagnosis. Because early antibiotic therapy (with streptomycin or gentamicin) can greatly reduce the lethality rate (26), an immediate diagnosis of an infection with *F. tularensis* is critical. Because of the virulence of *F. tularensis*, the severity of the disease, the rapidity of progression from initial onset to seriousness or death, and the high mortality rate, several agencies (27) have been

concerned about the weaponization of *F. tularensis*, especially the use of aerosolized bacteria by terrorists as an airborne pathogen (28-30). *F. tularensis* has been designated as one of the ten organisms most likely to be engineered for bioterrorism (31), and one of the six "category A bioterrorism organisms" (19, 29, 32-34).

3.2 Current diagnostic techniques for *F. tularensis*

Bacteriological methods can be used for detecting *F. tularensis* (35), but culturing the organism is difficult (20, 22, 28, 36), time-consuming (sometimes taking several days), and is potentially hazardous to laboratory personnel (37). Moreover, several studies have shown (38) that the sensitivities and specificities of these methods are low (note: "specificity" means that there are no "false positives" – i.e., the test gives a positive result ONLY for the target organism).

Since culturing *F. tularensis* is difficult, serological tests such as the bacterial microagglutination (MA) test, have been used to diagnose tularemia, but this takes 1 week for measurable levels of antibodies to develop, and an additional week for sufficient antibody levels for a reliable test (28, 39-42). Antibodies against Francisella may cross react with other organisms such as Brucella, Proteus OX19, and Yersinia spp. (28), which reduces the specificity and could lead to false positives. More recently, another type of serological test, an enzyme-linked immunosorbent assay (ELISA), achieved a detection limit of 10^3 bacteria/mL in PBS and 10^4 bacteria/mL in human serum (43). However, when samples are contaminated with other microorganisms (44), accurate diagnosis with ELISA or culturing methods is very difficult due to their low specificities.

PCR is a molecular method for detection of *F. tularensis* that usually only takes several hours per reaction. A hand-held assay (HHA) PCR (43) could detect *F. tularensis* in 3 hours, and achieved the analytical sensitivity of 100 bacteria/mL PBS or 10^3 – 10^4 bacteria/mL serum (45). A real-time Taq-Man PCR assay, combining multiple individual assays, showed a detection limit of one organism (46). However, PCR may give false positives from contamination with other DNA, which lowers its specificity. Moreover, simultaneous assaying for multiple species or virulence factors by PCR is difficult (3).

3.3 iMALDI for *F. tularensis*

The peptide-based immuno tandem mass spectrometry (iMALDI) assay circumvents many of the problems associated with PCR and ELISA, and is distinguished by its ability to provide absolute specificity, absolute quantitation, and high sensitivity.

The iMALDI assay for detection of *F. tularensis* is based on capture of the *F. tularensis* IglC aa 49-61 peptide. This assay is capable of fast, safe, sensitive, and specific detection of *F. tularensis* in environmental samples. It can be used for absolute quantitation of target peptides and, therefore, for absolute quantitation of their parent proteins. It is also demonstrated that iMALDI is applicable to the detection of *F. tularensis* in clinical samples, such as human plasma and nasal swabs.

3.4 Materials and Methods

3.4.1 Target protein

The 23kDa protein, IglC, from *F. tularensis* bacteria is encoded by iglC, whose sequence has no significant homology to any other gene present in the GenBank database (47). Live Vaccine Strain (LVS) bacteria, at a concentration of $2.25x10^9$ CFU (Colony Forming Units)/mL in 70% ethanol, were used. The total protein concentration of the bacteria sample was 700ng/mL as determined with an ELX800 Universal Microplate Reader.

3.4.2 Nasal swab samples

Mice were inoculated intranasally with 1×10^5 CFU LVS. Two, five and seven days post inoculation, a small, moist, alginate swab was used to rub across the nostrils of the mice. The swab was then swirled in a tube containing 100 µL PBS. Next, 100 µL of 95% ethanol was added to the sample to ensure inactivation.

3.4.3 Tryptic digestion

Digestion of human plasma (UNC blood bank) was carried out in 25mM ammonium bicarbonate (Sigma) at 37^{o}C overnight. A ~1:10 enzyme:substrate ratio of trypsin (Sequencing-grade modified trypsin, Promega) to protein was used. The digested human plasma was spiked with *F. tularensis* peptides or a bacterial digest to mimic clinical samples. Before digestion, the nasal swab solution was diluted with ammonium bicarbonate until the final concentration of ethanol was 38%, which is compatible with tryptic digestion.

3.4.4 Antibody production and immobilization of antibodies on beads

This MALDI-MS-based technique to detect bacterial proteins from *F. tularensis* uses a customized antibody that was raised against an *F. tularensis* IglC peptide, selected because of its high sensitivity in MALDI-MS (**Figure 2**).

Four *F. tularensis* IglC tryptic peptides, which are absolutely unique to, and thus diagnostic of *F. tularensis*, were selected for their high sensitivity in the MALDI-MS mode (**Figure 2**). These four peptides were synthesized by Sigma-Genosys. (Note: a cysteine residue was added to some peptide sequences because of ready conjugation with carrier proteins). Antibodies were raised against each peptide, and tested by ELISA to determine the detection sensitivities/efficiencies. The peptide that showed the highest binding efficiency was selected for use in the Francisella peptide chip, and the corresponding antibody was purified by Sigma-Genosys.

The anti-peptide antibody was then immobilized on CNBr-activated sepharose (Amersham Pharmacia) according to the manufacturer's instructions (48). Briefly, the CNBr beads react with the primary amine groups on the antibody, thereby covalently linking the antibody to the beads. Excess binding sites on the beads are blocked by incubation with an amine-containing buffer, (in this case, Tris), and the process is completed by a series of washes at alternating pH.

3.4.5 Immuno-adsorption protocol

An aliquot of settled antibody-bead slurry (1-5 µL) was added to a compact reaction column (USB) and washed 4-5 times with 400 µL of 0.1x PBS. To mimic an environmental sample, a bacterial digest or synthetic *F. tularensis* IglC peptide (CNIVAIEGGEDVTK, aa 49-61) was dissolved in water/buffer and diluted to a series of concentrations, e.g. 25 ng/µL, 5 ng/µL, 0.5 ng/µL, etc. A 20 µL aliquot of each

solution was incubated separately with a small amount (1~5 µL) of antibody-beads to immuno-adsorb the epitope-containing peptide. PBS was incubated with the antibody beads or unconjugated agarose beads as negative controls. *F. tularensis* bacteria spiked into a plasma digest mixture were used to mimic clinical samples. PBS was incubated with the antibody beads or unconjugated agarose beads as a negative control. Three negative control experiments were performed by incubating (a) unconjugated agarose beads with PBS; (b) unconjugated agarose beads with synthetic peptide or bacteria digest sample; and (c) antibody-beads with PBS, respectively.

After incubation for 2-4 h at room temperature with end-to-end rotation on a "Labquake" rotator (Lab Industries), the beads were washed 6 times with 400 µL of freshly-made 50 mM ammonium bicarbonate, or 3 times with 400mM of NaCl (Sigma) followed by 3 times with 50 mM ammonium bicarbonate in case high levels of non-specific binding was expected in complex samples such as plasma. The beads were re-suspended in a small volume of 50 mM ammonium bicarbonate (1-5 µL) and an aliquot of the beads (0.5 µL) was spotted directly onto the MALDI target as described in the Results and Discussion section.

3.4.6 Isotopic labeling for absolute quantitation

The *F. tularensis* IglC aa 49-61 peptide ^{49}NIVAIEGGED<u>V</u>TK^{61} containing an isotopically-labeled valine at position 59 (underlined) (the "heavy" peptide) was synthesized at the UNC Peptide Synthesis Facility, using a ^{13}C-labeled fmoc Valine purchased from Isotec/Sigma-Aldrich. The increase in mass from the unlabeled

("light") *F. tularensis* IglC aa 49-61 peptide was 6 Da. The synthesis was performed according the fmoc approach described in details elsewhere (49).

3.4.7 Absolute quantitation

The “heavy” peptide, dissolved in water (HPLC grade, Pierce) was used as an internal standard. Heavy peptide was spiked into a *F. tularensis* bacterial digest in various amounts, immuno-adsorbd on anti-*F. tularensis* IglC peptide antibody beads, and quantitated by MALDI-MS analysis of the beads on the MALDI target.

3.4.8 Mass spectrometric analysis

MALDI-MS experiments were performed on Bruker Daltonics’ (Billerica, MA) Reflex III and Ultraflex MALDI-TOFs, using Bruker’s Anchor-chip MALDI-target plates (400 or 600 µm/384 spot format). MS/MS analyses were carried out on an Applied Biosystems Voyager 4700 (Framingham, MA) MALDI-TOF/TOF. The matrix used for all experiments was α-cyano-4-hydroxycinnamic acid (HCCA) (Sigma) after re-crystallization from hot methanol. A saturated solution of HCCA in 50:49.9:0.1 acetonitrile (Caledon Laboratories):water (HPLC grade, Pierce):trifluoroacetic acid (Pierce) was used. Ammonium bicarbonate (ABC) solution (50mM) was added for easy placement of the beads on the MALDI target. Following placement of the antibody beads on the target, 0.3-0.5 µL of HCCA matrix was added, and the spot was allowed to dry at room temperature.

3.5 Results and Discussion

3.5.1 Sensitivity

3.5.1.1 Detection of *F. tularensis*/bacteria in PBS solution

By direct MALDI-MS analysis of the beads, the detection limit for the synthetic *F. tularensis* IglC peptide (cysteine was added for antibody production) was determined to be in the low attomole range (14 attomoles) in buffer (HPLC-grade water or PBS). Without enrichment by iMALDI, in MALDI-MS analysis, the detection sensitivity of the peptide in buffer was in the low femtomole range (17 femtomoles) (**Figure 3**). In the enrichment process, the peptides are bound to antibody beads, which greatly reduces losses due to adsorption by tubes and tips. Moreover, when spotted on the target plate, even after elution by the matrix solution, the peptides do not spread out all over the spot area. Instead, they tend to aggregate at “hot spots”, and when the laser is focused at these “hot spots”, greatly increased detection sensitivities can be obtained.

The *F. tularensis* iMALDI assay, based on the anti-*F. tularensis* IglC, aa49-61 antibody, can also detect the target *F. tularensis* peptide from a bacterial digest. The target *F. tularensis* peptide was determined in a bacterial digest equivalent to 80 bacteria. The bacteria were digested and incubated with a small aliquot (1-5 µL) of anti-aa 49-61 NIVAIEGGEDVTK antibody beads. One tenth of the antibody beads were spotted on the MALDI target plate and analyzed directly. Although there were signals from some unknown impurities or non-specifically bound peptides from, for example, the bacterial digest, the target peptide was still detectable by its mass. The singly-charged, epitope-containing tryptic peptide (aa 49-61 NIVAIEGGEDVTK) from the IglC protein was observed at m/z = 1344.7 **(Figure 4).** Therefore the detection limit for bacteria in PBS solution is determined to be 8 CFU bacteria on

target by using 50% of the antibody beads incubated with 50µL of a bacterial solution in PBS, at a concentration of 320 CFU mL^{-1}, which is as low as the most sensitive detection systems yet developed (46, 50). It should be noted that 8 CFU does not represent the lowest possible detection limit in real applications, but is an estimate of the lowest possible detection limit of our method, as the tryptic digestion of the protein equivalent of 8 CFU might be difficult.

3.5.1.2 Detection of *F. tularensis* bacteria in blood

The following detection limits were determined for the synthetic *F. tularensis* IglC, aa49-61 and *F. tularensis* bacteria spiked into blood samples (plasma): 69 attomoles of the *F. tularensis* IglC aa49-61 peptide and 800 CFU of bacteria (**Figure 5**) on target by using 50% of the antibody beads incubated with 125µL of spiked plasma, at a concentration of 1 femtomole mL^{-1} IglC aa49-61 peptide and 1280 CFU mL^{-1} bacteria, respectively. Although this method is not as sensitive for the detection of *F. tularensis* bacteria in blood as it is for the detection of bacteria in PBS solution, the detection limit is still comparable to most ELISA methods (43, 51). However, our technology has the advantage of greater safety because we are detecting bacterial peptides after the bacteria have been inactivated by lysis and proteolysis. In addition, our technology has higher specificity, since tandem mass spectrometry is a molecular approach with sequencing capabilities to provide absolute identification of the target molecule.

Due to the high virulence of bioterrorism pathogens, high sensitivity is one of the most important requirements in order to avoid “false negative” results. The

sensitivity in plasma (800 CFU) is lower than in PBS solution (ca. 10 CFU bacteria), may be due to incomplete digestion and to high levels of non-specific binding of other plasma proteins which can suppress the ion signal from the target peptide. Optimized sample preparation protocols may alleviate this problem. In particular, a major advantage of this MS/MS-based technique is that the proteins which are the source of these non-specifically-bound peptides can be identified from the MS/MS spectra of their peptides, and depletion protocols can be specifically designed to remove these proteins prior to digestion and affinity capture of the target peptides (52). Improved sample preparation protocols, and improvements in mass spectrometric detection technology, should lead to routine detection sensitivities of ~10-100 attomoles of peptide.

3.5.1.3 Detection of *F. tularensis* bacteria in Nasal swab samples

The *F. tularensis* iMALDI assay is also usable for nasal swab analyses. Nasal swabs are widely used in clinical tests, and are a simple and useful method for collecting a wide range of respiratory viruses. The collection of a nasal swab is easy and painless, and it can easily be performed in remote locations (53). In the mouse nasal swab samples collected 2 days after inoculation, a very low level of target peptide (m/z=1344.7) was detected, while in the uninfected mouse samples the signal for this peptide was not detected (**Figure 5**). No bacteria were observed in a culture of the nasal swab solution from the infected animal (which later developed tularemia), thus demonstrating that iMALDI could detect *F. tularensis* at levels too low for successful culturing of the bacteria.

3.5.2 Specificity

By “specificity” of the assay, we mean that the target peptide is unique to the organism (as determined by a BLAST search of the peptide sequence). Thus the detection of this peptide, at the appropriate MW and with the correct sequence is a positive indication of the presence of *F. tularensis*. The iMALDI can provide this specificity by combining two molecular characteristics of the epitope-containing peptides: i) the molecular weight, typically measured by MALDI-MS within an error of ≤100 ppm and ii) the amino acid sequence, determined by performing MS/MS on the same sample.

We have demonstrated that this iMALDI technology is able to accurately determine the molecular weight of an immunoaffinity-enriched *F. tularensis* peptide from a proteolytic digest of *F. tularensis* bacteria in PBS solution and blood. The molecular weight determination was accurate to within 60 ppm. Sequence information on the affinity-bound peptide was also obtained by MALDI-MS/MS (**Figure 6A**). MS/MS analysis produces high-abundance sequence-specific ions, resulting in an almost complete amino acid sequence of the peptide. These sequence-specific ions allow confident assignment of the target peptide (**Figure 6B**). Using the existing NCBInr database (2006.02.16), searching with only MS data, we retrieved 74,528 hits out of more than 3 million entries; in contrast, searching with combined MS and MS/MS data, resulted in only one hit (**Figure 6C**). This demonstrates the specificity of this iMALDI technique for detecting *F. tularensis*. Here, we used polyclonal antibodies because of their ready availability. Polyclonal antibodies often contain more than a single epitope within a particular stretch of

amino acids, and therefore, are able to capture even modified peptides since they are likely to contain an unmodified stretch of amino acids. This makes this detection method tolerant of small sequence variations. On the other hand, the use of polyclonal antibodies might lead to cross-reactivity with peptides from other species. This could decrease specificity and/or the detection limit of our method. This issue could be resolved by using a several monoclonal antibodies, or perhaps even both types of antibodies, for the final clinical version of this technique

3.5.3 Quantitation

To determine the absolute concentration of a given peptide in a sample (and, therefore, the absolute concentration of the original protein, which should correlate with the number of bacteria in the sample), synthetic stable-isotopically labeled "heavy" peptide is used as an internal standard. These heavy peptides are identical in amino acid sequence to the native epitope-containing "light" peptides from the protein in the sample, but are higher in mass and are thus distinguishable by mass spectrometry. The heavy peptide is added to the proteolytically-digested protein sample prior to incubation with the immobilized anti-peptide antibody. Following affinity enrichment of the target peptides, MALDI-MS analysis of the antibody beads shows doublets of ion signals from the light and heavy peptides, whose intensity ratios can be used for absolute quantitation.

For quantifying *F. tularensis* IglC protein, the *F. tularensis* IglC aa 49-61 peptide ^{49}NIVAIEGGEDVTK^{61} was synthesized with an isotopically-labeled valine at position 59 (underscored). This "heavy" peptide (H) is identical in amino acid sequence to the native epitope-containing "light" peptide (L), but is 6 Da higher in

mass (m/z=1350.7) due to 6 ^{13}C's in place of 6 ^{12}C's. In these experiments, the heavy peptide was added in various amounts (100 fmol – 5 pmol) to a proteolytic digestion of *F. tularensis* bacteria in buffer, and was immuno-adsorbd using the anti-*F. tularensis* IglC aa49-61 antibody, immobilized on affinity beads (**Figure 7A-D**). Analysis of an aliquot of these beads spotted directly on the MALDI target reveals monoisotopic protonated molecular ions from the two peptides, separated by 6 Da. Figure 7E shows a linear correlation (R^2=0.9988) between the ratio of the two peptides (H/L) and the amount of H spiked in as internal standard over near two orders of magnitude (from 100 fmol to 5 pmol). The nearly perfect linear correlation demonstrates the accuracy of iMALDI^2 for the absolute quantitation of F.tularensis peptides/proteins using an internal standard.

3.5.4 Conclusion

It has been shown that the combination of two mass spectrometric approaches (MALDI-MS and MALDI-MS/MS), inherent in the iMALDI approach, can unambiguously identify affinity-bound peptides, and therefore, permits specific detection of *F. tularensis* bacteria. The *F. tularensis* iMALDI can detect *F. tularensis* bacteria in environmental samples at a sensitivity of better than 10 CFU of bacteria. The high sensitivity of this technique means a low rate of "false negatives", and the high specificity means a low rate of "false positives", since both the molecular weight and the sequence of the bacterial peptide are determined at high accuracy. In addition, the *F. tularensis* iMALDI assay is adequate for accurate and absolute quantitation of the target protein concentration. With the addition of robotic liquid handling systems, it would be an even safer technique, as well as being capable of

high-throughput analyses. This would make iMALDI an extremely valuable tool for the early detection of *F. tularensis* and other bioterrorist agents.

CHAPTER 4

4 DEVELOPMENT AND APPLICATION OF iMALDI FOR EGFR DIAGNOSIS

4.1 EGFR and cancer

Cancer is a complex disease, caused by uncontrolled, abnormal cellular growth through oncogenic and anti-apoptotic pathways with multiple mechanisms. One key player of cell growth is the epidermal growth factor receptor (EGFR), a member of the ErbB family of membrane-associated receptor tyrosine kinases. EGFR, a 170 kDa transmembrane glycoprotein, consists of an extracellular ligand-binding domain, a membrane-spanning domain, and an intracellular cytoplasmic tyrosine kinase domain (54). In response to a number of ligands, EGFR and its ErbB family members can homo- and heterodimerize, eliciting a variety of signaling transduction events that can affect cell growth and adhesion, migration, differentiation, and apoptosis (55). EGFR up-regulation is frequently observed in a number of cancers, such as prostate, lung, ovary, and breast cancers (56-59) . This up-regulation can deregulate multiple signaling pathways, which lead to increased proliferation and inhibited apoptosis. High EGFR level has been related to poor response to treatment and decreased survival times (58, 60).

4.2 Currrent techniques

Immunohistochemistry (IHC) is frequently used to determine EGFR protein expression. However, IHC techniques have limited sensitivity and quantitation

capability of EGFR expression (54). IHC cannot quantitate the expression and modification of multiple proteins in a single, high-throughput assay format. Other methods, such as Western blot analysis and enzyme-linked immunosorbent assay (ELISA), have limitations the same as IHC, such as limited sensitivity and quantitation capability. Northern blotting or quantitative reverse transcription polymerase chain reaction (RT-PCR) assess mRNA levels, but there may be problems caused by RNA degradation and contamination. Furthermore, mRNA levels cannot predict post-translational modifications and do not always correlate with actual cellular protein levels or activity (61, 62).

New proteomics technologies, such as surface-enhanced laser desorption/ionization MS (SELDI), are used to identify potential cancer biomarkers (61-65). In SELDI, bait proteins, such as antibodies, are immobilized on chemically modified surfaces to capture intact analyte proteins for MS analysis. However, proteins, extremely sensitive to surface substrate physico-chemical properties, are easily denatured. MS analysis of full-length proteins suffers from limited resolution, preventing identification based solely on mass. When detecting proteins in complex biological samples is attempted, the heterogeneity, hydrophobicity, and limited solubility of proteins may introduce significant loss-associated bias.

4.3 iMALDI for EGFR

The iMALDI assay for detecting EGFR is based on affinity capture of the tryptic peptide aa 963-975 from EGFR. This assay circumvents many problems described above, and is able to detect EGFR in mammalian breast cancer cell lines and tumors sensitively, specifically, and quantitatively.,

4.4 Materials and methods

4.4.1 EGFR

EGFR protein (human) was purchased from SIGMA (Saint Louis, MI).

4.4.2 Breast cancer cell lines and tumor lysates

ME16C, SUM102 (http://www.asterand.com/Asterand/BIOREPOSITORY/102PT.aspx), and MCF-7 cell lines, and the BR97-0137B tumor sample, were obtained from the Perou lab (LCCC, UNC-CH) as cell lysates, extracted from a fresh frozen tumor sample of BR97-0137B and cell lines using Pierce's Tissue (T-PER) and cell line (M-PER) protein lysate extraction kit (Pierce). The protein concentration of the whole cell lysate was determined using Pierce's Micro BCA assay according to the manufacturer's protocol.

4.4.3 Tryptic digestion

Digestion was carried out in 25mM ammonium bicarbonate (Sigma) at 37^{o}C overnight, with an enzyme:substrate ratio of trypsin (Sequencing-grade modified, Promega) to protein of around 1:10.

4.4.4 Antibody Production and Immobilization of antibodies on beads

One of the tryptic peptides from EGFR with high sensitivity in the MALDI-MS mode (**Figure 8**) was seleced as the target, against which an antibody was raised.

This antibody was then covalently immobilized on CNBr-activated Sepharose beads (Amersham Pharmacia) according to the manufacturer's instructions (48).

4.4.5 Immuno precipitation Protocol

Affinity binding was carried out in compact reaction columns (CRC) (USB). An aliquot of antibody beads (1-5 uL) was loaded into a CRC, and then washed 4-6 times with 400 µL of 0.1x PBS. The synthetic peptide, C^{963}MHLPSPTDSNFYR^{975} (Biosynthesis) with an additional N-terminal cysteine residue which was added for ready conjugation with carrier proteins,.was dissolved in HPLC water, and then diluted to a series of concentrations, e.g. 150 ng/µL, 15 ng/µL, 1.5 ng/µL, etc. A 10 µL of each solution was incubated with an aliquot (1~5 µL) of antibody-beads to immuno-adsorb the epitope-containing peptide. Immuno-adsorption of tryptic digests of the EGFR protein, cell lysates or tumor biopsy samples was carried out similarly. Incubation of PBS and conjugated or unconjugated agarose beads were used as negative controls.

The beads were incubated for 2-4 h at room temperature with end-to-end rotation on a "Labquake" shaker (Lab Industries), and then washed 6 times with 400 µL of freshly-made 50 mM ammonium bicarbonate, or 3 times with 400mM of NaCl (Sigma) followed by 3 times with 50 mM ammonium bicarbonate in cases where high levels of non-specific binding was expected. After resuspended in a small volume of 50 mM ammonium bicarbonate (1-5 µL), an aliquot of the beads (0.5 µL) was spotted directly onto the MALDI target for analysis.

4.4.6 Isotopic Labeling for absolute Quantitation

The heavy EGFR aa 963-975 peptide (^{963}MHL̲PSPTDSNFYR^{975}) containing an isotopically-labeled leucine at position 965 (underscored) was synthesized with a ^{2}H-labeled FMOC leucine purchased from C/D/N ISOTOPES (Quebec, Canada) at the UNC peptide synthesis facility (49). The increase in mass was 10 Da from the unlabeled EGFR aa 963-975 peptide.

4.4.7 Absolute Quantitation

The heavy peptide, used as an internal standard, was accurately weighed on a microbalance and dissolved in water (HPLC grade, Pierce). Various amounts of the heavy peptide was spiked into the tryptic digest of SUM102 cell lysate, incubated with anti-EGFR peptide antibody beads, and quantitated by MALDI-MS analysis of the beads deposited directly on the MALDI target.

4.4.8 Mass Spectrometric Analysis

All MALDI-MS and MS/MS experiments were carried out on a MALDI-TOF instrument (Ultraflex) from Bruker Daltonics (Billerica, MA) with their Anchor-chip™ MALDI-target plates (400 or 600 μm/384 spot format). α-cyano-4-hydroxycinnamic acid (HCCA) (Sigma) re-crystallized from hot methanol was used as matrix for all experiments. The solvent for HCCA was a mixture of 50% acetonitrile (Caledon Laboratories), 49.9% water (HPLC grade, Pierce), and 0.1% trifluoroacetic acid (Pierce) (v/v/v).

After the antibody beads were placed on the target, 0.5 μL of a saturated solution of HCCA matrix was added, and the spot was air-dried at room temperature.

4.5 Results and Discussion

4.5.1 Sensitivity Studies – synthetic peptide

The iMALDI technology was able to detect the synthetic EGFR peptide as low as one attomole in buffer. In contrast, without enrichment by iMALDI, MALDI-MS analysis of the peptide solution could only detect the peptide in the low femtomole range in buffer (5 femtomole) (**Figure 9**). This decreasing in detection sensitivity is likely due to the peptide absorption by the walls of the tubes and by the pipette tips during the sample preparation process. While using the enrichment procedure, the peptides are bound to the antibody beads, greatly reducing the absorption and therefore the peptide loss.

The S/N ratio of the signal from the 1 attomole with iMALDI (**Figure 9B**) is higher than that of the 10 femtomole and 100 attomole levels, because the co-crystallization is not uniform, and for the 1 attomole level, apparently a better crystal was sampled than for the 10 femtomole and 100 attomole levels.

4.5.2 Breast Cancer Cell Lines

Both EGFR-expressing mammalian breast cancer cell lines, SUM102 is a tumor-derived basal-like cell line (66), and ME16C is a mammary epithelial cell line immortalized with hTERT with characteristics of the basal-like subtype(66).

4.5.2.1 SUM102 Cell Lysate

EGFR was detected in a SUM102 cell lysate equivalent to 10 cells. The cell lysate digest was incubated with a small aliquot (1-5 µL) of anti-aa963-975 EGFR

antibody beads, one tenth of which were spotted directly on the MALDI target plate and analyzed. The epitope-containing target peptide (aa 963-975 MHLPSPTDSNFYR) with an m/z of 1564.7 was observed (**Figure 10**).

EGFR was also quantitated in SUM102 absolutely. Unlike in "relative quantitation," where the ratios of the peptide concentrations in two samples are compared, in "absolute quantitation," the absolute amount of the peptide was determined. Before incubation with the immobilized anti-peptide antibody, the synthetic stable-isotopically labeled peptide (heavy peptide), is added to the proteolytically-digested protein sampleas internal standard. MALDI-MS analysis of the antibody beads after immuno-adsorption shows doublets of ion signals from the light and heavy peptides, whose intensity ratios are used for absolute quantitation.

The heavy peptide (H) EGFR aa 963-975 peptide ^{963}MH<u>L</u>PSPTDSNFYR^{975}, is identical in amino acid sequence to the native epitope-containing peptide (light peptide, L, m/z=1564.7), but is 10 Da higher in mass (m/z=1574.7). The heavy aa 963-975 peptide (H) was added in various amounts to the tryptic digest of SUM102 cell lysate, and immuno-adsorbd using the immobilized anti-EGFR aa 963-975 antibody (**Figure 11A-B**).

Analysis of an aliquot of the beads (ca. 10%) shows that the monoisotopic ion signals of the two peptides can be distinguished from each other by a mass difference of 10 Da. The logarithmic plot (**Figure 11C**) shows a linear correlation (slope is approximately one) between the signal intensity ratio of the two peptides (H/L) and the amount of the internal standard (H) over near two orders of magnitude, demonstrating the accuracy of this assay for the absolute quantitation of EGFR peptides by using an internal standard.

With this standard curve, the amount of EGFR in SUM102 cells was determined. The amount of EGFR peptide is equal to the amount of digested EGFR protein in the cell lysate, which is equal to the amount in the original cell lysate and cells, assuming 100% efficiency of the digestion reaction and the protein extraction upon cell lysis. The concentration of EGFR was determined to be 0.65 attomoles/cell, or approximately 390,000 EGFR molecules per SUM102 cell, Which is consistent with the EGFR number (~50,000) in HeLa cell in the literature(67).

4.5.2.2 ME16C Cell Lysate

The iMALDI assay was also able to detect EGFR aa963-975 peptide in the ME16C cell lysate (Data not shown).

4.5.2.3 MCF-7 Cell Line

The assay was repeated for the MCF-7 cell lysate, which is a tumor-derived luminal cell line that does not express EGFR. The target peptide was not detected (Data not shown).

4.5.3 Human Breast Cancer Tumor Biopsy Sample

An estrogen receptor negative and HER2-negative breast tumor biopsy sample, BR97-0137B, was analyzed. BR97-0137B had been previously shown to be a grade III infiltrating ductal carcinoma that has gene expression characteristics of the basal-like subtype and high gene expression of EGFR (68).

Ion signals of the target peptide (m/z=1564.7) was detected from the digest of BR97-0137B tumor tissue lysate with an S/N>5. (**Figure 12**) It is important to note that less than 1/25 of the biopsy sample was consumed.

4.5.4 Specificity

The molecular weight of the EGFR peptide was determined to be within an error of 75 ppm or better. In addition, sequence information of the affinity-bound EGFR aa963-975 peptide from the ME16C cell lysate digest was obtained by MALDI-MS/MS (**Figure 13**). Tandem MS analysis provides sequence-specific ions, such as the highly abundant y_1, y_5 and y_{12}, and several lower abundant ones. The mass accuracies of these sequence-specific ions are similar to that obtained in the MS mode, allowing confident assignment. The absences of y_7 and y_9, produced by C-terminal fragmentation of Pro, increases confidence in the assignment. In the existing NCBI database, searching with MS data only, 33,129 hits were obtained out of 2 million entries, which means about 33,129 out of 2 million peptides have masses around 1344.7. While searching with both MS and MS/MS data, one hit was obtained. This data clearly demonstrates that the combination of the two mass spectrometric approaches (MALDI-MS and MALDI-MS/MS) inherent in the iMALDI assay, is capable of unambiguously identifying affinity-bound peptides, permitting highly specific detection of EGFR.

Based on affinity capture by the antibody, this technique may induce false negatives due to target sequence variation. If a key amino acid residue in the epitope-containing region of the peptide is substituted, the antibody may fail to

capture the peptide, resulting in a “false negative.” To eliminate this problem, other antibodies can be used at the same time in the assay to target multiple epitopes, providing more coverage of the protein. Moreover, polyclonal antibodies are used in this assay. Because more than a single epitope may be contained within a particular stretch of amino acids, modified peptides are likely to contain enough unmodified parts to be captured by the polyclonal antibodies. Mutations in the DNA could lead to unexpected amino acids in the target peptide sequences, which would be detected by the MS/MS sequencing of the captured peptides, revealing the homology. In addition, current database searching programs, such as the MASCOT^{TM} software, can identify peptides with substitutions and/or deletions. All these will reduce “false negatives”.

4.6 Concluding remarks

The iMALDI assay for EGFR using the anti-EGFR aa 963-975 antibody, detected EGFR in mammalian breast cancer cell lines ME16C and SUM102 where it is known present, and not in the MCF-7 cell line where it is known NOT present. It also detected EGFR in a human breast tumor biopsy sample. This iMALDI has achieved sensitivities in the low attomole range, high specificity, and absolute quantitation of target peptides with a linear dynamic range of over two orders of magnitude. The sensitivity of the EGFR iMALDI assay allows the detection of EGFR from the cell lysate corresponding to a few ($\leqq$10) SUM102 cells, and the specificity of the assay ensures a low rate of false positives.

CHAPTER 5

5 FUTURE DIRECTIONS

5.1 Protocol optimization

The protocols for samples preparation can be further optimized in order to achieve higher sensitivity and decreased assay times.

5.1.1 Pre-depletion of plasma

Sensitivity is one of the most critical parameters affecting the utility of the iMALDI assay, especially for diagnosis of infectious pathogens such as *F. tularensis*. Insufficient sensitivity to detect the pathogens at trace levels can lead to “false negative” results. Although this iMALDI assay can detect as few as ten CFU *F. tularensis* bacteria in buffer (environmental samples), its sensitivity of bacteria in plasma (800CFU) might not be enough for clinical diagnosis. This may be due to the complexity of plasma. Other more abundant peptides from the tryptic-digested plasma may compete with the low level of target peptide during the immunoaffinity capture by the antibody beads. The high level of non-specific binding of other peptides greatly suppresses the ion signal from the target peptide. This problem can be eliminated through optimizing the protocols for sample preparation. In particular, the proteins which are the source of these non-specifically-bound peptides will be identified from their MS/MS spectra, and these proteins will be depleted from the

plasma using commercially-available or customized depletion kits prior to affinity capture of the target peptides.

5.1.2 Digestion

Currently the digestion is carried out at 37^{o}C overnight. The use of long incubation times and elevated temperatures inevitably leads to more digestion artifacts like nonspecific cleavage, which will lead to failure of affinity capture of the targets by the antibody beads, giving rise to “false negatives”. By using immobilized trypsin, digestion can be done at the room temperature. Because the effective protease concentration on the solid support is very high, digestion efficiency can be strongly enhanced and digestion times will be greatly reduced.

The digestion efficiency of proteins can also be enhanced by the use of organic solvents during digestion. This is because proteins tend to denature in the presence of organic solvents such as acetonitrile, thereby increasing cleavage-site accessibility.

5.1.3 Use of nanoparticles

Antibody beads can be made by nanoparticles. Compared to the micro affinity beads that are used now, the nanoparticle has a larger surface area-to-volume ratio. The higher density of antibodies on the surface of the nanoparticles, will lead to improved rate constants for the association of analytes with the antibody-conjugated nanoparticles, and therefore improved sensitivity, and reduced incubation time.

5.1.4 Smaller spot

After the incubation for affinity capture, using the robotic spotting machine, the antibody beads can be deposited onto smaller spots on the target, like 400um and 200um (currently 600um). This is expected to increase the surface concentration of the analytes, which will also improve the sensitivity.

5.1.5 More sensitive mass spectrometer

Technology is still developing. There will be more sensitive mass spectrometers. Our collaborator, Bruker Daltonics, will develop specialized mass spectrometers for analysis of beads.

5.2 Multiple targets

With any antibody-based technique there is the possibility of false negatives due to strain variation or gene mutation. If there has been a substitution of a key amino acid residue in the epitope-containing region, the protein or peptide may not be captured by the antibody, resulting in a “false negative”. To eliminate this problem, multiple target proteins can be selected. For each target protein, several peptides can be selected as the targets. This would provide more coverage of each targeted protein. Polyclonal antibodies against 12-17 amino acid peptides can be used instead of monoclonal antibodies, and more than a single epitope will be contained within this stretch of amino acids. Therefore, even modified peptides are likely to contain enough of an unmodified stretch to be captured by these polyclonal antibodies. Moreover, it is highly unlikely that all target peptides would be mutated, so several characteristic peptides should still be detected by this assay. Mutations

in the DNA could lead to failure of the PCR-based assays and unexpected amino acids in the target peptide sequences. But these would be detected by the MS/MS sequencing of the captured peptides. In addition, current database searching programs, such as the MASCOT software, can identify peptides with substitutions and/or deletions. If determination of bacterial strains proves to be of clinical interest, the iMALDI is an extremely flexible format, and beads containing antibodies raised against peptides which are characteristic of the different strains could simply be added to the chip.

5.3 Automation& high throughput

The enrichment capability of using immobilized antibodies allows the detection of bacteria at low concentrations by using more sample and by using fewer beads, thereby increasing the number of bound peptides per bead. However, it becomes very difficult to handle a low amount of beads manually, but this limitation can be circumvented by using automated robotic systems. Automation is essential for bioweapons diagnosis because of the danger inherent in handling these organisms, and is also an essential component of high-throughput analysis. Biomachines, one of our collaborators, is developing the state-of-the-art robotic systems, based on the MALDI spotting machine, to adapt this iMALDI technology for automated high throughput analysis. It will provide a routine detection sensitivity of ~10-100 attomoles of peptides and increased sample throughput. It will be able to operate in a safe environment, which will reduce both the possibility of the operator's exposure to the pathogen, and the possibility of contamination of the samples.

The proof-of-principle study has demonstrated the possibility to integrate and fully automate the iMALDI protocols using a robot designed for a different intended use. A smaller clinical quality processor will be designed and built specifically for iMALDI.

The key components of the iMALDI Processor will be a vision-guided small-volume liquid handling robot, including a multi-purpose station for incubating, mixing, and washing, disposable reaction vessels, a MALDI spotting station, and a storage station for chilling and resuspending reagents. All parts will be enclosed and equipped with HEPA (high-efficiency paniculate air) filtered airflow to ensure sterility. The vision system will be used for interactive teaching during set-up to guide precision deposition of the beads, and for post process inspection and clinically required quality control. The disposable reaction vessel can be a single chamber; or for high volume processing, a multiwell filter plate, from a 96-well filter plate to a 384-well filter plate, depending on the required throughput; or for low volume work, similar to unitized micro chromatography columns, held in a rack placed in the same multi-purpose chamber as the filter plate. Samples, trypsin and beads are stored in a chiller which also keeps the beads in suspension by mixing. All surfaces will be easily accessible and made of inert materials such as stainless steel so that they can be aggressively cleaned to inactivate and remove contaminating materials.

The disposable reaction vessel(s) are loaded into a central multi-purpose station for incubating, mixing, and washing: 1) pathogens such as bacteria are inactivated by ethanol followed by boiling; 2) the whole proteome is proteolytically digested at 37^{o}C with gentle mixing; 3) beads with immobilized antibodies are added and incubated with the digested sample at room temperature; 4) unbound materials

are removed by washing. In Step 5, resuspended beads are deposited onto a MALDI target with matrix and then analyzed. For complete automated diagnosis, the only manual part will be introducing the sample to the robotic system and inserting the MALDI target into the mass spectrometer, like a “push button”. Thus, the operation does not require any specific knowledge or skills in microbiology, protein chemistry, mass spectrometry, or computational sciences, and can be operated by low-level technicians.

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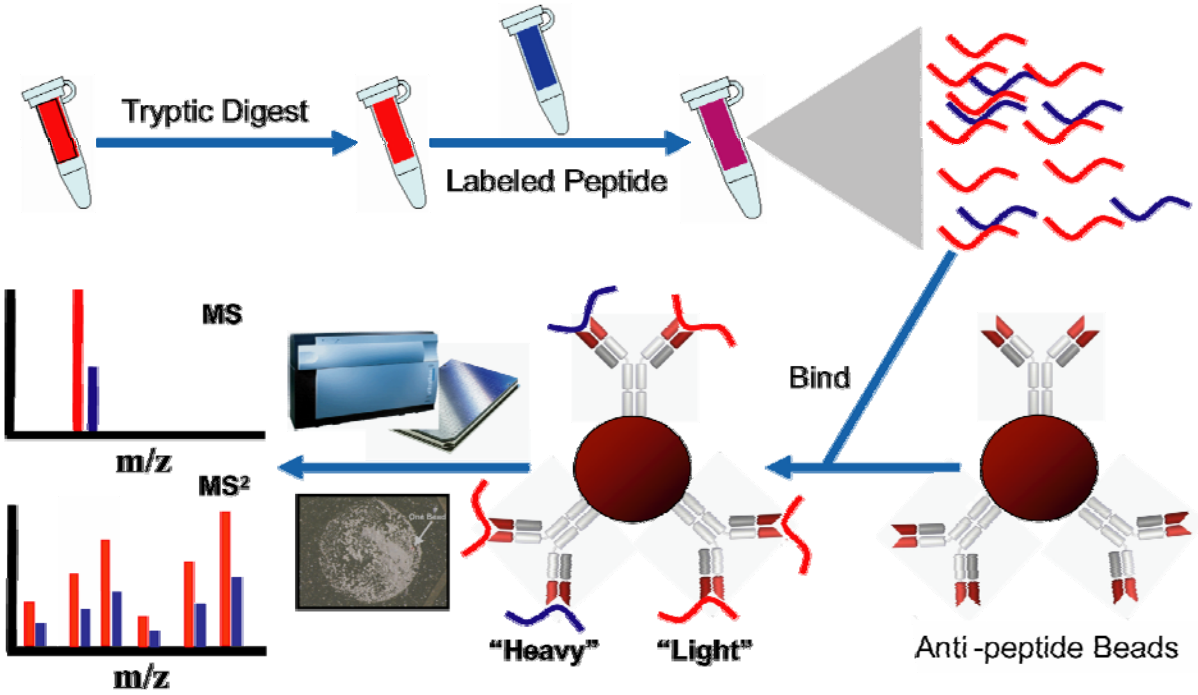
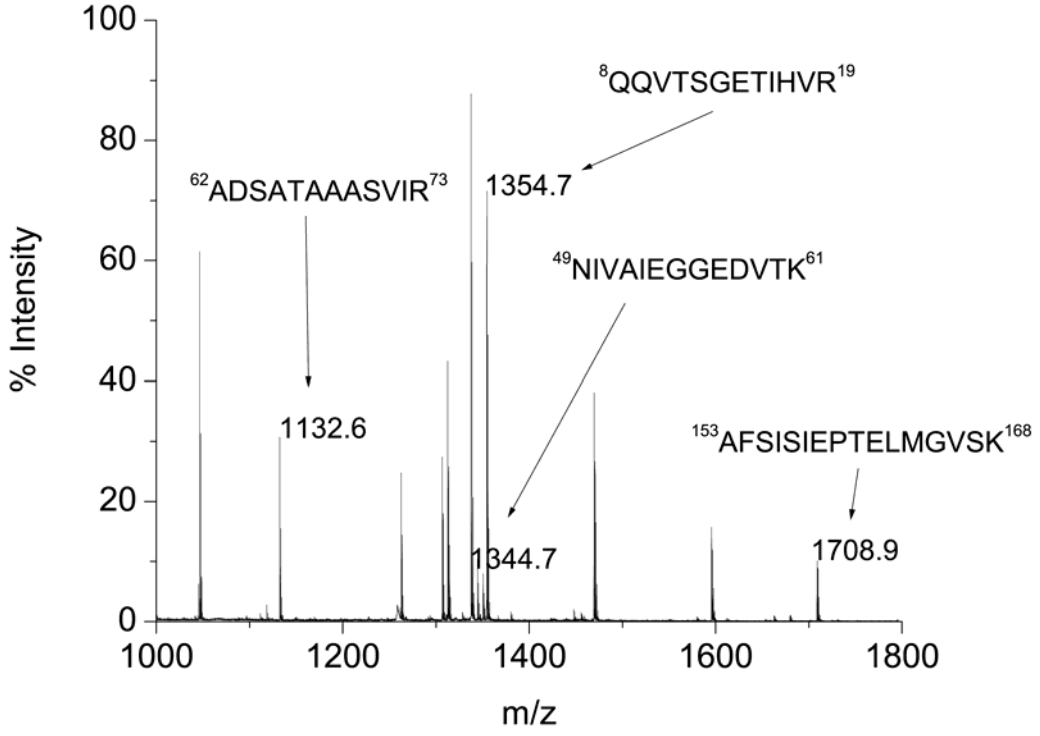


Figure 1: Analytical scheme of the iMALDI assay.

A



B

Tryptic F.t. p23 peptides used antibody production	Anti-(F.t. p23) peptide Antibodies	ELISA response (titer)
[153]AFSISIEPTELMGVS[167]	[(F.t. p23) aa153-167]	1/50,000
[8]QQVTSGETIHVR[19]	[(F.t. p23) aa8-19]	1/30,00
[48]C*NIVAIEGGEDVTK[61]	[(F.t. p23) aa48-61]	1/500,000
[62]ADSATAAASVIR[73]	[(F.t. p23) aa62-73]	1/10,000

Figure 2: Selection of *F. tularensis* IglC peptides for raising antibodies to be used for the Francisella tularensis iMALDI assay. A) MALDI-MS of proteolytic *F. tularensis* IglC peptides obtained by in-solution digestion of IglC with trypsin. Four "true" (C-terminal cleavage of K or R residues) tryptic peptides of IglC (shown in blue) were selected for antibody production based on the high sensitivity in the MALDI-MS. B) Affinity determination of the four anti-(*F. tularensis* IglC) peptide antibodies against their epitope peptide by ELISA. The cysteine residue marked with asterisk has been added to the native sequence because of ease of conjugation with the carrier protein.

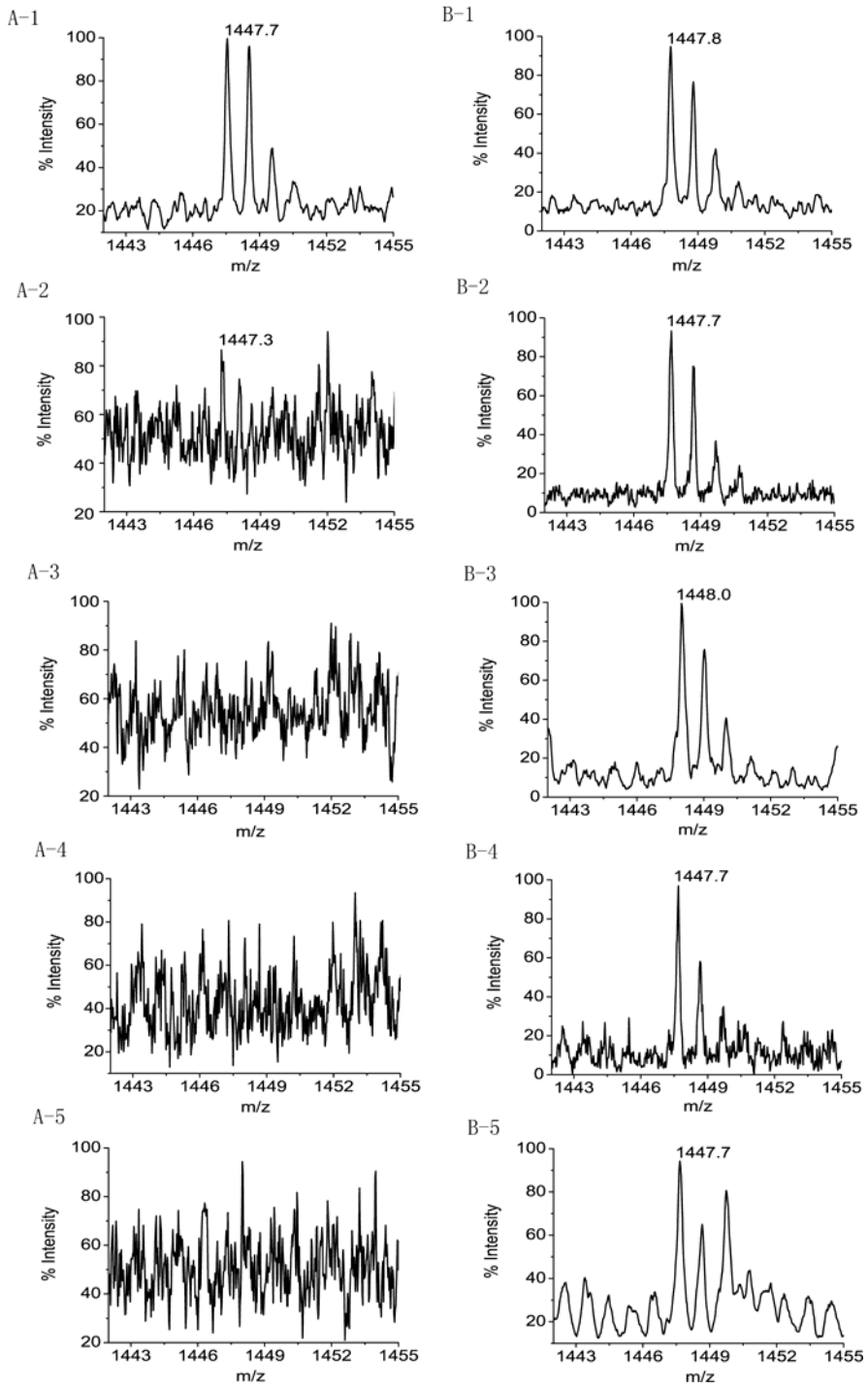


Figure 3: Detection sensitivity of synthetic *F. tularensis* IglC peptides in solution and using the *F. tularensis* iMALDI assay. A) MALDI-MS spectrum of synthetic *F. tularensis* IglC peptides CNIVAIEGGEDVTK in solution: A-1) 100 femtomole; A-2) 10 femtomole; A-3) 1 femtomole; A-4) 100 attomole; A-5) 10 attomole. B) MALDI-MS spectrum of synthetic *F. tularensis* IglC peptides CNIVAIEGGEDVTK affinity-bound to anti-aa48-61(*F. tularensis*p23) antibody beads: B-1) 138 femtomole; B-2) 13.8 femtomole; B-3) 1.38 femtomole; B-4) 138 attomole; B-4) 13.8 attomole.

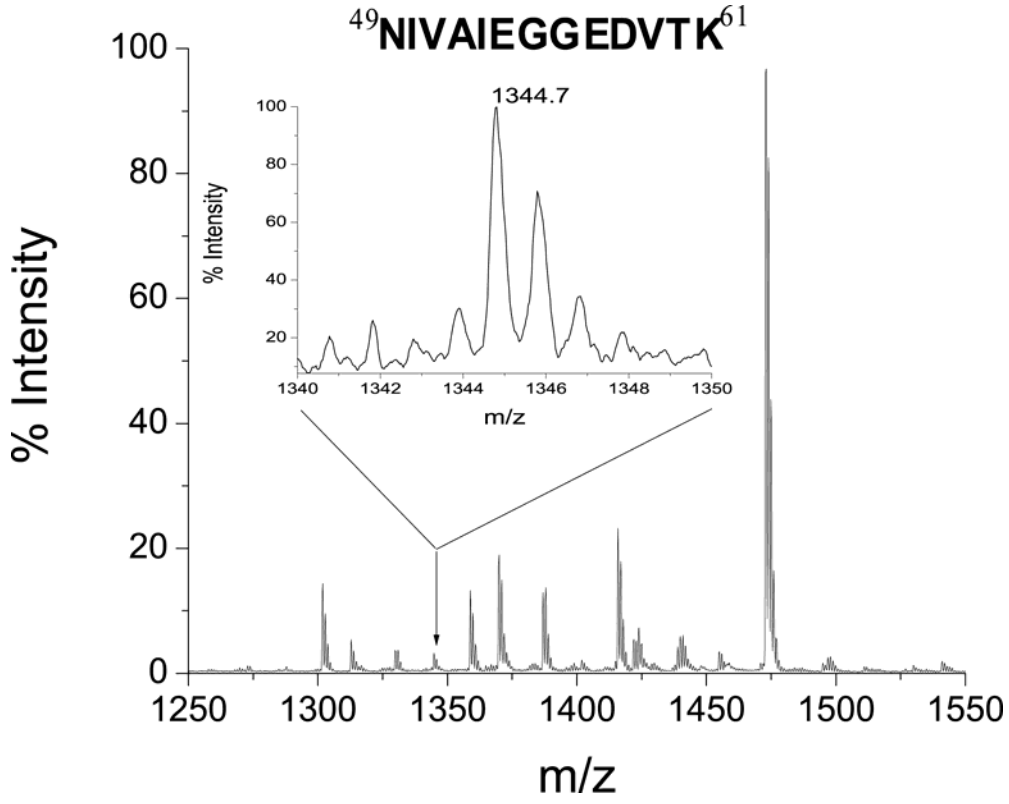


Figure 4: Detection of *F. tularensis* bacteria in environmental samples using the *F. tularensis* iMALDI assay. MALDI-MS spectrum of peptides obtained after proteolysis of *F. tularensis* bacteria in buffer, affinity-bound to anti-aa49-61 (*F. tularensis* IglC) antibody beads. The singly-charged epitope-containing tryptic peptide (aa 49-61 *F. tularensis* IglC protein) is observed at m/z = 1344.7 (see inset). The spectrum represents the amount of IglC peptide equivalent to 8 CFU for *F. tularensis* bacteria.

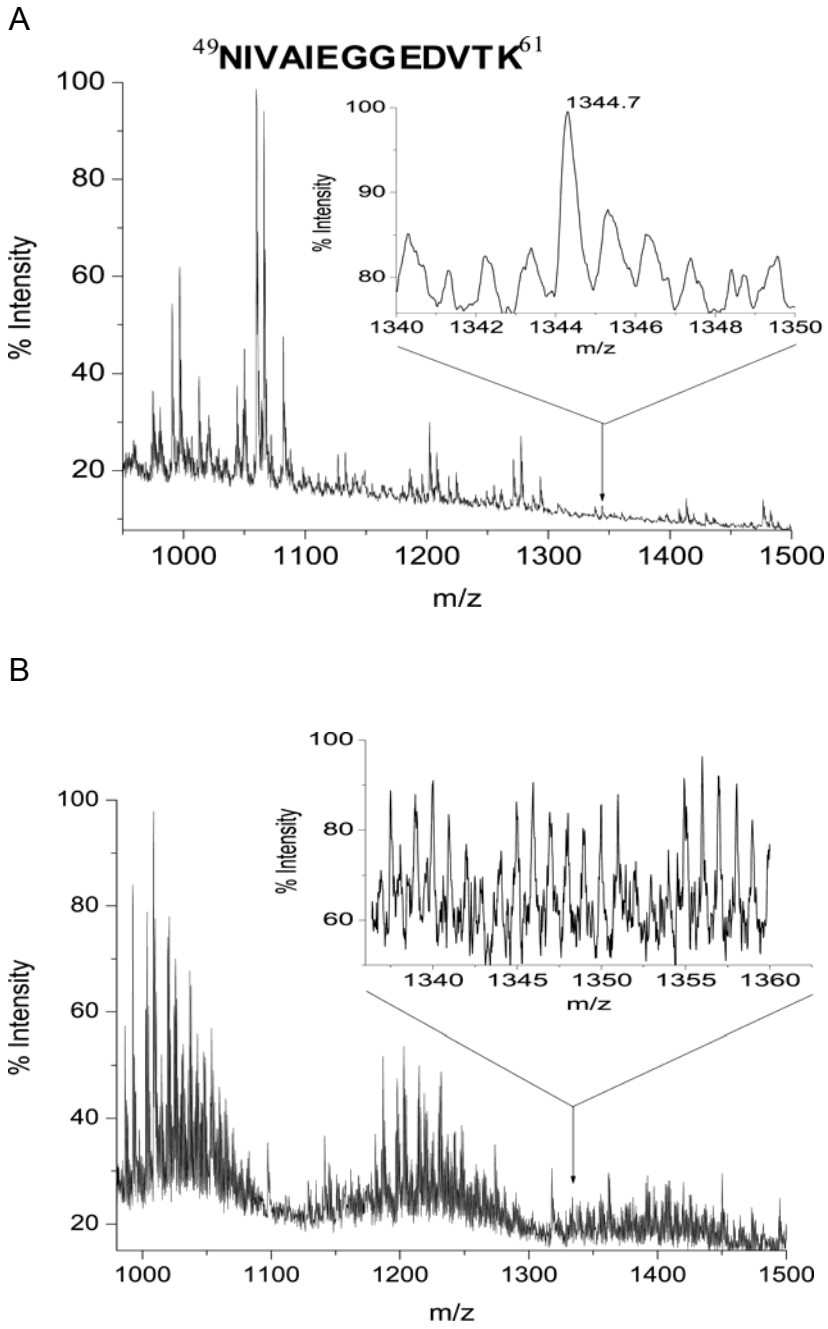
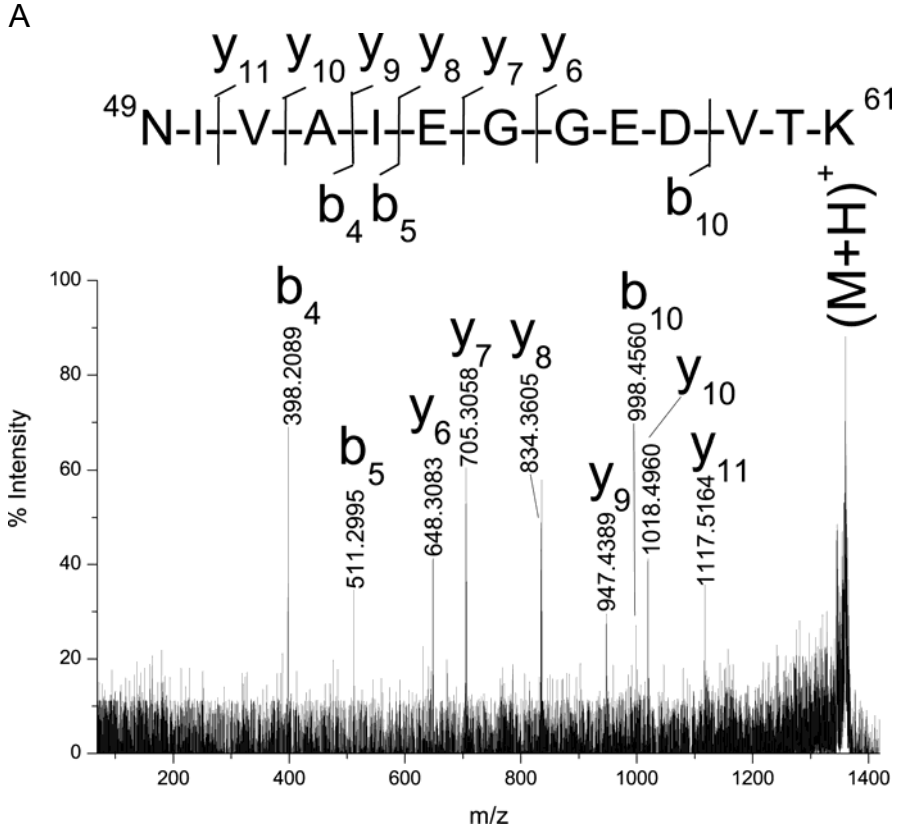


Figure 5: Detection of *F. tularensis* bacteria in nasal swab samples using the *F. tularensis* iMALDI assay. A) MALDI-MS spectrum of peptides affinity-bound to anti-aa49-61 (*F. tularensis* IglC) antibody beads, obtained after proteolysis of mouse nasal swab extracts collected two days after the mice were inoculated with *F. tularensis* The singly-charged, epitope-containing, tryptic peptide, NIVAIEGGEDVTK, corresponding to aa 49-61 of the *F. tularensis* IglC protein, was observed at m/z = 1344.7 (see inset). B) MALDI-MS spectrum of anti-aa 49-61 (*F. tularensis* IglC) antibody beads obtained after proteolysis of nasal swab extracts collected from uninfected mice on the same day.

A



B

Fragment ions	m/z observed	m/z theoretical	Mass accuracy (ppm)
b_4	398.2089	398.24	-79
b_5	511.2995	511.32	-49
b_{10}	998.4560	998.48	-24
y_6	648.3083	648.32	-19
y_7	705.3058	705.34	-51
y_8	834.3605	834.38	-29
y_9	947.4389	947.47	-31
y_{10}	1018.4960	1018.51	-9.5
y_{11}	1117.5164	1117.57	-52

C

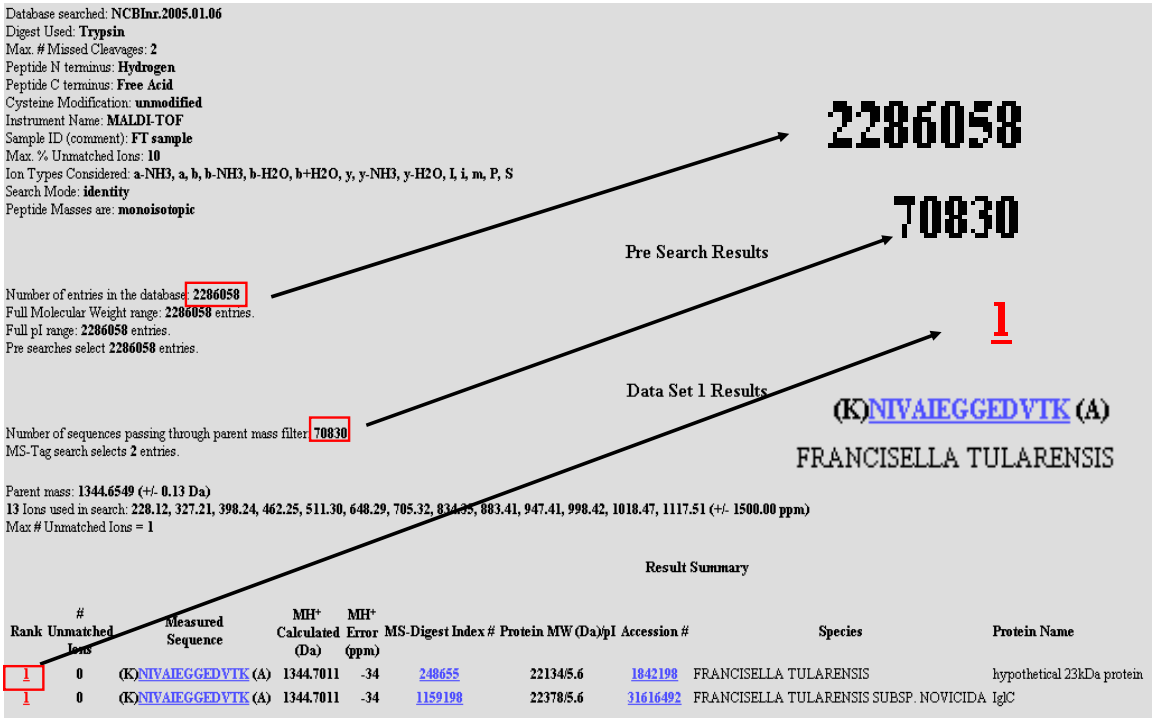


Figure 6: Highly specific detection of *F. tularensis* bacteria in environmental samples by mass spectrometric sequencing of the immunoaffinity-enriched *F. tularensis* IglC aa49-61 peptide, using the *F. tularensis* iMALDI assay. A) MALDI-MS/MS spectrum of the peptide at m/z = 1344.7 affinity-bound to anti-[aa49-61(*F. tularensis* IglC)] antibody beads, obtained after proteolysis of *F. tularensis* bacteria in buffer. All of the abundant ions can be assigned to sequence-specific y- and b-ions of the *F. tularensis* IglC peptide aa49-61, resulting in unambiguous identification of the immunoaffinity-enriched peptide. B) This table shows the mass accuracies of the fragment ions, demonstrating that the assignment of these ion signals is correct. This therefore demonstrates that, using our *F. tularensis* peptide chip, *F. tularensis* bacteria can be detected with high specificity and correspondingly low false positive rates. C) Database search results for the peptide NIVAIEGGEDVTK in the NCBI database with MALDI-MS and MALDI-MS/MS data.

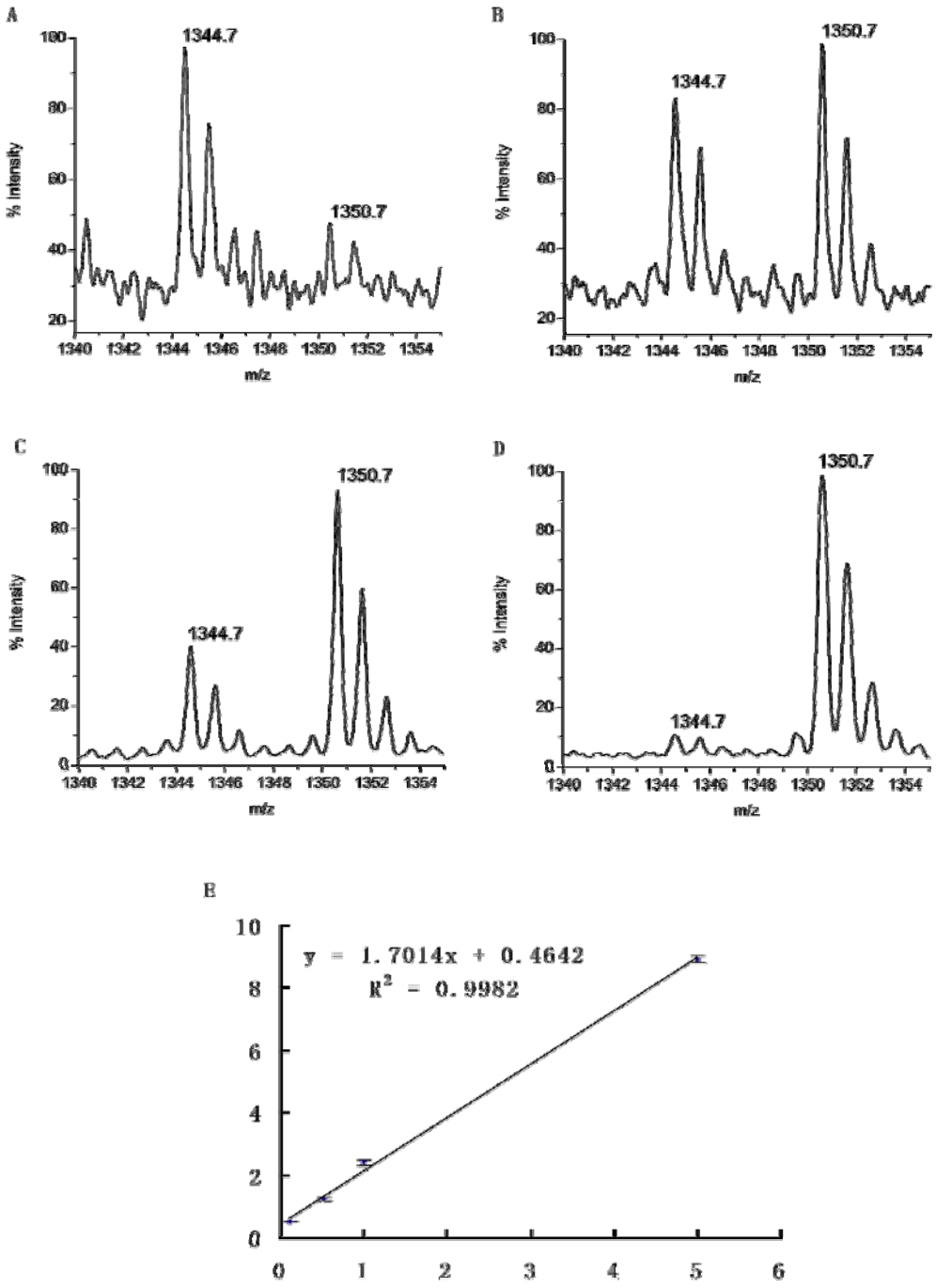


Figure 7: Quantitation of *F. tularensis* bacteria using the *F. tularensis* iMALDI assay. Absolute quantitation of the IglC peptide NIVAIEGGEDVTK (aa 48-61) (L, light peptide, m/z = 1344.7) in a bacterial sample. *F. tularensis* bacteria were digested and incubated with different amounts of heavy peptides (H, m/z =1350.7) as internal standards: A) 0.1 pmol, B) 0.5 pmol, C) 1 pmol, D) 5 pmol, E) Plot of the observed ratios of monoisotopic abundances of H and L in the MALDI-MS spectra (A-D) versus the absolute amount of H added. Note, only a 10% aliquot has been used for the analysis.

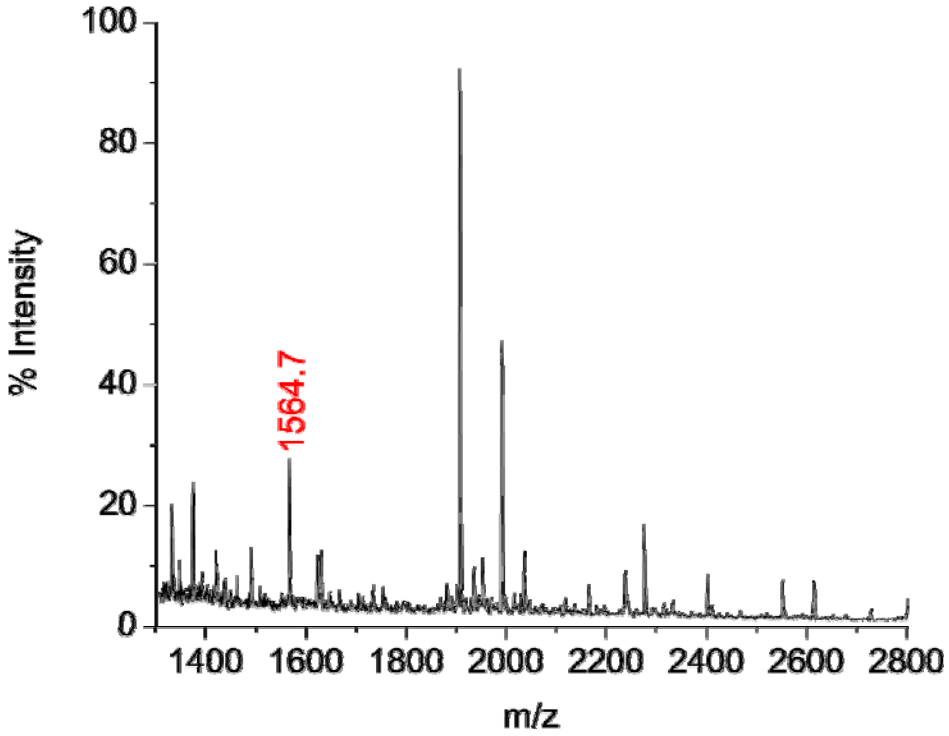


Figure 8: Selection of EGFR peptides to be used for raising antibodies for the EGFR iMALDI assay. MALDI-MS of proteolytic EGFR peptides, obtained by in-solution digestion of EGFR with trypsin. The “true” (C-terminal cleavage of K or R residues) tryptic peptide of EGFR of m/z 1564.7 (sequence shown in inset) was selected for antibody production based on its high sensitivity in the MALDI-MS mode.

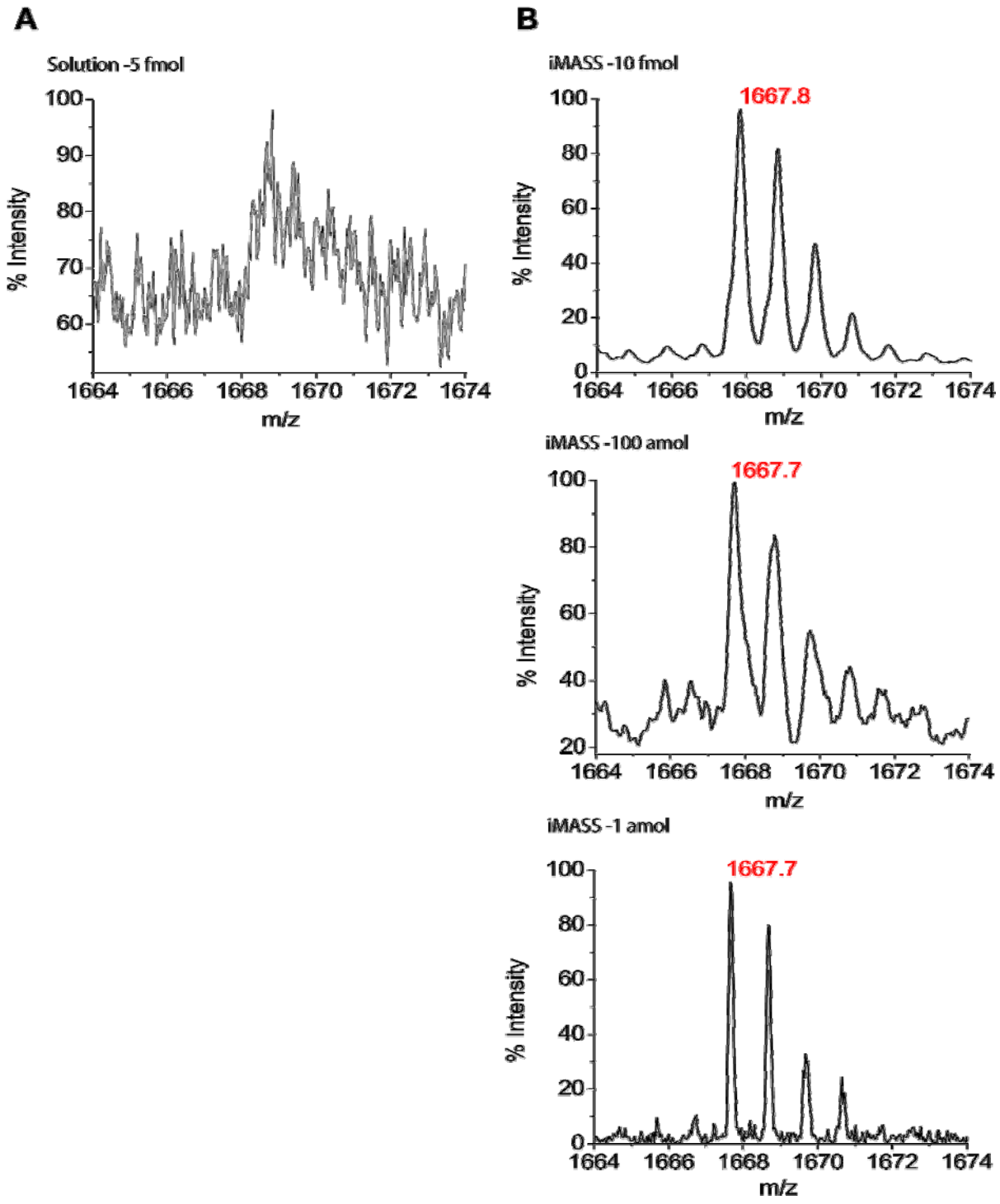


Figure 9: Comparative study to determine the sensitivity of MALDI-MS analysis of the EGFR peptide aa 963-975 A) from solution and B) using our iMALDI technology. The EGFR peptide aa963-975 of m/z = 1667.7 was used for raising antibodies, and is identical to the native EGFR peptide aa963-975, with the addition of an N-terminal cysteine residue (C^{963}MHLPSPTDSNFYR^{975}) to facilitate conjugation with carrier proteins.

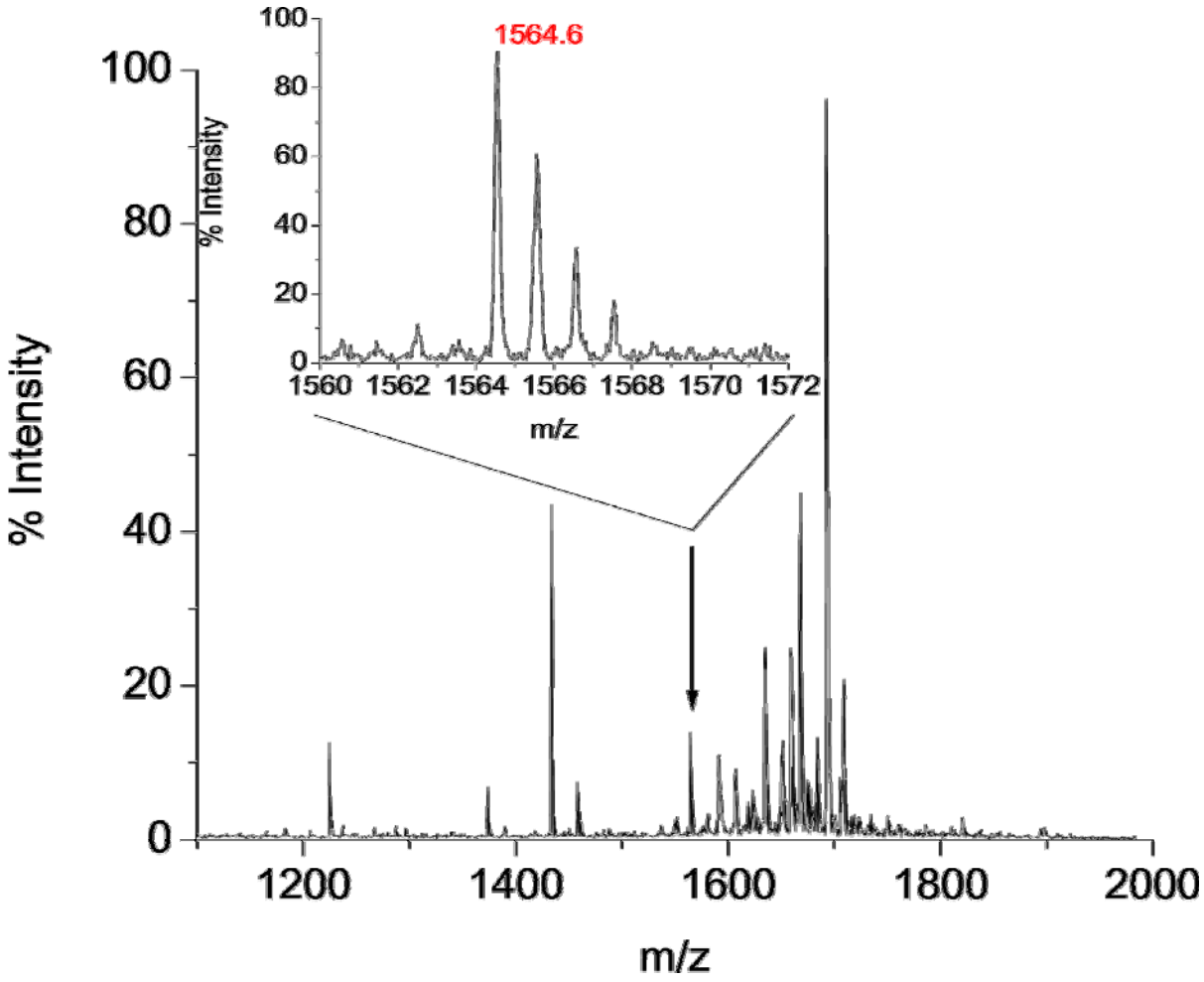


Figure 10: Detection of EGFR using the EGFR iMALDI assay in mammalian breast cancer cells. MALDI-MS spectrum of peptides affinity-bound to anti-aa963-975 EGFR antibody beads obtained after lysis of SUM102 cells, followed by proteolysis with trypsin. The singly-charged, epitope-containing, tryptic peptide aa 963-975 MHLPSPTDSNFYR of the EGFR protein is observed at m/z = 1564.7 (see inset). The spectrum represents the amount of EGFR peptide on MALDI target equivalent to one SUM102 cell.

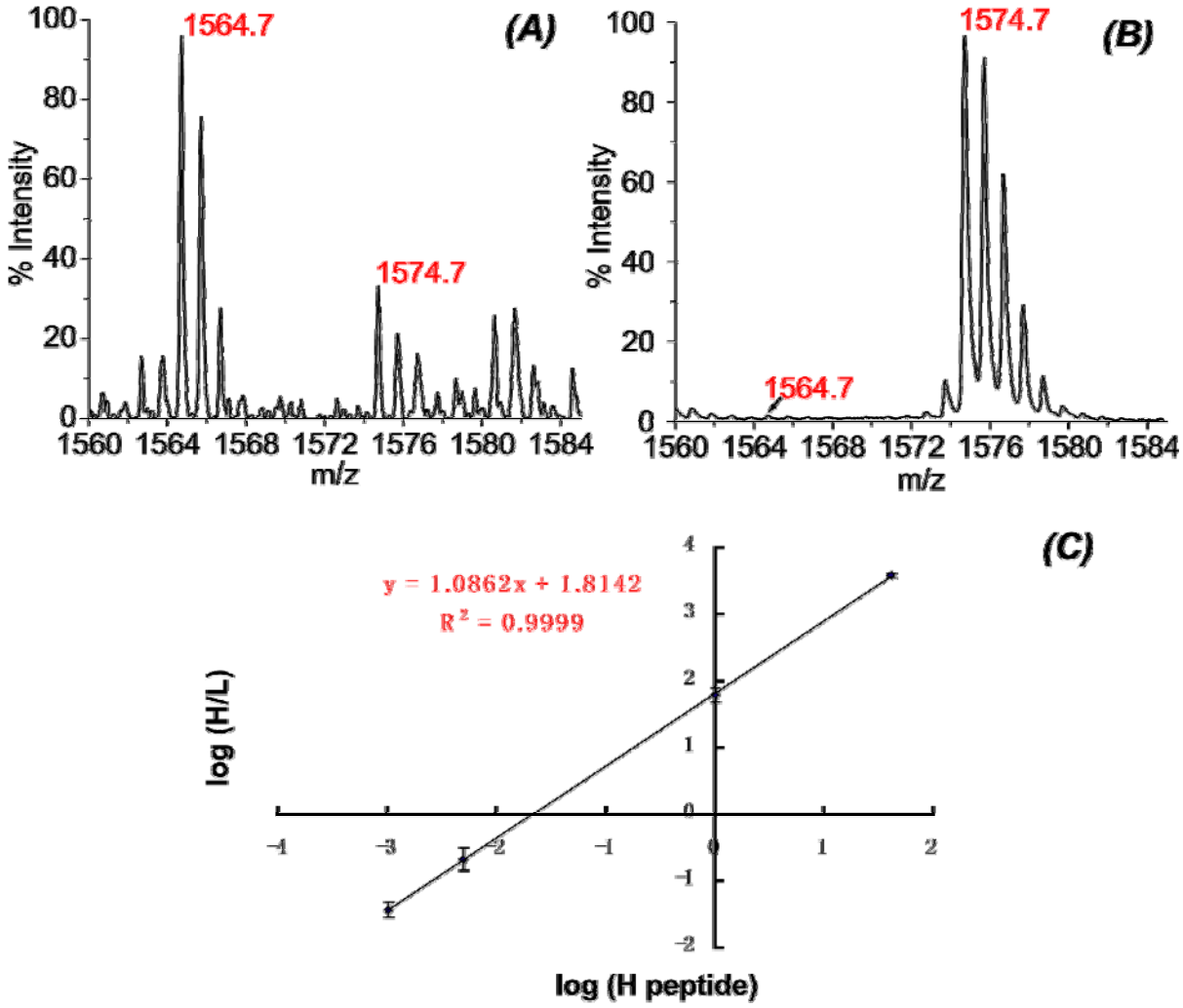


Figure 11: Quantitation of EGFR in SUM102 cells using the EGFR iMALDI. Absolute quantitation of the EGFR peptide MHLPSPTDSNFYR (aa 963-975) (L, light peptide, m/z = 1564.7) in SUM102 cells. Cells were lysed, digested, and incubated with known amounts of heavy peptides (H, m/z =1574.7) as internal standards: A) 0.1pmol, B) 1 pmol of H. C) Logarithmic plot of the observed ratios of monoisotopic ion abundances of H and L in the MALDI-MS spectra versus the absolute amount of H added.

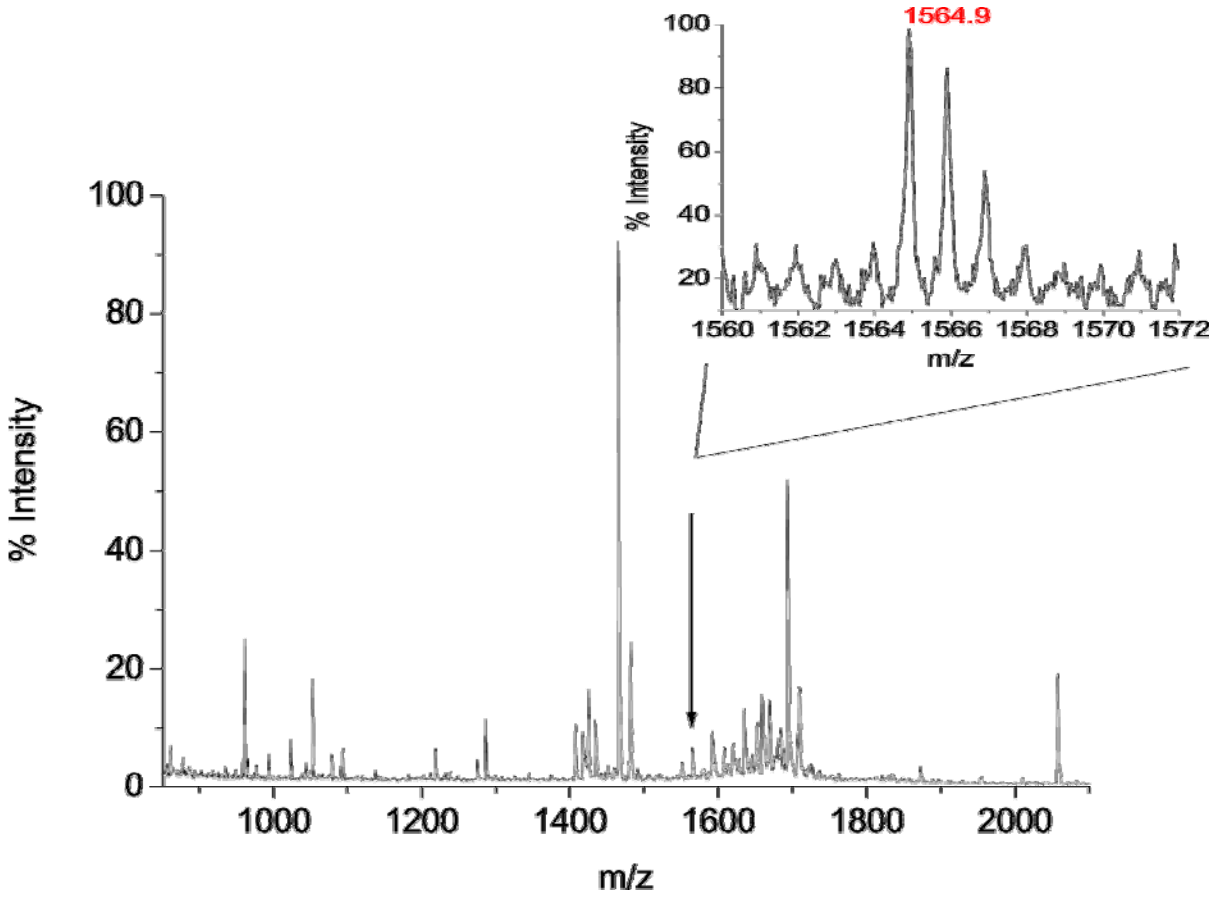


Figure 12: Detection of EGFR using the EGFR iMALDI assay in the basal-like primary human tumor BR97-0137B. MALDI-MS spectrum of peptides affinity-bound to anti-aa963-975 EGFR antibody beads obtained after lysis of BR97-0137B cells, followed by tryptic proteolysis. The singly-charged, epitope-containing, tryptic peptide aa 963-975 MHLPSPTDSNFYR of the EGFR protein can be observed at m/z = 1564.7 (see inset).

Fragment ions	m/z observed	m/z theoretical	Mass accuracy (ppm)
y1	174.884	175.120	-1348
b2	268.785	269.107	-1197
y2	337.799	338.183	-1135
b3	381.839	382.191	-921
y4	598.908	599.294	-644
y5	685.887	686.326	-640
y8	998.796	999.454	-658
y12	1433.062	1433.681	-432

Figure 13: Highly-specific detection of EGFR in ME16C cells by mass spectrometric sequencing of the immunoaffinity enriched EGFR aa963-975 peptide using the EGFR iMALDI. The sequence-specific b and y-ions of the peptide at m/z = 1564.7 affinity-bound to anti-aa963-975 EGFR antibody beads observed in MALDI-MS/MS spectrum after lysis and proteolysis of ME16C cells.