Doctoral Dissertation

Study on pulse-heating ion source and its application for bio-molecules

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Contents

CHAPTER 1 Introduction

Abstract

Mass spectrometry (MS) system as a high sensitivity and high specificity analytical device has attracted extensive attention, especially in the field of biomolecule analysis. With the introduction of the “soft” ionization technique, such as matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI), the MS system is capable of measuring very large even intact biomolecules. Despite many advantages, the deployment of MS system has been limited inside the laboratory, due to the size and power requirements. Recently, the development of miniaturized mass spectrometry (MS) system, which is light weight and low cost, makes it possible to obtain molecular information on-site with minimal sample pretreatment and without sending to a laboratory. In this chapter, the most widely used ionization techniques for mass spectrometry, including electrospray ionization (ESI), matrix-assisted laser desorption/ionization (MALDI), and electron impact ionization (EI), have been briefly reviewed. Their ion formation mechanism and application in the miniaturized mass spectrometry have been also discussed. By comparing the advantages and disadvantages of these conventional ionization techniques, a novel on-chip pulse-heating desorption/ionization method has been proposed to realize the miniaturized mass spectrometry system, in which only a low voltage pulse supplied thermal energy is used instead of high voltage or laser light. The advantages and disadvantages of pulse-heating ion source are discussed. Then the objective of this thesis is proposed followed by the thesis organization.

1.1. General introduction of the ion source

Mass spectrometry (MS) is one of the most widely used analytical tools, and it is valuable in numerous research fields. MS is a high-accuracy, high-sensitivity, and high-throughput methodology to provide qualitative and molecular structural information by measuring the mass-to-charge ratio (*m/z*). Generally, the MS system consists of three elementary parts: the ion source, the mass filter, and the ion detector. There are some optional components that can be coupled with the MS system for enhancing performance.

Figure 1.1 shows a general flow chart for an MS system with varying configurations [25]. In most cases, the sample is introduced to the ion source after purification. Several sample separation and purification techniques are commonly used, such as gas chromatography (GC), liquid chromatography (LC), and capillary electrophoresis (CE). The ion source is used to ionize the sample. The generated ions are accelerated and then separated by one or several mass filters according to their *m/z* values. Finally, the ions are detected and converted to signals by the detector. To obtain the mass spectrum, the ion signals are analyzed by a data processing program.

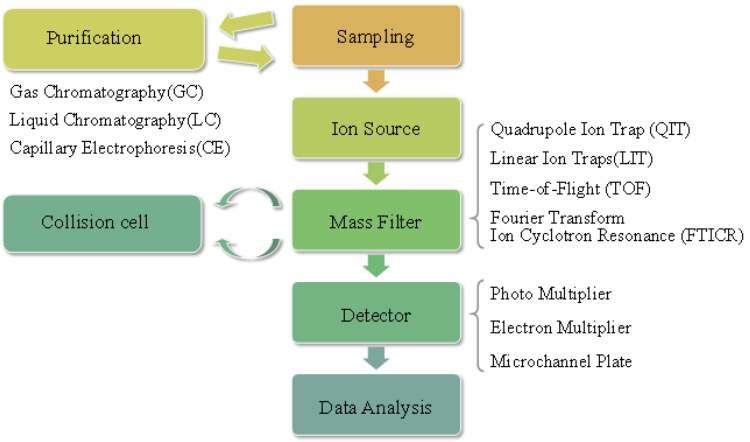

Figure 1.1. The configuration of MS system.

A high-vacuum system is needed for an MS system, especially for the mass filter, to prevent collisions between gaseous molecules. The most commonly used mass filters and ion detectors are also listed on Fig.1.1. With the various configurations, the MS system is capable for various applications. The MS system has been extensively used to various fields including physics, geochemistry, chemistry, and biology. In the field of biology, MS has become a major analytical technique during the last 30 years. The introduction of "soft" ionization methods, including matrix-assisted laser desorption/ionization (MALDI) [1-2] and electrospray ionization (ESI) [3], allow for the analysis of large intact molecules, such as proteins and peptides, by the MS system.

The ion source is one of the most important parts of an MS system as it must be properly matched with the type of analyte. There are many ways to ionize a sample, and each has different applications, advantages, and disadvantages. In other words,

the choice of the ion source can significantly affect MS analysis. Table 1.1 shows a summary of common ionization techniques and their applications. The sampling method and the features are also discussed. For instance, the electron ionization (EI) source is generally used for gas phase samples, where ions are generated through sample evaporation and ionization. For solid or liquid samples, the sample molecules are converted into gas phase ions during desorption and ionization process.

In relation to the generated ions, the ionization techniques are divided into two categories, namely "hard" ionization and "soft" ionization. For the "hard" ionization method, the molecules are cleaved to generate fragmentation ions. In "soft" ionization, the intact molecules are ionized with less fragmentation ions.

Table 1.1. Common ionization techniques and their applications [4].

Ion source	Mass range (Da)	Sampling method	Features
MALDI	< 500,000	Co-crystallized with a matrix	Singly charged ions; Qualitative; Poor reproducibility.
ESI	< 500,000	Sample in solution	Multiple-charged ions; Low back ground;
TI	< 220	Directly place onto a cold filament	Isotope signals; Surface emission; Isotope ratio;
EI	<1000	gas phase molecules ; organic molecules	Mainly fragment ions; Isotope ratio; Gaseous sample only.

In this chapter, the most commonly used ionization techniques for biomolecule analysis, including matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI) will be briefly introduced. For comparison, the electron ionization (EI), thermal ionization (TI), and plasma ionization (PI) will also be outlined. The ion formation mechanism of both positively and negatively charged ions will additionally be discussed.

1.1.1. Electrospray ionization

Electrospray ionization (ESI) is a widely used ionization method, and it can be used to analyze a wide range of compounds from inorganic samples, polymers, and nucleic acids, to peptides and proteins. The ESI source mainly produces multi-charged ions. In this method, a liquid sample is introduced into a spray capillary or needle with a flow rate of 1-1,000 μL/min [5-6]. Under atmospheric pressure, the sample solution is nebulized to form highly charged small droplets by applying a strong electric field. A potential difference (3-6 kV) between the spray point and the counter electrode is used to supply the electric field. These are generally spaced at a distance of 0.3-2 cm [7]. Figure 1.2 shows the schematic image of a typical ESI source. Spray efficiency can be improved using a heated inert gas, such as nitrogen. Before entering the mass filter, the small charged droplets pass through an evaporation chamber for solvent evaporation by a drying gas.

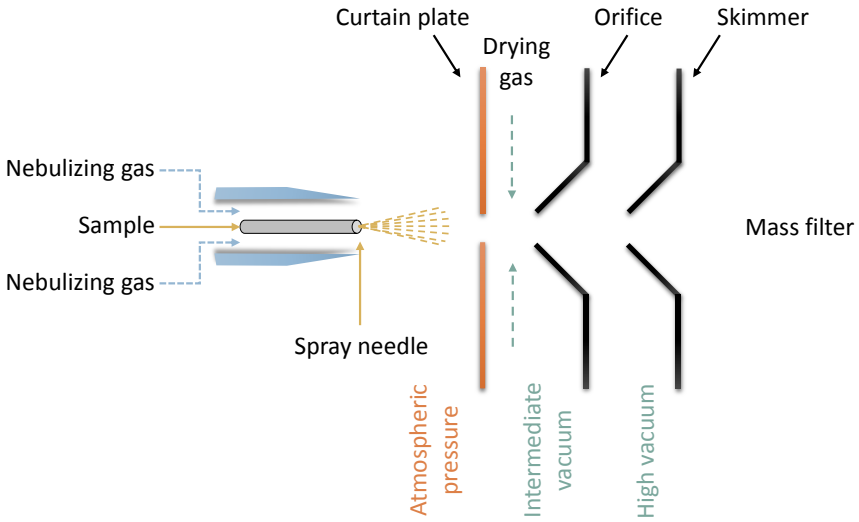

Figure 1.2. Schematic image of a common ESI source.

The polarity of the generated ions is determined by the polarity of the applied voltage. By changing the polarity of the electric field, the ESI source can be used to generate positively charged ions or negatively charged ions. There are two possible ion formation mechanisms for ESI sources, as illustrated in Figure 1.3 [8]. The upper track in Figure 1.3 show an ion evaporation model [9]. This model proposes that the ions are formed due to solvent evaporation and Coulombic explosion of the highly charged small droplets. Since the solvent vaporization process is very fast, there is no time for the charged droplets to reach the equilibrium state. Therefore, as the size of the droplet decreases, the charge density inside the droplet increases until the repulsive force of the droplet surface is larger than the cohesive force of the surface tension.

The lower track in Figure 1.3 shows a charge residual model [3]. In this model, the molecule does not desorb from the charged droplet. In other words, after the solvent is completely evaporated, the charged molecule remains. It is proposed that this phenomenon is more likely to occur with a large molecule [10].

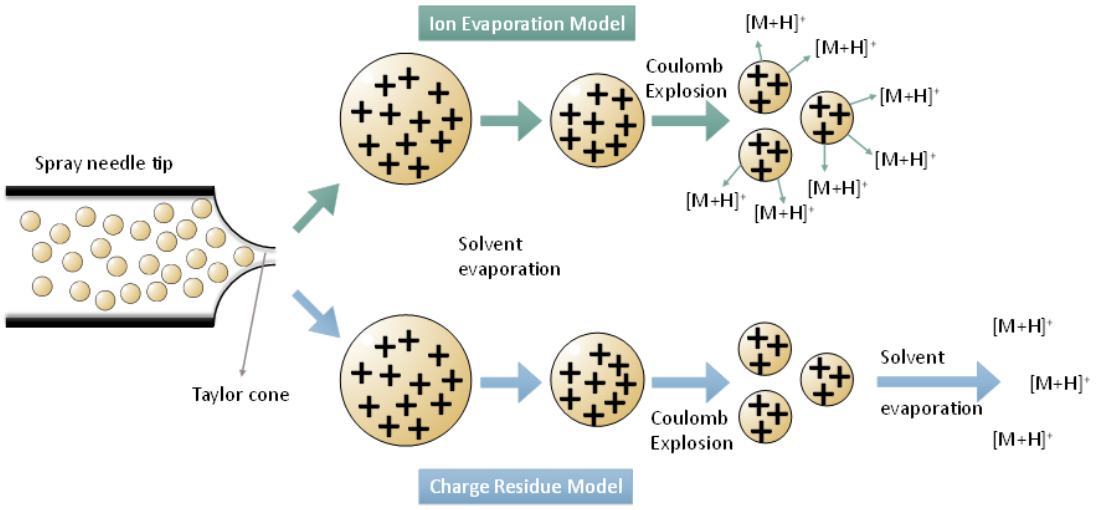

Figure 1.3. Two ion formation mechanisms using an ESI source.

The ions are formed due to the electrochemical process at the probe tip. The formation of multiple-charged ions makes the ESI source compatible with quadrupoles acting as mass filters with limited *m/z* range. Since the sample ions are formed directly from the sample solvent, the ESI source can be coupled with a liquid separation technique, such as HPLC [11].

Despite the many advantages of ESI, there are two shortcomings that cannot be ignored. One is sample wastage. Although the ESI source is more sensitive to sample

concentration than to sample amount, some sample solution is still wasted during the spray process. A pulsed ESI source [12] and a high-duty-cycle mass filter [13] have been reported to solve this problem.

The other shortcoming is the ion suppression effect. The ionization efficiency may decrease due to high concentrations of the analytes competing for limited charges. Additionally, the nonvolatile compounds affect the efficacy of droplet formation and evaporation. For protein samples, a desalting process is needed before analysis using an ESI source because the high salt concentration usually hinders the analyte ion formation [14].

1.1.2. Laser desorption ionization

For laser desorption ionization (LDI), the sample is usually in the solid state. The laser can provide large energy density within a small area. The sample molecules are radiated by a laser pulse that produces the ions. Generally, a matrix is used to absorb the laser energy. It then passes the energy to the sample, leading to ionization.

There are mainly two kinds of matrices. One is composed of organic compounds, which are usually co-crystallized with analyte samples in solution and dried on an ionization plate. This is the most extensively used method, and it is named matrix-assisted laser desorption ionization (MALDI). The other matrix is a modified

ionization plate with nanostructured materials, and it is named surface-assisted laser desorption ionization (SALDI).

In MALDI, one first finds an appropriate matrix, and then one mixes the matrix solution with a target sample solution. Many factors may affect the mass spectrum obtained from MALDI. These include the wavelength of the laser, the pulse energy, the length of the laser, and the sample preparation method. Table 1.2 shows a summary of commonly used laser sources with the wavelengths, photon energies, and the typical pulse widths. [15] The nitrogen laser is the one most frequently used, and it has the wavelength of 337 nm.

The choice of matrix is the most important step for MALDI. The analyte-matrix mixture is dried and co-crystallized on the ionization plate before analysis. The pulse laser beam irradiates the mixture, and the laser energy is absorbed by the matrix followed by sample desorption and ionization. Singly charged ion production is typical for MALDI; however, the ionization mechanism of the ion formation is still unclear.

Generally, the ion formation mechanism is divided into two steps: the primary ionization and the secondary ionization [16]. The primary ionization refers to the first ion formation from the neutral molecule. Generally, the first ions are generated from the matrix. Several different kinds of mechanisms have been reported for explaining

the primary ion formation of MALDI, and they can be divided into two groups: the excited state model and pre-formed ion model [17].

The pre-formed ionization model is the most widely accepted ion formation mechanism. It proposes that proton transfer occurs in the solid phase instead of in the gas phase or during the desorption process in the expanding plume from photon ionized matrix molecules [7]. The secondary ionization refers to the ion being formed in the gas phase. The generated ions will interact and collide with neutral molecules where the charges are transferred between each other. In the gas phase, the generated ions are accelerated by a potential difference before entering the mass filter. The details of the mechanism will be discussed in chapter 3.

Table 1.2. Typical MALDI laser sources [15].

Laser	Wavelength	Photon energy (eV)	Typical pulse width
Nitrogen	337 nm	3.68	<1 ns - few ns
NdYAG×3	355 nm	3.49	5 ns
NdYAG×4	266 nm	4.66	5 ns
Excimer (XeCl)	308 nm	4.02	25 ns
Excimer (KrF)	248 nm	5.00	25 ns
Excimer (ArF)	193 nm	6.42	15 ns
Er.YAG	2.94 μm	0.42	85 ns
CO_2	10.6 μm	0.12	100 ns + 1 μs tail

Normally, the MALDI is operated under vacuum. In 2000, an atmospheric-pressure (AP) MALDI was developed for ionization [18]. This method produces ions under atmospheric conditions from the analyte-matrix mixture sample by

irradiating the sample with a laser beam. Figure 1.4 shows the schematic image of a typical AP-MALDI source. The ionization principle is similar to that of conventional MALDI. A matrix is needed for ionization, and analytes mixed with the matrix are dried on the ionization plate. The only difference is that the ionization process occurs under atmospheric conditions, whereas vacuum conditions are needed for conventional MALDI. The mass filter part is still in a vacuum chamber. Therefore, the generated ions are transferred into the vacuum using an atmospheric-pressure interface (API). Similar to the conventional MALDI, the AP-MALDI also produces mainly singly charged molecule ions. Because the ion's internal energy is more efficient under atmospheric conditions compared to under vacuum conditions, the AP-MALDI shows a softer behavior compared with the conventional MALDI. Generally, no fragmentation ions can be observed in the AP-MALDI.

Despite the advantages of MALDI, there are also some drawbacks. One is the difficulty in coupling with the mass filter since the ions are generated by a pulse rather than continuously. Therefore, only a few mass filters, such as time-of-flight (TOF) and ion trap (IT), are easily coupled with MALDI. Another disadvantage is that it is difficult to analyze a small molecule with a molecular weight less than 1,500 Da with MALDI as the presence of the matrix may result in some signals in the low mass region. However, this problem can be solved by other LDI methods, namely SALDI. The detail of SALDI will be discussed on chapter 4.

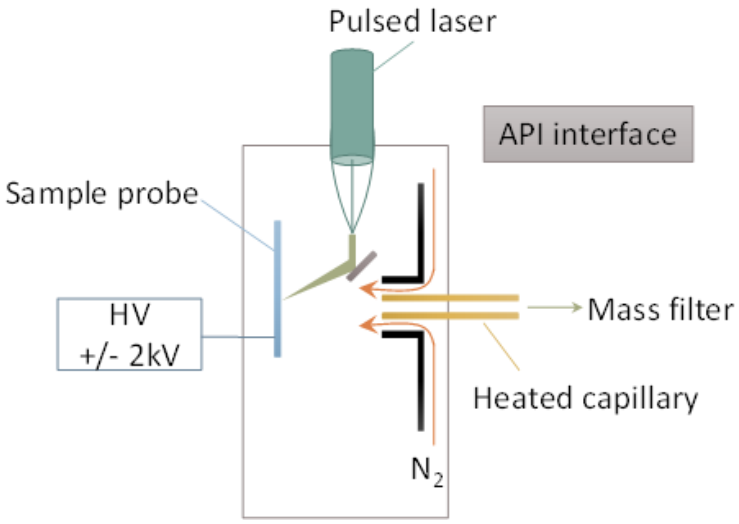

Figure 1.4. Schematic image of a typical AP-MALDI.

1.1.3. The electron impact ion source

The electron impact (EI) ion source uses an electron beam to ionize a gas phase molecule or atom. The EI source has been extensively used in the fields of atmospheric monitoring [19-21], production quality control [22], industrial gas waste inspection [23], and food and drug quality testing [24-25]. Since a gas sample is used, the EI source can couple with a gas chromatography (GC) system. The EI source is also extensively used for the ionization of nonpolar or volatile components. Figure 1.5 shows the schematic for an EI source.

The main component of the EI source is the heated filament used to emit electrons. The gaseous molecules of the analyte sample are injected into the source, and there they collide with the electrons. For sample analysis, gaseous and high-

vapor-pressure samples can be directly introduced into the source, whereas liquid and solid samples need to be heated to convert them into the gas phase. The emitted electrons are accelerated by a potential difference.

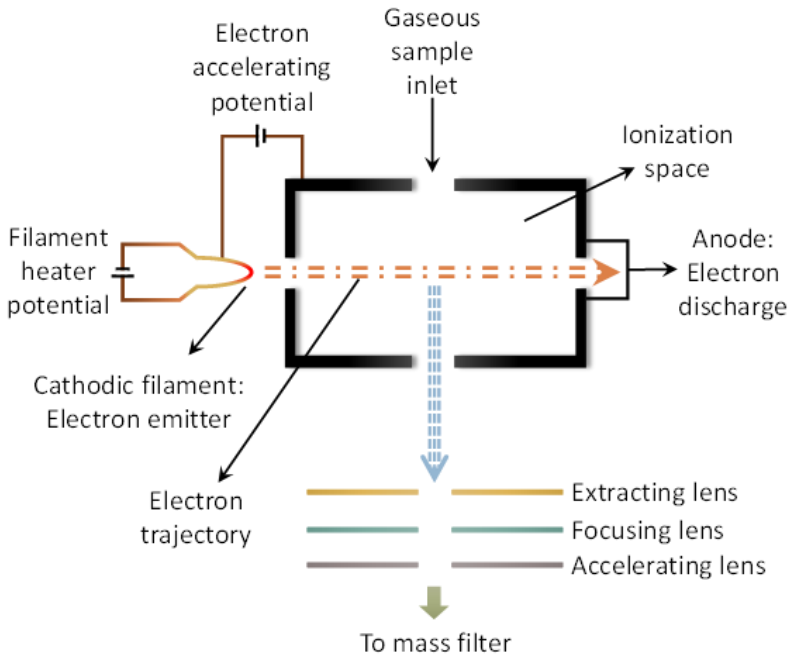

Figure 1.5. Diagram of an electron ion source.

The EI source is commonly used for analysis the organic samples and gaseous samples. EI is also capable of isotope analysis of a sample. In the EI source, the molecular ions and fragmentation ions are produced by bombardment of the analyte by an electron beam. The ion formation mechanisms for both positive and negative ions are shown in Figure 1.6.

The filament is usually made of metallic materials such as tungsten. Heating the filament produces electrons. The generated electron beam is accelerated by an electric field and undergoes collision with the gaseous sample. Electrons are ejected from the sample molecules resulting in positively charged radical cations. Because large molecules are easily destroyed by the electron beam, this ionization method is generally not suitable for them. With some compounds, negatively charged ions also can be generated.

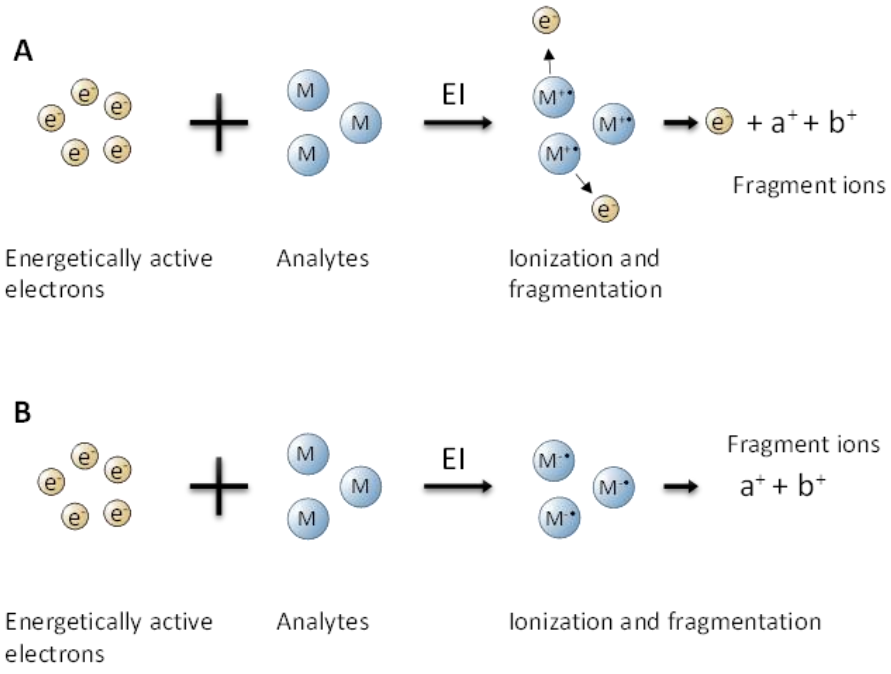

Figure 1.6. Ion formation mechanisms with an EI source: A. positive ion generation; B. negative ion generation.

1.1.4. Thermal ionization

The thermal ionization (TI) method produces atomic or molecular ions by contact with the hot surface of a metal filament [26-27]. It is one of the most powerful techniques for isotopic sample analysis, and it has been widely used in the fields of geochemistry, environmental analysis, and material analysis.

For the TI source, the sample is deposited on a metallic filament (such as tungsten, platinum, or rhenium), and the metal is heated to a high temperature with an electric current. For positively charged ion formation, the electrons transfer from the atom to the filament. In the generation of negatively charged ions, the electrons are transferred from the filament to the atom. In this process, the sample needs to be fixed onto the filament. This is generally accomplished by depositing the sample solution on the surface of the filament and then drying it. The electrodeposition method can also be used for preparing the sample.

The filament is the main component of the TI source. One or more filaments can be used in one TI source. For the TI source with only one filament, the filament is used not only for evaporation of the sample, but it is also used for sample ionization. For TI sources that consist of two or more filaments, the first filament is used to evaporate the sample while the other filament(s) is/are used to ionize the sample. In this way, the sample evaporation rate and the ionization temperature can be controlled independently. This method is particularly suitable for elements with high

vapor pressures. When increasing the temperature of the filament, the sample molecules are evaporated before an appropriate ionization temperature can be reached. The TI source which has three filaments is used to obtain the signal from two different samples at the same time. In such cases, the two kinds of sample can be compared under the same conditions.

For the TI source, both positively and negatively charged ions can be obtained. The positively charged ions are obtained from samples with low ionization potentials, while the negatively charged ions are obtained from atoms or molecules with high electron affinity. Due to their physical and chemical characteristics, metal molecules can form the positively charged ions, while the nonmetallic, semi-metallic and transition metallic samples and their oxides usually form the negatively charged ions. For the different TI sources, the singly charged atomic ions are usually abundantly generated. Under common ionization conditions, there are no multiple-charged ions, and very few cluster ions are observed. However, some metallic samples readily form metal oxide ions.

For TI sources, the ionization efficiency depends on the target elements. By using a thermal ionization cavity (TIC) source, the ionization efficiency can be improved significantly [28]. Figure 1.7 shows the schematic image of the TIC source. The filament is replaced by a refractory metal tube for sample evaporation and ionization. The thermal energy is supplied by an electron beam bombarding the tube. This causes the temperature of the tube to increase to a point where it is high enough

to cause ionization. Along with the sample evaporation process, the ions are produced by gaseous atom interactions. The interaction process includes atom-atom interactions and interaction of the atoms with the inner walls of the cavity. Comparing the conventional TI sources with the filament, the TI source with the cavity shows higher ionization efficiency with orders-of-magnitude enhancement.

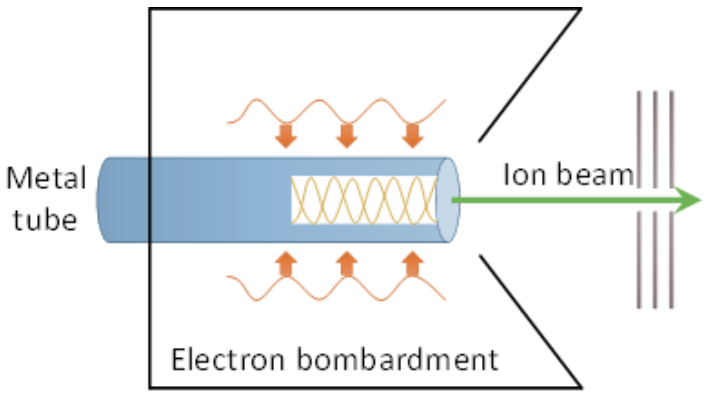

Figure 1.7 Diagram of a typical TI source.

Figure 1.8 shows the schematic image of the thermal-spray (TSP) source [29]. Firstly, the sample solution containing the sample and a salt is introduced into a steel capillary. Secondly, the capillary is quickly heated to a high temperature. Thirdly, the supersonic liquid sample passes through a vacuum chamber and reaches the nozzle. Finally, the sample solution, containing ions, solvent and sample molecules, forms a fine-droplet mist and sprays out.

The charged droplets consist of both the solvent molecules and the target molecules. The droplets are then accelerated by a repelling electrode and are

extracted into the mass filter. The vaporization process for the sample solution is not necessary, since the ions are directly generated from the sample solution. The corona discharge process is used to increase the charge of the droplets which is of benefit to the ion extraction process. The droplets are pumped out continuously as a supersonic beam towards the outlet. This allows for avoidance of a large vapor volume of the solvent. Under vacuum conditions, the heating process is needed to prevent droplet freeze. The heating processes are determined by monitoring the beam temperature with a thermocouple under vacuum. When a current passes through the capillary, the sample solution is heated and also acts as heating resistance.

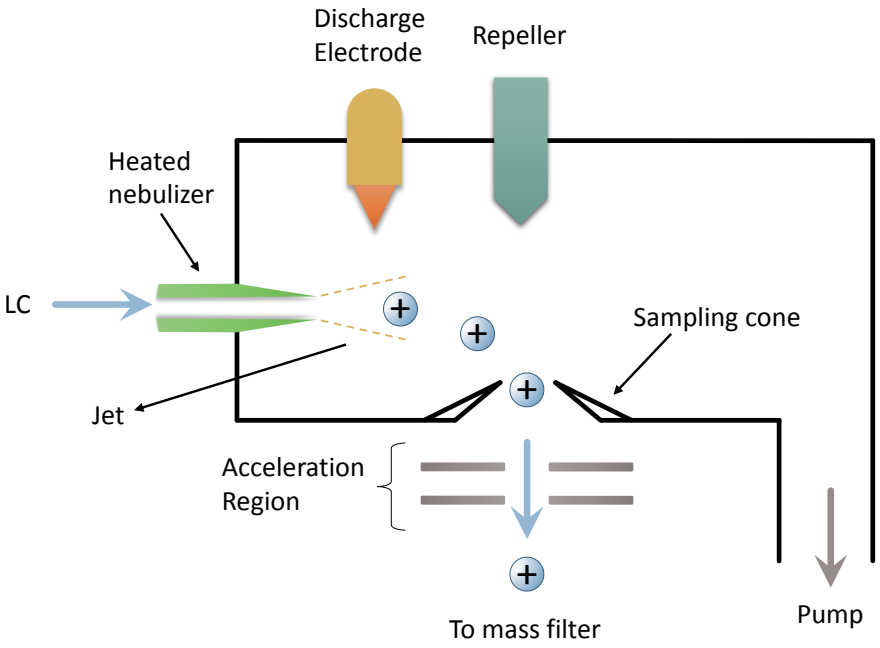

Figure 1.8 Diagram of the thermal spray ion source.

1.1.5. Plasma ionization

Plasma desorption (PD) was developed to ionize a whole intact molecule [7]. It is one of the earliest "soft" ionization methods, and it can be used for biomolecule analysis [30]. The PD source is based on field ionization and field desorption methods [31-32]. For a PD source, the sample is deposited on an aluminized nylon foil. The ionization energy is supplied by the fission fragments of ^{252}Cf which have energies of several megaelectronvolts. The sample is exposed to the fission fragments of ^{252}Cf, resulting in thousands of fragmentations per second. Shock waves are used to induce desorption of neutral and ionic species.

1.2. The miniaturized ion source

The conventional MS system is usually bulky, expensive, and power-consuming. The development of miniaturized mass spectrometry (MS) systems has made it possible to obtain molecular real-time information on-site with minimal sample pretreatment. The goals for miniaturization are the development of a compact size and a low power consumption. In the past 15 years, efforts have been made to miniaturize the components of a variety of MS components, including sample introduction techniques [33-34], ion sources [35-37], mass filters [38-39], ion detectors [40-41], and vacuum systems [42-43]. This has resulted in many MS systems that are portable [44-46], with many being even hand-held [47]. As discussed in 1.2, the ion source is one of the most important parts of the MS system since it determines the analytes that

can be examined. Therefore, there are several approaches to miniaturization of the ion source.

1.2.1. Miniaturized electron ionization

As introduced in 1.1.3, the electron ionization (EI) source is used for analysis of gas samples. A micro-fabricated tungsten filament was developed for a hot-cathode EI source coupled with a miniaturized TOF mass filter [48]. However, the lifetime of the integrated filament was short due to the high temperature. This was then modified and coupled to a cold-cathode EI source [39]. Other modifications then included using a nickel filament [49] and carbon nanotubes (CNTs) as the electron sources. Carbon-based materials have been intensively reported as field emitters for EI sources due to their great mechanical structure and chemical stability. Kornienko et al. reported a diamond-coated silicon whiskers array for a cold cathode EI source [50]. Carbon nanotubes were also reported as field emitters for ionization [51-54].

1.2.2. Miniaturized electrospray ionization

Electrospray ionization (ESI) is a soft ionization method which can be used for a wide range of samples from small molecules to large biomolecules under atmospheric conditions. Many studies have been done regarding the miniaturization of the ESI source. The initial work toward miniaturization of the ESI source used

nanospray ionization [55-56]. Smaller spray tips with lower sample flow rates were developed for the nanospray method.

The introduction of nanospray allows for the ion source to be integrated with a microfluidic separation device. Using an on-chip emitter as an EI source coupled with a microfluidic separation device allowed for direct spray from the planar chip edge for ionization [57-58]. This on-chip EI source was fabricated with glass which resulted in sample spillage from the edge of the chip. To overcome this drawback, a modification of spray orifices was undertaken. One method used a hydrophobic coating to increase the wettability of the surface. Another method changed the material to a hydrophobic bulk polymer such as polydimethylsiloxane (PDMS) [59] or polyethyleneterephthalate (PET) [60]. Unfortunately, these modifications made for complicated fabrication processes, and the polymers also suffered from short lifetimes. Additionally, the polymer materials were also limited by their instability in organic solvent, and they generally showed heavy background signals in the mass spectra [61].

The desorption electrospray ionization (DESI) source is a modification of the ESI source. It is one of the most intensively investigated ambient ionization techniques. In the DESI source, the spray probe is attached to a mobile x-y stage to control the position of the spray nozzle on the sample film. A schematic image of a typical DESI source is shown in Figure 1.9. The ion formation mechanism is similar to that of the ESI source. The small charged droplets are sprayed to form a thin solvent layer. Solid

phase analytes are then extracted into the thin film. Collison of the primary droplets with the thin film results in ejection of secondary droplets which contain the analytes. Although the high gas and solvent flows are not conducive to miniaturization, a DESI source with a sampling probe has been reported for a miniaturized MS system [62].

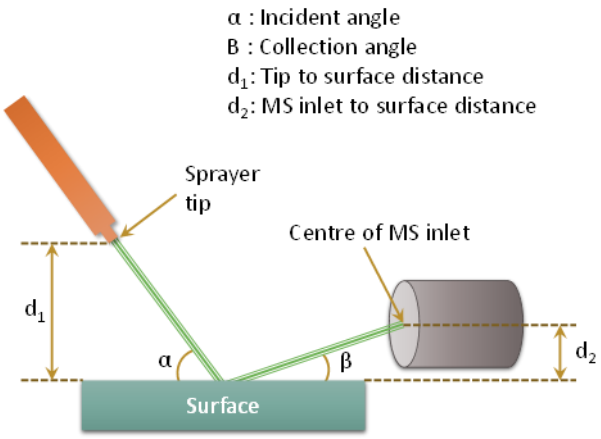

Figure 1.9. Schematic image of typical DESI source.

Paper spray ionization (PSI) is another variant of an ESI source [63-65]. It is also commonly used for a miniaturized MS system. The biggest advantage of PSI is that the paper is available at very low cost. Therefore, this method is especially suitable for diagnostics. The fabrication of a PSI source is also very simple in that one only needs to cut the paper into a triangle shape. The sample consumption is also very low (~ 10 μL) compared with a conventional ESI source. Ions are generated by applying a high voltage to the paper. It has been reported that the PSI source is

useful with a wide variety of analytes, including small organic samples, peptides, and proteins [65].

1.3. The pulse-heating ion source

In our previous study, a novel ionization technique named pulse-heating desorption/ionization (PHDI) was reported for protein analysis [66]. This ion source consists of a metallic layer (Cr/Pt) on a Si/SiO_2 substrate. It is fabricated by a lift-off process. Figure 1.10 shows the schematic image of the PHDI source. The narrow part is used as the ionization zone. By applying a low voltage pulse (width: 500 ns) to the ion source chip, the center part is heated. The thermal energy supplied by the ionization zone is used for sample evaporation and ionization. In an early study, bovine serum albumin (BSA), a protein sample, was analyzed by the PHDI source.

The sample preparation method is similar to conventional MALDI in that a matrix is needed. The protein is dissolved in a water solution and is mixed with the matrix solution. The mixture (0.5 μL) is then dropped on the center part of the ion source chip followed by drying under vacuum. The protein sample cocrystallizes with the matrix and forms a sample layer. Several kinds of matrices, which can also be used for conventional MALDI, have been investigated to obtain a clear mass spectrum with singly-charged ion signals. These include 2,5-dihydroxybenzoic acid (DHB), sinapic acid (SA), and 2,5-dihydroxyacetophenone (DHAP). Among these matrices, only the thin film of the sample with DHB showed a singly charged ion

signal. With SA or SHAP, the grain-like morphology of the sample only produced multiple-charged and fragmentation ion signals.

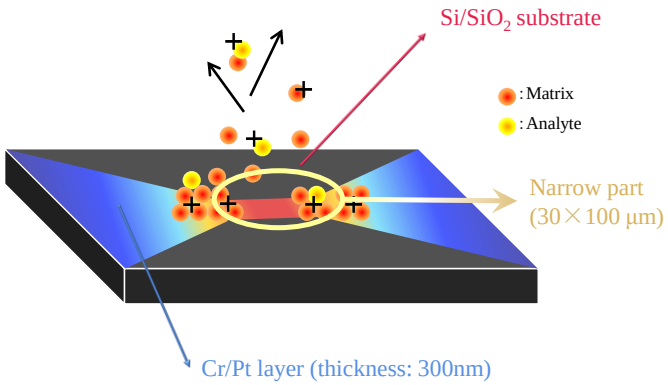

Figure 1.10. Schematic image of pulse-heating ion source.

With the pulse-heating ion source, a miniaturized MS system has been built with the on-chip ion source and a very short home-made time-of-flight (TOF) mass filter (8 cm). Compared with other miniaturized ion sources, the PHDI only consumes a very small amount of the sample (~ 750 mmol) [67]. The ionization efficiency is very high compared with ESI or MALDI since the sample can be ionized with only one pulse. In addition, this PHDI source can be directly coupled with a TOF mass filter and therefore can be used for protein analysis. The observation of singly charged signals indicates that soft ionization occurs. Furthermore, the fabricated on-chip PHDI source can be used to further reduce the MS system size and cost since it does not need a laser, high voltage, or a heated ambient gas.

Despite the many advantages, this PHI method suffers from the problems of poor reproducibility and the presence of fragmentation signals. The mass spectra obtained from the PHI source show many fragmentation signals and multiple-charged ion signals, thus creating complications in the interpretation of the spectrum. The undesirable fragmentation and poor reproducibility limit the application of this on-chip ion source in a miniaturized MS system. Although several matrices have been investigated, the main factor necessary for obtaining a clear mass spectrum with only singly charged ion signals and high signal-to-noise (S/N) ratio is still unclear. To overcome these difficulties, it is important to find the factors important for obtaining a clear mass spectrum by the PHI method.

Different from the existing ionization method, the PHDI is a novel way for ionization. Therefore, a better understanding of the ionization mechanisms are very important. With only a BSA sample having been investigated and measured, the sample capability of PHDI is still unclear. In this thesis, efforts to increase reproducibility and to produce clearer mass spectra with the PHDI-MS system are discussed. In addition, the ability of the PHDI in the analysis of different kinds of samples is explored.

1.4. Objective and organization.

The aim of this study is to solve the problem of poor reproducibility and to find the key influencing factors of the PHDI source. An ionization mechanism is proposed

for understanding the ion formation pathway. Then the capability of different samples are investigated.

The chapter 1 reviewed the common ionization techniques and their recent advances in miniaturization. Advantages, disadvantages, and current issues of the PHDI method are carefully discussed. Based on such considerations, the objectives of this dissertation is proposed.

The chapter 2 discusses the possible reasons for the poor reproducibility. Some modifications on the system and sample preparation method such as alignment system and oxygen plasma treatment for sample surface are proposed. Effects of such modifications on the signal reproducibility and mass spectral quality are carefully evaluated. As a result, highly reproducible mass spectra with ultralow background signal are able to be obtained.

The chapter 3 proposes an ion formation mechanism for the pulse-heating desorption/ionization method. By introducing a negative ion detection mode to the MS system, the formation of negative ions can be observed. Comparing the positive and negative ion mass spectra for variety of samples, a possible ion formation pathway based on supplied thermal energy is discussed.

Chapter 4 shows a novel functionality of the pulse-heating desorption/ionization method for matrix-free ionization. A variety of biomolecules are tested without

matrix by the PHDI and conventional MALDI methods for comparison. The mass range of matrix-free pulse-heating desorption/ionization method is estimated.

Chapter 5 summarizes all the work done in this thesis and perspectives of the PHDI method.

Reference

1. Tanaka, K., et al., Protein and polymer analyses up to m/z 100 000 by laser ionization time-of-flight mass spectrometry. Rapid communications in mass spectrometry, 1988. **2**(8): p. 151-153.
2. Karas, M. and F. Hillenkamp, Laser desorption ionization of proteins with molecular masses exceeding 10,000 daltons. Analytical chemistry, 1988. **60**(20): p. 2299-2301.
3. Dole, M., et al., Molecular be an MS of macroions. The Journal of Chemical Physics, 1968. **49**(5): p. 2240-2249.
4. Herbert, C.G. and R.A. Johnstone, Mass spectrometry basics2002: CRC press.
5. Clark, A.E., et al., Matrix-assisted laser desorption ionization–time of flight mass spectrometry: a fundamental shift in the routine practice of clinical microbiology. Clinical microbiology reviews, 2013. **26**(3): p. 547-603.
6. Fenselau, C., Peer Reviewed: MALDI MS and Strategies for Protein Analysis. Analytical Chemistry, 1997. **69**(21): p. 661A-665A.
7. El-Aneed, A., A. Cohen, and J. Banoub, Mass spectrometry, review of the basics: electrospray, MALDI, and commonly used mass analyzers. Applied Spectroscopy Reviews, 2009. **44**(3): p. 210-230.

8. Kiendler, A. and F. Arnold, Unambiguous identification and measurement of sulfuric acid cluster chemiions in aircraft jet engine exhaust. Atmospheric Environment, 2002. **36**(11): p. 1757-1761.
9. Fenn, J., et al., Electrospray ionization for mass spectrometry of large biomolecules. Science. **246**(4): p. 926.
10. Mann, M., C.K. Meng, and J.B. Fenn, Interpreting mass spectra of multiply charged ions. Analytical Chemistry, 1989. **61**(15): p. 1702-1708.
11. De Hoffmann, E. and V. Stroobant, Mass spectrometry: principles and applications2007: John Wiley & Sons.
12. Awad, H., M.M. Khamis, and A. El-Aneed, Mass Spectrometry, Review of the Basics: Ionization. Applied Spectroscopy Reviews, 2015. **50**(2): p. 158-175.
13. Iribarne, J. and B. Thomson, On the evaporation of small ions from charged droplets. The Journal of Chemical Physics, 1976. **64**(6): p. 2287-2294.
14. Dole, M., et al., Gas phase macroions. Macromolecules, 1968. **1**(1): p. 96-97.
15. Glish, G.L. and R.W. Vachet, The basics of mass spectrometry in the twenty-first century. Nature Reviews Drug Discovery, 2003. **2**(2): p. 140-150.
16. Berggren, W.T., M.S. Westphall, and L.M. Smith, Single-pulse nanoelectrospray ionization. Analytical chemistry, 2002. **74**(14): p. 3443-3448.
17. Lu, Y., et al., Pulsed electrospray for mass spectrometry. Analytical chemistry, 2001. **73**(19): p. 4748-4753.
18. Cech, N.B. and C.G. Enke, Practical implications of some recent studies in electrospray ionization fundamentals. Mass Spectrometry Reviews, 2001. **20**(6): p. 362-387.
19. Hamdan, M.H. and P.G. Righetti, Proteomics today: protein assessment and biomarkers using mass spectrometry, 2D electrophoresis, and microarray technology. Vol. 18. 2005: John Wiley & Sons.
20. Zenobi, R. and R. Knochenmuss, Ion formation in MALDI mass spectrometry. Mass Spectrometry Reviews, 1998. **17**(5): p. 337-366.

21. Bae, Y.J. and M.S. Kim, A thermal mechanism of ion formation in MALDI. Annual Review of Analytical Chemistry, 2015. **8**: p. 41-60.
22. Laiko, V.V., M.A. Baldwin, and A.L. Burlingame, Atmospheric pressure matrix-assisted laser desorption/ionization mass spectrometry. Analytical Chemistry, 2000. **72**(4): p. 652-657.
23. Santos, F. and M. Galceran, Modern developments in gas chromatography-mass spectrometry-based environmental analysis. Journal of Chromatography A, 2003. **1000**(1): p. 125-151.
24. Santos, F. and M. Galceran, The application of gas chromatography to environmental analysis. TrAC Trends in Analytical Chemistry, 2002. **21**(9): p. 672-685.
25. Llamas, A.M., C.B. Ojeda, and F.S. Rojas, Process Analytical Chemistry-Application of Mass Spectrometry in Environmental Analysis: An Overview. Applied Spectroscopy Reviews, 2007. **42**(4): p. 345-367.
26. Cook, K.D., K.H. Bennett, and M.L. Haddix, On-line mass spectrometry: a faster route to process monitoring and control. Industrial & engineering chemistry research, 1999. **38**(4): p. 1192-1204.
27. Lisec, J., et al., Gas chromatography mass spectrometry–based metabolite profiling in plants. Nature protocols, 2006. **1**(1): p. 387-396.
28. Careri, M., F. Bianchi, and C. Corradini, Recent advances in the application of mass spectrometry in food-related analysis. Journal of Chromatography A, 2002. **970**(1): p. 3-64.
29. Meyer, M.R., et al., Beta-keto amphetamines: studies on the metabolism of the designer drug mephedrone and toxicological detection of mephedrone, butylone, and methylone in urine using gas chromatography–mass spectrometry. Analytical and bioanalytical chemistry, 2010. **397**(3): p. 1225-1233.
30. Inghram, M.G. and W.A. Chupka, Surface ionization source using multiple filaments. Review of Scientific Instruments, 1953. **24**(7): p. 518-520.

31. Heumann, K.G., et al., Precision and accuracy in isotope ratio measurements by plasma source mass spectrometry. Journal of Analytical Atomic Spectrometry, 1998. **13**(9): p. 1001-1008.
32. Johnson, P., A. Bolson, and C. Henderson, A high temperature ion source for isotope separators. Nuclear Instruments and Methods, 1973. **106**(1): p. 83-87.
33. Vestal, M.L., Ionization techniques for nonvolatile molecules. Mass Spectrometry Reviews, 1983. **2**(4): p. 447-480.
34. McNeal, C.J. and R.D. Macfarlane, Observation of a fully protected oligonucleotide dimer at m/z 12637 by californium-252 plasma desorption mass spectrometry. Journal of the American Chemical Society, 1981. **103**(6): p. 1609-1610.
35. Beckey, H., Field desorption mass spectrometry: A technique for the study of thermally unstable substances of low volatility. International Journal of Mass Spectrometry and Ion Physics, 1969. **2**(6): p. 500-502.
36. Inghram, M.G. and R. Gomer, Mass spectrometric analysis of ions from the field microscope. The Journal of Chemical Physics, 1954. **22**(7): p. 1279-1280.
37. Wright, S., et al., MEMS-Based Nanospray-Ionization Mass spectrometry. Journal of Microelectromechanical Systems, 2010. **19**(6): p. 1430-1443.
38. Anthony, S.N., D.L. Shinholt, and M.F. Jarrold, A simple electrospray interface based on a DC ion carpet. International Journal of Mass Spectrometry, 2014. **371**: p. 1-7.
39. Chen, F., et al., Single-cell analysis using drop-on-demand inkjet printing and probe electrospray ionization mass spectrometry. Analytical chemistry, 2016. **88**(8): p. 4354-4360.
40. Vigne, S., et al., Optimization of an electron impact ion source on a MEMS time-of-flight mass spectrometry. Sensors and Actuators B: Chemical, 2017. **243**: p. 690-695.

41. Qing, J., et al., A miniaturised electron ionisation time-of-flight mass spectrometry that uses a unique helium ion removal pulsing technique specifically for gas analysis. Analyst, 2013. **138**(12): p. 3394-3401.
42. Chaudhary, A., F.H.W. van Amerom, and R.T. Short, Experimental evaluation of micro-ion trap mass spectrometry geometries. International Journal of Mass Spectrometry, 2014. **371**: p. 17-27.
43. Tassetti, C.-M., et al., A MEMS electron impact ion source integrated in a microtime-of-flight mass spectrometry. Sensors and Actuators B: Chemical, 2013. **189**: p. 173-178.
44. Hadjar, O., et al., Preliminary demonstration of an IonCCD as an alternative pixelated anode for direct MCP readout in a compact MS-based detector. Journal of The American Society for Mass Spectrometry, 2012. **23**(2): p. 418-424.
45. Hanay, M., et al., Single-protein nanomechanical mass spectrometry in real time. Nature nanotechnology, 2012. **7**(9): p. 602-608.
46. Chen, C.-H., et al., Design of portable mass spectrometrys with handheld probes: aspects of the sampling and miniature pumping systems. Journal of the American Society for Mass Spectrometry, 2015. **26**(2): p. 240-247.
47. Sugiyama, K., Y. Ukita, and Y. Takamura, Development of on-chip vacuum generation by gas–liquid phase transition. Sensors and Actuators A: Physical, 2012. **176**: p. 138-142.
48. Gao, L., et al., Design and characterization of a multisource hand-held tandem mass spectrometry. Analytical chemistry, 2008. **80**(19): p. 7198-7205.
49. Hendricks, P.I., et al., Autonomous in situ analysis and real-time chemical detection using a backpack miniature mass spectrometry: concept, instrumentation development, and performance. Analytical chemistry, 2014. **86**(6): p. 2900-2908.
50. Yang, M., et al., Development of a palm portable mass spectrometry. Journal of the American Society for Mass Spectrometry, 2008. **19**(10): p. 1442-1448.

51. Gao, L., et al., Handheld rectilinear ion trap mass spectrometry. Analytical chemistry, 2006. **78**(17): p. 5994-6002.
52. Yoon, H.J., et al., Fabrication of two types of micro ion sources for a micro time-of-flight mass spectrometry. Journal of Micromechanics and Microengineering, 2007. **17**(8): p. 1542.
53. Velasquez-Garcia, L.F., B.L.P. Gassend, and A.I. Akinwande, CNT-based MEMS/NEMS gas ionizers for portable mass spectrometry applications. Journal of Microelectromechanical Systems, 2010. **19**(3): p. 484-493.
54. Kornienko, O., et al., Field-emission cold-cathode EI source for a microscale ion trap mass spectrometry. Analytical chemistry, 2000. **72**(3): p. 559-562.
55. Bower, C.A., et al., On-chip electron-impact ion source using carbon nanotube field emitters. Applied Physics Letters, 2007. **90**(12): p. 124102.
56. Hwang, J.S., et al. The Micro Mass spectrometry with A Carbon Nano Structure Ion Source. in 2006 1st IEEE International Conference on Nano/Micro Engineered and Molecular Systems. 2006.
57. Chen, L.-Y., et al., A microionizer for portable mass spectrometrys using double-gated isolated vertically aligned carbon nanofiber arrays. IEEE Transactions on Electron Devices, 2011. **58**(7): p. 2149-2158.
58. Park, S.-J., J. Eden, and K.-H. Park, Carbon nanotube-enhanced performance of microplasma devices. Applied physics letters, 2004. **84**(22): p. 4481-4483.
59. Wilm, M.S. and M. Mann, Electrospray and Taylor-Cone theory, Dole's beam of macromolecules at last? International Journal of Mass Spectrometry and Ion Processes, 1994. **136**(2-3): p. 167-180.
60. Wilm, M. and M. Mann, Analytical properties of the nanoelectrospray ion source. Analytical chemistry, 1996. **68**(1): p. 1-8.
61. R an MSey, R. and J.M. R an MSey, Generating electrospray from microchip devices using electroosmotic pumping. Analytical Chemistry, 1997. **69**(6): p. 1174-1178.

62. Xue, Q., et al., Multichannel microchip electrospray mass spectrometry. Analytical chemistry, 1997. **69**(3): p. 426-430.
63. Huikko, K., R. Kostiainen, and T. Kotiaho, Introduction to micro-analytical systems: bioanalytical and pharmaceutical applications. European journal of pharmaceutical sciences, 2003. **20**(2): p. 149-171.
64. Rohner, T.C., J.S. Rossier, and H.H. Girault, Polymer microspray with an integrated thick-film microelectrode. Analytical chemistry, 2001. **73**(22): p. 5353-5357.
65. Sikanen, T., et al., Microchip technology in mass spectrometry. Mass spectrometry reviews, 2010. **29**(3): p. 351-391.
66. Chen, C.-H., et al., Real-time sample analysis using a sampling probe and miniature mass spectrometry. Analytical chemistry, 2015. **87**(17): p. 8867-8873.
67. Liu, J., et al., Development, characterization, and application of paper spray ionization. Analytical chemistry, 2010. **82**(6): p. 2463-2471.

CHAPTER 2 Improved Sampling Method for Pulse-heating Ion Source

Abstract

For the PHDI source, the sampling method and alignment of ion source and mass filter were found related to the reproducibility of mass spectra. Therefore, in this chapter, the sampling method and alignment process has been improved. For the sampling method, the sample solution has been dropped onto the center of the ion source chip and then dried in a vacuum, previously. To obtain a thin and uniform sample film, the oxygen plasma treatment process has been introduced for pre-treatment of the ion source chip. The sample consumption has been reduced from 500 nL to 250 nL. As a result, the thickness of sample film has been reduced from 300 nm to 20 nm. To improve the accuracy of alignment between ion source and mass filter, a chip holder has been designed and added to the mass spectrometry system. The results show that the miss alignment may cause a shift of the ion signal position. With a precise alignment, reproduced mass spectra have been obtained. By investigating the position dependence of ionization zone, a calibration method for the on-chip ion source mass spectrometry has been established.

2.1. Introduction

Sample preparation process plays an important role in ionization technique for the detection sensitivity and data acquisition [1]. In the pulse-heating desorption/ionization, the sample film is formed by crystallization. The thermal energy is supplied from the bottom side of the sample film. Therefore, the thickness and uniformity of sample film affects the thermal transfer efficiency and thermal energy distribution, significantly. Besides, the inhomogeneous sample surface may lead to signals variation. Since the analyte molecules are co-crystallized, the size of the crystal is also an important factor. Figure 2.1 shows the schematic image of the effect of crystal size on the obtained mass spectra. The size of crystal also affects to the signals position. Compared with the small size of crystals, the large size of crystals are hard to form a uniform and thin sample film.

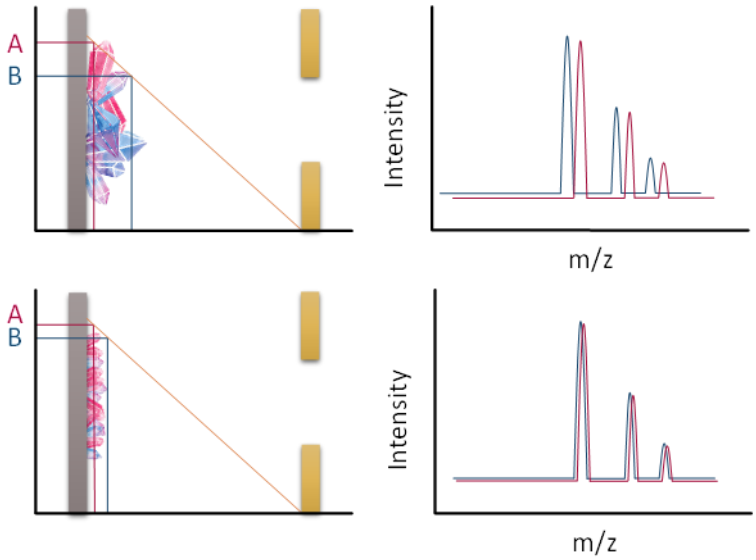

Figure 2.1. Schematic image of the effect of crystal size.

The sample preparation method of pulse-heating ion source is similar with conventional matrix-assisted laser desorption/ionization (MALDI) source. For MALDI source, a lot of studies have been done for developing the sample preparation method with a uniform sample film [2-4]. Tasso Miliotis et al reported an airbrush device to generate a fine spray for making a thin and uniform sample film for MALDI [5]. Acetone was chosen as the solvent for a fast evaporation, resulting in very small crystals of sample was obtained. The electrospray method was also used to obtain a uniform sample film [6-7]. The dried droplet method is the first and most widely used sample preparation method [8]. In the dried droplet method, the sample is mixed with matrix in an appropriate solvent and dropped the pre-mixed solution on the ionization zone, then dried in air or in vacuum. It has been reported that a higher analyte sensitivities could be obtained by a matrix sublimation process [9]. Under a vacuum condition, the sample could be purified by the sublimation process. It also has been reported that the smaller and uniform distributed sample crystals show a higher spot-to spot reproducibility [10].

The morphology and thickness of sample film may affect to the reproducibility. Similar with MALDI source, the sample forms a film for ionization [11]. Therefore, the uniformity of the sample film is one of the factor. Since the ion is accelerated by an electric field, the accuracy of the electric fields relative to ideal fields. The precision of the electrodes becomes another factor. The imperfections, apertures, and non-ideal shapes may affect to the mass signal and result in the mass shifts. This is because the ions experience a high energy collision and higher order nonlinear

resonances, especially when the ions are away from the center of the field, which causes the shift of their motion frequency, resulting in ejection at a slightly shift of the *m/z* [11].

2.2. Objective

In this chapter, the improvements of the sampling process are described to obtain a clear mass spectrum with high signal-to-noise ratio (S/N), less background, and better reproducibility. First, the fabrication process of the ion source was improved by using a glass mask instead of the film mask for fabricating a precise electrode. Second, the sample preparation process was improved by adding an oxygen plasma treatment process to obtain a thin and uniform sample film by increasing the wettability of chip surface. Third, a chip holder was fabricated for fixing the position of the ion source chip in order to maintain accurate alignment between the ionization and the inlet of the mass filter.

2.3. Experimental

2.3.1. Fabrication of on-chip ion source

The ion source chip consisting of a Pt/Cr micro metal electrode has been fabricated by a lift-off processing on a SiO_2/Si substrate (20 mm × 20 mm). Same with previous report, the Cr layer with a thickness of 220 nm and the Pt layer with

a thickness of 30 nm has been deposited by sputtering (ULVAC, MNS-2000, Japan) [12]. The schematic image of on-chip ion source is shown in Figure 2.2. The center part of Pt/Cr micro metal electrode (size: 30 μm × 100 μm) works as the ionization zone. In order to reduce the interaction of each ionization zone during ionization, the number of ionization zone within one ionization chip has been reduced from 5 to 4 and the space for separating the ionization zone has been increased from 1 mm to 3 mm. For the new design, the size of one ion source is 16 mm × 2 mm. For one chip, there are 4 ion sources. Both ends of the ion source are connected with wires by soldering. The length of each ion source is increased from 12 mm to 16 mm for easy soldering.

In order to fabricate a neat and uniform edge of ionization zone, the mask used for the on-chip ion source fabrication has been changed from film mask to glass mask. The glass mask has been fabricated by electron-beam lithography system (ELIONIX ELS-3700) with a dose of 10^{-4} C/cm^2 and a beam current of 3 nA. Figure 2.3 shows the microscopic images of the ionization zone fabricated by different masks. It can be easily seen that the edge of ionization zone of the left one is not neat enough. With the serrated edge, the resistance of the ionization zone is increase due to the smaller cross-sectional area. Therefore, when apply high thermal energy, it easy broke due to the inhomogeneous thermal energy distribution.

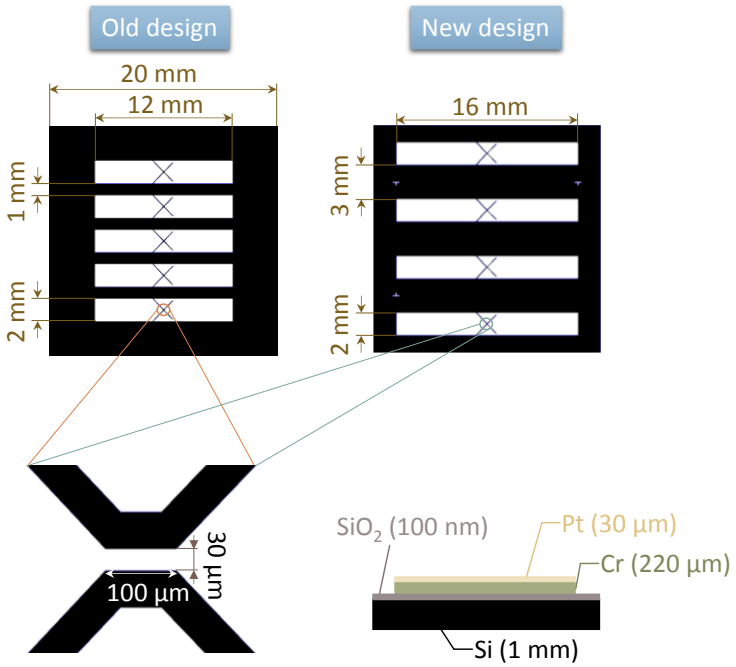

Figure 2.2. Schematic images of on-chip ion source.

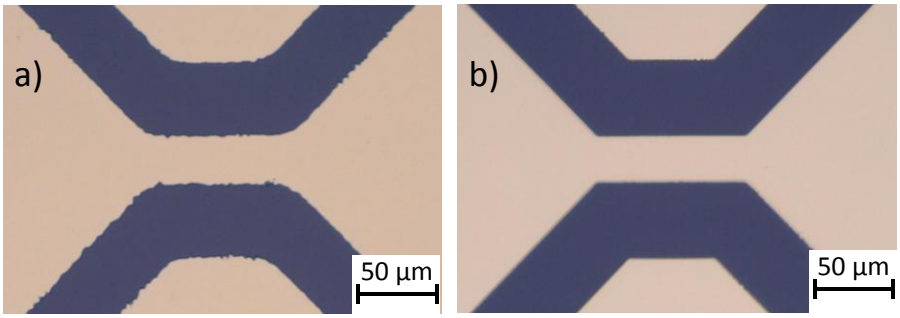

Figure 2.3. Microscopic images of the ionization zone with a) rough edge and b) neat edge.

2.3.2. Oxygen plasma treatment

Before sampling process, there are two steps to pretreat the ion source chip. The first step is cleaning. After soldering with wires, the ion source chip was carefully cleaned by following process. (1) Place the ion source chip into a clean container and pour in enough acetone (99.5%, Wako, Japan) to submerge the whole ion source chip. Then place the container in the ultrasonic bath and sonicate for 5 minutes. (2) Repeat the above steps but change the solvent to ethanol (99.9%, Wako, Japan). (3) Repeat the above steps but change the solvent to pure water (Millipore Co., USA). (4) Dry the ion source chip using a stream of high-purity nitrogen. Store the ion source chip in a dust-free environment.

The second step is treatment with oxygen plasma. In the previously study, a SiO_2 layer has been used to improve the wettability of the chip surface. However, the introduction of the SiO_2 layer reduces the efficiency of thermal conduction. In this study, an oxygen plasma treatment process was introduced instead of the SiO_2 layer to improve the wettability of the chip surface. It has been reported that the surface wetting properties of platinum (Pt) increases after oxygen plasma treatment [13]. After oxygen plasma treatment, a platinum oxide film has been formed on the surface of the platinum layer, resulting in the increased hydrophilicity. Since the Pt is a kind of catalytic material, a variety of chemical substances can be adsorbed on the platinum surface [14]. The longer the exposure time in the air, more and more substances (such as CO, carbohydrate or other organic compounds) are adsorbed on

the Pt surface. Then the surface free energy of Pt surface decreases. Therefore, the sample was dropped on the center part (ionization zone) of each on-chip ion source. Then dry the sample in a vacuum. The solvent is sublimated under a vacuum condition. It has been reported that the initial ion velocity increases due to the sublimation process [15]. The sublimation process also contributs to the formation of thin and uniform sample film.

The contact angle experiments were performed to confirm the increasing of water wettability. This is the conditions used for oxygen plasma treatment. For the ion source chip pre-treatment, 10 min was used to ensure the treatment was completed. After the treatment, place the chip in the air for 2 hours, the surface is still wet. Figure 2.4 shows microscopic images of the water droplet on the ion source chip surface. With the oxygen plasma treatment process, the contact angle is strongly reduced.

Figure 2.4. Schematic image of on-chip ion source.

In this experiment, sodium chloride (NaCl, 99.5%) obtained from Wako Pure Chemical (Japan) has chosen as the analyte for testing the system. NaCl has been dissolved in Milli-Q water with a concentration of 4 mg/mL. 2,5-dihydroxybenzoic acid (DHB, 98%) sinapic acid (SA), and 2,5-dihydroxyacetophenone (DHAP) have been used as the matrix obtained from Wako Pure Chemical (Japan). The matrices were dissolved in acetone with the concentration of 10 mg/mL. Cytochrome C (Cyt C) purchased from MP Biomedicals (France) was prepared by dissolving in Milli-Q water (Millipore, Billerica, MA, USA) with the concentration of 1 mg/mL. Subsequently, the protein sample was mixed with the matrix solution with a ratio of 1:1. 250 nL of sample solution was dropped on the micro electrode and dried in a vacuum.

2.3.3. Fabrication of chip holder

The alignment of the ionization zone relative to the mass filter inlet is very important for the performance of the pulse-heating ion source. Because the generated ion might be scattered by the electric field between the ion source chip and the acceleration zone, and thus cannot be effectively sucked in mass filter. In order to reduce the loss of ions and to improve its accuracy, the ionization zone requires precise alignment. For other ion sources, the position of the ion source is fixed or aligned using a microscope. Therefore, the alignment between the ion source and the mass filter could be ensured. However, the pulse-heating ion sources is fixed on a chip. And the chip position should be fix to obtain a better alignment between

the ion source and the mass filter. Therefore, a chip holder is needed to fix the position of ion source chip and the distance between the chip and mass filter inlet, in order to achieve precise alignment of ionization zone.

Considering the cost and fabrication time, a poly-dimethyl siloxane (PDMS) and poly methyl 2-methylpropenoate (PMMA) based chip-holder was designed and fabricated. The chip holder developed in this study includes two layers (Figure 2.5). The PDMS was attached onto a 40 mm × 30 mm PMMA plate. The PDMA layers are used for holding chip and for fixing the position of the chip. The thickness of the PDMS holder is determined by the height of chip and must be fit for chip to keep a constant distance to the time-of-flight mass filter (TOF) part. Liquid Sylgard 184 (Dow Corning Toray Co., Ltd, Japan) was thoroughly mixed with curing agent in a weight volume ratio of 10:1 to make PDMS gel. The process for making chip holder is as follows. First, cut a SiO_2/Si substrate with a same size of ion source chip and put the SiO_2/Si substrate on the center of PMMA plate as a mold. Second, poured the glue mixture to create PDMS sheet. After curing for approximately 3 hours at 75 ^{o}C, in the third step, cut down three sides of the PDMS sheet to the same size as the chip and let one side cover the edge of the chip. After that, took the chip mold out, the remained PDMS become a stage to fix the chip on the holder. Finally, affixed the chip holder on a single-axis operating manipulator to change different ionization spots.

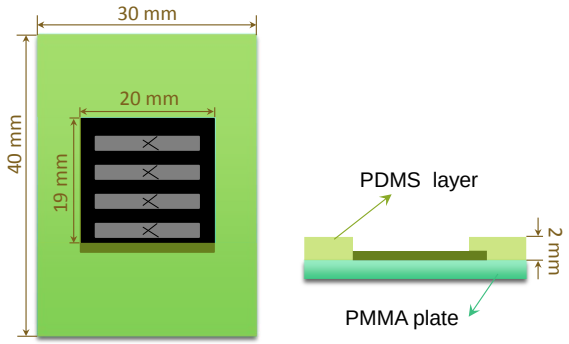

Figure 2.5. Schematic image of on-chip ion source.

2.3.4. Experiment setup

The main components of the miniaturized mass spectrometry, including an on-chip ion source, a liner time-of-flight (TOF) mass filter (8 cm) and a channeltron electron multiplier (CEM) as the detector (Detector Technology, Model 414, USA), are placed in a vacuum chamber (~10^{-3} Pa) [12]. Figure 2.6 shows the schematic image of the miniaturized mass spectrometry (MS) system.

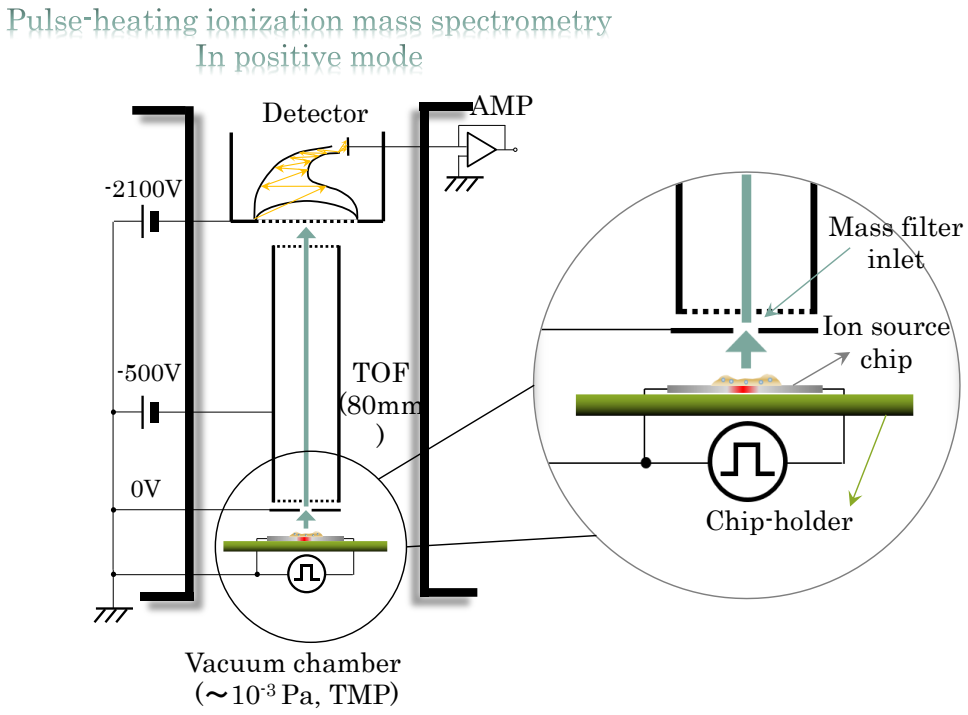

Figure 2.6. Schematic image of on-chip ion source.

The chip holder was installed on a single-axis operating manipulator. A single pulse voltage (20-40 V, width: 500 ns) is applied to the on-chip ion source for ionization by a function generator (AFG3251, Tektronix Inc., USA). The generated ions are extracted and accelerated by using the potential difference between the surface of the ion source and the inlet of the TOF. The TOF signals detected by the CEM are amplified by an amplifier unit (C9663, Hamamatsu photonics, Japan), then recorded by an oscilloscope (TDS2022C, Tektronix Inc., USA). The mass spectra is calculated from the TOF signals as previously reported [12].

2.4. Results and discussion

2.4.1. Simple inorganic sample ionization

As discussed previously, there are some multiple charged ion signals and fragmentation signals are observed from the mass spectra for protein analysis. To confirm these signals, mass spectra of a pristine chip (without sample) and a chip with pure acetone are measured. No remarkable signals are seen indicating that the background signal is negligible. Since the width of ionization pulse is 500 ns, the signal shift is inevitable. Therefore, a calibration process is needed. There are two basic types of calibration methods, namely external and internal. For the external calibration, the standard sample and the analyte are measured separately. The parameters obtained from the standard sample are used for analyte sample calibration. For the internal calibration, the standard sample is mixed with the analyte sample. The signal from the standard sample is identified, then used to calibrate the entire spectrum. The internal calibration is more accurate than the external calibration, but has the following drawbacks. One is that a prior knowledge of the ratio of the standard sample to the analyte sample is needed. If the quantities of standard sample are not enough, the standard signals may not be identified. On the other hand, the sample signal may be overlapped or suppressed due to excessive proportion of standard samples. Here, the simple inorganic sample, namely NaCl has been chosen for external calibration. Figure 2.7 shows the mass spectra of NaCl (the inset shows an optical microscope image of the fabricated chip with the NaCl

sample). As clearly seen on the surface of the ion-source chip, the NaCl sample is uniformly distributed, which is very important for obtaining reproducible high-quality mass spectra. The intense Na^+ peaks have been observed in the mass spectrum with a high signal-to-noise ratio. Furthermore, there are no undesirable signal in the high mass region of the spectrum suggesting the NaCl is suitable for calibration.

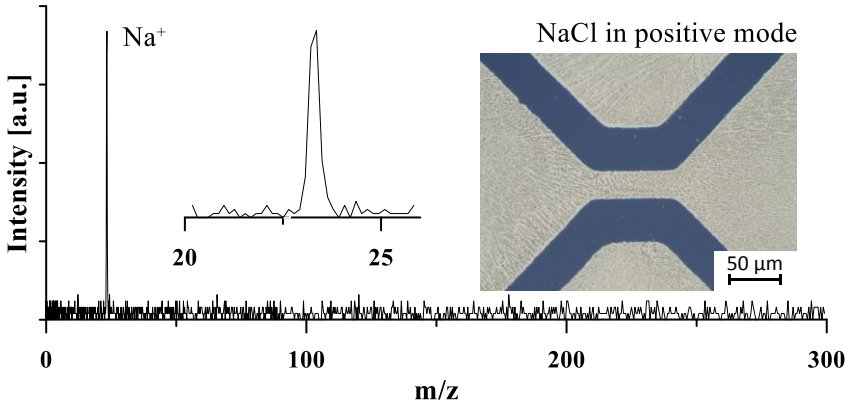

Figure 2.7. Mass spectra and microscopic image of NaCl.

2.4.2. Effect of alignment

It has been reported that the alignment of MALDI plume relative to the mass spectrometry inlet is very important for Atmospheric pressure (AP) MALDI performance. The misaligned of the plume reduces the ion count rates due to that the ions cannot be efficiently drawn in by the vacuum and may scattered by the electric fields in this region [16]. To analyze the position effect of the alignment between ionization zone and the inlet of TOF mass filter, track of the ions has been

numerically calculated by COMDOL 4.2a. The simulation model is based on previous study. The following formulas are used to calculate the electric potential and ion trajectory.

$$E = -\nabla \mathrm{V}$$

$$\frac{\mathrm{d}(m_p v)}{\mathrm{dt}} = qzE$$

Where, m_p is mass of one ion, v is ion velocity, q is elementary charge, and z is ion valence. Here, the protein molecule (Cyt C) is used, and only singly charged ion is considered. Therefore, the m_p value has benn fixed at $12{,}000 \times 1.602 \times 10^{-27}$ kg. Figure 2.8 shows the potential distribution and the ion movement trajectory. In ideal case, all generated ions research to the detector with a precise alignment. When the ionization zone off the center 0.1 mm, a few ions is scattered by the electric fields and lost in the free flight region. When the ionization zone off the center 0.5 mm, only a few ions can research to the detector. When the ionization zone off the center is more than 1.0 mm, the entire MALDI plume has been consumed by the chamber. In the real case, the mesh and the hole of inlet gasket also may affect to the ion flight track.

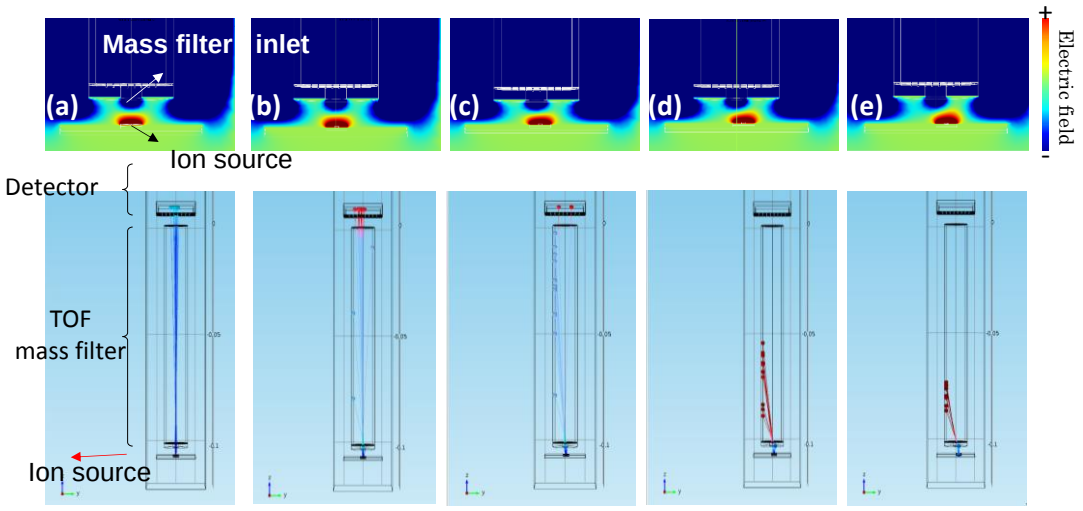

Figure 2.8. Numerical simulation of electric potential and ion trace with different position of on-chip ion source. (a) Center; (b) Y-axis translation 0.1 mm; (c) Y-axis translation 0.5 mm; (d) Y-axis translation 1.0 mm; (e) Y-axis translation 1.5mm.

After adding the chip holder for fixing the chip position, the position effect has been investigated. The diameter of the mass filter inlet is 3.0 mm. The ion source ship has been fixed on the chip holder and the chip holder has been fixed on a single-axis operating manipulator. To obtain the mass spectra, the pulse-voltage has repeatedly applied with the same ionization zone in one chip but the different position. Figure 2.9 shows the mass spectra of NaCl sample with the different position. The chip has been deliberately misaligned by 0.3, 0.6, and 0.9 mm. The signal of Na^+ shows a shift. When moving the chip off the center more than 0.9 mm, no signal has been observed suggesting the generated ions has been consumed before reaching to the detector. This result indicates that the precise alignment is very important not only for calibration process, but also for preventing the ion losses for the pulse-heating desorption/ionization method.

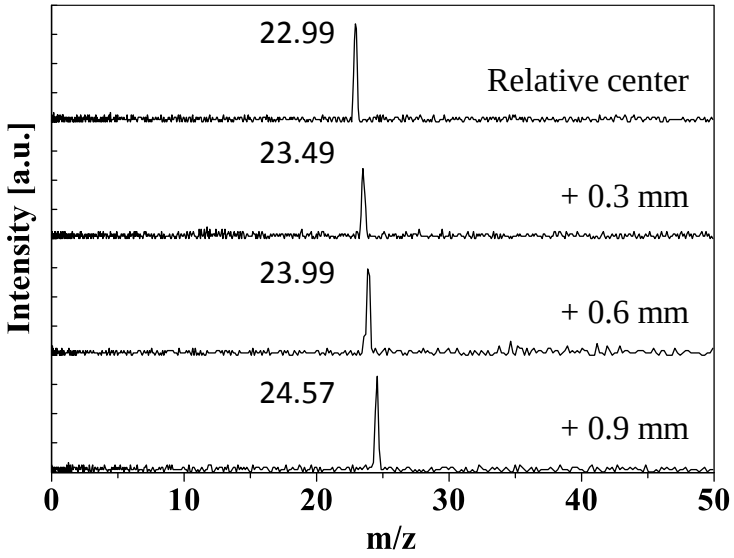

Figure 2.9. Mass spectra of NaCl in positive mode with different chip positive voltage.

2.4.3. Reproducibility of on-chip ion source

With a precise alignment, the reproducibility of the pulse-heating desorption/ionization source has been investigated. Three different ion source chips each of which consists of three ionization zones have been used for NaCl sample. Only one pulse has been used for sample ionization. Figure 2.9 shows the obtained mass spectra. A singly charged Na^+ ion signal has been obtained with good reproducibility.

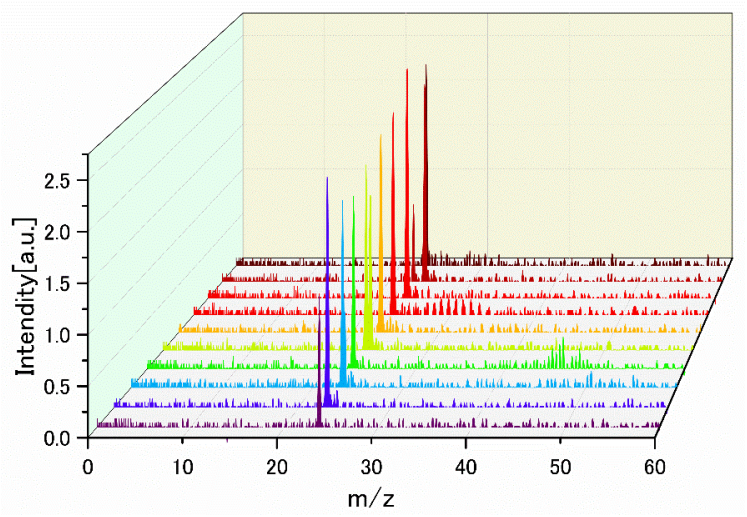

Figure 2.10. Reproducibility of NaCl in positive mode.

2.4.4. Effect of the matrix

It has been reported that a thin-film like morphology of sample layer is desired to transfer thermal energy effectively and to obtain the singly-charged ion signal [12]. Without the SiO_2 insulating layer, the surface of the ion source chip is hydrophobic. The hydrophobic chip surface is benefit to fabricate a thin and uniform sample layer due to fast evaporation of sample solvent. At first, the thin-layer sample preparation method was used for Cyt C.

The thin-layer sample preparation method includes two steps. First, the matrix solution is dropped on the chip. The sample fast spreads on the chip since the viscosity of sample solvent is very low (acetone). The acetone evaporates within a few seconds and a thin and uniform matrix layer is formed. Second, drop the analyte-matrix mixture solution is dropped on the matrix layer. Then the sample is dried in

a vacuum condition. Within minutes, a uniform sample layer is formed with a thickness approximately 300 nm.

The standard matrices [17-20], including DHB, DHAP, and SA have been chosen for Cyt C. The molecular weight of Cyt C is 12 kDa. DHB can be used for MALDI analysis of a wide variety pf peptides, proteins, polymers and carbohydrates. DHAP is a matrix used for preparation of protein with a mass of 8-100 kDa. And SA is also a good choice for protein analysis. It is suitable for lager protein (10-150 kDa) and some polymers.

Figure 2.11 shows the microscopic images of sample layer before and after pulse-heating process by using the thin-layer sample preparation method. 250 nL of matrix and 250 nL of analyte-matrix mixture has been used. By using DHAP as the matrix, a film-like sample layer has been obtained. There are many small crystals evenly distributed on the sample layer. For the SA, granular crystals have been observed. The DHB as the matrix has formed a film-like sample layer but not uniform. After pulse-heating, all the sample in the ionization zone has been vanished.

Figure 2.12 shows the mass spectra obtained by these samples. Similar results have been obtained with the Bovine serum albumin (BSA) sample [21]. The singly-charged ion signal can be obtained from film-like sample layer. Besides, there are multiple charged ion signals and fragmentation signals.

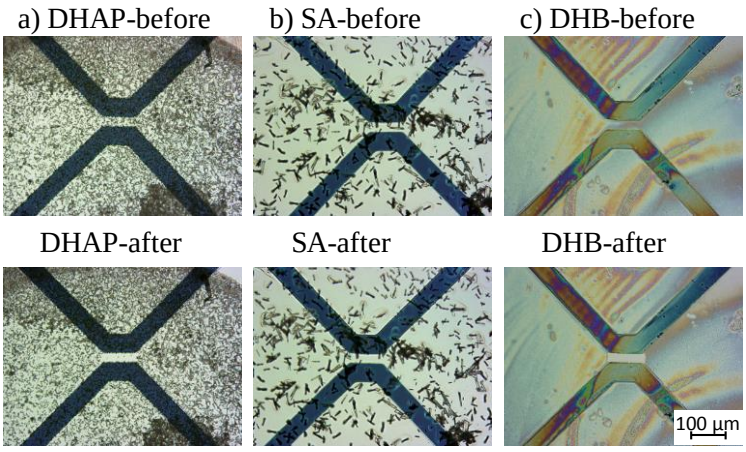

Figure 2.11. Microscopic images of Cyt C sample prepared by thin-layer method.

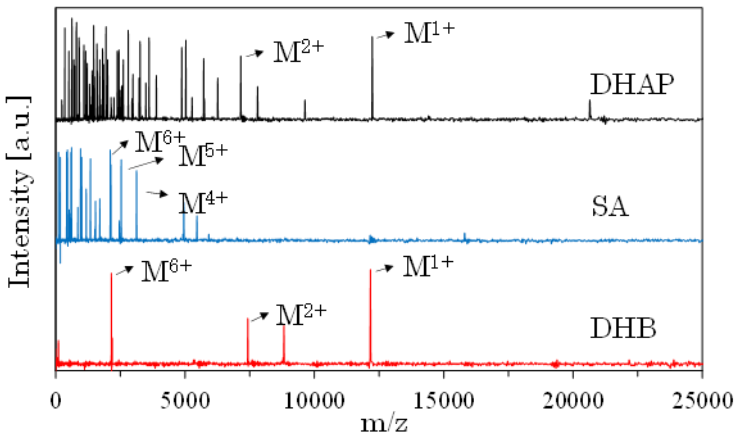

Figure 2.12. Mass spectra of Cyt C sample prepared by thin-layer method.

2.4.5. Morphology of the sample film

As discussed in 2.3.2, the wettability of chip surface can be increased by introducing of oxygen plasma treatment. After pretreatment of the ion source chip surface, the fast spreading of sample can be predicted since there is 50% of water

contained in the analyte-matrix mixture. Then the sample is dried in a vacuum condition. Figure 2.13 shows the microscopic images of sample layer. Compared with the thin-layer sample preparation method (see Figure 2.6), a much uniform sample layer is observed. For Cyt C mixed with DHAP sample, a much smaller crystal particles are present and form a uniform film sample layer. Although there are a few crystals present, the Cyt C mixed with SA sample has also formed a film-like sample. Furthermore, the Cyt C mixed with DHB sample shows a very uniform sample film. Therefore, DHB is used as the matrix for protein analysis in all the experiment.

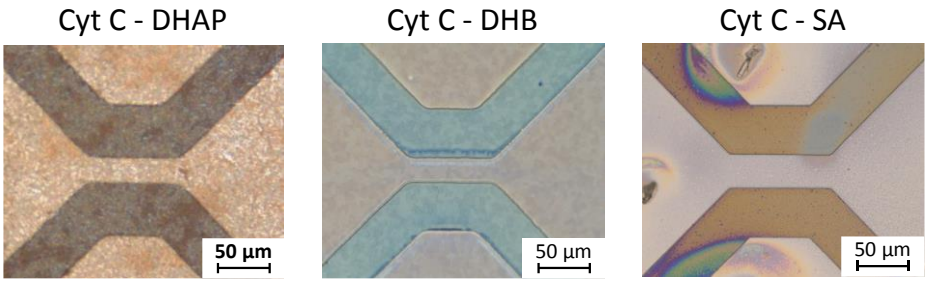

Figure 2.13. Microscopic images of Cyt C sample prepared after oxygen plasma cleaning.

Figure 2.14 (a) shows the thickness measurement of Cyt C mixed with DHB. After one pulse-heating, the sample in the ionization zone has been evaporated. Then the thickness has been measured by a surface profilometer (P-15 Stylus Profiler, KLA-Tencor Corp., USA) from the ionization zone to the sample layer. And the profiling result is show in Figure 2.13 (b). Compared with thin-layer sample preparation method, the thickness of sample layer is strongly reduced form 300 nm to 20 nm.

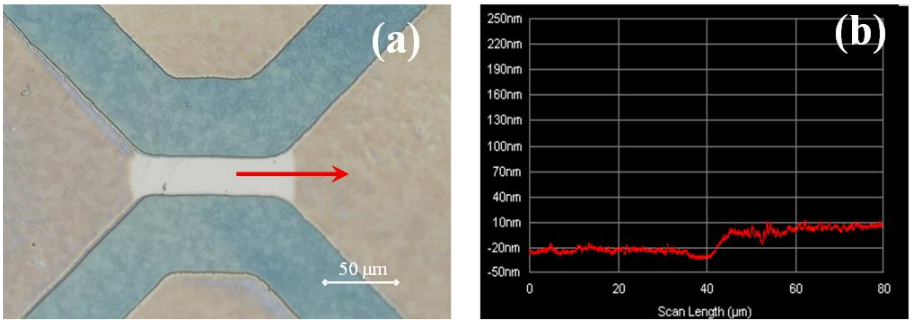

Figure 2.14. The thickness measurement of Cyt C mixed with DHB sample. (a) The microscope image of ion source chip after experiment. (b) The surface profile of ion source chip.

There are several key points for a thinner uniform sample film fabrication. The first key point is the sample consumption. For thin-layer method, there are two layer of sample which may increase the sample layer thickness. Besides, the total sample consumption for thin-layer method is 500 nL, whereas 250 nL for pre-mixed sample after oxygen plasma treatment. The second key point is the cleanliness of the sample surface. It is difficult to form a uniform sample film if there are dusts on the ion source chip. The third key point is fast spread and fast dry. Since the wettability is increased by the oxygen plasma treatment, the liquid sample spreads on the ion source chip in the initial step of sample preparation. Then a thin liquid sample layer is formed on the surface of ion source chip. Since the sample was dried in a vacuum condition, the solvent is easy to evaporate. Actually, the drying process of sample layer is very fast due to a relatively large surface area. And the ion source surface is hydrophilic, the liquid sample layer is dried before shrinkage. Therefore, a thinner and uniform sample film could be obtained.

Figure 2.15 shows the mass spectra in different sample film conditions. The microscopic image is inset. Under optimized condition, only singly-charged ion signal has been obtained with a thinner sample layer (upper one), whereas several fragmentation ion signals have been also obtained with the relatively thick sample layer (lower one). It has been reported that the small particle size of sample may hinder the heat diffusion [15]. It could be suspected that the thermal energy distributed more uniform with a thinner and uniform sample layer compared with a thick sample layer when the same thermal energy is supplied by the ion source chip.

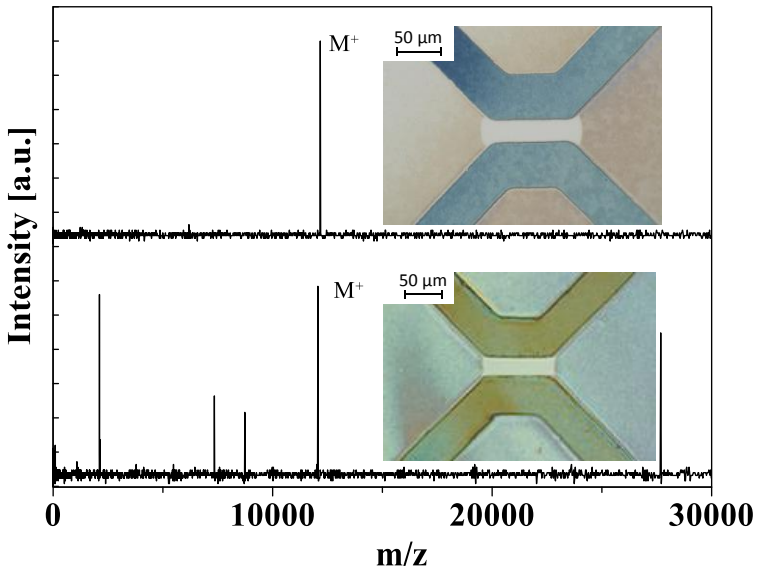

Figure 2.15. Mass spectra in different sample film conditions.

2.5. **Summary**

A chip holder for fixing ion source and maintaining distance to analyzer has been designed and fabricated successfully. As a result, a precise alignment between

the ion source and TOF part has been achieved, leading to the improvement of reproducibility and MS signals. The sample preparation of pulse-heating desorption/ionization method has been improved by introducing the oxygen plasma process. For protein analysis, the premixed sample has been dropped on the ion source chip and dried in vacuum to form the sample layer. A much thinner sample film with a thickness of 30 nm has been obtained.

Reference

1. Vorm, O., P. Roepstorff, and M. Mann, Improved resolution and very high sensitivity in MALDI TOF of matrix surfaces made by fast evaporation. Analytical Chemistry, 1994. **66**(19): p. 3281-3287.
2. Billeci, T.M. and J.T. Stults, Tryptic mapping of recombinant proteins by matrix-assisted laser desorption/ionization mass spectrometry. Analytical chemistry, 1993. **65**(13): p. 1709-1716.
3. Nicola, A.J., et al., Application of the fast-evaporation sample preparation method for improving quantification of angiotensin II by matrix-assisted laser desorption/ionization. Rapid communications in mass spectrometry, 1995. **9**(12): p. 1164-1171.
4. Cohen, S.L. and B.T. Chait, Influence of matrix solution conditions on the MALDI-MS analysis of peptides and proteins. Aanlytical Chenistry –Washinton DC-, 1996. **68**: p. 31-37.
5. Miliotis, T., et al., Ready-made matrix-assisted laser desorption/ ionization target plates coated with thin matrix layer for automated sample deposition in high -density array format. Rapid communications in mass spectrometry, 2002. **16**(2): p. 117-126.

6. Axelsson, J., et al., Improved Reproducibility and Increased Signal Intensity in Matrix-ssisted Laser Desorption/Ionization as a Result of Electrospray Sample Preparation. Rapid communications in mass spectrometry, 1997. **11**(2): p. 209-213.
7. Preisler, J., et al., Capillary Electrophoresis-Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry Using a Vacuum Deposition Interface. Analytical chemistry, 2000. **72**(20): p. 4785-4795.
8. Karas, M. and F. Hillenkamp, Laser desorption ionization of proteins with molecular masses exceeding 10,000 daltons. Analytical chemistry, 1988. **60**(20): p. 2299-2301.
9. Röhl, H., S. Goethel, and K. Reihs, MPep MALDI Chips for high-sensitivity and high-throughput peptide analysis by MALDI-TOF MS. Nature Methods, 2005. **2**(6).
10. Önnerfjord, P., et al., Homogeneous sample preparation for automated high throughput analysis with matrix-assisted laser desorption/ ionisation time-of-flight mass spectrometry. Rapid communications in mass spectrometry, 1999. **13**(5): p. 315-322.
11. Snyder, D.T., et al., Miniature and Fieldable Mass spectrometrys: Recent Advances. Analytical Chemistry, 2016. **88**(1): p. 2-29.
12. Li, Z., et al., Surface properties of platinum thin films as a function of plasma treatment conditions. Surface Science, 2003. **529**(3): p. 410-418.
13. Hubbard, A.T., Electrochemistry at well-characterized surfaces. Chemical Reviews, 1988. **88**(4): p. 633-656.
14. Jaskolla, T.W., et al., Comparison between vacuum sublimed matrices and conventional dried droplet preparation in MALDI-TOF mass spectrometry. Journal of the American Society for Mass Spectrometry, 2009. **20**(6): p. 1104-1114.

15. Schneider, B.B., C. Lock, and T.R. Covey, AP and vacuum MALDI on a QqLIT instrument. Journal of the American Society for Mass Spectrometry, 2005. **16**(2): p. 176-182.
16. Beavis, R.C., B.T. Chait, and H. Fales, Cinnamic acid derivatives as matrices for ultraviolet laser desorption mass spectrometry of proteins. Rapid Communications in Mass Spectrometry, 1989. **3**(12): p. 432-435.
17. Beavis, R.C., B.T. Chait, and K. Standing, Factors affecting the ultraviolet laser desorption of proteins. Rapid Communications in Mass Spectrometry, 1989. **3**(7): p. 233-237.
18. Cohen, S.L. and B.T. Chait, Influence of matrix solution conditions on the MALDI-MS analysis of peptides and proteins. Analytical chemistry, 1996. **68**(1): p. 31-37.
19. Mank, M., B. Stahl, and G. Boehm, 2,5-Dihydroxybenzoic acid butylamine and other ionic liquid matrixes for enhanced MALDI-MS analysis of biomolecules. Analytical chemistry, 2004. **76**(10): p. 2938-2950.
20. Sugiyama, K., Development of on-chip mass spectrometry with pulse-heating desorption/ionization source. 2014, Japan advance institute of science and technology: Nomi. p. 148.
21. Technology, D. 2010; Available from: http://www.detechinc.com/technotes.html#multOp.

CHAPTER 3 Positive and Negative Ion Generation Mechanism for Pulse-heating Ionization

Abstract

A thermally determined electron and proton transfer process is proposed to explain the primary ion formation mechanism of the pulse-heating desorption/ionization. A simple electron transfer model is suggested from observation of anion and cation generated by simple inorganic sample. Then the model has verified and refined by the matrix and protein mixed with matrix sample analyzed in both positive and negative modes. The key factor for ion formation has been found to be the supplied thermal energy. By only thermal energy supplying, the sample has been evaporated and sublimated, and the ion has been formed. At the same time, the ion is formed according to the electron and proton affinity. A nearly ideal mass spectrum, only singly charged molecular weight ions with high signal-to-noise ratio, has been obtained by only one low-voltage pulse for not only simple inorganic sample, but also protein sample in both positive and negative modes.

3.1. Introduction

The development of miniaturized mass spectrometry (MS) system made it possible to obtain molecular information on-site without sending to a laboratory in real time and with minimal sample pretreatment. Recently, a lot of studies have been reported related to the miniaturized MS system, not only the components, including sample introduction [1-2], ion source [3-5], mass filter [6-7], ion detector [8-9], and vacuum systems [10-11], but also MS systems which are portable [12-14] and even hand-held [15]. In the previous study, pulse-heating on-chip ion source was established to protein detection for a miniaturized MS system. This on-chip ion source shows the ability to ionize the protein sample by only applying thermal energy from a single low voltage (less than 40 V) pulse without the laser irradiation, high voltage or heated ambient gas [16]. However, the obtained mass spectra were suffering from the undesirable fragmentations and peak position shifts, which limited the miniature MS system application of this on-chip ion source.

In this study, the pulse-heating ion source MS system have been extended to negative ion detection mode (negative mode). Not only protein and matrix sample, but also simple inorganic sample has been tested by pulse-heating desorption/ionization system. By discussing the differences and similarities in ion formation of organic and inorganic samples in both positive and negative modes, the key factor of ion generation has been found. Then based on the observation of ion

formation, we have proposed an ion formation model based on thermal energy for pulse-heating desorption/ionization method.

3.2. Objective

To find the key factor for ion formation, different kinds of samples have been tested. In order to understand the ion formation mechanism of the pulse-heating desorption/ionization, the ion signals from both positive and negative modes are discussed. The electron transfer process is proposed for the simple organic sample ionization. Then, the energy dependence of matrix and protein sample is investigated. Under different thermal energy supplied, the signals of the sample are discussed to confirm the electron transfer process theory. Finally, a thermal energy based charge (electron and proton) transfer ionization mechanism is proposed for pulse-heating desorption/ionization source.

3.3. Experimental setup

3.3.1. Negative ion detection mode

The setup of the MS system in the positive detection mode was the same as the description in chapter2 - 2.3.4. For analysis of both negative and positive ions, the MS system has been extended to negative detection mode. Figure 3.1 shows the schematic diagram of experimental setup. The MS system consists of an on-chip ion

source, a Time-of-flight (TOF) mass filter and a detector (Detector Technology, Model 414, USA). A low-voltage pulse (20-40 V) was supplied by a function generator to generate heat on the ionization zone. Within 500 ns (the width of pulse), the sample on the ionization zone is sublimated and ionized. A chip-holder was used for fix the position of the on-chip ion source, and the distance between TOF mass filter and ion source (2 mm). By switching the polarity of the acceleration voltage, the positive and negative charged ions could be extracted separately. The TOF mass filter is a free flight region with no potential difference. The electron multiplier detector has been operated according to the "manual" [17] and the "Multiplier Mode" has been used for both positive and negative ion detection. The ion source, mass filter and detector were placed in a vacuum chamber with the pressure approximately 10^{-3} Pa supplied by a turbo-molecular pump (HiCube 300 Classic, Pfeiffer Vacuum, Germany). The acceleration voltage is supplied by the electric field of the entrance of time-of-flight (TOF). The polarity of the acceleration voltage for negative ion should be positive, and vice versa, since the ion is accelerated by the electric field. Therefore, this system can be utilized in both positive and negative ion detection modes only by switching the polarity of the acceleration voltage. The acceleration voltage used for all experiments was 450V. Moreover, a chip holder is added to fix the chip position to improve the accuracy of alignment between ion source and mass filter in order to reduce the signal misalignment. All the mass spectra have been obtained by applying only one low-voltage pulse for ionization.

	Positive mode	Negative mode
Detection voltage (V)	2100	2100
Acceleration voltage (V)	- 450	450
Ionization voltage (V)	20-40	20-40

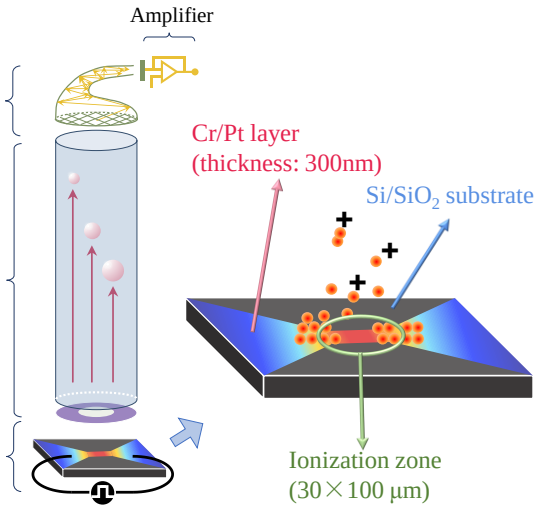

Figure 3.1 The schematic view of the experimental setup.

3.3.2. Sample preparation method

The on-chip ion source consisting of a Pt/Cr micro metal electrode layer has been fabricated by a lift-off processing on a Si/SiO_2 substrate [16]. A narrow part (length: 100 μm, width: 30 μm) of the Pt/Cr electrode layer works as the ionization zone. In previous study, a SiO_2 insulating layer covering the ionization zone was used to form a uniform hydrophilic surface. However, the insulating layer may affect the efficiency and uniformity of thermal conduction. Alternatively, oxygen plasma treatment has been introduced for cleaning the surface and increasing the water wettability of ion source chip before sample film formation. After each experiment, the chip has been carefully cleaned by sonication in ethanol and pure water for 3 min, respectively.

Sodium chloride (NaCl, 99.5%) and 2,5-dihydroxybenzoic acid (DHB, 98%) are obtained from Wako Pure Chemical (Japan). NaCl is dissolved in Milli-Q water with a concentration of 4 mg/mL. DHB is used as a matrix dissolved in acetone with the concentration of 10 mg/mL. Cytochrome C (Cyt C) purchased from MP Biomedicals (France) is prepared by dissolving in Milli-Q water (Millipore, Billerica, MA, USA) with the concentration of 1 mg/mL. Subsequently, the protein sample is mixed with the matrix solution with a ratio of 1:1. After O_2 plasma treatment, 0.25 μL of sample solution is dropped on the micro electrode and dried in a vacuum. All compounds are used without further treatment or purification.

3.4. Results and discussion

To understand the ion generation mechanism, the experimental observations have been first discussed from the thermodynamic point of view since only thermal energy has been supplied for the ionization. As reported in our previous study [16], when the 1.68×10^{-2} μJ/μm^2 of pulse-heating energy is applied, the temperature of the ionization zone is approximately 2180 K (the melting point of Cr). The actual temperature should be lower than this due to thermal diffusion and evaporative cooling. Other than this, when the 1.20×10^{-2} μJ/μm^2 of pulse-heating energy is applied, the temperature should be higher than 478 K (the sublimation point of DHB) [18] since all the sample is vanished. It has been reported that the effective temperature of MALDI is around 500K [19].

Recently, a review related to thermal ionization mechanism of MALDI has reported that the primary ion formation of DHB is 1,900 K in the gas phase [20]. Therefore, the thermal energy supplied by the pulse-heating desorption/ionization is comparable with MALDI. In case of MALDI, it has been reported that around 80% of the initially absorbed UV laser energy is converted to thermal energy [18]. While, our system directly utilizes thermal energy for ionization, which is generated by applying a low-voltage pulse to a micro metal electrode. In addition, a molecular weight ion signal of protein sample is observed in both cases. Therefore, the mechanism of the pulse-heating desorption/ionization method is similar with MALDI to some extent. However, there is still no agreement on a simple ion formation model for the MALDI technique.

There are several models are reported to elaborate the mechanism of MALDI. Almost all the proposed MALDI mechanism models agree with the "two-steps" process, namely primary and secondary ion formation. The primary ion formation pathway shows how the initial ion forms. It can be classified into two groups: the excited ion model and the pre-formed ion model [20]. The excited ion model claims that the matrix molecules [21-22] or excitons [23-24] will absorb the energy from photons supplied by laser, whereas the pre-formed ion model, including pre-formed ion emission [25-26] and the cluster model [27-29], considers that the ions are pre-formed in the solid sample and then are emitted by laser ablation. However, both models are unable to explain the mechanism of pulse-heating desorption/ionization. In contrast to MALDI, the pulse-heating desorption/ionization method does not use any laser

light which result in neither photoexcitation nor photon transfer during ionization. On the other hand, if the ions are pre-formed in solid sample, some ion signals should be observed when the thermal energy is enough for sublimation. However, no signal has been observed as a low thermal energy applied although the sample has been sublimated. Therefore, another ion formation model should be considered for the pulse-heating ion source which is only based on thermal energy.

3.4.1. Inorganic sample ionization

There is a few type of ions that may be generated by the pulse-heating desorption/ionization method. For examples, cation (M^+), anions (M^-), protonated ion ($[M+H]^+$), and deprotonated ion ($[M-H]^-$) (here, M represent molecule). To understand the mechanism of ionization, one of the simplest inorganic sample NaCl has been chosen for the initial experiment since only cation and anion can be generated. Figure 3.2 shows positive and negative mode TOF mass spectra of NaCl ions without matrix. With the exception of polarity, both spectra have been recorded under identical, optimized experimental conditions. With only one pulse, the single charged Na^+ and Cl^- ion signals have been observed in the positive and negative mode, respectively. To generate ions from neutral molecules is an endothermic process, since the charges are separated from the Coulomb attraction. To separate the Na^+ and Cl^- in gas phase costs 4.8 eV, theoretically [22]. It indicates that the ionization processes almost impossible occur in the gas phase since it needs very high temperature. It can be suspected that the electron is more inclined to close to the Cl

in the NaCl molecule in the solid phase. This phenomenon can be explained by the electron affinity difference of Na and Cl elements. The thermal energy supplied by the ion source is used for sample sublimation. When the sample converted from solid to gaseous, the ions are generated. Hence, we propose an ion formation mechanism based on thermal assisted electron transfer process to produce ions by the pulse-heating ion source.

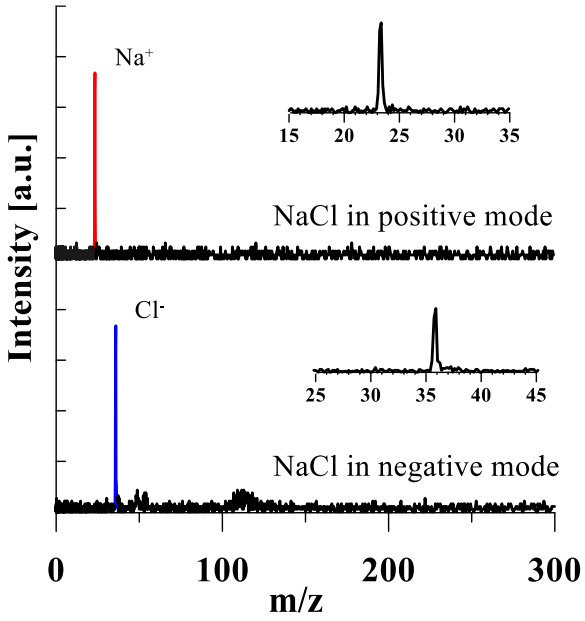

Figure 3.2 The mass spectra of NaCl in both positive and negative mode

3.4.2. Matrix sample ionization

For protein, the ionization mechanism should be much more complex compared with simple inorganic sample. Therefore, the matrix sample has been analyzed first to verify this mechanism is compatible, or can be refined to become compatible with

the protein analysis. DHB has been chosen as a matrix sample since it is well known as a standard and efficient matrix of MALDI which can be used for various samples from protein, peptide to nucleotides [30]. Although DHB is an acidic matrix which favor the formation of protonated analytes, DHB also works well in negative ion mode [31]. DHB has been first tested with different pulse energies by using pulse-heating desorption/ionization method in the negative mode. As can be seen in Figure 3.3, the single charged matrix signal can be obtained under an appropriate pulse-heating energy in both positive ion mode and negative ion mode, whereas some other signals (fragmentation and/or multiple-charged ions) have been observed with a higher pulse energy. No signal has been obtained under 1.20×10^{-2} μJ/μm^2 of pulse-heating energy, although almost all the sample in the ionization zone has been vanished. Even if increasing the voltage of the second pulse applied to the sample, there has been still no ion signal indicating all the sample in the ionization zone has been sublimated by the first pulse. With an appropriate energy (1.20×10^{-2} μJ/μm^2) supplied, the DHB has been also tested with positive mode for comparison. A clear mass spectra with single charged molecular weight ion signal in both positive and negative mode have been obtained, which indicates that the positive ion and negative ion of DHB sample presents at the same time.

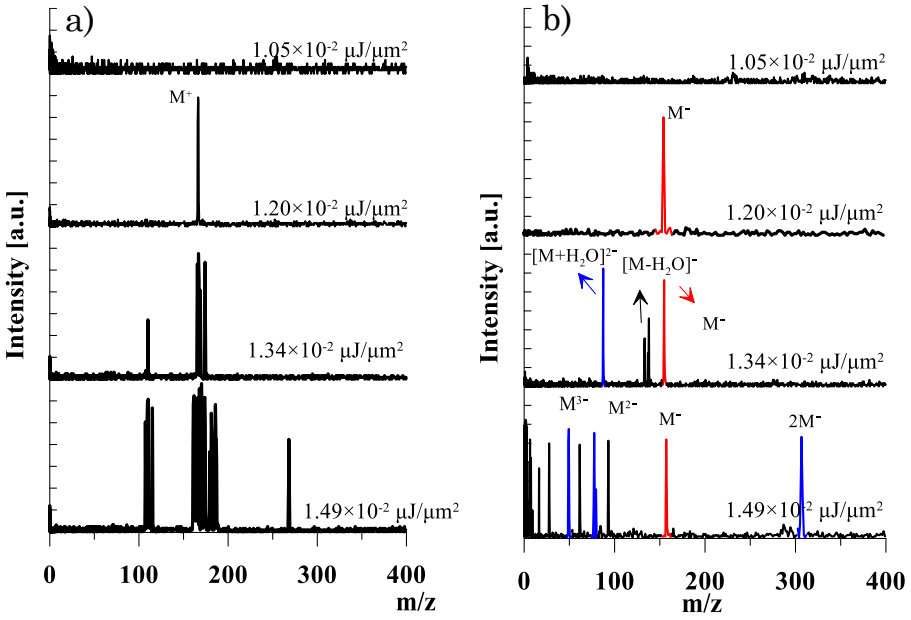

Figure 3.3 Mass spectra of DHB with different thermal energies. a) Positive ion mode; b) Negative ion mode.

3.4.3. A mechanism for pulse-heating desorption/ionization

Suppose all the DHB molecules in the ionization zone are sublimated within 500 ns (the pulse width), the molecules should be dispersed in the gas phase. In the gas phase, the thermal energy absorbed by the matrix may result in plume expansion, thus the distance among molecules increases. Since the sample consumption in the ionization zone is very less (~750 amol), the behavior of individual molecule is possibly considered. For the structure of molecule, the C-H or O-H bond lengths are very short (~0.1 nm). As a result, to remove the H^+ from DHB, it is required a high Coulomb energy of 13.8 eV [22]. For the physical-chemical properties of DHB, the neutral DHB electron affinity is 0.42 eV [34], the proton affinity is 8.90±0.15 eV

(854±14 kJ/mol) [32], while the ionization potential of DHB is 8.05 eV [22]. Therefore, the electron transfer ionization initially occurs followed by proton transfer reactivity the same as MALDI-MS [21]. Then the mechanism is refined as that the thermal energy transferred to the sample molecules leading to sample sublimation, and the ionization (electron or proton transfer) process occurs according to the characteristics of the molecule.

The schematic image of pulse-heating desorption/ionization mechanism is shown in Figure 3.4. Under the lower pulse-heating energy supplied, the matrix molecules absorb the thermal energy and sublimate into gas phase. Only neutral molecules exist, thus no signal could be observed. When the energy increases, the extra thermal energy is used to dissociate electrons from the matrix during the sublimation process. The neutral molecules generating the M^+ and M^- ions during a very short time of the plume expansion. During this period, a small proportion of electrons with large amount of neutral molecules co-exist, resulting in only one single charged ion signal. With a higher energy, not only electrons but also protons are dissociated leading to a higher density of charges present in the gas phase. This phenomenon can be used to explain why the various ion types are observed at the high energy. In addition, it has been reported for DHB that M^+ and $(M+H)^+$ can be converted [33], and same conversion can be observed with M^- and $(M-H)^-$ [21]. The energy required for inter-conversion of different ion type for DHB was speculated around 0.31 eV (30 kJ/mol) within kT in the plume. The $M-H_2O$ might also be formed

due to thermal or non-thermal decay products [21]. These ions were observed at the energy higher than 1.20×10^{-2} μJ/μm² (see Figure 3.3).

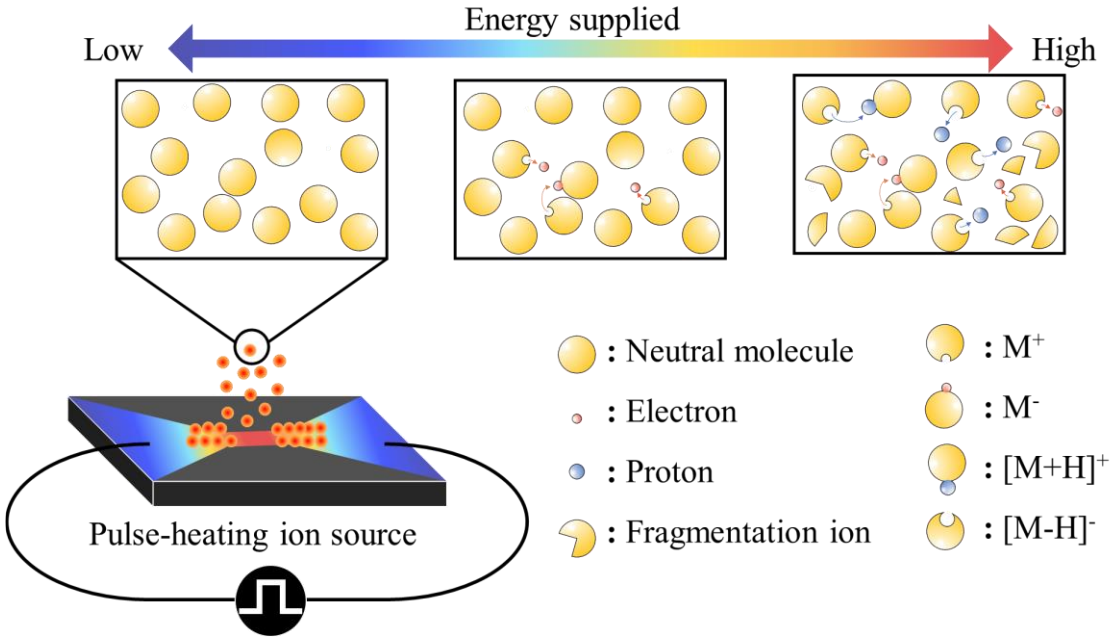

Figure 3.4 Schematic image of pulse-heating desorption/ionization mechanism.

3.4.4. Protein sample ionization

Cyt C as the protein sample mixed with DHB as the matrix (1:1, v/v) have been studied by the pulse-heating ion source MS system. Under the optimized condition, the single charged Cyt C ions have been produced with both positive and negative modes as shown in the Figure 3.5. The inset figure is an optical microscope image of the ion source chip after one pulse-heating, which shows a uniform protein sample distribution.

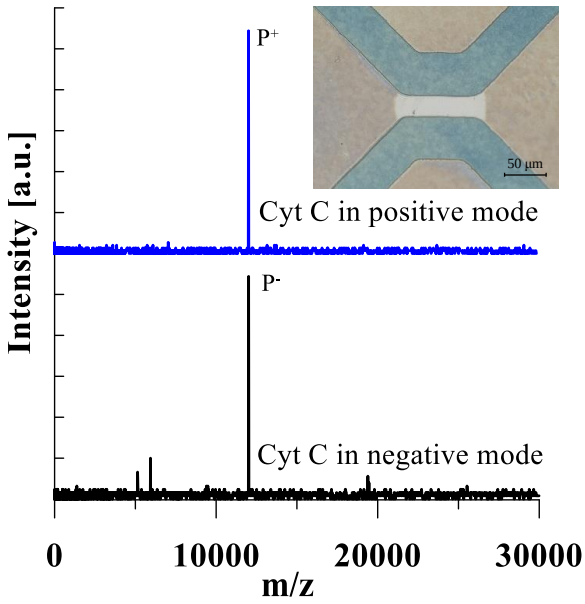

Figure 3.5 The mass spectra of Cyt C in both positive and negative mode.

For comparison, the mass spectrum obtained by conventional MALDI were shown in Figure 3.6. Compared with the conventional MALDI, the mass spectra obtained by pulse-heating desorption/ionization method show less background, especially in the low mass region. It is important to note that the mass spectra with a high signal-to-noise ratio (S/N) are obtained by only single pulse. In addition, almost all the sample on the ionization zone is evaporated or ionized with the single pulse, and no signal can be found with a second pulse.

Much higher S/N is obtained by the pulse-heating desorption/ionization method, indicating a higher ionization efficiency as compared to MALDI. Although the matrix suppression phenomenon is also observed in the conventional MALDI-MS [34-35], the appearance of multiple-charged ions and matrix ions can be completely suppressed under optimized conditions. The generation of fragmentation ions strongly depends

on the pulse energy (see Figure 3.7). Under optimized conditions, a nearly ideal mass spectrum, only signal from analyte without other signal, can be obtained under optimized condition.

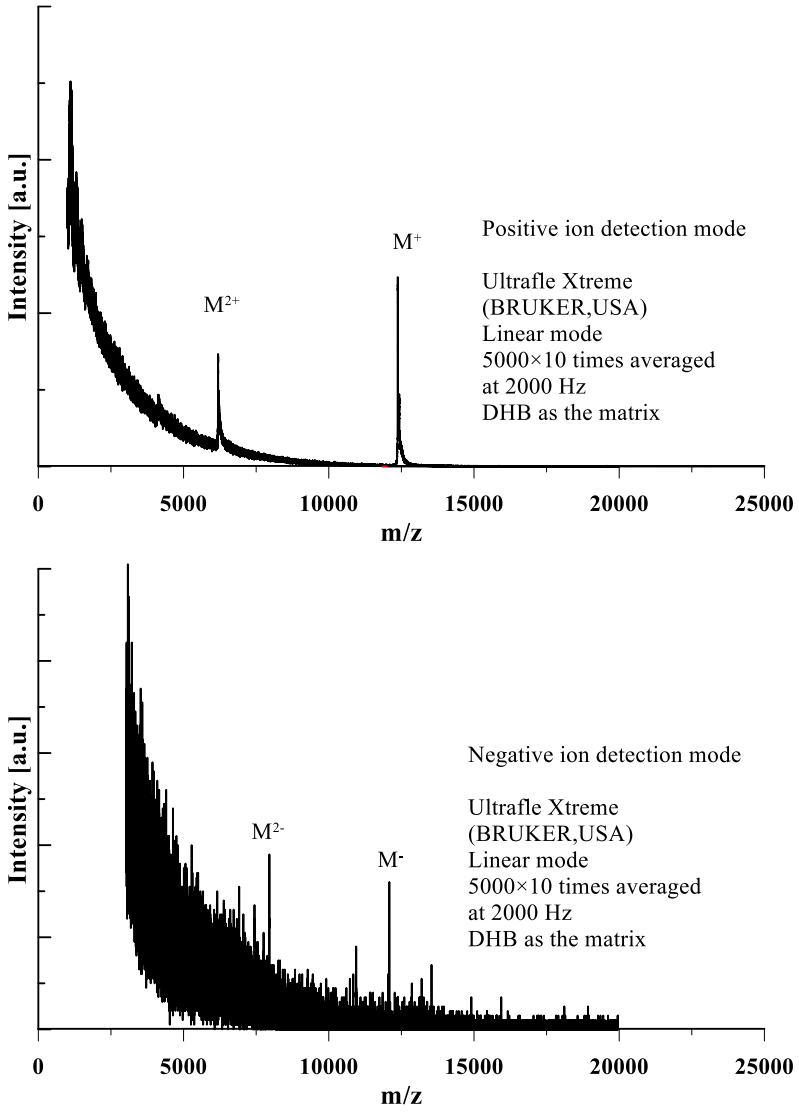

Figure 3.6 Mass spectrum of Cyt C in the positive detection mode with conventional MALDI-MS.

Similar with the conventional MALDI MS system, the ionization process is desorption/ionization process. A two-step ionization pathway is considered to elaborate the ion formation pathway of protein. In the first step, the charge (electron and/or proton) association/dissociation of matrix molecule takes place. In the second step, the generated charges are transferred to protein molecules. As the lower energy supplied, only neutral molecules exist in the gas phase due to evaporation or sublimation, resulting in no signal. With an appropriate energy supplied, a few electrons dissociated by the matrix molecules co-exist with a large amount of neutral molecules in the beginning of ionization process. Then the protein reacts with matrix ions (here, P represent protein molecule):

$$M^{+/-} + P \rightarrow M + P^{+/-}$$

When a higher energy is supplied, protein molecules may undergo collisions before being extracted into the drift region of the MS due to the dense charges dissociated from the matrix in the gas phase. That is why the multiple-charged and fragmentation ion signals are observed. Signal from the matrix is not observed. This might be because of the fact that small matrix ions are radially ejected from the sample plume due to Coulomb repulsion [34].

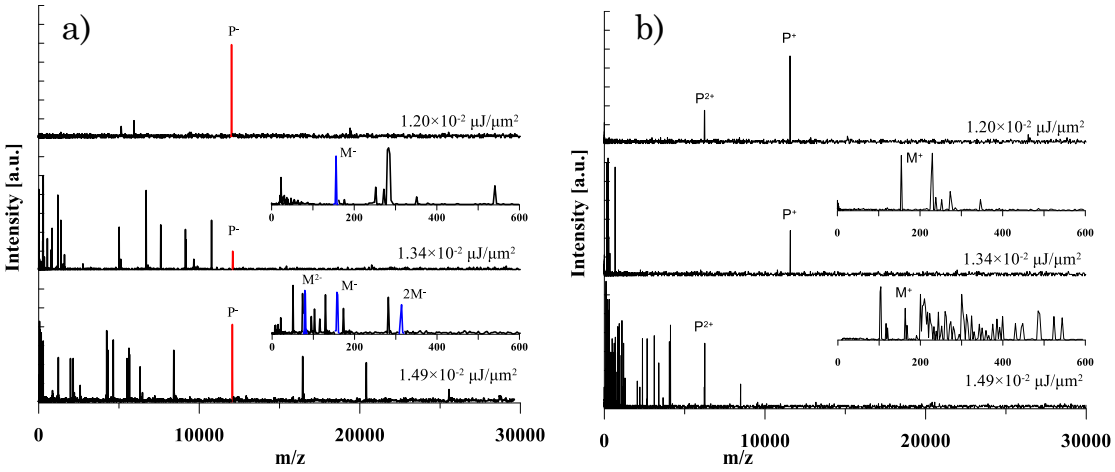

Figure 3.7 Energy dependence of Cyt C. a) Negative ion mode; b) Positive ion mode.

3.5. **Summary**

On-chip pulse-heating ion source MS system has been developed for molecular analysis in both negative and positive mode. With appropriate ionization energy, the single-charged ion signal can be obtained not only for the protein sample, but also for the inorganic sample. Here, we propose an ionization pathway of single-charged ion formation based on the electron transfer among analyte molecules. The neutral molecules can integrate with any charge to form ions. The mechanism of the PHDI method is considered to be somewhat similar to the thermal ionization occurring in the MALDI. However, further studies are needed for understanding the mechanism of the PHDI. The proposed mechanism can be used to explain most observations in the pulse-heating desorption/ionization, and also may contribute to understanding the mechanism of MALDI. The only single signal from analyte will be beneficial to analysis of the complex biological system, thereby offering the possibility of point-of-care application with the miniaturized MS system.

Reference

1. Wright, S.; Syms, R. R. A.; Moseley, R.; Hong, G. D.; O'Prey, S.; Boxford, W. E.; Dash, N.; Edwards, P., MEMS-Based Nanospray-Ionization Mass spectrometry. J Microelectromech S, 2010. **19** (6), 1430-1443.
2. Anthony, S. N.; Shinholt, D. L.; Jarrold, M. F., A simple electrospray interface based on a DC ion carpet. Int J Mass Spectrom **2014,** 371, 1-7.
3. Chen, F.; Lin, L.; Zhang, J.; He, Z.; Uchiyama, K.; Lin, J.-M., Single-cell analysis using drop-on-demand inkjet printing and probe electrospray ionization mass spectrometry. Anal Chem **2016,** 88 (8), 4354-4360.
4. Vigne, S.; Alava, T.; Tassetti, C.-M.; Duraffourg, L.; Progent, F., Optimization of an electron impact ion source on a MEMS time-of-flight mass spectrometry. Sensors and Actuators B: Chemical **2017,** 243, 690-695.
5. Qing, J.; Huang, Z.; Zhang, Y.; Zhu, H.; Tan, G.; Gao, W.; Yang, P.-y., A miniaturised electron ionisation time-of-flight mass spectrometry that uses a unique helium ion removal pulsing technique specifically for gas analysis. Analyst **2013,** 138 (12), 3394-3401.
6. Chaudhary, A.; van Amerom, F. H. W.; Short, R. T., Experimental evaluation of micro-ion trap mass spectrometry geometries. Int J Mass Spectrom **2014,** 371, 17-27.
7. Tassetti, C.-M.; Mahieu, R.; Danel, J.-S.; Peyssonneaux, O.; Progent, F.; Polizzi, J.-P.; Machuron-Mandard, X.; Duraffourg, L., A MEMS electron impact ion source integrated in a microtime-of-flight mass spectrometry. Sensors and Actuators B: Chemical **2013,** 189, 173-178.
8. Hadjar, O.; Fowler, W. K.; Kibelka, G.; Schnute, W. C., Preliminary demonstration of an IonCCD as an alternative pixelated anode for direct MCP readout in a compact MS-based detector. Journal of The American Society for Mass Spectrometry **2012,** 23 (2), 418-424.

9. Hanay, M.; Kelber, S.; Naik, A.; Chi, D.; Hentz, S.; Bullard, E.; Colinet, E.; Duraffourg, L.; Roukes, M., Single-protein nanomechanical mass spectrometry in real time. Nature nanotechnology **2012,** 7 (9), 602-608.
10. Chen, C.-H.; Chen, T.-C.; Zhou, X.; Kline-Schoder, R.; Sorensen, P.; Cooks, R. G.; Ouyang, Z., Design of portable mass spectrometrys with handheld probes: aspects of the sampling and miniature pumping systems. Journal of the American Society for Mass Spectrometry **2015,** 26 (2), 240-247.
11. Sugiyama, K.; Ukita, Y.; Takamura, Y., Development of on-chip vacuum generation by gas–liquid phase transition. Sensors and Actuators A: Physical **2012,** 176, 138-142.
12. Gao, L.; Sugiarto, A.; Harper, J. D.; Cooks, R. G.; Ouyang, Z., Design and characterization of a multisource hand-held tandem mass spectrometry. Anal Chem **2008,** 80 (19), 7198-7205.
13. Hendricks, P. I.; Dalgleish, J. K.; Shelley, J. T.; Kirleis, M. A.; McNicholas, M. T.; Li, L.; Chen, T.-C.; Chen, C.-H.; Duncan, J. S.; Boudreau, F., Autonomous in situ analysis and real-time chemical detection using a backpack miniature mass spectrometry: concept, instrumentation development, and performance. Anal Chem **2014,** 86 (6), 2900-2908.
14. Yang, M.; Kim, T.-Y.; Hwang, H.-C.; Yi, S.-K.; Kim, D.-H., Development of a palm portable mass spectrometry. Journal of the American Society for Mass Spectrometry **2008,** 19 (10), 1442-1448.
15. Gao, L.; Song, Q.; Patterson, G. E.; Cooks, R. G.; Ouyang, Z., Handheld rectilinear ion trap mass spectrometry. Anal Chem **2006,** 78 (17), 5994-6002.
16. Sugiyama, K.; Harako, H.; Ukita, Y.; Shimoda, T.; Takamura, Y., Pulse-heating ionization for protein on-chip mass spectrometry. Analytical chemistry **2014,** 86 (15), 7593-7597.
17. Technology, D. http://www.detechinc.com/technotes.html#multOp.

18. Koubenakis, A.; Frankevich, V.; Zhang, J.; Zenobi, R., Time-resolved surface temperature measurement of MALDI matrices under pulsed UV laser irradiation. The Journal of Physical Chemistry A **2004,** 108 (13), 2405-2410.
19. Mowry, C. D.; Johnston, M. V., Internal Energy of Neutral Molecules Ejected by Matrix-Assisted Laser Desorption. The Journal of Physical Chemistry **1994,** 98 (7), 1904-1909.
20. Bae, Y. J.; Kim, M. S., A thermal mechanism of ion formation in MALDI. Annual Review of Analytical Chemistry **2015,** 8, 41-60.
21. Knochenmuss, R., Ion formation mechanisms in UV-MALDI. Analyst **2006,** 131 (9), 966-986.
22. Zenobi, R.; Knochenmuss, R., Ion formation in MALDI mass spectrometry. Mass Spectrometry Reviews **1998,** 17 (5), 337-366.
23. Knochenmuss, R.; Zhigilei, L. V., Molecular dynamics model of ultraviolet matrix-assisted laser desorption/ionization including ionization processes. J Phys Chem B **2005,** 109 (48), 22947-22957.
24. Knochenmuss, R.; Zhigilei, L. V., Molecular dynamics simulations of MALDI laser fluence and pulse width dependence of plume characteristics and consequences for matrix and analyte ionization. Journal of Mass Spectrometry **2010,** 45 (4), 333-346.
25. Krüger, R.; Pfenninger, A.; Fournier, I.; Glückmann, M.; Karas, M., Analyte incorporation and ionization in matrix-assisted laser desorption/ionization visualized by pH indicator molecular probes. Anal Chem **2001,** 73 (24), 5812-5821.
26. Zhu, Y.; Lee, K.; Tang, K.; Allman, S.; Taranenko, N.; Chen, C., Revisit of MALDI for small proteins. Rapid communications in mass spectrometry **1995,** 9 (13), 1315-1320.
27. Karas, M.; Krüger, R., Ion formation in MALDI: the cluster ionization mechanism. Chemical reviews **2003,** 103 (2), 427-440.

28. Karas, M.; Glückmann, M.; Schäfer, J., Ionization in matrix-assisted laser desorption/ionization: singly charged molecular ions are the lucky survivors. Journal of Mass Spectrometry **2000,** 35 (1), 1-12.
29. Jaskolla, T. W.; Karas, M., Compelling evidence for lucky survivor and gas phase protonation: the unified MALDI analyte protonation mechanism. Journal of the American Society for Mass Spectrometry **2011,** 22 (6), 976-988.
30. Jessome, L.; Hsu, N. Y.; Wang, Y. S.; Chen, C. H., Matrix-assisted laser desorption/ionization mechanism study with dihydroxybenzoic acid isomers as matrices. Rapid Communications in Mass Spectrometry **2008,** 22 (2), 130-134.
31. Gao, J.; Cassady, C. J., Negative ion production from peptides and proteins by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Rapid Communications in Mass Spectrometry **2008,** 22 (24), 4066-4072.
32. Steenvoorden, R.; Breuker, K.; Zenobi, R., The gas phase basicities of matrix-assisted laser desorption/ionization matrices. European Mass Spectrometry **1997,** 3 (5), 339-346.
33. Asfandiarov, N. L.; Pshenichnyuk, S. A.; Fokin, A. I.; Lukin, V. G.; Fal'ko, V. S. Electron capture negative ion mass spectra of some typical matrix-assisted laser desorption/ionization matrices Rapid Communications in Mass Spectrometry Volume 16, Issue 18 Rapid Communications in Mass Spectrometry [Online], 2002, p. 1760-1765. http://onlinelibrary.wiley.com/doi/10.1002/rcm.787/abstract (accessed 30).
34. Knochenmuss, R.; Dubois, F.; Dale, M. J.; Zenobi, R., The matrix suppression effect and ionization mechanism in MALDI. Rapid Commun Mass Spectrom **1996,** 10, 871.
35. Knochenmuss, R.; Karbach, V.; Wiesli, U.; Breuker, K.; Zenobi, R., The matrix suppression effect in matrix-assisted laser desorption/ionization: Application to negative ions and further characteristics. Rapid communications in mass spectrometry **1998,** 12 (9), 529-534.

CHAPTER 4 Matrix-free ionization by Pulse-heating Ion source

Abstract

A matrix-free pulse-heating desorption/ionization method is proposed for biomolecule analysis. A wide range of samples including carbohydrate, amino acid, peptide, and protein, are tested. Compared with conventional matrix-assisted laser desorption/ionization (MALDI), the pulse-heating desorption/ionization method shows extremely high-yield and singly-charged ion, with very less fragmentation and background. It is believed that this work offers a new technique for matrix-free biomolecule analysis which overcomes the limitations of the conventional MALDI.

4.1. Introduction

Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry has been extensively used for biomolecule analysis, due to its rapid, accurate and high sensitivity. This ionization method need matrix, usually refers to some organic small molecular compounds, for absorb and transfer energy for ionization. However, the matrix used for conventional MALDI resulting a serious matrix signal interference at the low mass region [1].

To avoid the effect of matrix signals and make MALDI capable for low molecular weight compounds, a lot of studies have been done. There are mainly three approach. Firstly, to find a matrix which is no interfere with the analyte and then pick up the analyte signals from the mass spectra. However, this method is laborious and time-consuming, since finding a suitable matrix itself is a hard task for conventional MALDI. Secondly, to use a high molecule weight compound as matrix, such as polymer. However, the polymer often does not co-crystallized with the analyte. In addition, the polymer matrix is incompatible with a similar molecular weight compound which means that this method still cannot be used for the entire range of molecular weight for analysis. Thirdly, to find an ionization method which do not need a matrix for ionization. Only in this way, can we find an ionization method for the entire range mass analysis.

Matrix-free sample analysis, in another words, find another material to absorb and transfer energy for ionization. Till now, there are several matrix-free methods which have been reported for MALDI-MS. The initial one is using inorganic matrix. Tanaka et al reported in 1988 that using cobalt powder mixed with glycerol as the matrix for protein and polymer analysis [2]. Zenobi group reported in 1997 that directly deposited the tryptophan for ionization [3]. However, this method is limited to analytes with a molecular weight less than 2000, and it is strongly affected by the properties of the analytes. Furthermore, more fragmentation ions are generated due to the higher laser energy required by the direct ionization without the organic matrix.

Another method is using a modified sample plate with nanostructured substrates to absorb and transfer energy. This method is also known as surface-assisted laser desorption ionization or solid-assisted laser desorption ionization (SALDI) [1, 4]. There mainly three kinds of nanostructured substrates are used to replace the function of matrix, the carbon based nanoparticles, and the semiconductor based nanoparticles and the metallic nanoparticles. However, the SALDI methods are generally not capable with the large molecules which molecular weight is large than 3,000. If the mass range can be extended, then the matrix-free method may be capable for peptide and even protein sample. In addition, preparation of nanostructured materials affects to the MS performance as well [5]. Such limitations make the SALDI method not a general ones. Hence, a paramount

challenge still remains in matrix-free analysis that is capable of a large mass range of biomolecules.

In previous study, an on-chip pulse-heating desorption/ionization method with high ionization efficiency was proposed for protein analysis with matrix [6]. However, the matrix was used for the pulse-heating desorption/ionization also showing signals which may overlap with the analyte signals, resulting in the difficulty of low molecule weight biomolecule analysis. Since a uniform thin film can be obtained, the pulse-heating desorption/ionization is applied successfully to inorganic sample ionization without matrix (the second chapter). According to the ionization mechanism of the pulse-heating desorption/ionization proposed in the chapter 3, the ionization is due to thermal energy. Therefore, we suspected that under an optimized condition, not only the inorganic sample, but also organic sample including peptide and proteins can be ionized by this pulse-heating desorption/ionization method.

Herein, we report a novel matrix-free ionization process by on-chip PHDI with a large variety of samples, including amino acids, carbohydrates, peptide, and proteins to prove this concept. In the early stage of this study, a uniform and thin film of sample is found to offer significant advantages for ion generation. Therefore, an oxygen plasma treatment process has been introduced for on-chip ion source pre-treatment, leading to a remarkable decrease in sample film thickness. With such a uniform thin film of sample, the PHDI shows excellent capability for matrix-free biomolecular ionization.

To demonstrate the capabilities of the PHDI method for ultrahigh yield matrix-free biomolecules ionization, in this work we first have investigated matrix-assisted pulse-heating desorption/ionization, and then extended the method to matrix-free ionization of a wide range of biomolecules. This on-chip PHDI method is quite different from existing ionization approaches in that an ultrahigh yield singly-charged sample ions are generated only by thermal energy. Without the need of laser, high voltage or heated gas, the PHDI method is able to dramatically reduce the size and weight of the whole MS system. Without the need of matrix, the "sweet spot" effect is circumvented. The unique advantage of the on-chip PHDI is that only one pulse is needed for sample desorption and ionization. Furthermore, the on-chip PHDI ion source can be combined with other miniaturized components (pump [7, 8], mass filter [9-11], detector [12, 13] et al) to realize one-chip MS system.

4.2. **Objective**

In this chapter, a matrix-free biomolecule analysis method is established by pulse-heating ion source. A wide range of samples including carbohydrate, amino acid, peptide, and protein, are tested. It is believed that this work offers a new technique for matrix-free biomolecule analysis which overcomes the limitations of the conventional MALDI.

4.3. Experimental

4.3.1. Experimental setup

The experimental setup is the same as 2.3.4 in chapter 2. The positive ion detection is used for all experiments. The NaCl sample is used for calibration since a high sensitive and reproducible mass spectrum of NaCl is easily obtained. The oxygen plasma cleaning process is utilized for pre-treatment of the chip.

4.3.2. Sample preparation

Glutamic acid (99%), Glycine (99%), Lysine (97%), and Histidine (98%) as the amino acid samples were from Wako (Japan). Glucose (anhydrous, 98%) and α-Cyclodextrin (97%) as the carbohydrate samples were purchased from Wako (Japan). Angiotensin I (97%) as the peptide sample was purchased from Sigma-Aldrich (Japan). Cytochrome C (Cyt C), and α-Lactalbumin (α-Lac) as the protein samples were purchased from MP Biomedicals (France), Protea Biosciences (US) and Sigma-Aldrich (Japan), respectively. All the sample are dissolved in pure water at a concentration of 1 mg/mL for all experiment. Table 4.1 summarizes all the analytes with the molecular weight. The chip is prepared as same as chapter 3. After oxygen plasma treatment, 250 μL of sample solution is dropped onto the center part of the ion source chip and dried in a vacuum condition.

Table 4.1. The molecular information of analytes.

Analyte	Formula	Molecular weight (Da)	Category
Glutamic acid	$C_5H_9NO_4$	147.13	Amino acid
Glycine	$C_2H_5NO_2$	75.07	Amino acid
Lysine	$C_6H_{14}N_2O_2$	146.19	Amino acid
Histidine	$C_6H_9N_3O_2$	155.15	Amino acid
Glucose	$C_6H_{12}O_6$	180.16	Carbohydrate
α-Cyclodextrin	$C_{36}H_{60}O_{30}$	972.84	Carbohydrate
Angiotensin I	$C_{62}H_{89}N_{17}O_{14}$	1296.48	Peptide
Cytochrome C	--	12,000	Protein
α-Lactalbumin	--	14,178	Protein

4.4. Results and discussion

By using the PHDI method, not only protein mixed with matrix sample, but also matrix-free protein sample can be ionized. Compared with the conventional MALDI, an ultrahigh ion yield, singly charged, very-less fragmentation and large S/N mass spectrum can be obtained with only one pulse by the PHDI for a wide variety of biomolecules. In conventional MALDI, the matrix is used to absorb the laser energy for ionization while in the PHDI, the thermal energy supplied by the low voltage pulse is directly used for ionization. Therefore, the matrix maybe not needed for the PHDI.

Previously, ionization of BSA sample by the matrix-free PHDI was once attempted, however only fragmentation signals were observed at low-mass region. There are two possible reasons. One is the thickness and uniformity of the sample film. In the PHDI method, the thermal energy is introduced from the bottom side of sample layer, thus a thin and uniform sample film is highly desired. The thermal energy supplied by the pulse-heating can be effectively transferred and uniformly distributed to a homogeneous thin film which has a higher surface-to-target thermal conductivity than a thick film. On the other hand, for the thick film the thermal distribution in the vertical direction is non-uniform. In particular, more energy absorbed by bottom layer of the sample film may result in fragmentation or collision dissociation.

The thickness of protein mixed with matrix sample was reduced to approximately 20 nm instead of the non-uniform and thicker one (300 nm) in previous study. Another reason is the thermal energy supplied for ionization. The ionization energy for matrix-free protein sample should be higher than protein mixed with matrix sample. For protein mixed with matrix sample, the protein molecules are dispersed by the matrix molecules and the protein ions can also be formed by a charge transfer process with the matrix ions.

4.4.1. Ionization of protein sample

For the initial investigation, bovine serum albumin (BSA) and cytochrome c (Cyt c) are used as protein samples and subjected to analysis with matrix by the PHDI method; the mass spectra are compared with the results from MALDI. Three commonly used MALDI matrices are tested, including 2,5-dihydroxyacetophenon (DHAP), 2,5-dihydroxybenzoic acid (DHB), and sinapic acid (SA). For protein analysis, 1.20×10^{-2} μJ/μm2 of pulse-heating energy is supplied. As shown in Fig. 4.1 for BSA and Fig. 4.2 for Cyt c, singly charged ion signals can be obtained by both methods with a suitable matrix. It is important to mention that only one pulse-heating shot is applied for the PHDI (Fig. 4.1 c), while over 10,000 times shots are utilized for MALDI (Fig.1 b and Fig.4.2 a).

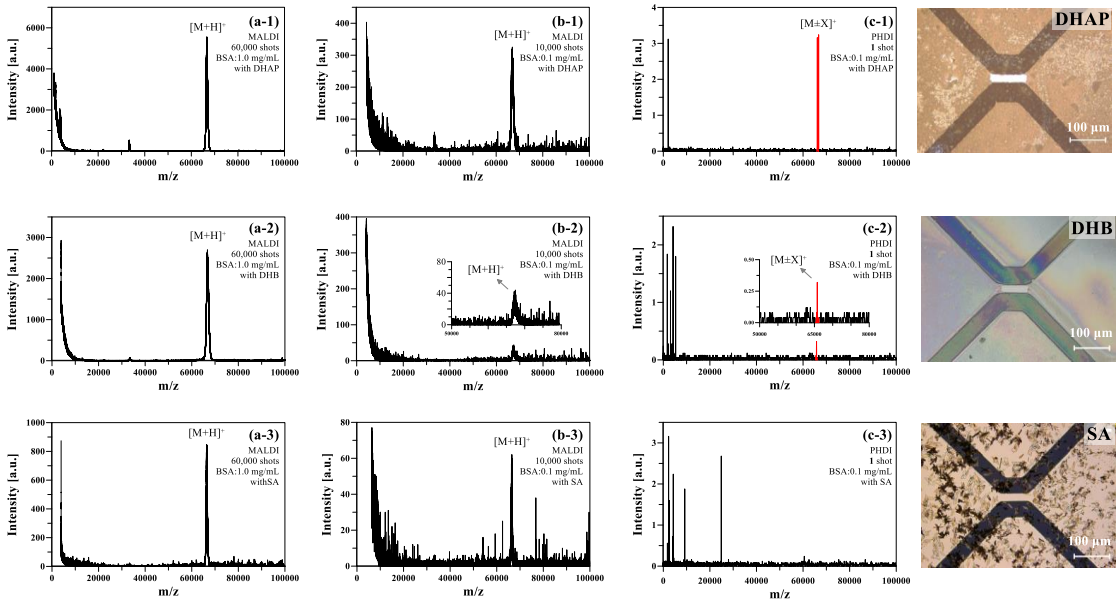

Figure 4.1. Comparison of conventional MALDI and PHDI method. (a) MALDI mass spectra obtained from BSA (1.0 mg/mL) mixed with DHAP (a-1), DHB (a-2), and SA (a-3) by 60,000 shots averages. (b) MALDI mass spectra obtained from BSA (0.1 mg/mL) mixed with

DHAP (b-1), DHB (b-2), and SA (b-3) by 10,000 shots averages. (c) PHDI mass spectra obtained by BSA (0.1 mg/mL) mixed with DHAP (b-1), DHB (b-2), and SA (b-3) by 1 shot. Inset figures show the microscopic image of the PHDI source. After one pulse-heating shot, the sample at the center (ionization zone) is desorbed and ionized.

With the same concentration of BSA sample solution, a low-intensity $[M+H]^+$ signal is observed by MALDI (Fig. 4.1 b), while a relatively high signal-to-charge ratio (S/N) signal is obtained by PHDI with DHAP as the matrix (Fig. 4.1 c-1). From the S/N value, the limit of detection is estimated to be 0.01 mg/mL for Fig.4.1 b-1, and 0.003 mg/mL for Fig.1 c-1.

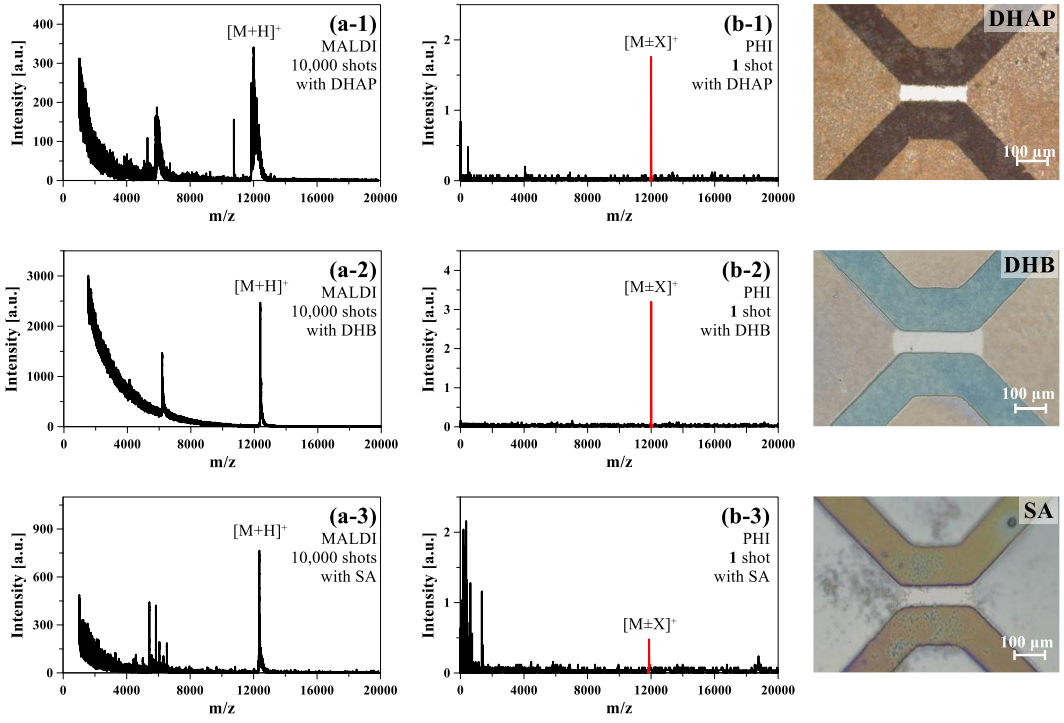

Figure 4.2. Comparison of conventional MALDI and PHDI method. (a) MALDI mass spectra obtained from Cytochrome C (Cyt c) (1.0 mg/mL) mixed with DHAP (a-1), DHB (a-2), and SA (a-3) by 10,000 shots averages. (b) PHDI mass spectra obtained by Cyt c (1.0 mg/mL) mixed with DHAP (b-1), DHB (b-2), and SA (b-3) by 1 shot. Inset figures show the microscopic image of PHDI source. After one pulse-heating shot, the sample at the center (ionization zone) is desorbed and ionized.

Increasing the number of laser shots or the sample concentration is another way for increasing the signal intensity (Fig. 4.1 a). Besides, the strong background noise is observed in the MALDI method, especially in the low mass region. In this paper, the $[M\pm X]^+$ is used to represent the singly charged ion since the ion species generated by the PHDI method is still unclear.

4.4.2. Ionization of protein carbohydrates

Carbohydrates as a kind of functional biomolecule play many critical roles in biological processes, such as cell-cell recognition, protein targeting, and nutrition [14]. Development of a method which can analyze carbohydrates intracellular is becoming more and more important for understanding their biological functions. For conventional MALDI source, the 2,5-dihydroxybenzoic (DHB) is usually used as the matrix for carbohydrate analysis. However, the DHB has a strong interfere and a low ionization efficiency. Some new matrices are designed for carbohydrates, such as 1-Naphthylhydrazine hydrochloride [15] and quantum dots [16]. In the preliminary application test of the capability of pulse-heating desorption/ionization method for carbohydrates, glucose has been chosen as representative and analyzed without any matrix. Figure 4.3 shows mass spectra of glucose with different pulse energy supplied. With a lower energy supplied, no signal is obtained. The molecule weight ion signal is observed with 2.01×10^{-2} kJ/μm^2 of energy supplied, whereas the fragmentation signals are observed with a higher energy supplied. A possible fragmentation is shown as an inset [8].

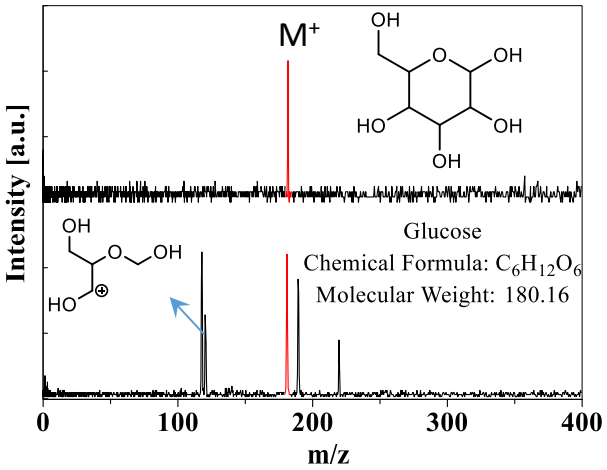

Figure 4.3. Mass spectra of Glucose. (Upper) under optimized condition. (Lower) Higher energy supplied.

Figure 4.4 shows the reproducibility of glucose analysis. Under optimized conditions, only singly charged ion signal is obtained with a reproducible mass spectrum. These results show that there is little or no fragmentation of the glucose sample, illustrating the excellent performance of the pulse-heating desorption/ionization method, especially in the low mass region. The average signal-to-noise ratio (S/N) is 65 for 10 times parallel experiments.

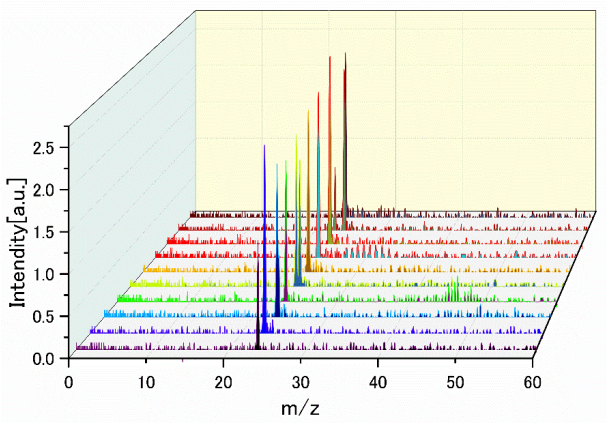

Figure 4.4. Reproducibility mass spectra of Glucose.

Figure 4.5 shows the mass spectrum of α-Cyclodextrin sample. A much higher energy is needed for the ionization of α-Cyclodextrin. Not only the singly charged α-Cyclodextrin molecular, but also the singly charged glucose unit signal is also observed.

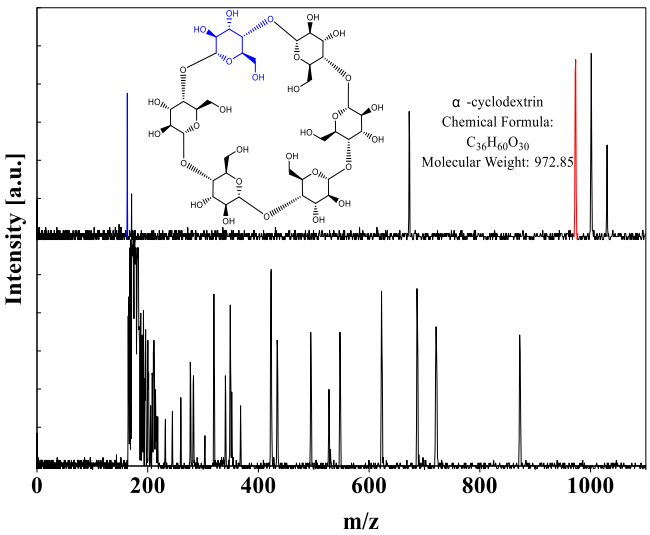

Figure 4.5. Mass spectra of α-Cyclodextrin. (Upper) under optimized condition. (Lower) Higher energy supplied.

4.4.3. Ionization of amino acids

Amino acids play important roles in building blocks of peptides and proteins. Besides, the single amino acid also participates in the cell and energy metabolism. Therefore, an effective method for amino acids analysis is important for the study of biotechnology and clinical biochemistry. Three kinds of amino acids are tested and their mass spectra are obtained without matrix, including glutamic acid (E), Glycine (G), and Histidine (H). The amino acid is a zwitterion in that at least a basic amine group and an acidic carboxylic acid group exist. According to the isoelectric point, the amino acids can be classified into three group, acidic, basic and neutral amino acid. In this experiment, E is chosen as the acidic amino acid. There are two carboxyl groups making it acidic. Figure 4.6 shows the mass spectrum of E with different energy supplied.

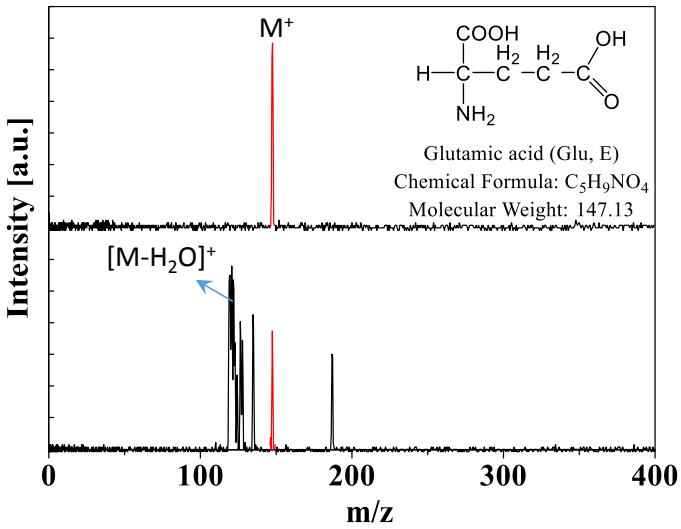

Figure 4.6. Mass spectra of Glutamic acid. (Upper) under optimized condition. (Lower) Higher energy supplied.

G is chosen as the neutral amino acid. It is the simplest amino acid. Figure 4.7 shows the mass spectrum of G with different energy supplied.

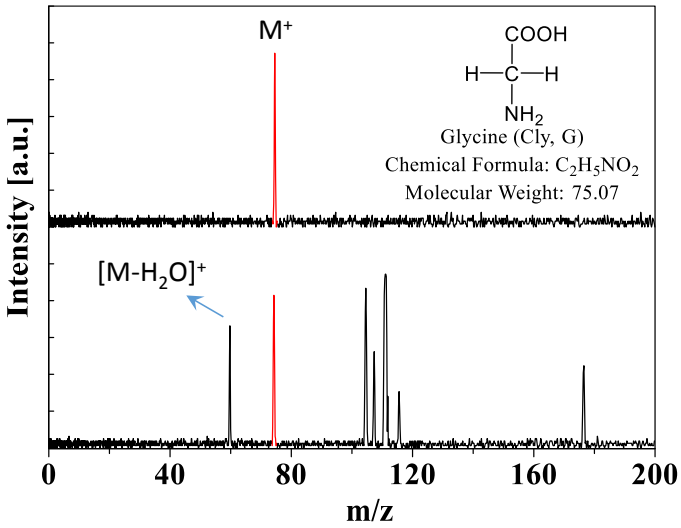

Figure 4.7. Mass spectra of Glycine. (Upper) under optimized condition. (Lower) Higher energy supplied.

H is chosen as the basic amino acid, which has positive charge on the side chain. Besides, all these three kinds of amino acid are soluble in water. The energy dependence is systemically investigated. Figure 4.8 shows the mass spectrum of H with different energy supplied.

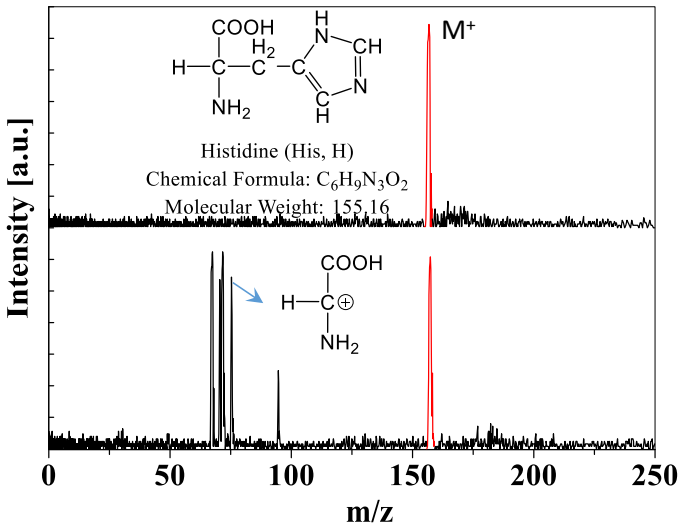

Figure 4.8. Mass spectra of Histidine. (Upper) under optimized condition. (Lower) Higher energy supplied.

For all three kinds of amino acid samples, the singly-charged ion signals are observed. Under optimized conditions, a high signal-to-noise ratio (S/N) molecular weight signal is obtained with very clean background. When the ionization energy is increased, the fragmentation signals are observed. For the amino acid E and G, the signals of $[M\text{-}H_2O]^+$ are observed. The signals presenting larger than the molecular weight ion signal maybe due to the ionization of cluster. For the amino acid H, there are only fragmentation signals observed.

The amino acid sample is also tested by the conventional MALDI for comparison. The Voyager-DETM RP MALDI system is used in linear and positive mode. The mass spectra are obtained by 100 shots. As shows in Figure 4.9, the matrix-free samples and mixed with matrix samples are measured. Three kinds of common matrices are used. Although the resolution of conventional MALDI is higher than the

miniaturized one, it is difficult to separate the molecular weight signal form the matrix signal when the molecular weight is similar. Besides, the fragmentation signal generation is strongly depend on the sample molecule itself for matrix-free sample.

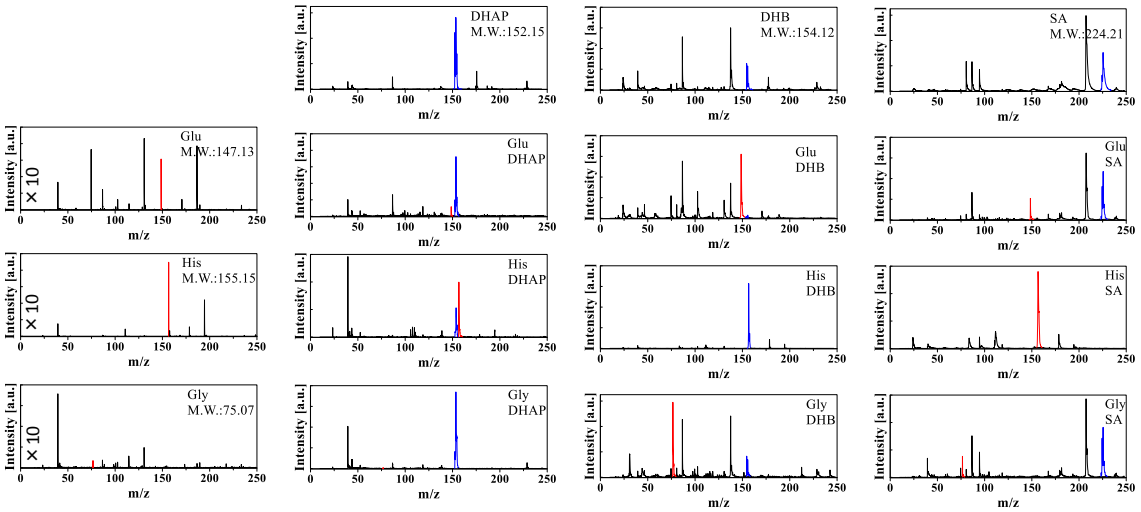

Figure 4.9. Mass spectra of amino acids by conventional MALDI.

4.4.4. Ionization of peptide

Encouraged by the above results, Angiotensin I with a molecular weight of 1296.5 Da is also examined. Figure 4.10 shows the positive ion mass spectra of Angiotensin I. Surprisingly, the singly-charged molecular ion signal is also observed under an optimized conditions. With a higher energy supplied, the double charged ion signal is also observed with several fragmentation ion signals.

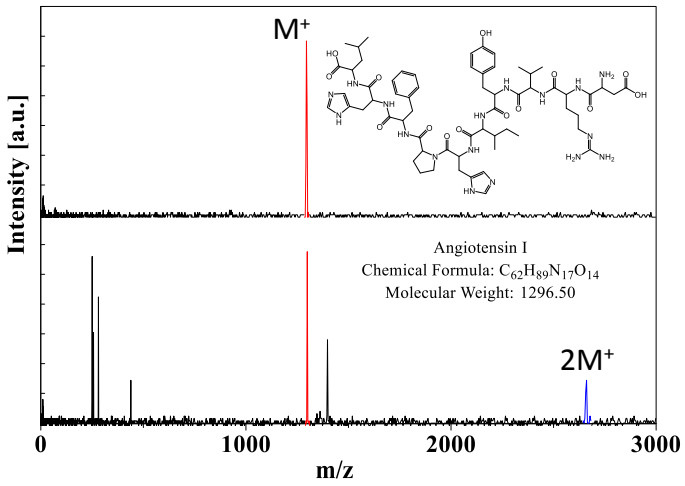

Figure 4.10. Mass spectra of Angiotensin I. (Upper) under optimized condition. (Lower) Higher energy supplied.

4.4.5. Ionization of protein

Then the Cyt C is chosen as a protein sample and examined by the matrix-free pulse-heating desorption/ionization method. And the obtained mass spectra are shown in Figure 4.11. Similar with other sample, the singly charged molecular weight ion signal is obtained under an optimized condition with very less back ground.

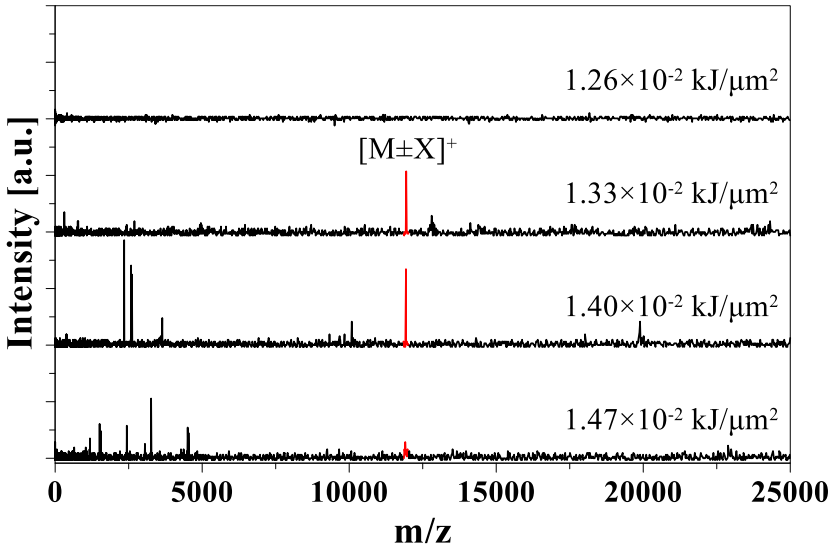

Figure 4.11. Mass spectra of Cyt C with different pulse-heating energy.

However, the intensity is not as high as the small molecular weight sample. Along with the increase of the pulse voltage, the intensity of molecular weight ion signal is decreased. And the fragmentation ion signals appear. Further increase the applied pulse voltage, the smaller fragmentation ion signals are observed. To confirm the capability for protein analysis, α-Lactalbumin is also examined by the matrix-free pulse-heating desorption/ionization method. Similar tendency is shown with α-Lac and the mass spectra is shown in Figure 4.12.

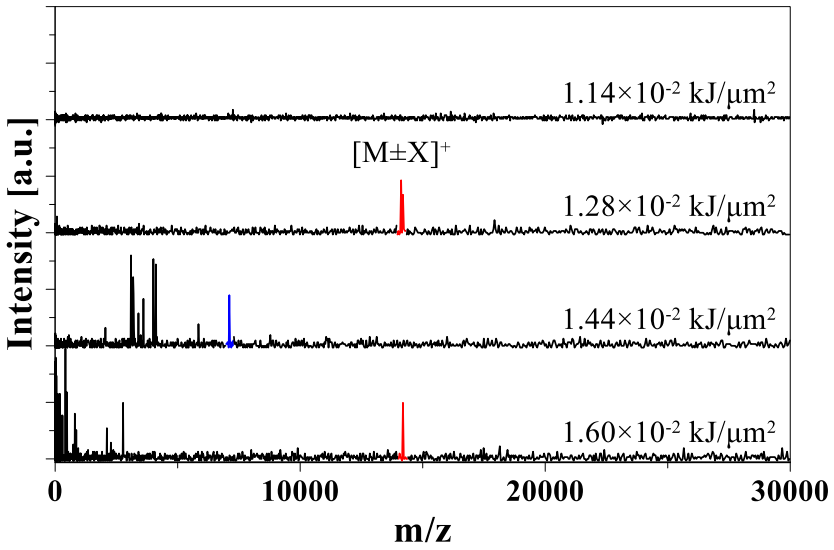

Figure 4.12. Mass spectra of α-Lac with different pulse-heating energy.

4.5. Summary

As compared to a previous study, the present experiments yield a much clear mass spectrum with very few fragmentation ion signals. One possible reason is that a more homogeneous ion generation process is enabled by the uniform and thin sample film, as opposed to the non-uniform and thick (300 nm) BSA film used in the previous study. In this study, oxygen plasma treatment of the micro-heater surface is introduced before sample layer formation, so that the water wettability of the PHDI chip can be increased and the sample film thickness is significantly reduced to approximately 20 nm. Thus, the thermal energy supplied by the micro-heater is effectively transferred and uniformly distributed over the thin sample film. This is in contrast to a previous study, where the thermal distribution was less uniform due to the non-uniform and thick film, which could resulted in fragmentation. In addition,

the reproducibility of the mass spectrum was greatly improved by the uniform sample film.

Regarding the ionization yield, the PHDI method gives a series of quantitative peaks with a single shot of pulse-heating, and clear peaks are observed even with low sample concentrations, which give a small peak in conventional MALDI; thus, the ionization yield with PHDI is not poor. For further discussion, the sensitivity of the PHDI method is estimated from the sample consumption per pulse. Taking the BSA sample as an example, a dried sample spot (~2 mm in diameter) is formed with 250 nL BSA solution (0.1 mg/mL). For each pulse-heating, the sample in the ionization zone (30 μm × 100 μm) is completely desorbed and ionized. Therefore, approximately 360 amol BSA is consumed per pulse-heating shot, which is comparable with the sample consumption (23-1000 amol) per laser shot in conventional MALDI.

As shown in Fig. 4.1, a relatively high S/N is obtained by the PHDI method with only one pulse, as opposed to the MALDI method, which required 10,000 shots. Therefore, the ionization yield for PHDI is considered to be much higher than that for the MALDI method with each pulse (shot). A possible reason is as follows. In our previous study, desorption and ionization occurred at a limited range of higher pulse-heating energy (from 1.45×10^{-2} to 1.65×10^{-2} μJ/μm^2), and only desorption occurred at a wider and lower range of pulse-heating energy (from 1.02×10^{-2} to 1.45×10^{-2} μJ/μm^2). If a similar phenomenon is assumed to occur in MALDI, it is considered that only

the sample at the center of the laser spot is desorbed and ionized, while the other part is desorbed only because the laser energy varies strongly in the laser beam profile. On the contrary, the thermal energy of PHDI is uniformly distributed over the ionization zone. Therefore, the PHDI method is more efficient than the MALDI under the optimized conditions.

The capabilities of matrix-free on-chip PHDI paradigm have been unfolded successfully for a variety of biomolecules, which indicates that the matrix is not essential for the PHDI method. For proteins such as Cyt c, relatively high thermal energy is required for the matrix-free PHDI (Fig. 4.11) as compared with the matrix PHDI (Fig.4.1). This result suggests that the matrix assists ionization to some extent and probably donates protons to the protein molecules, similar to the conventional MALDI. For BSA, singly charged ion signal has not been observed without matrix. For such large proteins, singly charged ion signals may be obtained with the matrix. Besides, further optimization of the sample concentration and thickness may help to obtain singly charged ion signals without matrix. In short, the matrix-free PHDI method is suitable for small molecules, while the analysis of large molecules requires a matrix to obtain the singly-charged ion signals.

The supplied thermal energy is an important parameter for the ion generation mechanism in the PHDI method. Desorption occurs at a lower thermal energy, and with an increase in the supplied thermal energy, ionization starts. At the appropriate level of thermal energy, only singly charged ions are generated. At a higher energy

supplied, fragmentation ion signals may be observed due to decomposition and fragmentation. As reported in our previous study, when 1.68×10^{-2} μJ/μm^2 of pulse-heating energy is applied, the temperature of the ionization zone is approximately 2180 K (the melting point of Cr), which is comparable with that in MALDI. The mechanism of the PHDI method is considered to be somewhat similar to the thermal ionization occurring in the MALDI. However, further studies are needed for understanding the mechanism of the PHDI more clearly.

In conclusion, this study has demonstrated the feasibility of using the PHDI method for matrix-free ionization of a variety of biomolecules, including carbohydrates, which are difficult to analyze directly by the conventional MALDI. With a uniform and thin sample film, the on-chip PHDI method shows high yield and affords singly charged ions with very less fragmentation and background with one pulse-heating shot. Without the matrix, the sample preparation could be simplified for the on-chip PHDI source, making it suitable for application to on-site and rapid analysis.

Reference

1. Guinan, T.; Kirkbride, P.; Pigou, P. E.; Ronci, M.; Kobus, H.; Voelcker, N. H., Surface ‐assisted laser desorption ionization mass spectrometry techniques for application in forensics. Mass spectrometry reviews **2015,** 34 (6), 627-640.
2. Tanaka, K.; Waki, H.; Ido, Y.; Akita, S.; Yoshida, Y.; Yoshida, T.; Matsuo, T., Protein and polymer analyses up to m/z 100 000 by laser ionization time-of-flight

mass spectrometry. Rapid Communications in Mass Spectrometry **1988,** 2 (8), 151-153.

3. Zenobi, R., Laser-assisted mass spectrometry. CHIMIA International Journal for Chemistry **1997,** 51 (10), 801-803.
4. Peterson, D. S., Matrix‐free methods for laser desorption/ionization mass spectrometry. Mass spectrometry reviews **2007,** 26 (1), 19-34.
5. Guinan T, Kirkbride P, Pigou PE, Ronci M, Kobus H, Voelcker NH. Surface-assisted laser desorption ionization mass spectrometry techniques for application in forensics. Mass spectrometry reviews **2015**,34, 627-640.
6. Sugiyama, K.; Harako, H.; Ukita, Y.; Shimoda, T.; Takamura, Y., Pulse-heating ionization for protein on-chip mass spectrometry. Analytical chemistry **2014,** 86 (15), 7593-7597.
7. Doms M, Muller J. A micromachined vapor-jet vacuum pump. In: Solid-State Sensors, Actuators and Microsystems Conference **2007**. Transduces 2007. International. IEEE (2007).
8. Sugiyama K, Ukita Y, Takamura Y. Development of on-chip vacuum generation by gas-liquid phase transition. Sensors and Actuators A: Physical **2012**, 176, 138-142.
9. Siebert P, Petzold G, Hellenbart A, Müller J. Surface microstructure/miniature mass spectrometer:. processing and applications. Applied Physics A: Materials Science & Processing 67 **1998**,155-160.
10. Wapelhorst E, Hauschild J-P, Müller J. Complex MEMS: a fully integrated TOF micro mass spectrometer. Sensors and actuators A: Physical **2007**, 138, 22-27.
11. Wright S, O'Prey S, Syms RR, Hong G, Holmes AS. Microfabricated quadrupole mass spectrometer with a Brubaker prefilter. Journal of Microelectromechanical Systems **2010**, 19, 325-337.

12. Darling RB, Scheidemann AA, Bhat K, Chen T-C. Micromachined Faraday cup array using deep reactive ion etching. Sensors and Actuators A: Physical **2002**, 95, 84-93.
13. Naik A, Hanay M, Hiebert W, Feng X, Roukes M. Towards single-molecule nanomechanical mass spectrometry. Nature nanotechnology **2009**, 4, 445-450.
14. Bibi, A.; Ju, H., Quantum dots assisted laser desorption/ionization mass spectrometric detection of carbohydrates: qualitative and quantitative analysis. Journal of Mass Spectrometry **2016,** 51 (4), 291-297.
15. He, Q.; Chen, S.; Wang, J.; Hou, J.; Wang, J.; Xiong, S.; Nie, Z., 1-Naphthylhydrazine hydrochloride: A new matrix for the quantification of glucose and homogentisic acid in real samples by MALDI-TOF MS. Clinica Chimica Acta **2013,** 420, 94-98.
16. Beneito-Cambra, M.; Bernabé-Zafón, V.; Herrero-Martínez, J. M.; Ramis-Ramos, G., Study of the Fragmentation of D-Glucose and Alkylmonoglycosides in the Presence of Sodium Ions in an Ion-Trap Mass spectrometry. Analytical Letters **2009,** 42 (6), 907-921.

CHAPTER 5 Conclusions

In this thesis, the performance of PHDI ion source is improved. To obtain a thin and uniform sample film, the oxygen plasma treatment process is introduced for pre-treatment of the ion source chip. The thickness of sample film is reduced from 300nm to 20 nm. To improve the accuracy of alignment between ion source and mass filter, a chip holder is designed and added to the mass spectrometry system. With a precise alignment and uniform sample film, a reproduced mass spectra are obtained. By investigating the position dependence of ionization zone, a calibration method for the on-chip ion source mass spectrometry is established.

Then the pulse-heating desorption/ionization MS system is extended to negative ion detection mode. A thermally determined electron and proton transfer process is proposed to explain the primary ion formation mechanism of the pulse-heating desorption/ionization. A simple electron transfer model has been suggested from observation of anion and cation generated by simple inorganic sample in the gas phase. Then the model is verified and refined by the matrix and protein mixed with matrix sample analyzed in both positive and negative mode. The key factor for ion formation has been found that it is the supplied thermal energy. By only thermal energy supplying, the sample was evaporated and sublimated, and the ion is formed according to the electron and proton affinity. A nearly ideal mass spectrum, only singly charged molecular weight ions with high signal-to-noise ratio, is obtained by only one low-voltage pulse for not only simple inorganic sample, but also protein sample in both positive and negative mode.

According to the mechanism proposed, the sample can be ionized only based on the thermal energy supplied. Then the matrix maybe not needed. Therefore, a matrix-free pulse-heating desorption/ionization method is proposed for biomolecule analysis. A wide range of samples including carbohydrate, amino acid, peptide, and protein, are tested. Compared with conventional matrix-assisted laser desorption /ionization (MALDI), the pulse-heating desorption/ionization method shows extremely high-yield and singly-charged ion, with very less fragmentation and background. It is believed that this work offers a new technique for matrix-free biomolecule analysis which overcomes the limitations of the conventional MALDI.

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Achievements

JOURNAL PAPER

Xi Luo, Phan Trong Tue, Kiyotaka Sugiyama, et al. High yield matrix-free ionization of biomolecules by pulse-heating ion source. Scientific Reports. 2017, 7, 15170.

[Under preparation]

Xi Luo, Phan Trong Tue, Kiyotaka Sugiyama, et al. Positive and Negative ion formation of on-chip pulse-heating desorption/ionization method.

Xi Luo, Phan Trong Tue, Kiyotaka Sugiyama, et al. Matrix-free amino acid analysis by pulse-heating ion source for miniaturized mass spectrometry.

CONFERENCE PAPERS

1. Xi Luo, Phan Trong Tue, Yuzuru Takamura. Matrix-free Biomolecule Analysis by a Miniaturized On-chip Pulse-heating Ionization Mass Spectrometer. The 21st International Conference on Miniaturized Systems for Chemistry and Life Sciences (MicroTAS 2017, Savannah, USA. October 22-26, 2017).
2. Xi Luo, Phan Trong Tue, Yuzuru Takamura. Matrix-free Carbohydrates Analysis by On-chip Pulse-heating Ionization Mass Spectrometer. The 79th JSAP Autumn Meeting, (Fukuoka, Japan, September 5-8, 2016).
3. Xi Luo, Phan Trong Tue, Yuzuru Takamura. A Novel Strategy for Amino Acid Analysis by Pulse-heating Ion Source. 9th International Conference on Molecular Electronics and Bioelectronics (ME&B 2017, Kanazawa, Japan. June 26-28, 2017).
4. Xi Luo, Phan Trong Tue, Yuzuru Takamura. The Mechanism of On-chip Pulse-heating Ionization. The 65th Annual Conference on Mass Spectrometry, (Tsukuba, Japan, May 17-19, 2017).

5. Xi Luo, Phan Trong Tue, Yuzuru Takamura. Matrix-free Protein Ionization by Pulse-heating Ion Source. The 64th JSAP Spring Meeting, (Yokohama, Japan, March 14-17, 2017).

6. Xi Luo, Phan Trong Tue, Yuzuru Takamura. Pulse-heating Ionization for Inorganic Material. The 78th JSAP Autumn Meeting, (Niigata, Japan, September 13-16, 2016).

7. Xi Luo, Phan Trong Tue, Yuzuru Takamura. Development of Mass Spectrometer with Pulse-heating Ion Source. The 10th Bio-related Chemistry Symposium, (Kanazawa, Japan, September 7-9, 2016. IN JAPANESE).

8. Xi Luo, Phan Trong Tue, Kiyotaka Sugiyama, Yuzuru Takamura. Negative Ion Behavior in Pulse-heating Ion Source Mass Spectrometry. 21st International Mass Spectrometry Conference (IMSC 2016, Toronto, Canada. August 20-26, 2016).

9. Xi Luo, Phan Trong Tue, Kiyotaka Sugiyama, Yuzuru Takamura. Reproducibility Improvement of Pulse-heating Ionization Source for On-chip Mass Spectrometry. 8th Asia-Pacific Conference of Transducers and Micro/Nano Technologies (APCOT 2016, Kanazawa, Japan, June 26-29, 2016).

10. Xi Luo, Kiyotaka Sugiyama, Yuzuru Takamura. Effects of the Matrix Solution Composition on Cytochrome C Detection by On-Chip. 32nd CHEMINAS, (Kitakyushu, Japan, November 26-27, 2015).

11. Xi Luo, Kiyotaka Sugiyama, Yuzuru Takamura. Matrix Effect on Mass Spectra of Cytochrome C in Pulse-heating Ionization for On-chip Mass Spectrometry. 28th International Microprocesses and Nanotechnology Conference (MNC 2015, Toyama, Japan, November 10-13).

12. Kiyotaka Sugiyama, Xi Luo, Yuzuru Takamura. On-chip Protein Mass Spectrometry with the Pulse-heating Ionization Source and the Miniaturized Electrostatic Ion Lens. 19th International Conference on Miniaturized Systems for Chemistry and Life Sciences (MicroTAS 2015, Gyeongju, Korea, October 25-29, 2015).