analytical chemistry

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Pulse-Heating Ionization for Protein On-Chip Mass Spectrometry

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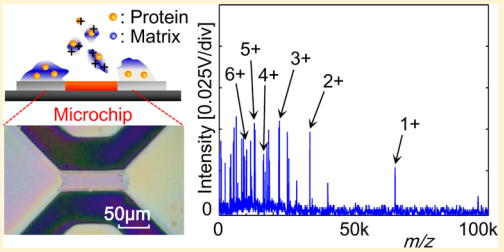
Supporting Information

ABSTRACT: An on-chip pulse-heating ionization source for protein samples was developed for the realization of miniaturized mass spectrometry. A protein analyte was ionized on a chip by applying only thermal energy to the solid phase sample without a laser, high voltage, or heated ambient gases. A fabricated ionization source consisting of a Pt/Cr microheater (width: 30 μm; length: 100 μm) on a silicon substrate was coupled with a time-of-flight mass filter to analyze a protein sample of bovine serum albumin (BSA, M = 66 kDa). A singly charged BSA ion and other multiply charged BSA ions were generated in the presence of 2,5-dihydroxybenzoic acid as a matrix. To detect the singly charged BSA ion, the required surface energy density of 1.65 $\times$ 10^{-2} μJ/μm^2 was applied to the microheater for 500 ns. The use of the 2,5-dihydroxyacetophenone matrix resulted in the generation of the multiply charged protein analyte, while the use of the sinapic acid matrix showed abundant peaks in the low m/z region.



Mass spectrometry (MS) is among the highly sensitive and highly selective analytical methods for examining multiple analytes in biological and environmental samples. Recently, MS comes into use for clinical and more practical applications by rapid and portable devices as well as research. For example, desorption electrospray ionization (DESI)[1−3] and direct analysis in real time (DART)[4,5] are applied to the rapid screening of chemicals in liquids[6] and in solids[7] and the rapid identification of biological tissues in clinical surgeries[8,9] under ambient conditions. On the other hand, miniaturization of MS[10−23] is also a significant challenge for highly sensitive on-site analysis by portable devices. The miniaturized mass spectrometer consists of a mass filter,[10−17] an ionization source,[18−22] an ion detector,[23] and a vacuum pump.[24−26] Ion separation in the microchannel is an interesting topic. Miniaturized mass filters using magnetic fields[10] and electric fields,[11] a quadrupole filter,[12,13] a cylindrical ion trap filter,[14,15] and a time-of-flight (TOF) filter[16,17] have been demonstrated on a chip. The on-chip ionization of gaseous compounds was performed using ionization sources with a microformed carbon nanotube (CNT)[18] and with a Ni filament.[22] In order to ionize an aqueous solution containing low levels of chemical compounds, microfluidic miniaturized electrospray ionization (ESI)[19−21] was employed. Additionally, miniaturized detectors such as Faraday cup arrays[23] and miniaturized vacuum pumps,[24−26] which are important components for MS, were fabricated.

Improvement of the miniaturized ionization source is particularly important for miniaturized MS to be suitable for a wide range of applications because the types of compatible samples are restricted by the ionization method. Field emission ionization sources and hot electron emitters are typically used for the ionization of gaseous samples. Microfabricated ESIs, capillary electrophoresis coupled ESIs,[21] and paper-based ESIs[27,28] have been developed for the analysis of the solution with organic compounds and peptides. To obtain the mass spectrum of a sample with a mass-to-charge ratio, m/z, ranging up to 2000, these methods can analyze the peptides from a tryptic digest of cytcrome c[20] or bovine serum albumin[21] as well as therapeutic drugs in whole blood.[28] On the other hand, desorption methods[29,30] for nonvolatile compounds with the assistance of heat have been studied, before the development of laser ionization[31] and matrix assisted laser ionization/desorption (MALDI).[32] In these methods, the techniques of rapid heating[29] and of volatility enhancement[30] were used only for evaporation of the nonvolatile compounds before ionization and were studied to desorb the molecules with little fragment. Electron impact ionization[29] or chemical ionization such as proton transfer ionization with NH_4^+ followed[30] after evaporation. Photochemical ionization by the laser source and MALDI were developed for the mass spectrometry of peptides and proteins in 1987 and 1988, respectively. While matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) has the advantage of analyzing proteins with m/z up to 100 000[32] without the decomposition of the samples, the present miniaturized MS does not allow the analysis of proteins. Recently, novel research studied the ionization of nonvolatile compounds by the assistance of heated capillary inlet[33−36] or matrix evaporation in a vacuum.[37−39] In those reports, highly charged protein ions were produced without the use of high voltage, a laser, or additional heat.[35] These matrix and vacuum assisted soft ionization techniques have great

Received: April 17, 2014
Accepted: July 17, 2014
Published: July 17, 2014

   dx.doi.org/10.1021/ac501407c | Anal. Chem. 2014, 86, 7593−7597

potential for the analysis of fragile molecules and molecular complexes in living organisms as well as high-throughput analysis directly from the multiwall plates. If the production of lowly charged protein ions occurs by applying pulse-heating to the sample instead of the heated inlet of the mass analyzer, miniaturization of the ionization source for the protein can be realized. In this paper, a method for the on-chip ionization of protein analyte with a simple device is discussed. We speculated that ionization might occur because of thermal energy applied to the solid state sample by pulse-heating with a microheater. The miniaturized pulse ionization source was coupled with the TOF mass filter for the analysis of protein samples. The effects of the matrix on the pulse-heating ionization are also reported.

EXPERIMENTAL SECTION

Chip Fabrication. In order to rapidly apply thermal energy to the protein sample on a chip, microfabricated heaters were used. Figure 1a shows the microheaters fabricated by lift-off

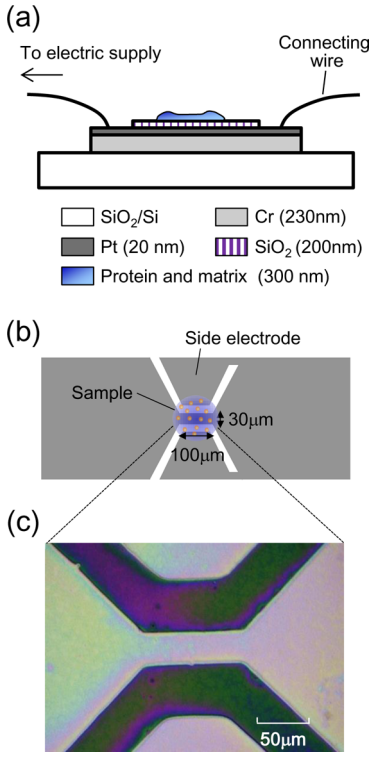


Figure 1. Design of the microfabricated pulse-heating ionization source. (a) Cross section view of the ionization chip. (b) Structure of the ionization source. (c) Microscopic image of the sample-applied region of the ionization chip after applying BSA and 2,5-DHB matrix. The sample was uniformly covered on the microheater.

processing on a silicon substrate with a thermally grown 100 nm-thick SiO_2 layer. The heater electrode had a narrow part with a length of 100 μm and a width of 30 μm, where the electric field was concentrated, and two connection pads with a length of 5 mm and a width of 2 mm as shown in Figure 1b. Side electrodes were fabricated for the realization of a uniform electric field above the ionization source. The convergence of ions in the entrance of the TOF mass filter was improved due to the presence of the side electrode (see Figure S1 of the numerical simulation in the Supporting Information). A SiO_2 insulation layer was formed on the center of the electrode to cover the heating region. This SiO_2 layer functioned to form a uniform hydrophilic surface on the microheater. Moreover, the insulation layer prevented thermal electron emission from the Pt surface and the electric field from affecting the ionization. Subsequently, an aqueous sample mixture of bovine serum albumin (BSA, M = 66 kDa) as a protein analyte and 2,5-dihydroxybenzoic acid (2,5-DHB), sinapic acid (SA), or 2,5-dihydroxyacetophenone (2,5-DHAP) as a matrix was applied onto the center part of the ionization source and dried in a vacuum chamber. Figure 1c shows a typical microscopic image of the fabricated microheater after drying the sample solution of BSA and 2,5-DHB. A transparent thin-film like layer was formed.

Experimental Method. Figure 2 shows the experimental setup for investigation of the capability of the ionization and

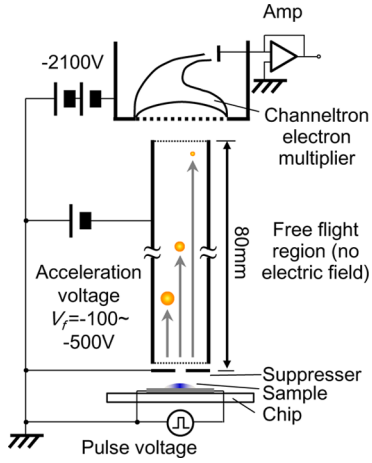


Figure 2. Schematic view of the experimental setup. These components were placed in a vacuum chamber. All experiments were conducted at a pressure of approximately 10^{-3} Pa.

mass spectrometry. The fabricated ionization chip was coupled with a home-built TOF mass filter and a commercially available detector. The free flight region with no electric field was adjusted to be 80 mm long between the suppressor and the exit of the free flight region. Fabricated chip and the detector were aligned with a gap of 1 mm from the free flight region, respectively. These components were placed in a vacuum chamber with a turbo-molecular pump (HiCube 300 Classic, Pfeiffer Vacuum, Germany). Pressure in the vacuum chamber was approximately 10^{-3} Pa in all experiments. To apply a single pulse voltage to the microheater for pulse-heating, the fabricated electrode was connected to a commercially available MOS FET (2sk2382, TOSHIBA, Japan) and a logic IC for pulse control. The full width at half-maximum of the pulse voltage was set at 500 ns for all experiments. The applied voltage was varied in the range of 22 to 40 V for the pulse-heating ionization. After pulse-heating, generated positive ions above the microheater were extracted using the difference in potential between the surface of the chip and the entrance of the TOF mass filter at the leading edge of the input pulse voltage. Accordingly, accelerated ions traveled within an 80 mm length of the TOF region at a constant velocity. Positive ions were captured and amplified by a factor of approximately 10^4 by a negatively biased channeltron electron multiplier. The electron multiplier was connected to an amplifier unit (C9663, Hamamatsu photonics, Japan), which had a conversion factor of 4 mV/μA. Time flight signals were recorded by an oscilloscope (TDS2022C, Tektronics Inc.,

dx.doi.org/10.1021/ac501407c | Anal. Chem. 2014, 86, 7593−7597

USA) for the analysis of the mass spectrum. Calibration of home-built TOF mass analyzer was evaluated by the detection of vapored copper particles (see Figure S2 in the Supporting Information).

Sample Preparation. In this paper, bovine serum albumin was used to evaluate the potential for the ionization of protein samples. The BSA was purchased from Sigma-Aldrich (Japan). In a process similar to conventional MALDI-MS,[40,41] a matrix was mixed with the sample to disperse the protein molecules uniformly on the surface of the microheater. 2,5-DHB, SA, and 2,5-DHAP (Wako Pure Chemical, Japan) were used as a matrix in the experiments. All aqueous solutions were prepared using ultrapure water (18.2 MΩcm) from a Milli-Q system (Millipore, Billerica, MA, USA). Protein samples were prepared by dissolving 100 μg/mL or 1 μg/mL BSA in Milli-Q water. The matrices were dissolved in acetonitrile/water mixtures (1:1, v/v) at a concentration of 10 mg/mL. Subsequently, the protein sample was mixed with the matrix solution at a ratio of 1:1. A 0.5 μL sample solution was dropped onto the center of the microheater by pipetting and then was rapidly dried in a vacuum. The thickness of the deposited layer of BSA and 2,5-DHB matrix on the microheater was approximately 300 nm (see Figure S3 in the Supporting Information).

RESULTS AND DISCUSSION

To evaluate the possibility of the ionization of protein analyte by applying only pulse-heating, we studied the detection of charged molecules with a TOF mass analyzer. Figure 3 shows

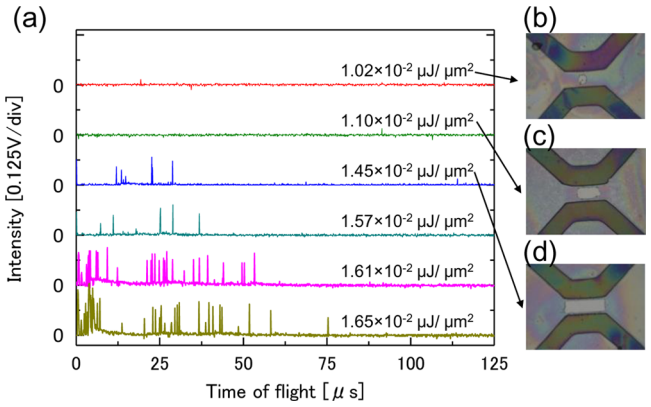


Figure 3. Time-of-flight signals of ionized sample of BSA and 2,5-DHB matrix and microscopic images of the ionization source. (a) Time-of-flight signals obtained by the electron multiplier with different pulse-heating energies. Typical microscopic images after a single pulse-heating in the case of (b) 1.02 × 10^{-2} μJ/μm^2, (c) 1.10 × 10^{-2} μJ/μm^2, and (d) 1.45 × 10^{-2} μJ/μm^2.

the time-of-flight signals as well as microscopic images after a single pulse-heating. As shown in Figure 3a, the applied pulse-heating energy was varied as 1.02 × 10^{-2}, 1.10 × 10^{-2}, 1.45 × 10^{-2}, 1.57 × 10^{-2}, 1.61 × 10^{-2}, or 1.65 × 10^{-2} μJ/μm^2. The voltage applied to the microheater was changed while the pulse width was fixed at 500 ns. The signals were measured using the samples, which consisted of 100 μg/mL BSA and 10 mg/mL 2,5-DHB matrix at a ratio of 1:1 (v/v). The acceleration voltage of the generated positive ions was −300 V. Desorption of the sample was observed at the center part of the microheater in the bright part of the microscopic images when 1.02 × 10^{-2} μJ/μm^2 of pulse-heating energy was applied, as shown in Figure 3b. The ionization is not shown when 1.02 × 10^{-2} μJ/μm^2 of pulse-heating was applied. In contrast to this, some peaks are detected when pulse-heating energy exceeding 1.45 × 10^{-2} μJ/μm^2 was applied, indicating the generation of positively charged molecules. Remarkably, these results showed not only the occurrence of desorption (Figure 3d) but also the generation of ions. Fewer than ten peaks are observed when a single pulse voltage was applied with pulse-heating energy less than 1.57 × 10^{-2} μJ/μm^2. The number of the peaks increased drastically when pulse-heating energy greater than 1.61 × 10^{-2} μJ/μm^2 was applied (quantitative analysis of desorption and ionization is available in Figure S4 in the Supporting Information). After a second pulse-heating, the number of detected peaks was drastically reduced because most samples on the heating region vanished. If pulse-heating energy exceeding 1.68 × 10^{-2} μJ/μm^2 was applied for 500 ns, the surficial SiO_2 layer cracked and then the Cr layer melted. It is estimated that the temperature of the microheater increased up to nearly the melting temperature of chromium.

The mass spectrum was analyzed to convert the flight time of the obtained peaks into a mass-to-charge ratio, m/z. The aim of the mass spectrum analysis was to determine whether the detected charged molecules originated in the BSA protein. The mass-to-charge ratio, m/z, was calculated using the free flight time according to the following equation.

$$\frac{m}{z} = \frac{t^2}{l^2}\frac{2V_f e}{u} \quad (1)$$

Here, m is the mass of ions, z is the valence of ions, t is the time-of-flight, l = 0.08 m (the free flight distance in this experiment), V_f is the difference in potential between the chip and the entrance of the TOF mass filter, e = 1.602 × 10^{-19} C (the elementary charge), and u = 1.660 × 10^{-27} Da (the atomic mass unit). Figure 4 shows the mass spectra of 2,5-DHB, BSA,

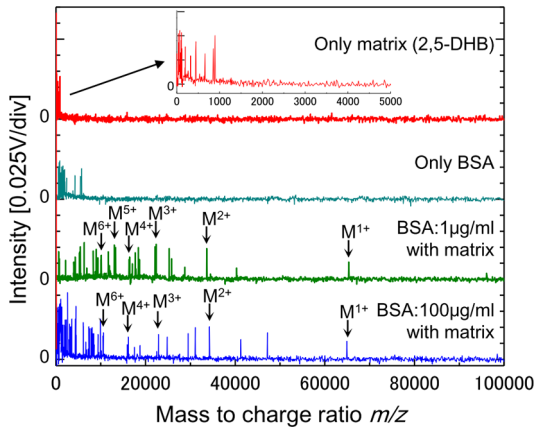


Figure 4. Mass spectra of only 2,5-DHB matrix, only BSA analyte, and BSA mixed with 2,5-DHB matrix at the pulse-heating energy of 1.66 × 10^{-2} μJ/μm^2. 100 or 1 μg/mL BSA samples were mixed with a 10 mg/mL matrix at a ratio of 1:1. 0.5 μL of the sample solution was applied to the ionization source and was analyzed by a TOF mass analyzer with the acceleration voltage of −500 V.

and BSA mixed with 2,5-DHB matrix at pulse-heating energy of 1.65 × 10^{-2} μJ/μm^2 for 500 ns. An acceleration voltage of −500 V was used to minimize the velocity variation of the accelerated ions due to its initial velocity distribution. The mass spectra were obtained by averaging three times pulse-heating experiments. The chip was washed with ethanol and plenty of deionized water, and then, fresh sample was applied onto the

dx.doi.org/10.1021/ac501407c | Anal. Chem. 2014, 86, 7593−7597

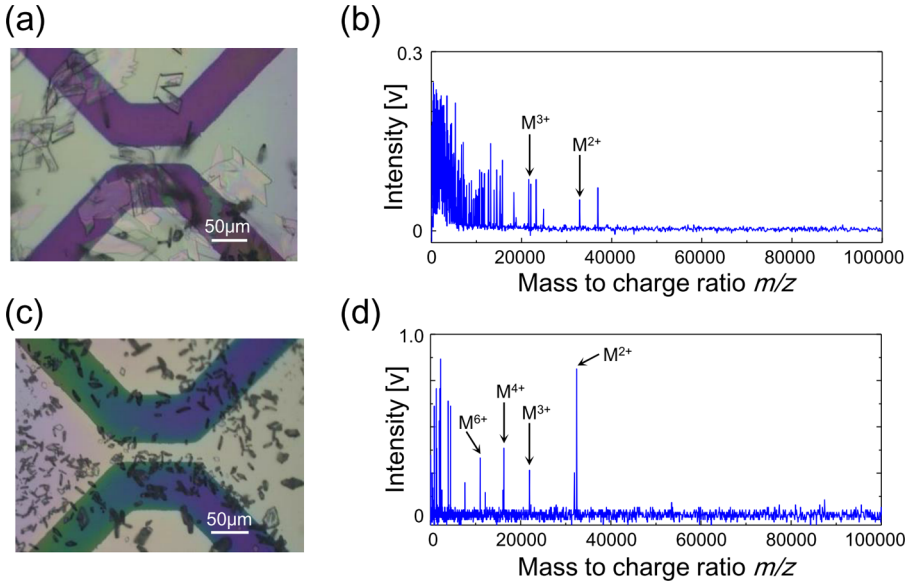


Figure 5. Pulse-heating ionization of BSA analyte with (a, b) SA matrix and (c, d) 2,5-DHAP matrix. (a, c) Typical microscopic images of the ionization source after drying of the sample solution. (b) Mass spectra of BSA with SA matrix. (d) Mass spectra of BSA with 2,5-DHAP matrix.

chip between each experiment. The mass spectrum of the 2,5-DHB matrix shows that the peaks appeared in the range of m/z of 0−1000. In the case of BSA without the presence of any matrix, ionized molecules are detected in the range of m/z of 0−10 000. In contrast to this, mass spectra of BSA mixed with 2,5-DHB matrix indicate signals over the m/z of 10 000 including M^{1+}, M^{2+}, M^{3+}, M^{4+}, M^{5+}, and M^{6+} BSA ions. The clear difference in the presence or absence of the matrix suggests that the matrix assistance plays a significant role in the pulse-heating ionization. Unlike ESI, singly charged ion was obtained, similar to the conventional MALDI-MS of BSA.[40]

Figure 5 shows the microscopic images of the chip and BSA mass spectra with SA or 2,5-DHAP as a matrix. The mass spectra were obtained in a similar manner. Figure 5a,b shows the results of the use of SA matrix which is a typical matrix for MALDI of proteins. Here, grain like morphology of the matrix with the size of approximately 50 μm was shown on the chip. Thin-film like morphology as shown in Figure 1c was not formed in both cases of drying in a vacuum and the atmosphere. The mass spectrum shows that the singly charged ion is not detected, while highly charged ions including M^{2+} and M^{3+} ions are observed. Many peaks closely distributed in the region of m/z of 0−10 000 caused difficulty for further analysis of the mass spectrum. Figure 5c,d shows the results of the use of 2,5-DHAP matrix. The grain like morphology of the matrix with the size of approximately 10 μm was observed in the microscopic image as shown in Figure 5c. In the mass spectrum, M^{2+}, M^{3+}, M^{4+}, and M^{6+} ions are clearly detected. Interestingly, the fragment-like peaks at m/z = 18 000, 24 000, 42 000, and 48 000 in Figure 4 for 2,5-DHB matrix are not observed in Figure 5d for 2,5-DHAP matrix.

From these results, pulse-heating ionization clearly shows that the photochemical process is not indispensable for the ionization of singly charged protein by pulse-heating with the assistance of a proper matrix. From Figure 3, it is suggested that that desorption and ionization for higher m/z peaks required higher thermal energy transfer from the microheater to the matrix. It has been reported by McEwen et al.[33] that 2,5-DHAP as the matrix has an advantage of significantly lower ion transfer temperature than 2,5-DHB in inlet ionization. If it is due to high efficiency of energy transfer of 2,5-DHB, in our method in Figure 5, the 2,5-DHAP matrix is expected to ionize higher m/z peaks than 2,5-DHB in Figure 4, but these are not the results. The higher m/z peaks are not observed in the mass spectrum with SA matrix either. As a possible cause, the grain like morphology of SA and 2,5-DHAP matrix are considered to have less effective heat transfer from the microheater than the thin-film like matrix layer of 2,5-DHB.

Advantages of pulse-heating ionization over the previously reported microfabricated ESI[19−21] and CNT-based miniaturized ionizer[23] are as follows: (1) direct coupling with a TOF mass analyzer is possible, which provides a mass spectrum of molecular ions exceeding 10 kDa, such as proteins; (2) a singly charged ion is observed without heated inlet or heated nitrogen gases; (3) no complicated process is required to fabricate the miniaturized ionization source. Another advantage of the pulse-heating ionization method over MALDI is that further miniaturization of the ionization source into the nanoscale will be expected by the photolithography process. In our ionization method, pulse-heating energy was applied to the whole sample on the heating region from the boundary between the bottom of the sample and the microheater. When the deposited sample formed a layer with a thickness of a few micrometers, desorption and ionization were not caused by the increase in the thermal conductance of the sample itself.

■ CONCLUSIONS

For the development of a miniaturized mass spectrometer, we studied a novel on-chip ionization method involving pulse-heating of a protein sample. The ionization source was fabricated by a Pt/Cr microheater (width: 30 μm; length: 100 μm) on a silicon substrate. The fabricated ionization source was directly coupled with a time-of-flight mass filter to analyze bovine serum albumin mixed with the matrix. The protein sample was ionized by pulse-heating on a chip, and the 2,5-DHB matrix assistance played a key role in obtaining a singly charged ion of BSA. To the best of our knowledge, this is the first report of time-of-flight mass spectrometry of protein with

dx.doi.org/10.1021/ac501407c | Anal. Chem. 2014, 86, 7593−7597

an on-chip ionization source. The use of 2,5-DHAP as a matrix showed the mass spectrum with the peaks of multiply charged protein ions. The chip-based pulse-heating ionization source has the potential for significant miniaturization because it does not require an external laser light apparatus, a high voltage source, or heated ambient gases.

■ ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org/.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by JSPS KAKENHI Grant Number 26600059, 26-7894.

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