Mass Spectrometry

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Rapid Removal of Matrices from Small-Volume Samples by Step-Voltage Nanoelectrospray**

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Many fundamental researches in life sciences involve small volume analysis.[1–6] For example, the analysis of single cell or special physiological fluids,[6–11] which could help further our understanding of important physiological procedures from organism to molecular level,[12–16] involves the analysis of nanoliter or even picoliter samples.[4] Nanoelectrospray ionization mass spectrometry (Nano-ESI-MS) is a sensitive method with low sample consumption,[17] which is especially suitable for small and even ultra-small volume analysis.[18,19] Unfortunately, many biological samples are very complicated, and the matrices, especially those containing salts, have a significant ion suppression effect, which enormously reduces the sensitivity of Nano-ESI-MS.[20,21] Moreover, the salts in samples can form complicated compounds through molecular association and coordination, which makes it quite difficult to clearly identify the target compounds in the mass spectrum. It is even worse when using Nano-ESI-MS for the analysis of a single cell owing to its very small sample volume.[10,15] Although the separation methods, such as liquid chromatograph (LC),[22–25] capillary electrophoresis (CE)[1,7,26–29] and solid phase micro-extraction (SPME),[30–31] have been frequently applied for salt removal to reduce the interference from the matrix, the process is relatively tedious. Moreover, the separation procedures for LC or CE may cause a sample dilution during elution, especially for ultra-small volume of samples. More recently, Huang et al. used induced nESI to control ion flow in solution for the ultra-high sensitive analysis of small-volume samples, an approach which showed a strong tolerance to matrices. Samples such as serum and urine could be analyzed without any pre-treatment, which made high-throughput analysis of complex samples possible.[18] However, salts might still be introduced with analytes into the mass spectrometer and the salt peaks recorded in mass spectra would cause interference.

Herein, we report a step-voltage nanoelectrospray ionization method (SV-Nano-ESI) to significantly remove the interference of matrices in the analysis of a small-volume sample (Figure 1). The step voltage is shown in Figure 1b. A voltage of 5.2 kV was first applied on the emitter for 30 s, and

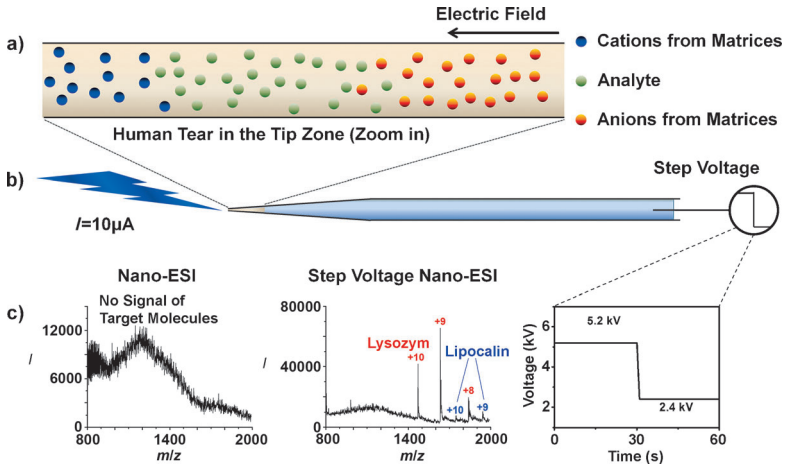

Figure 1. a) The ion electromigration for the separation of matrices and analyte. Owing to their different electromigration speeds the salt cations and anions in the matrices migrated to the leading band and tailing band, respectively, while the analyte remained still in the middle band. b) Schematic representation of SV-Nano-ESI. The emitter (orifice = 15 ≈ 20 μm) was loaded with sample solution by a micropipette. A metal wire conductor was then inserted into the capillary. Step voltage was applied to the emitter: 5.2 kV for matrix separation (for the first 30 s, a current of 10 μA was recorded, indicating the action of ions under the strong electric field) then a +2.4 kV voltage was applied for electrospray and MS analysis in a positive ion mode. c) The mass spectra of human tear sample by using conventional nanoelectrospray and SV-Nano-ESI, the matrices interferences is removed apparently.

effective separation between analytes and matrices generated through ion electromigration in the tip zone of the emitter. Subsequent nanoelectrospray was carried out at a voltage of 2.4 kV for the sample analysis. Under the action of strong electric field, salt cations and anions in matrices would rapidly migrate in opposite directions in the tip zone, forming the leading band and tailing band. Owing to the low electromigration rates, the target proteins remained almost in the middle band and could be analyzed without matrix interference and a high signal-to-noise ratio was achieved.

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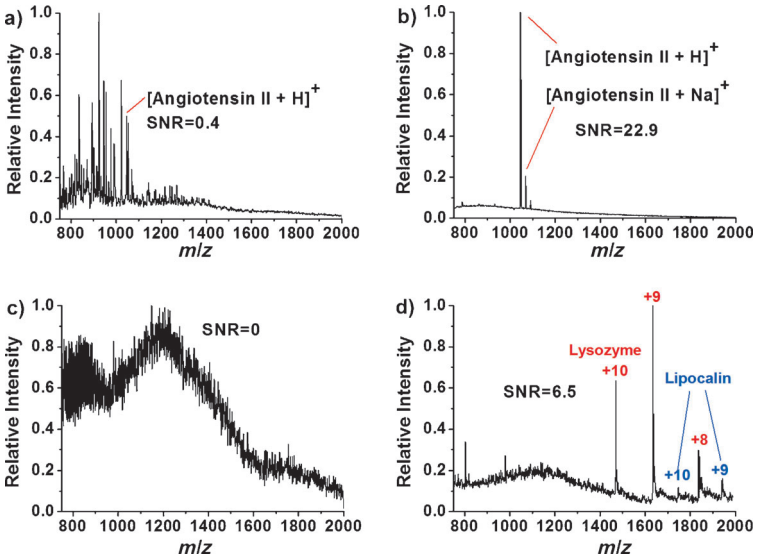

Figure 2. The performance of the conventional nanoelectrospray (a,c) and SV-Nano-ESI (b,d) in the analysis of different samples. a) and b) are mass spectra of 10 ppm angiotensin Tris buffer solution ($[M+H]^+$, m/z 1047; $[M+Na]^+$, m/z 1069). c) and d) are spectra of human tear samples. Each time, 100 nL sample was loaded for analysis and a 10 points average was used to acquire the mass spectrum. By comparison, the SNR of angiotensin II in Tris buffer solution increased from 0.4 to 22.9. The SNR of tear lysozyme (+10 m/z 1470; +9 m/z 1634; +8 m/z 1838, the m/z is in accordance to the result achieved by electrospray laser desorption/ionization (ELDI).[32]) increased to 6.5 by using SV-Nano-ESI compared to no signals by using conventional Nano-ESI.

To demonstrate the efficiency of the method on matrices removal, Tris buffer solution (Supporting Information, Figure S1) of angiotensin II, maltoheptaose, lysozyme and human urine and tear samples were analyzed. To quantitatively evaluate the interference from the matrices, signal-to-noise ratio (SNR = peak intensity of the molecule of interest/the strongest peak intensity of the matrices) was defined. As shown in Figure 2 and Figure S2, the SNRs of angiotensin II, maltoheptaose, and lysozyme in Tris buffer solution are all below 0.4, indicating that the molecules of interest were hardly observed. However, when SV-Nano-ESI was applied, the SNR of the three samples increase dramatically. For angiotensin II and maltoheptaose, although the target molecular ion peaks can be observed with conventional nanoelectrospray, the peak intensity was very weak and submerged by other peaks from the complicated matrices. With the utilization of SV-Nano-ESI, the SNR for the molecules of interest increased by more than 50 times, and the peaks became the dominant ones in the spectrum, whereas the relative intensity of the peaks from the matrices was very weak. With the SV-Nano-ESI for analysis of angiotensin II in Tris buffer solution, the sensitivity was improved one order of magnitude relative to the conventional nanoelectrospray (Figure S3). For the lysozyme sample, the ion suppression effect resulting from matrices is much clearer, and the target molecule cannot be detected with the conventional nanoelectrospray. But by coupling SV-Nano-ESI with mass spectrometry for analysis the ion peaks of lysozyme were detected with SNR = 4.7. Urine and tear are both very complex biological samples, which are hard to analyze by nanoelectrospray without any pre-treatment. However, adopting SV-Nano-ESI gave a SNR of 4.3 for angiotensin II in a urine sample, and a SNR of 6.5 for lysozyme in a human tear. These results suggest that SV-Nano-ESI improves the analysis sensitivity of the target compounds in these samples.

For SV-Nano-ESI, the high voltage applied on the emitter is a crucial factor for matrix removal. This voltage must be higher than a critical voltage for separation to occur. Table S1 shows the correlation between high voltage and SNR. When the voltage applied was less than 4.8 kV, the separation of analyte from the matrices could not be observed. With the increase of the voltage higher than 4.8 kV, effective separation of the analyte occurred in the tip zone of the emitter. (Supporting Information, Figure S4). The optimal voltage was in the range of 5.2 kV to 5.6 kV. To elucidate the difference between both cases, a current meter was used to measure the current at the tip during spray. It was found that the current with SV-Nano-ESI (10 μA) was much higher than that from the conventional nanoelectrospray, and is attributable to the occurrence of discharge.

The efficiency of the matrix removal is also highly dependent on the duration of the high voltage, namely the discharge time. In the present study, the removal efficiency was evaluated with the SNR value. As shown in Figure 3 a), the SNR value monotonically increases with the extension of

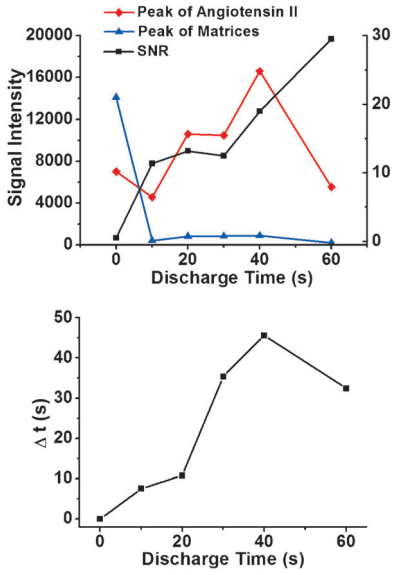

Figure 3. The correlation between discharge time and performance. 10 points average was used to acquire the mass spectrum after discharge. a) the base peak of matrices and angiotensin II and SNR after discharge, the signal intensity of matrices reduced dramatically when discharge was applied while the signal intensity of angiotensin II increased 2 to 3 times when 30 s to 40 s of discharge applied. The SNR increased with discharge time. b) The correlation of Δt and discharge time, 30 s to 40 s discharge time achieved the longest Δt.

discharge time. The maximum peak intensity from matrices sharply decreased from 14000 to 800 as the discharge time increased from 0 to 10 s. With further increase of discharge time from 10 to 40 s, the signal from matrices maintain almost constant, whereas the peak intensity of angiotensin II gradually increased up to 16000, followed by decreasing. The results indicate that the appropriate extension of discharge time suppressed signal from matrices, and enhanced the peak intensity of angiotensin II. Also, the discharge time has a great influence on Δt of the delay time of the analyte and matrices (the definition of delay time and Δt is shown in Figure S5), in turn, the separation efficiency between the analyte and the matrices. As shown in Figure 3b and Figure S6, the Δt value increased up to 45.6 s with the change of the discharge time from 10 to 40 s. However, further increase of the discharge time up to 60 s led to a decrease of Δt to 32.4 s. Taking into account the effect of discharge time on SNR and Δt, 30 to 40 s discharge time was regarded as the best value for analysis.

From the above experiments, we infer that the mechanism of matrix removal in the micro zone of the emitter tip is based on the electromigration of ions with the analyte and salts having different electromigration rates. Ion electromigration and solution migration are two important processes during ESI.[33–35] Taking the positive voltage mode as an example, ion electromigration causes cations to migrate to the tip of emitter and anions to migrate to the anode. The solution migration, which is forced by the formation of a jet flow at tip, causes continuous solution consumption. For the conventional nanoelectrospray (e.g., 2.4 kV), solution migration caused by jet flow is faster than ion electromigration in the emitter. So the separation effect could not be observed in the conventional nanoelectrospray. For SV-Nano-ESI, however, ion electromigration is much faster than solution migration. So the separation of ions with different electromigration rates could be achieved efficiently. This hypothesis is confirmed by the results as shown in Figure 4. For the matrices, cations and anions carried high apparent charge(s) and migrated fast, in opposite directions, to zone 1 and zone 4 with delay times of 2 s and 42 s, respectively, leaving a 40 s long zone without matrix interference. Angiotensin II has a relatively low apparent negative charge in the solution because it is an amphoteric molecule with an isoelectric point (pI) of 6.7.[36] So it migrated to the anode slowly with a delay time of 19 s. Maltoheptaose appeared to be a neutral molecule in the solution with a very low apparent charge, so its ion-migration rate is very slow with a delay time of 5 s. Therefore, the molecules with different apparent charge(s) were separated from zone 1 to 4 after discharge. (Figure 4b)

The hypothesis is further confirmed by utilizing SV-Nano-ESI to analyze angiotensin II with different pH values. The charge state of angiotensin II could be controlled by adjusting the solution pH value. Four samples with pH value of 3, 5, 7, and 9 were examined. There is a significant difference between the delay time of angiotensin II below and above its pI, as shown in Figure 5. When the pH value is above

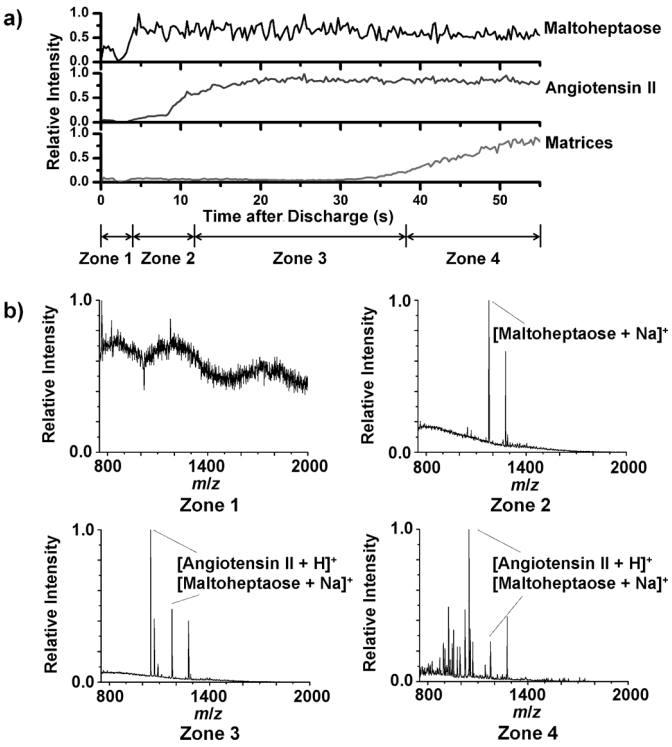

Figure 4. a) the extracted ion chronogram of different ions in the tip zone after high voltage applied in positive mode. The pH value of the solution is 9. The extremely high electric field forced different ions to migrate, salt cations and anions from the matrices migrated the fastest and distributed more in zone 1 and zone 4; maltoheptaose and angiotensin II migrated slowly and distributed more in the zone 2 and zone 3. b) The mass spectra of zones 1–4. The ratio of intensity of angiotensin II and maltoheptaose in zone 3 was chosen to evaluate the stability. The root-mean-squared standard deviation is 2.3% (under 7 times analysis).

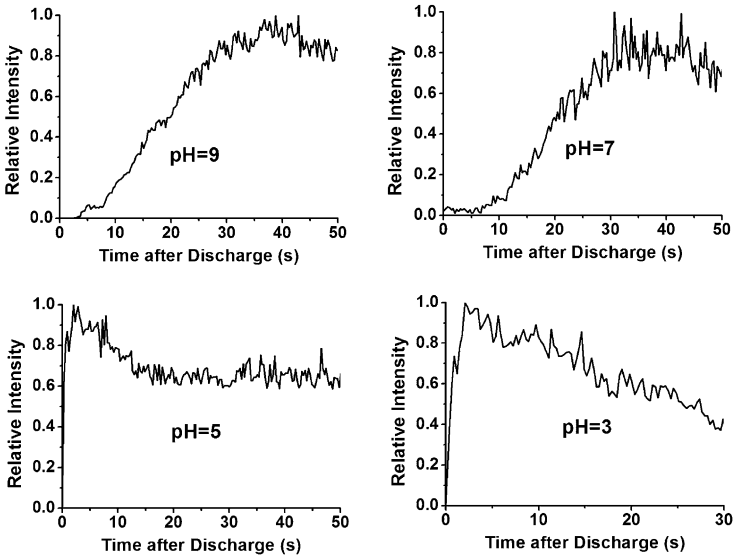

Figure 5. The extracted ion chronogram of m/z 1046.8 of 10 ppm angiotensin II with Tris buffer solution of different pH values. When the pH value of the solution is higher than the pI, angiotensin migrated to the anode and the EIC is upward; otherwise, angiotensin migrated to the emitter tip and the EIC is downward.

its pI, angiotensin II in the solution carried negative apparent charge and migrated to the tailing band. So the delay time of angiotensin II is 40 s for pH 9, and 36 s for pH 7. On the contrary, when the pH value is below its pI, angiotensin II in the solution carried positive apparent charge and migrated to the leading band. Therefore, the delay time of angiotensin II was significantly reduced to 4 s when pH 5, and 2 s when pH 3.

In conclusion, a novel method has been developed for matrix removal from small-volume samples by SV-Nano-ES, which potentially has broad applications in life sciences, such as proteomics, metabonomics, and single-cell analysis. Furthermore, no pre-treatment is required for the analysis of complicated samples, which makes it possible to achieve high throughput and rapid analysis. Although the mechanism of SV-Nano-ES is not fully understood, it does not hinder the application of this method. By controlling the high voltage and discharge time, separation could be simply achieved. The changing of fixed voltage nanoelectrospray to step-voltage nanoelectrospray to integrate separation and ionization together is a novel approach. Moreover, this method would not be limited in mass spectrometry but also other related techniques.

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