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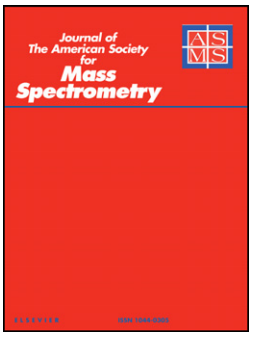

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Improving the Signal Intensity and Sensitivity of MALDI Mass Spectrometry by Using Nanoliter Spots Deposited by Induction-based Fluidics

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Abstract

A new contact-free, small droplet deposition method using induction-based fluidics (IBF) technique to dispense nanoliter drops is described and evaluated for sample preparation in matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS). The signal intensities available when using nanoliter spots are greater than those obtained with normal, microliter spots when the same amount of analyte is used. When using an ionic-liquid matrix, the improvement in sensitivity is equal to the concentration enhancement that was achieved by using smaller quantities of matrix. When using a conventional solid matrix, however, the increase in signal intensity shows a more complicated relationship to concentration. The approach of nL deposition also supports multiple spotting to increase sample concentration and, hence, sample signal intensity. Nanoliter spotting not only improves the signal intensity and sensitivity achieved by MALDI MS but also allows a major fraction of trace samples to be saved for other experiments, thus expanding the application of MALDI MS to biological studies where sample quantity is limited.

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Introduction

Although MALDI–MS has wide applicability to biological and synthetic polymers, obtaining high quality MS signals when the quantity of sample is small is difficult, which is endemic in biological studies. A limited sample amount gives rise to poor signal intensity, hampering spectra interpretation, unknown identification, and quantification, hence limiting the application in biological studies. One way to increase the signal intensity without increasing the sample amount is to make MALDI spots smaller and more concentrated.

Small scale sample preparation on a MALDI target has been achieved in several ways. One approach minimizes dispersion of sample materials by etching or drilling small channels on the target [1, 2] or using hydrophobic materials as MALDI plate surfaces [3-6]. A second approach employs micro dispensing, including use of capillary tubes [7-10], piezoelectric techniques [11-14], electrodynamic methods [15], and acoustic ejection [16]. This general approach improves MALDI for imaging [14, 16], on-line analysis [13, 17], and concentration enhancement by microextraction [10]. Common in both approaches [1-12, 15, 17] is the concept that the sensitivity of MALDI-MS increases when small sample spots are used and that the search for "hot spots" within the sample area becomes more productive when solid matrices are used. Our approach of using small volumes of concentrated analytes is motivated by the concept of purposefully creating a "hot spot".

Herein, we describe an approach by which a nanoliter syringe and induction-based fluidics (IBF) deposit small-volume droplets (in nanoliter range) in a non-contact, flight-

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controlled way. Using this technique, we first investigated the relationship between the spot size and signal intensity with an ionic liquid matrix (ILM), taking advantage of the homogeneity of ILM and subsequent ion-signal reproducibility that they permit [18-20]. This is the first report that compares quantitatively the signal intensity enhancement achieved by nanoliter spotting with that by normal spotting using both ILM and solid matrices. Furthermore, we introduce concentration enhancement of nanoliter spots by using multiple spotting whereby small amounts of sample and matrix are built up into a single spot of relatively high concentration that should yield improved signal intensities.

Experimental Section

Materials

Butylamine, α-cyano-4-hydroxycinnamic acid (CHCA, ≥99.0%), bradykinin (BK) and HPLC-grade organic solvents were obtained from Sigma-Aldrich (St. Louis, MO). All chemicals were used without further purification. The ionic liquid matrix (ILM) butylammonium α-cyano-4-hydroxycinnamate (Bu-CHCA) was prepared, as previously reported [18]. Its composition and purity were verified by ^{1}H NMR spectroscopy.

Sample Preparation

Bu-CHCA and CHCA were dissolved in acetonitrile/water (1:1, v:v) at the concentrations of 0.5 M [18, 19] and 10 mg/mL, respectively. BK (MW 1059.6) was chosen as the test analyte. It was dissolved in acetonitrile/water (1:1, v/v) at a concentration of 1 mM, which was further diluted to different concentrations by using the same solvent mixture.

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An aliquot of a BK solution was mixed with the same volume of Bu-CHCA or CHCA solutions to give the final spotting solutions.

MALDI Spot Preparation

The conventional spots of normal size (0.5 μL/spot) were deposited onto a 192-well MALDI plate by an Eppendorf pipettor (scale 0.1-2 μL). The nanoliter spots were made by the IBF Nanoliter Spotter (Figure 1, Nanoliter LLC, Henderson, NV). A nanoliter syringe with the sample was placed in the nanoliter wave's inductor. After the growth of a specific accurate volume on the capillary tip, controlled by the aligned micrometer, the needle was energized to charge the drop and launch it to the MALDI plate, effecting spherical droplet transmission to a specific location on the MALDI target. Six deposits were made for each test, and each result was the average of these measurements.

Mass Spectrometry

An ABI 4700 Proteomics analyzer (Applied Biosystems, Framingham, MA) equipped with an Nd:YAG laser (355 nm, 3-7 nsec pulses) was operated in the reflector, positive-ion mode. The acceleration voltage was 20 kV. The laser was operated at a fixed fluence ~5% above the threshold for ionization; its rate was 200 Hz, and the laser-firing pattern was set to "uniform." Both sample plate and laser were aligned before spectral acquisition. For each spot, spectra were obtained from 1000 laser shots (40 subspectra in different positions, 25 shots per subspectrum) and averaged to form a single spectrum. Spectra were smoothed by "noise filter" and baseline corrected with Data Explorer 4.0.

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Results and Discussion

Induction-Based Fluidics for Nanoliter Spotting

Induction-based fluidics (IBF) transports liquids by inductively charging them. During charging, capacitors form between the nanoliter wave inductor and syringe plunger and also between the capillary tip and MALDI plate. The inductive charging facilitates a launch that does not fragment the droplet, permitting manipulation of sub microliter drops [21] without using any moving parts. The electrical force directs the droplet flight, but buoyancy, drag, and gravity are also important given that the droplet deposition process occurs in the atmosphere.

Unlike electrospray, which uses conduction to charge liquids, IBF employs electric induction to charge droplets. The ability to launch droplets to targets of all types, to direct dynamically the liquids in flight, and to count them make IBF different from other technologies for transporting small liquid volumes [21, 22].

Nanoliter Spotting of Ionic Liquid Matrices

To investigate the relationship between the spot size and signal intensity when the same analyte concentration is used, we chose the ionic liquid butylammonium α-cyano-4-hydroxycinnamate (Bu-CHCA) as one matrix; its use affords good reproducibility and sensitivity, as was determined previously [18, 19]. Conventional spots (0.5 µL) and nanoliter spots (200, 100, 50, 20, 10 nL) were made from 5 µM BK solution. Although the nanoliter spotter can dispense volumes on the pL level, 10 nL was chosen as the

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smallest size of spots studied because the resulting spot has a diameter close to that of the MALDI laser beam. Microscope examination shows that Bu-CHCA spots are homogeneous and transparent [18, 20]. Spots of the same volume have nearly identical sizes, and spots of different volumes have diameters roughly proportional to the cube roots of the volumes, attesting to the good volume control (see Supplementary Material).

The first question we asked was "do spots of different volume but with the same concentration produce signals of the same intensity when using an ILM?" All the spots afford reproducible mass spectra and the signals from spots of different volumes are of nearly identical intensity (Supplementary Material), underscoring an advantage of ILMs [18-20]. Further, the ILM spots, although small, are durable for exposure to the laser beam [20], allowing a high number of repetitive laser shots without compromising signal intensity.

To compare the signal intensities from conventional and nanoliter spots that contain an equal amount of analyte, we spotted solutions with different BK concentrations in volumes of 500, 50, and 20 nL to give final BK amounts from 0.1 to 1.25 pmol per spot. For the sample spots containing the same amount of analyte, the 20 and 50 nL spots showed enhancement of signal intensity compared to conventional spots, presumably owing to the increased concentration (Figure 2). The effect is quantified by the ratio of the slopes of the linear fits: the slopes are in the ratio of 1.0 : 9.1 : 23, whereas the concentration ratio is 1 : 10 : 25. The signal enhancement is nearly proportional to the

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concentration enhancement, consistent with the good signal dynamic range that is achievable with an ILM [19, 23].

Multiple Spotting of Nanoliter Drops

Nanoliter spotting should allow for "on-plate" concentration enhancement when the analyte amount is so small that, even when dissolved in a small volume, the spotting solution is still too dilute. This problem is difficult to solve by using microliter volumes because the resulting spot is large, dispersed, and produces ion signals with poor S/N. Multiple spotting of nanodroplets at the same position interspersed by short delays for solvent evaporation permits accumulation of the analyte in a single, small spot. Analyte concentration is possible here because the droplet flight can be accurately directed, permitting deposition of one drop upon another with the IBF nanoliter spotter. To demonstrate, we accumulated a small spot (Figure 3) by depositing ten 20 nL droplets at the same position (3B). The sample deposited in the multiple-spotting approach afforded a signal intensity that was nearly 10 times greater than that of a conventional spot made by depositing one normal size drop (3A) containing 2.5 times the amount of analyte. For comparison purposes, we tested 20 nL spots (3C) with a concentration that was 10 times higher than those in (3A) and (3B) and found they gave similar signal intensities as the samples prepared by the multiple spotting technique (3B), indicating clearly that the ten overlaid depositions enhanced the concentration by 10 times. These results show the efficacy of analyte accumulation to give concentration enhancement and increased signal intensity while using a smaller amount of analyte than that used for conventional spotting.

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Nanoliter Spotting with Conventional Solid Matrices

Solid matrices are widely used in MALDI, especially in proteomics. Therefore, we applied nanoliter spotting of solutions with α-cyano-4-hydroxycinnamic acid (CHCA) as a solid matrix. We prepared conventional spots (0.5 μL) and nanoliter spots (100 and 50 nL), using the same approach as with ILM, and compared the resulting mass spectra from the spots containing the same sample amounts.

For each group of identically sized spots, the signal intensities correlate linearly with the analyte amount, as they do when an ionic liquid is used. The reproducibility of the data and the R^2 terms are not as good as those achieved with the ILM spots (see Supplementary Material). We found significant signal enhancement for 100 and 50 nL compared to that achieved for conventional spots (0.5 μL) containing the same amount of analyte. This confirms that MALDI signal intensities can be improved by using nanoliter spots of both ILM and conventional solid matrices. The signal enhancements for use of a solid matrix are 1 : 6.9 : 10 (Supplementary Material), which are ratios of the three line slopes that represent 500, 100 and 50 nL spots, respectively.

Although the observed ratios agree reasonably with the concentration ratios in the three groups of spots (1 : 5 : 10), the signal enhancement for the 100 nL spotting is ~ 40% greater than the concentration enhancement. This is not the case when 100 nL spots are made using Eppendorf pipettors. We attribute this additional enhancement to a "depth effect". This occurs because IBF deposition creates 100 nL spots that have ~65% of the surface area of a 100 nL spot from a pipettor and hence greater depth (the nanoliter

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spotter has a capillary tip with a much smaller diameter than that of a normal pipet tip, giving a more "focused" spot shape). The crystals formed from the IBF spotter have a denser distribution, coalescing and stacking the "sweet spots" within a smaller area and making them easier to be sampled by the laser. Higher average signal intensity and higher laser absorption efficiency are thus achieved, leading to the additional signal enhancement.

The quantitative signal enhancement, however, does not apply well to nanoliter CHCA spots with volume ≤ 20 nL, presumably owing to an "ablation effect". Perforations occur with significant laser ablation of the thin matrix layer [15], causing intensity to decrease with more laser shots. This problem can be resolved by decreasing the number of laser shots but at the expense of S/N. When the laser power was set at 5% above the ionization threshold, we achieved an improved detection limit (as low as ~ 60 attomole for BK with a S/N of 5 : 1) with 20 nL or less. The resulting sensitivities, however, don't obey the quantitative relationship we reported above. For 50 nL spots, however, the signal enhancement (10 times when compared to normal size spots with the same amount of analyte) is consistent with the concentration enhancement (10 times); this agreement may be result from a compromise of the "depth effect" (dominant in 100 nL spots) and "ablation effect" (dominant in ≤ 20 nL spots).

In summary, IBF nanoliter spotting leads to better signal intensity and sensitivity than when using microliter spots with the same quantity of material. This new approach may

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have the potential for improving the analysis of proteins and other biological samples especially when they are isolated in limited quantities.

Acknowledgement

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Figure Legends

Figure 1. Schematic of a portable IBF nanoliter spotter for droplet deposition: (a) power supply module, (b) micrometer, (c) nanoliter wave inductor, (d) stainless steel MALDI plate, (e) electrical wire connected to ground, (f) electric wire connected to high voltage, (g) syringe holder, (h) syringe with a chemically treated capillary tip, (i) x-y adjustment stage.

Figure 2. Comparison of peak intensities for normal-size spots deposited by an Eppendorf pipettor (open circles) and nanoliter spots deposited by the IBF spotter (50 nL, solid squares; 20 nL, open triangles) containing the same bradykinin amounts for MALDI using Bu-CHCA as the matrix. The three lines show the dynamic range for absolute quantitation of bradykinin based on molecular ion signal intensity. The slopes of these three lines are 2,680 (lower), 24,400 (middle) and 61,000 (upper).

Figure 3. Signal intensities from (A) 0.5 μL spots by using a Eppendorf pipettor using 1 μM BK solution (0.5 pmol BK per spot); (B) nanoliter spots produced by ten multiple spottings (using IBF nanospotter) of 20 nL each of 1 μM BK solution (0.2 pmol in each final spot). The final spot size was nearly identical to that of a 20 nL spot prepared in a single IBF deposition; (C) 20 nL nanoliter spot prepared by a single IBF spotting of 10 μM BK. The final Bu-CHCA concentration was 0.5 M in all three cases. Photographs of spots were taken using a Leica EZ4 microscope (Leica Microsystems Ltd, Switzerland). The light-colored circles are ridges outlining the spot area.

* Graphical Abstact

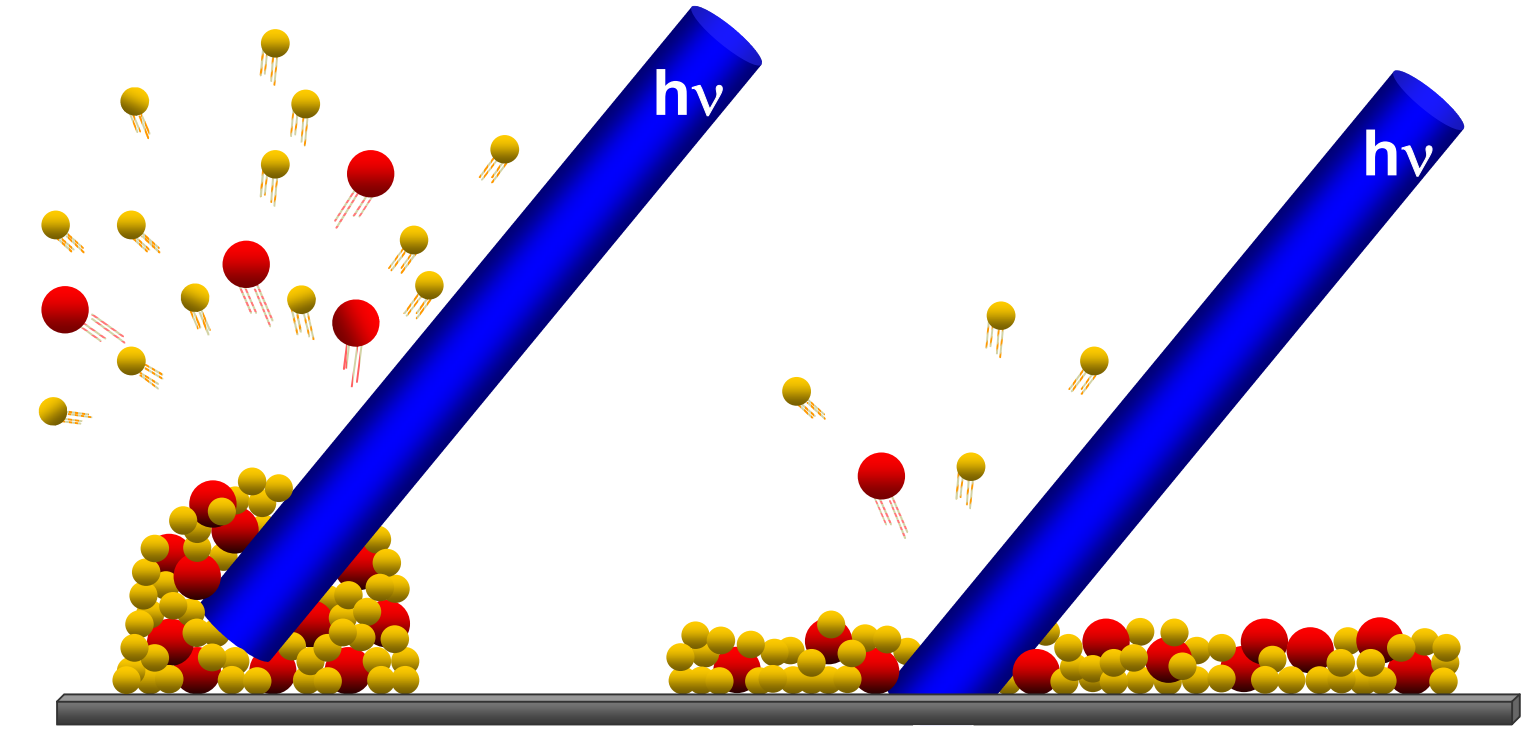

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Figure 1.

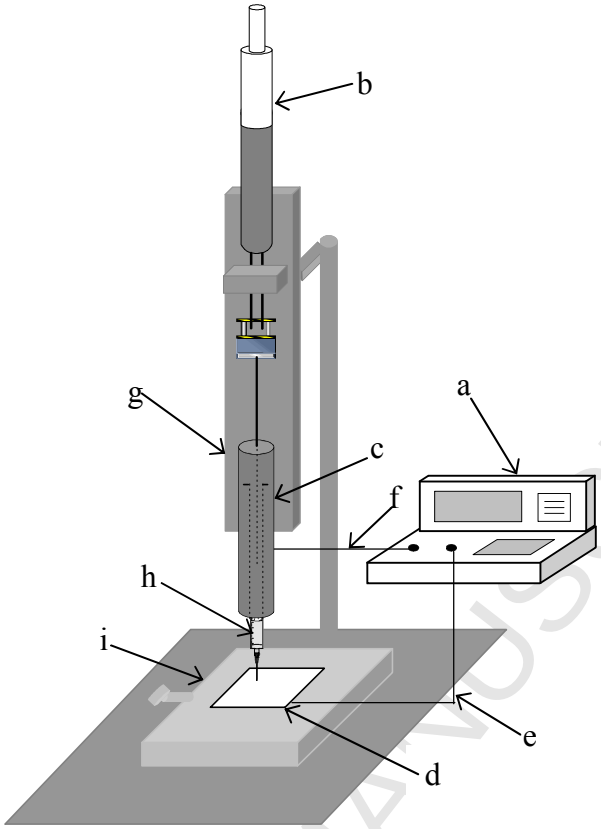

Figure 2.

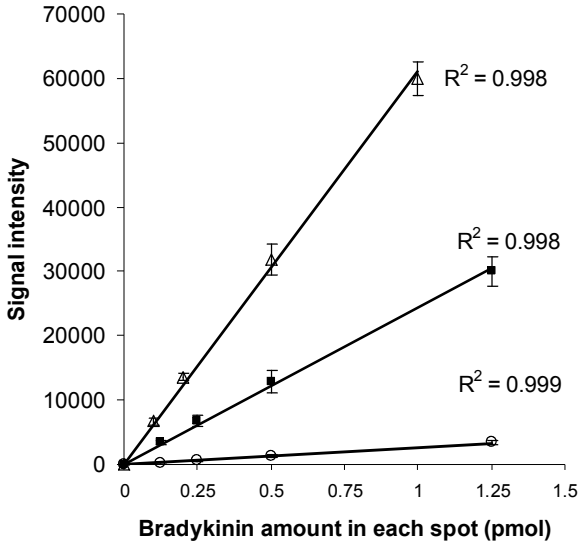

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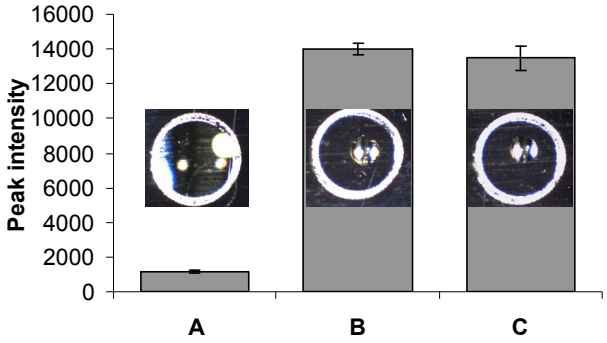
Figure 3.



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Sample Deposition Volumes	Deposition Methods	Spot diameters (mm)	Relative Average Signals	Relative standard deviation
0.5 µL	Pipettor	1.5	1.00	12%
200 nL	IBF spotter	1.2	0.98	4%
100 nL	IBF spotter	0.9	0.98	9%
50 nL	IBF spotter	0.6	0.96	7%
20 nL	IBF spotter	0.5	1.02	7%
10 nL	IBF spotter	0.4	0.97	10%

Table S-1 The relative signal intensities from spots with different volumes but the same analyte concentration. All the spots had a final bradykinin concentration at 5 µM and ILM concentration as 0.5 M. Spot diameters were determined relative to the known inner diameter of the sample well on the MALDI plate.

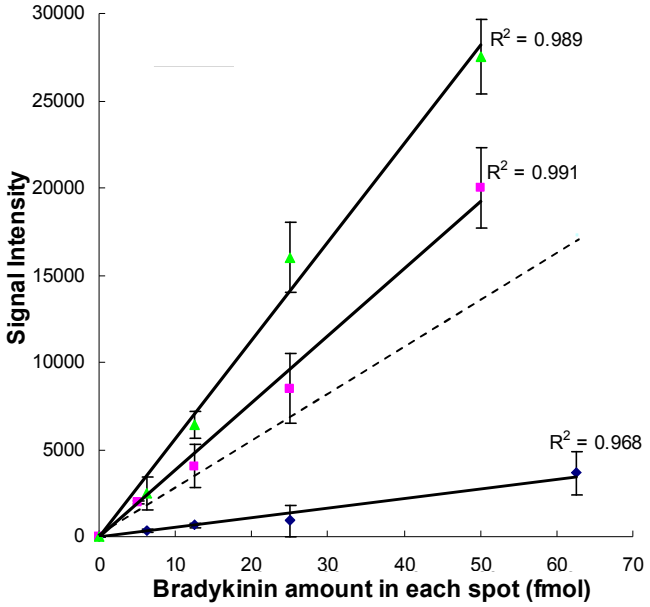

Figure S-1 Comparison of peak intensities for normal-size spots deposited by an Eppendorf pipettor (blue diamond) and IBF nanoliter spots (100 nL, pink square; 50 nL, green triangles) containing the same bradykinin amounts for MALDI using CHCA as the matrix. The three solid lines show the dynamic range for absolute quantitation of bradykinin based on the molecular ion signal intensity. The slopes of these three lines are 55 (lower), 384 (middle) and 563 (upper). To show the expected slope, the dashed line is also shown with a slope 5 times of that of normal-size spot line (see supplemental text for details).