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An Automated Noncontact Deposition Interface for Liquid Chromatography Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry

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A new multichannel deposition system was developed for off-line liquid chromatography/matrix-assisted laser desorption/ionization mass spectrometry (LC/MALDI-MS). This system employs a pulsed electric field to transfer the eluents from multiple parallel columns directly onto MALDI targets without the column outlets touching the target surface. The deposition device performs well with a wide variety of solvents that have different viscosities, vapor pressures, polarities, and ionic strengths. Surface-modified targets were used to facilitate concentration and precise positioning of samples, allowing for efficient automation of high-throughput MALDI analysis. The operational properties of this system allow the user to prepare samples using MALDI matrixes whose properties range from hydrophilic to hydrophobic. The latter, exemplified by α-cyano-4-hydroxycinnamic acid, were typically processed with a multistep deposition method consisting of precoating of individual spots on the target plate, sample deposition, and sample recrystallization steps. Using this method, 50 amol of angiotensin II was detected reproducibly with high signal-to-noise ratio after LC separation. Experimental results show that there is no significant decrease in chromatographic resolution using this device. To assess the behavior of the apparatus for complex mixtures, 5 μg of a tryptic digest of the cytosolic proteins of yeast was analyzed by LC/MALDI-MS and more than 13 500 unique analytes were detected in a single LC/MS analysis.

Although numerous sophisticated approaches have been developed to explore the nature and function of genes on a global scale,[1−3] similar studies of their protein products, i.e., the proteome, present far greater challenges,[4,5] due to the possibility of multiple gene products.[6]

Currently, 2D gel electrophoresis followed by mass spectrometric analysis of proteolytic digests of individually excised gel bands remains the most common technique for performing large-scale protein analyses,[7] despite its well-recognized limitations.[8−10]

These limitations have prompted the exploration of alternative analysis platforms based on multidimensional liquid chromatography or capillary electrophoresis combined with MS or tandem MS methods.[11,12] The separations are required to help minimize ion suppression effects that occur during the analysis of complex mixtures. They can be performed at either the peptide[13] or the protein level,[14] although protein identifications are usually based on tandem MS analyses of protein digests.[15] Using these methodologies, the identification of proteins present in affinity complexes, organelles, and even whole organisms has been performed.[16,17] Further refinements for reducing the complexity of the analyte mixture based on the presence of particular amino acids[18−20] or specific PTMs[21,22] have been reported.

The overwhelming majority of these studies have employed electrospray ionization to directly couple the separation system to the mass spectrometer. Although straightforward to implement, the time constraints imposed on the analysis by the on-line nature of this configuration imposes significant limitations on the thorough analysis of highly complex samples. More comprehensive analysis is possible if multiple LC/MS experiments are

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performed. However, sample consumption is increased and system throughput reduced if such an approach is taken.[23,24]

Alternatively, off-line deposition of separated analytes on a target plate and subsequent MS analysis using laser desorption/ionization methods such as matrix-assisted laser desorption/ionization (MALDI)[25] or DIOS[26,27] would resolve the problems imposed by coupled separations/MS analyses. The resulting "permanent record" of the separation can quickly be scanned in the MS mode to produce an overview of the whole sample. From this overview, species can be selected for further characterization,[19] thus increasing the effective throughput of the system. In addition, these sample records can be stored for months under appropriate conditions,[28] allowing the efficient characterization of additional species in subsequent experiments without additional sample processing. Furthermore, off-line sample deposition avoids the situation encountered in "peak parking",[29,30] where the resolution and concentration of the eluting species are compromised to allow more time for the mass spectrometer to analyze the eluates.

Several different approaches for the off-line coupling of column separations to MALDI-MS have recently been reported. "Heart fractionation" methods achieved either by off-target fraction collection and subsequent processing or direct deposition onto a target plate are readily implemented using commercially available equipment but may sacrifice some chromatographic resolution.[31,32] Alternatively, the effluent of a separation column have been sprayed directly onto a target plate as a continuous stream,[33,34] deposited as a series of small droplets using a piezoactuated microdispenser,[35−37] or applied as a continuous trace using a specialized liquid junction-coupled subatmospheric pressure deposition chamber.[38,39] Ultimately, the successful implementation of any MALDI-based analysis platform requires a highly robust deposition system that can handle large numbers of highly complex samples with minimal preprocessing, while still maintaining the separation efficiency required for successful MS analysis.

These considerations clearly favor a separation strategy based on LC, because most chromatographic modes can concentrate dilute samples and possess the comparatively high loading capacities required to address the large dynamic range of protein expression in biological samples. Liquid chromatography is also highly robust, compatible with most labeling methodologies, and amenable to target plate deposition. The continued development of ultrahigh-pressure LC[40] promises to further improve chromatographic separation.

In this report, we describe a new noncontact, liquid deposition system that transfers the eluents of multiple chromatographic columns onto MALDI target plates, as a series of droplets, using a precisely controlled pulsed electric field. The flexibility of this system allows the co-deposition of eluents with MALDI matrixes that range from hydrophobic to hydrophilic. Although completely compatible with nanoscale chromatography, highly robust yet equally sensitive separations can be performed using μHPLC columns in conjunction with surface-modified target plates that facilitate on-target sample concentration. In addition, the deposition system can readily handle high ionic strength solutions and pure organic solvents, enabling its use in various modes of chromatography as well as novel, on-plate, automated recrystallizations. The performance of this system coupled off-line to both MALDI time-of-flight MS (MALDI-TOF MS) and MALDI Fourier transform ion cyclotron resonance MS (MALDI-FTICR MS) is demonstrated using standard peptides, tryptic digests of model proteins, and, finally, the cytosolic fraction of whole yeast extracts.

EXPERIMENTAL SECTION

Chemicals. All protein and peptide standards, 1,4-dithio-DL-threitol (DTT), and iodoacetamide (IAA) were obtained from Sigma (St. Louis, MO) in the highest purity available and used without further purification. 2,5-Dihdroxybenzoic acid (DHB), α-cyano-4-hydroxycinnamic acid (HCCA), acetic and formic acids (HPLC grade), trifluoroacetic acid (TFA, protein sequencing grade), and various buffers were also obtained from Sigma. HPLC grade acetonitrile (ACN) and matrix solvents were obtained from Fisher Chemicals (Fairlawn, NJ), and sequencing grade trypsin was obtained from Promega (Madison, WI).

DHB was recrystallized before use from ethanol and water (1:1 v/v) and then dissolved in 0.1% v/v TFA (in water) to a final concentration of 16 mg/mL. HCCA was recrystallized from ethanol and water (1:1 v/v), dissolved in *N*,*N*-dimethylformamide (DMF) to a final concentration of 1 mg/mL and used for precoating MALDI target spots or dissolved in 75:25 ACN/water and 0.1% v/v TFA (in water) to a final concentration of 0.2 mg/mL for use in on-plate recrystallizations.

Preparation of Total Yeast Extracts from *Saccharomyces cerevisiae*. Yeast total lysates were prepared as described previously[41] with some modifications. *S. cerevisiae* strain L40[42] was grown to an optical density at 600 nm (OD_{600}) of 2.7 in 500 mL of YPD media (reference or quantities of ingredients/L) in 2-L flasks at 30 °C and shaken at 250 rpm. The cells were pelleted at 4000*g*,

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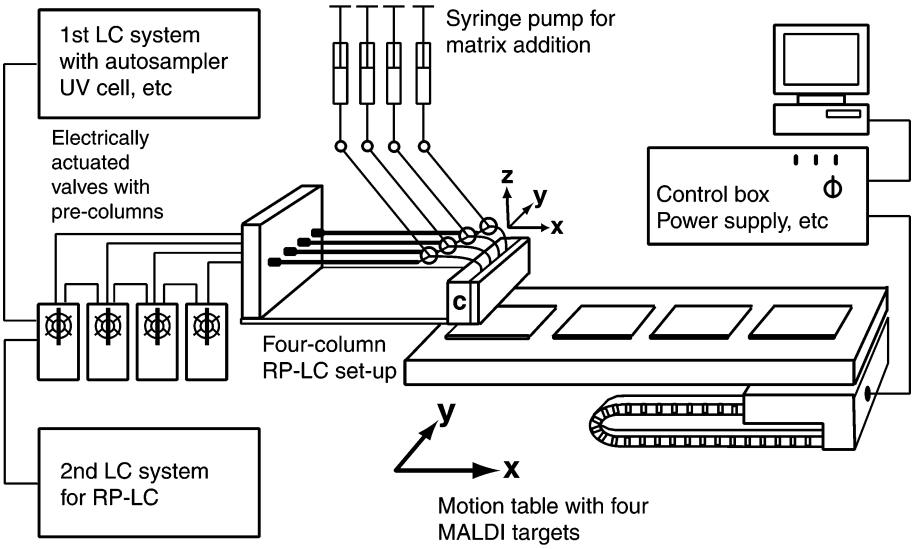

Figure 1. Schematic overview of the off-line LC/MALDI deposition system.

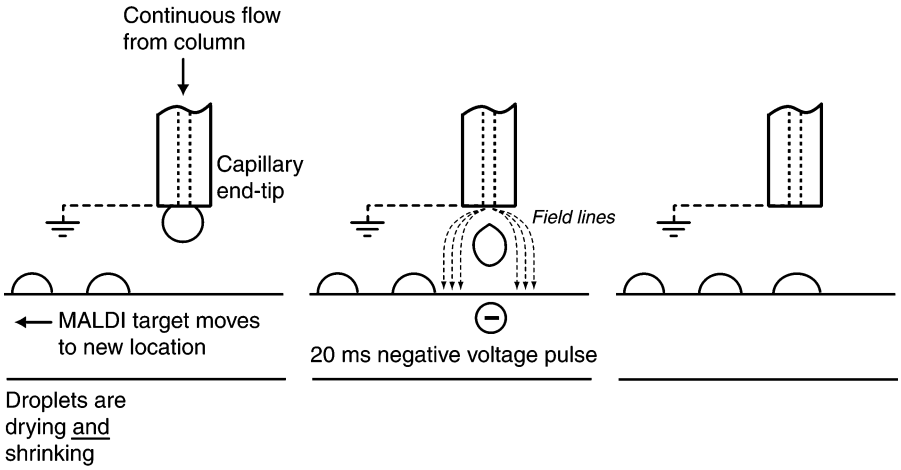

Figure 2. Principle of the electrically mediated liquid deposition.

and the pellets were weighed after washing once with water. Cells were then incubated with shaking for 15 min at 30 °C in Tris-DTT buffer (0.1 M Tris, pH 9.4, 10 mM DTT). Subsequently, the cells were washed in buffer A (1.2 M sorbitol, 20 mM potassium phosphate, pH 7.4) and incubated for 30 min at 30 °C with shaking in 2 mL of buffer A/g of yeast pellet and Zymolyase 20T (ICN pharmaceuticals, Costa Mesa, CA) at 2.5 mg/g of yeast pellet. The resulting spheroplasts were washed twice at 4 °C with buffer A and collected by centrifugation at 4000*g*. Spheroplasts were then lysed in lysis buffer (50 mM Tris pH 7.5, 100 mM NaCl, 1% nonidet P40 (NP-40), 20 μg/mL aprotinin, 20 μg/mL leupeptin, 1 mM Perfabloc, 10 mM β-glycerophosphate, 1 mM EDTA, and 10% glycerol (all from Sigma). The lysate was centrifuged at 12000*g* for 15 min at 4 °C. Supernatant was stored at −80 °C at a protein concentration of ∼10 mg/mL.

Enzymatic Digestion. Protein samples were digested using a standard protocol.[43] Briefly, soluble proteins were denatured in a solution of 100 mM NH_4HCO_3 and 8 M urea (pH 8.0), reduced with DTT, alkylated with IAA, and, after dilution to 2 M urea, digested overnight with trypsin in a ratio of 1:20 (w/w) at 37 °C. For dried-droplet experiments, urea and salts were first removed from the sample using either Peptide MicroTraps (Michrom BioResources, Inc, Auburn, CA) or a ZipTip (Millipore Corp., Bedford, MA).

Noncontact Deposition Device. A diagram of the deposition system is shown in Figure 1, while the mechanism of deposition is illustrated in Figure 2. Close-up photos of the deposition head itself and the resulting sample spot after deposition onto hydrophilic/hydrophobic targets are shown in Figure 3. The outlet capillaries (260-μm o.d., 50-μm i.d.) of four HPLC columns (see below) were fixed vertically in a capillary clamp (C) in Figure 1 (also seen in Figure 3A) such that their ends were located 2−5 mm above the surface of a MALDI target plate. Four plates were arranged in series on top of a linear motor-driven translational stage that physically moved the plates beneath the outlets of the columns. The target plates possessed arrays of well-defined circular hydrophilic regions on an otherwise hydrophobic surface[44] (Anchor Plate, 1536 spots, 400-μm o.d., Bruker Daltonics, Billerica, MA). Initial fine adjustments of the clamp height relative to the target plate was achieved using an *x*,*y*,*z* angle-adjustment stage

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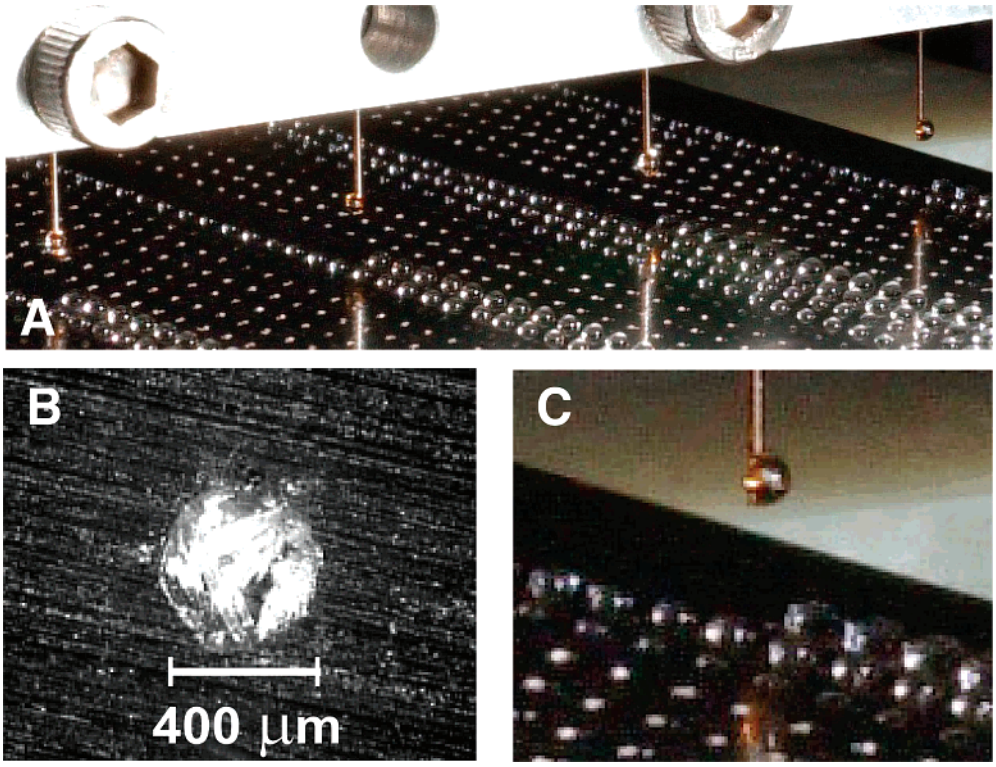

Figure 3. (A) Photographs of the automated deposition of the eluents from four LC separations mixed with DHB onto a 1536 target. (B) The eluents and matrix are concentrated, dried, and cocrystallized on arrays of hydrophilic islands of 400-μm o.d. having well-defined coordinates. (C) A droplet of ~650 nL just before being pulled down onto the target surface. Focused and dried sample/matrix spots can be seen in the foreground.

(P100-T-1, Newport Corp., Irvine, CA) and thereafter remained constant for repeat experiments. The deposition device was built out of a linear motion table for the *x*-axis and a stepper motor-driven cross-roller table for the *y*-axis. Tables, controllers, and a power supply were from Parker Automation (Parker Daedal Division, Irwin, PA). The entire system was operated using software written with the supplied programming tools (Parker Compumotor Motion Planner, Parker-Hannifin Corp., Irvin, PA), which enabled control over the voltage pulse duration, total deposition time for a given position, and both the number and locations of deposition positions on a plate. The high-voltage pulse applied to the target plate through the plate holder was generated by switching the dc input power to a proportional dc−dc converter (G05, ECMO High Voltage, Sutter Creek, CA) "on" and "off" with the help of a relay triggered through the stage controllers. The amplitude of the output pulse was controlled manually by adjusting the input voltage level to the dc-dc converter.

Chromatographic System. Four capillary columns (PepMap, 300 μm i.d. × 15 cm with 3-μm, 100-Å, C18 beads, LC Packings, Amsterdam, The Netherlands) were used to perform the LC separations. Precolumns (300 μm i.d. × 0.5 cm) having the same PepMap stationary phase were connected to the analytical columns via electrically actuated valves (Valco Instruments Co. Inc., Houston, TX). This setup allowed both independent injections onto any column using the autosampler and simultaneous chromatography on all four columns with an Agilent 1100 HPLC system (Agilent Technologies Inc., Palo Alto, CA).

Reproducible flow rates at low volumetric flows were obtained by connecting a splitter capillary upstream to the precolumns such that the flow rate through the pre- and analytical columns was 3 μL/min. Mobile phase A consisted of 0.1% TFA in water, and mobile phase B consisted of ACN with 0.1% TFA. The solvent gradient used was 3−10% of mobile phase B in 10 min, 10−50% B from 10 to 60 min, and 50−95% B from 60 to 65 min. Sample injections were performed with a second Agilent 1100 HPLC system equipped with an autosampler. During a separation with solvent gradient, 384 fractions per column were deposited at a rate of 0.1 Hz onto the hydrophilic spots of the target plates. When fraction deposition was performed at a rate of 1 Hz, a single column was used and approximately half of the gradient was collected as 1536 fractions.

Matrix can be added before, during, or after the deposition of the analytes. When added during the course of the separation, DHB solutions were infused using a syringe pump (kdScientific, New Hope, PA) via low-dead volume, postcolumn Y-connections (Upchurch Scientific, Oak Harbor, WA). The syringe pump typically delivered the matrix solution at a flow rate of 1 μL/min.

For the more hydrophobic HCCA, the preferred method was precoating of the individual spots on the MALDI targets. In this case, 100 nL of a 1 mg/mL solution of HCCA dissolved in DMF was first deposited at a rate of 2 Hz, onto the individual target locations. This was immediately followed by deposition of the eluents of the columns. The dried fractions were then recrystallized by the addition of 50 nL of a 0.2 mg/mL solution of HCCA in 75% ACN and 0.1% TFA using the same deposition system.

Mass Spectrometry. MALDI-TOF measurements were performed using a Bruker Biflex III mass spectrometer operated in the reflectron mode. MALDI-FTICR experiments were performed at 9.4 T with a modified Apex II FTICR system (Bruker Daltonics, Billerica, MA). Modifications included the replacement of the

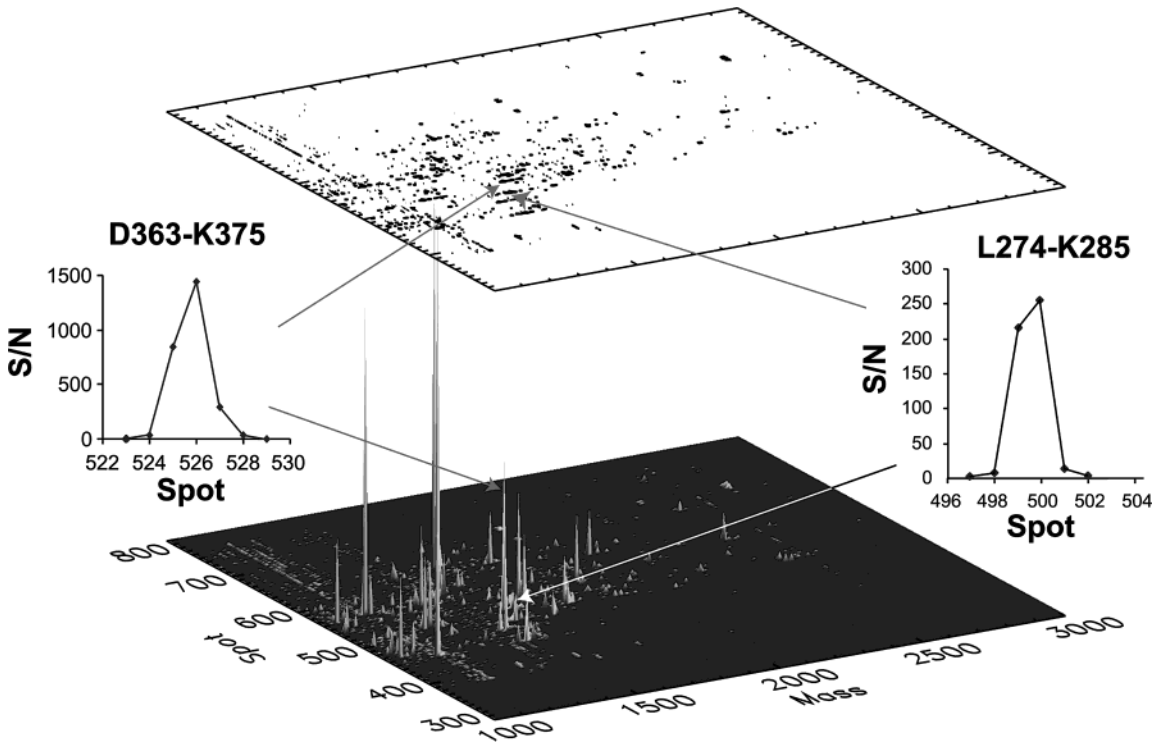

Figure 4. 3D plot of the reversed-phase LC/MALDI-MS analysis of 200 fmol of a BSA tryptic digest with extracted elution profiles (◆) from two peptides. A DHB solution was introduced to the column eluents via postcolumn Y-connections, and droplets were spotted down once every 10 s.

analyzer cell and ion-transfer optics with in-house designs as the addition of a custom intermediate-pressure MALDI source (manuscript in preparation). All spectra acquired on the MALDI-FTICR mass spectrometer were internally calibrated using a novel method that employs gas-phase mixing of sample and calibrant ions in a hexapole ion storage guide. This allows mass spectra to be aquired routinely with a mass tolerance of 3 ppm rms. All data used for definition of unique analytes and peptide assignments were acquired using MALDI-FTICR MS. A customized implementation of the THRASH algorithm[45] that allows internal calibration of mass spectra, reduction of spectra to monoisotopic peak lists, and condensation of peak lists to single entries along the time dimension of the separations was used as part of the data analysis. Masses within a 10 ppm-wide m/z window detected in one or several spots, possessing a signal-to-noise ratio greater than 3, and observed to form an elution profile were combined to define a unique analyte. Tryptic peptide assignments of digested proteins were assigned to masses calculated by THRASH using the PAWS program (ProteoMetrics, New York, NY) with a highly conservative mass accuracy of 10 ppm.

RESULTS AND DISCUSSION

Principle of the Electrically Mediated Deposition. The approach used for deposition of eluent of a separation column onto a target plate is critical to the overall performance of an off-line LC/MALDI system. In theory, continuous deposition should most accurately maintain the fidelity of a separation process. However, care must be taken to minimize the dispersal of the analyte on the target, and some resolution may still need to be sacrificed in order to produce sample regions with sufficient signal for detection.[34] In addition, the mass spectrometer has to query the entire length of a continuous trace since the locations of the different analytes are not known, potentially decreasing the throughput of the system. Alternatively, the deposition of column eluent onto well defined, localized regions enables the mass spectrometer to analyze a maximum number of analytes distributed over a minimal number of fractions while maintaining the ability to assess the chromatographic performance of the system. More importantly, the use of surface-patterned target plates effect analyte concentration after the chromatographic step enables the use of significantly more robust μHPLC columns while sensitivities typical of nano-HPLC techniques are maintained.

The deposition process for each droplet consists essentially of three steps as shown in Figure 2. The process starts, at the capillary tip, with the formation of an electrically grounded droplet acting as the point in a point-plane electrode configuration. A short negative voltage pulse (−2 kV) of 20 ms applied to the target stage causes polarization of the droplet, which is then pulled to the target surface by the electric field. This strategy prevents undesirable electrospray deposition because of the pulsed nature of the applied voltage and the low field strength at the droplet surface due to its comparatively large radius. Electrospray would distribute the column eluent over a much wider area, decreasing sensitivity and causing potential cross-contamination between adjacent sample rows in high-density arrays. In addition, the pulsed nature of the electric field minimizes unwanted electrochemical side reactions.

Characterization of the Performance of the Deposition System. A tryptic digest of BSA was used to characterize the fundamental performance of the LC/MALDI deposition device.

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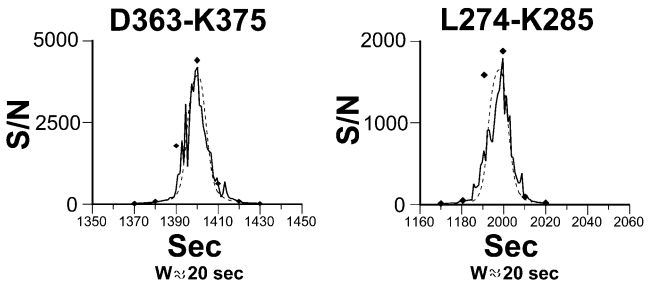

Figure 5. Higher resolution elution profiles obtained by deposition every second during the analysis of 5 pmol of a BSA tryptic digest. The dashed line is an estimate of the real peak shapes and *W* is the chromatographic peak width at the baseline.

A total of 200 fmol of digested BSA was loaded onto each of four columns, and the reversed-phase separations were performed in parallel. These samples were introduced as 20-μL aliquots of a 10 fmol/μL digest solution. The eluents from the four columns were simultaneously co-deposited with DHB every 10 s onto hydrophilic spots on the MALDI target plate and analyzed using the MALDI-FTICR instrument. Figure 4 shows a 3D view of one of the resulting mass chromatograms as well as the extracted signal-to-noise profiles (◆) for the peptides D363-K375 and L274-K285 of BSA. The average of the four experiments gave a sequence coverage for BSA of 78.4% (457/583) from 48 assigned peptides with a total length of 619 amino acids counting overlapping missed−cleaved peptides. Depositing column eluents as discrete fractions every 10 s onto surface-patterned target plates provided clear advantages by concentrating analytes and decreasing the total number of MS spectra to be acquired while the ability to assess the chromatographic performance was still maintained. Figure 5 shows the extracted signal-to-noise profiles for the same two BSA peptides (5 pmol of digest initially loaded onto the columns) obtained by depositing the eluent every second instead of every 10 s. Base peak widths (*W*) of ~20 s were observed for these as well as most other peptides. Such values are typical for the stationary phase and the solvent gradient employed, confirming that the postcolumn addition of DHB, as performed in this experiment using a Y-connector, had virtually no effect on chromatographic resolution. More importantly, the signal-to-noise ratio profiles obtained by depositing every 10 s (◆) were similar to those obtained using a more frequent sampling rate but required less than one-tenth the amount of sample.

By comparison, a dried-droplet preparation of 200 fmol of the same BSA digest (concentrated on a ZipTip and directly eluted onto an Anchor Plate) gave an amino acid sequence coverage of 59.9% (348/583) from 27 assigned peptides with a total length of 365 amino acids, again counting overlapping missed−cleaved peptides. This reduced sequence information for the dried-droplet experiment is likely due to the enhanced cocrystallization of the more hydrophobic peptides with the MALDI matrix.[46] The reversed-phase separation prior to deposition mitigated this effect by ensuring that the peptides that elute concurrently from the column had similar hydrophobicity.

Use of the Deposition Device with Nonpolar Matrixes. While highly water-soluble matrixes such as DHB can readily be co-deposited with column eluent as discrete droplets across the entire range of ACN/water concentrations employed in reversed-phase chromatography, other matrixes commonly used in the analysis of peptides such as HCCA create considerable operational difficulties. HCCA is significantly more hydrophobic and begins to crystallize immediately upon deposition, especially at the beginning of the reversed-phase gradient where the water content is higher. These varying conditions under which matrix crystallization occurs can prevent efficient localization and concentration of analytes on the surface-patterned target plates.[44]

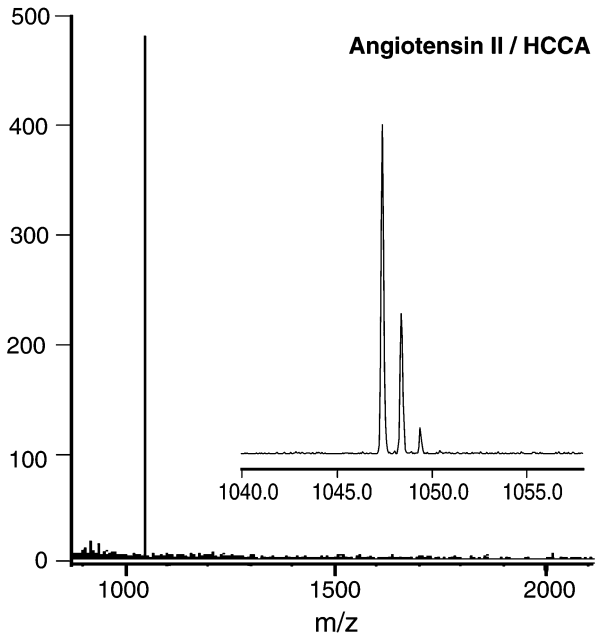

Figure 6. LC/MALDI-TOF MS analysis of 50 amol of angiotensin II deposited onto the target using the multistep deposition process with HCCA.

The unique properties of our deposition system facilitate novel solutions to such operational incompatibilities. For example, a fully automated, multistep deposition process readily enabled the use of HCCA in conjunction with both reversed-phase chromatography and surface-patterned targets. A 1 mg/mL HCCA solution in DMF was first deposited onto the hydrophilic spots (100 nL/spot), and the reversed-phase separations were then performed such that the eluents were deposited on top of the initial DMF solutions. The low vapor pressure and favorable solvent properties of DMF served to prevent the premature crystallization of HCCA without substantially increasing the overall time required for the combined droplets to shrink onto the spots. Then, a 0.2 mg/mL HCCA solution in 75% ACN and 0.1% TFA was added to each dry sample position (50 nL/spot), enabling all the sample preparations to be recrystallized under identical conditions. Parallel deposition of the various HCCA solutions caused only a minimal increase in overall sample preparation time, since well-defined volumes of either solution can readily be added to 1536 positions in less than 1 min using our deposition system.

The effectiveness of this multistep deposition process was demonstrated by loading 200 fmol of the same tryptic digest of BSA as described above onto each of four capillary columns and simultaneously depositing the four eluents using the described multistep process. Despite depositing ~0.5 μL of eluent onto each position, the HCCA matrix and analyte were fully localized within the 400-μm-i.d. anchors across the entire range of the solvent

(46) Cohen, S. L.; Chait, B. T. *Anal. Chem.* **1996**, *68*, 31−37.

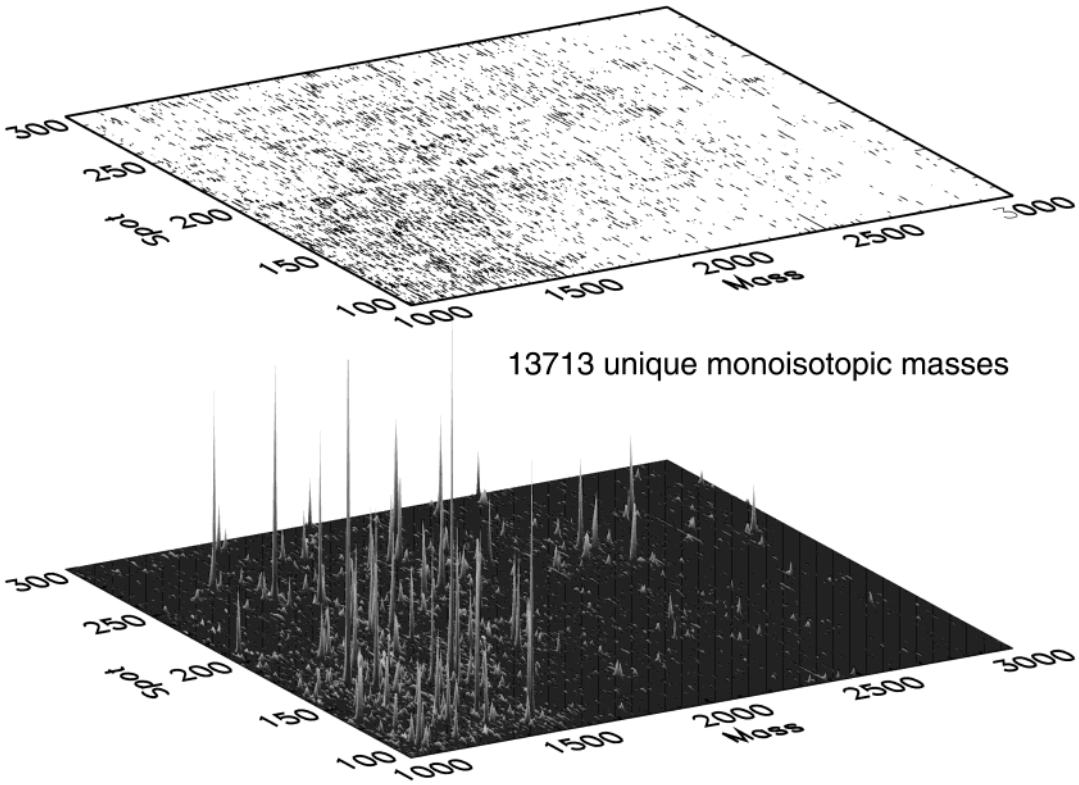

Figure 7. 3D plot of the reversed-phase LC/MALDI-MS analysis of a tryptic digest of the cytosolic proteins in yeast.

gradient. Using a MALDI-TOF mass spectrometer with a mass accuracy of 100 ppm for peptide assignments, an average amino acid sequence coverage of ~70% was obtained for the four column separations after recrystallization compared to 30% obtained by the dried-droplet method.

The sensitivity of the system was tested using the standard peptide angiotensin II. Figure 6 shows that a clear signal was obtained when 50 amol of angiotensin II was loaded onto a single column, separated by reversed-phase chromatography, deposited using the multistep deposition process with HCCA, and analyzed by MALDI TOF MS.

Using the Deposition Device To Process Highly Complex Mixtures. Although useful for assessing the fundamental performance of a given deposition system, standard peptides or single protein digests do not reflect the difficulties presented by samples derived from living cells. These samples display large variations in complexity, concentration, and cleanliness, thus presenting operational challenges for any high-throughput analysis platform. To test our system's performance using biological relevant samples, extracts of soluble yeast proteins were prepared and digested with trypsin. Five micrograms of this digest was loaded onto a single column, and the eluent of the reversed-phase chromatography was automatically deposited every 10 s with DHB onto a surface-patterned target plate. Automated analysis of the resulting trace using MALDI-FTICR MS found over 13 500 unique analytes (Figure 7), of which more than 50% where detected in two or more consecutive spots. While additional modes of separation such as strong cation exchange chromatography are clearly required to begin to address the true complexity of such a sample, the highly automated and robust nature of this system allows fast parallel processing of the multiple fractions resulting from such an initial fractionation.

Other Applications for This System. This deposition strategy performed well with a wide variety of solvents possessing significantly different viscosities, vapor pressures, and polarities. These included saturated sucrose solutions, 2-butoxyethanol, hexanes, and even high ionic strength solutions such as a 1 M NaCl if parameters were adjusted accordingly. Thus, this deposition system can function with different modes of chromatography or perform automated on-plate recrystallizations with various solvents, providing significantly more flexibility than methodologies limited to reversed-phase solvent systems. The current implementation of our deposition system is also useful for other applications such as dispensing of volumes as small as 20 nL at a rate of 20 Hz, which can be accomplished by adjustment of the deposition parameters on the current device.

CONCLUSIONS

We have demonstrated a new noncontact liquid deposition system and its application for off-line LC/MALDI-MS analyses. The system is fully automated and enables the simultaneous chromatographic separation of multiple samples and their subsequent deposition onto MALDI targets using an electric field. The unique properties of the deposition system facilitate the usage of the full range of typical MALDI matrixes as well as novel on-plate recrystallization methodologies. Additionally, the discrete deposition of column eluents onto surface-patterned target plates enables highly sensitive analyses to be performed while the high sample loading capacity and robustness of μHPLC-scale separations are maintained. These attributes make our system well suited to handle the challenges posed by complex biological samples.

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